INVESTIGATION OF UNIQUE COENZYME A BIOSYNTHETIC AND REDOX
FUNCTIONS IN Bacillus anthracis

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“Our deepest fear is not that we are inadequate. Our deepest fear is that we are powerful beyond measure. It is our light, not our darkness that most frightens us. We ask ourselves, who am I to be brilliant, gorgeous, talented, fabulous? Actually, who are you not to be? You are a child of God. Your playing small does not serve the world. There is nothing enlightened about shrinking so that other people won't feel insecure around you. We are all meant to shine, as children do. We were born to make manifest the glory of God that is within us. It's not just in some of us; it's in everyone. And as we let our own light shine, we unconsciously give other people permission to do the same. As we are liberated from our own fear, our presence automatically liberates others.”

- Marianne Williamson
ACKNOWLEDGEMENTS

September 26, 2008 marks a total of 162,345,600 seconds, 2,705,760 minutes, 45,096 hours, 268 weeks or 5 years, 1 month and 21 days since I started working towards my goal of obtaining a Ph.D. This journey has been one for the record books mounting 11,083.52 miles traveled. While the burden was tough, and the road was long, I am proud to say that with God’s grace and mercy I am that I am.

This accomplishment would not have been possible were it not for the trust that Dr. Al Claiborne, my advisor, had in me to go forth and develop a project fit enough for a dissertation. When I first entered Al’s laboratory, I was certain that I wanted to study the physiological roles of various proteins in *Bacillus anthracis*. Unfortunately, the laboratory was not set up for such experimentation. Al placed numerous phone calls and emails to make my scientific interests a reality and I am indebted to him for that. I have been able to foster bonds of friendship with people that will only serve assets in my career development. Al, I thank you!

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there in the beginning helping to acclimate me to the Claiborne lab. I thank you for providing me with a solid bench work foundation and always being there to answer all of my questions. Dr. Jacquelyn Fetrow, you were the first to introduce me to the world of bioinformatics. In Spring 2004, I had no idea how I would be able to use this information in my research efforts; however, I am happy to say that your class ignited the interest and I am now convinced that bioinformatics is a strong tool to be used in parallel with basic science. Finally, Dr. Nathan Fisher, your diligence as a graduate student made available a genetic tool that I was able to implement and resulted in my very first, first-authored publication. Also, your expertise in *B. anthracis* is unparalleled to that offered by my home institution.

My dedication and steadfast spirit while pursuing this degree, is attributed to the appreciation and prayers of my family. Mom, you are the reason that I work as hard as I do. In fact, it is all I can do to repay you for the sacrifices you made for me. Xavier, please look at the path Mom and I have set before you. I challenge you to leave your own imprint in this world, but do so remembering that we love you and are always here for you. Lastly, Mr. Lawrence R. Anderson, I am grateful to have such an understanding, thoughtful, considerate, respectful and loving mate in you. Thank you for wavered all of my emotional inconsistencies during this process. Thank you for reminding me just how capable I am in pursuing this endeavor. Thank you for traveling to all of the destinations my training has led me. Thank you, thank you and thank you for all of the support that worlds alone just cannot explain. And to a whole host of family and friends, I give gratitude!
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LIST OF ABBREVIATIONS

PA, protective antigen; LF, lethal factor; EF, edema factor; MAPKK, mitogen-activated protein kinase kinase; ATR, anthrax toxin receptor; CaM, calmodulin; SOD, superoxide dismutase; CysSSCys, oxidized cystine; GSSG, oxidized glutathione; GSH, glutathione; GCL, glutamate cysteine ligase; GS, glutathione synthase; GR, glutathione reductase; MSH, mycothiol; CoASH, coenzyme A; Grx, glutaredoxin; Trx, thioredoxin; TrxR, thioredoxin reductase; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; AP, alkaline phosphatase; DSR, disulfide reductase subgroup; tDBDF, two dinucleotide binding domains flavoproteins superfamily; PanK, pantothenate kinase; Pan, pantothenate; EcPanK, *E. coli* type I pantothenate kinase; SaPanK, *Staphylococcus aureus* type II pantothenate kinase; BsPanK, *Bacillus subtilis* type III pantothenate kinase; HpPanK, *Helicobacter pylori* type III pantothenate kinase; PaPanK, *P. aeruginosa* type III pantothenate kinase; TmPanK, *Thermatoga maritima* type III pantothenate kinase; MIC, minimal inhibitory concentration; CoADR, coenzyme A-disulfide reductase; CoAD, coenzyme A-disulfide; SACoADR, *S. aureus* coenzyme A-disulfide reductase; DPA, dipicolinic acid; CLE, cortex lytic enzymes; protein-SSCoA, soluble proteins S-thiolated with CoASH; Npx, NADH peroxidase; POR, NADH peroxidase/oxidase and CoAD reductase subgroup; Nox, NADH oxidase; Cys-SOH, cysteine-sulfenic acid; BACoADR, *B. anthracis* coenzyme A-disulfide reductase; CoADR-RHD, coenzyme A-disulfide reductase-rhodanese homology domain protein; RHD, rhodanese homology domain; BaPanK, *B. anthracis* type III pantothenate kinase; AMPPNP, 5′-adenylimidodiphosphate; N5-Pan, *N*-pentylpantothenamide; mBBr, monobromobimane; NEM, *N*-
ethylmaleimide; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; SeMet, selenomethionine; MAD, multiwavelength anomalous dispersion; TIGR, The Institute for Genomic Research; ASKHA, Acetate and Sugar Kinase/Hsc70/Actin; BHI, Brain Heart Infusion; LB, Luria-Bertani; IPTG, isopropyl-β-D-thiogalactopyranoside; RT, reverse transcriptase; COAXd, B. anthracis conditional mutant carrying the coaX::pNFd13 genotype; RACE, rapid amplification of cDNA ends; COAXd(Su), B. anthracis suppressor mutant carrying the coaX::pNFd13, constitutive Pspac promoter characteristics; PNDOR, pyridine nucleotide disulfide oxidoreductase; NCS, non-crystallographic symmetry; EH₂, two-electron reduced enzyme; EH₄, four-electron reduced enzyme; L-san, Lactobacillus sanfranciscensis; HMM, hidden Markov model;
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ABSTRACT

Coenzyme A (CoASH) replaces the common tripeptide thiol glutathione as the major low molecular-weight thiol in the human pathogen *Bacillus anthracis*. A novel type III pantothenate kinase (PanK) catalyzes the first committed step in the CoASH biosynthetic pathway in *B. anthracis*. The 2.0 Å crystal structure of the *B. anthracis* PanK (*BaPanK*) demonstrates that it is a new member of the Acetate and Sugar Kinase/Hsc70/Actin (ASKHA) superfamily and modeling the pantothenate and ATP substrates into the active-site cleft provides a clear rationale for the absence of CoASH feedback inhibition. In addition, bioinformatics analyses indicate a widespread distribution of type III PanK isoforms. The gene encoding *BaPanK*, *coaX*, is transcribed as part of a tricistronic operon including *hslO* and *cysK-1* loci, which encode the redox-regulated heat shock protein Hsp33 and cysteine synthase A, respectively. A conditional *coaX* mutant, in the absence of inducer, demonstrates exponential growth after a lag period of 8 h; this unanticipated result is due to a guanine to adenine suppressor mutation identified in the *lac* operator. Transcription of the tricistronic *coaX-hslO-cysK-1* mRNA is observed in the suppressor mutant, as is observed with wild-type *B. anthracis*. Therefore, these data support *BaPanK* as an essential enzyme, thus contributing to its validation as a new antimicrobial target.

The intracellular cellular thiol-disulfide redox status of *B. anthracis* is maintained by coenzyme A-disulfide reductase (BACoADR), which catalyzes the NAD(P)H-dependent reduction of coenzyme A-disulfide (CoAD) to 2 CoASH. *B. anthracis* also contains an additional CoADR isoform, coenzyme A-disulfide reductase-rhodanese homology domain (CoADR-RHD), which does not catalyze the reduction of CoAD and
is implicated in to function in sulfur metabolism. In order to test the physiological contributions of BACoADR and CoADR-RHD in thiol-disulfide redox homeostasis, as well as germination and outgrowth of the \textit{B. anthracis} endospore, in-frame deletion mutants of their respective encoding genes, \textit{cdr} and \textit{cdrX}, were constructed, singly and in combination. Both BACoADR and CoADR-RHD appear to have a role in the morphological transition from the endospore to the vegetative cell. However, CoADR-RHD appears to be the primary enzyme responsible for protection against diamide-induced disulfide stress. CoADRs represent unique members of the pyridine nucleotide disulfide oxidoreductase (PNDOR) family due to a single active-site cysteine, present as a stable Cys-SSCoA mixed disulfide. In order to distinguish CoADRs from the closely related NADH (per)oxidases and enable more accurate identification of CoADR proteins among its larger superfamily, hidden Markov model-based bioinformatics analyses have led to the development of CoADR-specific functional motifs.
CHAPTER I

INTRODUCTION: THIOL-DISULFIDE HOMEOSTASIS AND COENZYME A-DEPENDENT REDOX BIOLOGY IN THE PATHOGEN *Bacillus anthracis*
Pathogenesis of disease caused by *Bacillus anthracis*. Comparative genome hybridization analysis of *Bacillus anthracis* Ames and 19 *Bacillus cereus* and *Bacillus thuringiensis* strains demonstrated a high degree of chromosome identity between all three organisms (1). Additionally, comparisons of 16S rRNA sequences (2) or 16S-23S rRNA spacer regions (3) could not adequately distinguish between these organisms, supporting the taxonomic classification of the *Bacillus cereus* group, which includes *B. anthracis*, *B. cereus* and *B. thuringiensis* (4). Currently, there are 28 sequenced strains of the *B. cereus* group available for genome analysis (Table 1). While the chromosome diversity among members of the *B. cereus* group is minimal, variability exists within extra-chromosomal genetic elements (1), which are thought to provide the difference in disease manifestations [e.g., *B. anthracis* plasmids pXO1 and pXO2 (see below)]. *B. anthracis* is a mammalian pathogen and the causative agent of anthrax disease (5), *B. thuringiensis* is a biological insecticide (6), and *B. cereus* is referred to as an opportunistic pathogen (7). There are usually rare, nonfatal diseases associated with *B. cereus*, such as endophthalmitis following eye trauma and food poisoning (7). However, *B. cereus* G2941 has been implicated in a lethal infection similar in clinical presentation to *B. anthracis*, due to the expression of plasmid-encoded anthrax toxin genes (8).

*B. anthracis* is a Gram-positive, non-motile, aerobic rod that grows as long chains of vegetative cells and is capable of forming dormant endospores upon nutrient exhaustion. The *B. anthracis* endospore has no measurable metabolism, no ATP production, and represents the anthrax infectious contagion (9). Anthrax infection occurs in humans by three routes of exposure: cutaneous, gastrointestinal, and inhalational (5).
Table 1. Sequenced strains of the *B. cereus* group.

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Cutaneous anthrax is the most common naturally occurring form of anthrax disease; endospores enter through small skin lesions (Figure 1). There are an estimated 2000 cases of cutaneous anthrax reported annually worldwide (5), representing approximately 95% of all reported cases (16). Cutaneous anthrax is characterized by the formation of a small papule at the site of inoculation; after several days the papule ulcerates to form a distinctive painless black eschar. In most cases the disease is self-limiting and resolves without treatment. However, in a small subset (10-20%), cutaneous anthrax leads to systemic disease, which is almost always fatal. Gastrointestinal anthrax is the rarest, least common form and is typically only found in developing countries when *B. anthracis* gains access to the gastrointestinal tract via ingestion of contaminated food sources (5).

Due to the fact that gastrointestinal anthrax is difficult to diagnose, the mortality rate has been reported to be as high as 50%, even with treatment (16). Inhalational anthrax is the most serious form of the disease, in which endospores enter the alveolar spaces of the lung. The disease symptoms progress from a mild fever to extensive pleural effusion, mediastinal widening and septicemia/toxemia that is normally fatal within 24 hours (16). The mortality rate is very close to 100% because of the rapid progression to systemic infection, although it should be noted that because of heightened awareness and the consequent rapid response to and treatment of suspect cases, the mortality among the victims diagnosed with inhalational anthrax in the 2001 postal bioterrorism attacks was only 45% (16). In all three routes of acquisition, the systemic disease manifestation is due to the morphological transition of the *B. anthracis* endospore to the vegetative cell (termed germination). The encapsulated, replicating bacilli are protected from phagocytosis and release toxins, which ultimately lead to toxemia and septicemia (5).
Figure 1. Diagram highlighting the three major routes of human exposure to the *B. anthracis* endospore, leading to anthrax disease. Cutaneous anthrax is acquired when endospores enter through a skin abrasion (16). Gastrointestinal anthrax develops as a result of inadequate processing of food sources contaminated with endospores and/or vegetative cells (5). Inhalational anthrax occurs on inoculation of endospores into the airways to the lungs of a susceptible host (5).
The major pathogenic determinants of *B. anthracis* are encoded on two plasmids, both of which are required for virulence (1). The structural genes for the tripartite anthrax toxin components, protective antigen (PA), lethal factor (LF), and edema factor (EF), are present on pXO1 [185 kilobase-pairs (kb)]. The three toxin components combine to form two A-B type exotoxins, whereby the “B” (binding) moiety attaches to the target cell and facilitates entry of the “A” (catalytic or effector) moiety into the host cell cytoplasm (17). Anthrax toxin is composed of two effector proteins, LF and EF, and a single binding protein, PA, which is required for toxin entry (Figure 2). LF is translocated into the mammalian host cell, where it functions as a zinc-metalloprotease, inactivating mitogen-activated protein kinase kinase (MAPKK) via proteolytic cleavage (18). This results in the inhibition of cell signaling pathways, which slows the immune response. EF, a calmodulin-dependent adenylate cyclase, increases the intracellular concentration of cyclic AMP, leading to immunosuppression and clinical edema (18). A positive regulator of anthrax toxin production, AtxA, is also encoded on pXO1 (19). AtxA initiation of toxin production at 37°C and 5% CO₂, conditions provided by the human host (20). The second plasmid, pXO2 (95 kb) encodes genes responsible for the synthesis and degradation of the poly-D-glutamic acid capsule (21). The capsule prevents opsonization and phagocytosis of vegetative cells, largely by virtue of its negative charge (5). *B. anthracis* 34F₂, also known as the Sterne strain, lacks pXO2 and is used as an attenuated vaccine strain (11). The experiments described in this dissertation are implemented utilizing *B. anthracis* 34F₂ as the parental strain.

*B. anthracis* is a facultative anaerobe and, while the anthrax toxins (PA, LF, and EF) are critical to proper establishment and progression of the disease, they play no role
Figure 2. Mechanism(s) of action for *B. anthracis* toxins, lethal factor (LF) and edema factor (EF). Vegetative cells of *B. anthracis* express (encoded on pXO1) proteins for protective antigen (PA), LF and EF. PA interacts with the host cell surface through the anthrax toxin receptor (ATR). Once bound, PA is cleaved by the host cell protease furin, resulting in a 63-kDa form that oligomerizes into heptamers, exposing the LF and EF binding sites. The receptor, cleaved PA, and bound toxin are clustered onto lipid rafts and internalized via the clathrin-dependent endocytosis pathway. Toxins are then released through an endosomal pore where the action of calmodulin (CaM)-dependent EF ultimately results in edema, while LF inhibits signaling pathways via proteolytic cleavage of mitogen-activated protein kinase (MAPK) causing necrosis and hypoxia. This figure was adapted from ref (22) with permission of Future Medicine Ltd.
in protection of the vegetative cell from the toxic by products of oxygen metabolism that are produced during aerobic growth. Superoxide dismutase (SOD) catalyzes the conversion of superoxide anion (O$_2^\cdot$) to hydrogen peroxide and molecular oxygen. SOD thus acts as a scavenger of O$_2^\cdot$ and provides protection against this potential source of cellular damage (23). Therefore, in an effort to determine the role(s) of the four chromosomally-encoded superoxide dismutases (sod15, sodC, sodA1 and sodA2) in B. anthracis, mutant strains were generated and tested for growth under normal and oxidative stress conditions, both in vitro and in mouse infection models (23). The results of this analysis demonstrated that SODA1 is most important of the four B. anthracis SOD proteins for protection against O$_2^\cdot$ and is the predominant protective enzyme during aerobic growth. Spore inoculation with the ΔsodA1 and Δsod15 mutants in inhalational mouse models resulted in slight attenuation. Passalacqua et al. proposed that the basis for the decreased virulence observed could be attributed either to bacterial killing early in infection or to a lack of efficient bacterial metabolism. As such, this study provides a platform for the investigation of redox homeostasis as a potential factor in the virulence of B. anthracis. In addition to superoxide dismutase, B. anthracis encodes multiple antioxidant enzyme systems, such as catalases and peroxidases (1), underscoring the complex defensive strategies that this important pathogen has developed in order to cope with variably generated oxidative stresses. Although not well established in B. anthracis, thiol-disulfide redox homeostasis systems may provide another mechanism for protection against the potential harmful effects of oxidative environments. Therefore, the primary goals of this dissertation are to investigate thiol-disulfide redox systems in B. anthracis.
and to elucidate the mechanisms by which these systems contribute to the cell life cycle, from sporulation to germination, and to virulence.

**Cytoplasmic Thiol-Disulfide Redox Systems in *Escherichia coli***. Antioxidant defense is a major process required in all aerobic and/or aerotolerant organisms, as the accumulation of potentially toxic oxygen by products such as H$_2$O$_2$ and O$_2^-\,$ hinders normal cellular activities through damage to DNA, lipids, and proteins (24). The bacterial cytoplasm generally provides a strong reducing environment; the number of cytoplasmic proteins containing oxidized cystine (CysSSCys) disulfides in this steady state has been reported to be relatively small (25). The catalytic reduction of such protein disulfides [and of important low-molecular weight disulfides, e.g. oxidized glutathione (GSSG)] and reoxidation of the resulting thiols are mediated by thiol-disulfide oxidoreductase enzymes. Proper control of cytosolic redox status is not only integral to maintaining a reduced intracellular environment, but is also critical for protein function, regulation of both enzyme and DNA transcriptional activities, and for protection of important cysteine residues from overoxidation.

*Escherichia coli* serves as a model organism for studying the mechanisms of thiol-disulfide homeostasis, where protection against oxidative damage is in part facilitated by the major low-molecular weight thiol, in combination with specific disulfide oxidoreductases. The major low-molecular weight thiol in *E. coli* is glutathione (GSH; Figure 3), which is present at approximately 5 mM (26). GSH biosynthesis occurs in a two-step enzymatic process catalyzed by glutamate cysteine ligase (GCL) and GSH synthase (GS); in *Streptococcus agalactiae*, GSH is synthesized by a single bifunctional enzyme (27). As the principal thiol-disulfide redox buffer, the ratio of GSH/GSSG in the
Figure 3. Structures of the low-molecular weight thiols glutathione (GSH) (26), mycothiol (MSH) (29), and coenzyme A (CoASH) (30). The chemical constituents that make up each thiol are labeled in green. The 4′-phosphopantetheine and 3′,5′-ADP moieties of CoASH are denoted by a dashed line.
E. coli cytoplasm is >200 (28). Glutathione reductase (GR; encoded by gor) functions to maintain the high GSH/GSSG ratio by catalyzing the NADPH-dependent reduction of intracellular GSSG (Figure 4). GSH is in turn reoxidized in providing reducing equivalents for the glutaredoxins (Grx) that act on protein disulfides (31). There are three Grx isoforms in E. coli, Grx1 (encoded by grxA), Grx2 (encoded by grxB) and Grx3 (encoded by grxC). In addition to the GSH redox system, E. coli also contains the thioredoxin system that functions to maintain thiol-disulfide homeostasis through complementary and sometimes overlapping roles (28). Thioredoxin (Trx) is ubiquitous; it is conserved in all organisms from archaea to humans (25). The two Trx isoforms in E. coli are Trx1 and Trx2, encoded by trxA and trxC, respectively. Thioredoxin reductase (TrxR; encoded by trxB), which is unable to reduce any of the Grx proteins (32), catalyzes the NADPH-dependent reduction of the Trx protein disulfide.

Investigations of the roles of Trx and GSH-dependent redox systems in E. coli demonstrate contributions from both systems in formation and the reduction of disulfides that are essential for enzyme activity as part of their catalytic mechanism (Figure 4) (33). For example, both Trx and Grx serve as external electron donors for the reduction of ribonucleotide reductase, which generates the deoxyribonucleotides required for DNA synthesis (34). Reduction of both 3′-phosphoadenosine-5′-phosphosulfate (PAPS) reductase (35) and methionine sulfoxide reductase (36) has also been demonstrated to occur via Trx and Grx enzymes. As an additional example, hydrogen peroxide-mediated oxidative stress leads to the activation of the OxyR transcription factor (37), via the formation of an intramolecular disulfide (38) (Figure 4). OxyR has been demonstrated to induce the expression of a number of genes (the oxyR regulon), including both gor and
Figure 4. Cytoplasmic thiol-disulfide homeostasis in *E. coli*. A) There are two major intracellular redox systems in *E. coli* that function synergistically to maintain a primarily reducing environment. NADPH serves as the reducing substrate for both glutathione reductase (GR) and thioredoxin reductase (TrxR). GSH is the principal thiol-disulfide redox buffer, providing reducing equivalents to Grx enzymes and being regenerated by GR. TrxR functions to reduce Trx. B) Grx and Trx act directly to reduce intramolecular and protein-SSG disulfides that accumulate during oxidative stress in *E. coli*.
Grx1 functions in this autoregulatory circuit to reduce the OxyR disulfide leading to its inactivation.

Protein S-thiolation, or the formation of mixed disulfides between protein-SH and low-molecular weight thiols under oxidizing conditions, is a reversible mechanism thought to protect proteins from irreversible thiol oxidation (to Cys-sulfenic and/or sulfonic acid states) and to modulate protein function. In *E. coli*, protein S-glutathionylation can occur via (i) direct interaction between cysteine-sulfenic acid and GSH and (ii) thiol-disulfide exchange between protein thiols and GSSG (39). The removal of the GSH moiety from the resulting protein-SSG disulfide is facilitated by both Trx and Grx proteins (Figure 4), which are also S-glutathionylated in response to oxidative stress (39).

In an effort to analyze the role of the thiol-disulfide oxidoreductase systems in the *E. coli* cytoplasm, Prinz *et al.* assessed intracellular disulfide bond formation in various mutants that lack components of both the TrxR-Trx and GR-GSH-Grx systems, either singly or in combination (28). The cytoplasmic oxidative state was measured by the enzymatic activity of an alkaline phosphatase (AP) variant that contained an N-terminal deletion to remove the periplasmic translocation signal peptide and therefore, remains in the cytoplasm. In summary, the mutants analyzed grow normally under nutrient rich conditions but exhibit increased sensitivity during either aerobic growth in minimal medium or in the presence of the thiol-specific oxidant diamide (28). Mutants that lack either GSH or GR in combination with one of the proteins of the Trx system allow a substantial amount of disulfide bond formation in the cytoplasm when grown in minimal medium; this is similar to the level of active, oxidized AP observed in the *trxB* deletion
mutant under the same conditions (Table 2). In general, the effect of diamide on the mutant strains demonstrates a strong correlation; mutants with increased cytosolic AP activity, reflecting a lower capacity to reduce cytoplasmic disulfide bonds, are also characterized by diamide-induced growth inhibition. Overall, Prinz et al. concluded that eliminating certain combinations of components within the TrxR-Trx and GR-GSH-Grx systems results in high levels of disulfide bond formation (28). In contrast, deletion of the gene encoding the single Trx in Bacillus subtilis is lethal (40), perhaps highlighting differences in thiol-disulfide homeostasis systems among Gram-negative and Gram-positive bacteria.

**Major Low-molecular Weight Thiols Function in Roles Analogous to GSH.** GSH metabolism is thought to have evolved as a protective mechanism against the adverse effects of oxygen in early prokaryotic organisms (41). However, most of the bacteria that lack GSH rely on other “novel” low-molecular weight thiols that function in analogous roles (42). Novel thiols are defined as low-molecular weight thiols that are present at substantial levels in some, but not all organisms. For example, GSH is absent in a number of actinomycetes, which have been demonstrated to produce mycothiol (MSH; Figure 3) as the principle thiol-disulfide redox buffer (29). MSH biochemistry is of importance to bacterial metabolism because the actinomycetes include roughly one-half of the known bacterial species identified in soil (42). Furthermore, MSH levels are especially high in mycobacteria, including the human pathogen *Mycobacterium tuberculosis*. This organism is able to defend against the oxidative burst used by host macrophages to kill most bacteria, and it has been suggested that MSH may play a direct role in its ability to
Table 2. Summary of AP activities observed in various *E. coli* mutants grown aerobically in minimal medium (28).

<table>
<thead>
<tr>
<th>Relevant genotype</th>
<th>AP Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>90</td>
</tr>
<tr>
<td><em>trx</em>A</td>
<td>130</td>
</tr>
<tr>
<td><em>trx</em>B <em>trx</em>A <em>grxC</em></td>
<td>140</td>
</tr>
<tr>
<td><em>trx</em>A <em>grxA</em> <em>grxC</em></td>
<td>200</td>
</tr>
<tr>
<td><em>trx</em>B <em>trx</em>A <em>grxA</em></td>
<td>230</td>
</tr>
<tr>
<td><em>gor</em></td>
<td>260</td>
</tr>
<tr>
<td><em>grxC</em></td>
<td>290</td>
</tr>
<tr>
<td><em>trx</em>B <em>trx</em>A <em>grxA</em> <em>grxC</em></td>
<td>290</td>
</tr>
<tr>
<td><em>trx</em>B <em>trx</em>A</td>
<td>310</td>
</tr>
<tr>
<td><em>trx</em>B <em>grxA</em></td>
<td>420</td>
</tr>
<tr>
<td><em>trx</em>A <em>gsh</em>A</td>
<td>760</td>
</tr>
<tr>
<td><em>trx</em>B <em>grxC</em></td>
<td>860</td>
</tr>
<tr>
<td><em>trx</em>A <em>gor</em></td>
<td>860</td>
</tr>
<tr>
<td><em>trx</em>B</td>
<td>870</td>
</tr>
</tbody>
</table>

*The ability of disulfide bonds to form in the cytoplasm of these strains was assessed by determining the extent to which *E. coli* alkaline phosphatase (AP) is able to form disulfide bonds in the cytoplasm of the mutants. AP is a periplasmic homodimeric enzyme that contains two intrachain disulfide bonds in each monomer. These bonds are required for AP to be enzymatically active. AP is synthesized with an N-terminal signal sequence, which targets it for export to the periplasm. When AP is expressed with a defective or missing signal sequence, it is not exported to the periplasm, but remains in the cytoplasm. In this compartment, AP does not form disulfide bonds and cannot fold into an enzymatically active conformation. However, in the cytoplasm of *E. coli* mutants missing thioredoxin reductase a fraction of a signal sequenceless version of AP (ΔAP2-22) does forms disulfide bonds and folds into an active conformation. Thus, ΔAP2-22 can be used to assess the potential for disulfide bond formation in the cytoplasm of the mutants missing components of the thioredoxin and glutaredoxin systems.\(^{\text{a}}\)*
survive and grow within the macrophage (29). MSH homeostasis is maintained by the NADPH-dependent mycothione reductase (43), which like GR is a member of the disulfide reductase (DSR) subgroup of the “two dinucleotide binding domains” flavoproteins (tDBDF) superfamily (44).

In contrast to utilizing novel thiols to replace GSH, some organisms take advantage of common thiols, those that are present in all organisms, to function in this capacity (42). In fact, B. subtilis, B. cereus, Bacillus megaterium, Deinococcus radiodurans, Staphylococcus aureus, and Staphylococcus epidermidis all were demonstrated to lack GSH and to contain coenzyme A (CoASH; Figure 3) or its precursors as the major low-molecular weight thiol (30, 41, 45-47). This discovery supports an expanded role for CoASH and its precursors in thiol-disulfide homeostasis in these representative Gram-positive organisms.

Consistent with the evidence for protein S-glutathionylation described previously for E. coli, CoASH and its precursors have been demonstrated to function similarly. In B. subtilis, the OhrR transcription factor has a complex thiolate switch for regulation (48). Studies have shown that the sole cysteine in OhrR (Cys15) is oxidized to Cys-SOH; the Cys15-SOH intermediate, in the presence of low-molecular weight thiols, generates the corresponding OhrR-mixed disulfides. Formation of these OhrRCys15-SSR forms is sufficient to promote dissociation from the ohr operator. The Cys15 modifications also protect against overoxidation, and provide redox-regulated pathway(s) for regeneration of the active repressor involving disulfide reduction. Recovery of OhrR disulfide forms from B. subtilis identifies three distinct Cys15-SSR species, including the respective mixed disulfides with a novel 398-Da thiol [now identified as bacillithiol, N(L-cysteinyl)-
D-glucosamine linked via glycosidic bond to malate-2-OH, cysteine, and CoASH. In addition, S-cysteinylation has been reported as a general mechanism for thiol protection of *B. subtilis* proteins during disulfide stress (49). Utilizing an [\[^{35}\text{S}\]cysteine labeling approach, in the presence of chloramphenicol to inhibit protein synthesis, cells treated with diamide showed an approximate 6-fold increase in the amount of [\[^{35}\text{S}\]cysteine incorporated into protein-SSCys disulfides when compared to control cells. Treatment of the \[^{35}\text{S}\]-labeled protein extracts with disulfide-reducing agents resulted in an 81% loss of label, indicating that most of the \[^{35}\text{S}\]-protein labeling involves mixed protein-SSCys disulfides.

**Coenzyme A Biosynthesis and Pantothenate Kinase.** CoASH is a ubiquitous and essential cofactor that functions as the major acyl group carrier in the intermediary metabolism of all living systems (50). It is synthesized in a five-step pathway from pantothenate. The enzyme pantothenate kinase (PanK), which catalyzes the ATP-dependent phosphorylation of pantothenate (PAN) to 4’-phosphopantothenate, is responsible for the first committed step in CoASH biosynthesis (50).

Currently, three bacterial PanK isoforms have been characterized (types I, II and III); these exhibit very limited conservation of primary structure (~15% sequence identity between respective types), and differ in substrate affinities, regulation, and three-dimensional structures (Figure 5). The type I PanK has been most extensively studied in *E. coli*; *EcPanK* is encoded by *coaA* (51). Kinetic analysis supports an ordered sequential mechanism with ATP binding before PAN; \(K_m\) values are 136 µM and ~36 µM for the ATP and PAN substrates, respectively (52). *EcPanK* is stringently controlled by CoASH
E. coli type I PanK

S. aureus type II PanK

B. anthracis type III PanK
Figure 5. Crystal structures for the three bacterial pantothenate kinase types. All three isoforms are dimeric; however, the overall structures are very distinct. The *E. coli* type I PanK (PDB entry: 1ESM) is a member of the P-loop kinase superfamily (54). While both the *S. aureus* type II PanK (PDB entry: 2EWS) and the *B. anthracis* type III PanK (PDB entry: 2H3G) are members of the Acetate and Sugar Kinase/Hsc70/Actin structural superfamily, with similar structural folds for the respective monomers, the dimerization domains are different, providing distinct catalytic properties (55, 56).
as a competitive feedback inhibitor \( (K_i = 4.3 \ \mu M) \) with respect to ATP \((53)\). This feedback regulation of CoASH biosynthesis in \textit{E. coli} provides the cell with a mechanism for controlling the intracellular concentration of free CoASH.

The \textit{S. aureus} PanK (\textit{Sa}PanK) is the prototypical type II enzyme; its primary structure, while distinct from type I and III isoforms, is more similar to the eukaryotic and human PanKs \((55)\). However, unlike \textit{EcPanK} and the human PanK enzymes, \textit{Sa}PanK is refractory to feedback inhibition by CoASH \((57)\). A comparison of the \textit{Sa}PanK structure with that of human PANK3 in complex with the inhibitor acetyl-CoA demonstrates that residues Ala337 and Trp340 of human PANK3 allow for favorable interaction and binding of acetyl-CoA \((58)\). These residues are replaced by Tyr240 and Arg244 in \textit{Sa}PanK, disrupting the hydrophobic pocket necessary for CoASH binding and thereby providing the difference in regulation by CoASH and/or acetyl-CoA \((58)\).

Utilizing an \textit{E. coli} temperature-sensitive mutant defective in PanK activity, the type III PanK was first identified in \textit{B. subtilis}; the \textit{B. subtilis} coaA (type I) and coaX (type III) genes were independently cloned \textit{in trans} and effectively restored growth to the \textit{E. coli} coaA(Ts)mutant \((59)\). Additionally, the \textit{B. subtilis} coaA mutant displayed a normal growth phenotype, while a double mutant defective in both coaA and coaX genes was not viable. Therefore, Yocum and Patterson concluded that \textit{B. subtilis} contains two genes that encode PanK activity \((59)\). The type III PanKs, \textit{B. subtilis} (\textit{Bs}PanK) and \textit{Helicobacter pylori} (\textit{Hp}PanK), are insensitive to feedback inhibition by CoASH \((60)\), as observed with \textit{Sa}PanK. Crystal structures of the type III PanKs from \textit{Pseudomonas aeruginosa} (\textit{Pa}PanK) \((55)\) and \textit{Thermotoga maritima} (\textit{Tm}PanK) \((61, 62)\) demonstrate that the lack of CoASH feedback inhibition is due to the presence of loop a or “Pan cap,” which
becomes ordered upon binding of PAN and thus blocks accommodation of the β-
aminoethanothiol moiety of CoASH.

Due to the central metabolic role played by CoASH, its biosynthesis has been identified as a prime target for the development of novel broad-spectrum antimicrobial agents (63, 64). In particular, the pantothenate kinase step is an attractive therapeutic target, as the type I and type III PanKs exhibit very limited sequence homology to the human enzyme (65). The N-alkylpantothenamides are a series of compounds first demonstrated to inhibit growth of *E. coli* with minimal inhibitory concentrations (MIC) ranging between 0.6-200 µg/ml (66). Specifically, N-pentylpantothenamide acts as an alternate substrate for both *EcPanK* and *SaPanK* enzymes; the phosphorylated product continues through the CoASH biosynthetic pathway, ultimately resulting in inactive holoacyl carrier proteins containing 4′-phospho(pentyldethia) pantetheine and causing cessation of fatty acid synthesis (57, 67-69). However, in contrast to *EcPanK* and *SaPanK* enzymes, *BsPanK* and *HpPanK* demonstrate no activity with this alternate substrate, at concentrations as high as 0.5 mM, indicating that these compounds are not recognized by type III PanKs. Moreover, the *TmPanK* (61, 62), *PaPanK* (55), and *B. anthracis* PanK (chapter II of this dissertation) provide evidence that the Pan cap also prevents the binding of *N*-alkylpantothenamides. Thus, the structural basis explaining the absence of CoASH feedback inhibition in the type III PanKs also accounts for the failure to recognize alternate pantothenamide substrates.

**Coenzyme A-Dependent Thiol-Disulfide Homeostasis.** CoASH appears to represent an essential component of intracellular thiol-disulfide homeostasis in those organisms where
it serves as the major low-molecular weight thiol, and coenzyme A-disulfide reductase (CoADR) is responsible for maintaining high cytosolic CoASH/coenzyme A-disulfide (CoAD) ratios. This has been demonstrated to be the case in the human pathogens *S. aureus* (70) and *Borrelia burgdorferi* (45). The *S. aureus* enzyme (SACoADR) utilizes NADPH as a reducing substrate to reduce CoAD, providing an intracellular ratio of CoASH/CoAD of ~450 (70). While the details of the intracellular redox environment during *S. aureus* infection are unknown, SACoADR has been implicated in contributing to virulence, suggesting that the [CoASH]/[CoAD] redox status is an important factor.

Signature-tagged mutagenesis, which utilizes transposons tagged with unique oligonucleotides, was employed to identify genes impacting the growth and survival of *S. aureus* in murine systemic and abscess models (71). A second independent study implemented differential fluorescence induction technology to identify *S. aureus* virulence factors (72). In both cases, mice challenged with CoADR-deficient strains of *S. aureus* demonstrated strong virulence attenuation. However, an additional report analyzing genetic loci by screening for loss-of-function mutants in a *Caenorhabditis elegans* killing assay failed to demonstrate a role for CoADR (73). While CoADR was not identified in this latter study, this result stands to emphasize that no single infection model is a good predictor for identifying genetic loci that are universally important for virulence and for maintaining infection. Furthermore, given that *S. aureus* can infect and persist in many different host environments, these analyses illustrate the complexity of gene expression during *S. aureus* infection.
Roles of CoASH and CoADR in Sporulation and Germination. Endospore formation is a characteristic of species from the Bacillus and Clostridium genera that has most likely evolved as a method that provides resistance to many severe environmental stresses (e.g., heat, radiation, dehydration and chemical exposure); this mechanism involves a form of cellular differentiation that leads to the metabolically dormant endospore (74). This morphological change and the underlying development program (Stages 0-VII) have been extensively studied in B. subtilis (75). Briefly, vegetative growth is defined as stage 0 (Figure 6). During stages 0-II, sporulating cells synthesize a number of enzymes (mainly extracellular), including α-amylase, proteases, and nucleases. Stage II comprises the formation of a septum at one pole of the cell, resulting in distinct compartments, each with its own chromosome. The larger compartment is termed the mother cell or sporangium, while the smaller compartment is termed the forespore. As stage II proceeds, the forespore compartment is engulfed by the mother cell, resulting in stage III. In stage IV, peptidoglycan is deposited between the forespore outer membrane to create the germ cell wall and cortex. Spore coat proteins are deposited on the outside of the developing spore in stage V. Finally, during stage VI the spore matures to develop full heat resistance, and in stage VII the mother cell lyses, releasing the mature endospore (75).

Endospore germination can be induced by sensing environmental signals (nutrient-triggered germination) or by the degradation of protective spore structures (non-nutrient-triggered germination) (74). The latter is considered a default mechanism that allows for an attempt at vegetative growth when an endospore experiences damage that may affect its survival. In contrast, nutrient-triggered germination is considered the process by which endospores sense conditions conducive for vegetative growth. During
Figure 6. Successive morphological stages of sporulation. Starting with the vegetative cell (Stage 0), sporulation proceeds in a step-wise process to result in release of a dormant endospore (Stage VII) (75).
germination, endospores initiate a cascade of events leading to the return of a vegetative morphology (Figure 7); this process occurs in two distinct phases: germination and outgrowth (74). Germination in turn is a two-stage biophysical and biochemical process in which the spore core is first hydrated, followed by a release of calcium and dipicolinic acid in stage one (biophysical). The degradation of the cortex then follows sequentially in stage two (biochemical). The resumption of metabolic activity, including the new synthesis of vegetative cell components, is considered outgrowth.

As stated previously, CoASH is the major low-molecular weight thiol in *B. megaterium*; in mature endospores 25% of the total CoASH is present as CoAD, 43% is in protein-SSCoA disulfides (soluble proteins *S*-thiolated with CoASH) and the remaining 32% is present as the free thiol (47). These protein-SSCoA disulfides were shown to accumulate during sporulation and were rapidly reduced early in spore germination. Reduction of at least 75% of the protein-SSCoA (to protein-SH and CoASH) occurs within the first minutes of spore germination. Two proposed functions for the accumulation of protein-SSCoA during sporulation are: 1) maintaining the metabolic dormancy of the spore via reversible inactivation (redox regulation) of key Cys-dependent spore enzymes, and 2) contributing to the heat and radiation resistance of the spore. The formation and reduction of these protein-SSCoA disulfides also correlates with the availability of reduced pyridine nucleotides. In the endospore, there is no measurable concentration of NAD(P)H; however, during the first few minutes of germination there is an observed increase (76). These analyses are consistent with independent analyses in *B. cereus* and *Clostridium bifermentans* (47).
Figure 7. Germination and outgrowth of dormant endospores. The endospore is composed of an inner core, germ cell wall, cortex and coat layer. Once stimulated, germination proceeds in a two-step process (74). First, the biophysical stage is characterized by the release of calcium, dipicolinic acid (DPA), core hydration, and ion exchange. The biochemical or second stage of germination is the degradation of the cortex layer by cortex lytic enzymes (CLE) and core expansion. The morphological transition of the endospore to the vegetative cell culminates with outgrowth, which includes the onset of metabolic activity (74).
The enzyme most likely responsible for the reduction of these protein-SSCoA disulfides during germination in *B. megaterium* was identified as an NADH-dependent flavoprotein disulfide reductase specific (in *in vitro* assays) for disulfide substrates containing pantethine-4′-4″-diphosphate (77, 78). As measured in cell-free extracts, the enzyme was present at low levels (specific activity, units per mg protein) in vegetative cells, and a 10-fold increase in specific activity was observed during sporulation. Analysis of the draft sequence generated from the 10X coverage of the *B. megaterium* chromosome\(^1\) has identified an NADH-dependent CoADR (*E*-value 5e\(^{-75}\)) with 33% sequence identity to SACoADR. Thus, *B. megaterium* CoADR is considered responsible for the direct or indirect reduction of both CoAD and protein-SSCoA disulfides during endospore germination.

**CoADR is a member of the “Two Dinucleotide Binding Domain” Flavoprotein (tDBDF) Superfamily.** Functionally-characterized members of the tDBDF superfamily catalyze oxidoreductase reactions utilizing an FAD coenzyme and a pyridine nucleotide [NAD(P)^+ or NAD(P)H] substrate. As recently described by Ojha *et al.*, the tDBDF superfamily can be categorized into nine distinct subgroups; however, sequence and structural analyses suggest an evolutionary relationship among all subgroups based on cofactor-imposed constraints (44). Although each subgroup exhibits limited homology with the others, most members of the tDBDF superfamily maintain conserved sequence motifs associated with FAD and NAD(P)^+/NAD(P)H binding. These include two GxGxxG dinucleotide binding motifs and the ATG, GxxP and GD motifs (79, 80).

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\(^1\) [www.bios.niu.edu/b_megaterium/index.html](http://www.bios.niu.edu/b_megaterium/index.html)
Specific to this work, the NADH peroxidase (Npx), NADH oxidase (Nox), and coenzyme A-disulfide reductase (POR) subgroup of enzymes differs from all other tDBDF subgroups based on the presence of a single redox-active cysteinyl derivative. Nox and Npx enzymes both contain a stable Cys-SOH redox center and catalyze the NADH-dependent reduction of molecular oxygen and hydrogen peroxide, respectively (81, 82). In contrast, CoADR contains a Cys-SSCoA redox-active disulfide (identical to the soluble protein-SSCoA linkage identified in B. megaterium spores) and is the only flavoprotein known to carry out reduction of a low-molecular weight disulfide substrate with a single catalytically essential cysteine (83).

**Identification of CoADR homologs in Bacillus anthracis.** Based on the recent crystal structure of SACoADR (84), the covalently-bound CoAS- yielded a set of sequence motifs useful in identifying other members of the CoADR family. As such, utilizing SACoADR as a query to search microbial genomes in the NCBI non-redundant database led to the identification of two CoADR isoforms in B. anthracis Ames. The B. anthracis CoADR (BACoADR, BA1263) maintains the same domain organization as SACoADR with 33% sequence identity (E-value 7e-69). However, in addition to the conserved CoADR module, the second isoform, coenzyme A-disulfide reductase-rhodanese homology domain protein (CoADR-RHD, BA0774), contains a 106-residue C-terminal RHD extension. The CoADR module of CoADR-RHD is 37% identical to SACoADR (E-value 3e-81). Transcriptional profiling of the B. anthracis life-cycle indicated that 36% of the genome is regulated in a growth-phase dependent manner (85); the regulated genes were clustered into five distinct temporal waves correlated with the physiological state of
the cell. The genes encoding BACoADR and CoADR-RHD are up-regulated during waves III and V, respectively, and the BACoADR protein was identified in the spore by proteomics methods (85, 86). In an independent study, BACoADR was also identified in the cytoplasmic proteome of B. anthracis UM23C1-2 during exponential growth in Luria-Bertani (LB) broth (87). These observations support the hypothesis that BACoADR is being packaged in the dormant spore, in anticipation of a substrate-regulated (availability of NAD(P)H, CoAD, and possibly protein-SSCoA) reduction; following germination and outgrowth the enzyme also maintains the reduced state of CoASH in the vegetative cell.

The C-terminal domain of CoADR-RHD represents a “catalytic” rhodanese domain; thus, CoADR-RHD is a new member of the “multidomain proteins-Group III” of the rhodanese superfamily (88) and the first disulfide reductase linked to an RHD. As characterized, the active-site Cys of rhodanese forms a persulfide intermediate on reaction with a sulfane sulfur (S°) donor; the distal sulfur is then transferred to a nucleophilic acceptor (89). In vitro assays of CoADR-RHD indicate that it does not catalyze the reduction of CoAD, and this is further supported by the crystal structure, which shows that the RHD prevents access to the CoADR active site by a molecule as large as CoAD2. Recent analysis identified a CoADR-RHD, from Shewanella loihica PV-4, which has been further characterized as an NADH-dependent, coenzyme A-activated polysulfide reductase (Npsr) (90). In addition, the Pelobacter carbinolicus CoADR-RHD gene is upregulated five-fold during sulfur-dependent growth (91). Therefore, based on

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these observations, it is likely that CoADR-RHD in *B. anthracis* functions in sulfur reduction; however, the physiological donor and acceptor of sulfur has yet to be elucidated.

**Scope of Study.** Within this dissertation, a multidisciplinary approach utilizing biochemical, genetics, and bioinformatics techniques has been implemented to better define CoASH-dependent redox biology, using *B. anthracis* as a model organism. A recent comprehensive bioinformatics survey of the phylogenetic distribution of type I, II, and III PanKs reveals that type III PanKs are present in 12 of the 13 major bacterial groups (61). This widespread distribution of type III PanKs, combined with their very distinct structural relationship with the human enzyme and the attractiveness of enzymes in the CoASH biosynthetic pathway as targets for antimicrobial development, underscores its significance. These analyses led to the identification of type II and type III PanK sequences in *B. anthracis*. Chapter II provides biochemical evidence demonstrating that the type II homolog in *B. anthracis* is not a functional PanK and that the type III PanK (*BaPanK*) is the only enzyme responsible for the phosphorylation of pantothenate in CoASH biosynthesis. Chapter II also describes the crystal structure of the *BaPanK*, establishes the presence of CoASH as the major low-molecular weight thiol in *B. anthracis*, and gives a summary of the distribution of CoASH-dependent organisms based on the absence of GSH biosynthetic genes, the presence of a type II or III PanK, and the presence of a CoADR protein.

As previously mentioned, CoASH has an essential role in the intermediary metabolism of all organisms, and enzymes involved in CoASH biosynthesis have been
described as targets for the development of new antimicrobials (64). Chapter II demonstrates that BaPanK serves as the sole enzyme responsible for catalyzing the first committed step for CoASH biosynthesis in B. anthracis. Therefore, deletion of the coaX gene that encodes BaPanK should be lethal. Chapter III identifies coaX as the first gene of a tricistronic operon including hslO, which encodes the redox-regulated chaperone Hsp33, and cysK-1, which encodes cysteine synthase. A conditional mutant in which coaX was cloned downstream of an inducible promoter provides the basis for establishing that BaPanK is essential for growth in B. anthracis and thus, a valid target for drug design.

Coenzyme A-dependent redox biology is operationally defined by the absence of GSH-biosynthetic genes, the presence of a type II or III PanK and the presence of a CoADR protein. As such, B. anthracis fits these criteria for utilization of CoASH in thiol-disulfide homeostasis. The mechanism by which the reduced states of CoASH in vegetative cells and perhaps, in germinating spores, are maintained has been suggested to involve the newly identified BACoADR. Sequence analyses of BACoADR reveal an ambiguous conservation of residues within the NAD(P)H-binding motif of this tDBDF POR subgroup enzyme. In contrast with the canonical preference for NADH versus NADPH, chapter IV describes experiments demonstrating a conformational change in BACoADR that supports the dual specificity observed with BACoADR. Additionally, comparison of SACoADR and BACoADR active sites display consistencies among residues important for the recognition of CoASH; however, the interactions with the CoASH moiety are different due to altered binding modes.
CoADR is the newest member of the POR subgroup within the tDBDF protein superfamily (44). Due to the sequence similarity between CoADR and Npx/Nox enzymes, CoADR sequences are commonly misannotated. The structures of SACoADR and BACoADR provide a basis for generating CoADR-specific motifs that allow for accurate differentiation from Npx/Nox sequences; ultimately, such a set of motifs will aid in the identification of organisms that utilize CoASH-dependent redox biology. Chapter V describes the bioinformatics approach used to determine the CoADR-specific motifs and evaluate the phylogenetic distribution of CoADR protein homologs. CoADR enzymes have been demonstrated to function in redox control during vegetative growth, and in the redox-mediated process of endospore germination and outgrowth. Also, the CoADR-RHD isoform has been linked to contribute to sulfur trafficking. Therefore, chapter V also provides the evidence for physiological characterization of BACoADR and CoADR-RHD isoforms through construction of B. anthracis strains lacking BACoADR and CoADR-RHD, either singly or in combination. These mutant phenotypes have been examined in germination and outgrowth and in cultures treated with the thiol-specific oxidant diamide.
REFERENCES


CHAPTER II

STRUCTURE OF THE TYPE III PANTOTHENATE KINASE FROM Bacillus anthracis AT 2.0 Å RESOLUTION: IMPLICATIONS FOR COENZYME A-DEPENDENT REDOX BIOLOGY

Nathan I. Nicely, Derek Parsonage, Carleitta Paige, Gerald L. Newton, Robert C. Fahey, Roberta Leonardi, Suzanne Jackowski, T. Conn Mallett, and Al Claiborne

The following manuscript is reproduced with permission, Biochemistry, volume 46, pages 3234-3245, copyright 2007 American Chemical Society. Stylistic variations are due to the requirements of the journal. Nicely solved the BaPanK crystal structure and performed kinetic assays with BaPanK and BA2901. Parsonage cloned genes for BaPanK and BA2901 into pET28a plasmid and purified both proteins. Paige was responsible for bioinformatics analyses performed utilizing the TIGR-Comprehensive Microbial Resource to identify bacteria that contain both, CoASH-refractory PanKs and CoADR-like proteins. Newton and Fahey performed all experiments for the identification of low molecular-weight thiols. Leonardi and Jackowski analyzed 30 phylogenetic classes of bacteria for CoASH-dependent redox markers utilizing the Integrated Microbial Genomes resource. Mallet provided oversight during X-ray diffraction data collection and structure refinement. Claiborne acted in an editorial capacity.
ABSTRACT

Coenzyme A (CoASH) is the major low-molecular weight thiol in *Staphylococcus aureus* and a number of other bacteria; the crystal structure of the *S. aureus* coenzyme A-disulfide reductase (CoADR), which maintains the reduced intracellular state of CoASH, has recently been reported [Mallett, T.C., Wallen, J.R., Karplus, P.A., Sakai, H., Tsukihara, T., and Claiborne, A. (2006) *Biochemistry* 45, 11278-11289]. In this report we demonstrate that CoASH is the major thiol in *Bacillus anthracis*; a bioinformatics analysis indicates that three of the four proteins responsible for the conversion of pantothenate (Pan) to CoASH in *Escherichia coli* are conserved in *B. anthracis*. In contrast, a novel type III pantothenate kinase (PanK) catalyzes the first committed step in the biosynthetic pathway in *B. anthracis*; unlike the *E. coli* type I PanK, this enzyme is not subject to feedback inhibition by CoASH. The crystal structure of *B. anthracis* PanK (*BaPanK*), solved using multiwavelength anomalous dispersion data and refined at a resolution of 2.0 Å, demonstrates that *BaPanK* is a new member of the Acetate and Sugar Kinase/Hsc70/Actin (ASKHA) superfamily. The Pan and ATP substrates have been modeled into the active-site cleft; in addition to providing a clear rationale for the absence of CoASH inhibition, analysis of the Pan-binding pocket has led to the development of two new structure-based motifs (the PAN and INTERFACE motifs). Our analyses also suggest that the type III PanK in the spore-forming *B. anthracis* plays an essential role in the novel thiol/disulfide redox biology of this category A biodefense pathogen.
INTRODUCTION

CoASH has been classified as a “common” thiol (1), as it occurs in all prokaryotes as well as eukaryotes. Although GSH plays the central role in maintaining thiol/disulfide homeostasis and providing an important line of antioxidant defense in, for example, *Escherichia coli* (2), GSH is clearly absent in a number of bacteria (3, 4) as well as all archaea (5, 6). CoASH has been shown to be the major low-molecular weight thiol in the human pathogens *Staphylococcus aureus* (7) and *Borrelia burgdorferi* (8), in the spore-forming *Bacillus megaterium* (4) and the radiation-resistant *Deinococcus radiodurans* (3), and in the hyperthermophile *Pyrococcus furiosus* (6) and other archaea. Coenzyme A-disulfide reductases (CoADR) have been characterized in most of these prokaryotes (4, 7-9), reinforcing the concept that CoASH assumes the intracellular redox function of GSH in these organisms, and the crystal structure of the *S. aureus* CoADR has recently been reported (10).

In *E. coli*, pantothenate kinase (CoaA, or type I PanK)\(^1\) is the key regulatory enzyme in CoASH biosynthesis (Scheme 1); kinetic analysis supports an ordered sequential mechanism, with ATP binding first (11, 12). CoASH is a competitive feedback inhibitor (\(K_i = 4.3 \mu M\)) with respect to ATP (13), and crystal structures are

\(^1\) The bacterial and eukaryotic pantothenate kinases have traditionally been distinguished as the CoaA and PanK proteins, respectively. With the identification of two distinct new bacterial pantothenate kinase classes in 2005, different investigators have used either type I, -II, and -III CoaAs (e.g., type II *Staphylococcus aureus* CoaA) or PanK-I, -II, and -III nomenclature to identify the three bacterial enzyme classes. In this report we refer to the three bacterial enzyme classes as bacterial type I, type II, and type III PanKs; CoaA is synonymous with the type I PanK.
Scheme 1. Pathway for CoASH Biosynthesis in *E. coli*.
available for the $Ec$PanK complexes with CoASH and with the non-hydrolyzable ATP analog AMPPNP, respectively (12), as well as for the ternary complex with ADP and Pan (14). There are now three types of bacterial PanK enzymes; the type I PanK found in $E. coli$ is a P-loop kinase [kinase family and fold group 2 (15)], and Pan binding leads to a significant conformational change, resulting in closure of a “lid” over the bound substrate (14). $Ec$PanK effectively phosphorylates the Pan antimetabolites (alternate substrates) $N$-penty1- and $N$-heptylpantothenamide; $K_m$ values are only three-fold higher than that of the natural substrate (14). The phosphorylated products are carried through the CoASH biosynthetic pathway, resulting in the accumulation of inactive holo-acyl carrier proteins (16). Modeling studies indicate that the pantothalamides can orient into the Pan binding site, with their extended alkyl chains interacting with a hydrophobic “dome” over the substrate pocket.

In 2005, the bacterial type II and type III PanKs were identified in $S. aureus$ (17), and in $Helicobacter pylori$ and $Bacillus subtilis$ (18), respectively. Neither isoform is similar in sequence to the type I $Ec$PanK, nor is either subject to feedback inhibition by CoASH. Analysis of the respective kinetic parameters revealed a broad range for $K_m$(ATP) values, from 34 µM for $Sa$PanK to 3.1 mM for $Bs$PanK. The type III $Bs$PanK also exhibited a five-to-six-fold increase in $K_m$(Pan) relative to $Ec$PanK and $Sa$PanK; this correlates with the observation that N5-Pan is neither an alternate substrate for nor a competitive inhibitor of $Bs$PanK. Limited bioinformatics analyses suggested that both type II and type III PanKs belonged to the ribonuclease H-like family [kinase family and fold group 4 (15)] of kinases (18). Very recently, Hong et al. (19) and Yang et al. (20) have confirmed these predictions with crystal structures for the type II $Sa$PanK-AMPPNP
complex and for the type III PanKs from *Pseudomonas aeruginosa* (PaPanK) and *Thermotoga maritima* (TmPanK), as well as the PaPanK-Pan complex.

Global sequence identities between PaPanK and TmPanK, respectively, and the type III PanK from the category A biodefense pathogen *Bacillus anthracis* are 25% and 37%. *P. aeruginosa* is a Gram-negative aerobe that also contains the GSH/GSSG/glutathione reductase thiol/disulfide redox system (21-23); *T. maritima* is an anaerobic rod-shaped hyperthermophilic bacterium for which the ability to synthesize GSH has not been demonstrated (23). The CoADR enzymes, such as that from *S. aureus* (7, 24, 25), are a key part of the intracellular machinery for those bacteria and archaea in which CoASH serves as the major low-molecular weight thiol. Our structure-based bioinformatics analysis (10) suggests that CoADR may be a more common activity than previously recognized and one that is significantly misannotated in databases. In this report we demonstrate that CoASH is the major low-molecular weight thiol in the Gram-positive facultative anaerobe *B. anthracis*. We also describe the crystal structure for the type III PanK from this spore-forming organism, as refined at 2.0 Å resolution. The recent report of Yang et al. (20) included a bioinformatics analysis which demonstrates that the type III PanK is also widely distributed among bacteria. Here we provide an extended bioinformatics analysis that provides a genome-based link for the absence of GSH biosynthesis, the presence of the type III PanK, and the presence of CoADR-like enzymes in a number of bacteria (Gram-positive and Gram-negative) and spirochetes. Taken together, these observations suggest that the CoASH-refractory type III PanK plays a special role in maintaining higher intracellular CoASH levels in order to
accommodate additional functions for the coenzyme in thiol/disulfide redox homeostasis in a diverse group of bacteria.
EXPERIMENTAL PROCEDURES

Analysis of Thiols from *B. anthracis*. A 1-L culture of *B. anthracis* Sterne was grown in Brain Heart Infusion medium (Becton, Dickinson, and Co.) and harvested in exponential-phase growth. Four pellets (~0.5 mL each) were extracted with 2.5 mL of 50% acetonitrile in 20 mM N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid], pH 8, containing 2 mM mBBr (Calbiochem), and incubated at 60°C for 15 min (26). The extract was cooled on ice and acidified with 5 µL of 5 M methanesulfonic acid. The pellet was removed by centrifugation and dried in a vacuum oven to constant weight. Four more pellets were extracted identically, except that 5 mM NEM was substituted for mBBr. A 0.5-mL aliquot of one NEM-treated pellet was reacted with 2 mM mBBr for 15 min at 60°C and acidified with methanesulfonic acid, as given above; this sample served as a control for the thiol samples. The remaining ~2 mL of this sample was reacted with one eq of β-mercaptoethanol (based on NEM) and concentrated to 0.6 mL on a Speed Vac. This extract was reduced with 2 mM DTT for 20 min at 23°C and then reacted with mBBr (8 mM overall). The sample was acidified with methanesulfonic acid prior to HPLC analysis. A DTT sample was prepared as above, without extract, to serve as a control for the disulfide sample.

The extracts were analyzed following HPLC protocols 1 and 2 (26) for thiols and disulfides. Single point calibration standards were used for quantitation (50 pmol) of the bimane derivatives of Cys, N-acetyl-Cys, GSH, thiosulfate (SSO$_3$$^2$), H$_2$S, mycothiol, CoASH, 3´-dephospho-CoASH, pantetheine, and 4´-phosphopantetheine. The bimane derivative of CoASH hydrolyzes over time in acidic extracts to give the 3´-dephospho-CoASH-adduct and is quantitated as the sum of the two (CoASH + 3´-dephospho-
CoASH) from HPLC protocol 2. The bimane derivatives of Cys, U12 (an unidentified *B. anthracis* thiol), pantetheine, 4′-phosphopantetheine, and H₂S were best analyzed with protocol 1.

**Expression and Purification of BaPanK and BA2901.** Genomic DNA from *B. anthracis* Ames was provided by Dr. Arthur Friedlander, U.S. Army Medical Research Institute of Infectious Diseases. *B. anthracis* PanK (NP_842634; "transcriptional activator, putative, Baf family") was expressed with a C-terminal His-tag provided by the pET28a plasmid vector in *E. coli* C41(DE3) cells. These cells were grown in a 3-L culture of tryptone-yeast extract-phosphate medium (27) supplemented with 30 mM glucose and 30 µg/mL kanamycin at 37°C. Cultures were induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside at an A₆₀₀ of 0.9 and allowed to grow overnight at 37°C. Following centrifugation at 5,000 x g for 15 min, the washed cell pellets were resuspended in ~100 mL 25 mM sodium phosphate, pH 7.0, containing 2 mM EDTA and 5 mM DTT, and lysed via disruption with a pneumatic cell homogenizer (Avestin EmulsiFlex-C5). After centrifuging at 20,000 x g, streptomycin sulfate was added (1% w/v) to the supernatant, with stirring, for 15 min. The supernatant obtained on subsequent centrifugation was filtered and loaded onto a Q-Sepharose HP column equilibrated in 25 mM sodium phosphate, pH 7.0, with 5 mM β-mercaptoethanol. The protein was eluted with a 0→1 M NaCl gradient in the loading buffer; fractions were analyzed by SDS-polyacrylamide gel electrophoresis. The BaPanK pool was brought to final concentrations of 0.25 M NaCl and 20 mM imidazole, and the pH was adjusted to 8.0 with 1 M K₂HPO₄. The protein was loaded onto a Ni-NTA Superflow (Qiagen) column equilibrated in 50 mM sodium phosphate, pH 8.0, containing 0.3 M NaCl and 5
mM β-mercaptoethanol. The His-tagged *Ba*PanK was eluted by increasing the imidazole concentration to 100 mM. The pure protein (calculated *m* = 29,915 Da for the His-tagged *Ba*PanK) was buffer-exchanged into 25 mM Tris-HCl, pH 8.0, containing 100 mM KCl, 0.5 mM EDTA, and 0.5 mM DTT and concentrated to ~20 mg/mL, based on a calculated ε280 = 23,510 M⁻¹cm⁻¹ [ProtParam (28)]. After adding glycerol to 25% (v/v), 1-mL aliquots were stored at -80°C. The SeMet derivative of *Ba*PanK was expressed from the pET28a-*Ba*PanK vector in the *E. coli* B834(DE3) strain (10); cultures were grown overnight in the auto-inducing medium PASM-5052 (29). The purification protocol for SeMet *Ba*PanK was essentially identical to that described for the native protein, except that the SeMet protein was eluted from the Ni-NTA column with 0.25 M imidazole. Yields for the native and SeMet *Ba*PanKs were ~200 mg per L of culture and ~30-35 mg per L of culture, respectively.

The *B. anthracis* homolog (NP_845240; "hypothetical protein BA2901") of the type II *Sa*PanK was expressed, with either N-terminal or C-terminal His-tags provided by the pET28a plasmid vector, in *E. coli* B834(DE3) cells. These cells were grown in tryptone-yeast extract-phosphate medium, as described above for the *Ba*PanK expression and purification. N-terminal His-tagged BA2901 was purified in the same way as *Ba*PanK, except that an SP-Sepharose HP column replaced the Q-Sepharose HP column in the first step, given the higher calculated pI of 8.7 for BA2901. For the C-terminal His-tagged protein, the pET28a clone was expressed in *E. coli* B834(DE3) at 25°C. The protein was purified with a combination of Q-Sepharose HP and Ni-NTA column steps and was stored at -80°C in 50 mM Tris-HCl, pH 7.4 (at 25°C), containing 1 mM DTT and 20% glycerol (v/v).
**PanK Assays.** The enzymatic activity of purified *BaPanK* was measured as recently described for *PaPanK* (19); the standard assay included 60 mM NH₄Cl to allow for activation of the enzyme in the presence of the monovalent cation. The purified BA2901 protein was assayed following the protocol described for *SaPanK* (17). We also tested the ability of BA2901 to complement the *E. coli* mutant strain DV70 [coaA(Ts)] (30) that is unable to grow at 42°C due to the inactivation of *EcPanK*. Control strains were obtained by transforming strain DV70 with either pSC4, a pET28a plasmid harboring the *S. aureus* coaA gene (17), or with the empty vector. All strains grew very well at 30°C, but only pSC4/DV70 grew (36 h) at 42°C.

**Crystallization and Data Collection.** Thawed aliquots of the native and SeMet *BaPanK* proteins were buffer-exchanged into 10 mM sodium N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] to give final protein concentrations of 22-24 mg/mL. The native protein as concentrated (pH 7.0) also included 50 mM KCl; the SeMet protein sample (pH 7.5) contained 10 mM KCl. Crystals were grown in 24-well sitting-drop plates over reservoirs of 0.5 mL 24-26% ethylene glycol, in drop sizes of either 2 + 2 or 4 + 4 µL. Crystals were flash-frozen in a nitrogen stream at 100K directly after being removed from the drop. Data sets for MAD phasing were collected at National Synchrotron Light Source beamline X12C.

**Phasing and Structure Refinement.** Images in each data set were indexed, integrated, and scaled in d*TREK* (31). Phasing, chain tracing, and density modification were performed with SOLVE/RESOLVE (32-34). Model refinement was carried out in CNS (35), with manual rebuilding in COOT (36). The latter stages of refinement were
performed with REFMAC5 (37). The final model was validated with MOLPROBITY (38) and with the built-in tools from COOT.

**Bioinformatics.** Sequence analyses with *Ba*PanK were performed with the NCBI (39) and SEED (40) databases using blastp. Multiple sequence alignments were performed with CLUSTALW (41). Secondary structural motifs were identified with DSSP (42), and structural homology searches were performed with the Combinatorial Extension [CE (43)] and DALI (44) web servers. Structural alignments and analyses were carried out in COOT, and structure-based sequence alignments were exported with SSM SUPERPOSE (45). The TIGR-Comprehensive Microbial Resource (46) Version 2.3 (Genomes Region Comparison tool; Data Releases through 19.0) and the Integrated Microbial Genomes Version 1.6 (phylogenetic distribution tool) were used to identify proteins similar to those in the test set, in other bacteria. The test set included *E. coli* GshA (NP_417173), *E. coli* GshB (NP_417422), *Streptococcus agalactiae* γ-glutamylcysteine synthetase-glutathione synthetase (NP_688811), *E. coli* type I PanK (NP_418405), *S. aureus* type II PanK (NP_646871), *B. subtilis* type III PanK (AAW83041), and *S. aureus* CoADR (YP_493573). Strong evidence for homology is provided by a sequence identity of ≥25% (47); alignments for determining pairwise identities were performed using LALIGN [default parameters (48)].
RESULTS AND DISCUSSION

CoASH in *Bacillus anthracis*. In order to analyze thiols from *B. anthracis*, samples were taken from a 1-L culture of *B. anthracis* Sterne harvested in exponential-phase growth; bimane derivatives of low-molecular weight thiols resolved by reverse phase HPLC are given in Figure 1. *B. anthracis* produces CoASH, cysteine, and H$_2$S; the H$_2$S is likely derived from iron-sulfur proteins and is not a low-molecular weight thiol. This profile is qualitatively similar to that reported for *B. subtilis*, but differs quantitatively in the relative amounts of CoASH and cysteine. GSH, pantetheine, and 4’-phosphopantetheine were not detectable [≤0.03 μmol/g (dry residual weight)]. Total CoASH was estimated at 0.87 ± 0.11 μmol/g (dry residual weight), very similar to the value of 1.1 ± 0.1 μmol/g (dry residual weight) reported for *S. aureus* (7). A new unknown thiol (U12) was also identified in the *B. anthracis* extract; purification and mass spectrometric analysis gave a mass of 397 Da for U12$^2$ (corrected for the bimane contribution); this thiol could not be identified by comparison with the retention times for known thiols as analyzed in HPLC protocols 1 and 2 (see "Experimental Procedures"). Parallel analyses for the corresponding low-molecular weight disulfides [CoA-disulfide ≤0.035 μmol/g (dry residual weight)] gave an intracellular ratio of CoASH/CoA-disulfide >50. The method used to determine disulfides involves treatment of thiol-blocked extracts with DTT, in order to reduce the disulfide to its thiol form for mBBr derivatization and analysis. Since DTT can also release CoASH from acyl-CoAs by

$^2$ A mixed disulfide form of the oxidized OhrR repressor has very recently been identified that is formed in *B. subtilis* cells on treatment with cumene hydroperoxide; the novel thiol component also gives a mass of 397 Da (J. Helmann, personal communication).
Figure 1. Reverse phase HPLC of *B. anthracis* thiols. The two chromatograms correspond to the thiol sample (A), in which cell extracts were reacted with mBBr, and the control sample (B), in which cell extracts were reacted first with NEM and then with mBBr. Peaks that appear in the thiol sample but not the control sample are bimane derivatives of cellular thiols. mBOH, hydrolysis product of mBBr; U12, unidentified *B. anthracis* thiol (bimane derivative) eluting at 12 min in HPLC protocols 1 and 2.
transacetylation \((49)\), the value determined in this way represents an upper limit for the disulfide content; the estimated CoASH/CoA-disulfide ratio therefore represents a lower limit. Using a value of 2 \(\mu\text{L/mg dry weight}\) for the cell volume, we estimate the concentration of CoASH in \(B.\ anthracis\) cells to be \(~0.44\ \text{mM}\). The concentration of CoASH in \(E.\ coli\) (1.5 mol/mg dry weight) can similarly be estimated at \(~0.4\ \text{mM}\) \((22)\).

**PanKs in \(B.\ anthracis\).** The conversion of Pan\(\rightarrow\)CoASH requires three gene products, in addition to the type I PanK, in \(E.\ coli\) (Scheme 1): the bifunctional phosphopantothenoylcysteine synthetase/decarboxylase (phosphopantothenate + cysteine\(\rightarrow\)\(4'\)-phosphopantetheine; \(\text{coaBC}\)), phosphopantetheine adenylyltransferase \((\text{coaD})\), and dephospho-CoASH kinase \((\text{coaE})\). With specific reference to CoASH biosynthesis in \(B.\ anthracis\), Gerdes et al. \((50)\) published an analysis of potential broad-spectrum antimicrobial drug targets among cofactor biosynthetic pathways; all four \(\text{coa}\) gene products in \(E.\ coli\) were concluded to be individually “essential,” as was the \(\text{acpS}\) gene product which synthesizes the holo-acyl carrier protein from CoASH + apo-acyl carrier protein \((51)\). Using the ERGO database and the \(B.\ anthracis\) Ames genome sequence, the predicted CoASH biosynthetic pathway for the anthrax pathogen was analyzed with the aid of the known \(E.\ coli\) pathway \((50)\). All components of the \(\text{de novo}\) Pan biosynthetic pathway, as well as the \(\text{Na}^+/\text{Pan}\) transporter, were identified (presence of the orthologous gene); similar results were found for the pathway from Pan\(\rightarrow\)CoASH, except for the type I PanK. The locus identified in this case was the ortholog of the eukaryotic \(\text{panK}\) gene ["hypothetical protein BA2901" \((\text{NP}_845240)\)], and the properties of the BA2901 protein are described below. Choudhry et al. \((52)\) later demonstrated that the type II \(S.\ aureus\) PanK was closely related to BA2901 as well (35% identity), and
both Choudhry et al. (52) and Genschel et al. (53) have suggested that this *B. anthracis* protein, which is also related to the human PanK, has likely arisen as the result of lateral gene transfer. We expressed and purified recombinant BA2901 with N- and C-terminal His-tags, respectively; the mass of the former (33.6 kDa) was verified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Bruker Autoflex; sinapinic acid matrix). Both purified proteins were inactive in the *in vitro* PanK assay, under conditions in which the purified *SaPanK* is fully active. In addition, the BA2901 pET28a expression plasmid failed to complement the *E. coli coaA*(Ts) strain DV70 under conditions where *S. aureus coaA*-pET28a restored growth at 42°C. A homology model for BA2901 based on the type II *SaPanK* structure (19) does not provide an explanation for the observed lack of activity. No crystal structure for the *SaPanK*-Pan complex is available; the model described by Hong et al. (19) represents a "loose fit" for bound Pan. The critical residues involved in Pan binding have not been strictly identified or tested. Similarly, a putative mammalian PanK (PanK4) was originally identified based on its very favorable comparison with the mammalian PanK1 sequence, but this protein is also inactive. We conclude that BA2901 is very likely a kinase of the ribonuclease H-like family, but Pan is not the acceptor substrate. Genetic studies already in progress3 are designed to provide a more definitive answer to this question. If BA2901 does lack compensating PanK activity, deletion of the BA0065 locus should be lethal to *B. anthracis* Sterne (see below).

3 C. Paige, P. Hanna, and A. Claiborne, unpublished experiments.
The absence of a type I PanK (and perhaps a functional type II enzyme as well, see above) in \textit{B. anthracis} is distinct from the situation in \textit{B. subtilis} and \textit{Bacillus halodurans}, where functional type I and III enzymes are found \cite{18, 54}. In \textit{B. subtilis}, deletion of the \textit{coaX} (type III) gene gave a normal growing phenotype, but \textit{coaX} could not be deleted from a strain containing a \textit{coaA} deletion; these results suggested that simultaneous deletion of both genes was lethal to \textit{B. subtilis}. The \textit{B. anthracis} Ames genome \cite{55} also yields a type III PanK homolog, annotated as "putative Baf family transcriptional activator BA0065" (NP_842634), which is 76\% identical to the functional \textit{BsPanK}. Of those bacterial species referred to earlier, known to lack GSH and containing CoASH as their major low-molecular weight thiol, \textit{B. burgdorferi}, \textit{D. radiodurans}, and \textit{B. megaterium} all have type III PanK homologs \cite{20}; all lack both type I and II PanK genes. All members of the \textit{Bacillus cereus} group lack \textit{coaA} orthologs, and all completed genomes of this group (\textit{B. anthracis}, \textit{B. cereus}, and \textit{Bacillus thuringiensis}) contain type III PanK homologs 95-100\% identical to that of the \textit{B. anthracis} Ames strain. The bacterial type II and III PanK enzymes examined thus far are refractory to inhibition by CoASH \cite{17-19}, suggesting that bacteria dependent on CoASH as the major intracellular thiol tend to possess CoASH biosynthetic pathways insensitive to feedback inhibition.

The C-terminal His-tagged BA0065 protein (\textit{BaPanK}) was expressed and purified from recombinant \textit{E. coli}; in contrast to the BA2901 protein, \textit{BaPanK} is active in the \textit{in vitro} enzyme assay, as examined in parallel with the type III \textit{PaPanK}. \textit{BaPanK} gives

\footnote{J. Ravel, personal communication.}
60% conversion of D[1-14C]pantothenate to 4'-phosphopantothenate under conditions where PaPanK gives 40% conversion.

Structure Solution of BaPanK. The structure of *B. anthracis* PanK was solved by three-wavelength MAD phasing with the SeMet-substituted protein. Statistics for the data sets and final structure model are shown in Tables 1 and 2, respectively. Ten Se sites were identified, and the experimental MAD-phased electron density map calculated at 2.0 Å showed continuous electron density for the protein backbone, with the exception of a stretch of seven residues (Arg164-Arg170), and allowed identification of most side chain positions. The $2F_o-F_c$ map in the region of the PHOSPHATE1 motif (see below) also illustrates the accuracy of the final refined model (Figure 2). The C-terminus, including residues Asn255-Glu262 plus the His$_6$ tag, is not visible in the electron density map; the model is truncated at Ala254. The crystal structure of BaPanK has one monomer in the asymmetric unit. Residues 164-170 are part of a long loop spanning residues Ser163-Asn183; the length of this loop, its location on the surface of the protein, and the absence of electron density for a part of it suggest that the entire loop is flexible. Yang et al. (20) have described the equivalent loop in *TmPanK* as comprising the "Pan cap."

The two constituent monomers of the BaPanK dimer are related by crystallographic symmetry (a two-fold rotation axis). Each monomer interacts with the other through a region consisting of $\beta3'$, $\alpha a$, $\alpha1'$, and the C-terminal part of the loop between $\beta a$ and $3_{10}b$ (Figure 3). As expected, structural superpositions with the BaPanK monomer and either PaPanK (19) or TmPanK (20) reveal nearly identical folds for the three type III PanKs; $C_\alpha$ rmsd values of 2.1 and 1.6 Å result with the respective A monomers (for 217 and 232 atoms, respectively). The type III PanKs have been
Table 1. Data Collection and Phasing Statistics

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<td>Cell (Å)</td>
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<td>90 90 90</td>
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<td>( I/\sigma )</td>
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<td>Figure of merit</td>
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<sup>a</sup>Collected at beamline X12C of the National Synchrotron Light Source (Brookhaven National Laboratory) using an ADSC Q210 CCD detector.

<sup>b</sup>Values in parentheses are for the highest resolution shell.
Table 2. Crystallographic Refinement Statistics

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</tr>
</tbody>
</table>

\(^a\)Values in parentheses are for the highest resolution shell.
\(^b\)Refined against all data in the resolution range.
\(^c\)Ethylene glycol.
Figure 2. Final $2F_o - F_c$ map, in stereo, for residues Leu5-Thr10 of the PHOSPHATE1 motif, together with the refined model. All residues are color-coded by atom type. The depicted contour level is $1\sigma$.

Figure 3. Elements involved in BaPanK dimerization, in stereo. Prime symbols designate secondary structural units in domain II [of monomer B (cyan), in this case]; lettered designations (e.g., $\alpha_a$) indicate insertions relative to the shared core fold. These monomer B elements are overlaid with a surface representation (color-coded by electrostatic potential) of monomer A. The segment 164'-170' missing electron density in monomer B is part of the long loop (163'-183') connecting $\alpha_a$ and $\alpha 1'$ in an area above the monomer A Pan-binding pocket.
identified as members of the ASKHA kinase superfamily [ribonuclease H-like, group 4 (15)]. Figure 4 gives a structure-based sequence alignment for the five known functional type III PanKs. As described, all share the three major sequence-structure motifs (56) attributed to the ASKHA superfamily (PHOSPHATE1, PHOSPHATE2, and ADENOSINE), as well as the interdomain helix-to-helix CONNECT elements (Ala116 and Gly245 in BaPanK). The three major ASKHA motifs correspond to elements involved in ATP recognition and binding as well as divalent metal coordination for the relevant phosphotransferase (PanK) activity (15, 56).

**PAN and INTERFACE Motifs.** BaPanK has few insertions to its core ASKHA fold. Other members of the ASKHA superfamily, including the type II SaPanK (17) and the eukaryotic PanKs (15), have several such insertions which adopt structures and motifs responsible for the formation of unique substrate-binding sites. Based on the structure for the PaPanK-Pan complex [2F9W (19)], a model of the BaPanK-Pan complex was developed. Most of the interactions between protein and Pan occur with residues that are outside the ASKHA motifs; two insertions to the core fold in BaPanK merit special attention. Broadly speaking, Pan binds in a pocket formed between β3′ and the loop connecting 310b and α3′ (Figure 5); this loop bears the conserved residues Gly107, Asp109, and Arg110 [the \(hGhDR\) sequence; \(h = \) hydrophobic (Figure 4)] and constitutes the new PAN motif. Strand β3′ bears the conserved residues Gly147, Gly148, and Ile150, which play roles in Pan binding, as well as Pro152 and Gly153, which are important in the context of the dimer interface; together, these residues constitute the new INTERFACE motif [the GGxlxPG sequence (Figure 4)]. These new PAN and INTERFACE motifs are found in all type III PanK proteins and serve to distinguish this
Figure 4. Structure-based sequence alignment for BaPanK and four known functional type III PanKs (references given in the text). Structures have now been determined for BaPanK (this work), PaPanK (19), and TmPanK (20). Residue numbering and assignments correspond to BaPanK and include the 164-170 segment (\[8888\]) for which electron density is absent. This segment constitutes a part of the loop (extending through Asn183; \[8888\]) that exhibits different conformations in the three structures. Red and yellow blocks correspond to conserved residues and conservative substitutions, respectively. ASKHA motifs are highlighted in green boxes (Ala116 and Gly245 represent CONNECT1 and CONNECT2 elements), and the new PAN and INTERFACE motifs, as well as Thr184, are highlighted in magenta boxes. Blue underscores represent residues involved in direct contacts across the dimer interface.
Figure 5. Topology diagram for the *BaPanK* monomer. Domains I and II represent an internal duplication of the RNase H-like fold, and helices $\alpha_3$ and $\alpha_3'$ cross over to form the base of an interdomain cleft. Insertions to the core ASKHA fold are hatched; motifs indicated in Figure 4 are labeled in red (PHO, PHOSPHATE; INT, INTERFACE; ADO, ADENOSINE), as is the Pan cap segment. The diagram is represented as a rainbow from blue (N-terminus) to red (C-terminus).
from other ASKHA families; an inspection of the eukaryotic PanK family alignment (15) reveals that the PAN and INTERFACE motifs are unique to the type III sequences. 

*BaPanK* Asp109 forms side chain hydrogen bonds to the Pan C2′- and C4′-hydroxyls (Figure 6); this Asp is conserved within the PAN motif in the type III PanK sequences. 

*BaPanK* Gly107-N [this Gly is also conserved within the PAN motif (Figure 4)] forms a hydrogen bond with the Pan C1-carboxylate. In addition, the absence of a side chain here avoids a potential steric clash with the bound substrate. *BaPanK* Arg110 of the PAN motif (conserved in all known functional type III PanKs) and Tyr100 also interact with the Pan carboxylate. The Asp109 and Arg110 side chain rotamers, which form hydrogen bonds with Asp6 and Thr135, respectively, in the apoenzyme, are adjusted in the *BaPanK-Pan* model to optimize the interactions with bound Pan. Inspection of the *TmPanK* structure (20) and the *TmPanK-Pan* model reveals that a change in the apoenzyme Arg106 rotamer would also be necessary for optimization of that Pan-binding interaction. 

Within the INTERFACE motif, Gly147 and Gly148 provide space at the end of the binding pocket for the Pan carboxylate. Any side chain at either of these positions would produce steric clashes with the bound Pan. Ile150 makes van der Waals contacts with bound Pan, while Pro152 and Gly153 are conserved, not for Pan binding, but for their roles in dimerization as described earlier. Conserved regions in the type II *SaPanK* (17) and the eukaryotic PanKs (15) do not correlate with either the PAN or INTERFACE motifs; Hong et al. (19) have demonstrated that, although the *SaPanK* and *PaPanK* monomers have similar structures (SSM SUPERPOSE gives a Cα rmsd = 2.9-3.1 Å for 195 atoms), the respective homodimers assemble with distinctly different architectures.
Figure 6. LIGPLOT representation of protein interactions with Pan in the PaPanK-Pan complex (amino acid residues labeled in orange; equivalent BaPanK residues in black), using default parameters for geometry and hydrogen-bonding distance. Carbon atoms for monomer A residues are colored magenta, while those for Thr180' (monomer B) are in cyan. Pan is color-coded by atom type, with carbon atoms in black; note that Pan atom numbering here follows that for the bound Pan ("DPA") in the Protein Data Bank file 2F9W.
The SaPanK-Pan model suggests that Glu70, which is structurally equivalent to PaPanK Asp101, interacts with the Pan C4′-OH; this Glu residue does not, however, appear within any PAN-like motif. SaPanK Arg113, implicated in contact with the Pan C1-carboxylate, follows by 43 residues in the sequence (in contrast to the hGhDR motif in the type III PanKs). Further inspection of the SaPanK sequence reveals that Arg113 is followed closely by Gly115-Gly116; these Gly residues are structurally equivalent to the conserved Gly147-Gly148 pair described for the INTERFACE motif of the type III PanKs. In the context of the dimer, the importance of another region in the type III PanKs becomes apparent; this region represents a unique insertion element, as compared with the type II SaPanK. The segment corresponding to BaPanK Leu161-Val180 (TmPanK 159-178 and PaPanK 153-176) is observed to exhibit different conformations in the three structures (Figure 7). Helix αa is present in all three structures to some degree, but the subsequent loop leading to helix α1′ shows significant differences. Similarly, residues 164-170 lack electron density in the BaPanK structure. Yang et al. (20) describe several residues in this region as part of the "Pan cap" which contributes to the conserved hydrophobic pocket described earlier (including elements of the INTERFACE motif) that accommodates the two C3′-methyl groups of bound Pan. This cap is also proposed to protect the Pan-binding site - and the bound Pan substrate - from solvent.

The differential ordering/disordering of this region in the three distinct type III PanK structures cannot be explained in the context of the respective crystal lattices. Furthermore, the ordering of the region in monomer B of the apo-PaPanK structure (versus its disordering in monomer A) is nearly identical to that seen in both monomers.
Figure 7. Ordered versus disordered Pan caps in PaPanK and BaPanK (stereo). The $\alpha_a$ and $\alpha_1'$ helices of the BaPanK-Pan model (cyan) are overlaid with a surface rendering (color-coded by electrostatic potential) of the Pan-binding pocket of monomer A. Bound Pan is represented with a space-filling model. Bound ATP is also shown, as modeled based on the structure of the type II SaPanK-AMPPNP complex. The $\alpha_a$ and $\alpha_1'$ helices of PaPanK monomer B (Pan complex, in yellow), with the Leu153'-Pro176' loop, are shown from a superposition.
of the *PaPanK*-Pan structure, so the conformational differences cannot be explained by the simple absence or presence of bound substrate. These observations have led to a preliminary examination of the conformational dynamics for the Pan cap. Our structural model for the *BaPanK*-Pan complex is stable under energy minimization. In the same short molecular dynamics simulations, the Pan substrate diffuses out of its binding site in the *BaPanK* dimer, suggesting that the unresolved loop nearby plays a role in binding the substrate. In addition to local rearrangements expected in the active site on Pan binding, domain closure may play a role in strengthening the interactions within the enzyme-substrate complex, as well as in excluding solvent from the phosphotransferase reaction center.

A conserved Thr residue near this flexible region shows a specific interaction with the bound Pan. In the model of the *BaPanK*-Pan complex, though a large part of the long loop is missing electron density, Thr184' is in nearly the same relative position as *PaPanK* Thr180' (Figure 6), suggesting that this residue is conserved in order to stabilize Pan in its binding site. Thr180' of *PaPanK* monomer B forms a hydrogen bond with the Pan carboxylate bound to monomer A.

**Interactions with CoASH.** CoASH is an important feedback inhibitor of the type I PanK, exhibiting a $K_i$ of 4.3 µM (13); binding is competitive with respect to ATP, and crystal structures of the *EcPanK*-AMPPNP and -CoASH complexes (12) demonstrated that the α- and β-phosphates of CoASH occupy the same site as the β- and γ-phosphates of ATP. In both cases, Lys101 is essential in mediating the interactions with the

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5 F.R. Salsbury, N.I. Nicely, and A. Claiborne, unpublished experiments.
phosphate moieties (11). His177, Phe247, and Arg106 are unique and essential to CoASH binding and inhibition; H177Q, F247V, and R106A EcPanK mutants are all refractory to any inhibition at [CoASH] ≤ 160 µM, nor do the mutants bind CoASH in equilibrium dialysis experiments (57). In the crystal structure of the EcPanK-CoASH complex, the CoASH β-mercaptoethylamido moiety extends into the hydrophobic dome over the Pan-binding pocket; the same hydrophobic residues that interact with the extended alkyl chain of N5-Pan also form the binding groove for the β-mercaptoethylamido moiety of CoASH (12). The thiol group is tightly sealed from solvent by weakly polar interactions with four aromatic residues from the hydrophobic dome and also participates in a hydrogen bond with the CoASH adenine 6-NH₂ group.

Although many of the protein elements defined in the EcPanK-CoASH interaction are absent in BaPanK, the most direct explanation for the failure of the type III PanKs to exhibit feedback inhibition by CoASH lies in the tight Pan-binding pocket seen in the BaPanK-Pan model (Figure 7). The absence of the hydrophobic dome results in an inability to accommodate the β-mercaptoethylamido moiety of CoASH, in parallel with the failure to accommodate the alkyl substituents of N5-Pan and N-heptylpantothenamide. An identical conclusion regarding the absence of CoASH inhibition with the type III TmPanK has recently been given by Yang et al. (20). As the type II SaPanK Pan-binding pocket is fully exposed and can bind the pantothenamides (19), other factors must explain the resistance of SaPanK to CoASH feedback inhibition. The biological consequences of these analyses are considered in the following section.

Type III PanKs and CoASH-Dependent Redox Biology. In the specific case of S. aureus, GSH is absent, as described earlier (7). CoASH is the major intracellular thiol,
and a unique NADPH-dependent CoADR functions to maintain the appropriate intracellular thiol/disulfide redox balance, analogous to the GSH/GSSG/glutathione reductase system in *E. coli* (10, 24, 25); in *S. aureus*, the ratio of CoASH/CoA-disulfide is about 450. The presence of the type II PanK in *S. aureus* (17, 19), which is refractory to inhibition by CoASH, allows CoASH levels to rise to an upper limit (calculated to be ~0.55 mM) likely controlled only by the supply of Pan, thus contributing to this organism's unique CoASH-dependent redox biology. Bacterial type II PanK homologs have been identified only in *Staphylococcus*, in the *B. cereus* group, and in *Oceanobacillus iheyensis* (20). In this work, however, we have been unable to demonstrate the catalytic ATP-dependent phosphorylation of Pan *in vitro*, using two different His-tagged versions of the purified recombinant type II PanK homolog from *B. anthracis* (BA2901); nor does the BA2901 expression plasmid complement the temperature-sensitive *E. coli coaA* mutant strain DV70. Our working conclusion for *B. anthracis* is that the type III PanK may represent the sole functional enzyme for synthesis of 4′-phosphopantothenate; genetic studies already in progress\(^4\) are designed to provide a conclusive answer to this question. The bioinformatics analysis of Copley and Dhillon (23), combined with biochemical analyses reported by the Fahey laboratory over the past 30 years (1), demonstrate that GSH biosynthesis is found "only rarely" in Gram-positive bacteria. The demonstration that functional CoADR enzymes are present in *S. aureus* (7), in *B. megaterium* (4) and *B. anthracis\(^6\)*, and in *B. burgdorferi* (8) correlates perfectly

\(^6\) J.R. Wallen and A. Claiborne, unpublished experiments
with the presence of CoASH as the major low-molecular weight thiol, and with the presence of a type II or type III PanK.

In *B. megaterium* spores (58), 43% of the total CoASH was shown to be present as soluble protein-SSCoA disulfides; >75% of this protein-SSCoA disulfide pool was reduced within the first minutes of germination. Two likely functions for these protein-SSCoA disulfides were considered: 1) reversible inactivation of key thiol-dependent spore enzymes, maintaining metabolic dormancy in the spore, and 2) reversible protection of labile protein thiols in the spore, thereby contributing to heat and radiation resistance. The protein-SSCoA disulfide pool in vegetative *B. megaterium* accounted for <2% of the total CoASH but increased dramatically to ~45% of the total CoASH during sporulation. Formation and reduction of these protein-SSCoA disulfides (during spore formation and germination, respectively) has been attributed to a CoASH-mediated redox control process. The effective absence of reduced pyridine nucleotides (NADH and NADPH) in dormant spores of *B. megaterium* (59) corresponds to a dramatic decrease in the intracellular [CoASH]/[CoA-disulfide] redox state, which corresponds in turn to maximal protein-SSCoA disulfide formation. The rapid accumulation of NADH during the first minutes of spore germination (59) has been proposed to lead to reduction of the protein-SSCoA disulfide pool, either directly by a CoADR-like enzyme or indirectly, via a CoASH-mediated process (4).

The potential importance of such a CoASH-mediated germination control mechanism in *B. anthracis* is clear, as spore germination and outgrowth are fundamental to proliferation (60). The analysis of the transcriptome activated during growth and sporulation of *B. anthracis* Sterne has provided additional support for this hypothesis.
The *coaX* gene encoding *BaPanK* is up-regulated in wave II, as is the *coaD* locus encoding phosphopantetheine adenylyltransferase; the *coaBC* and *coaE* genes corresponding to the bifunctional phosphopantothenoylcysteine synthetase/decarboxylase and depophospho-CoASH kinase, respectively, are up-regulated in waves I and III. Bergman et al. (61) have suggested, that since waves I and II are the phases of the *B. anthracis* life cycle that occur within the infected host, genes up-regulated specifically within this temporal frame may be particularly useful as therapeutic targets. The *B. anthracis* CoADR (BA1263) and CoADR-rhodanese homology domain (BA0774) proteins were expressed in waves III and V, respectively, and the presence of CoADR in the fractured spore was confirmed by proteomics analysis (62). At least four gene products essential for biosynthesis of the CoASH precursors Pan (BA1562, BA1563, and BA1564) and cysteine (BA1831) were also expressed in wave V. As described by Bergman et al. (61), waves IV and V seem to represent the final stages of sporulation in *B. anthracis* and include a large number of sporulation-associated loci as well as loci involved in oxidative stress responses.

In collaboration with Dr. Jacques Ravel of TIGR, we have undertaken a bioinformatics analysis using 1) the *E. coli* *gshA* and *gshB* genes (23), along with the bifunctional γ-glutamylcysteine synthetase-glutathione synthetase (SAG1821) gene from *Streptococcus agalactiae* (63), 2) the *S. aureus* CoADR gene (24), and 3) the *E. coli* type I (64), *S. aureus* type II (17), and *B. subtilis* type III (18) PanK genes, together with the TIGR Comprehensive Microbial Resource (version 2.3; Data Releases through 19.0). Table 3 lists those bacteria (genomes available as of April, 2006) that lack the ability to synthesize GSH, contain a type II or type III PanK, and contain a CoADR-like protein.
Table 3. Codistribution of CoASH-refractory PanKs and CoADR-like Proteins in Bacteria. *

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<th>Bacterium</th>
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</tr>
<tr>
<td><em>Bacteroides thetaiotamicron</em></td>
<td><em>Staphylococcus haemolyticus</em></td>
</tr>
<tr>
<td><em>Borrelia burgdorferi</em></td>
<td><em>Staphylococcus saprophyticus</em></td>
</tr>
<tr>
<td><em>Borrelia garinii</em></td>
<td><em>Streptomyces avermitilis</em></td>
</tr>
<tr>
<td><em>Clostridium tetani</em></td>
<td><em>Streptomyces coelicolor</em></td>
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<tr>
<td><em>Deinococcus radiodurans</em></td>
<td><em>Symbiobacterium thermophilum</em></td>
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<tr>
<td><em>Fusobacterium nucleatum</em></td>
<td><em>Thermoanaerobacter tengcongensis</em></td>
</tr>
<tr>
<td><em>Geobacillus kaustophilus</em></td>
<td><em>Thermotoga maritima</em></td>
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<tr>
<td><em>Geobacter sulfurreducens</em></td>
<td><em>Thermus thermophilus</em></td>
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<tr>
<td><em>Nocardia farcinica</em></td>
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</table>

*Analyzed with TIGR-Comprehensive Microbial Resource as described in "Experimental Procedures." *b*Contains homologs to both type II and III PanKs. *c*Known to lack GSH and to contain CoASH as major low-molecular weight thiol. *d*Contains homologs to both type I and III PanKs. *e*J. Ravel, personal communication. *f*Ref. (20). *g*Contains only type II PanK. *h*Known to lack GSH; mycothiol is major low-molecular weight thiol.
Of the 27 species represented, for which we conclude that a CoASH-dependent redox biology pertains, only the four staphylococcal species lack the type III PanK. The absence of GSH has been confirmed for seven of the 27 species, and CoASH is known to be the major low-molecular weight thiol in six of these. It is of interest to note that even though mycothiol is the major low-molecular weight thiol in *Streptomyces coelicolor* and other streptomycetes (22), the level of intracellular CoASH is similar to that in *S. aureus*. CoADR-like proteins (E values = 4e\(^{-41}\)-6e\(^{-39}\)) are present in both *S. coelicolor* and *Streptomyces avermitilis*, suggesting a redundancy in low-molecular weight thiol/disulfide redox systems for this organism. As CoASH has been identified as an important component of the sporulation process in *B. megaterium* (see above), the CoASH/CoA-disulfide/CoADR system may also function in spore formation for streptomycetes.

In a parallel analysis, using the Integrated Microbial Genomes resource (65), 14 phylogenetic classes of bacteria (among the 30 classes analyzed) were identified as lacking the three marker proteins for GSH biosynthesis (GshA, GshB, and \(\gamma\)-glutamylcysteine synthetase-glutathione synthetase), and we conclude that these organisms lack the ability to synthesize GSH. All 14 classes also lack the type I PanK, and 13 of these classes have the CoASH-refractory type III PanK; eight classes have both the type III PanK plus a CoADR-like protein. It should, of course, be emphasized that other important bacteria such as *Pseudomonas aeruginosa* have been demonstrated to contain millimolar levels of GSH (22); still they express only the type III PanK (19, 20). Analysis of the *Helicobacter pylori* genome has been reported to reveal the absence of *gshA*, *gor*, and *grx* genes (66), implying the lack of a GSH/GSSG/glutathione reductase
thiol/disulfide redox system. Thus, of the five known functional type III PanKs, only one is derived from a GSH-dependent microorganism (P. aeruginosa).
ACKNOWLEDGMENTS

We would like to thank Dr. Arthur Friedlander for kindly providing *B. anthracis* genomic DNA, Dr. Fred Salsbury for performing the molecular dynamics calculations, Dr. Don Guiney for providing *B. anthracis* Sterne cultures, Dr. Hee-won Park for providing coordinates prior to publication, Mr. William Boles for support during data collection at the National Synchrotron Light Source, and Ms. Sumana Choudhury for excellent technical assistance in expressing the *BaPanK* and BA2901 proteins. We also thank Dr. Jacques Ravel for hosting one of us (C.P.) in his laboratory and contributing to the bioinformatics analysis.
REFERENCES


CHAPTER III

THE TYPE III PANTOTHENATE KINASE ENCODED BY coaX IS ESSENTIAL FOR GROWTH OF Bacillus anthracis

Carleitta Paige, Sean D. Reid, Philip C. Hanna, and Al Claiborne

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ABSTRACT

Coenzyme A (CoASH) is the major low-molecular weight thiol in *Bacillus anthracis*; the crystal structures of the type III pantothenate kinase from *B. anthracis* (BaPanK), which catalyzes the first committed step in CoASH biosynthesis, and the coenzyme A-disulfide reductase from *B. anthracis*, which maintains the reduced intracellular state of CoASH in vegetative cells, have recently been reported [Wallen, J.R., Paige, C., Mallett, T.C., Karplus, P.A., and Claiborne, A. (2008) Biochemistry 47, 5182-5193, and references therein]. We have now demonstrated that the coaX gene encoding BaPanK is transcribed as part of a tricistronic operon including the hslO and cysK-1 loci, which encode the (thiol-disulfide) redox-regulated heat shock protein Hsp33 and cysteine synthase A, respectively. In order to test whether BaPanK plays an essential role in the novel CoASH-dependent redox biology of this spore-forming pathogen, we have analyzed the growth characteristics of a conditional coaX mutant. Exponential growth of the conditional mutant, in the absence of inducer, is observed after a lag of 8 h; this unanticipated result is due to a guanine→adenine suppressor mutation identified in position 5 of the lac operator, which has been shown to be critical for lac repressor binding within the inducible P_{spac} promoter. The stable suppressor mutant that results produces the same tricistronic coaX-hslO-cysK-1 mRNA as is observed with wild-type *B. anthracis*. This study demonstrates that the recently identified type III pantothenate kinase in *B. anthracis* is an essential enzyme, thus contributing to its validation as a new antimicrobial target that may provide for the development of therapeutically useful molecules.
INTRODUCTION

The very recent report of Hochgräfe et al. (1) has been taken to support the conclusion that \( S \)-thiolation by Cys represents a general, reversible mechanism for protection and regulation of protein-SH groups during disulfide (and other oxidative) stresses in Bacillus subtilis strain 168. The tripeptide thiol glutathione (2) is absent in all species of Bacillus analyzed to date (3-5); while Cys is the major low-molecular weight thiol in B. subtilis (3), we have recently reported (6) that coenzyme A [CoASH (7)] is the major low-molecular weight thiol in the category A priority pathogen Bacillus anthracis. While three of the four proteins responsible for the conversion of pantothenate (Pan; vitamin B5) to CoASH in Escherichia coli are conserved in B. anthracis (8, 9), a novel type III pantothenate kinase [BaPanK; BA0065 (6)] catalyzes the first committed step in the biosynthetic pathway in B. anthracis. The type III PanKs, first characterized in 2005 from B. subtilis and Helicobacter pylori (10), exhibit two particularly significant differences from the type I E. coli PanK (7); the type III enzymes are not subject to feedback inhibition by CoASH, and they do not recognize the Pan antimetabolite (alternate substrate) \( N \)-pentylpantothenamide. This has been attributed (6) to the absence of any structural equivalent to the hydrophobic dome found in the type I PanK substrate-binding pocket; this element accommodates both the \( \beta \)-mercaptoethylamido moiety of CoASH and the extended alkyl substituent of \( N \)-pentylpantothenamide (11, 12).

CoASH biosynthesis has very recently been reviewed as an antimicrobial drug target (13), and there are several observations supporting a central, if not somewhat unusual, role for CoASH in the redox biology of B. anthracis and other Bacillus species such as Bacillus megaterium (6). Setlow and Setlow (14) demonstrated that the effective
absence of reduced pyridine nucleotides (NADH + NADPH) in dormant *B. megaterium* spores led to a dramatic increase in soluble protein-SSCoA, from <2% of the total CoASH pool in vegetative cells to ~45% of the total CoASH in spores. Reduction of the spore protein-SSCoA pool (to protein-SH + CoASH), early in germination, has been linked to an NADH-dependent coenzyme A-disulfide reductase (5); in *B. anthracis*, this enzyme (BACoADR) has been demonstrated to function via a Cys42-SSCoA intermediate (15). Furthermore, in their transcriptional profiling of the *B. anthracis* life cycle, in which five distinct temporal waves of gene expression were identified from germination through sporulation, Bergman et al. (16) showed that the *coaX, coaBC* (BA4007), and *coaD* (BA4139), genes encoding the first three enzymes in the Pan→CoASH pathway (7), are up-regulated in waves I and II. The *cdr* gene encoding BACoADR and *coaE* (BA4828) are expressed in wave III. The *coaX* gene was also up-regulated >2-fold between 1 and 2 h postinfection within host macrophages (17), and both *coaX* and *cdr* genes have now been identified in the 5x draft sequence of the *B. megaterium* chromosome (J. Ravel, personal communication). It should be emphasized that this scheme is quite distinct from that found in *B. subtilis* (see above), which has both type I and type III PanKs (10, 18) but lacks coenzyme A-disulfide reductase (15).

Given the growing interest in this novel type III PanK, crystal structures are now available for the enzymes from *B. anthracis* (6), *Pseudomonas aeruginosa* (19), and *Thermotoga maritima* (20, 21) and include binary complexes with Pan and the 4′-phosphopantothenate product, as well as the ternary complex with Pan and ADP. These have led to the identification of new motifs for the Pan-binding pocket and suggest, based on differences in the binding modes for both Pan and ATP substrates [relative to the type
II human PanK (22)], potential modes of design for new inhibitors specifically targeting the type III enzymes. Bioinformatics analyses (6) have demonstrated a strong correlation among the absence of glutathione biosynthesis, the absence of the type I PanK, and the presence of the type III enzyme for 14 phylogenetic classes of bacteria. The distribution of type III PanKs among major human pathogens (e.g., *B. anthracis*, *Mycobacterium tuberculosis*, *Clostridium botulinum*, and *Francisella tularensis*) has underscored the significance of this potential target for the design of therapeutically useful molecules (6, 20).

In order to provide further support for the conclusion (6) that *Ba*PanK is the sole functional enzyme for synthesis of 4′-phosphopantothenate in *B. anthracis*, and to test whether this enzyme plays an essential role in the novel CoASH-dependent redox biology of this spore-forming pathogen, we have characterized the *coaX* transcriptional unit, as defined in exponential growth, and used genetic approaches to determine whether *Ba*PanK is important for the survival of *B. anthracis* during normal growth.
EXPERIMENTAL PROCEDURES

**Bacterial strains and antibiotics.** Cultures of *B. anthracis* Sterne 34F2 were grown and maintained in BHI broth (Difco) and on solid media containing 15 g/l of agar. *E. coli* DB3.1 (23), cultured and maintained in LB broth or solid media containing either 50 µg/ml kanamycin sulfate or 300-400 µg/ml erythromycin, was used for cloning and propagation of the pNFd13 (23) and pBKJ236 (24) plasmids, respectively. For preparation of parental and mutant endospores of *B. anthracis* Sterne, cultures were grown on BHI plates containing antibiotics as appropriate and at temperatures of either 37°C or 39°C (pNFd13 derivative only). A single colony was inoculated into 3 ml of BHI broth containing antibiotics as required, and this culture was taken as the inoculum (5%, v/v) for 75 ml of fresh modified G (sporulation) medium (25) without antibiotic, after 8-12 h of growth. After 4 days' growth at the appropriate temperature endospores were collected by centrifugation, and residual vegetative cells were killed by a 30-min incubation at 65°C. Pellets were washed 3-4 times in deionized water, and endospores were stored (in deionized water) at ambient temperature. The purity of the spore preparations was confirmed by phase-contrast microscopy, and spore titers were determined by serial dilution. For complementation studies with the pNFd13 derivative, 10 mM IPTG (Sigma) was added to the sporulation culture at the time of inoculation.

**RNA isolation.** Cells were harvested at an *A*₆₀₀ of 0.4-0.6 from BHI cultures of *B. anthracis* Sterne, and RNA was isolated (26) using the Ambion RiboPure-Bacteria Kit according to the manufacturer's instructions; final RNA purification with the QIAGEN RNeasy Mini Kit employed a DNase digestion step as described by the manufacturer. Qualitative analysis of RNA preparations, purified simultaneously from three parallel
cultures, included (1) $A_{260}/A_{280}$ measurement, using a SpectraMax M2 Microplate Reader (Molecular Devices), and (2) electrophoretic analysis on a denaturing formaldehyde agarose gel (1 µg RNA).

**Endpoint RT-PCR.** $coaX$ expression was analyzed by endpoint RT-PCR (26); ~750 ng of RNA was used per reaction with the Invitrogen SuperScript III One-Step RT-PCR System, following the manufacturer's directions. Sequence-specific primers (all primer sequences are available on request) were designed to yield products of 200-500 bp. Parameters for both RT and PCR reactions were nearly identical to those used by Passalacqua et al. (26), and endpoint PCR product (5 µl) was run on 0.8% agarose gels and visualized by ethidium bromide staining. Appropriate controls, both with genomic *B. anthracis* Sterne DNA and without RT, were done with each experiment.

**Disruption of $coaX$ by temperature-dependent plasmid-insertion mutagenesis.** On the basis of the *B. anthracis* Sterne genome sequence (AE017225), oligonucleotide primers complementary to a 500-bp sequence beginning with the ribosome-binding site and extending into the $coaX$ coding sequence were used for PCR amplification, and the product was cloned into pNFd13 (23) to give pNFd13:$coaX'$. Following electroporation and recovery of *B. anthracis* Sterne transformants at 30°C, the plasmid was integrated into the target locus by growing *B. anthracis* Sterne(pNFd13:$coaX'$) overnight at 30°C in BHI broth plus 50 µg/ml kanamycin sulfate. The culture was then back-diluted 1:1000 into 75 ml of fresh BHI broth. After incubating at 30°C with shaking at 225-250 rpm until an $A_{600}$ of 0.2-0.3 was reached, the growth temperature was shifted to 39°C, with continued shaking. At $A_{600} = 0.9$ the culture was subjected to several rounds of strain enrichment at 39°C, and the resulting integrants were confirmed by PCR; primers specific
to the integrated plasmid locus, to the intact coaX gene, and to the independently replicating pNFd13:coaX' plasmid were used in this analysis. The B. anthracis Sterne conditional mutant (COAXd) carrying the coaX::pNFd13 genotype was then sporulated at 39ºC as described above.

**Isolation of COAXd genomic DNA and sequencing of the P\textsubscript{spac} promoter region.** COAXd spores were plated on LB-kanamycin in the presence and absence, respectively, of IPTG and incubated overnight at 39ºC. Four colonies were selected from each growth condition (presence and absence of IPTG) and were used as templates for colony PCR, with primers specific to the P\textsubscript{spac} promoter region of coaX::pNFd13 (primers complementary to a region beginning ~200 bp upstream of the P\textsubscript{spac} promoter and extending to the coaX stop codon). The purified pNFd13 P\textsubscript{spac} region was amplified by PCR as a positive control. All PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN) and sequenced by the University of Michigan DNA Sequencing Core.

**Temperature-dependent gene disruption with pBKJ236.** Internal coding sequences (~500 bp) of the B. anthracis Sterne hslO and cysK-1 loci were PCR-amplified using Invitrogen Platinum Taq DNA Polymerase High-Fidelity with primers that provided NotI and BamHI restriction sites at the respective 5'- and 3'-ends of the product. Each product was cloned into pBKJ236 (24), and E. coli INV110 (Invitrogen; dam- and dcm-deficient) transformants (pBKJ236:hslO' and pBKJ236:cysK-1') were selected on BHI-erythromycin plates. The respective pBKJ236:hslO' and pBKJ236:cysK-1' plasmids were transferred into B. anthracis Sterne via conjugation, with selection on BHI-polymyxin B plus erythromycin plates, and B. anthracis Sterne(pBKJ236:hslO') and
-(pBKJ236:cyS-K-1′) transformants were grown overnight in BHI broth plus erythromycin at ambient temperature. These cultures were back-diluted 1:1000 into fresh BHI broth plus erythromycin; overnight incubation at 37°C selected against those transformants that had not integrated the respective pBKJ236 derivative into the chromosome. Chromosomal integrants were then analyzed (hslO::pBKJ236 and cyS-K-1::pBKJ236) for the ability to grow on BHI-erythromycin plates and were confirmed by PCR analysis using primers specific to the integrated plasmid locus.

5′-Rapid amplification of cDNA ends (RACE). The 5′-region preceding the coaX coding sequence was analyzed using the 5′/3′ RACE Kit (Roche Applied Science) with B. anthracis Sterne RNA, prepared as described above, and following the manufacturer's protocol. Using one coaX-specific primer, the cDNA was prepared by RT and purified with the Roche High Pure PCR Product Purification Kit. After addition of a poly(A) tail to the 3′-end of the coaX cDNA, a second coaX-specific primer was used with the oligo dT-anchor primer provided for PCR amplification. After analysis by agarose gel electrophoresis the product was purified with the QIAquick kit and sequenced (GENEWIZ). RACE analysis was performed with each of the three B. anthracis Sterne RNA preparations, in parallel, and gave identical results.

Bioinformatics. Sequence analyses were performed with BLASTP (27), using the NCBI protein databases, with the SEED annotation and analysis tool (9), and with LALIGN (28), using default parameters. Consensus nucleotide sequences (e.g., σA-dependent promoter) were analyzed with the GeneQuest module of Lasergene 6.0 (DNASTAR, Inc.).
RESULTS AND DISCUSSION

Bioinformatics of the *B. anthracis* coaX gene cluster. Sequence analysis of the *Ba*PanK locus (9) indicated that *coaX* might be linked with the *hslO* (encoding the Hsp33 heat shock protein) and *cysK-1* (encoding cysteine synthase A) genes (BA0066 and BA0067, respectively) in a transcriptional unit (Figure 1A). Analysis of the 225-bp intergenic region separating the *ftsH* and *coaX* loci in *B. anthracis* Sterne revealed the presence of a predicted rho-independent transcription terminator, consisting of a 10-bp inverted repeat; this element is separated from the *ftsH* stop codon by only six bases. The intergenic distances between *coaX-hslO* and *hslO-cysK-1* are 7 and 105 bp, respectively (29), and additional bioinformatics analysis demonstrates that the same *coaX-hslO-cysK-1* clusters are conserved in some other *Bacillus* species, in *Geobacillus kaustophilus*, and in several strains of the human pathogen *Listeria monocytogenes*. In *B. subtilis*, the *yacD* protein-encoding gene [limited sequence identity with the *B. subtilis* PrsA peptidyl-prolyl isomerase (30)] is inserted within the *coaX* cluster (Figure 1A), between the *hslO* and *cysK* loci. As *B. subtilis* has both type I (*coaA*) and type III (*coaX*) PanKs, that *coaX* gene can only be deleted from *B. subtilis* strains with the intact *coaA* locus (18).

Table 1 summarizes details of the structural and functional annotations for each of the genes in the *B. anthracis* coaX cluster. The Hsp33 protein, originally characterized in *E. coli* (31), is a redox-regulated chaperone that functions to protect cells against the lethal effects of harsh oxidizing conditions (such as the increasingly toxic environment of the developing spore). Four conserved Cys residues comprise a sensitive redox switch; in the inactive reduced form, these Cys-SH coordinate one Zn$^{2+}$. Under oxidizing conditions, these Cys residues form two intramolecular disulfides, leading to the
Figure 1. coaX is part of a tricistronic operon during exponential growth of *B. anthracis* Sterne. (A) Genomic contexts (9) for *coaX* in *B. anthracis* Sterne (upper) and *B. subtilis* (lower). Primer sets 1-5 for endpoint RT-PCR (arrows) are indicated above the *B. anthracis* open reading frames and correspond to 1, *ftsH-coaX*; 2, *coaX-hslO*; 3, *hslO-cysK-*1; 4, *cysK-*1 inner; and 5, *cysK-*1-*pabB*. (B) Endpoint RT-PCR shows that *coaX* is transcribed as part of a contiguous transcript during exponential growth. The gel shows contiguous products (at A₆₀₀ = 0.4-0.6) from primer sets 2-4, but not from pairs 1 or 5. Controls with genomic DNA and without RT were done for each experiment. (C) Partial sequence of the intergenic region between *B. anthracis* Sterne *ftsH* and *coaX*. Elements of the σₐ*-dependent promoter sequence (-35, TG, and -10) are underlined, as is the ribosome binding site. The *coaX* transcription start site, as determined by 5′ RACE analysis, is indicated by a bent arrow; the start codon (ATG) is boxed.
Table 1. Bioinformatics of the *B. anthracis* coaX gene cluster

<table>
<thead>
<tr>
<th>Function/PDB entry (representative)</th>
<th>Functional homolog in <em>B. subtilis</em></th>
<th><em>B. anthracis</em> sporulation transcriptome&lt;sup&gt;b&lt;/sup&gt;</th>
<th><em>B. anthracis</em> spore proteome&lt;sup&gt;c&lt;/sup&gt;</th>
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<tr>
<td>coaX type III pantothenate kinase/2H3G</td>
<td>BSU00700 75% identity, E value $2 \times 10^{-98}$</td>
<td>Wave II</td>
<td>Not identified</td>
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<tr>
<td>hslO heat shock protein 33/- (1VZY; <em>B. subtilis</em>)</td>
<td>BSU00710 61% identity, E value $2 \times 10^{-101}$</td>
<td>Wave II</td>
<td>Present</td>
</tr>
<tr>
<td>cysK-1 cysteine synthase A/- (1OAS; <em>S. typhimurium</em>)</td>
<td>BSU00730 82% identity, E value $7 \times 10^{-138}$</td>
<td>Wave III</td>
<td>Present</td>
</tr>
</tbody>
</table>

<sup>a</sup> Obtained with BLASTP using the Protein Data Bank (PDB) proteins database.  
<sup>b</sup> Ref (16). coaX and cysK-1 are also up-regulated >2-fold between 1 and 2 h postinfection in host macrophages (17). <sup>c</sup> Ref (32). CysK-1 is also present in the cytoplasmic proteome of cells growing exponentially in LB medium (33).
chaperone-active form. Both the *B. subtilis* Hsp33 [Protein Data Bank entry 1VZY (34)] and the *B. anthracis* hslO gene product conserve the two pairs of redox-regulated Cys residues. Functional cysteine synthase A enzymes, producing Cys from bisulfide (HS\(^-\)) and O-acetylserine, have been characterized in *Salmonella typhimurium* (35) and, more recently, in *B. subtilis* (36) and *M. tuberculosis* (37). CysK-1 and BACoADR were among the proteins identified within the cytoplasmic proteome of *B. anthracis* UM23C1-2 during exponential growth in LB medium (33), and both Hsp33 and CysK-1, as well as BACoADR, are present in the *B. anthracis* spore proteome (32). In *B. subtilis* CysK is also among a number of proteins whose expression levels are significantly increased during disulfide stress (38).

The *coaX*, *hslO*, and *cysK*-1 genes are expressed in *B. anthracis* during waves II and III of sporulation (16), and *coaX* and *cysK*-1 are also up-regulated >2-fold between 1 and 2 h postinfection within host macrophages [Table 1 (17)]. Wave II has been noted (16) to correspond with a time frame during which *B. anthracis* cells progress through germination (from spore inocula) and early outgrowth and reach a relatively rapid growth rate. Because this time frame corresponds to the phases of the *B. anthracis* life cycle that occur within the infected host, genes up-regulated specifically within this interval {e.g., *coaX*, *hslO*, and *coaD* [*coaD* is also up-regulated during infection of host macrophages (17)]} may be particularly significant as potential targets for the design of therapeutically useful molecules, as suggested by Bergman et al. (17).

**coaX is part of a tricistronic operon during exponential growth.** In order to test the suggestion that *coaX*, *hslO*, and *cysK*-1 are transcribed as a single mRNA unit in *B.
*B. anthracis*, we performed RT-PCR (Figure 1B) with RNA isolated from exponentially growing BHI cultures of *B. anthracis* Sterne. These results revealed, as suggested by the bioinformatics analyses, that *coaX* is the first gene in a tricistronic operon. This operon assignment is also consistent with the results obtained with the operon prediction algorithm developed by Bergman et al. (29).

RACE analysis was performed with *B. anthracis* RNA in order to analyze the 5′-region preceding the *coaX* coding sequence (Figure 1C). The *coaX* transcription start site is located 54 bp upstream of the ATG initiation codon, and the -35 and -10 elements of the σ^A^-dependent promoter sequence conserve three (CTGATT) and five (TATGAT) bases, respectively, of the consensus hexanucleotide sequences identified for *B. subtilis* (39). The two promoter elements are separated by a 16-base spacer region, which includes the TG dinucleotide motif at positions -16,-15. The predicted ribosome binding site (AGTGG), deduced by comparison with the highly conserved pentanucleotide sequence described for *B. subtilis* genes (40), is also indicated in Figure 1C. For the 12-bp region containing this motif (the spacing from GTGG to the ATG initiation codon is seven bases), eight bases are complementary to the 3′-end of 16S ribosomal RNA, including one stretch of five consecutive (GGTGA).

Given that CysK-1 was identified within the *B. anthracis* cytoplasmic proteome (33), and in consideration of the longer 105-bp intergenic region separating *hslO* and *cysK-1* (29), we examined the possibility that *cysK-1* could be transcribed independently in *B. anthracis*. The *in vitro* expression values for *coaX* and *hslO* are very related; the Pearson correlation coefficient is >0.9 (N. Bergman, personal communication). However, the correlation coefficient for *hslO* and *cysK-1* (0.63) indicates a much weaker
relatedness, resulting from divergence during exponential growth and early stationary phase or sporulation. For the corresponding 5′ RACE analysis, we prepared total RNA from the hslO::pBKJ236 mutant strain constructed as described in "EXPERIMENTAL PROCEDURES." The chromosomal integration of the 6.3 kb plasmid is expected to have a strong polar effect on downstream transcription of cysK-1 from the coaX promoter, but it would not be expected to interfere with monocistronic transcription of cysK-1 from its own promoter. RACE analysis with cysK-1-specific primers should only yield product from the predicted monocistronic transcript; there should be no tricistronic transcript from the mutant strain. However, analyses of RNA preparations from cultures harvested either at $A_{600} = 0.4$ in BHI broth [known to give the tricistronic coaX-hslO-cysK-1 transcript (Figure 2B)] or at $A_{600} = 1.0$ in LB broth [known to give CysK-1 protein expression in B. anthracis UM23C1-2 (2)] failed to provide evidence for any cysK-1 transcript (data not shown).

In B. subtilis a monocistronic cysK mRNA is induced in the presence of diamide [disulfide stress (38)], in a ΔcymR mutant lacking the cysteine metabolism repressor [CymR (41)], by the Spx global transcriptional regulator (42), and during growth on Met as sulfur source (43). The promoter region ($\sigma^A$-dependent) was mapped for the B. subtilis cysK locus, and the CymR repressor was shown to bind a cysK promoter fragment. Although direct evidence for independent cysK-1 transcription is lacking in B. anthracis Sterne, we were able to identify consensus -35 and -10 elements in the 5′-region preceding the cysK-1 locus (Figure 2A); the two segments match the B. subtilis $\sigma^A$-dependent consensus in 10 of 12 total positions. However, the spacer length of 22 bp is significantly different from the $17\pm1$-bp average reported by Helmann (39); in his
Figure 2. Comparison of experimentally-characterized and putative promoter and CymR-binding elements for *B. subtilis cysK* and *B. anthracis* Sterne *cysK*1, respectively, with the corresponding consensus sequences. Sequence numbering is given for the *B. subtilis cysK* promoter and transcript. (A) $\sigma^\alpha$-dependent promoter sequences for *cysK* (experimental) and *cysK*1 (putative) are compared with the consensus [Ref. (39); SS = transcription start site). Spacer lengths are given in parentheses; the putative *cysK*1 promoter sequence lack the TG dinucleotide in position -14, -15, and its 22-bp spacer length is longer than the average of 17±1 bp. (B) CymR-binding motifs are identified in the 5′-regions preceeding both *cysK* and *cysK*1 and are compared with the consensus sequence as established in Ref. (41). For that consensus, W represents T or A, M represents C or A, and N represents any base, as in (A) above. Specific designations represent bases present in at least six of the seven CymR target promoter regions analyzed (41). (C) Ribosome binding sites are compared with the “ideal” *B. subtilis* sequence give in Ref. (40). Initiation codons (ATG) are given in each case.
analysis of 142 experimentally-characterized \textit{B. subtilis} $\sigma^A$-dependent promoter sequences, none had spacer lengths greater than 20 bp. The canonical TG dinucleotide, which was identified in 45\% of those promoters, is absent from the putative \textit{B. anthracis} Sterne \textit{cysK-1} promoter as well. In their analysis of CymR target genes in \textit{B. subtilis}, Even et al. (41) developed a 27-bp consensus binding motif. Our sequence analysis also identifies the CymR-binding motif (Figure 2B), which lies adjacent to the putative \textit{cysK-1} ribosome binding site. The \textit{B. anthracis} motif matches the consensus in 22 of 27 positions; the \textit{B. subtilis} \textit{cysK} CymR-binding sequence matches the consensus in 23 positions (41). A homolog of the \textit{B. subtilis} CymR repressor has been identified in the \textit{B. anthracis} protein database (BA4627; 79\% identity, \textit{E} value $6e^{-37}$); in addition, a relatively weak consensus ribosome binding site (Figure 2C) does provide five consecutive bases (AGGAG) complementary to the 3′-end of 16S ribosomal RNA and gives the proper spacing (7 bp) preceding the \textit{cysK-1} initiation codon.

\textbf{Construction and analysis of \textit{coaX} mutants.} We repeatedly failed to construct a markerless deletion mutant of \textit{coaX} in \textit{B. anthracis}, suggesting that \textit{BaPanK} is essential. We therefore decided to construct a conditional mutant by placing \textit{coaX} under the control of \textit{P_{spac}}, following the temperature-dependent, pNFd13 insertion mutagenesis protocol developed by Fisher and Hanna (23). PCR was used to assess the genomic structure of the \textit{coaX::pNFd13} mutant (COAXd) and the absence of replicating plasmid at 39°C; these results are given in Figure 3A. Unlike the markerless gene replacement protocol (24), the pNFd13 insertion mutagenesis method is expected to introduce polar effects on the downstream \textit{hslO} and \textit{cysK-1} genes. The chaperone function of Hsp33 is redox-regulated (31, 34); the reduced form of the \textit{E. coli} protein is inactive, and we would not
Figure 3. Construction of the COAXd mutant strain and the effect of IPTG addition on growth. (A) Lanes 2-5; at the permissive temperature of 30°C, PCR analyses confirm the presence of the independently replicating pNFd13:coaX’ plasmid (lane 2), the intact chromosomal copy of coaX (lane 3), and the coaX::pNFd13 integrant (lanes 4 and 5). Lanes 6-9; at the restrictive temperature 39°C, PCR analyses confirm the absence of both the independently replicating plasmid (lane 6) and the intact coaX locus (lane 7), as well as the presence of the chromosomal integrant (lanes 8 and 9). Lane 1 is a 1-kb ladder. The four primer combinations used, both for lanes 2-5 and for lanes 6-9, were NFd-FOR/NFd-REV; US-FOR/DS-REV (coaX) US-FOR/NFd-REV; and NFd-FOR/DS-REV. Sequences are available upon request. (B) Effects of IPTG on the B. anthracis Sterne COAXd mutant strain. The COAXd strain, in which the expression of coaX is controlled by Pspac, was grown in the presence of 50 µM IPTG and diluted 40-fold into fresh prewarmed BHI broth with (■) or without 50 µM IPTG. The wild-type strain (□) is included as a control. At 4 h, different concentrations of IPTG were added (indicated by the arrow) to those cultures inoculated without IPTG: ●, COAXd without IPTG; ○, COAXd with 5 µM IPTG; △, COAXd with 10 µM IPTG; and ▲, COAXd with 50 µM IPTG. The COAXd(Su) suppressor mutant is responsible for growth observed in the absence of IPTG.
expect the *B. anthracis* protein to be functional in the highly reduced intracellular environment accompanying vegetative growth. In addition, BLASTP analysis of the *B. anthracis* genome, using the *B. anthracis* CysK-1 as the query, identifies both CysK-2 (59% identity, *E* value 4e\(^{-101}\)) and CysM (44% identity, *E* value 1e\(^{-70}\)) homologs. The existence of multiple cysteine synthase forms would be expected to provide a level of redundancy that makes it unlikely that CysK-1 is essential for growth, particularly in rich media. Nonetheless, we tested these possibilities for the *hslO* and *cysK-1* loci, respectively, via insertional inactivation. Both *hslO::pBKJ236* and *cysK-1::pBKJ236* mutant strains grew on BHI-erythromycin plate; Figure 4 gives the PCR results confirming the genomic structures of the respective chromosomal integrants. Neither *hslO* nor *cysK-1* gene products are essential for *B. anthracis* growth under these conditions; the results presented for COAXd (see below) are solely attributable to the conditional disruption of the *coaX* gene and/or the stable suppressor mutation. This conclusion is also supported by the earlier failure experienced in attempting to construct markerless *coaX* deletion mutants.

In BHI cultures containing kanamycin, with 50 µM IPTG added at the time of inoculation (with heat-activated spores), the *coaX::pNFd13* (COAXd) mutant strain grows similarly to the wild-type Sterne strain (Figure 3B). When different concentrations of IPTG (5-50 µM) were added to cultures incubated initially (for 4 h) without IPTG, growth was also observed. However, we were very surprised to observe growth of the COAXd strain, in the absence of IPTG, ~7 h after inoculation. On solid LB media containing kanamycin, in the absence of IPTG, ~10 single colonies appeared (from COAXd spores) after overnight (~12 h) incubation; these colonies were distinctly smaller.
Figure 4. Construction of *hslO* and *cysK-1* mutants. Diagnostic PCR confirming the correct integrations of pBKJ236:*hslO*′ (lanes 2 and 3) and of pBKJ236:*cysK-1*′ (lanes 4 and 5) into *hslO* and *cysK-1* loci, respectively. Lane 1 is a 1-kb ladder. The respective primer combinations used (sequences available upon request) were: lane 2, US-FOR/BKJ-REV (*hslO*); lane 3, BKJ-FOR/DS-REV (*hslO*); lane 4, US-FOR/BKJ-REV (*cysK-1*); lane 5, BKJ-FOR/DS-REV (*cysK-1*).
in size than those observed for COAXd in the presence of 10 mM IPTG. Figures 5A and 5B contrast the confluent growth observed from COAXd spores in the presence of IPTG versus the small number of morphologically distinct colonies observed in the absence of inducer. After an additional ~18 h of incubation, the several small colonies (Figure 5B) develop into near-confluent growth in the absence of IPTG (Figure 5C). In order to test the possibility that the slow growth from COAXd on solid media in the absence of IPTG could be due to a stable suppressor mutation, a single colony from the overnight growth was used to inoculate a fresh LB-kanamycin plate. The confluent growth observed after ~12 h incubation (Figure 5D) is very similar to that observed originally in the presence of IPTG and supports the working hypothesis that a stable suppressor mutation allows growth in the absence of IPTG in both liquid (Figure 3B) and solid media.

**COAXd growth is due to a stable suppressor mutation.** The unexpected growth observed from COAXd spores in the absence of IPTG, both in liquid BHI cultures and on LB agar plates, displayed characteristics still very different from either the wild-type Sterne strain or even from COAXd in the presence of IPTG. An ~8-h lag precedes exponential growth in BHI broth in the absence of IPTG, in contrast to the 1-h lag (from spores) in the presence of IPTG. The stability of this adapted growth phenotype for COAXd suggests the isolation of a stable suppressor mutant. We considered two plausible explanations, the first of which involves a possible gain-of-function mutation in the inactive *B. anthracis* homolog ["hypothetical protein BA2901;" *E* value 4e-45 (6)] of the functional *S. aureus* type II PanK (19, 44). However, under the selective pressure to produce CoASH in a temperature-sensitive *E. coli* coaA mutant, attempts to complement with the BA2901 protein-encoding gene were unsuccessful. This is consistent with our
Figure 5. COAXd growth in the absence of IPTG is due to a stable suppressor mutation. Washed COAXd spores were plated on LB-kanamycin with (A) and without (B) 10 mM IPTG. Both plates were incubated overnight at 39°C. (C) The overnight incubation for COAXd in the absence of IPTG was continued for a total of 30 h. (D) A single colony from (B) was used to streak a fresh LB-kanamycin plate, which was incubated overnight in the absence of IPTG at 39°C. The confluent growth observed demonstrates that a stable suppressor mutant, COAXd(Su), is responsible.
earlier report (6) that the purified recombinant BA2901 protein is inactive in the in vitro PanK assay; as with the recently characterized yeast type I PanK homolog YFH7 (45), we conclude that the BA2901 protein is an ATP-dependent small molecule kinase, but Pan is not the acceptor substrate.

The second scenario considered, however, involves an altered expression mutation within the Pspac promoter employed in pNFd13 mutagenesis (23). We considered the possibility that a stable coaX suppressor mutant [COAXd(Su)], specifically involving the lac repressor:operator interaction, might have arisen under the selective pressure to produce CoASH. We evaluated this suggestion by isolating multiple clones of COAXd, grown in the presence of IPTG, and of COAXd(Su), grown in the absence of IPTG. Representative sequence results for the Pspac regions from pNFd13, COAXd, and COAXd(Su) are given in Figure 6A. The pNFd13 and COAXd Pspac sequences (four independent COAXd clones) are identical. However, the sequences of four independent COAXd(Su) clones reveal a single base substitution of adenine for guanine at base pair 5 [lac operator base numbering system of Sadler et al. (46)] of the lac operator. That transcription of the wild-type coaX operon is indeed occurring in COAXd(Su) is demonstrated in Figure 6B, where RT-PCR results using the same primers described for Figure 2B are given for COAXd and for COAXd(Su). The same tricistronic coaX transcripts are present in both cases.

While Scharf et al. (47) reported no growth for the conditional (Pspac-regulated) B. subtilis trxA mutant for at least 12 h in the absence of IPTG, Prágai and Harwood (48) observed exponential growth for the Pspac-controlled B. subtilis yscC (putative GTP-binding protein) mutant, but only after a lag of more than 20 h. The lac operator of Pspac
Figure 6. The COAXd(Su) suppressor mutant carries a single-base substitution within the lac operator and constitutively produces the tricistronic coaX transcript. (A) Multiple alignment of partial sequences for the Pspac promoter regions of pNFd13 (Pspac), the COAXd conditional mutant grown in the presence of IPTG, and the COAXd(Su) suppressor mutant isolated in the absence of IPTG. Details are given in "Materials and Methods;" four individual colonies were analyzed for both COAXd and COAXd(Su), with internally identical results. σ^A-dependent promoter and lac operator sequences are boxed. The COAXd(Su) lac operator carries a guanine→adenine mutation in position 5; this position is boxed and indicated with an asterisk (*). (B) Total RNAs were isolated from COAXd and COAXd(Su) as described in "EXPERIMENTAL PROCEDURES" for wild-type B. anthracis Sterne, but cultures were grown in the presence and absence, respectively, of IPTG and at 39˚C. Samples were analyzed by endpoint RT-PCR using the primer sets described in Figure 2. The gels show contiguous coaX transcripts for both samples.
consists of a 20-bp palindromic sequence with perfect twofold symmetry (46); each operator binds one dimer of the tetrameric lac repressor. In the \textit{B. subtilis} \textit{trxA}::pHV501 conditional mutant, for example, repressor bound to the \textit{lac} operator within P_{spac} blocks \textit{trxA} transcription in the absence of IPTG, and no growth is observed. The presence of IPTG leads to repressor dissociation, \textit{trxA} transcription, and growth. The delayed growth of the \textit{B. subtilis} \textit{ysxC}::pYSXCF conditional mutant in the absence of IPTG was linked to a stable suppressor mutation within that P_{spac} lac operator [thymine for cytosine at base pair 10 (numbering system described above)], resulting in constitutive \textit{ysxC} transcription and growth.

Sadler et al. (46) first designed, constructed, and evaluated the completely symmetric synthetic version of the \textit{E. coli} \textit{lac} operator that is used in pNFd13 and pYSXCF and in their pMUTIN4 precursor (49); in this early work, the authors also summarized the results of previous studies showing that substitution of adenine for guanine at base pair 5 of the natural \textit{lac} operator, equivalent to the mutation identified in COAXd(Su), resulted in a \textit{lac} repressor affinity 1\% that of the wild-type operator. The identical base change at the symmetric position (cytosine at position 17→thymine) resulted in 6\% wild-type repressor affinity, indicating that \textit{lac} repressor:operator interactions involving the left half of the natural operator sequence contribute more to overall binding. Taken together with the strong evidence that at least two repressor subunits contact the operator in similar ways, the combination of DNase I protection assays and additional symmetric mutations added to the conclusion that the left operator half involved in the COAXd(Su) mutation was the unit optimally recognized and bound
by a repressor subunit. In a subsequent report (50), the simultaneous substitution of adenine for guanine at position 5 (numbering system described above) and thymine for cytosine at position 16 was shown to result in a repression value of 3, in contrast to the value of >200 for the ideal synthetic operator. This decreased repression was reflected in a >100-fold increase in β-galactosidase activity in the presence of lac repressor.

On the basis of these independent analyses of both ideal and natural lac operator mutants, we conclude that the guanine to adenine mutation in the COAXd(Su) lac operator reduces repressor affinity, allows transcription of the wild-type coaX operon (as demonstrated in Figure 6B) and CoASH synthesis, and restores growth in the absence of IPTG. The reports of Scharf et al. (47) and Prágai and Harwood (48) represent two cases of pMUTIN-based conditional mutants involving essential genes in B. subtilis, and other investigators have noted that pMUTIN constructs are often unstable under these circumstances (U. Gerth, personal communication). The major distinction in the respective experimental protocols with the trxA and ysxC mutants is the extended time course of 30 h in the latter case; it is possible that a trxA suppressor mutant would have initiated exponential growth beyond 12 h. The Pspac elements in the two conditional mutants did carry different versions of the lac operator, and the ysxC mutant also carried the lacI gene on a separate multicopy plasmid.

CoASH biosynthetic enzymes, in particular PanK, continue to be recognized as possible new antimicrobial targets that may provide for the development of therapeutically useful molecules (13). As distinguished from the type II human PanK (22), the type III PanKs share very limited sequence identity and exhibit distinct kinetic properties and substrate preferences (10, 19); they also reveal drastic differences in
binding modes for both ATP and Pan substrates (21). Yang et al. (21) have concluded that these differences should be exploited in the development of new inhibitors specifically targeting BaPanK and other type III enzymes. This work provides evidence supporting the conclusion that the type III PanK is an essential enzyme in B. anthracis, thus contributing to its validation as a target for such development.
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CHAPTER IV

PYRIDINE NUCLEOTIDE COMPLEXES WITH Bacillus anthracis COENZYME A-DISULFIDE REDUCTASE: A STRUCTURAL ANALYSIS OF DUAL NAD(P)H SPECIFICITY

Jamie R. Wallen, Carleitta Paige, T. Conn Mallett, P. Andrew Karplus, and Al Claiborne

The following manuscript is reproduced with permission, Biochemistry, volume 47, pages 5182-5193, copyright 2008 American Chemical Society. Stylistic variations are due to the requirements of the journal. Wallen performed kinetic assays with wild-type and mutant BACoADR proteins, and solved the crystal structure of the native enzyme, as well as in complex with pyridine nucleotides. Paige cloned genes for BACoADR and mutant proteins into pTHCm plasmid and purified each to homogeneity. Mallet provided oversight during X-ray diffraction data collection and structure refinement. Karplus and Claiborne acted in editorial capacities.
ABSTRACT

We have recently reported that CoASH is the major low-molecular weight thiol in *Bacillus anthracis* [Nicely, N.I., Parsonage, D., Paige, C., Newton, G.L., Fahey, R.C., Leonardi, R., Jackowski, S., Mallett, T.C., and Claiborne, A. (2007) Biochemistry 46, 3234-3245], and we have now characterized the kinetic and redox properties of the *B. anthracis* coenzyme A-disulfide reductase (CoADR, BACoADR) and determined the crystal structure at 2.30 Å resolution. While the *Staphylococcus aureus* and *Borrelia burgdorferi* CoADRs exhibit strong preferences for NADPH and NADH, respectively, *B. anthracis* CoADR can use either pyridine nucleotide equally well. Sequence elements within the respective NAD(P)H-binding motifs correctly reflect the preferences for *S. aureus* and *Bo. burgdorferi* CoADRs, but leave questions as to how BACoADR can interact with both pyridine nucleotides. The structures of the NADH and NADPH complexes at ca. 2.3 Å resolution reveal that a loop consisting of residues Glu180-Thr187 becomes ordered and changes conformation on NAD(P)H binding. NADH and NADPH interact with nearly identical conformations of this loop; the latter interaction, however, involves a novel binding mode in which the 2′-phosphate of NADPH points out toward solvent. In addition, the NAD(P)H-reduced BACoADR structures provide the first view of the reduced form (Cys42-SH/CoASH) of the Cys42-SSCoA redox center. The Cys42-SH side chain adopts a new conformation in which the conserved Tyr367′-OH and Tyr425′-OH interact with the nascent thiol(ate) on the flavin *si*-face. Kinetic data with Y367F, Y425F, and Y367,425F BACoADR mutants indicate that Tyr425′ is the primary
proton donor in catalysis, with Tyr367′ functioning as a cryptic alternate donor in the absence of Tyr425′.
INTRODUCTION

In *Bacillus subtilis*, diamide-mediated disulfide stress affects the transcription of more than 20% of all genes (1); the inductions observed for the genes encoding thioredoxin, thioredoxin reductase, cysteine synthetase A, and other putative Cys biosynthesis genes have been taken to suggest that free Cys may play a role in *B. subtilis* analogous to that played by GSH in other organisms. Indeed, *B. subtilis* lacks GSH, and Cys is the major low-molecular weight thiol (2). Very recently, Hochgräfe et al. (3) demonstrated a six-fold increase in protein-associated $^{35}$S radioactivity in *B. subtilis* cells stressed with diamide in the presence of $[^{35}S]$Cys (and chloramphenicol, inhibiting protein synthesis); over 80% of the $^{35}$S label incorporated into proteins could be removed by treatment with disulfide reductants. These results have been taken to support the conclusion that S-thiolation by Cys represents a general, reversible mechanism for protection and regulation of protein-SH groups during disulfide (and other oxidative) stresses in *B. subtilis*. A complementary report (4) showed that the redox-regulated *B. subtilis* OhrR protein was S-thiolated on Cys15 in cells challenged with cumene hydroperoxide; in addition to Cys, mixed disulfides were also identified with CoASH and with a new 398-Da thiol of as-yet-unknown structure. In contrast, in other *Bacillus* species, CoASH rather than Cys is the major low-molecular weight thiol, with ~45% of the total CoASH in *Bacillus megaterium* spores being present as soluble protein-SSCoA (5). Also, mature *B. megaterium* spores were shown (6) to contain a flavoprotein disulfide reductase that catalyzed the NADH-dependent reduction of CoA-disulfide (CoAD) $\rightarrow$ 2CoASH; the 75% reduction of protein-SSCoA (to protein-SH plus CoASH) observed during spore germination (5) was linked to this enzyme.
Very recently, Ojha et al. (7) have classified coenzyme A-disulfide reductase (CoADR) as one of the prototype enzymes of the NADH Peroxidase/Oxidase and CoAD Reductase (POR) subgroup [also previously identified as Group 3 of the PNDOR family (8)] of the "two dinucleotide binding domains" flavoproteins superfamily. A critical distinction between CoADR and all other PNDOR enzymes, including the Group 1 and Group 2 enzymes (7), lies in the fact that CoADR is the only disulfide reductase that uses a single active-site Cys in catalysis (9-11). The recent crystal structure for Staphylococcus aureus CoADR [SACoADR (12)] revealed the resting state of the enzyme as containing a mixed disulfide of this Cys (SACoADR Cys43) with CoASH; this nonflavin redox center plays an essential role in catalysis. The structure also identified two Tyr residues in the active site, Tyr361′ and Tyr419′, that were proposed to be important in catalysis.

Furthermore, a set of sequence motifs was developed to allow identification of other members of the CoADR class. On this basis, we have now identified the NADH-dependent CoADR in B. megaterium (E value 5e-75)\(^1\) CoADRs have also been identified in Bacillus anthracis and other members of the Bacillus cereus group (12), but not in B. subtilis 168.\(^2\) In B. anthracis Ames, we identified a second, multimodular CoADR isoform as well, in which the CoADR module is linked to a C-terminal Rhodanese Homology Domain [RHD (12)]. Recent transcriptome analyses (13) have shown that

\(^1\) http://www.bios.niu.edu/b_megaterium/index.html

\(^2\) The four lowest E-values range from 7e-26 to 3 e-8; the essential Cys43 equivalent is absent in the two closest matches, and alignments with two more distantly related proteins are limited to the NADPH-binding domain of S. aureus CoADR.
BACoADR (BA1263) and CoADR-RHD (BA0774) are expressed in waves III and V, respectively, during growth and sporulation of *B. anthracis* Sterne. The presence of BACoADR in the fractured spore was confirmed by proteomics analysis (14), providing an additional link with the NADH-dependent CoADR purified from *B. megaterium* spores (6). BACoADR was also identified within the cytoplasmic proteome of *B. anthracis* UM23C1-2 during exponential growth in rich medium (15), suggesting that it plays a general role in thiol-disulfide homeostasis. Other multimodular proteins that include a CoADR module have been identified in the category A biodefense pathogen *Clostridium botulinum* [CoADR-RHD-SirA-COG2210 (16)] and in the anti-tumor agent *Clostridium novyi-NT* [CoADR-RHD (17)].

As in *S. aureus* (9), and in an interesting contrast with respect to *B. subtilis* (2), CoASH is the major low-molecular weight thiol in *B. anthracis* (18), *B. megaterium* (6), and *B. cereus* (19), as well as the human pathogen *Borrelia burgdorferi* (20). While the *S. aureus* CoADR expresses a preference for NADPH, the enzymes from *B. megaterium* and *Bo. burgdorferi* prefer NADH. In contrast, an analysis of the NAD(P)H-binding motif for the *B. anthracis* CoADR was suggestive of a dual specificity for the pyridine nucleotide substrate. We have undertaken kinetic and spectroscopic studies of BACoADR and selected active-site mutants and also present the crystal structures for oxidized wild-type BACoADR and for reduced enzyme complexes with the product CoASH and either NADH or NADPH. These results add to our understanding of catalysis by this PNDOR Group 3 disulfide reductase, demonstrating that BACoADR exhibits dual specificity with respect to the NAD(P)H substrate and revealing how
residues that serve to discriminate between NADH and NADPH in other PNDOR enzymes serve to promote recognition of both pyridine nucleotides in BACoADR.
EXPERIMENTAL PROCEDURES

Expression and Purification of Wild-Type and Mutant BACoADRs. Genomic DNA from *B. anthracis* Ames was provided by Dr. Arthur Friedlander, U.S. Army Medical Research Institute of Infectious Diseases. *B. anthracis* CoADR (NP_843735; "pyridine nucleotide-disulfide oxidoreductase, class I") was expressed with an N-terminal His-tag provided by the pTHCm plasmid vector in *Escherichia coli* B834(DE3) cells. These cells were grown in an 8-L culture (10 x 800 mL in 2.8-L Fernbach flasks) of tryptone-yeast extract-phosphate medium (21) supplemented with 30 mM glucose and 100 μg/mL chloramphenicol at 37°C. Cultures were induced with 1 mM isopropyl-β-D-thiogalactopyranoside at an $A_{600}$ of 1.0 and allowed to grow 4-8 h at 37°C. Following centrifugation at 6,220g for 20 min, the washed cell pellets were resuspended in a minimal volume of 25 mM sodium phosphate, pH 7.0, containing 2 mM EDTA. A small amount of DNase was added, and cells were lysed via disruption with a pneumatic cell homogenizer (Avestin EmulsiFlex-C5). After centrifuging at 39,200g, streptomycin sulfate was added (2% w/v) to the supernatant, with stirring, for 20 min. The supernatant obtained on subsequent centrifugation was filtered and loaded onto a Q-Sepharose HP column equilibrated in 25 mM sodium phosphate, pH 7.0. The protein was eluted with a 0.1 M NaCl gradient in the loading buffer; yellow fractions were pooled and loaded onto a Ni-NTA Superflow (Qiagen) column equilibrated in 50 mM sodium phosphate, pH 8.0, containing 0.5 M NaCl and 20 mM imidazole. The His-tagged BACoADR was eluted by increasing the imidazole concentration to 100 mM. The BACoADR pool was dialyzed into 50 mM potassium phosphate, pH 7.0, containing 0.5 mM EDTA, concentrated to 10 mg/mL, and flash-frozen in liquid nitrogen as 0.02-0.5-mL aliquots and stored at -80°C.
The expression and purification protocol for SeMet BACoADR was essentially identical to that described for the native protein, except that cultures were grown in MOPS medium (EZ Rich Defined Medium, Teknova), with SeMet replacing Met. Yields for the native and SeMet BACoADR were ca. 15 mg/L of culture.

Mutant BACoADR were generated with the QuikChange Site-Directed Mutagenesis Kit (Stratagene), using the wild-type and Y367F (for the Y367,425F double mutant) expression plasmids as templates. The presence of only the desired mutations was confirmed by sequence analysis. Protocols and yields for the BACoADR mutant proteins were very similar to that described for the native protein, except that 250 mM imidazole was used to elute the Tyr→Phe mutant proteins from the Ni Sepharose High Performance (GE Healthcare) column.

**BACoADR Assays and Anaerobic Titrations.** The activities of wild-type and mutant BACoADR were measured as described earlier for SACoADR (11), using a Cary 50 spectrophotometer (Varian) thermostated at 25°C; initial rates of ∆A_{340} were corrected for NAD(P)H oxidase activities measured in parallel. Stopped-flow measurements of BACoADR activity were carried out with the Applied Photophysics DX.17 MV stopped-flow spectrophotometer (11), as described previously with SACoADR, except that the final BACoADR concentration on mixing was 50 nM. CoAD concentrations were varied over the range 0.5-40 μM, at fixed NAD(P)H concentrations ranging from 0.5-20 μM, and initial rates (six measurements each) were analyzed as described by Cornish-Bowden (22) in order to determine steady-state kinetic parameters.

Extinction coefficients for wild-type and C42S BACoADR proteins (ε_{453} = 11,100 M^{-1}cm^{-1} and ε_{440} = 13,100 M^{-1}cm^{-1}, respectively) were determined by standard methods.
Anaerobic titrations followed established protocols using Agilent model 8453 and Hewlett-Packard model 8452A diode-array spectrophotometers. The anaerobic gas train used to remove residual oxygen from the nitrogen supply has been updated to include 2 Oxiclear disposable gas purifier cartridges connected in-line with copper tubing and an Oxiclear indicating oxygen trap (23).

**Crystal Preparation and Data Collection.** Thawed aliquots of the native and SeMet BACoADR proteins were buffer-exchanged into 10 mM sodium N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid, pH 7.2, to give final protein concentrations of 10 mg/mL. Large, single crystals grew best in the presence of 2 mM NAD⁺ or NADP⁺, in 24-well sitting-drop plates over reservoirs of 0.5 mL 16-26% 2-methyl-2,4-pentanediol, 0.2 M magnesium acetate, and 0.1 M sodium cacodylate, pH 6.5, at 15°C; drop sizes were 4 + 4 μL. Crystals appeared within 1 day, growing to full size in 1 week, and were flash-frozen in a nitrogen stream at 100 K after being removed from the drop. To obtain structures for the NADH and NADPH complexes, a 150 mM NAD(P)H stock solution [freshly prepared in 10 mM sodium N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid, pH 7.2] was first diluted 3-fold into a suitable cryoprotectant solution (based on the concentration of 2-methyl-2,4-pentanediol used with that crystal). The crystal was soaked in 10 μL of this solution for 1-5 min, during which time a color transition from yellow→faint red occurred (24); the crystal was then flash-frozen in a nitrogen stream at 100 K. Data sets for native BACoADR and for the BACoADR-NADH complex were collected on a Rigaku Saturn-92 CCD detector using Cu-Kα radiation from a MicroMax-007 rotating anode X-ray generator; data sets for MAD
and/or single-wavelength anomalous dispersion phasing with SeMet BACoADR and for the BACoADR-NADPH complex were collected at beamline X26C of the National Synchrotron Light Source, using an ADSC Quantum-4 CCD detector. The SeMet BACoADR and BACoADR-NADH data sets were indexed, integrated, and scaled in d*TREK (25); the native and BACoADR-NADPH data sets were processed using MOSFLM and SCALA (26) and HKL2000 (27), respectively. Table 1 summarizes data collection statistics for native and SeMet BACoADR and for the BACoADR-NAD(P)H complexes.

**BACoADR Phasing and Structural Refinement.** We initially pursued a molecular replacement strategy using the CoADR module (35% identity) from *B. anthracis* CoADR-RHD as the search model. (The structure of CoADR-RHD has been determined in this laboratory at 2.1 Å resolution with an *R*-free of 21.8% and will be the subject of a separate report.)  

Using MOLREP (28) and the native BACoADR data set (to 3 Å), the CoADR portion of CoADR-RHD, with side chains changed to Ala, yielded an apparent solution for two molecules in the asymmetric unit. $2F_o - F_c$ and $F_o - F_c$ electron density maps calculated after initial rounds of rigid body refinement and simulated annealing using CNS (29) suffered from model bias, and attempts to refine the BACoADR model stalled at *R*-factor and *R*-free of 44 and 50%, respectively. Side chains conserved in CoADR-RHD and BACoADR were added to the poly-Ala model during this stage of refinement.

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**Table 1.** Data Collection Statistics for Native Oxidized and SeMet BACoADR and Native Complexes with NADH and NADPH

- **SEM**e BACoADR peak
- **SEM**e BACoADR edge

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*Numbers in parentheses represent data for the highest resolution shell.*
A three-wavelength MAD data set was collected with a single SeMet BACoADR crystal, but attempts to locate the 13 Se sites per monomer with SOLVE (30) were unsuccessful. However, an anomalous difference map calculated at 2.0 Å resolution, using the peak wavelength data set from the MAD experiment and the initial molecular replacement phases from the BACoADR model, allowed the location of 21 of the 26 expected Se sites per dimer in anomalous difference peaks (of >7σ). These sites were then used to phase the structure with SOLVE, using either the peak wavelength (single-wavelength anomalous dispersion, to 3 Å) or the complete MAD data set (to 2.5 Å). Density modification and automatic building using RESOLVE (31, 32) with the MAD SOLVE phases allowed 85% of the dimer to be built, and electron density maps from both the single-wavelength anomalous dispersion and MAD SOLVE/RESOLVE phases (figure of merit = 0.75) were used to build the remaining residues manually with O (33). At this point residues Arg181-Thr187 were not visible; these were only added in the final stages of refinement. Refinement was carried out at 2.5 Å resolution using CNS with rounds of manual rebuilding, simulated annealing, energy minimization, and group B-factor refinement; FAD and CoAS- were added during these rounds, which led to a model with $R$-factor = 27.5% and $R$-free = 29.8%. Phase extension and density modification with SOLOMON (34) incorporated the 1.90 Å resolution data. During further refinement in CNS, water molecules were identified using a 3σ difference Fourier cutoff. Final rounds of refinement were performed using REFMAC5 (35) and COOT (36), resulting in $R$-factor and $R$-free of 19.9% and 23.0%, respectively.

The final SeMet BACoADR structure was used to determine the native BACoADR by molecular replacement, and this structure was also refined in REFMAC5.
with manual rebuilding in COOT. Waters (in $2F_o-F_c$ peaks greater than 1σ) were also added. Both BACoADR-NADH and BACoADR-NADPH structures were determined using oxidized BACoADR as the molecular replacement model; initial refinements using CNS were followed with TLS (37) plus restrained refinement in REFMAC5. Refinement statistics for all models are summarized in Table 2. NCS restraints were not used in refinement.
Table 2. Crystallographic Refinement Statistics for Native Oxidized and SeMet BACoADR and Native Complexes with NADH and NADPH.

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*BACoADR dimer.*

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130
RESULTS AND DISCUSSION

Catalytic, Spectral, and Redox Properties of BACoADR. Protocols for expression and purification of recombinant wild-type, C42S, and Y367F, Y425F, and Y367,425F BACoADRs differed from that used with SACoADR, in that all proteins carried an N-terminal His-tag. Earlier SACoADR assays with 100 μM NADH replacing NADPH (K_m ~0.2 μM) gave apparent k_cat = 4-5 s^-1, or ~17% that with NADPH (11). A more recent analysis of the Bo. burgdorferi CoADR (20) yielded apparent k_cat values of 14 s^-1 and 0.3 s^-1 with NADH and NADPH, respectively, indicating a pyridine nucleotide preference opposite that of SACoADR. Stopped-flow initial velocity measurements with BACoADR over a range of [CoAD] at several fixed [NADH] or [NADPH] yield a series of lines intersecting on the y-axis, consistent with a substituted-enzyme mechanism (22). k_cat with NADH = 27 s^-1, with K_m(NADH) = 1 μM and K_m(CoAD) = 2 μM; with NADPH the respective parameters are 28 s^-1, 3 μM, and 6 μM. The specificity constants for BACoADR with NADH and NADPH are 2.7 x 10^7 M^-1s^-1 and 9.3 x 10^6 M^-1s^-1, respectively. These results demonstrate that BACoADR exhibits dual specificity with respect to the pyridine nucleotide substrate, in contrast to the CoADRs from S. aureus and Bo. burgdorferi.

Dithionite titration of BACoADR provides spectral evidence for an asymmetric EH_2 species in which one FAD per dimer is reduced; there is a 55% decrease in A_453 on addition of 1.2 equiv of dithionite/FAD. Although there is a small increase in A_525 at this

---

^4 Preparations of the NADH-dependent CoADR enzyme from B. megaterium were reported to be inactive with NADPH, in addition (6).
point, there is not a convincing EH$_2$ charge-transfer (Cys-S$^-$→FAD) intermediate (8, 38, 39), and complete flavin reduction is observed with 2 equiv of dithionite/FAD. The titration behavior observed with NADH is significantly different (Figure 1); the maximal decrease in $A_{453}$ (only ca. 28% reduction, where 100% reduction represents zero absorbance at 453 nm) coincides with addition of 1.1 equiv of NADH/FAD. Judging from the isosbestic point at 346 nm (determined from difference spectra over the titration range 0-0.7 equiv of NADH), stoichiometric oxidation of added NADH is observed at this stage. Figure 1 also indicates that a significant Cys-S$^-$→FAD charge-transfer component is present. As NADH is added, to 2.1 equiv/FAD, this $A_{525}$ component effectively doubles, commensurate with the observed increase in $A_{453}$. Essentially identical spectral changes are observed on titration with NADPH, and in both cases the dimeric EH$_2$ form is stabilized in the presence of up to 4 equiv of NAD(P)H/FAD (Figure 1 and Scheme 1). Since the intramolecular redox event observed as E(FADH$_2$) oxidation with wild-type BACoADR requires the addition of the second equiv of NADH, it is reasonable to suggest that NADH is tightly bound to one or both subunits in the EH$_2$ form. Direct evidence in support of this is provided with the crystal structures described in a later section. For the BACoADR C42S mutant, dithionite titration yields complete FAD reduction, as expected (40), with 1 equiv/FAD. However, titrations of the mutant with either NADH or NADPH fail to yield full flavin reduction on addition of even 10-13 equiv of NAD(P)H/FAD. Absorbance changes at 340 nm and beyond 500 nm are suggestive of E[FAD-NAD(P)H] complex formation, and the limiting spectral form
Figure 1. Anaerobic titration of wild-type BACoADR with NADH. The enzyme (40.8 μM, in 1.0 mL of 50 mM potassium phosphate, pH 7.0, plus 0.5 mM EDTA) was titrated with a 5.4 mM solution of NADH. Spectra shown, in order of increasing absorbance at 525 nm, correspond to the addition of 0 (-), 1.05 (---), and 2.1 (···) equiv of NADH/FAD. The end point for the first phase, taken from absorbance changes at 453 and 344 nm, is 1.03 equiv of NADH/FAD; the end point for the second phase, from absorbance changes at 453 and 525 nm, is 1.97 equiv of NADH/FAD.
Scheme 1. Reductive Intermediates on NAD(P)H Titration of BACoADR

Roman numeral designations for EH$_2$ species correspond to those used in Figure 7 of Ref 40.
observed with 10.8 equiv of NADPH/FAD exhibits a largely oxidized (66% of the starting $A_{440}$) component.

We interpret the redox behavior of wild-type BACoADR as follows, and as adapted from the model proposed by Wilkinson and Williams for *E. coli* lipoamide dehydrogenase (41). The dithionite titration at 1 equiv/FAD yields ca. 50% of the enzyme subunits with reduced flavin (FADH$_2$), corresponding to species IIIB (see Figure 7 of Ref 40). Since no convincing charge-transfer intermediate corresponding to species II is observed, we attribute the remaining 50% of the BACoADR subunits to species I (A or B), where the single active-site Cys42-SH of BACoADR is equivalent to the proximal Cys in lipoamide dehydrogenase. BACoADR and all PNDOR Group 3 enzymes lack the distal Cys residue that represents the interchange thiol (8). Given the asymmetric behavior documented for SACoADR on interaction with pyridine nucleotides (11), we interpret these BACoADR results in a similar asymmetric scheme, with the two-electron (dithionite) reduced BACoADR represented as $E(\text{FADH}_2, \text{Cys42-SSCoA})/E(\text{FAD, Cys42-SH})$. Complete dithionite reduction yields the $EH_4$ enzyme form: $E(\text{FADH}_2, \text{Cys42-SH})$. This behavior with dithionite, at the $EH_2$ state, contrasts with that reported for SACoADR, as no FAD reduction occurs with 1 equiv of dithionite in the latter case (11).

At 1 equiv of NADH/FAD, there is convincing evidence for ca. 50% of the total charge-transfer absorbance ultimately observed with an excess of reductant (Scheme 1); this corresponds to species II in lipoamide dehydrogenase (see Figure 7 of Ref 40). There is significant flavin reduction as well, but any $E(\text{FADH}_2, \text{NAD}^+)\text{ component}$ is spectrally silent. Although we cannot be certain, it is likely that a significant species I
component is also present. Addition of the second equiv of NADH/FAD leads to quantitative appearance of species II.

The major differences in redox behavior for SACoADR and BACoADR with NAD(P)H are 1) the presence of strong \( \text{EH}_2 \) charge-transfer absorbance in BACoADR [with 2 equiv of NAD(P)H/FAD] and 2) the effective absence of an \( E(\text{FADH}_2) \) component in BACoADR titrated with 2 equiv of NAD(P)H/FAD. Finally, while the SACoADR C43S mutant is reduced directly with 1 equiv NADPH/FAD to give the dimeric \( E(\text{FADH}_2-\text{NADP}^+) \) species (11), the equivalent BACoADR C42S mutant is refractory to complete reduction in titrations with either NADPH or NADH. It is of interest to note that the equivalent C44S mutant of \textit{E. coli} lipoamide dehydrogenase was also resistant to NADH reduction in static titrations; only ca. 15% FAD reduction was observed with 1 equiv/FAD (41). Independent measurements of the redox potentials (FAD/FADH\(_2\) couples) for the wild-type \( (\text{EH}_2/\text{EH}_4) \) and C44S mutant lipoamide dehydrogenases demonstrated that the flavin potential is 65 mV lower for the mutant (42).

**NAD(P)H Dinucleotide-Binding Motifs.** Among the four conserved sequence motifs identified by Dym and Eisenberg (43) as being shared by all NAD(P)H-dependent members of the GR\(_1\) subfamily [which includes all of the PNDOR enzymes (38, 39)], one represents part of the \( \beta\alpha\beta \) Rossmann NAD(P)H dinucleotide-binding motif. This specific motif has most recently been applied in the description and functional analysis of the "two dinucleotide binding domains" flavoproteins superfamily (7) containing both FAD- and NAD(P)H-binding motifs. Figure 2 presents a structure-based sequence alignment for the NAD(P)H-binding motifs of SACoADR (NADPH),
Figure 2. Structure-based sequence alignment for the NAD(P)H-binding motifs of selected PNDOR enzymes. Specific enzymes are identified in the text; BBCoADR represents the enzyme from *Bo. burgdorferi*. The secondary structure corresponds to SACoADR (12), while sequence numbering corresponds to BACoADR. Enzymes are grouped according to NAD(P)H substrate preference; red blocks represent NADPH, and blue blocks represent NADH. BACoADR and L-san Nox exhibit dual NAD(P)H specificity. Asterisks designate conserved residues, and positions conserving hydrophobic residues are indicated by "ϕ." The conserved (in GR, Npx, and Nox) Tyr is highlighted in cyan.
GR [NADPH (44)], BACoADR (NADH/NADPH), *L-san* Nox [NADH/NADPH (45)], Npx [NADH (46)], and the *Bo. burgdorferi* CoADR (NADH). Studies have shown that GR Ala and Arg residues at positions 162, 181, and 187 (BACoADR numbering used here for simplicity) contribute to NADPH specificity (44, 47), and that Npx Gly and Asp residues at positions 162 and 180 contribute to NADH specificity (46). The BACoADR NAD(P)H-binding motif reflects a hybrid sequence and includes both Glu180 and Arg181. In addition, the presence of Gly162 should enable recognition of NADH while retaining facile recognition of NADPH, because in *E. coli* GR $K_m$(NADPH) actually decreased two-fold in the corresponding Ala $\rightarrow$ Gly mutant (47), while $K_m$(NADH) decreased by 40-fold. These inferences are supported by the recently (48) reported structures of glutathione amide reductase, which is closely related to GR but prefers NADH (49), and its complex with NAD$^+$. 

**Overall Structure of Oxidized BACoADR.** Although the 1.90 Å resolution of the refined SeMet BACoADR model is better than the 2.30 Å resolution for native oxidized BACoADR, the Cys42-SSCoA disulfide is partly reduced (~50%) by synchrotron radiation in the SeMet structure. We therefore discuss the native oxidized BACoADR structure, which includes residues 1-444 for both chains A and B of the biological dimer (with residues from chain B being designated by a prime symbol), two FAD cofactors, two covalently-bound CoAS-, and 442 solvent waters. It has an $R$-factor of 19.2% ($R$-free = 23.8%) with reasonable geometry (Table 2). There is no electron density for the residues of the N-terminal His-tag. Although NCS restraints were not used in refinement, the two subunits of the dimer are essentially identical with a 0.2 Å C$_\alpha$ rmsd [DALILITE (50)]. As expected, BACoADR is very similar to SACoADR (PDB code 1YQZ) with a
$C_\alpha$ rmsd = 1.7 Å for 841 atoms (COOT) in the dimer (33% identity; Figure 3). Since the SACoADR structure has already been described in detail (12), here we highlight the features of BACoADR that differ from SACoADR.

The first area of difference focuses on the covalently-bound CoAS-. In the SACoADR structure (12), it was noted that the active-site CoAS- for chain B was less well-ordered than that for chain A and exhibited an alternate conformation for the pyrophosphate moiety. In addition, the CoAS-3′-phosphate (both chains) was observed to be entirely solvent exposed. In oxidized BACoADR, alternate conformations also exist for the CoAS-pyrophosphate in both chains, but the alternate conformations differ from those in SACoADR. More importantly, however, the CoAS-3′-phosphate in BACoADR is not fully exposed to solvent, but is anchored in the protein, connecting via hydrogen bonds with Gln18, Arg21, Arg308, and Arg441′ side chains (Figure 4); Arg21, Arg61, and Arg441′ provide for strong electrostatic interaction with the CoAS-pyrophosphate, and Arg442′ provides a cation-π stacking interaction with the adenine moiety of the bound CoAS-. Although Gln18 and Arg21 are conserved in SACoADR, they play completely different roles in CoAS- recognition in the two enzymes. As shown in Figure 4, the SACoADR Gln19 side chain provides three hydrogen bonds to the adenine and pyrophosphate components of the covalently-bound CoAS-; BACoADR Gln18 instead provides a single hydrogen bond to the CoAS-3′-phosphate. And, while SACoADR Arg22 is engaged via both a cation-π stacking interaction with the CoAS-adenine and a single hydrogen bond with the ribose-2′-hydroxyl, BACoADR Arg21 provides three hydrogen bonds with the CoAS-pyrophosphate and 3′-phosphate.
Figure 3. Superposition of the BACoADR dimer (slate blue) with that of SACoADR (magenta); both proteins are rendered as 50% transparent. FAD and CoAS- are color-coded by atom type, with carbon atoms colored as follows: FAD, yellow; CoAS- of BACoADR, white; CoAS- of SACoADR, gray. The view down the crystallographic twofold symmetry axis corresponds to a 90° forward rotation of the SACoADR dimer given in Figure 2 of Ref 12.
Figure 4. Stereoview comparing protein-CoAS- interactions in BACoADR and SACoADR. BACoADR helix α1 and the ADP-3’-phosphate moiety of CoAS- (pantetheine moiety omitted for clarity) are colored slate blue, as are carbon atoms for chain A residues; those for Arg441’ and Arg442’ (chain B) are light blue. SACoADR helix α1 and that CoAS- ADP-3’-phosphate are colored magenta, as are carbon atoms for chain A residues; those for Lys427’ (chain B) are cyan. Protein-CoAS- hydrogen-bonding interactions are given in black and red dashes for BACoADR and SACoADR, respectively. All side chains are color-coded by atom type; all elements of SACoADR are rendered as 30% transparent.
A second major structural difference focuses on the respective NAD(P)H-binding motifs. In oxidized BACoADR, the Glu180-Thr187 loop is poorly-ordered, with weak electron density and high B-factors (near 45 Å² for chain A); the equivalent segment of the NADPH-binding motif in SACoADR is well-ordered and adopts a distinct conformation. This segment in fact represents the most significant main chain difference between the two structures. Disorder involving this region of the NAD(P)H-binding motif (Figure 2) has not been seen for other PNDOR enzymes (44, 46). Superpositions of the oxidized BACoADR structure with those of the GR(GSSG + NADP⁺) and Npx Cys42-SO₃H-NADH complexes indicate that either a BACoADR protein conformational change must occur to allow binding of NAD(P)H, or NAD(P)H must bind to BACoADR in a conformation very different from those observed in other PNDOR enzymes.

The Reduced BACoADR-NADH and NADPH Complexes. Soaks of oxidized BACoADR crystals with NADH and NADPH led to reduction of the crystalline enzyme as evidenced by color changes of the crystals (see "Experimental Procedures"). The two models were refined at near 2.3 Å resolution to reasonable R-factors (Table 2). Each of the final refined models includes an NADH (Figure 5; or NADPH) in each active site and a reduced Cys42-SSCoA center, with the CoASH product remaining tightly bound and the nascent Cys42-SH side chain adopting a new conformation in which -SG interacts with Tyr367'-OH and Tyr425'-OH. The new Cys42-SG to CoASH-S distance is 4.0 Å; each CoASH is now well-ordered in the respective structure, with no alternate conformation for the CoASH-pyrophosphate as described in oxidized BACoADR.
Figure 5. Stereoview of the BACoADR-NADH complex, focusing on the active site. The refined model is shown for the NADH-reduced Cys42-SSCoA center and includes FAD, bound NADH, and the Tyr residues from chain B. 2$F_o$ - $F_c$ density contoured at 1σ is included for all elements except FAD, and portions of the FAD, CoAS-, and NADH have been omitted for clarity. Color-coding is by atom type, as in Figure 3; carbon atoms for NADH, Cys42, and Tyr are colored gray, wheat, and sand, respectively.
For both complexes, the two subunits of the respective dimer are essentially identical (rmsd ~0.2 Å).

Comparing the BACoADR-NADH complex with oxidized BACoADR (rmsd = 0.8 Å), the largest movement occurs for the Glu180-Thr187 loop (Figure 6A), which becomes ordered and shifts up to 8 Å (maximal Cα displacement at Asp183). This change in conformation allows the formation of many new hydrogen bonds with both protein and NADH groups (Table 3). Three of the new hydrogen bonds made with NADH are from the Glu180 and Asn182 side chains to the adenine-ribose hydroxyls (Figure 6B). The conformation adopted by Glu180-Thr187 in the BACoADR-NADH complex is very similar to that found within the NADPH-binding motif of both oxidized SACoADR (12), and the Npx Cys42-SO₃H-NADH complex [with the BACoADR Glu180 side chain matching Npx Asp179, as inferred from Figure 2 (46)].

This simple binding mode for NADH raises the question as to how NADPH is recognized, since the interactions of the Glu180 and Asn182 side chains with the 2′-hydroxyl in the BACoADR-NADH complex would be expected to discriminate against the 2′-phosphate of a canonically-bound NADPH (44, 47). The structure of the BACoADR-NADPH complex demonstrates that binding is achieved by small shifts in the adenine-ribose pucker that allow the 2′-phosphate to point away from the enzyme surface (Figure 6B). The hydrogen bond between the Glu180-carboxylate and the adenine-ribose 3′-hydroxyl is preserved, and Asn182-ND2 retains its interaction with the bridging oxygen of the 2′-phosphate (i.e., the 2′-hydroxyl in NADH) and develops a new hydrogen bond with one of the phosphate oxygens. Arg181 also shifts its side chain
Figure 6. Comparison of the Glu180-Thr187 loop conformations in oxidized and NADH-reduced BACoADR. (A) Shown in stereo are the oxidized and reduced (Cys42-SH + CoASH) forms of the Cys42-SSCoA center and the two FAD centers (portions omitted for clarity), respectively, and the full bound NADH of the BACoADR-NADH complex. The Glu180-Thr187 loop conformations for oxidized and BACoADR-NADH complex forms are colored slate blue and wheat, respectively. All other protein elements are rendered in white; NADH, FAD, and Cys42-SSCoA color-coding are as in Figure 5. The superposition was performed with the respective dimers. (B) Glu180-Thr187 loop side chain conformations and NAD(P)H interactions in the BACoADR-NADH and -NADPH complexes. Color-coding is by atom type; protein carbon atoms for the complexes are colored wheat and forest green, respectively; NAD(P)H carbons are colored as in Figure 5. Hydrogen bonds involving NADH and NADPH are given in black and red dashes, respectively. As in (A), the superposition was performed with the respective dimers.
Table 3. Hydrogen Bonding Interactions for the Glu180-Thr187 Loop in Oxidized BACoADR and in the NADH Complex

<table>
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<tr>
<th>Oxidized BACoADR protein atoms</th>
<th>D...A (Å)</th>
<th>BACoADR-NADH complex protein and NADH atoms</th>
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conformation to allow hydrogen bonds with two of the 2′-phosphate oxygens. Finally, a new hydrogen bond is formed between Thr187-OG1 and the 3′-hydroxyl of bound NADPH. Scrutton et al. (47) examined the contributions of seven residues in *E. coli* GR, with respect to the preference for NADPH versus NADH. In addition to four of the human GR residues (some of these are not conserved in the *E. coli* enzyme) highlighted in Figure 2 [Ala162, Arg181, His182, and Arg187 (BACoADR sequence numbering)], Ala166, Ile180, and Asp183 were included in that analysis. In addition to the analyses of BACoADR Glu180, Arg181, Asn182, and Thr187 described above, we also evaluated BACoADR Gly162, Ala166, and Asp183 for their contributions to cofactor discrimination in the BACoADR-NAD(P)H complexes. In summary, none of these residues appear to make an active contribution; the closest approach for Gly162 (Gly162-N) gives a distance of ~4.1 Å from the cofactor pyrophosphate. Ala166-Cα is ~11 Å from the nearest atom of bound NAD(P)H, and Asp183 is not conserved among GRs; it is replaced by His in the *E. coli* enzyme. In BACoADR Asp183 is exposed to solvent as well.

**Dual NAD(P)H Specificity in *Lactobacillus sanfranciscensis* Nox.** Lountos et al. (45) recently reported that the Nox from *Lactobacillus sanfranciscensis* (*L*-san) oxidizes NADH and NADPH with approximately equal affinity, although an apparent 3-4-fold preference for NADH over NADPH was noted. Calculations of kcat/Km for NADH and NADPH from the data given yield values of 2.7 x 10^7 M⁻¹s⁻¹ and 8.5 x 10^6 M⁻¹s⁻¹, respectively. Electron density interpreted as an active-site ADP in the *L*-san Nox crystal structure, combined with the identification of ADP by mass spectrometry, led to the
conclusion that ADP is bound to and blocks the canonical NADH-binding site. This inference led to the heterodox proposal that ADP remains bound throughout the catalytic cycle; as such, NAD(P)H access to the flavin occurs not at the expected binding site, but via an unprecedented large, re-face channel which lacks the determinants of coenzyme specificity (see Figure 8 of Ref 45). This is taken further to support the conclusion that "the presence of the ADP ligand in L.san-Nox2 appears to be responsible...for the lack of substrate preference by this homologue."

Not finding this explanation attractive, our attention was drawn to the sequence of the NAD(P)H-binding motif for L-san Nox. The pyridine-nucleotide binding motif for L-san Nox (Figure 2) is entirely consistent with a subtle mechanism for dual NAD(P)H specificity similar to that described above for BACoADR. Although there is no basic residue equivalent to BACoADR Arg181, there are basic residues equivalent to Asn182 (Nox His181) and Thr187 (Nox Lys187) that could support dual coenzyme specificity for L-san Nox. To test the plausibility of this reinterpretation of the L-san Nox data, we have superimposed the BACoADR-NADPH structure with that of the L-san Nox-ADP complex (chain A; PDB code 2CDU). Without invoking the controversial conclusions that 1) ADP remains bound throughout the catalytic cycle, and 2) NAD(P)H accesses the flavin through a promiscuous re-face channel, we can demonstrate that this alternate NADPH conformation positions the 2′-phosphate away from the Nox protein interior. Nox His181 is indeed structurally equivalent to BACoADR Asn182 and, with a change in the side chain rotamer, can provide a strong hydrogen bond (2.99 Å) with the bridging oxygen (O2'A) of the NADPH 2′-phosphate in this model. Nox Lys187 is structurally
equivalent to BACoADR Thr187 [and to GR Arg224 in the GR(GSSG + NADP\(^+\)) complex (44)] and can also interact with the 2′-phosphate on adjustment of the side chain rotamer. And, while Nox Lys213 is not structurally equivalent to BACoADR-NADPH Arg181, Lys213-NZ is proximal to the Arg181 guanidinium moiety in the overlay. Its distance of \(\sim 5\ \text{Å}\) from the 2′-phosphate would require some change in the Nox-NADPH conformation in order to establish a favorable interaction.

We should also note that the putative bound ADP in the Nox crystal structure (45) may in fact be NAD\(^+\) or a mixture of NAD\(^+\) and ADP. A major 686-Da species observed in the mass spectrometric analysis but not discussed (see Figure S1 of Ref 45), fits the \(m/z\) calculated for the sodium (Na\(^+\), 23 Da) adduct of NAD\(^+\) (663 Da). In this light, half or more of the "ADP" modeled into the Nox structure could represent a bound NAD\(^+\) in which only the ADP portion is ordered; such partial ordering of nicotinamide dinucleotides is very common among PNDOR enzymes, as is illustrated by the GR-(GSSG + NADP\(^+\)) crystal structure (44), for which only the 2′, 5′-ADP moiety of bound NADP\(^+\) is included in the final refined model. In concluding, and without offering any support for the \(L\)-san Nox kinetic analysis, the BACoADR mechanism for NAD(P)H recognition may also provide for the dual specificity reported for \(L\)-san Nox. One distinct advantage in this model is that it avoids other controversial, and in our view, unnecessarily heterodox proposals for PNDOR enzyme-substrate recognition.

**The BACoADR Ala160-for-Tyr Replacement.** Although not a specificity determinant for NAD(P)H recognition, GR Tyr197 does play an important role in NADPH binding (44). From the alignment given in Figure 2, this Tyr is not conserved in BACoADR but
is replaced by Ala. This Tyr is conserved in Npx and in all known functional NADH oxidases [Nox; PNDOR Group 3, see above (12, 45)] and GRs (51), but not in other PNDOR enzymes; it is present in several of the CoADRs identified in bacteria (including SACoADR and the Bo. burgdorferi and B. megaterium CoADRs) and archaea (12) but is often replaced with Ala or other aliphatic residue. Replacement of this conserved (in GR, Npx, and Nox) Tyr residue with Ala raises an important question about the molecular spring mechanism for hydride transfer (51), as it relates to BACoADR. The molecular spring hypothesis was derived from the observation that the GR reductive half-reaction is seriously compromised in the Y197S mutant. The apparent $k_{cat}$ is 14% that of wild-type GR, and the rate constant for the limiting step in reduction (conversion to the EH$_2$ charge-transfer intermediate) is decreased 20-fold. The $k_{cat}$ values for SACoADR (Tyr158) and BACoADR (Ala160) with NADPH are 27 s$^{-1}$ (11) and 28 s$^{-1}$, respectively; although the point has not been confirmed experimentally (e.g., by mutagenesis), it appears that any molecular spring function for SACoADR Tyr158 has a minimal impact on turnover.

**Structure of the Reduced Cys42-SSCoA Center.** As described in earlier studies with SACoADR (9, 11, 12), a key mechanistic contrast with GR and other PNDOR Group 1 and 2 enzymes (8) involves the single active-site Cys (BACoADR Cys42), which both receives electrons from the flavin during Cys42-SSCoA reduction (and serves as the charge-transfer donor to FAD in the EH$_2$ form; Figure 1), and engages the CoAD substrate as the nucleophile in the thiol-disulfide exchange reaction. In both the NADH and NADPH complexes with BACoADR, while the respective nicotinamides are stacked on the *re*-face of the flavin isiallooxazine, the Cys42-SSCoA centers on the flavin *si*-face are reduced. In these structures (Figures 5 and 7), the CoASH product remains well-
ordered in its binding pocket, suggesting that it binds rather tightly; in solution assays, the
dissociation of CoASH product may be enhanced by excess CoAD. There are no crystal
contacts involved in this CoASH:protein interaction. A slight shift (∼1.7 Å) in position
of the bound CoASH-S accompanies reduction, such that CoASH-S occupies the pocket
filled by Cys42-SG in oxidized BACoADR. The nascent Cys42-SH side chain
accommodates the CoASH-S shift by adopting a new conformation away from the flavin
and interacting with the conserved active-site Tyr367′-OH and Tyr425′-OH at distances
of 3.0 and 2.9 Å, respectively. As a result of the slight shift in position of the CoASH-S
on reduction and the Cys42-SH conformational change, the two sulfur atoms are
separated by 4.0 Å in the NADH and NADPH complexes (Figure 7). While the
interaction of Cys42-SH with Tyr367′ and Tyr425′ should stabilize the conjugate thiolate
base, this external conformation, which gives a Cys42-SG to FAD-C4a distance of 5.1 Å,
is not expected to support the EH₂ charge-transfer absorbance observed in NAD(P)H
titrations with BACoADR. While the external conformation observed for Cys42 may
well be populated to some extent during catalysis, and while this aspect of the reduced
structure gives detailed insight into a Cys42-SG position that interacts strongly with the
conserved Tyr residues, we conclude that this conformation results from the tightly-
bound, reduced CoASH (CoASH-I). CoASH dissociation is expected to allow Cys42 to
return to the side chain conformation, very similar to that in oxidized BACoADR, which
optimizes the EH₂ charge-transfer interaction and promotes the nucleophilic attack on
CoAD (see below). Interestingly, a similar "out" conformation for Cys44-SH in the E.
coli lipoamide dehydrogenase EH$_2$ form could correspond to the fluorescent species I (see Figure 7 of Ref 40) identified in dithionite titrations.

The environment of Cys42 in the BACoADR-NAD(P)H complex is reminiscent of that for Cys15 in the B. subtilis OhrR repressor, as deduced from structural analyses of reduced wild-type OhrR and of the C15S mutant (52). Very recently S-thiolation of Cys15, via a Cys15-sulfenic acid intermediate, has been demonstrated in vivo with Cys, CoASH, and with a novel 398-Da thiol (4). In each case S-thiolation leads to dissociation of the OhrR:operator complex. The apparent $pK_a$ of Cys15-SH is 5.2; the thiolate species is stabilized in part by a positive helix dipole, but there are no basic residues within 7 Å of Cys15-SG. Hydrogen-bonding interactions with Tyr29′-OH and Tyr40′-OH are thought to contribute to stabilization of Cys15-S$^-$; a very recent, extensive analysis of Tyr29 and Tyr40 mutants (53) demonstrates that both residues are important factors in the peroxide reactivity of Cys15. In addition to their potential effects on Cys15-SH $pK_a$, Tyr29′ and Tyr40′ may facilitate Cys-sulfenic acid formation either by restricting the orientation of the Cys15 side chain or by protonating the ROH product formed with cumene- and other alkyl hydroperoxides.

Solely on the basis of the refined 1.54 Å structure of oxidized SACoADR, in which Tyr361′-OH interacts strongly with the active-site chloride ion, we proposed (12) that this residue might serve a primary role in protonating the nascent CoAS-II thiolate during nucleophilic attack of Cys43-S$^-$ on CoAS-I of the CoAD substrate. Although the external conformation observed for Cys42 in the reduced BACoADR complex does
interact strongly with Tyr367' and Tyr425', it does not provide an appropriate geometry for nucleophilic attack on CoAS-I; in fact, Cys42-SG of the BACoADR-NADH complex is in contact violation (2.2 Å) with the position of the SACoADR chloride ion (the modeled CoAS-II sulfur of CoAD) in the corresponding overlay. Figure 7 also includes the modeled CoAD substrate as developed with the oxidized SACoADR structure (I2). As indicated, the CoAS-II sulfur lies 3.9 Å from Cys42-CB and is close to the limiting van der Waals distance for the two atoms. Tyr367'-OH and Tyr425'-OH (oxidized BACoADR) are 3.0 and 3.5 Å, respectively, from the CoAS-II sulfur. There are key similarities (Scheme 2) between the reduction of CoAD by BACoADR (Cys42) and that of GSSG by GR [Cys58 (8, 44)]. BACoADR Tyr425 is structurally equivalent to GR His467 [His439 in E. coli GR (54)]; in the GR(GSSG + NADP⁺) crystal structure (44), the optimal hydrogen-bonding potential for His467'-NE2 lies with the GS-I sulfur. NE2 points most directly at the GS-I sulfur, at a distance of 3.4 Å, in the GSSG complex; there is no interaction with either Cys58-SG or the GS-II sulfur. As Cys58-SG proceeds to attack GS-I, His467'-NE2 has been proposed to become protonated; this proton is subsequently transferred to the GSH-I product that appears as Cys63-SG reforms the protein disulfide with Cys58. The E. coli GR H439A mutant has 0.3% activity, supporting its critical role as an acid-base catalyst in turnover. It should be noted that an alternate interpretation of the GR mutant data (54) concludes that His439' protonates the GS-II sulfur, preventing the facile back-reaction. The GR(GSSG + NADP⁺) and GR Cys58-SSG(GSH) structures (44), however, support the His role in GS-I protonation and product release.
Scheme 2. Proton Donors and their Proposed Roles in GR and BACoADR
Although certain aspects of the model presented in Figure 7 (e.g., Tyr-OH
distances to CoAS-II sulfur in reduced BACoADR, geometry of CoAD disulfide bond
relative to Cys42-S) require further analysis, the present work does suggest that Tyr367’
and, in particular, Tyr425’ may be important in BACoADR catalysis. To test this idea,
we have evaluated the roles of Tyr367’ and Tyr425’ in BACoADR in standard assays
with the corresponding Tyr—►Phe mutants and in the Y367,425F double mutant. With
NADPH as the reducing substrate the Y425F mutant is 1% as active as wild-type
BACoADR (Table 4), but the Y367F enzyme is more active by an order of magnitude
(18% as active as wild-type enzyme). BACoADR Tyr367’ certainly does not perform
any essential acid-base function in catalysis. The much lower activity for the Y425F
mutant is suggestive of a more critical role for Tyr425’ in catalysis, consistent with its
hydrogen-bonding distance (to Cys42-SG) of 2.9 Â. Furthermore, the Y367,425F double
mutant has no measurable activity in the CoADR assay. Very recently, Tu et al. (55)
have identified two distinct proton donors in the complex flavoprotein 2,4-dienoyl-CoA
reductase, Tyr166 and Glu164. While Tyr166 had earlier been considered essential for
the proton transfer step during substrate reduction, the Y166F mutant has 27% activity
(similar to the BACoADR Y367F mutant). The Y166F/E164Q double mutant, however,
has no detectable (<0.01%) activity; in consideration of these results and the crystal
structure of an enzyme-product analog complex, the authors conclude that 1) Tyr166 is
not an essential proton donor, 2) Glu164 is a cryptic alternate proton donor, providing
this function only in the absence of Tyr166, and 3) His252 interacts with both residues
Figure 7. Tyr367’ and Tyr425’ interactions with Cys42-SG in the BACoADR-NADH complex and with modeled CoA-disulfide substrate. This view of the oxidized versus NADH-reduced BACoADR overlay (originally described in Figure 6) focuses on the respective FAD centers (with a portion of bound NADH depicted on the distal re-face), the reduced and oxidized forms of the Cys42-SSCoA center, and the Tyr residues from chain B. All color-coding is as previously described, with carbon atoms for Cys42 and both Tyr colored as in Figures 4 (oxidized BACoADR) and 5 (BACoADR-NADH). Hydrogen bonds between both Tyr and Cys42-SG in the NADH-reduced structure are given in black dashes. The modeled CoAD substrate (see Figure 7 of Ref 12) was introduced by overlaying the dimeric model of the oxidized SACoADR-SSCoA-II complex with oxidized BACoADR. The position of the CoAS-II sulfur is identical to that of the SACoADR chloride ion (12). The position of the CoAS-I sulfur (relative to oxidized BACoADR) was adjusted to allow for the proper CoAD disulfide bond length. The distances between the two Tyr-OH (oxidized BACoADR) and the CoAS-II sulfur of modeled CoAD are indicated by cyan dashes, and that between the CoAS-II sulfur and Cys42-CB (oxidized BACoADR) is given with red dashes.
Table 4. Kinetic Parameters for Wild-Type and Mutant\textsuperscript{a} BACoADRs

<table>
<thead>
<tr>
<th>BACoADR</th>
<th>K\textsubscript{m}[NAD(P)H]</th>
<th>K\textsubscript{m}(CoAD)</th>
<th>k\textsubscript{cat}</th>
<th>k\textsubscript{cat}/K\textsubscript{m}[NAD(P)H]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(NADH)</td>
<td>1 \textmu M</td>
<td>2 \textmu M</td>
<td>27 s\textsuperscript{-1}</td>
<td>2.7 x 10\textsuperscript{7} M\textsuperscript{-1}s\textsuperscript{-1}</td>
</tr>
<tr>
<td>(NADPH)</td>
<td>3 \textmu M</td>
<td>6 \textmu M</td>
<td>28 s\textsuperscript{-1}</td>
<td>9.3 x 10\textsuperscript{6} M\textsuperscript{-1}s\textsuperscript{-1}</td>
</tr>
<tr>
<td>Y367F</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(NADH)</td>
<td></td>
<td></td>
<td>7 s\textsuperscript{-1}</td>
<td></td>
</tr>
<tr>
<td>(NADPH)</td>
<td></td>
<td></td>
<td>5 s\textsuperscript{-1}</td>
<td></td>
</tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(NADH)</td>
<td></td>
<td></td>
<td>0.9 s\textsuperscript{-1}</td>
<td></td>
</tr>
<tr>
<td>(NADPH)</td>
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<td></td>
<td>0.3 s\textsuperscript{-1}</td>
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</tbody>
</table>

\textsuperscript{a}Turnover numbers for the mutants were determined in the standard spectrophotometric assay at fixed NAD(P)H and CoAD concentrations.
and is an important factor in facilitating their respective proton donor roles. Although Tyr367′-OH and Tyr425′-OH are separated by a distance of 3.8 Å in the BACoADR-NADH complex, each residue interacts with Cys42-SG. Our results, in view of the proton-transfer dyad identified in 2,4-dienoyl-CoA reductase, suggest that Tyr425′ may be the primary proton donor in BACoADR (Scheme 2), protonating the nascent CoAS-II thiolate; in the absence of Tyr425′, this function can be provided by Tyr367′. In this model, the 1% residual activity in the Y425F mutant is thus attributed to Tyr367′ operating as the cryptic alternate donor to the CoAS-II thiolate. The 18% residual activity in the Y367F mutant suggests that it plays a minor role in ensuring the proper environment, perhaps positioning the bound CoAD and/or Tyr425′-OH (Tyr367′-OH···Tyr425′-OH distance = 3.4 Å in oxidized BACoADR). The latter interaction could, for example, reflect a mechanism by which Tyr367′ could facilitate proton transfer from Tyr425′, similar to the role of His252 in 2,4-dienoyl-CoA reductase. In either case, the Y367,425F double mutant lacks any source of the proton required for CoASH-II release and has no detectable CoADR activity.
ACKNOWLEDGMENTS

This work was supported by National Institutes of Health (NIH) Grant GM-35394 (A.C.), by a grant from the Southeast Regional Center of Excellence for Biodefense and Emerging Infections (SERCEB; to A.C.), and by National Science Foundation grant MCB-9982727 (P.A.K.). C.P. was the recipient of a Graduate Fellowship from the U.S. Department of Homeland Security (DHS). SERCEB is supported by an award from the NIH (National Institute of Allergy and Infectious Diseases; NIAID). The DHS Scholarship and Fellowship Program is administered by the Oak Ridge Institute for Science and Education (ORISE) through an interagency agreement with the U.S. Department of Energy (DOE). ORISE is managed by Oak Ridge Associated Universities under DOE contract number DE-AC05-06OR23100. The findings, opinions, and recommendations expressed in this paper are those of the authors and are not necessarily those of NIAID, SERCEB, NIH, DHS, DOE, or ORISE. Data for this study were measured at beamline X26C of the National Synchrotron Light Source. Financial support comes principally from the Offices of Biological and Environmental Research and of Basic Energy Sciences of the DOE, and from the National Center for Research Resources of the NIH.
REFERENCES


Chapter V

FUNCTIONAL MOTIFS ALLOW FOR IDENTIFICATION OF COENZYME A-DISULFIDE REDUCTASE ISOFORMS: IMPLICATIONS FOR ROLES IN ENDOSPORE GERMINATION AND DIAMIDE-INDUCED DISULFIDE STRESS IN Bacillus anthracis

Carleitta Paige, Sunil Ojha, Sean D. Reid, Philip C. Hanna, Patricia C. Babbitt, and Al Claiborne

The following manuscript is in preparation for submission to Proteins: Structure, Function, and Bioinformatics. Stylistic variations are due to the requirements of the journal. Paige performed bioinformatics analyses, genetic manipulations, biochemical analyses of mutant strains, and prepared the manuscript. Ojha provided oversight during bioinformatics analyses. Reid, Hanna, Babbitt, and Claiborne acted in advisory and editorial capacities.
ABSTRACT

NADH peroxidase (Npx), NADH oxidase (Nox) and coenzyme A-disulfide reductase (CoADR) define the POR subgroup within the “two dinucleotide binding domain” flavoproteins (tDBDF) superfamily. These enzymes differ from all other tDBDF subgroups based on the absence of a redox-active cystine-disulfide that is replaced with a single cysteinyl derivative. Npx and Nox enzymes both contain a stable Cys-SOH redox center and catalyze the NADH-dependent reduction of hydrogen peroxide and molecular oxygen, respectively. In contrast, CoADR contains a Cys-SSCoA active-site mixed disulfide and is the only flavoprotein disulfide reductase known to catalyze the reduction of a low-molecular weight disulfide substrate with a single catalytically essential cysteine. The combination of hidden Markov model (HMM)-based bioinformatics and phylogenetic analyses reveal that the POR subgroup consists of two distinct classes, CoADR and Npx/Nox, demonstrated by unique clustering of experimentally characterized enzymes. Furthermore, sequence analyses of the experimentally characterized Staphylococcus aureus and Bacillus anthracis CoADRs has led to the discovery of functional motifs, which will allow more accurate identification of CoADR isoforms. We have recently reported the crystal structure for the oxidized B. anthracis CoADR (BACoADR), and for the reduced enzyme in complex with NADH and NADPH [Wallen, J. R., Paige, C., Mallett, T. C., Karplus, P. A., and Claiborne, A. (2008) Biochemistry 47, 5182-5193]. In addition to BACoADR, B. anthracis also contains a second CoADR isoform, coenzyme A-disulfide reductase-rhodanese homology domain (CoADR-RHD). Based on the function of BACoADR in thiol-disulfide homeostasis and the role implicated for CoADR-RHD in sulfur
metabolism, in-frame deletion mutants of genes encoding each enzyme were generated and tested for germination efficiency, as well as sensitivity to disulfide stress. We report that both BACoADR and CoADR-RHD contribute to endospore germination. However, only CoADR-RHD appears to provide protection against disulfide stress.
INTRODUCTION

The structural constraints imposed by adenine dinucleotide cofactors and substrates, FAD and NAD(P)H, on the evolution of new functions in the “two dinucleotide binding domains” flavoprotein (tDBDF) superfamily has recently been assessed (1). These ligands, as bound, are stabilized by specific interactions with protein residues from several motifs distributed among both dinucleotide-binding domains and adopt conserved conformations optimal for stereospecific hydride transfer. Hidden Markov model (HMM)-based sequence analyses demonstrate that members of the tDBDF superfamily can be divided into nine subgroups; the alkylhydroperoxide reductase (AHR), disulfide reductase (DSR), and NADH peroxidase/oxidase and CoA-disulfide reductase (POR) subgroups are of primary relevance to the present work, as these enzymes collectively represent the pyridine nucleotide disulfide oxidoreductase (PNDOR) family (2). The AHR subgroup enzymes have a redox active disulfide in the NAD(P)H-binding domain, as represented by the E. coli thioredoxin reductase (TrxR) (3). Enzymes of the DSR subgroup share a common redox-active disulfide, but present in the FAD-binding domain. Glutathione reductase (GR) is a member of the DSR subgroup and catalyzes the NADPH-dependent reduction of glutathione disulfide (GSSG) (4). In contrast, enzymes of the POR subgroup lack the primary cystine-disulfide redox center, which is replaced by a single active-site Cys-SX residue within the FAD-binding domain.

POR subgroup members NADH peroxidase (Npx) and NADH oxidase (Nox), which catalyze the reductions of hydrogen peroxide and molecular oxygen, respectively, contain the single active-site Cys-SX residue present as a stable cysteine sulfenic acid (Cys-SOH) (5). The crystal structure of oxidized Npx (PDB entry: 1JOA) demonstrates
that the active-site Cys-SOH is stabilized by His10 and Arg303 via a hydrogen-bonding network (6). The “newest” member of the POR subgroup, coenzyme A-disulfide reductase (CoADR), also contains a single active-site Cys. The first CoADR to be extensively studied was the *Staphylococcus aureus* CoADR (SACoADR). In contrast with Npx and Nox, the single active-site Cys43 of SACoADR is present as a Cys43-SSCoA mixed disulfide (7). SACoADR catalyzes the NADPH-dependent reduction of CoA-disulfide (CoAD) (8) and is therefore the only PNDOR enzyme known to reduce a low-molecular weight disulfide substrate with a single catalytically-essential Cys. The SACoADR crystal structure (PDB entry: 1YQZ) also demonstrates that Ala11 and His299 replace the His10 and Arg303 residues (9), which are important in the stabilization of the Cys-SOH of Npx, demonstrating a difference in active-site chemistries among enzymes of the POR subgroup.

*S. aureus* replaces GSH with CoASH as its major low-molecular weight thiol (10). Consistent with the working conclusion that CoASH functions as a GSH substitute, SACoADR functions to maintain an intracellular CoASH/CoAD ratio of ~450 (10). In addition, SACoADR has been implicated as contrubiting to the pathogenesis of *S. aureus*, suggesting that the thiol-disulfide balance is an important factor in virulence. Two independent studies have demonstrated that virulence in mouse models is strongly attenuated in SACoADR-deficient strains of *S. aureus* (11, 12). However, SACoADR did not appear to contribute to *S. aureus* virulence, as measured in a *Caenorhabditis elegans* killing assay (13). Given that *S. aureus* can infect and persist in many different host environments, it is probable that its virulence program is complex, but also different depending on the site of infection.
Utilizing the SACoADR protein sequence in BLASTP analysis led to the identification of two CoADR isoforms in another Gram-positive pathogen, *Bacillus anthracis*. The first, *B. anthracis* CoADR (BACoADR; encoded by *cdr*), reduces CoAD using NAD(P)H as a reducing substrate (14). The second isoform, coenzyme A-disulfide reductase-rhodanese homology domain (CoADR-RHD; encoded by *cdrX*), maintains the CoADR module linked to a C-terminal rhodanese homology domain (RHD). Under standard CoADR assay conditions (7), CoADR-RHD does not catalyze CoAD reduction.\(^1\) The recent report of Lukose et al. (15) indicates that the *Shewanella loihica* PV-4 CoADR-RHD functions as an NADH-dependent, coenzyme-A activated polysulfide reductase (reaction: \(\text{NADH} + \text{H}^+ + \text{S}\text{n}_{2^-} \rightarrow \text{NAD}^+ + \text{S}_{n-1^{2^-}} + \text{H}_2\text{S}\)) and maybe involved in dissimilatory sulfur reduction. In addition, the upregulation of genes encoding CoADR-RHD-SirA-DsrE (CoADR isoform not present in *B. anthracis*) and CoADR-RHD in *Pelobacter carbinolicus* during growth on Fe(III) suggests possible roles for these proteins in the indirect sulfur-dependent reduction of Fe(III) (16). Furthermore, the transcriptional profiling of *B. anthracis* Sterne cultured in sporulation-inducing medium identified five distinct temporal waves of gene expression (17). The genes encoding BACoADR and CoADR-RHD were upregulated during waves III and V, respectively, both of which correspond to formation of the dormant endospore. BACoADR has been identified both in the cytoplasmic proteome of vegetative cells (18) and in the dormant spore (19). These data are also consistent with reports described of a flavoprotein disulfide reductase from *B. megaterium*, capable of CoAD reduction, which

\(^1\) Wallen, J. R., Mallett, T. C., Boles, W., Parsonage, D., Furdui, C. M., Karplus, A., and Claiborne, A., unpublished experiments.
was linked to the reduction of protein-SSCoA disulfides during spore germination (20, 21).

Given that the tDBDF superfamily, in particular the POR subgroup, is marked by the fact that only a small minority of proteins have been experimentally characterized, we have utilized a bioinformatics approach to assess the functional and structural constraints imposed by the CoASH cofactor. In this work, the functional inferences of the POR subgroup have been refined, specifically within the CoADR family, to develop motifs for the accurate identification of CoADR and its distinction from Npx/Nox. In addition, the transcriptome and proteome analyses of *B. anthracis* provide indirect support for a proposal for a role of BACoADR and/or CoADR-RHD in maintaining redox homeostasis during vegetative growth, as well as in redox regulation, with respect to germination and outgrowth of the dormant endospore. In order to test these proposals, the markerless gene replacement method (22) was utilized to construct *B. anthracis* strains lacking the genes encoding BACoADR and CoADR-RHD, both singly, and in combination. Together these approaches demonstrate a wide distribution of CoADR isoforms and provide insight into their physiological contributions.
**EXPERIMENTAL PROCEDURES**

**Bacterial strains and growth conditions.** $10^7$ spores of *B. anthracis* Sterne wild-type, $\Delta cdr$, $\Delta cdrX$, and $\Delta cdr\Delta cdrX$ strains were added to 200 µl of BHI medium at 37°C, with shaking, and allowed to germinate and grow for 12 h; $A_{600}$ was monitored at 5-min intervals, using the kinetic read function on the SpectraMax M2 microplate reader (Molecular Devices). For growth curves testing the response to disulfide stress, 1 mM diamide (Sigma) was added either to the initial spore inoculum or to exponentially growing cultures ($A_{600} = 0.4$ to 0.6). Growth curves were performed with three different spore preparations on different days, and all measurements were performed in triplicate. All strains were sporulated as follows. In short, overnight BHI broth cultures were diluted 1:20 into modified G (sporulation) medium and shaken at 300 rpm for 3 days at 37°C. The cultures were pelleted by centrifugation at 3,000 x g for 30 min and washed 4 times in 45 ml of sterile Milli-Q water. Pellets were resuspended in Milli-Q water, heat-treated at 65°C for 30 min to kill any remaining vegetative bacilli, and then centrifuged again before transferring into 1.5-ml screw-cap tubes. The purity of the spore preparations was confirmed by phase-contrast microscopy, and concentrations were determined by serial dilution.

**Construction of $\Delta cdr$ and $\Delta cdrX$ mutants by markerless gene replacement.** In-frame deletion mutants of the genes encoding BACoADR ($cdr$; locus tag BAS1170) and CoADR-RHD ($cdrX$; locus tag BAS0736) were constructed with markerless gene replacement strategy, which utilizes the pBKJ236 and pBKJ223 plasmids (22). Briefly, modified chromosomal segments containing a deletion of $cdr$ or $cdrX$ with flanking upstream and downstream regions were cloned into pBKJ236. Following propagation in
E. coli INV110 (Invitrogen; dam' and dcm' deficient), plasmids harboring the gene deletion constructs (cdr::pBKJ236 and cdrX::pBKJ236) were conjugated into B. anthracis Sterne and identified by selection for erythromycin resistance. After growth at room temperature overnight, isolation of cdr::pBKJ236 and cdrX::pBKJ236 chromosomal integrants was performed by a shift to the restrictive temperature (37°C) for plasmid replication, while maintaining selection for erythromycin resistance. Next, plasmid pBKJ223 was electroporated into cdr::pBKJ236 and cdrX::pBKJ236 strains of B. anthracis, and transformants were grown on BHI agar plates plus tetracycline. The pBKJ223-encoded I-SceI restriction enzyme is expressed and cleaves the corresponding site on the cdr::pBKJ236 and cdrX::pBKJ236 plasmids. This introduces a double-stranded break in the B. anthracis chromosome that is repaired via the bacterial host recombination repair systems. Repair of the chromosomal break can occur via homologous recombination, resulting in either regeneration of the wild-type locus or deletion of the gene of interest. After screening for the desired mutation by PCR amplification, positive clones were streaked and maintained for two passages on solid BHI media to promote loss of pBKJ223, then scored for tetracycline sensitivity. The ΔcdrΔcdrX strain was constructed following the same protocol, utilizing the Δcdr strain for the initial conjugation step.

Germination analysis. Germination was analyzed by the decrease in $A_{600}$ as previously described (23). Spores of B. anthracis Sterne (wild-type, Δcdr, ΔcdrX, and ΔcdrΔcdrX mutant strains) were placed in a 96-well plate to an $A_{600} = 0.3$ in 200 µl of germination buffer (100 mM L-alanine in phosphate-buffered saline [phosphate-buffered saline; pH 7.4; Gibco]) at 37°C. The decrease in $A_{600}$ was measured at 30-s intervals over a time
course of 60 min, using the SpectraMax M2 microplate reader (Molecular Devices), as described above. Germination is represented as the percent decrease in $A_{600}$ for the test mixture versus time. Percent decrease in $A_{600}$ is calculated using:

$$\% \text{ decrease in } A_{600} \text{ at time } t (t_i) = \left[ \left( \frac{A_{600} \text{ at } t_i - A_{600} \text{ at } t_{end}}{A_{600} \text{ at } t_i} \right) \times 100 \right]$$

As shown previously, a decrease in $A_{600}$ of ~60% correlates with the loss of heat resistance in >99% of the cultures (23). All experiments were performed with three different spore preparations, and all measurements were performed in triplicate.

**Bioinformatics.** Sequence searches were performed using the HMMer search (http://selab.janelia.org) and BLAST against the 90% identity-filtered non-redundant protein database (nrd90, updated in July 2008). A training set of CoADR homologs was established to generate a CoADR-specific HMM. The training set sequences were obtained from the POR subgroup identified earlier and were evaluated for the presence of CoADR-specific residues as identified in the *S. aureus* and *B. anthracis* CoADRs (9, 14). Each sequence from the training set was analyzed in SHOTGUN (24) to identify any divergently related sequences. The SHOTGUN results were negative (no sequences were identified), supporting a high confidence level for the CoADR training set. The training set was then used to create a CoADR-specific HMM. Searching the nrd90 database, the HMM identified a total of 2,850 sequences with $E$-values $<10^{-5}$. Individual sequences within the POR subgroup were validated by the presence of the conserved single active-site Cys. This collection included a total of 155 sequences. The evolutionary history of the POR subgroup and CoADR family was inferred using the Neighboring-Joining method (25). The bootstrap consensus tree inferred from 500 replicates (26) was taken to

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2 Ojha, S. and Babbitt, P. C., unpublished data
represent the evolutionary history for those sequences analyzed. Phylogenetic analyses were conducted in MEGA4 (27). In order to identify new CoADR functional motifs, a CLUSTALW (28) alignment of the S. aureus (9), B. anthracis (14), and Borrelia burgdorferi (29) CoADR enzymes was performed. The Enterococcus faecalis Npx (6) and Streptococcus pneumoniae Nox (30) sequences were then used to validate the conserved (i.e., excluding conservative substitutions) CoADR motifs. The S. aureus, B. anthracis and Bo. burgdorferi sequences were analyzed by MEME (31) to verify these functional motifs as specific to CoADR and not Npx/Nox. Protein Homology/analogY Recognition Engine (PHYRE) was used to generate the homology model structure (32).
RESULTS AND DISCUSSION

CoADR represents a separate family within the POR subgroup. In the HMM-based analyses of the entire tDBDF superfamily ~70 sequences were identified as putative members of the POR subgroup. In order to narrow the scope and to focus primarily on the CoADR family, crystal structures of the SACoADR (PDB entry: 1YQZ) and BACoADR (PDB entry: 3CGC) enzymes initially revealed conservation of the following very minimal set of residues (replaced in Npx and Nox, as described above): 1) Ala or Gly11 (SACoADR numbering), 2) Arg22, 3) Tyr361, and 4) Tyr419 (9, 14). Therefore, utilizing the ~70 sequences identified in the initial analysis, a training set was compiled consisting of 21 sequences (Table 1), all of which contain these four CoADR-specific (versus Npx/Nox) residues. The training set sequences were then used to generate a CoADR HMM to search against the 90% filtered non-redundant database (nrd90, updated July 2008). The HMM-based search results identified 155 sequences as putative members of the POR subgroup based solely on the conserved single active-site Cys; most of these were annotated as Nox, but only eight represented proteins that have been characterized experimentally.

While the Npx, Nox and CoADR enzymes all are members of the POR subgroup, a more critical investigation of their evolutionary relationships, using phylogenetic analyses demonstrated that the POR subgroup could be divided into separate families, supporting a clear distinction between CoADR and Npx/Nox. The set of 155 sequences identified by the CoADR-specific HMM was used to generate a phylogenetic tree (Figure 1). First, the Npx/Nox family is characterized by the clustering of the experimentally
Table 1. Training set of CoADR sequences

<table>
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<th>GI Number</th>
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<td>15923960</td>
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<td><em>Streptomyces coelicolor</em></td>
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<td><em>Borrelia burgdorferi</em></td>
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<td><em>Vibrio vulnificus</em></td>
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<td><em>Pyrococcus abyssi</em></td>
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<td><em>Pyrococcus horikoshii</em></td>
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<td><em>Pyrococcus furiosus</em></td>
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<td>20093415</td>
<td><em>Methanosarcina acetivorans</em></td>
</tr>
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</table>
Figure 1. Sequence relationships within the POR subgroup. Nodes that define the CoADR and Npx/Nox families contain at least one experimentally characterized member and are identified by circles.
characterized Npx and Nox enzymes. Within the Npx/Nox clade, all of the sequences maintain His10 (Enterococcus faecalis Npx numbering), which interacts with the Cys42-SOH redox center. Npx His10 mutants exhibit significantly lower $k_{cat}/K_m(H_2O_2)$ values, reflecting the decreased second-order rate constant for catalytic $H_2O_2$ reduction; these mutants also exhibit increased sensitivity to $H_2O_2$ inactivation (33). The Cys42-OH and the chemistry of $H_2O_2$ reduction involving His10 are not utilized in CoADR enzymes; therefore, CoADR replaces His10 with Ala or Gly11 and represents the second family within the POR subgroup. Of the 155 sequences obtained utilizing the CoADR HMM, 122 (or ~78% of the total) maintain conservation of CoADR-specific residues, thus reflecting the requirement for new functional motifs capable of both distinguishing CoADR from Npx/Nox and supporting Cys43-SSCoA redox chemistry.

In previous studies, blastp searches of the NCBI proteins database, utilizing SACoADR as the query, identified novel CoADR isoforms, CoADR-RHD and CoADR-RHD-SirA-DsrE (9). Therefore, in order to evaluate the relationships of the CoADR isoforms, the 122 sequences that make up the CoADR family were used to construct a phylogenetic tree (Figure 2). These data indicate two separate clades within the CoADR family, supporting the identification of a CoADR class (those sequences corresponding to ~450 residue polypeptides like SACoADR) and the CoADR-extended class (those sequences that have C-terminal extensions) from the CoADR module. This finding is consistent with the divergent catalytic functions of these enzymes, where CoADR has been demonstrated to catalyze the NAD(P)H-dependent reduction of CoAD (8, 14, 29) and CoADR-RHD does not. Kinetic assays of the Sh. loihica PV-4 CoADR-RHD have demonstrated a polysulfide reductase activity that could be enhanced 4 to 6-fold in the
Figure 2. Sequence relationships within the CoADR family. Nodes defining the CoADR and CoADR-extended classes are identified by circles.
presence of 0.5mM to 1 mM CoASH (15). Currently, the function of CoADR-RHD-SirA-DsrE is unknown; however, a recent report documents the dramatic (>20-fold) upregulation of this encoding gene in *P. carbinolicus* grown on Fe(III), in the presence of sulfur, suggesting that CoADR-RHD-SirA-DsrE may function indirectly in Fe(III) reduction (16).

**CoASH-binding highlights CoADR-specific functional motifs.** The crystal structures of SACoADR and BACoADR have provided a platform for developing CoADR-specific motifs that allow for structure-function evaluation and differentiation from the Npx/Nox family. The structures of both enzymes identify the covalently-bound CoASH- in an extended conformation, although differences are observed in the conformation of the 3’-phosphate (9, 14). In SACoADR, the adenine base is partially solvent exposed, and the 3’-phosphate is fully exposed. Gln19 forms three hydrogen bonds to the adenosine 3’-phosphate 5’-pyrophosphate moiety of CoASH. In addition, Arg22 interacts with CoASH- via cation-π stacking interactions with the adenine and hydrogen bonding to the 2’-OH of the ribose. In BACoADR, the 3’-phosphate is anchored in the protein matrix through an extensive hydrogen-bonding network. Gln18 and Arg22 (SACoADR numbering) are conserved; however, the interaction of BACoADR Gln18 involves a single hydrogen bond to the 3’-phosphate, and Arg21 is networked through three hydrogen bonds to both the 5’-pyrophosphate and 3’-phosphate components of the 3’-phospho-ADP moiety. Due to the fact that Npx/Nox do not bind CoASH, these residues are not conserved (Figure 3). As such, we conclude that the QxxR (Gln19-Arg22) motif is critical to the binding of CoASH-.
Figure 3. Multiple sequence alignment of experimentally characterized POR subgroup enzymes. The red and yellow boxes indicate conserved residues and conservative substitutions, respectively. The blue boxes indicate the conservation of CoADR specific motifs among the CoADR family, which are absent in Npx/Nox. The representative sequences include: *S. aureus* CoADR (9), *B. anthracis* CoADR (14), *Bo. burgdorferi* CoADR (29), *Enterococcus faecalis* Npx (34), and *Streptococcus pneumoniae* Nox (30).
The SY(F)xxC active-site motif is a sequence identifier for the POR subgroup and can be used to recognize both Npx/Nox and CoADR families. Sequence and structural evidence support the extension of this motif within the CoADR family to be redefined as SF(Y)A(G)xCG(A)LPY. Ala41 (SACoADR numbering) replaces Leu40, which is conserved in the Npx/Nox family. This substitution in CoADR satisfies local steric requirements that aid in CoAS- recognition. Also, the C-terminal Leu, Pro, and Tyr residues of this motif are present within a hydrophobic pocket where SACoADR Tyr47 provides stacking interactions with Tyr361′ (residues from the complementary subunit designated with a prime symbol), which also contributes to the interface domain of CoADR.

The original 2.16 Å resolution crystal structure of Enterococcus (formerly Streptococcus) faecalis Npx indicated the presence of two Phe residues (Phe366′ and Phe424′) in the active-site (35); subsequent analyses show these Phe residues to be conserved in functional members of the Npx/Nox family (Figure 3). Phe424′ helps to shield the FAD isoalloxazine from solvent and is hydrogen-bonded (Phe424′-O) to FAD-N3F. In SACoADR and BACoADR, in contrast, Phe366′ and Phe424′ are replaced with Tyr361′ and Tyr419′, respectively (Figure 3). Kinetic analyses of BACoADR Y367F (equivalent to SACoADR Tyr361), Y425F (equivalent to SACoADR Tyr419), and Y367,425F mutants in the standard CoADR assay demonstrate diminished enzyme activity (14). The Y367F mutant is 18% as active as wild-type, Y425F is 1% as active as wild-type, and Y367,425F has no measurable activity. In addition, structural evidence supports the role of these Tyr residues in stabilizing the reduced Cys42-S- during BACoADR catalysis. Tyr361′ and Tyr419′ are conserved as YYP (Tyr361-Pro363) and
YA(S)PPY(F)S(N)xxW(K)D (Tyr419-Asp428) motifs, respectively. Proper orientation of the YYP motif appears to be mediated by the SACoADR Tyr47 stacking interaction with Tyr361′. Also, Asp428 forms an intrasubunit salt bridge with Arg395 of the DKR motif, stabilizing interactions within the CoADR interface domain. These CoADR-specific motifs are summarized in Table 2.

**Functional predictions.** Functional evolution within the POR subgroup, led by the diversification of protein-protein interactions via cofactor constraints, allows us to predict functional and mechanistic aspects of less-well-characterized members. As noted earlier, most of the sequences identified in the POR subgroup are annotated as “NOX” enzymes and have not been experimentally validated. For instance, analysis of the *Methanocaldococcus* (formerly *Methanococcus jannaschii*) genome identified a gene encoding a putative “NADH oxidase” (MJ0649) (36). The corresponding protein sequence maintained the canonical single active-site Cys; however, phylogenetic analysis demonstrated greater similarity to SACoADR than to Nox enzymes; for example, the MJ0649 sequence lacked the His10 residue conserved in Npx/Nox sequences (8).

The CoADR HMM search analysis also identified MJ0649; the 448-residue sequence has 28% and 34% sequence identity compared to SACoADR and BACoADR, respectively. While there is conservation of the active-site cysteine, the active-site motif of MJ0649 is altered (AYSxCAIPY; Figure 4). The substitutions in MJ0649 of Ser39 (SACoADR numbering) with Ala, Ala41 with Ser, and Leu45 with Ile are conservative and are not expected to alter active-site chemistry or conformations. The QxxR motif, in which both Gln and Arg contribute to CoAS′ binding, is present as TxxK in MJ0649. Again, the substitutions in MJ0649 are not drastic and the chemical conservations
Table 2. CoADR-specific functional motifs.

<table>
<thead>
<tr>
<th>Motif&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Residue Position (SACoADR numbering)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q(K)xxR</td>
<td>19-22</td>
<td>CoAS- binding</td>
</tr>
<tr>
<td>SF(Y)A(G)xCG(A)LPY</td>
<td>39-47</td>
<td>Active-site</td>
</tr>
<tr>
<td>YYP</td>
<td>361-363</td>
<td>Ty361′ alternate CoASH-proton donor</td>
</tr>
<tr>
<td>YA(S)PPY(F)S(N)xxW(K)D</td>
<td>419-428</td>
<td>Tyr419′ primary proton donor</td>
</tr>
<tr>
<td>DKR</td>
<td>393-395</td>
<td>Interface domain</td>
</tr>
</tbody>
</table>

<sup>a</sup>The residues given in parentheses appear in one of the three test CoADR sequences.
Figure 4. Partial sequence alignment of MJ0649 and experimentally characterized CoADRs focusing on the CoASH-specific functional motifs. A red box indicates conserved residues and a yellow box highlights conservative substitutions.
would allow for similar interactions with bound CoAS\(^{-}\) in either conformation observed in SACoADR or BACoADR. The motifs including the active-site tyrosine residues are also maintained.

These sequence observations are further supported by the generation of an MJ0649 homology model obtained by threading this sequence on the BACoADR structure. Figure 5 demonstrates a superposition of the dimeric BACoADR protein and the MJ0649 model. The overall global C\(\alpha\) backbone structures superimpose with an rmsd = 1.894 Å. The secondary structures appear to align well, while the variation in structures is apparent in the flexible loop regions. While the location of the residue side chains in the MJ0649 structure are not supported by calculated electron density, the homology model does support favorable stabilization of the Cys thiolate formed during catalysis (Figure 6) and the recognition and binding of the CoASH cofactor (Figure 7). The model for MJ0649 only predicted a monomeric structure and therefore, the contributions from the second monomer could not be visualized. However, the predicted position of the MJ0649 Cys supports the conformation for the oxidized enzyme. Additionally, the proposed side chain interactions for CoASH recognition appear favorable.

All archaea lack the common tripeptide GSH, and CoASH has been shown to be the major low molecular-weight thiol in *Pyrococcus furiosus*, *Thermococcus litoralis*, and *Sulfolobus solfataricus* (37). This is consistent with a role for CoASH in the redox biology of these “sulfur-reducing archaea, and the characterization of CoADR proteins in archaea supports this hypothesis. The *Pyrococcus horikoshii* CoADR enzyme catalyzes the reduction of CoAD; however, there is evidence suggesting that the CoADR activity of
Figure 5. Superposition of the BACoADR crystal structure and MJ0649 homology model. The homology model was determined using PHYRE (32). The dimeric BACoADR crystal structure (PDB entry: 3CGC) is in salmon and the monomer model of MJ0649 is in violet.
Figure 6. Active-site comparison of BACoADR and the MJ0649 model. The CoASH cofactor is colored by atom with carbon atoms as gray. The FAD cofactor is colored by atom with carbon atoms yellow. Side changes for BACoADR are salmon and those for MJ0649 are violet.
Figure 7. CoAS- binding interactions for BACoADR and the MJ0649 model. CoASH is colored by atom with carbon atoms in gray. The side chains for BACoADR are salmon and MJ0649 in violet.
this enzyme is a partial reaction of its true physiological function as a CoASH-dependent S° reductase (38, 39). Identification of CoADR in *M. jannaschii* provides a basis for delineating the function of this protein as a CoADR or a CoASH-dependent S° reductase. Overall, the implementation of the CoADR-specific functional motifs and homology modeling has provided complementary evidence that MJ0649 is likely a functional CoADR although further biochemical analysis is required.

**CoADR and CoADR-RHD have roles in *Bacillus anthracis* endospore germination and outgrowth.** Species of the genera *Clostridia* and *Bacillus* are capable of differentiation between two distinct cellular morphologies, the endospore and the vegetative cell. Endospore formation (sporulation) is initiated in response to suboptimal growth conditions, allowing for the survival of the organism over extended periods of time and under otherwise stressful conditions (40). However, upon the return to a more favorable growth environment, endospores are able to stimulate the morphological transition into metabolically active vegetative cells (germination) (41). Germination includes a complex series of biophysical and biochemical processes; however, the mechanism(s) by which metabolism ensues (outgrowth) in *B. anthracis* remains poorly understood.

In *Bacillus megaterium*, the accumulation of soluble protein S-thiolated with CoASH (protein-SSCoA) disulfides during sporulation and its reduction (to protein-SH plus CoASH) early in germination have been attributed to CoADR (20, 21). Transcriptional profiling of *B. anthracis* Sterne cultured in a sporulation-inducing medium demonstrated an upregulation of genes encoding BACoADR (*cdr*) and CoADR-RHD (*cdrX*) (17). BACoADR was also identified in the dormant spore, as well as in the
cytoplasmic proteome of the vegetative cell (18, 19). Therefore, we have proposed that BACoADR and/or CoADR-RHD were being expressed and packaged during development of the endospore, in order to contribute to the reduction of CoAS-specific disulfides on subsequent germination. In order to analyze the roles of BACoADR and CoADR-RHD in *B. anthracis*, in-frame deletion mutant strains were generated either singly or in combination, utilizing a markerless gene replacement method (22). The growth characteristics of the Δcdr, ΔcdrX, and ΔcdrΔcdrX mutant strains under aerobic conditions in BHI medium was the same as that of the corresponding isogenic wild-type Sterne strain, as demonstrated in Figure 8. In addition, sporulation efficiency of the mutant strains was compared to that of the wild-type Sterne strain via spore counts, and no measurable differences were observed. This suggests that neither BACoADR nor CoADR-RHD is essential for growth of *B. anthracis* in rich medium; deletion, singly and in combination, of the corresponding genes did not result in any marked advantage or disadvantage for either vegetative growth or spore formation.

As germination is critical to the establishment of anthrax, and identification of targets that can be exploited to diminish this virulence potential is of interest, we sought to test the role of BACoADR and CoADR-RHD in germination of *B. anthracis* endospores utilizing Δcdr, ΔcdrX, and ΔcdrΔcdrX strains. L-Alanine has been identified as the only small molecule nutrient capable of initiating germination of *B. anthracis* endospores, in the absence of a cogerminant (23). Therefore, utilizing 100 mM L-alanine, we observed impaired germination efficiency in both Δcdr and ΔcdrX strains in comparison to the wild-type Sterne strain (Figure 9). As previously reported, the wild-type strain demonstrates a biphasic germination response with L-alanine characterized by
Figure 8. Growth of *B. anthracis* Sterne (wild-type), ∆cdr, ∆cdrX, and ∆cdr∆cdrX mutant strains. All strains were inoculated (10^7 spores) into BHI liquid broth and grown at 37°C, 300 rpm. $A_{600}$ was monitored with the SpectraMax M2 microplate reader. Growth curves shown are representative of three independent experiments performed from three separate spore stocks.
Figure 9. Germination of *B. anthracis* Sterne (wild-type), Δcdr, ΔcdrX, and ΔcdrΔcdrX mutant strains. Germination efficiency of endospores was monitored by the decrease in $A_{600}$ of an endospore suspension during incubation with 100 mM L-alanine in PBS at 37°C. (The results are averages from triplicate experiments on three independent spore preparations. Standard deviations are <10% of the mean.)
a 40% decrease in $A_{600}$ over 10 min; the decrease extends to 60% of the initial $A_{600}$ after 30 min (23). However, in Δcdr and ΔcdrX, strains after 10 min incubation in 100 mM L-alanine, the decreases in initial $A_{600}$ attributed to germination are only ~28% and 20%, respectively. After 30 min, the Δcdr strain plateaus at 40% of the initial $A_{600}$, while ΔcdrX is significantly compromised in germination, leveling off at an $A_{600}$ value decreased by only 30%. Interestingly, the ΔcdrΔcdrX strain does not demonstrate any statistically significant difference in germination efficiency as compared to the wild-type strain, suggesting possible upregulation of a redundant redox system in the absence of both CoADR and CoADR-RHD. Based on the diminished capacity for endospore germination exhibited by Δcdr and ΔcdrX B. anthracis strains, we conclude that both BACoADR and CoADR-RHD have roles in this cellular differentiation process.

**CoADR-RHD contributes to protection from diamide-induced disulfide stress.** In B. subtilis, transcriptome and proteomics analyses of the response to cellular thiol oxidation (disulfide stress), using the thiol-specific oxidant diamide [Scheme 1; (42)], reveals upregulation of ~350 genes, including genes encoding thioredoxin ($trxA$), thioredoxin reductase ($trxB$) and cysteine synthetase ($cysK$) (43). In these analyses, B. subtilis strain 168 was cultured aerobically at 37°C in minimal media and 1 mM diamide was added to exponentially-growing cultures. In general, Leichert et al. describe a complex response to diamide-induced disulfide stress that also supports an antioxidant role for free cysteine. More recently, diamide-induced disulfide stress in B. subtilis led to an increase in the reversible S-cysteinylation of protein thiols, as a protective mechanism (44). *In vivo* labeling of proteins with $[^{35}\text{S}]$cysteine, following diamide treatment (in the presence of the protein synthesis inhibitor chloramphenicol), revealed an ~six-fold increase in
Scheme 1. Mechanism of action for the thiol-specific oxidant diamide.
protein-bound $^{35}$S radioactivity; 81% of the $^{35}$S label was lost after incubation with a disulfide reducing agent. In addition, mass spectrometric analyses led to the identification of six proteins (argininosuccinate synthase, inosine-monophosphate dehydrogenase, cobalamin-independent methionine synthase, inorganic pyrophosphatase, phosphoglycerate dehydrogenase, and a protein similar in sequence to branched-chain amino acid aminotransferase) as being S-cysteinylated; three of these proteins are known to be S-glutathionylated in other organisms.

Similar to the results with B. subtilis, analyses of protein oxidation in response to 1 mM diamide added to exponentially growing cultures of S. aureus, resulted in the global oxidation of cysteine residues on intracellular proteins and a shift in the intracellular protein thiol pool from a reduced to a reversibly-oxidized state, as measured by $^{35}$S- and fluorescence labeling (45). The identification of a low molecular-weight thiol associated with the observed thiol oxidation in S. aureus has yet to be elucidated. However, in contrast to B. subtilis, S. aureus contains CoASH as its principal thiol-disulfide redox buffer (10) and utilizes CoADR to maintain a high intracellular [CoASH]/[CoAD] redox state (8). To address the direct contribution of CoADR during diamide-induced disulfide stress with S. aureus, Northern blot analyses have indicated maximal expression of the cdr gene during exponential growth; RT-PCR analyses demonstrate an upregulation of the cdr gene 15 min after treatment with 4 mM diamide during mid-exponential, late-exponential, and stationary phases. In addition, a CoADR-deficient strain of S. aureus was tested for its sensitivity to diamide; these results did not reveal any significant difference in growth, however, from the wild-type strain. 3

3 Pope, M., and Davies, J., unpublished experiments.
Consistent with the hypothesis that CoASH functions in thiol-disulfide homeostasis, we investigated the possibility that BACoADR and CoADR-RHD would contribute to stabilizing the redox balance in *B. anthracis* and that Δcdr, ΔcdrX, and ΔcdrΔcdrX mutant strains would demonstrate enhanced sensitivity to diamide. With the addition of 1 mM diamide to exponentially growing cultures in BHI medium, all strains showed a transient growth arrest (Figure 10A), as observed for *B. subtilis* cultures grown in minimal medium (43). However, all three mutants revealed an extended lag in growth (~45 min, suggesting that both BACoADR and CoADR-RHD contribute to the resumption of exponential growth after diamide exposure. In order to evaluate the roles of BACoADR and CoADR-RHD in disulfide stress during the morphological transition from endospore to vegetative cell, 1 mM diamide was also added to spore inocula, following the conditions described earlier in Figure 9. Again, all strains demonstrated a pronounced lag in approaching exponential growth as compared to the control culture (Figure 10B): wild-type, 2 hr; Δcdr, 3 hr; ΔcdrX, 5 hr, and ΔcdrΔcdrX, 3.5 hr. The ΔcdrX strain exhibited a significantly more pronounced growth arrest in response to diamide treatment. In either disulfide-stress condition tested, the ΔcdrX strain exhibited more sensitivity suggesting that CoADR-RHD, not BACoADR, is the primary protein (of the two) involved in the ability of *B. anthracis* to overcome diamide-induced disulfide stress.
Figure 10. Effect of 1 mM diamide on *B. anthracis* Sterne (wild-type), ∆cdr, ∆cdrX, and ∆cdr∆cdrX mutant strains. A) Diamide added to vegetative cell cultures in BHI medium. B) Diamide was added at the time of endospore inoculation.
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CHAPTER VI:

CONCLUSIONS AND FUTURE DIRECTIONS
Coenzyme A (CoASH) has been demonstrated to have a novel role in the intracellular thiol-disulfide redox homeostasis of prokaryotes that lack the common tripeptide thiol glutathione (GSH); CoASH is the major low-molecular weight thiol in many bacteria and, apparently, in all archaea (1-5). In the GSH-dependent *Escherichia coli*, the first step of CoASH biosynthesis is catalyzed by the type I pantothenate kinase (PanK) (6); this PanK is subject to feedback inhibition by CoASH (7). In organisms where CoASH serves as the principal thiol/disulfide redox buffer, type II and III PanK isoforms are refractory to this feedback inhibition (8-11). Consistent with this function for CoASH in antioxidant defense, coenzyme A-disulfide reductase (CoADR) maintains a high CoASH/CoAD ratio and has been identified and characterized in several Gram-positive organisms (3, 12, 13). As such, CoASH-dependent redox biology is operationally defined as 1) absence of genes required for GSH biosynthesis, 2) presence of either type II or III PanK, and 3) presence of a CoADR homolog (10). The results outlined in this dissertation combine crystallographic, genetic, and bioinformatics strategies that provide insight into CoASH-dependent redox biology within the category A priority pathogen *Bacillus anthracis*. While these findings support a novel role for CoASH in *B. anthracis*, they also raise additional subjects for investigation, including, 1) drug design with respect to the type III PanK, 2) the possibility of CoASH-dependent S-thiolation during sporulation of *B. anthracis*, and 3) the role(s) of CoADR isoforms in virulence.

**Chapter II: Bacillus anthracis Type III Pantothenate Kinase.** CoASH accumulates to an intracellular concentration of approximately 0.44 mM in *B. anthracis* and represents the major low-molecular weight thiol. Bioinformatics analyses of the *B. anthracis*
genome, using the type II \textit{S. aureus} and type III \textit{B. subtilis} PanKs as queries, identified two PanK homologs, BA2901 and BA0065. The purified recombinant BA2901 protein was inactive in the \textit{in vitro} PanK assay, under conditions where SaPanK was active. Additionally, BA2901 expressed \textit{in trans} failed to complement a temperature sensitive \textit{E. coli coaA} mutant; SaPanK, however, restore growth. Therefore, we concluded that while BA2901 may contain phosphotransferase activity, pantothenate is not the acceptor substrate. In contrast, BA0065 (BaPanK) is active in the \textit{in vitro} enzyme assay, as examined in parallel with the type III PanK from \textit{Pseudomonas aeruginosa}. The crystal structure of BaPanK was determined at 2.0 Å resolution; BaPanK is a new member of the Acetate and Sugar Kinase/Hsc70/Actin (ASKHA) superfamily. Two insertions within the core ASKHA fold in BaPanK provide for newly identified PAN and INTERFACE motifs, which contribute to both pantothenate binding and dimerization and are unique to the type III isoforms. The crystal structure also demonstrates the absence of a hydrophobic dome in the pantothenate-binding pocket; this active-site structural element in the type I PanKs accommodates $\beta$-mercaptoethylamido moiety of CoASH, explaining both the competitive mode of feedback inhibition observed with CoASH and recognition of N-alkylpantothenamides as alternative substrates. We conclude that BaPanK represents the sole functional enzyme for synthesis of 4′-phosphopanthothenate in \textit{B. anthracis}. Furthermore, bioinformatics analyses reveal a widespread distribution of type III PanKs, strongly correlated with the absence of GSH biosynthesis, supporting an expanded role for CoASH in redox biology.

\textbf{Chapter III: BaPanK is essential for growth of Bacillus anthracis.} The gene \textit{coaX} encodes BaPanK; unlike the situation in \textit{B. subtilis}, we have concluded that BaPanK is
the sole enzyme catalyzing the first step of CoASH biosynthesis in *B. anthracis*. As CoASH is essential, the deletion of *coaX* should be lethal. We have shown that *coaX*, is the first gene of a tricistronic operon that’s is expressed during exponential growth (followed by the downstream genes *hslO* and *cysK-1* loci). Bioinformatics analyses demonstrate that *hslO* and *cysK-1* encode the redox-regulated heat shock protein Hsp33 and cysteine synthase A, respectively. Insertional inactivations of both the *hslO* and *cysK-1* loci, respectively, demonstrated that neither gene is essential for growth of *B. anthracis* and, therefore, any phenotype observed in *coaX* mutant strains is solely attributable to the function of *BaPanK*. Several attempts to construct an in-frame *coaX*-deletion mutant in *B. anthracis* were unsuccessful, indirectly supporting the working conclusion that *BaPanK* is essential. Therefore, a conditional mutant strategy was implemented, where *coaX* was cloned downstream of an IPTG-inducible promoter. With the addition of IPTG to the spore inoculum, the *coaX* conditional mutant grew similarly to wild-type. Growth was also observed in conditional mutant strains when IPTG was added four hours after inoculation. However, an unanticipated growth of the conditional mutant, in the absence of IPTG, was observed ~seven hours after inoculation. This observation was further corroborated on solid BHI media; small, single colonies were observed after a 12-hour incubation in the absence of IPTG. The growth of the conditional mutant has been demonstrated to result from a stable suppressor mutation (guanine to adenine) within the *lac* operator region. The guanine to adenine mutation reduces the repressor affinity and allows for *coaX* expression. We conclude that the selective pressure to provide for CoASH leads to the selection for the suppressor mutation. This work provides evidence supporting the conclusion that *BaPanK* is an
essential enzyme in *B. anthracis* and contributes to its validation as a therapeutic drug target.

**Chapter IV: *Bacillus anthracis* Coenzyme A-Disulfide Reductase.** The recent crystal structure for *S. aureus* CoADR (SACoADR) provided a set of sequence motifs that allowed for identification of CoADR enzymes in other organisms. On this basis, we report the kinetic, redox, and structural properties of the *B. anthracis* CoADR (BACoADR). In contrast to the NADPH-dependent SACoADR, BACoADR exhibits dual specificity with respect to the pyridine nucleotide substrate; it is able to use both NADH and NADPH with comparable efficiency in the catalytic reduction of CoAD. Analysis of the pyridine nucleotide-binding motif of BACoADR identifies a hybrid sequence combining elements of motifs thought to specify NADH *versus* NADPH recognition; these include residues for NADH (e.g., Gly162 and Glu180) and NADPH (e.g., Arg181) recognition. The 2.30 Å resolution crystal structure of BACoADR reveals a loop (Glu180-Thr187) that changes conformation in order to allow pyridine nucleotide binding. The covalently bound active-site CoAS⁻ is bound in an alternate conformation; the CoAS-3'-phosphate is anchored in the protein *via* hydrogen bonds with Gln18, Arg21, Arg308, and Arg441', in contrast to the solvent exposed conformation of this moiety in SACoADR. The structure of the reduced BACoADR Cys42-SSCoA redox center (Cys42-SH + CoASH) demonstrates a shift in the CoASH-S, which now occupies the position filled by Cys42-SH in the oxidized enzyme. Cys42-SH interacts with conserved active-site Tyr367'-OH and Tyr-425'-OH, supporting the contribution of both Tyr residues in catalysis.
Chapter V: Coenzyme A-disulfide reductase functional motifs and implications in *Bacillus anthracis*. The POR subgroup of the tDBDF superfamily is marked by the absence of the cystine-disulfide redox center (observed in both the DSR and AHR subgroups) replaced with a single redox-active cysteinyl derivative. Npx and Nox enzymes both contain a stabilized Cys-SOH redox center, while CoADR has a Cys-SSCoA mixed disulfide center. As the active-site chemistries for Npx/Nox and CoADR are significantly different, we utilized hidden Markov model and phylogenetic analyses to evaluate relationships among sequences within the POR subgroup. We report that the POR subgroup can be divided into two distinct protein families, namely Npx/Nox and CoADR, based on the clustering of sequences representing experimentally characterized enzymes. In addition, the CoADR family can be further differentiated into, CoADR and “CoADR-extended” classes, this distinction is further supported by the differences in catalytic function demonstrated for CoADR and CoADR-RHD, for example.\(^1\) In addition, the crystal structures of the *S. aureus* and *B. anthracis* CoADRs provide a basis for the development of CoADR-specific functional motifs for accurate identification in protein databases. Investigation of CoADR enzymes reveal five functional motifs: QXXR, SF(Y)A(G)xCG(A)LPY, YYP, YA(S)PPY(F)S(N)xW(K)D, and DKR. In parallel with these bioinformatics approaches, genetic and biochemical analyses were performed to construct Δcdr (BACoADR), ΔcdrX (CoADR-RHD) and ΔcdrΔcdrX *B. anthracis* mutant strains and to assess the contributions of these enzymes in the germination and outgrowth of dormant endospores, and in thiol-disulfide redox homeostasis. These results

\(^1\) Wallen, J.R., Mallett, T.C., Boles, W., Parsonage, D., Furdui, C.M., Karplus, K., and Claiborne, A., unpublished experiments
demonstrate roles for both BACoADR and CoADR-RHD in germination; CoADR-RHD, but not BACoADR, contributes to protection against diamide-induced disulfide stress.

**Future Directions: BaPanK is a valid drug target.** Current treatment for anthrax includes the administration of antibiotics often prior to the development of symptoms (14). Other strategies include antitoxins and vaccines; however, questions remain on their effectiveness and toxicity. The two licensed human vaccines, anthrax vaccine absorbed (AVA) and the corresponding United Kingdom vaccine, both contain protective antigen as the principal antigen (14). Drawbacks to the use of these vaccines include the very lengthy and complicated dosing schedules for the development of protective titers. Thus, the development of new vaccines and other, more effective therapeutic agents is hampered by the difficulty in demonstrating their effectiveness in humans. Currently, research efforts for analyzing and identifying new drug targets are concentrated on components of the *B. anthracis* endospore, which, if/when inhibited, inactivate the spore (15). However, the genome sequence of the *B. anthracis* Ames strain (16) revealed additional chromosomally-encoded proteins (as opposed to the pXO1 and pXO2 plasmids) that may contribute to virulence. As such, Chapter III highlights the BaPanK protein, encoded by *coaX*, as a potential target for therapeutic treatment; BaPanK is essential for growth of *B. anthracis*.

In order to identify compounds that inhibit BaPanK, the first step involves optimizing an *in vitro* high-throughput screening (HTS) assay. BellBrook Labs has developed the Transcreener ADP Assay for monitoring the activity of enzymes (e.g., kinases) ATP-dependent phosphotransferase reactions (17). More specifically, the Transcreener ADP Assay is a competitive fluorescence polarization immunoassay based
on the detection of ADP (Scheme 1). The two-part endpoint assay consists of an Enzyme Reaction component followed by the addition of the ADP Detection Mixture. The ADP Detection Mixture contains an ADP Alexa Fluor633 tracer bound to an anti-ADP antibody; the tracer is displaced by ADP produced during the enzyme reaction. The correlation time of the displaced tracer is much shorter than that of the antibody-bound form resulting in a decrease in fluorescence polarization values, ADP production is thus proportional to the decrease in polarization. Following the identification of compounds that are consistent in their inhibition of BaPanK (Z’ value of >0.5), the BaPanK crystal structure can aid in the design of compounds to improve potency. Finally, these compounds can be scrutinized in more detail by determining the 50% inhibition concentration (IC50) and evaluating off-target toxicities.

One example of the application of the Transcreener ADP Assay involves acetyl-coenzyme A carboxylase (ACC). ACC exists in two isoenzyme forms, ACC1 and ACC2, which have critical roles in fatty acid biosynthesis and oxidation (I8). Although the two isoenzymes differ in tissue distribution and subcellular localization, both catalyze multi-step ATP-dependent reactions that result in the carboxylation of acetyl-CoA to produce malonyl-CoA. Due to the potent inhibitory activity of ACC2 on carnitine palmitoyltransferase (the rate-limiting enzyme of fatty acid β-oxidation), inhibitors of ACC2 may provide useful therapeutics. Liu et al. (I8) utilized two HTS assays to identify inhibitors of ACC2, one of which was the Transcreener fluorescence polarization-based ADP assay (FP ADP Assay). In this report, conditions were optimized for the FP ADP Assay for ACC2, demonstrating its kinetic profile with limiting substrate (Figure 1). Next, a library of >500,000 compounds was screened in the FP ADP HTS Assay for the
Scheme 1. Transcreener™ assay principle for kinases and ATPases.
Figure 1. Kinetic assay using FP ADP assay in the presence (closed circles) or absence (open circles) of ACC2. The plateau of activity is due to exhaustion of the limiting substrate, acetyl-CoA. Assays were carried out using ACC2 at 20 nM. This figure is reprinted with permission from ref (18).
inhibition of ACC2. The initial screen revealed 3,574 compounds with >70% inhibition. Of the 3,574 compounds identified in the initial screen, 834 were confirmed, and 322 compounds were subsequently found to exhibit an average IC$_{50}$ < 10 µM.

While BaPanK is essential for growth of *B. anthracis*, there are other microbial pathogens that contain the type III PanK isoform as the sole source of the 4′-phosphopantothenate intermediate in CoASH biosynthesis. Such organisms include: *Bacillus cereus* G9241 (anthrax-like pathogen), *Pseudomonas aeruginosa* (opportunistic pathogen), *Clostridium botulinum* (NIAID category A priority pathogen), *Bordetella pertussis* (whooping cough), *Burkholderia mallei* (category B priority pathogen), *Neisseria gonorrhoeae* (gonorrhoea), *Helicobacter pylori* (ulcerations), *Francisella tularensis* (tularemia), and *Borrelia burgdorferi* (Lyme disease) (11). Therefore, the identification of compounds that inhibit type III PanK activity may ultimately serve as an initial step toward the development of a broad-spectrum approach to treatment of bacterial infections.

**Role of Coenzyme A in Sporulation and Germination of Bacillus anthracis.** In *B. megaterium* endospores, 68% of the total CoASH was present in the oxidized state (5); 25% was present as CoA-disulfide (CoAD), and 43% was identified in protein-SSCoA disulfides (soluble proteins S-thiolated with CoASH). The remaining 32% of the total CoASH was present as free thiol. Within the first minutes of spore germination, >75% of the protein-SSCoA disulfides were reduced (to protein-SH + CoASH). The accumulation of protein-SSCoA disulfides during sporulation is thought to contribute to the ability of the endospore to survive harsh environments (e.g., heat, dehydration, radiation) and to
help maintain metabolic dormancy via reversible inactivation of key thiol-dependent spore enzymes. The enzyme most likely responsible for the reduction of these protein-SSCoA disulfides was identified as an NADH-dependent flavoprotein disulfide reductase specific for disulfide substrates containing such as CoAD, and pantethine-4’-4”-diphosphate (19). Analysis of the draft sequence generated from 10X coverage of the *B. megaterium* chromosome\(^2\) has identified an NADH-dependent CoADR (*E*-value \(5e^{-75}\)) with 33% identity to SACoADR. The *B. megaterium* CoADR is considered responsible for the direct or indirect reduction of both CoAD (to 2 CoASH) and protein-SSCoA disulfides (to protein-SH + CoASH) during endospore germination. This process of CoASH-dependent S-thiolation of soluble proteins during sporulation has yet to be demonstrated in *B. anthracis*. However, the contribution of both CoADR and CoADR-RHD proteins to germination of *B. anthracis* endospores (Chapter V) is suggestive of such a connection.

In order to investigate the potential role of CoASH-dependent protein thiolation during sporulation and germination of *B. anthracis*, our laboratory plans to utilize a CoASH-specific polyclonal antibody in Western blot analyses. To date, I have optimized a protocol for isolating cell-free extracts from vegetative cultures, as well as soluble protein from dormant spores of *B. anthracis*. Briefly, overnight BHI broth cultures (<12 h) are diluted 1:20 into fresh BHI medium and modified G medium (sporulation medium) for the vegetative cell and endospore cultures, respectively, and shaken at 37°C at 300 rpm. Vegetative cell cultures are monitored by \(A_{600}\) and harvested during exponential growth (\(A_{600} = 0.4-0.6\)). Endospore cultures are incubated for 3 days before harvesting. In

\(^2\) [www.bios.niu.edu/b_megaterium/index.html](http://www.bios.niu.edu/b_megaterium/index.html)
each case, the respective culture is harvested by centrifugation and pellets are suspended in 1 mL of ice-cold 0.1 M potassium phosphate pH 7 including 25 mM benzamidine (extraction buffer). While maintaining all samples and buffers on ice, each suspension is transferred to 2-mL collection tube, which contains glass beads pre-soaked with the extraction buffer. Samples are then shaken rigorously at least 8-12 times for 45 seconds, with 1-min incubations (on ice) between cycles. Samples are then centrifuged to remove cellular debris, and the supernatant, which represents the soluble protein, is collected; a 500 mL liquid culture (either vegetative cells or spores) averages a soluble protein concentration of ~2-3 µg/mL.

Following the isolation of soluble protein samples from exponentially growing cultures and dormant endospores, I performed one SDS-PAGE Coomassie stain and Western blot, combined experiment to visualize the results of this approach. SDS-PAGE was performed under non-reducing conditions utilizing NuPage (Invitrogen) Bis-Tris gels (separating gel pH = 7). Figure 2A demonstrates the results for the Coomassie-stained gel and displays distinctive protein profiles for the vegetative cell versus spore samples, consistent with the metabolic states of the two cell types. Extrapolating from the earlier published analyses for protein-SSCoA in B. megaterium, we anticipated that the Western blot experiments would demonstrate an increase in the number of proteins identified by the CoASH-antibody in the dormant spore extract as contrasted with the vegetative cell samples. Although densitometry analyses suggest comparable levels of total CoASH-modified protein in the two samples (data not shown), there are distinct CoASH containing protein bands present in the spore extracts that are absent in the vegetative sample (Figure 2B). From this one experiment, the results confirm that S-thiolation of
Figure 2. CoASH-modification of soluble protein during *B. anthracis* sporulation. A) Coomassie-stained gel of *B. anthracis* protein extracts, and B) Western-blot analyses with CoASH-specific antibody. Lanes 1, 2 and 3 in the two panels correspond to 15 µg of soluble vegetative protein, soluble endospore protein, and the MagicMark Western protein standards, respectively.
soluble proteins by CoASH during sporulation is a protein-specific phenomenon and, as expected, does not occur for all proteins present in the dormant spore. These experiments should be repeated, with *B. megaterium* protein extracts. The \(\Delta cdr\) and \(\Delta cdx\) mutants (Chapter V) should also be used to visualize any changes in the CoASH-thiolation pattern in the absence of BACoADR and CoADR-RHD. Finally, available BACoADR antisera should be applied in order to probe the covalent CoAS\(^-\) of BACoADR and CoADR-RHD, as an internal control.

**Inhalational anthrax mouse virulence model.** The intracellular redox state of *B. anthracis* during infection is poorly understood; however, investigation of superoxide dismutase (SOD) proteins in virulence provides one particular example in which the maintenance of redox homeostasis was described, in this context (20). The *B. anthracis* chromosome contains four genes that encode SOD proteins, *sodA1*, *sodA2*, *sodC*, and *sod15*. To determine whether any of the four SOD proteins are important for virulence, Passalacqua *et al.* performed survival studies on DBA/2 mice, a strain that is sensitive to infection with wild-type *B. anthracis* Sterne endospores. The intratracheal route of infection was chosen to closely mimic an inhalational route of spore entry. In these analyses, mice were infected with \(10^4\) spores of wild-type, \(\Delta sodA1\), \(\Delta sodA2\), \(\Delta sodC\), and \(\Delta sod15\) mutant strains. Higher rates of survival after 10 days were observed in mice challenged with \(\Delta sod15\) and \(\Delta sodA1\), with 44 and 56\% surviving, respectively. Analyses of these data failed to demonstrate statistical significance; however, although the trends were consistent, attenuation is only suggested in this case.

*In vitro* assays support some role for both CoADR and CoADR-RHD in germination of *B. anthracis* endospores as well as in providing protection and/or recovery
in diamide-induced disulfide stress (Chapter V). As germination is critical to pathogenesis, a strain demonstrating a decreased ability to germinate may be more sensitive to bacterial killing by host defense mechanisms. Therefore, it stands to reason that CoADR and CoADR-RHD may in fact contribute to the overall virulence of \textit{B. anthracis}. In collaboration with Dr. Philip Hanna, University of Michigan, our laboratory plans to test this hypothesis utilizing the $\Delta cdr$, $\Delta cdrX$ and $\Delta cdr\Delta cdrX$ mutant strains in intratracheal infections of mice. Such a study would indicate a role for thiol-disulfide redox homeostasis during infection.

Overall, the future experiments presented provide a multidisciplinary approach to address the questions that remain; the rational supporting these approaches is provided by the findings described. \textit{BaPanK} is essential for growth in \textit{B. anthracis}, and the crystal structure can now be exploited to develop type III PanK inhibitors as potential therapeutic agents. Establishing CoASH-dependent redox regulation, including protein S-thiolation, in \textit{B. anthracis} sporulation and germination would serve as an important step in understanding the molecular basis of this morphological transition. Finally, demonstrating a role for CoADR isoforms in the virulence of \textit{B. anthracis} would demonstrate how interfering with the “redox biology” of this pathogen could curtail virulence. While examining the CoASH/CoAD ratio in the $\Delta cdr$, $\Delta cdrX$ and $\Delta cdr\Delta cdrX$ mutant strains, evaluating transcriptome and proteome changes in the \textit{B. anthracis} mutants following diamide treatment, and identifying functional motifs for type II and III PanK isoforms represent viable lines of investigation, I also believe that the aforementioned experiments would provide a more significant contribution to the \textit{B. anthracis} pathogenesis and CoASH-dependent redox biology fields.
REFERENCES


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EDUCATION

Ph.D., Biochemistry and Molecular Biology, Wake Forest University School of Medicine, Winston-Salem, NC. Degree anticipated August 2008
Advisor: Dr. Al Claiborne, Professor of Biochemistry

Bachelor of Science (Magna Cum Laude), Biology, Virginia Union University, Richmond, VA May 2003

RESEARCH EXPERIENCE

Doctoral Research: Wake Forest University School of Medicine, Winston-Salem, NC. 8/2003-present.
Investigating coenzyme A-dependent biosynthetic and redox functions present in Bacillus anthracis. Physiological characterization of enzymes hypothesized to provide thiol-disulfide homeostasis in B. anthracis, as well as protect against oxidative stress.

Advisor: Dr. Al Claiborne, Department of Biochemistry and Molecular Biology

Utilized genetic manipulation tools to construct gene deletion or conditional mutants in B. anthracis Sterne and analyzed phenotypic differences between mutant and wild-type strains.

Advisor: Dr. Philip Hanna, Department of Microbiology and Immunology

Visiting Scientist: University of California San Francisco, San Francisco, CA. 1/2005
Employed several bioinformatics computational tools to generate a structure-based fingerprint/motif for a novel group of pyridine nucleotide disulfide oxidoreductase (PNDOR) enzymes and type I, II, and III pantothenate kinase isoforms.

Advisor: Dr. Patricia Babbitt, Department of Biopharmaceutical Sciences
Analyzed the evolution of a coenzyme A-dependent cycle in the biosphere. Searched all available sequenced genomes for the lack of glutathione biosynthesis genes, the presence of a type II or III pantothenate kinase genes and the presence of genes encoding a coenzyme A-disulfide reductase homolog, which are the biomarkers for the presence of a coenzyme A-dependent redox cycle.

Advisor: Dr. Jacques Ravel, Microbial Genomics group

**Internship:** Department of Homeland Security Fellow, Department of Homeland Security, National Biodefense and Countermeasures Center (NBACC), Walter Reed Army Institute of Research, Silver Spring, MD. 6/2005-11/2005
Prepared a pH6 antigen-deficient strain of *Yersinia pestis* CO92 to analyze macrophage interaction. Ultimately, the pH6 antigen-deficient strain will be used in animal models to determine if virulence associated with *Y. pestis* is attenuated.

Advisor: Dr. Luther Lindler, Senior Science Advisor

**Senior Research:** MARC U* STAR Trainee, Virginia Union University, Richmond, VA 8/2002-5/2003
Analyzed transcription of the silent phenoxazinone synthase gene of *Streptomyces lividans* in *Escherichia coli*.

Advisor: Dr. Anthony Madu, Associate Professor, MARC U* STAR Program Director

**Internship:** Ronald E. McNair Scholar, University of North Texas Health Science Center, Fort Worth, TX. 6/2002-8/2002
Introduced protein kinase C (PKC)-η into human breast cancer cells and rat embryonic kidney cells to examine the effects of PKC activators and inhibitors on PKC-η expression. This research was particularly focused on the PKC signal transduction pathway and its role in cell growth regulation.

Advisor: Dr. Alakananda Basu, Professor, Department of Molecular Biology and Immunology

**Internship:** Hope College, Holland, MI. 6/2001-8/2001
Determined the significance of Leu-17 and Arg-19 in the DNA binding domain of the GATA-1 transcription factor. Functional binding activity of GATA-1 mutants determined that substitutions to Leu-17, but not Arg19, retained binding activity.
Advisor: Dr. Michael Pikaart, Assistant Professor of Chemistry

ACADEMIC AWARDS
2007  Best Speaker Award, Genetics and Environmental Mutagenesis Society Fall Meeting
2007  Selected to participate in the Intramural NIAID Research Opportunities (INRO) Program
2005-2006 Sandy Lee Cowgill Scholar in Biochemistry Graduate Fellowship
2004-2007 Department of Homeland Security Graduate Fellowship
2003-2004 Wake Forest University Graduate School Fellowship
2002  Ronald E. McNair Scholar
2001  Annual Biomedical Research Conference for Minority Students Oral Presentation Award
2001-2003 Minority Access to Research Careers (MARC) Scholar
1999-2003 Virginia Union University Presidential Scholarship

ACADEMIC ORGANIZATIONS
2006  Association for Women In Science
2006-2008 American Society for Microbiology
2003  Alpha Kappa Mu Honor Society
2003  Beta Kappa Chi Scientific Honor Society

PROFESSIONAL ORGANIZATIONS
2000-present  Alpha Kappa Alpha Sorority, Inc.

ABSTRACTS/POSTER PRESENTATIONS


Functions in *Bacillus anthracis*: Crystallographic Analyses of Coenzyme A-Disulfide Reductase in Complex with Pyridine Nucleotides.”


**INVITED TALKS**


Pantothenate Kinase in *Bacillus anthracis* is a likely candidate for therapeutic intervention against anthrax infection.”

**Paige, C.** (2005) Virginia Union University, MARC U* STAR Seminar Series, Richmond, VA. “Success in the Sciences: A Personal Perspective.”


**PUBLICATIONS**

