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The Structure and Function of Novel Proteins of *Bacillus anthracis* and Other Spore-Forming Bacteria: Development of Novel Prophylactic and Therapeutic Agents

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ABSTRACT: The overall goal of this review is to summarize the current body of knowledge about the structure and function of major proteins of *Bacillus anthracis* and/or similar spore-forming organisms. *B. anthracis* is a key spore-forming biological threat agent, as well as human and animal Gram-positive bacterial pathogen. The structural information described here is limited to approximately the last 5 years. This information is then related to the role of the selected proteins in pathogenesis and in the possible development of novel vaccine and/or other antimicrobial agents against spore-forming organisms, including anthrax, a disease caused by *B. anthracis*.

Among spore-forming bacteria, *Bacillus* and *Clostridium* species are the predominant spore-forming bacilli that cause serious diseases. The biochemical properties and mechanism of catalysis of the novel spore germination protease that degrades small, acid-soluble proteins protecting DNA against damage, a cofactor independent phosphoglycerate mutase, NAD^+ synthetase, and the three know *B. anthracis* toxins, protective antigen, lethal factor, and edema factor are described. The studies described in this work review and unify selected information critical for the prevention of microbial diseases such as anthrax. A strategy for the structure-guided development of new prophylactic and therapeutic agents is discussed.

KEY WORDS: anthrax, bioterrorism, *Clostridium,* drug, threat agent, vaccine.

I. INTRODUCTION

A. Spore-Forming Bacteria

Certain bacterial organisms such as members of *Bacillus* and *Clostridium* genera have the ability to form spores (Slepecky, 1992). Spores are created by these organisms in a spore-forming process (termed sporulation) under conditions of limited nutrients and are dormant forms of regular vegetative bacterial cells with a totally different structure than the cells themselves (Kennedy *et al*., 1994). Spores contain only essential elements from the original bacterial cell that are necessary for the future development of vegetative cells when the conditions become more appropriate for cell survival. They can survive, even in harsh environments, for as long as millions of years (Cano and Borucki, 1995). In 1872 spores and their developmental cycle were first described in *Bacillus subtilis* (Slepecky, 1992) followed by the description of the *Bacillus anthracis* spore development in 1876 (Keynan and Sandler, 1983). Most of these organisms are saprophytic and are prevalent in soil. Members of the genus *Bacillus* are also found in water, air, and on vegetation, whereas *Clostridia* are also present in the intestinal tract of animals and humans. A major pathogenic organism of these two genera is *B. anthracis*, which is the causative agent of anthrax (Hanna and Ireland, 1999). This disease affects animals and humans and is a major component of current biological threat strategies (Meselson *et al*., 1994; Thorne, 1993).

Although most sporulating bacteria do not cause illness, some of them can cause serious diseases in animals as well as in humans. A major pathogenic organism among spore-formers is *B. anthracis*, which is the causative agent of anthrax (Hanna and Ireland, 1999). In 1996 *B. anthracis* spores and anthrax were considered the main biological risk to the world with *Clostridium botulinum* as a second major risk (Meselson *et al*., 1994; Thorne, 1993). Examples of other disease causing sporulating agents are

B. cereus (food poisoning), *C. tetani* (tetanus), *C. botulinum* (botulism), *C. perfringens* (gas gangrene), and *C. difficile* (pseudomembranous colitis).

B. *Bacillus anthracis* and Anthrax

Although anthrax is primarily a disease of noncarnivorous animals, humans can also be affected (Hanna, 1998). Birds, reptiles, amphibians, and carnivores are usually not affected by the disease. *B. anthracis* was first clearly recognized as a bacterial pathogen in 1850 in the blood of animals dying from anthrax (LaForce, 1994). *B. anthracis* spores infect usually through injured skin (wounds or lesions), insect bites (Ivins and Welkos, 1988), the gastrointestinal tract, or by inhalation of spores into the lungs (LaForce, 1994). The spores germinate, usually at the site of entry, and grow to vegetative cells with the formation of gelatinous edeme and congestion. The inhaled spores reach the bronchioles and alveoli of the lung where they become rapidly phagocytozed by alveolar macrophages (Guidi-Rontani, 2002). Once the spores reach phagolysosomes, they germinate and the created vegetative cells produce anthrax toxins: protective antigen, edema, and lethal factors. During this process the spores/cells migrate with the macrophages to the lymph nodes. Subsequent disruption of phagolysosome membrane allows for release of bacilli to macrophage cytoplasm followed by toxins' mediated loss of macrophage cell viability and spread to the bloodstream (Guidi-Rontani, 2002). Once in the bloodstream the bacteria can freely multiply causing death of the host (LaForce, 1994). During the initial incubation period of 11 to 17 days (Brookmeyer and Blades, 2002; Brookmeyer *et al*., 2001) they reach 10^7 to 10^8 bacterial cells per milliliter of blood (Dixon *et al*., 1999; Meselson *et al*., 1994). At the time of death as much as a third of the volume of blood can consist of bacilli. Cutaneous infections with *B. anthracis* are rarely lethal when treated properly with penicillin or other antibiotics (LaForce, 1994). Infections through inhalation are more serious, but proper treatment with a combination of antibiotics and a vaccine increases the survival chances (LaForce, 1994). The current anthrax vaccine, produced by the Michigan Biologic Products Institute, has been approved by the Food and Drug Administration (FDA) for use and has been used by livestock workers and veterinarians since the early 1970s (Ivins *et al*., 1992). The human anthrax vaccine has been used on laboratory workers in the military and approximately 150,000 soldiers during the Gulf War. This vaccine is a formalin-inactivated, dead form of *B. anthracis*. The moderate effectiveness of the current vaccine (Turnbull, 1991 and 2000), the increasing emergence of antibiotic-resistant strains (Lalitha and Thomas, 1997), and the possibility of use of engineered strains as biological threat agent, make the fast detection of the disease and the development of new therapeutics more urgent than ever. *B. anthracis*, as well as other sporulating organisms, can be genetically modified to contain toxic parts that can become equally or even more dangerous agents in biological threats (Steffen *et al*., 1997).

The molecular factors of *B. anthracis* directly implicated in the disease (virulence factors) consist of the capsular poly-D-glutamic acid as well as certain toxins, including edema factor, protective antigen, and lethal factor (Ezzell and Abshire, 1988; Keim *et al*., 1997). The genes encoding these virulence factors are located on plasmids called pXO1 and pXO2 but not on the chromosomal DNA, and only strains having both of these plasmids are fully virulent, whereas the strains containing only the chromosome

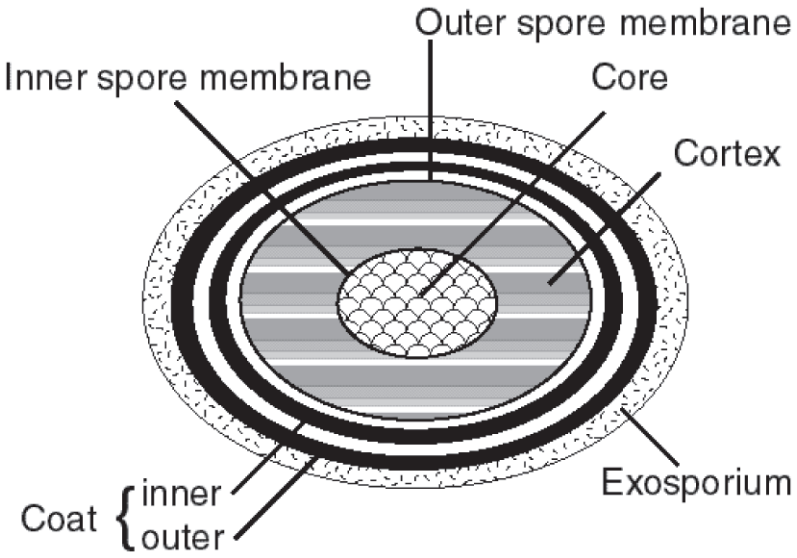
of *B. anthracis* are avirulent (Keim *et al.*, 1997).

C. Properties of Spores

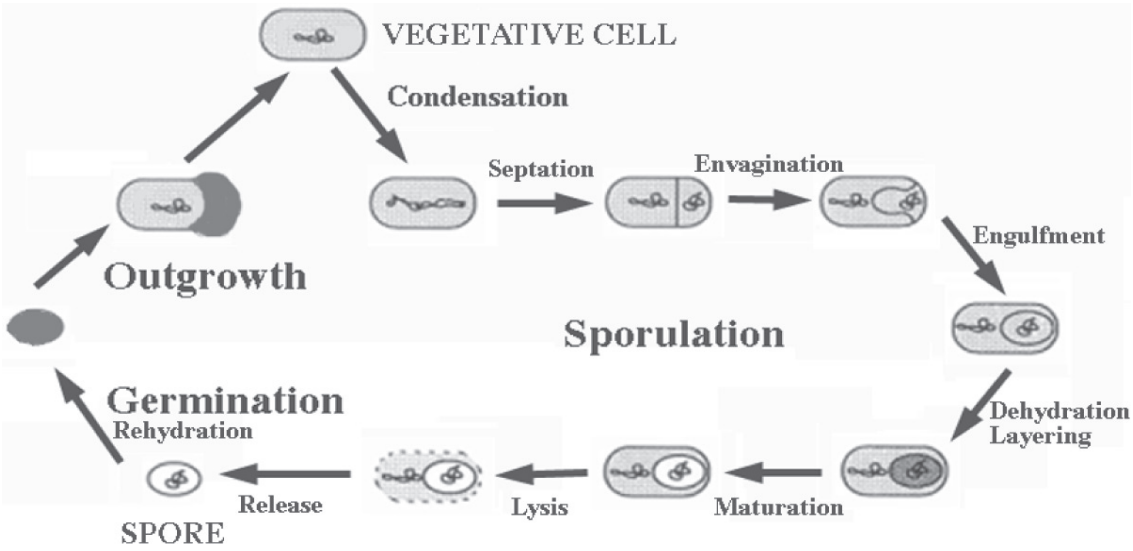
A spore represents a significantly different construct as compared to a normal bacterial cell. It is a dormant form, much like a seed, that consists of an exosporium on the outside with the multilayered spore coats below, a cortex with a structure similar but not identical to regular bacterial cell wall peptidoglycan, and a central core (Figure 1a). The exosporium consists of protein, lipid, and carbohydrate and is usually decorated with filamentous appendages that have been implicated in spore attachment to surfaces and ligands, much like other bacteria utilize pili (Mock and Fouet, 2001; Setlow, 1993a). The core contains most of the spore's enzymes, ribosomes, and DNA (Setlow, 1993a). Amino acids and amino acid biosynthetic enzymes are essentially absent in the spore. Enzymes that are present in the dormant spore core are inactive probably due to the very low water content in the core. Dormant spores are able to survive for a very long time, and they are resistant to adverse conditions including heat, UV and γ-radiation, pressure, and chemical agents (Setlow, 1993a and b, and 1992). Dormant state spores may survive for several million years (Cano and Borucki, 1995; Kennedy *et al.*, 1994). One mechanism that protects dormant spores against external damaging effects is the saturation of the spore chromosome with low molecular weight proteins termed small, acid-soluble proteins (SASP) (Setlow, 1993a and b). These proteins are synthesized midway in the sporulation process.

In a suitable environment or after activation by specific agents called germinants, spores initiate the conversion process to the vegetative cell (germination) followed by outgrowth that leads to the return to vegetative growth (Figure 1b). Amino acids for protein synthesis are supplied early in the germination process by degradation of SASP (Setlow, 1993a). This degradation process is initiated by a novel type of protease called germination protease (GPR), synthesized in parallel with SASP during sporulation (Setlow, 1993a and b). During spore germination this sequence specific protease initiates SASP degradation to amino acids and in doing so allows DNA transcription and provides amino acids for protein synthesis. In addition, the energy in the form of ATP is not available in spores even though small amounts of AMP is present. Therefore, significant energy for the early steps in spore germination is obtained by the metabolism of the spore's large deposits of 3-phosphoglycerate, a process leading to ATP synthesis, and by utilizing some of the amino acids generated by proteolysis of SASP. The lack of many chemical molecules and enzymes in the spore requires performing countless biochemical reactions to obtain all elements that are needed in the normal bacterial cell. NAD^+ synthesis seems to be one of the requirements as this molecule is needed in a variety of biochemical processes, in some of them only as a cofactor. The availability of appropriate levels of NAD^+ is absolutely essential for development of a new cell as well as for its survival.

These sources of dormant spore protection from diverse damaging effects in the environment, the removal of this protection to allow for transcription of DNA, and the supply of energy and NAD^+ during the early spore degradation stages are examples of absolutely essential processes for the dormant spore, spore germination, and spore outgrowth, and therefore these processes can be targeted in the development of therapeutics against them. Defining the mechanisms by which spores protect themselves and how



A



B

FIGURE 1. Spore structure, life cycle, and evolutionary relationship of members of *Bacillus* species. (A) Schematic diagram of a spore structure. The **core** contains the cytoplasm, the genome (Setlow, 1993a), and large amounts of Ca^{2+} and dipicolinic acid, and is surrounded by the inner spore membrane. The **cortex** is a peptidoglycan of somewhat different structure than in growing cells and is surrounded by the outer spore membrane. The **coat** consists largely of proteins and the **exosporium** present in some species consists of a lipoprotein layer (Setlow, 1995). **(B)** Life cycle of spore-forming bacteria. Under adverse conditions of nutrient limitations vegetative cells of *Bacillus* species have the ability to undergo transformation into a spore (sporulation process). Spores have totally different structure than a vegetative cell of this organism (see panel A of this figure) and are designed to survive for a long time under adverse conditions. When nutrients become available the spore is triggered into a germination process followed by outgrowth, which result in the synthesis of a vegetative cell. **(C)** Evolutionary tree for selected members of *Bacillus* species. Distances between various members the *Bacillus* genera correspond to their evolutionary relation and similarities. Branch lengths are drawn to scale.

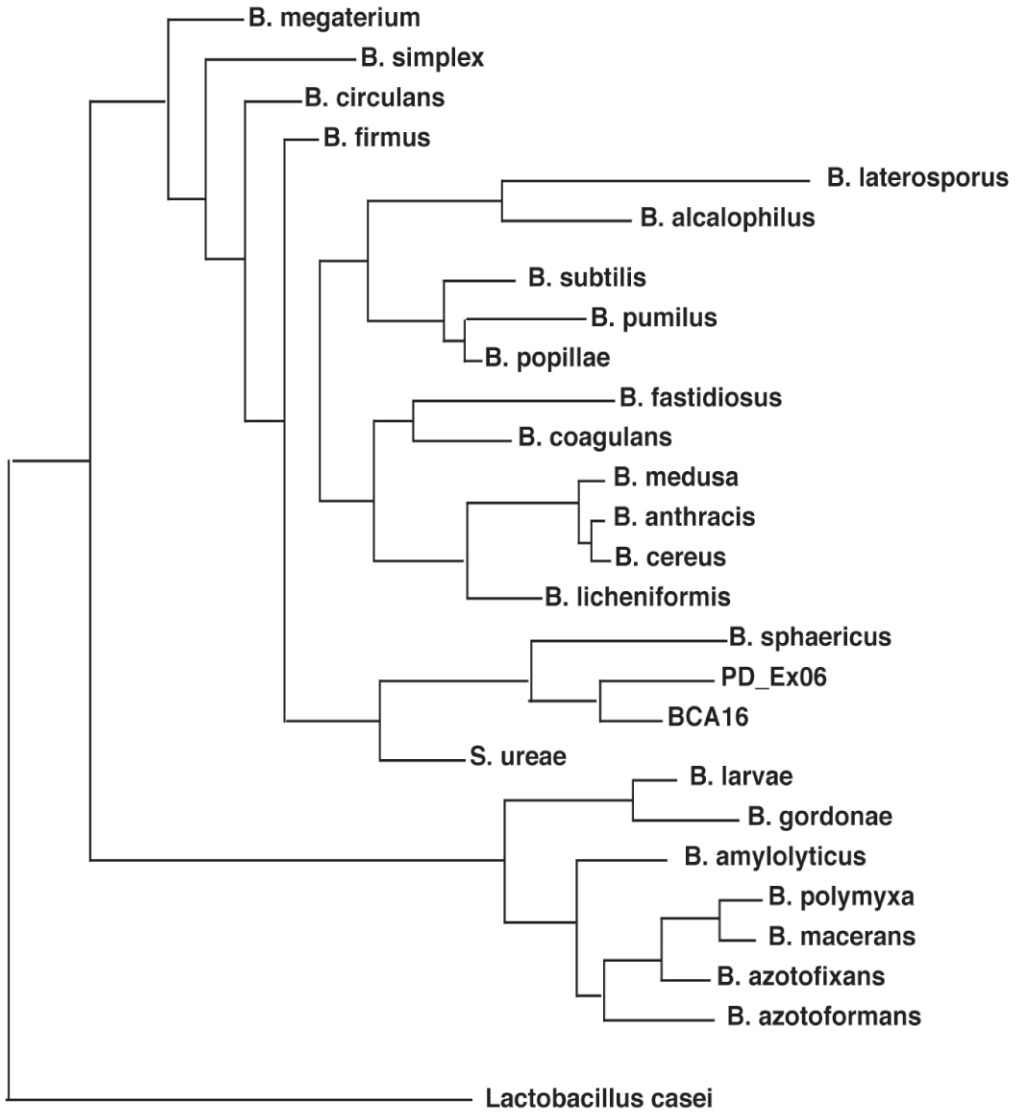


FIGURE 1C

they obtain energy will lead to a better understanding of how to limit spore-related illnesses.

D. Development of Novel and Improved Therapies Against Diseases Caused by Spore-Forming Bacteria

The current prophylactic agent and therapies against *B. anthracis* and anthrax are at best limited, especially for inhalation anthrax. The cutaneous or gastrointestinal forms of the disease are significantly less dangerous and are treatable with antibiotics. The inhalation anthrax, on the other hand, is difficult to treat successfully and usually involves treatment with a vaccine in parallel with antibiotics prescribed for an elongated time (LaForce, 1994). However, the current anthrax vaccine available is relatively rudimentary when compared with other vaccines on the market (Ivins *et al*., 1992) and the shortage of preclinical, clinical, pharmacological, safety data, and the possibility of undesired side effects makes it less than ideal (Turnbull, 1991 and 2000). On the other hand, the Institute of Medicine report declares 'the currently licensed anthrax vaccine to be acceptably safe and effective for the prevention of the disease', but the report also suggests the need for the development of a better product (Tranor, 2002). The recent Anthrax Vaccine Expert Committee, a civilian panel of physicians and scientists, did not identify unusually high frequency or pattern of serious adverse events due to this vaccine usage (Sever *et al*., 2002). The development of new prophylactic and therapeutic agents against anthrax such as a new vaccine and antibiotics is important, especially in light of their increased significance and demand due to the possible use of *B. anthracis* in bioterrorism or as biological threat agent. In addition, the basic knowledge about the spore-forming organisms and the understanding or their developmental properties, predominantly those of *B. anthracis*, are at best very limited. During recent years several processes indispensable for the members of *Bacillus* and *Clostridium* species were investigated in the author's laboratory using structural methods. Our increased understanding of the basic processes in the spore itself, followed by the understanding of germination process, and the continued development of spore during the outgrowth stage are essential. Such understanding is also critical for the development of novel cures for the disease. The understanding and the knowledge about the interactions between the fully developed vegetative cell of spore-forming bacterium and its environment, including the human host, are invaluable, especially the grasp of the role and function of virulence factors and toxins in pathogenesis.

The specific processes investigated and described in this work include the protection of spore's DNA against damage, sources of energy in the dormant spore, spore's ability to perform essential biochemical reactions, and, finally, the function of the virulence factors of these bacteria. The microbiological and biochemical investigations during recent decades were recently supplemented by the elucidation of the three-dimensional structures of proteins involved in the processes described above. The availability of structural data permits a detailed understanding of function as well as mechanisms. Such detailed knowledge provides the ability to influence or to modify these processes and possibly to develop a novel vaccine or antibacterial drug. Knowing positions of atoms in three dimensions in the active site of the enzymes or on protein epitopes allows for the detailed rational design of molecules, epitopes, or immuno-

gens that might interfere with the functional properties of these proteins. The majority of the proteins described here are critical for the survival of the bacterial organisms. For example, germination protease and toxins are specific to spore-forming bacteria, whereas others, such as phosphoglycerate mutase or NAD^+ synthetase, are more universal and are present in many other prokaryotic cells. These two enzymes from spore-forming organisms, however, have sufficient unique features that make them excellent targets for drug development specifically against spore formers.

Below are the details of the structure, function, and mechanism for germination protease, phosphoglycerate mutase, NAD^+ synthetase, protective antigen, lethal and edema factors are described and analyzed. Suggestions how such an understanding could be utilized in the development of novel therapeutics or prophylactics are also provided. Germination protease, phosphoglycerate mutase, NAD^+ synthetase represent potential targets for drug development, whereas protective antigen, lethal, and edema factors are, in addition to being drug targets, considered vaccine targets (protein-based novel generation of vaccines).

II. PROTECTION OF SPORES' DNA FROM DAMAGE

A. Protection by Small, Acid-Soluble Proteins

The DNA of dormant spores is saturated with a group of α/β-type small, acid-soluble proteins (SASP) that bind to the DNA surface (Setlow, 1991, 1992, and 1993a). These proteins are the main components responsible for the protection of spore DNA against damage and are important in spore resistance to desiccation, heat, oxidizing agents, and radiation (Setlow, 1995; Lindahl, 1993). SASP synthesis starts midway in the spore formation stages and occurs only in the developing spore (Setlow, 1993a). In *Bacillus* species there are two types of SASP that constitute 7 to 20% of total spore protein (Setlow, 1993c). The first type comprises up to seven α/β-type SASP that bind to DNA and are the source of its protection (Setlow, 1993b). The second type is a single γ-SASP, found only in *Bacillus* species, that does not bind DNA (Setlow, 1993c). Both types of SASP are, however, degraded by germination protease (GPR) during spore germination. In addition to the mechanisms that protect DNA in dormant spores, spores also have an efficient mechanism for DNA damage repair early in spore germination that is similar to that of normal bacterial cells, but not in the dormant spore (Setlow, 1993b).

B. Removal of Protection by Germination Protease

Germination Protease (GPR) initiates degradation of α/β- and γ-type SASP during the first minutes of the germination process for the *Bacillus* species. GPR is an amino acid sequence-specific enzyme that cleaves SASP at only 1 to 2 sites, followed by further degradation of the resulting oligopeptides by nonspecific peptidases. The consensus sequence motif recognized by GPR is X-E-$^{\downarrow}$I/F-A-S-E-X, with cleavage after the first glutamyl residue (marked in the sequence motif by an arrow). Sequences with slight deviations from this consensus sequence are also cleaved, but more slowly (Carrillo-Martinez and Setlow, 1994). GPR is synthesized in the developing spore as a zymogen termed P46, approximately in parallel with its SASP substrates (Loshon *et*

al., 1982). A few hours later after GPR synthesis, P46 converts to P41 by autoproteolytically removing 15 amino acids in *B. megaterium* or 16 amino acids in *B. subtilis* N-terminal residues (Loshon *et al*., 1982). Both P46 and P41 are tetramers (monomeric P41 is not active) (Loshon and Setlow, 1982). This autopro-cessing event is facilitated by a pH decrease in the developing spore, increasing levels of dipicolinic acid, and spore core dehydration (Illades-Aguiar and Setlow, 1994a). Although P41 is enzymatically active *in vitro*, it does not degrade SASP during sporulation or in the dormant spore. Significant dehydration inside the spore accounts for this lack of *in vivo* activity (Illades-Aguiar and Setlow, 1994a). However, as soon as spore germination commences, the spore core rehydrates and P41 starts rapid α/β- and γ-type SASP degradation that leads to the uncoating of the spore's DNA. Precise timing of the synthesis and processing of GPR is essential for the development of a fully resistant spore (Illades-Aguiar and Setlow, 1994b). Any deviation from the normal process results in a reduction of SASP levels in the spore with an attendant decrease in spore resistance and long-term survival (Illades-Aguiar and Setlow, 1994a and b).

C. Germination Protease is a Novel Proteolytic Enzyme

Mounting evidence from site-directed mutagenesis, use of protease inhibitors, chemical modification studies, and structural investigations suggest that GPR is a member of a new class of proteases. In fact, GPRs of *B. anthracis*, *B. megaterium*, *B. subtilis*, *C. acetobutylicum*, and *C. difficile* lack the characteristic signature sequences found in aspartic, cysteine, metallo- or serine proteases (Nessi *et al*., 1998). The results of sequence analysis were further investigated by a variety of methods. One such methods was site-directed mutagenesis of selected residues that were conserved at least in some of the known sequences for GPRs. The mutation studies were also supplemented by inhibitor studies and residue-specific chemical modifications of the possible catalytic residues such as cysteins, serines, and aspartates (Ponnuraj *et al*., 1999 and 2000; Nessi *et al*., 1998). These results reconfirmed that GPR is not a serine, cysteine, or aspartic protease.

The possibility that GPR might be an unusual metalloprotease, in the sense that it lacks the classic H-E-X-X-H sequence motif, was investigated by metal analysis using atomic absorption spectroscopy. These studies rule out the possibility of that the enzyme was a typical metalloprotease (Nessi *et al*., 1998). Finally, a trypsin-digested smaller form, termed P30, was generated that lacked the C-terminal 10-kDa segment (past Lys268). P30 was found also to be a tetramer and showed activity towards the autoconversion to a smaller form similar to the P46 to P41 conversion. These data suggest that the C-terminal ~10-kDa part of the protease past Lys268 is not essential for activity. The three-dimensional structure (see below) was also found to be unique in its fold organization and structure. Taken together, all information available suggests that GPR constitutes a new class of proteolytic enzymes.

D. Structure and Mechanism of Action of Germination Protease

The structure of the zymogen of germination protease from *B. megaterium* has been elucidated recently by X-ray crystallography (Ponnuraj *et al*., 1999 and 2000). The

zymogen is a tetramer assembled together as two sets of interacting dimes or a dimer of dimers (Plates 1a and b*). The structure is novel because it does not have any similarities to any known three-dimensional structures. The zymogen molecule has two distinct domains, a core structural domain accompanied by a smaller but more flexible cap domain. The core is built from mixed eight-stranded β-sheets surrounded on the outside by helices, whereas the cap domain consists of two α-helices and a significant amount of flexible loops that are not clearly observed in the structure determined. The enzymatically active functional form of the P46 molecule (i.e., having the ability to autoprocess to the P41 form) is a tetramer that, similar to the monomeric molecule, has a well-defined core structure that becomes more flexible and disordered toward the surface part of the molecule. The structural regions of the structure that are most distant from the center include the cap domains of the tetramer. The propeptide region built from amino acids 1 to 15 for the *B. megaterium* enzyme extends outside of the molecule toward the surrounding solvent region. The central part of the functional tetrameric unit forms a channel, ~18 Å in diameter (Plates 1b and c). The function of the channel is currently not known, but due to the highly electronegative surface in this region, it likely attracts a large number of positively charged divalent cations, such as Ca^{2+}, that could compensate for the electronegative character of the channel and make it more neutral in charge. The region of the propeptide location in the structure of P46 is likely the place where the catalytic center of the enzyme is located, that is, the place where the catalytic activity of the protease to convert P46 to P41 as well as SASP degradation by P41 takes place. The propeptide actually covers the cavity that contains the catalytic center that is exposed once the propeptide is removed. This region contains a large cavity located between the cap and core domains (Plate 1d). SASP molecules could be guided easily there by the electronegative potential generated by the electronegative surface of the enzyme as SASP molecules are either electropositive or at least less electronegative at the spores' pH than the GPR enzyme, especially for the cavity around the propeptide.

Although the precise mechanism of the GPR, P46, activation, and the catalytic mechanism of P41 are still somewhat speculative, three glutamic acid residues, Glu19, Glu26, and Glu202, all located in the propeptide cavity, have been implicated in this function (Ponnuraj *et al.*, 2000). Modeling studies have shown that SASP molecules can easily fit in this cavity where they could make contact with these Glu residues (M.J. Jedrzejas, unpublished results) (Plate 2*). If three Glu residues are indeed involved in catalysis, GPR would be the first glutamic acid (Glu) protease discovered (Ponnuraj *et al.*, 2000). However, definite answers will require structure determination of the active form of the germination protease, P41, preferably in a complex with its SASP substrate or product of degradation.

The close evolutional relation of the members of spore-forming organisms (Figure 1c) and the primary structure comparison of five GPR from *Bacillus* and *Clostridium* genera show high sequence similarity, suggesting a high structural similarity of all these enzymes (Figure 1c and Plate 3). Therefore, it is highly likely that the information gained from the structural and mechanistic studies of the *B. megaterium* protease that have been obtained are applicable to germination proteases from all spore-forming bacteria, especially *Bacillus* and *Clostridium* species. The active centers of similar GPRs will be easy to locate by the

* Plates appear following page 346.

sequence analysis or by the modeling studies of similar structures, including structures of *B. anthracis* P46 and P41. The studies of *gpr*⁻ mutant cells of *Bacillus subtilis* showed that it drastically compromises the ability of spores to make a vegetative cell, especially during the outgrowth stage of cell development (Sanchez-Salas *et al.*, 1992).

III. SUPPLY OF ENERGY FOR GERMINATING SPORE

The energy in the form of ATP is not available in spores even though small amounts of AMP, but not ATP, are present. Therefore, significant energy for the early steps in spore germination is obtained by the metabolism of the spore's significant deposits of 3-phosphoglycerate that were created during the sporulation process (see Introduction, Properties of Spores section) and by utilizing some of the amino acids generated by proteolysis of SASP. The utilization of the 3-phosphoglycerate deposits in spores leads to ATP synthesis as a consequence of the glycolytic pathway that involves phosphoglycerate mutase as well as other glycolytic enzymes. Compromising the function of phosphoglycerate mutase in bacterial cells lead to significantly compromised bacterial cells and ultimately to their death (Jedrzejas and Setlow, 2001; Jedrzejas, 2000).

A. Functional Properties of Phosphoglycerate Mutase

Phosphoglycerate mutases (PGMs) catalyze the transfer of a phosphate group between the carbon atoms of phosphoglycerates and are essential for glucose metabolism in most organisms. There are two general categories of phosphoglycerate mutases: bisphosphoglycerate mutases (bPGMs), whose role is to interconvert 1,3-diphosphoglycerate and 2,3-diphosphoglycerate (23PGA), and monophosphoglycerate mutases (mPGMs) that interconvert 2-phosphoglycerate (2-PGA) and 3-phosphoglycerate (3-PGA) (Chander *et al.*, 1999; Fothergill-Gilmore and Watson, 1989). Two types of mPGMs are known: one requires 23PGA for activity (dPGMs) and the other does not (iPGMs).

The iPGMs are found in all plants, selected invertebrates, certain fungi, selected algae, and some bacteria, predominantly Gram-positives, including spore-forming organisms (Grana *et al.*, 1995; Fothergill-Gilmore and Michels, 1993; Fothergill-Gilmore and Watson, 1989). These enzymes can have phosphatase activity toward 3-PGA, but, unlike dPGMs and bPGMs, do not have 23PGA phosphatase activity. The polypeptide chain length of iPGMs is much larger than that of dPGMs, and there is no significant sequence similarity between the iPGMs and either the dPGMs or bPGMs (Grana *et al.*, 1995; Fothergill-Gilmore and Watson, 1989). However, the primary sequences of all known iPGMs, even those from different kingdoms, are quite similar, suggesting that they have a common ancestor (Grana *et al.*, 1995). In addition, recent work has indicated that iPGMs have some limited sequence homology as well an evolutionary relationship with phosphopentomutases, alkaline phosphatases, and certain sulfatases (Galperin and Jedrzejas, 2001; Jedrzejas and Setlow, 2001; Jedrzejas, 2000).

It has been shown that iPGMs from spore-forming bacteria such as the various *Bacillus* and *Clostridium* species have characteristic and novel features that distinguish them from the rest of iPGMs in that the catalytic activity of these enzymes is extremely pH sensitive (maximum activity at

pH 8) and absolutely and specifically requires Mn^{2+} (Chander *et al*., 1998). These two properties appear to be related and are important in the regulation of enzyme activity during sporulation and germination of *Bacillus* species (explained in more detail below) and probably in *Clostridium* species as well (Chander *et al*., 1998).

Briefly, the sporulation process (the process of forming a spore from a vegetative cell), in opposition to germination, involves a pH drop in the developing spore to pH ~6.5 that decreases the activity of the pH-sensitive phosphoglycerate mutase PGM and results in the accumulation of large deposits of 3-PGA in the formed dormant spore (Magill *et al*., 1996; Kuhn *et al*., 1995). Later, when spores return to life via germination, the intraspore pH rapidly rises to approximately 8, thus reactivating the spore's PGM and allowing the utilization of the spore's 3-PGA deposits. The utilization of the 3-PGA deposits then provides a significant amount of the ATP needed in the early stages of spore germination. Although the biological significance of the Mn^{2+} requirement and pH sensitivity of the PGMs from Gram-positive spore formers is known, there is no understanding of the structural bases for these properties. Only recently was the three-dimensional structure of an iPGM from *Bacillus stearothermophilus* determined, and the availability of such structural data provided information necessary to determine how this and similar enzymes function (Jedrzejas *et al*., 2000a and b).

B. Structure and Mechanism of Spore's Phosphoglycerate Mutase

The structures of phosphoglycerate mutase from *B. stearothermophilus* have been elucidated in the functionally active form with both a substrate and a product, 3-PGA (Jedrzejas *et al*., 2000b) and 2-PGA (Jedrzejas *et al*., 2000a), respectively. The three-dimensional structure revealed a compact and globular structure of the enzyme with α/β topology so characteristic of glycolytic enzymes built from two domains (Plate 4a*). Both domains, A and B, are assembled with a central β-sheet flanked on the sides by α-helices. They interact via an extended surface-surface interaction and are connected only by two hinge-like loops that seem to give both domains a significant amount of structural flexibility with respect to one another. The cleft between these domains contains a well-defined and rigid in structure active site for the enzyme.

This catalytic site contains 15 amino acid residues from both domains strictly conserved across *Bacillus* species, two Mn^{2+} ions, phosphoglycerate, and a 'structured' water molecule that occupies a well-defined place in the structure of the enzyme. Each Mn^{2+} ion has five coordinating ligands, and they form a distorted square-pyramidal geometry around this ion, an arrangement that is common for Mn^{2+} ions. The coordination sphere of Mn1 includes interactions with three protein residues, Asp403, His407, His462, and interacts with two phosphate oxygen atoms of the phosphoglycerate (Plate 4b). The coordination sphere of Mn2, which is also square-pyramidal, involves only protein residues Asp12, Ser62 (implicated in formation of phosphoserine intermediate [see below]), Asp444, and His445. Both Mn^{2+} ions keep the active site residues in the appropriate orientation for tight binding of the phosphate group of the phosphoglycerate substrate and also participate in the catalytic reaction. Three histidine residues, His407, His445, and His462, are involved in the binding of the two Mn^{2+} ions. The pH-dependent properties of these histidines

* Plates appear following page 346.

likely cause the pH sensitivity of Mn^{2+} binding and catalysis (Kuhn *et al*., 1993 and 1995). The glycerate part of the substrate interacts in a bidentate fashion with Arg264, whereas the hydroxyl group interacts with Asp154 and Arg185. There is an ordered water molecule in the active site, Wat63, that directly interacts with the oxygen of the substrate carboxyl group and with the ester oxygen atom of the phosphate group. The position of this water molecule in close proximity to Mn1, as well as its interactions with the substrate, suggests a direct involvement of this molecule in the catalytic reaction through its polarization while coordinated with Mn1 during the catalytic cycle (see below). Two oxygen atoms of the phosphate group are hydrogen bonded with Arg261 in a bidentate fashion, and one of the phosphate's oxygen is also close to the backbone nitrogen atom of Ser62. The details of the interactions in the active site are provided in Plate 4b.

Based on the structure of the active site in *B. stearothermophilus* iPGM, mutation of the residues in the active site, and sequence comparison to *Escherichia coli* alkaline phosphatase, a mechanism of the interconversion of 3- and 2-PGA catalyzed by this iPGM has been proposed (Jedrzejas *et al*., 2000a and b). Such a mechanism proceeds via the formation of a phosphoenzyme intermediate (Breathnach and Knowles, 1977) on Ser62 (Plates 4b and c). The proposed catalytic mechanism involves phosphatase and phosphotransferase components. The five step mechanism starts with (**1**) 3-PGA substrate binding in the active site, followed by (**2**) formation of a phosphoserine intermediate (phosphatase reaction), (**3**) repositioning of the glycerate moiety, (**4**) transfer of the phosphate group back to the now repositioned glycerate to form a 2-PGA product (phosphotransferase reaction), and finally (**5**) dissociation of 2-PGA from the active site and exchange of protons with microenvironment, which makes enzyme available for the next round of catalysis. The reaction from 2-PGA to 3-PGA is equally probable.

The residues from domain A are responsible primarily for Mn^{2+} binding and formation of a phospho-serine enzyme intermediate, which is utilized for catalysis (Plate 4b). Residues from domain B are responsible for reorientation (isomerization) of the glycerate part of the substrate. The two Mn^{2+} ions are inherently involved in the formation and then dissociation of a phosphoserine intermediate (Plate 4c).

As is the case for GPR, the PGM enzymes of other spore-forming organisms are similar to one another (Plate 5), as they originate from evolutionarily related bacteria (Figure 1c). The primary sequence identity is 72%. Structural information for the *B. stearothermophilus* PGM is relevant to PGM enzymes of all spore-forming bacteria. All of these enzymes are probably Mn^{2+} metalloenzymes with high pH sensitivity. Sequence similarity of spore PGMs to other PGM from non-spore-forming Gram-positive bacterial organisms suggests that they might also be Mn^{2+} metalloenzymes. In this case such property leads to pH sensitivity that is physiologically not relevant. It suggests, however, that many bacterial organisms evolved from a common ancestor having the ability to form spores and some bacteria may have lost this ability during their evolution (Jedrzejas, 2000).

IV. BIOCHEMICAL REACTIONS IN THE GERMINATING SPORE

The lack of many chemical molecules and enzymes in the spore and the presence of only a few essential enzymes requires, starting from the early germination stage of spore degradation, transcription of multiple proteins, and synthesis of countless biochemical molecules to obtain all elements that are

needed in the normal bacterial cell. NAD^+ synthesis seems to be one of the requirements because this molecule is needed in a variety of biochemical processes, in some of them only as a cofactor. The availability of appropriate levels of NAD^+ is absolutely essential for development of a new vegetative cell as well as for its survival.

A. NAD^+ Synthetase and its Metabolic Importance

NAD^+ synthetase (EC 6.3.5.1) catalyzes the amidation of nicotinic acid adenine dinucleotide (NaAD) as the part of the last step in the synthesis of nicotinamide acid adenine dinucleotide (NAD), as shown: $NaAD + NH_3 + ATP + Mg^{2+} \Rightarrow NAD + AMP + PP_i + Mg^{2+}$ (Plate 6*). The enzyme belongs to the amidotransferase family of enzymes and through NAD, a ubiquitous coenzyme, is involved in countless biochemical processes such as oxidation-reduction reactions, substrate or cofactor in other reactions (Smith, 1995; Zalkin, 1985; Foster and Moat, 1980). It plays a central role in cellular metabolism. Compromising the activity of the enzyme results in significant impairing of cellular functionality. NAD can be obtained either through *de novo* pathway or using pyridine salvage. These two pathways merge at the level of synthesis of nicotinic acid adenine mononucleotide with two final steps of NAD synthesis being in common. The final step is performed by NAD^+ synthetase, which catalyzes the reaction outlined above. The *B. subtilis* enzyme has an absolute requirement for ammonia as amide donor (Nessi *et al.*, 1995). Finally, the *B. subtilis* enzyme has also been reported to be a general stress factor and the *outB* gene, coding this protein in *B. subtilis*, is strongly induced as a result of exposure to heat, salt, ethanol, or glucose deficiency (Antelmann *et al.*, 1997).

The enzyme has also been shown to be involved in germination and outgrowth of *B. subtilis* spores and is absolutely essential for these processes (Nessi *et al.*, 1995; Albertini *et al.*, 1987). NAD^+ synthetase was also found in dormant spores of this organism. Even though there are significant amounts of NAD stored in spores of *B. subtilis*, more NAD is needed for the development of the vegetative cell. Synthesis of NAD was observed as early as 30 min from the initiation of germination (Setlow and Setlow, 1977). The transcription of the *outB* gene was observed very early in the germination with detectable levels observed at 12 min (Albertini *et al.*, 1987 and 1988). The mutations of the *outB* gene were shown to cause spores to become temperature sensitive in the outgrowth stage of their development into a vegetative cell (Albertini *et al.*, 1987; Albertini and Galizzi, 1975). The enzyme has a natural ability to adapt to high temperatures, a property that might be reflected by the ability of spores to resist elevated temperatures (Nessi *et al.*, 1995). The mutations of this gene affect the ability for the vegetative cells to utilize various nitrogen sources such as asparagines, proline, glutamate, ammonium, or nitrate (Albertini and Galizzi, 1990). All mutants affecting NAD^+ synthetase activity impair cellular function and metabolism. Further evidence suggests that the product of the *outB* gene is not only required during the outgrowth of spores but is essential for the life of the cell (Albertini *et al.*, 1987).

B. Structure and Catalytic Mechanism of NAD^+ Synthetase

The NAD^+ synthetase from *B. subtilis* contains 271 amino acid residues, having a molecular weight of 30,240 Da (Nessi *et*

* Plates appear following page 346.

al., 1995). The structural information about the enzyme and its complexes with its natural substrates ATP, NaAD, and Mg^{2+} have been obtained by the means of X-ray crystallography. The functional unit of the enzyme is a compact homodimer with α/β subunit topology and with extended monomer-monomer interface (Devedjiev *et al*., 2001; Rizzi *et al*., 1996) (Plate 6a). The catalytic site is located in a deep cleft on the surface of the enzyme and extends across both subunits (Plates 6b, c, and d). It consists of a NaAD and ATP binding sites, and there are two separate catalytic sites per homodimer. These structural investigations suggest a mechanism of action of the enzyme in which an adenylated NaAD reaction intermediate is formed from ATP and NaAD substrates. This process involves at least two Mg^{2+} ions and possibly a monovalent cation that facilitate the formation and stabilization of the reaction intermediate (Rizzi *et al*., 1998). The utilization of specific interactions between the two substrates and the enzyme amino acid residues for this part of catalysis seems to be minimal as the enzyme primarily provides appropriately configured active site by which the enzyme fixes the placement and conformation of the reacting groups (Rizzi *et al*., 1998). The catalysis is performed on the properly positioned substrates in the active site of the enzyme solely by the cations present in its active site. These ions appear to weaken the α- to β-phosphate bond in ATP using the polarization of the respective atoms, allowing for its subsequent hydrolysis with formation of AMP and pyrophosphate (PPi), and the stabilization of the negatively charged transition state intermediate involving the now-adenylated NaAD (Plates 6c and e). The catalysis for this part of the reaction is mediated solely by the electron-withdrawing group of three metals, two Mg^{2+} ions, and one monovalent cation. Subsequent amidation of the reaction intermediate performed by nucleophilic attack of an ammonia molecule affords NAD^+ as a final product of the reaction (Plate 6e) (Spencer and Preiss, 1967). This process is started by initial binding of ammonium ion to the negative pocket in the enzyme prior to the deprotonation of this ammonium ion molecule, which affords the reactive ammonia needed to perform the nucleophilic attack. The Asp173 amino acid residue is well positioned to perform this proton abstraction and stabilization of the amino group of the transition state intermediate (Rizzi *et al*., 1998) (Plates 6e and 7). In contrast to the adenylation part of the reaction catalyzed by NAD^+ synthetase, in the amidation part of the reaction the amino acid residues of the enzyme, namely Asp173, play an active role in the catalytic process. The remaining byproducts of the reaction, AMP and PPi, are then released together with the NAD^+ product to permit the next round of catalysis (Rizzi *et al*., 1998). Magnesium ions stabilize the active site of the enzyme by coordinating to NaAD, and ATP substrates, and AMP, or PP_i products. The details of the stereochemistry of the substrate binding and analysis of the mechanism were analyzed utilizing the abundance of structures of the enzyme's complexes with substrates, products, and trapped intermediates at the various reaction conditions (Plates 6c and e). Such wealth of structural information allowed for detailed and precise functional and mechanistic conclusions and this, in turn, allowed for design of specific inhibitory compounds of the enzyme (Devedjiev *et al*., 2001; Rizzi *et al*., 1996 and 1998). By preventing the normal function of the enzyme, the inhibitors might become drug candidates against spore formers and other bacteria as the enzyme is essential in many biochemical reactions and is required for cell survival.

As is the case for GPR and PGM enzymes, NAD^+ synthetases of spore forming

organisms are similar to one another in their primary sequence (Plate 7) because they originate from evolutionarily related bacterial organism (Figure 1c). The overall primary sequence identity for the enzymes shown in Plate 7 is only 18%, but the amino acid residues essential for catalysis are strictly conserved, including the Asp173 residue responsible for binding of one of the substrates, an ammonium ion. Therefore, structural information for the *B. subtilis* NAD^+ synthetase is likely relevant to NAD^+ synthetases of all spore forming bacteria. Sequence similarity of spore NAD^+ synthetases to other NAD^+ synthetase from nonspore forming Gram-positive bacterial organisms also suggests their significant similarity (data not shown). The strict ammonia requirement for the bacterial enzyme and the ability of mammalian enzymes to use other nitrogen sources for this reaction strongly suggest that the design of specific inhibitors only against bacterial NAD^+ synthetases, not mammalian ones, should be possible.

C. *Bacillus anthracis* Toxins

The fully virulent strains of *B. anthracis* carry, in addition to chromosomal DNA, two plasmids, pXO1 and pXO2, which code among others for the primary virulence factors of this organism, including capsule formation and toxins (Brossier and Mock, 2001; Mock and Fouet, 2001). Their GC content is ~33%, which is of similar level as in the chromosome of *B. anthracis*. The pXO1 plasmid from the Sterne strain is 181,654 nucleotides long, and 143 open reading frames were identified accounting for ~61% of this plasmid's DNA (Okinaka *et al.*, 1999). Specifically, pXO1 includes genes that code for toxins: protective antigen (PA), lethal factor (LF), and edema factor (EF), regulatory elements, a resolvase and a transposase, and a three-gene germination operon gerX (Guidi-Rontani *et al.*, 1999). The toxins seem to act as binary combinations (Stanley and Smith, 1961). PA is a common cell-binding molecule that has the ability to interact with EF and LF. All these proteins were relatively recently elucidated on structural level using X-ray crystallography methods (Drum *et al.*, 2002; Pannifer *et al.*, 2001; Petosa *et al.*, 1997). The pXO2 plasmid from a Pasteur strain has also been sequenced (Mock and Fouet, 2001) and is 96,231 nucleotides long. A total of 85 open reading frames were identified in this plasmid, and these open reading frames include regulatory gene(s) and genes coding for proteins involved in capsule synthesis and degradation (Thorne, 1993).

VI. PROTECTIVE ANTIGEN

A. Functional Properties

Protective antigen is one of the primary virulence factors of *B. anthracis*, and it penetrates the physical defenses of the host by forming a host cells membrane-inserting heptamer after proteolytic activation by the host cells. This membrane heptamer translocates the other two toxic enzymes, LF and EF, into cytosol of the host cells, primarily microphages. PA is a ~83-kDa protein composed of 735 amino acid residues. The mature molecule is synthesized in an inactive form termed PA83. The proteolytic release of the N-terminal 20-kDa part, PA20 comprising residues 1 through 167, releases the fully active form of this molecule, which in turn is termed PA63. The activation of PA is performed *in vivo* by cell surface protease furin (Petosa *et al.*, 1997). The loss of PA20

leads to the formation of a heptamer built of PA63 molecular units (Milne *et al*., 1994). This heptamer is soluble in water-based solutions at neutral or basic pH but inserts itself in membranes at acidic pH, thus forming cation-selective channels in cellular membranes. Based on the structural studies, the size of the formed pores is approximately 160 Å in diameter, 85 Å in height, with the inner diameter of ~35 to 20 Å for the polar and negatively charged channel (Petosa *et al*., 1997).

B. Human Cellular Receptor

The receptor for PA in humans is called anthrax toxin receptor (ATR) and is a membrane protein with an extracellular component that binds to PA. The receptor was identified on human CHO-K1 cells and microphage cell lines (Bradley *et al*., 2001; Friedlander *et al*., 1993; Ezzell and Abshire, 1988), and at least one of its physiological functions is binding PA. This binding process leads to formation of heptamer and the transport of either LF or EF toxins into the cytosol of the host cells and causes serious toxic effects. The manifestation of such effects in animals when induced by the intravenous delivery of these toxins causes rapid death (Klimpel *et al*., 1994). This extracellular domain of ATR resembles a von Willebrand factor A (Bradley *et al*., 2001). The ATR protein is a ubiquitous protein expressed on cell surfaces (Friedlander *et al*., 1993; Escuyer and Collier, 1991). The receptor molecule was cloned and the protein consists from 368 amino acid residues, 27 of which compose a signal peptide, the following 298 amino acid residues compose an extracellular domain, the next 23 residues are included in a putative transmembrane part, and finally a few remaining amino acid residues compose a cytoplasmic tail of the receptor. The tail contains an acidic cluster of residues similar to that of furin (Bradley *et al*., 2001; Molloy *et al*., 1999). Residues 44 to 216 make up a von Willebrand factor type A domain known to be important for protein-protein interactions. This domain has been shown to be present on the variety of cell surfaces, including molecules such as integrin factors (Dickeson and Santoro, 1998). The similarity of PA receptor molecule to this factor is indicative of its functional properties. The protein also has a significant homology to TEM8 protein with unknown specific function, but it is known that TEM8 is upregulated in colorectal cancer endothelium (Bradley *et al*., 2001; St. Croix *et al*., 2000).

C. Three-Dimensional Structure

The PA structure has been elucidated by X-ray crystallography and shows an elongated and flattened molecule built from several distinct domains (Plate 8) (Petosa *et al*., 1997). Domain 1 contains amino acid residues 1 through 258 and is a β-sandwich with a few helices; two Ca^{2+} ions coordinate to the residues of this domain having an EF-hand motif. Domain 2 is comprised from residues 259 to 487 and has a β-barrel core structure with several long insertions such as a flexible loops implicated in PA membrane insertion. Domain 3, containing residues 488 to 595, is primarily a four-stranded, mixed β-sheet with some additions of α-helices and smaller β-sheet structures. Finally, domain 4 encompasses residues 596 to 735, and they form a fold similar to that of immunoglobulins with an additional insertion of a helix (Petosa *et al*., 1997). The first three domains interact with one another, whereas the fourth domain has a higher degree of separation from the rest (Plate 8a). Proteolytic activation of PA83 by furin leads to the truncation of domain 1 that after

truncation contains only amino acid residues 168 to 258 (termed domain 1'). The truncation process allows for the oligomerization of seven PA63 molecules to form a heptameric pore structure (Milne *et al*., 1994). The leaving PA20 molecule was shown to actively prevent the oligomerization of PA83 molecules because it causes the formation of significant steric conflicts during the oligomerization of PA; the removal of PA20 removes such steric constraints. The active PA molecules, PA63, pack like wedges with domains 1' and 2 on the inside of the ring and domain 3 and 4 on the outside of the pores ring structure (Plates 8a and b). There are no major structural and/or conformational changes to the PA63 monomers building the whole heptameric pore structure on pore formation. The residues located at the interface of the interacting monomers of the pore are highly conserved across different spore-forming organisms, indicating their importance for the oligomerization process (Petosa *et al*., 1997).

D. Model of Protective Antigen Membrane Insertion

The structure of domain 1' in the oligomer is stabilized by interactions with domain 2 and by the two Ca^{2+} ions which connect the new N-terminus of truncated PA, PA63, to the core structure of domain 1'. The release of PA20 creates a hydrophobic patch on the top of the heptameric pore structure, and this patch was directly implicated in binding of EF and LF toxins to PA63 (Leppla, 1995; Zhao *et al*., 1995). The evidence suggests that the PA63 pore is oriented in the membrane such that domain 1' is exposed to extracellular environment, making the domain 1' epitope available for LF and EF binding, and domain 4 together with bottom of domain 2 are placed next to the membrane (Petosa *et al*., 1997; Zhao *et al*., 1995). The exact mechanism of membrane insertion of PA heptamer is still speculative but a loop originating from domain 2 containing residues 302 to 325 was implicated in this process (Singh *et al*., 1994; Novak *et al*., 1992). There are some similarities of such loop with other similar structures of, for example, *Staphylococcus aureus* α-hemolysin (Song *et al*., 1996) and possibly even the tryptophan-rich loop of hemolysin of *Streptococcus pneumoniae*, pneumolysin O (Kelly and Jedrzejas, 2000a). The membrane-insertion process, similar to membrane insertion of, for example, hemolysin molecules, also involves changes of the secondary structure elements as well as changes in the three-dimensional structure of the building blocks of the pore (Kelly and Jedrzejas, 2000a and b; Petosa *et al*., 1997; Song *et al*., 1996). The membrane insertion process of PA63 pore is triggered *in vivo* by the acidification in endosomes; a similar process has been described for *Listeria monocytogene* listerolysin O, which lyses phagosomal membranes but not the cell membrane due to its slow pH activity optimum at around 5.5 (Geoffroy *et al*., 1987). Therefore, in general the whole physiological process of PA-mediated transport of *B. anthracis* toxin, LF and EF, inside the cytosol of the host cells consists of seven general steps: (**1**) binding of PA83 to the very recently discovered host cell receptor for PA, ATR; (**2**) proteolytic release of PA20 by furin and activation of PA by formation of PA63; (**3**) formation of a heptameric pore by PA63; (**4**) binding of the toxic enzymes, LF and EF, to PA63 heptamer; (**5**) endocytosis of the formed pore complex, followed by (**6**) acidification of the endosome triggering the membrane insertion of PA63 pore complex; and (**7**) translocation of LF and EF into the cytosol of the host cells (Petosa *et al*., 1997). After receptor binding an amphipathic β-hairpin of PA undergoes the insertion process into the cell membrane.

E. Hemolysins

Based on protective antigen structural and functional properties, PA shows some limited similarity to a larger group of proteins of pathogenic Gram-positive bacteria known as hemolysins (Plate 8). All of them are virulence factors for their organisms. Their mode of action is based on binding to the host cells' cytoplasmic membrane receptor (ATR for PA or a cholesterol molecule pneumolysin O), a process that is followed by insertion into the targeted membranes and formation of relatively large pores. Once the pores are formed, the targeted cell undergoes the lysis process or transport of toxins is initiated. One of the known exceptions to this standard mode of action for hemolysin-like molecules is the protein found in *Listeria monocytogenes*, termed listerolysin O, which lyses phagosomal membranes but not the cell membrane due to its slow pH activity optimum at around 5.5 (Geoffroy *et al*., 1987). All of these proteins have some similarities in their primary and overall three-dimensional structures as well as in their mode of action. Their structural properties seem to vary depending on their environment. Hemolysins often have a water-soluble form, a receptor-bound form, and a receptor plus membrane bound form. Each of these forms may differ in structure to a various degree. In the case of pneumolysin O, for example, its membrane pore assembly consists of 30 to 50 monomeric pneumolysin molecules, with a diameter of 350 to 450 Å (Kelly and Jedrzejas, 2000a; Rubins and Janoff, 1998). For PA, the membrane insertion process is slightly different and involves a formation of an intermediate heptamer pore built from individual molecules of activated form of PA.

F. Structures of Homologous Molecules

Other hemolisin-like, pore-forming molecules studied by structural X-ray crystallographic methods include aerolysin from *Aeromonas hydrophilia* (Parker *et al*., 1994), α-hemolysin from *Staphylococcus aureus* (Song *et al*., 1996), *Clostridium perfringens* perfringolysisn O (PFO) (Rossjohn *et al*., 1997), model of *S. pneumoniae* pneumolysin O (PLY) (Rossjohn *et al*., 1998), and finally the above-described protective antigen from *B. anthracis* (Petosa *et al*., 1997). All of these proteins share, in general, a similar fold with a characteristic domain-based structure, elongated shape, and high percentage of the β-sheet structure. After detailed comparison of these macromolecules, however, significant differences are obvious, mainly related to a different arrangement of the domains. The level of primary structure similarities among all these similar functionally proteins is also low.

VII. LETHAL FACTOR

A. Structure and Function

Yet another virulence factor of *B. anthracis* is the lethal factor. The three-dimensional crystals structure of this molecule has been elucidated recently by Pannifer *et al*. (2001). LF has a molecular weight of ~90 kDa, and it is a specific protease that cleaves members of the mitogen-activated protein kinase kinase family (MAPKK). The LF facilitated cleavage site is near the N-terminus of MAPKK and leads to inhibition of its signaling pathways (Vitale *et al*., 1998 and 2000; Duesbery, 1998). The main cell type affected by *B. anthracis* pathogenesis and LF is the macrophage (Hanna and Ireland,

1999; Hanna, 1998; Friedlander *et al*., 1993). Low levels of LF cleave MAPKK-3, inhibit the release of the proinflamatory mediators, NO, and tumor necrosis factor-α (Friedlander *et al*., 1993). On the other hand, high levels of LF cause lysis of macrophages. The data are consistent with the model suggesting that early in the infection LF may reduce or delay the immune system response, and at the late stages in infection it triggers macrophage lysis and the release of high levels of NO and tumor necrosis factor-α. Such a model is consistent with the symptoms observed before death, which are similar to those of septic shock (Pannifer *et al*., 2001).

The LF molecule is ~100 Å by 70 Å in size and is built from four distinct domains (Plates 9a and b). The N-terminal domain is involved in binding to PA (Gupta *et al*., 2001; Arora and Leppla, 1994). The middle part of the molecule contains a series of glutamate-rich repeats, which are essential for protein stability (Quinn *et al*., 1991), and the C-terminal part is the catalytic domain (Klimpel *et al*., 1994). Domain 1 comprises residues 1 to 254 and is a bit separated from the rest of the structure. This domain interacts only with domain 2. Domain 2 consists from a helix bundle packed against a mixed β-sheet. This domain, comprising residues 263 to 297 and 385 to 550, has a similar fold to that of the *B. cereus* VIP2 toxin (Pannifer *et al*., 2001). Domain 3 is also a small α-helical bundle and it comprises amino acid residues 303 to 382 of the mature protein. Finally, domain 4 is comprised of protein residues 552 to 776 and as domain 2 is built from a helix bundle packed against a β-sheet (Plate 9).

B. Zinc Metalloprotease Properties

Part of the domain 4 resembles the structure of a metalloprotease thermolysin and has the classical sequence motif for metalloprotease HExxH (Ponnuraj *et al*., 2000; Nessi *et al*., 1998; Klimpel *et al*., 1994). A Zn^{2+} ion was observed in the structure and shown to coordinate the trahedrally to a water molecule and three protein amino acid residues, His686, His690 from the signature sequence for metalloprotease, and Glu735. Another glutamic acid, Glu687, interacts with the ion coordinating water and is likely involved in this water molecule activation/polarization (Jedrzejas and Setlow, 2001; Jedrzejas, 2000). The Tyr728 residue was also implicated in the possible catalytic mechanism for this LF metalloenzyme as it also interacts with the same water molecule, as does the Glu735 residue (Pannifer *et al*., 2001). Mutation of any of the residues essential for zinc binding was shown to inactivate the protease (Klimpel *et al*., 1994). Such behavior of the LF enzyme further support that LF is a zinc protease. The structural studies by Pannifer *et al*. (2001) that involve a structure of the LF complex with the N-terminus of MAPKK clearly delineate the MAPKK binding site and allow for the identification of amino acid residues responsible for binding of substrate and also for the catalytic mechanism in the presence of Zn^{2+} ion (Plate 9). MAPKK pathway relays environmental signals to the cellular transcription machinery and modulates gene expression via a burst of protein phosphorylation (Brossier and Mock, 2001). Out of seven identified MAPKK proteins arranged in three pathways, LF cleaves all but the MAPKK5 (Vitale *et al*., 2000; Pellizzai *et al*., 1999; Widmann *et al*., 1999). The LF-induced cleavage takes place at the N-terminus of these signal transducing proteins, and specifically at the proline-rich region directly preceding the kinase domain. Such LF-induced cleavage disrupts direct interactions necessary for protein-protein interactions and for the assembly of signaling complexes necessary for the whole path-

way. However, some evidence suggests an additional substrate for LF that currently is unknown. That would explain additional properties of LF protease, such as its role in proteasome (Tang and Leppla, 1999). The binding site for the MAPKK substrate of LF is a broad and deep groove that is ~40 Å long and involves residues from domain 2 and from the interface between domains 2, 3, and 4. The groove is lined with largely electronegative residues. A 16 amino acid long of N-terminal part of the MAPKK-2 substrate was observed bound in this location in available the X-ray structure of the complex. The binding pocket has an open end at its N-terminal part, a feature likely allowing for a broad diversity of the chain lengths to be cleaved by this *B. anthracis* zinc metalloprotease.

VIII. EDEMA FACTOR

A. Functional and Structural Properties

The third virulence factor of *B. anthracis* described here is edema factor (EF). EF is a protein composed of ~800 amino acid residues, with an apparent molecular weight of ~89 kDa. It was the first of the *B. anthracis* toxins to be characterized biochemically (Leppla, 1982). Edema factor is a calmodulin-dependent adenylate cyclase that converts intracellular ATP into cAMP (Leppla, 1982). The toxic effect of EF in the host cells lead to the dramatic elevation of cAMP in these cells (Leppla, 1982 and 1995). It is likely the EF cooperates with LF within the host cells (Mock and Fouet, 2001). Even though macrophages are the key mediators of toxin-induced shock, the molecular mechanism and the sequence of events, especially involving EF, leading to death caused by anthrax are still unclear and controversial. EF toxin inhibits the phygocytic and oxidative burst (Mock and Fouet, 2001) and stimulates chemotaxis in human neutrophils and therefore has the ability to increase host susceptibility to infection.

The edema factor molecule has been shown biochemically to contain three conserved sequence ranges that are responsible for the catalytic process: 24 amino acid residue stretch from residues 303 to 339, including the GxxxxG(A)KS signature that is an ATP-binding recognition motif (Xia and Storm, 1990; Escuyer *et al*., 1988; Leppla, 1982); a calmodulin-binding site which is composed from two peptides, one from residue 499 to 532 and the second is the C-terminal 150 amino acid residues of the protein (Labruyere *et al*., 1991 and 1994; Munier *et al*., 1992); and a membrane association sequence (Guidi-Rontani *et al*., 2000; Wang *et al*., 1997). The 300 N-terminal amino acid residues of EF were shown to be involved in PA binding (Labruyere *et al*., 1994; Little *et al*., 1994).

B. Structure of *Bacillus anthracis* Edema Factor

The structure of catalytic part of *B. anthracis* EF (residues 291 to 800) has been elucidated recently by X-ray crystallography in its native form, in complex with calmodulin, and in complex with calmodulin together with 3'-deoxy-ATP (noncyclizable ATP) (Plates 10a and b) (Drum *et al*., 2001; and 2002). The structure of EF consists of three globular domains (domain 1, residues 294 to 349 and 490 to 622; domain 2, residues 350 to 489; and domain 3, residues 660 to 800). The first two domains constitute a catalytic part of the enzyme involved in adenylyl cyclase activity. This activity is placed at the interface between these two

domains. The third domain is all helical and is connected to domain 1 only by a 37 amino acid residue linker (residues 623 to 659). Domains 1 and 2 are built from α-helices and β-sheets.

Interestingly, in the complex structure with activator, calmodulin binds between the first and the third domain and effectively misplaces the helical domain (domain 3) out of its way to occupy the site of the third domain location in the native structure (Drum *et al*., 2002). The volume of calmodulin is surprisingly similar to that of the third domain. In order for this to take place the third domain is translocated by ~15 Å and rotated by 30° when compared with the native structure. Such large movement allows for the formation of clamp like structure by EF to nearly completely encircle the activator. After calmodulin binding, other significant parts of the EF molecule, in addition to the large movement of the helical domain, undergo significant structural rearrangement (Drum *et al*., 2002). As a final effect, calmodulin binding facilitate activation of EF catalysis, which increases EF activity ~1000 fold.

The structure of the EF active site responsible for ATP binding and catalysis is essentially unchanged in the structures of native enzyme or the one that is activated by calmodulin. The residues of catalytic base, the metal-binding residues, as well as residues proposed to be involved in stabilization of transition state have the same structural arrangement with respect to the substrate in the native and activated enzymes. The major differences between the native and activated enzymes in the substrate environment are proposed to involve primarily ATP binding residues not the residues performing catalysis.

The structural information about EF and its complexes with calmodulin and the noncyclizable ATP analog allowed for the formulation of a proposed mechanism of converting intracellular ATP into cAMP (Plate 10c). The interaction of His351 in the EF's active site with the 3'-hydrohyl group of the ribose ring of ATP substrate results in withdrawing of this OH group hydrogen by the deprotonated His351 residue. His351 acts, therefore, as a catalytic base. The remaining oxygen of the 3'-hydrohyl of ribose performs a direct nucleophilic attack on the α-phosphate of ATP leading to the cleavage of the α- to β-phosphate bond and the formation of cAMP product and PPi byproduct (Drum *et al*., 2002; Munier *et al*., 1992). The metal residue interacting with the enzyme active site and the ATP substrate, Mg^{2+}, together with Lys346 activate the α-phosphate group and also stabilize both, the α- and β-phosphate groups, and the resultant charges. During this process the proposed catalytic metal molecule, Mg^{2+} ion, is hexacoordinated to oxygens of the Asp491 and Asp493 residues and the oxygens of the α- and β-phosphates.

C. Structure of Eukaryotic Adenylate Cyclase

Contrary to previous beliefs, the *B. anthracis* EF structurally does not resemble membrane-associated eukaryotic adenylate cyclases (Plate 10d) (Mock and Fouet, 2001; Zhang *et al*., 1997). Mammalian adenylyl cyclases contain two conserved regions, C1 and C2, which are responsible for forskolin- and G-protein-stimulated catalysis. The structure of the C2 catalytic region of type II rat adenylyl cyclase was determined by the means of crystal X-ray diffraction (Zhang *et al*., 1997). The molecule has a α/β class fold in a wreath-like dimer, which has a central cleft (Plate 10d). In the structure reported by Zhang *et al*. (1997) two forskolin molecules were found to bind in hydrophobic pockets at both ends

of cleft. The central part of the cleft is lined by charged residues, which are implicated in ATP binding. Forskolin appears to activate mammalian adenylyl cyclases by promoting the assembly of the active dimer and by the direct interaction within the catalytic cleft. Other adenylyl cyclase regulators act at the dimer interface or on a flexible C-terminal region of the molecule. Even though the overall structures of prokaryotic, such as *B. anthracis* EF, and eukaryotic adenylyl cyclases are vastly different, the structural arrangement of the substrate-binding residues as well as the catalytic residues is surprisingly similar. In addition, both sets of enzymes require metal for catalysis and this metal is stabilized by two aspartates. The cluster of positively charged residues that are implicated in the stabilization of the transition state of the reaction is present in both groups of enzymes. Such similarities dissipate immediately outside of the active site area in the structures.

IX. CONCLUSIONS

A. Structure-Based Rational Development of Novel Prophylactic and Therapeutic Agents

The availability of three-dimensional structures for the selected proteins discussed in the review allows for a detailed understanding of their function as well as their mechanisms of action and shines some light on the general processes these proteins are involved in. Such detailed structural knowledge about the exact position of specific atoms of these proteins provides the ability to influence or modify the function and mechanism of these proteins and therefore provides the ability to influence the processes these proteins are involved in. As a consequence, structure-based rational development of a novel vaccine(s) by structurally mapping the important epitopes or antibacterial drug(s) by designing small molecules binding in the active sites of enzymes is possible. Such detailed knowledge of atomic positions should permit the design of molecules targeting specifically these proteins, not other proteins of the host, by the utilization of sometimes minute differences between the host and bacterial enzymes. For vaccines the structural information should allow for the determination of proper, most immunogenic, and well-defined epitopes to be included in new vaccine(s) to be developed.

B. Functional Importance of the Described Proteins

The precise mechanism for the activation of the germination protease as well as the mechanism of SASP degradation by the active form of this protease are still not fully known. However, the crystal structure of the zymogen of the protease revealed a novel fold of this enzyme that, together with other evidence, points to a novel proteolytic mechanism. On the other hand, the structure of the cofactor independent phosphoglycerate mutase revealed the mechanism of synthesis and degradation/isomerisation of 3-PGA. This catalytic process involves the unexpected formation of a manganese-catalyzed phospho-serine enzyme intermediate, and this process is extremely pH sensitive. This pH sensitivity allows for the regulation of 3-PGA deposits during spore formation and later during its degradation, a process necessary for the supply of energy in the early stages of germination. Similarly, the structural investigations of NAD^+ synthetase revealed more details about its

catalytic mechanism, and a cluster of three metal ions was implicated in the adenylation part of the reaction catalyzed by this enzyme. The studies of anthrax toxins, protective antigen, lethal factor and edema factor, have revealed the details of their function and related their functional properties to their structures. The PA serves as a vehicle transporting the LF and/or EF into cytosol of the host cells, primarily macrophages, which then allows these toxins to fully exert their destructive abilities on the host cells. The availability of the LF structure in large part facilitated the determination of the precise role of this multidomain enzyme in MAPKK degradation. This degradation leads to inactivation of the MAPKK pathway, which in turn leads to dramatic consequences for protein transcription and cell death followed by the death of the human or animal host. The availability of the structure of *B. anthracis* edema factor allowed for elucidation of the functional properties of this molecule (Drum *et al*., 2002) and the comparison to eukaryotic adenylate cyclase enzymes. As the three-dimensional structures of several eukaryotic adenylate cyclases are known (Mock and Fouet, 2001; Zhang *et al*., 1997), the comparison of the structure of *B. anthracis* EF to those of other adenylate cyclases' structures allows for confirmation of structural and mechanistic properties of EF.

C. Targeting the Spore and Spore-Degradation in the Development of Treatment Agents Against *B. anthracis* and Other Spore-Forming Bacteria

The proteins described in this study and the processes these proteins are involved in are absolutely essential for the survival of the spore-forming bacterial pathogens, especially *B. anthracis*, which is the main disease-causing bacterium of this group, a causative agent of anthrax. The importance of these proteins for various stages of the life cycle of *B. anthracis* and other spore formers can be utilized to develop an effective protection (prophylactic agent such as a vaccine) and treatment against infections (therapeutic agent such as a drug). It seems natural that targeting bacterial spore and preventing its development into a vegetative cell could be one approach for the development of a treatment against the infection, as it would prevent the formation of a vegetative cell. It is known that small molecules can penetrate external parts of spores and even reach the limited spore core enzymes. The vegetative cells cause multiple toxic and damaging effects in the host and its cells. The ungerminated spores are not known to be significantly toxic to the host. The prevention of the initiation of spore germination after, for example, spore inhalation by the host could be the first target for the development of prophylactic or therapeutic agent. The knowledge how this spore degradation process commences is, however, still limited, and more research needs to be completed to increase our understanding of these issues. The majority of the spore proteins involved in the detection of the signals for spore germination, and the subsequent transduction of this germination signal inside the spore are membrane proteins, and therefore their investigations are difficult and time consuming. The prevention of the onset of germination might also, however, make the spore difficult to eradicate from the host body.

The steps following the signal(s) for the spore to germinate involve the removal of DNA protection by GPR, supply of ATP-related energy by utilization of spore's depot of 3-PGA, and synthesis of essential cofactor for biochemical processes, NAD. The spores do not contain all necessary

enzyme or chemical molecules that are needed for the development of a vegetative cell. These enzymes and other molecules need to be synthesized using, in part, enzymes and the processes described above. All these individual enzymes, GPR, iPGM, and NAD^+ synthetase are plausible targets to either totally stop or at least very significantly delay the transformation of the spore through the early to late germination as well as through outgrowth processes. Targeting the enzymes involved by, for example, their inhibitory compounds would drastically compromise the germination and/or outgrowth processes likely leading to the prevention of the development of vegetative cells. The spores stopped at the various stages of the transformation into regular cells could likely be subsequently removed by appropriate host defense mechanisms and would prevent production of the toxins so important in invading the host and its defenses by the fully developed vegetative cells. The availability of the precise three-dimensional structural information will greatly facilitate the development of the inhibitory compounds for GPR, iPGM, and NAD^+ synthetase and will facilitate the development of these potent, novel drugs to combat spore infections, especially but not only of *B. anthracis* (as outlined throughout the text and in the Structure-based Rational Development of Novel Therapeutic and Prophylactic Agents section above).

In the first approximation such new drugs could be substrate analogs that could be competitive or even noncompetitive inhibitors of these enzymes. The metabolic importance of the iPGM and NAD^+ synthetase enzymes for bacterial organism (in particular in Gram-positive pathogens), the importance of GPR specifically for spore formers, as well as the absence of identical proteins in vertebrates, makes these proteins ideal targets for drug development in order to protect human population from these bacterial pathogens. The emergence of antibiotic-resistant strains, including spore formers and *B. anthracis*, necessitates the development of new and novel antibacterial drugs. In addition to the enzymes discussed here, the coat proteins of the spore coats should be considered targets as a possible vaccine or even for detection. The development of such spore coat-related vaccine might provide protection against spores before they develop into invasive cells. Inhibitors of iPGM, for example, are already under development and their initial testing *in vitro* shows promising results (M.J. Jedrzejas, personal communication).

D. Targeting Vegetative Cell in Development of New Cures

Finally, as the spore develops into a vegetative cell and invades the host, it produces toxins that for *B. anthracis* are known to be PA, LF, and EF. These three toxins working together in binary arrangements as PA-LF or PA-EF complexes cause the main damaging effects in anthrax. PA represents an excellent target to develop a new protein-based protective vaccine against anthrax for humans, as it has immunogenic properties, and such trials are already under way. PA is one of the major components of the currently available anthrax vaccine. The LF and EF toxins should be acceptable protein vaccine targets as well. The use of combined protein antigens such as all three toxins together, PA, LF, and EF, might provide the best target and the best protection. Also, these three toxins could be used as very reasonable targets for the development of small compounds inhibiting the function of LF or EF, or for the disruption of the binding between PA and LF or between PA and EF. These compounds or drugs facilitate the prevention of the toxic effects to the host

cellular physiology. The development of drugs inhibiting the catalytic function of LF and EF might be more straightforward instead of targeting the surface interactions involving PA. Similar to GPR, iPGM, and NAD^+ synthetase, the availability of the three-dimensional structures of PA, LF, and EF should greatly facilitate the development of both the vaccine and/or the novel drugs.

In addition, the utilization of tools of bioinformatics should readily provide a working, three-dimensional models for other enzymes possibly involved in essential processed of the vegetative cell even before true three-dimensional structure coordinates are available. The inhibitors should be specific for *B. anthracis* targets, and these targets should be somewhat different that the similar host proteins in order to avoid toxicity problems. Such bioinformatics-based approach, for example, was used successfully previously for structure determination of PhoE phosphatase from *B. subtilis* and *B. stearothermophilus* (Rigden *et al*., 2001 and 2002a) and endonuclease, penicillin-binding protein-related factor A (PrfA) also from both *B. subtilis* and *B. stearothermophilus* (D.J. Rigden and M.J. Jedrzejas, unpublished results; Rigden *et al*., 2002b). The modeled structures are often very reliable and essentially indistinguishable from those of the experimental structures.

Examples of the developments already well in progress toward new anthrax therapies include dominant-negative mutants of PA, polyvalent inhibitors blocking interactions between PA and EF or LF, and recombinant antibody fragments against PA. The identification of PA mutants that block translocation of toxins raised the possibility of their clinical applications. A group of such mutants that still has the ability of proteolytic activation and self-assembly was shown to coassemble with the wild-type PA to form hetero-heptamers. Some such mutants are dominant negative because their incorporation into heptamer prevents or at least impair translocation of toxins (Sellman *et al*., 2001). They were shown to strongly inhibit toxin action in cell culture and in an animal model. Such dominant negative mutants could possibly also be used as a vaccine component against anthrax. Wild-type PA is considered as the immunogen for a new vaccine being developed because it induces protective antibodies (Dixon *et al*., 1999). The utilization of dominant negative PA mutants might generate similar immunogenic protection as well as be a therapeutic drug (Sellman *et al*., 2001). In addition, polyvalent peptides weakly binding to PA are being developed for therapeutic use (Mourez *et al*., 2001). Using phage display selected peptides were identified that interfere with EF and LF binding to PA. Such peptides were linked to a polymeric backbone to increase their biologic activity, and they were shown to prevent the assembly of a full complex of PA heptamer with EF or LF enzymes *in vitro* and in an animal model (Mourez *et al*., 2001 and 2002). Finally, a panel of anthrax toxin neutralizing engineered antibodies was developed. Such antibodies are composed of synthetically linked single-chain variable domains of Fab fragments and similar constructs linked to constant κ domain of a human antibody (Maynard *et al*., 2002). The effectiveness against the disease of such constructs was shown effective *in vitro* and in a rat model of toxin challenge.

For the vaccine development, the three-dimensional structures described above could aid in identifying candidate vaccine components or epitopes instead of the whole proteins. For example, the current vaccine against anthrax already contains PA as its major immunogenic component (Friedlander *et al*., 1999). The enzymes discussed here could be used to obtain structural data on their complexes with antibodies generated

against them, for example. Such an approach would clearly identify the major immunogenic parts of such proteins, and only these parts could be of therapeutic utility. In addition to vaccine development utilizing the toxins, a possibility of using the *B. anthracis* poly-D-glutamic acid capsule or its components, or other virulence factors of this pathogen might be approached in the near future.

E. Final Remarks

The functional properties of all the proteins discussed above facilitate the understanding of significant aspects of either metabolism or pathogenesis of spore-forming bacteria that are essential in the colonization and/or invasion of the host tissues by these organisms. The evidence suggests that compromising the function of these proteins (by drugs or antibodies) will lead to either compromising pathogenicity or replication of these bacteria. At the current level of knowledge the best targets to combat diseases due to spore-forming bacteria, primarily *B. anthracis*, might be (**1**) anthrax toxin(s), mainly PA, or its cellular receptor for the vegetative cells and (**2**) iPGM, NADS, or GPR for the spore stage of the life cycle. Additional new developments such as a recent discovery of a bacteriophage lytic enzyme(s), for example, PlyG lysine isolated from the γ phage of *B. anthracis*, for the detection and killing of *B. anthracis* as well as related bacteria (Schuch *et al.*, 2002) might provide more avenues for finding the best cure.

The functional activities of the proteins can be compromised either by the utilization of specific drugs developed against these targets or by development of immunogenicity against them within the infected hosts by utilization of targeted novel vaccines. These two groups of therapeutic agents, prophylactic vaccines and therapeutic drugs, will likely be under development in the very near future. In addition, other proteins such as the lytic enzyme or truncated antibodies described earlier, might be of clinical use against the disease. Of special importance is the emergence of antibiotic-resistant strains and possible emergence of strains not protected by the currently available anthrax vaccine.

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