BACTERIAL INFECTIONS

Treatment of experimental anthrax with pegylated circularly permuted capsule depolymerase

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Anthrax is considered one of the most dangerous bioweapon agents, and concern about multidrug-resistant strains has led to the development of alternative therapeutic approaches that target the antiphagocytic capsule, an essential virulence determinant of *Bacillus anthracis*, the causative agent. Capsule depolymerase is a γ-glutamyltransferase that anchors the capsule to the cell wall of *B. anthracis*. Encapsulated strains of *B. anthracis* can be treated with recombinant capsule depolymerase to enzymatically remove the capsule and promote phagocytosis and killing by human neutrophils. Here, we show that pegylation improved the pharmacokinetic and therapeutic properties of a previously described variant of capsule depolymerase, CapD-CP, when delivered 24 hours after exposure every 8 hours for 2 days for the treatment of mice infected with *B. anthracis*. Mice infected with 382 LD_{50} of *B. anthracis* spores from a nontoxigenic encapsulated strain were completely protected (10 of 10) after treatment with the pegylated PEG-CapD-CP^{S334C}, whereas 10% of control mice (1 of 10) survived with control treatment using bovine serum albumin ($P < 0.0001$, log-rank analysis). Treatment of mice infected with five LD_{50} of a fully virulent toxigenic, encapsulated *B. anthracis* strain with PEG-CapD-CP^{S334C} protected 80% (8 of 10) of the animals, whereas 20% of controls (2 of 10) survived ($P = 0.0125$, log-rank analysis). This strategy renders *B. anthracis* susceptible to innate immune responses and does not rely on antibiotics. These findings suggest that enzyme-catalyzed removal of the capsule may be a potential therapeutic strategy for the treatment of multidrug- or vaccine-resistant anthrax and other bacterial infections.

INTRODUCTION

The major virulence factors of *Bacillus anthracis*, the causative agent of anthrax, are its capsule and two exotoxins. The capsule is the outermost layer surrounding the bacterium expressed during infection, and its synthesis can be induced in vitro in the presence of CO_2, bicarbonate, and serum (*1*, *2*). The capsule is composed of poly-γ-D-glutamic acid (PDGA) and is essential for virulence (*3*, *4*), and it enables the bacillus to resist phagocytosis and killing by innate immune cells (*5*, *6*) and disseminate throughout the host (*7*) in a manner similar to other encapsulated bacterial pathogens. The capsule can act as a physical barrier to prevent antibody and complement deposition on the surface of *B. anthracis* (*8*–*10*) and inhibit complement activity against other encapsulated bacteria (*11*). The *B. anthracis* capsule synthetase, capsule transporter, and capsule depolymerase (CapD; GenBank: AEG74409.1) are encoded within the *capBCADE* operon of the pXO2 plasmid (*12*, *13*). Loss of the pXO2 plasmid attenuates virulence, and the unencapsulated toxigenic Sterne-like strains lacking pXO2 are used throughout the world to protect livestock from anthrax and in countries of the former Soviet Union and China to protect humans (*14*).

Avery and Dubos (*15*) authored one of the earliest reports of the therapeutic use of enzymes to treat infections and the first use of capsule-degrading enzymes in the 1930s. Relatively crude preparations of capsule-degrading enzymes were used to remove the capsular polysaccharides from *Streptococcus pneumoniae* and were shown to be effective in treating experimentally infected mice, rabbits, and monkeys (*15*–*19*). Similar therapeutic approaches to remove capsules enzymatically to treat infections with bacterial and fungal pathogens have been reported for groups C and A *Streptococcus*, *Cryptococcus neoformans*, *Escherichia coli*, and *Klebsiella pneumoniae* (*20*–*23*). These enzymes do not kill the bacteria but render them susceptible to the host innate immune responses by removing the antiphagocytic capsule. This therapeutic strategy focused on targeting an essential virulence factor, could be categorized as an anti-infective rather than an antimicrobial because removal of the capsule is not bactericidal.

CapD is a γ-glutamyltransferase (GGT) that catalyzes endolytic cleavage of PDGA and degrades the capsular material to low–molecular weight multimers by catalyzing the transfer of a γ-glutamyl group to either water or an amino acid acceptor (*4*, *24*, *25*). The polymer of D-Glu is not present in humans.

In our previous studies, we extended the concept of removing capsule from bacteria to treat anthrax infections and showed that in vitro treatment of encapsulated bacilli with CapD removed the capsule and rendered the bacteria susceptible to phagocytic killing (*26*). Subsequently, we showed in mice that co-injection of CapD with either encapsulated bacilli from the toxigenic *B. anthracis* Ames or the nontoxigenic encapsulated *B. anthracis* ΔAmes strain markedly improved survival (*27*). More recent work by others confirmed that some protection was associated with treatment by enzymes from bacteria other than *B. anthracis* (*28*). In our studies, protection was also observed when recombinant CapD was administered 30 hours after infection with *B. anthracis* ΔAmes spores; however, protection was not achieved after challenge with *B. anthracis* Ames spores (*27*). To determine whether protection was limited by the

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stability and pharmacokinetic properties of the recombinant CapD, we developed a circularly permuted (CP) protein designed variant with greater stability (*29*).

GGT enzymes are N-terminal nucleophile hydrolases (Ntn-hydrolases). CapD must autocatalytically cleave itself internally to produce a free N-terminal Thr^{352}. Thr^{352} acts as the general acid, general base, and nucleophile in the reaction (*24*, *25*). The internal cleavage produces two fragments (residues 1 to 351 and 352 to 528), and the activity of GGT enzymes is limited by the efficiency of autoproteolysis. The circularly permuted construct (CapD-CP) shifts the nucleophilic Thr^{352} residue to the N terminus and removes the requirement of internal autoproteolysis, which improves the production of homogeneous protein. In addition, the CapD-CP has greater thermostability than native CapD (CapD-N) (*29*). In this study, we modified CapD-CP by introducing a cysteine (S334C) for pegylation, and we demonstrated that pegylated CapD-CP (PEG-CapD-CP^{S334C}) can protect mice against a lethal challenge with the fully virulent *B. anthracis* Ames strain.

RESULTS

Preparation and characterization of pegylated CapD-CP^{S334C}

In our previous work (*24*), we expressed the CapD-N sequence fused to maltose-binding protein (MBP-CapD-N) to enhance production of the soluble enzyme. MBP-CapD-N was obtained with high yields (~39 mg of the MBP fusion protein per liter of media); however, the activity of the enzyme was dependent on the degree of internal autocatalytic cleavage as the amino group of Thr^{352} has a proposed role in catalysis (*24*, *25*). Visual inspection of SDS–polyacrylamide gel electrophoresis (PAGE) gels revealed that ~60 to 70% of the purified MBP-CapD-N enzyme was autocatalytically cleaved by the end of purification. This observation suggested that the purified MBP fusion protein was a mixture of uncut and cut protein. Circular permutation of the N- and C-terminal domains and removal of the predicted signal peptide and other N-terminal residues (Fig. 1) resulted in expression of a soluble His-tagged protein with no additional fusion protein, referred to as CapD-CP. Cys residues are absent in MBP-CapD-N and CapD-CP; thus, Cys residues were introduced by site-directed mutagenesis to incorporate a pegylation site. Four pegylation sites on the surface of the enzyme were selected using the crystal structure (*30*) and tested in the MBP-CapD-N construct: S42C, S182C, S334C, and S475C (Fig. 1). Ser^{42} was only present in the full-length construct but not in the CP construct. The S334C variant had k_{cat} and K_m values most like the original construct, and minor reductions (<4-fold) in k_{cat} were observed for the other variants (table S1). Therefore, we introduced a cysteine at Ser^{334} for pegylation to the engineered CapD-CP designed by Wu *et al.* (*29*). Using the purification protocol described herein, we obtained 9.7 mg of purified C-terminally His-tagged CapD-CP^{S334C} per liter of media.

The S334C variant, CapD-CP^{S334C}, had a k_{cat} of 2.0 ± 0.2 min^{-1}, which was similar to MBP-CapD-N with a k_{cat} = 1.21 ± 0.05 min^{-1} (Table 1). The K_m of the CapD-CP^{S334C} variant was 3.1 ± 0.5 μM, which was also similar to the K_m of CapD-N (K_m = 3.3 ± 0.3). The specific activity (U/mg) was 1.6-fold higher for the CapD-CP^{S334C} variant (0.036 ± 0.003 U/mg) when compared with the CapD-N (0.022 ± 0.001 U/mg) variant that required autocatalytic internal cleavage. The higher activity indicates successful processing of the *f*-Met from the new N terminus, and production of active enzyme during cytoplasmic expression in *E. coli.*

To reduce immune clearance, denaturation, and proteolysis of the enzyme in vivo, a three-pronged (methyl-$PEG_{12})_3$-PEG_4-maleimide (molecular weight = 2361 g/mol) was covalently linked to Cys^{334}. The substrate-binding site of CapD was previously identified through modeling and site-directed mutagenesis (*24*, *25*). Kinetic parameters of the pegylated enzyme were less than threefold different from those of the nonpegylated enzyme, and no substantial reductions in activity or changes in the K_m were observed after pegylation (Table 1).

Effect of pegylation at S334C on melting temperature

For in vivo use, the enzyme must be stable for prolonged periods at body temperature (37°C) or slightly above (fever). Mutations that destabilize the enzyme by the removal of hydrogen bonds or interfere with correct protein folding can be detected by the measurement of the melting temperature (T_m) of the protein. We measured the T_m of CapD-N and its variants to find the least destabilizing mutation for pegylation (table S1). Thermal denaturation of the pegylated and nonpegylated enzymes was monitored by circular dichroism (CD) spectroscopy (Table 2). The crystal structure of CapD-N [Protein Data Bank (PDB) 3G9K] shows a large amount of α-helical content (*30*), so denaturation was monitored at 222 nm, and the T_m corresponds to the midpoint of the thermal denaturation curve and was measured for each protein in phosphate-buffered saline (PBS) (pH 7.4). Thermal denaturation of CapD-N without the MBP fusion protein produced a monophasic curve with a sharp transition and a T_m of 45.8° ± 0.1°C. The circularly permuted CapD-CP had a T_m of 50.6° ± 0.3°C, which was 4.8°C higher than CapD-N (Table 2). The S334C variant of CapD-CP had a T_m of 48.9° ± 0.5°C, which was 3.1°C higher than CapD-N (Table 2), and the curve was also monophasic (fig. S1). Thermal denaturation of PEG-CapD-CP^{S334C} produced a biphasic curve with a midpoint of 56.2° ± 0.1°C. In all cases, the denaturation of the protein was irreversible. The increase in T_m is consistent with stabilization of the

Table 1. Steady-state kinetic parameters for the CapD-N, circularly permuted CapD-CP^{S334C}, and pegylated CapD-CP^{S334C} using L-serine as an acceptor substrate in 25 mM Hepes (pH 7.4) and 0.1% Tween 20 at room temperature (22° ± 3°C).

Enzyme	V_{max} (U/mg)	k_{cat} (1/min)	K_m (μM)	k_{cat}/K_m (1/min*μM)
CapD-N (without MBP)	0.022 ± 0.001	1.21 ± 0.05	3.3 ± 0.3	0.37 ± 0.04
CapD-CP^{S334C}	0.036 ± 0.003	2.00 ± 0.20	3.1 ± 0.5	0.60 ± 0.10
PEG-CapD-CP^{S334C}	0.041 ± 0.002	2.30 ± 0.10	8.0 ± 1.0	0.29 ± 0.04

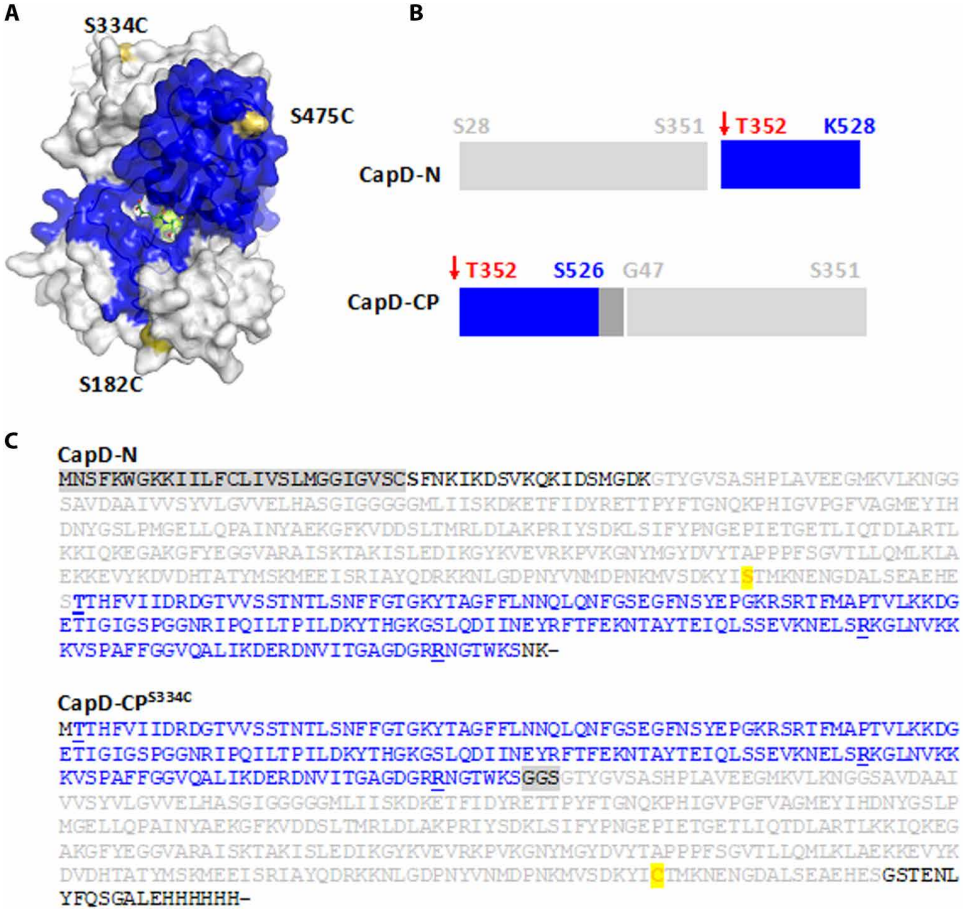

Fig. 1. Structure and organization of CapD-N and CapD-CP^{S334C}. (**A**) Structure of CapD-N (PDB 3G9K) (*30*). A docked Cys-Glu substrate is shown in the active site in ball and stick (green). The nucleophilic Thr^{352} is located behind the substrate (lime green). Four variants of MBP-CapD-N (S42C, S182C, S334C, and S475C) were tested for pegylation and are shown in yellow; Ser^{42} is in an unstructured region. After internal cleavage, CapD-N consists of two polypeptide chains (gray and blue). (**B**) Organization of CapD-N and the circularly permuted CapD-CP. The C-terminal portion of the polypeptide was moved to the N terminus and reconnected to the N-terminal portion of the enzyme with a short linker. (**C**) Protein sequences of CapD-N and CapD-CP^{S334C}. The serine shown in yellow corresponds to S334. The N-terminal sequence highlighted in gray in CapD-N is a predicted signal peptide sequence; this portion of the protein was omitted in the MBP-CapD-N construct. Residues shown in light gray and blue correspond to structured regions of the protein in PDB 3G9K. Residues in black are unstructured in PDB 3G9K (no observed density).

Table 2. Melting temperatures measured for CapD variants. Denaturation was monitored by CD spectroscopy.

Enzyme	T_m (°C)	Curve type
CapD-N	45.8 ± 0.1	Monophasic
MBP-CapD-N	56.4 ± 0.1	Broad and biphasic
CapD-CP	50.6 ± 0.3	Monophasic
CapD-CP^{S334C}	48.9 ± 0.5	Monophasic
PEG-CapD-CP^{S334C}	56.2 ± 0.1	Broad and biphasic

unfolded state and a reduction in irreversible processes such as aggregation. For all proteins, the T_m values were above body temperature, and no substantial unfolding was observed below 42°C.

Pharmacokinetics of CapD variants

We carried out an initial comparison between the CapD serum levels of mice injected intraperitoneally with either CapD-N or CapD-CP^{S334C} at 40 mg/kg and a second experiment compared CapD-CP^{S334C} with PEG-CapD-CP^{S334C} at 40 mg/kg. When the data from both experiments were analyzed together (Fig. 2), the geometric mean [geometric standard error (GSE)] of the area under the curve (AUC) of 1.47 (1.66) (μg × hour/ml) in CapD-N animals, versus 5.42 (1.27) in CapD-CP^{S334C} animals (P = 0.045), or versus 10.79 (1.62) in PEG-CapD-CP^{S334C} animals (P = 0.018) was statistically significant. These results indicate noticeable variability but suggest that PEG-CapD-CP^{S334C} persisted in the circulation longer than CapD-N or CapD-CP^{S334C}, although the difference between the nonpegylated and pegylated CapD-CP^{S334C} enzymes was not statistically significant (P = 0.210) (Fig. 2).

Protection afforded by PEG-CapD-CP^{S334C} after challenge with *B. anthracis*

Mice infected with spores of the encapsulated nontoxigenic *B. anthracis* ΔAmes strain were treated with PEG-CapD-CP^{S334C} or bovine serum albumin (BSA) as a control (each at 40 mg/kg) beginning 24 hours after infection at 8-hour intervals for 2 days. At ~32 hours, three mice from the BSA group had succumbed to the infection. Mice treated with PEG-CapD-CP^{S334C} were completely protected, and 100% (10 of 10) survived after 21 days, whereas only 10% of mice (1 of 10) survived after treatment with BSA (P < 0.0001, log-rank analysis) (Fig. 3).

In a second experiment, mice infected with spores of the encapsulated toxigenic *B. anthracis* Ames strain were similarly treated beginning 24 hours after infection with either PEG-CapD-CP^{S334C} or BSA (each at 40 mg/kg) at 8-hour intervals for 2 days. PEG-CapD-CP^{S334C} treatment protected 80% of the animals (8 of 10 survived) after 21 days, whereas only 20% (2 of 10) survived after treatment with BSA (P = 0.0125, log-rank analysis) (Fig. 4). This experiment was repeated and again showed significant protection in animals treated with PEG-CapD-CP^{S334C} compared to BSA (P = 0.0262, log-rank analysis).

DISCUSSION

The therapeutic use of capsule-degrading enzymes was pioneered by Avery and Dubos (*15*) in the 1930s. After the discovery of

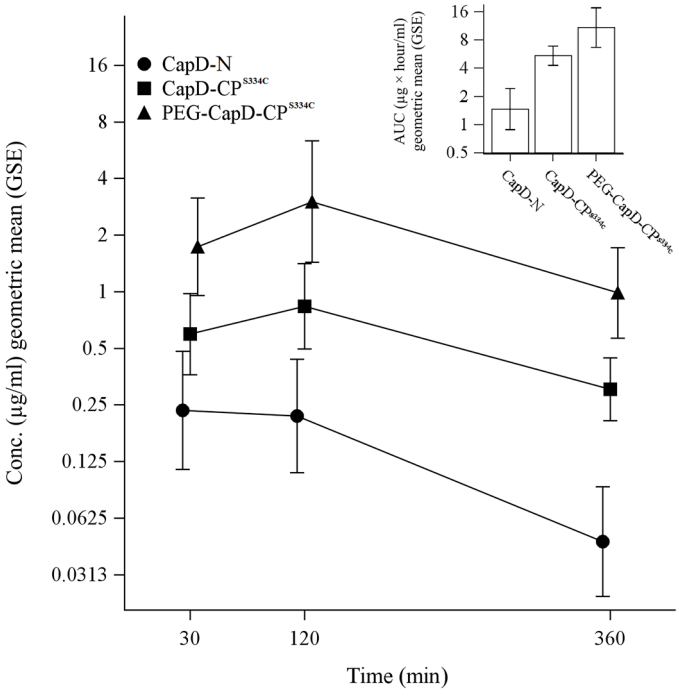

Fig. 2. Pharmacokinetics of CapD-N, CapD-CP^{S334C}, and PEG-CapD-CP^{S334C} in BALB/c mice. Time course of CapD serum concentrations (μg/ml) over 360 min after a single intraperitoneal injection. The data are presented as the geometric means (GSE). CapD-N (•; $n = 9$), CapD-CP^{S334C} (■; $n = 14$), and PEG-CapD-CP^{S334C} (▲; $n = 5$). In the inset are the geometric means (GSE) of the AUC. The geometric means (GSE) of the AUC of 10.79 (1.62) for PEG-CapD-CP^{S334C} and 5.42 (1.27) for CapD-CP^{S334C} were significantly greater than that of 1.47 (1.66) for CapD-N; $P = 0.018$ and $P = 0.045$, respectively, by two-way ANOVA). The difference in the AUC between PEG-CapD-CP^{S334C} and CapD-CP^{S334C} was not significant ($P = 0.210$).

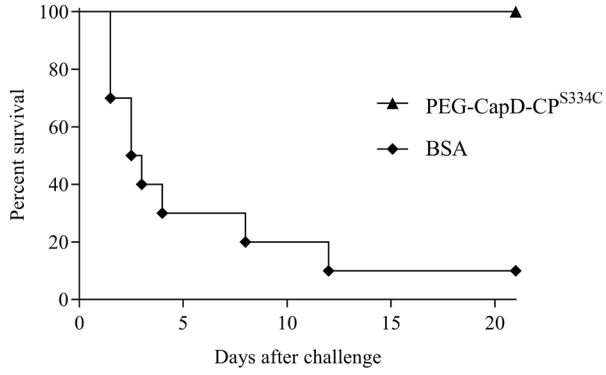

Fig. 3. Survival of BALB/c mice infected with *B. anthracis* ΔAmes spores. Mice (10 per group) were treated with either PEG-CapD-CP^{S334C} (▲) or BSA (♦; 40 mg/kg) 24 hours after infection. Survival was significantly better with PEG-CapD-CP^{S334C} treatment ($P < 0.0001$, log-rank analysis).

antibiotics in the 1940s, enzyme therapies were largely abandoned. However, the widespread use of antibiotics has led to resistance, and pathogens resistant to more than one broad spectrum antibiotic, so-called "superbugs," have since been isolated. Therapeutic enzymes capable of removing the *B. anthracis* capsule may be useful for treating antibiotic or vaccine-resistant strains that naturally arise or are intentionally developed, particularly multidrug-resistant strains.

The strength of this therapeutic enzyme strategy lies in the enzyme's insensitivity to the many evolved mechanisms of resistance to which small-molecule antimicrobials are ultimately subject (e.g., efflux pumps, mutation of binding sites, antibiotic degrading enzymes) (*31*). A "CapD-resistant" strain would essentially be an unencapsulated strain (like the attenuated Sterne vaccine strain) or a strain that had acquired new enzymes that alter its unusual PDGA capsule to render it uncleavable by CapD. In the former case, resistance to recombinant CapD could arise because of loss or damage of any component of the *capBCADE* biosynthetic operon; however, this would effectively attenuate the bacteria, reverting it to a more susceptible state in vivo. The selective pressure therefore lies in a favorable direction. In the latter case, the acquisition of new functional enzymes would be required, which would be unlikely to occur during a course of treatment.

Protein therapeutics are subject to proteolysis and removal by the immune system if they persist for >5 days. Their degradation and subsequent presentation to and removal by the immune system can be minimized by pegylation, which has been shown to prolong the half-life and reduce proteolysis of biologics in circulation (*32*), and by reducing the treatment period to <5 days. In this study, mice were treated for 2 days starting at 24 hours after infection. Under some conditions, individuals develop non–immunoglobulin E antibodies to enzymes during enzyme replacement therapies, and these antibodies do not necessarily reduce enzyme efficacy. In some cases, increased clearance of therapeutic enzyme can be overcome by increased enzyme dosing (*33*). The development of antibodies to CapD is not necessarily expected to lead to adverse effects, as CapD is a bacterial enzyme that is foreign to the host. It is encoded by the pXO2 plasmid of *B. anthracis*, and the enzyme is thought to be anchored to the membrane (*4*). During infection, the CapD-N enzyme is already present on the pathogen; thus, if antibodies were to arise, they might be expected to bind to a pathogenic bacterial cell surface protein.

Our early studies demonstrated that co-injection of CapD with encapsulated *B. anthracis* Ames in mice could improve survival by promoting phagocytosis (*26*, *27*), and subsequent work by others (*28*) confirmed these findings using a capsule-degrading enzyme called EnvD obtained from non-anthracis bacteria.

In our initial study, treatment with a single dose of CapD-N (400 μg) 30 hours after exposure to *B. anthracis* Ames spores did not lead to protection in mice (*27*). Recombinant CapD-N expressed in *E. coli* was obtained using a self-cleaving (intein) chitin-binding domain fusion, but yields were still relatively poor (*27*). The fusion of MBP to CapD-N led to a marked improvement in the yield of soluble enzyme (39 mg of the MBP fusion protein per liter of media) (*24*); however, the activity of the enzyme depended on the degree of internal autocatalytic cleavage as the amino group of Thr^{352} has a proposed role in catalysis (*24*, *25*). From visual inspection of SDS-PAGE gels, ~60 to 70% of the purified enzyme was autocatalytically cleaved by the end of the purification; thus, the purified MBP fusion protein was a mixture of uncut and cut protein (*24*). Circular permutation of the N- and C-terminal domains and removal of the predicted signal peptide and additional N-terminal residues (Fig. 1C) (*29*) led to expression of soluble His-tagged protein with no additional fusion protein. Only removal of the N-terminal *f*-Met residue was required for activity. Here, we obtained 9.7 mg of purified C-terminally His-tagged CapD-CP^{S334C} per liter of media, and the specific activity

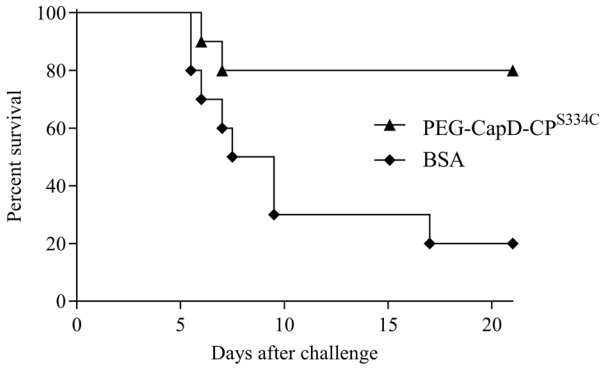

Fig. 4. Survival of BALB/c mice infected with *B. anthracis* Ames spores. Mice (10 per group) were treated with either PEG-CapD-CP^{S334C} (▲) or BSA (♦; 40 mg/kg) 24 hours after infection. Survival was significantly better with PEG-CapD-CP^{S334C} treatment ($P = 0.0125$, log-rank analysis).

was higher than that of CapD-N (Table 1). The improved yields and homogeneity of the purified enzyme are expected to make manufacturing the enzyme more efficient.

A large thiol-selective three-pronged PEG-maleimide molecule was used for a single site-specific pegylation. Several pegylated proteins have been approved by the U.S. Food and Drug Administration (*32*, *34*), and although pegylation can reduce enzyme activity in some cases, our study has shown that site-specific pegylation had a modest <2-fold effect on activity. Pegylation can also increase the in vivo half-life of a therapeutic enzyme (*32*, *34*). Our studies in mice showed markedly higher levels of CapD-CP^{S334C} in the blood compared with CapD-N, and the increased levels of PEG-CapD-CP^{S334C} compared to CapD-N was even greater, although the difference between the AUC of PEG-CapD-CP^{S334C} and CapD-CP^{S334C} did not reach statistical significance (Fig. 2). The dosing regimen of six injections of PEG-CapD-CP^{S334C} (40 mg/kg) given 24 hours after exposure every 8 hours for a period of 2 days achieved long-term protection (Figs. 3 and 4). Whereas other CapDs have been tested in vivo, pegylated CapDs have not been tested previously. Here, the enhanced thermostability of the pegylated and circularly permuted enzyme (fig. S1), as well as the dosing regimen, may have contributed to protection. The treatment period was also notably shorter than those used for traditional antibiotic regimens, and no antibiotics were required. Vaccination with licensed human vaccines that elicit antibodies to the protective antigen, to neutralize the lethal toxin and edema toxin, as well as other therapeutics are expected to be compatible with PEG-CapD-CP^{S334C} treatment. These may provide added benefit in a postexposure prophylaxis regimen, particularly with multidrug-resistant organisms.

The emergence of resistance against currently approved classes of antibiotics requires new therapeutic interventions. In addition, current therapeutic strategies do not degrade circulating capsule shed from the bacteria that can reach very high levels during anthrax infection and contribute to pathogenicity, as shown by its ability to suppress the innate immune responses and enhance the virulence of *B. anthracis* (*13*, *35*). Thus, the possible enzymatic cleavage of the circulating PDGA by PEG-CapD-CP^{S334C} may also have a favorable effect on survival. Protein therapeutics have limitations, and their use in treating infections such as anthrax would be reserved for multidrug-resistant strains for which there is no treatment. Their large molecular weight compared to antibiotics may reduce delivery to sites of infection, and their immunogenicity may limit their prolonged use.

Enzyme therapies may prove to be beneficial against bacteria that have acquired resistance due to enhanced efflux, increased expression of enzymes that metabolize antibiotics, mutations, or inaccessibility due to established biofilm growth. The results here demonstrate that enzymes can be effectively engineered for treatment of anthrax that may prove valuable against multidrug-resistant *B. anthracis* strains.

MATERIALS AND METHODS

Study design

The objective of the study was to develop and evaluate an improved CapD enzyme for treatment of experimental anthrax infection. On the basis of the crystal structure, four sites on CapD were selected to introduce a cysteine for pegylation. Thermal denaturation of the pegylated and nonpegylated enzymes was monitored by CD spectroscopy to find the least destabilizing mutation for pegylation, and this mutation, S334C, was introduced into the circularly permuted CapD-CP, which was pegylated and retained enzymatic activity. Pharmacokinetic studies of three CapD variants, CapD-N, CapD-CP^{S334C}, and PEG-CapD-CP^{S334C}, were performed in groups of mice ($n = 5$ to 14). Efficacy was then tested in experimentally infected mice using 10 animals per group with treatment begun 24 hours after infection. The animal studies were conducted under Institutional Animal Care and Use Committee (IACUC)–approved protocols in compliance with the Animal Welfare Act, Public Health Service (PHS) Policy, and other Federal statutes and regulations relating to animals and experiments involving animals.

Expression and purification of MBP-CapD-N

The MBP-fusion protein was expressed and purified from *E. coli* as previously described (*24*). MBP was removed with Factor Xa.

Expression and purification of circularly permuted *B. anthracis* CapD-CP

Plasmids expressing MBP-CapD-N (GenBank: AAT28993.2) and CapD-CP (GenBank: JF784156.1) were constructed and purified as previously described (*24*, *29*). The S334C mutation was introduced into CapD-CP using a QuikChange kit (Stratagene Inc., La Jolla, CA). CapD-CP^{S334C} was expressed and purified from *E. coli* BL-21(DE3). Cells (3 liters) were grown to an A_{600} of 1.0 and induced with 0.3 mM isopropyl-β-D-thiogalactoside (IPTG) overnight at 17°C. Cells were lysed [50 mM tris (pH 7.6), 500 mM NaCl, 30 mg of lysozyme, 25 U deoxyribonuclease, 2 mM β-mercaptoethanol (BME), and 35% BugBuster] and sonicated 10 to 20 times for 15 s. The lysate was clarified by centrifugation (30 min at 20,500*g* and 4°C) and then loaded onto a nickel-charged Chelating Sepharose column (Cytiva Inc., Marlborough, MA) equilibrated with 50 mM tris (pH 7.6), 500 mM NaCl, and 2 mM BME. The column was washed with buffer containing 60 mM imidazole, and the protein was eluted with buffer containing 300 mM imidazole. The protein was dialyzed against 0.5× PBS (pH 7.4) and 2 mM BME overnight at 4°C. Protein was loaded onto a Q Sepharose column (Cytiva Inc.) equilibrated with 0.5× PBS (pH 7.4) and 2 mM BME and eluted with a gradient (0 to 1 M NaCl). The protein was then dialyzed against 0.5× PBS (pH 7.4) and 2 mM BME overnight at 4°C, loaded onto a SP Sepharose

(Cytiva Inc.) column equilibrated with 0.5× PBS (pH 7.4) and 2 mM BME, and eluted with a gradient (0 to 1 M NaCl). Protein was concentrated using centrifugal ultrafiltration units, and dithiothreitol (DTT) was added to a final concentration of 25 mM. Before pegylation, the DTT was removed using a PD-10 column equilibrated with 1× PBS (pH 7.4) and then concentrated to ~24 mg/ml.

Pegylation

A 20-fold molar excess of PEG maleimide (#22361 from Thermo Fisher Scientific Inc., Waltham, MA) was added to CapD-CP^{S334C} and incubated at room temperature (23° ± 3°C) for 2 hours and then overnight at 4°C. The unreacted PEG maleimide was removed by a PD-10 column equilibrated with 2× PBS (pH 7.4). PEG-CapD-CP^{S334C} was stored in 1× PBS (pH 7.4) and 50% glycerol at −20°C. Protein was ≥95% pegylated after the incubation period.

Thiol titrations

To verify pegylation, the pegylated and nonpegylated proteins were incubated with 5,5-dithio-bis-(2-nitrobenzoic acid) (0.5 mM final concentration). The absorbance at 412 nm was read. Reduced glutathione was used to prepare a standard curve. The moles of SH versus moles of protein were plotted, and the number of free thiols was determined from the slope of the line.

Enzyme assays

CapD was assayed using a 5-mer poly-D-glutamate fluorescence resonance energy transfer (FRET) substrate containing EDANS and DABCYL (Biopeptide Co. Inc., San Diego, CA) (*4*, *24*). Cleavage was detected using an excitation wavelength of 340 nm and emission wavelength of 490 nm. An unquenched control peptide was used to make a standard curve. Inner filter effect corrections were applied to all data (*36*). Enzyme was assayed in 25 mM Hepes (pH 7.4), 0.1% Tween 20, and L-serine (0.5 mg/ml) at room temperature.

Sterilization of recombinant PEG-CapD-CP^{S334C} for in vivo use

Endotoxin levels were evaluated by using limulus amebocyte lysate (LAL) kit (Lonza Inc., Walkersville, MD) as directed. Endotoxin was removed by either treatment with Triton X-114 (*37*) followed by Pierce detergent removal resin (Thermo Fisher Scientific Inc.) or using Mustang E filters (Pall Life Sciences, Port Washington, NY) following the manufacturer's directions. Each disk was flushed with 1 ml of LAL water followed by 1 ml of CapD; each filter was used once.

Pharmacokinetic studies

Initial pharmacokinetic studies were performed comparing CapD-N and CapD-CP^{S334C}. Female BALB/c mice (20 to 30 g, Charles River, Frederick, MD) were bled 1 week before the start of each experiment and at 30, 120, and 360 min after intraperitoneal injection of either CapD-N or CapD-CP^{S334C} at 40 mg/kg. A total of nine mice per CapD construct were tested in four experiments. A comparison was also made between CapD-CP^{S334C} and PEG-CapD-CP^{S334C} in a second experiment. A pool of BALB/c serum served as the 0-hour time point. Mice were again bled at 30, 120, and 360 min after injection of 40 mg/kg of either CapD-CP^{S334C} or PEG-CapD-CP^{S334C} (n = 5 mice each) in two separate experiments. Blood was collected (100 to 200 μl) in BD Microtainer serum separator tubes (VWR Inc., Radnor, PA) from the saphenous vein or from the retro-orbital venous sinus from lightly anesthetized mice except for the final blood collection, which was by exsanguination of deeply anesthetized animals via cardiac puncture. Mice were anesthetized by intramuscular injection of 0.15 ml of ketamine (100 mg/ml), acepromazine (10 mg/ml), and xylazine (20 mg/ml). Serum was held at −70°C until testing. The activity of the CapD variants in the serum was measured using the FRET assay. CapD in the serum was quantified from standard curves for CapD-N, CapD-CP^{S334C}, and PEG-CapD-CP^{S334C} by plotting the relative velocity versus protein (μg/ml) using XLfit software (IDBS, Boston, MA). The AUC was determined from plots of serum protein concentration of CapD versus time using GraphPad Prism version 6 (San Diego, CA).

Preparation of spore suspensions

Spores from the nontoxigenic encapsulated *B. anthracis* ΔAmes (pXO1$^-$ and pXO2$^+$) strain and the toxigenic encapsulated *B. anthracis* Ames (pXO1$^+$ and pXO2$^+$) strain were prepared as previously described (*38*).

CapD-CP^{S334C} protection in mice

The therapeutic potential of PEG-CapD-CP^{S334C} was initially examined in BALB/c female mice that had been infected with *B. anthracis* ΔAmes spores, a strain that does not express the toxin but is encapsulated (pX01$^-$ and pX02$^+$). Mice are susceptible to nontoxigenic *B. anthracis* strains that express the capsule (*39*). Two groups of 10 female BALB/c mice (20 to 30 g) each were injected subcutaneously with 382 LD_{50} (median lethal dose) *B. anthracis* ΔAmes spores (LD_{50} = 17,300 spores). Twenty-four hours after infection, mice were injected intraperitoneally six times at 8-hour intervals with either PEG-CapD-CP^{S334C} or BSA diluted to 40 mg/kg brought up to a volume of 1 ml with Dulbecco's PBS (DPBS), calcium- and magnesium-free. Survival was noted for 21 days.

A therapeutic study was then performed in BALB/c female mice infected with the fully virulent *B. anthracis* Ames strain. Two groups of 10 female BALB/c mice each were injected subcutaneously with five LD_{50} Ames *B. anthracis* spores (LD_{50} = 9 spores). Twenty-four hours after infection, mice were injected intraperitoneally six times at 8-hour intervals with either PEG-CapD-CP^{S334C} or BSA diluted to 40 mg/kg brought up to a volume of 1 ml with DPBS, calcium- and magnesium-free. Survival was noted for 21 days.

This study was conducted under an IACUC-approved protocol in compliance with the Animal Welfare Act, PHS Policy, and other Federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) International and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011 (*40*).

Statistical analysis

The AUC of serum CapD levels was computed by trapezoid rule. Statistical differences in geometric mean AUC were determined by applying a two-way (experiment × treatment) block analysis of variance (ANOVA) to the log-transformed AUC values. Results were summarized as the geometric mean, estimated as the anti-log of the least squares mean, and GSE. Survival differences between treatment groups were determined by log-rank analysis of Kaplan-Meier survival curves. Analyses were conducted using SAS VER 9.4 (SAS Institute, Cary, NC).

SUPPLEMENTARY MATERIALS

www.science.org/doi/10.1126/scitranslmed.abh1682
Fig. S1
Table S1
Data file S1

View/request a protocol for this paper from *Bio-protocol*.

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Treatment of experimental anthrax with pegylated circularly permuted capsule depolymerase

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Disarming anthrax

Anthrax is a potentially fatal infection caused by *Bacillus anthracis* and has the potential to be used as a bioweapon. Although anthrax can be treated with existing antibiotics, a concern for drug-resistant anthrax has led to the development of different treatments. Legler *et al.* describe an alternative approach to disarming anthrax by disrupting the bacterial capsule. Previous work demonstrated that a recombinant capsule depolymerase (CapD) can enzymatically remove the capsule encasing anthrax bacilli and promote phagocytosis and bacterial clearance, but effectiveness was limited for the treatment of toxigenic Ames spores. Here, they modified CapD by pegylation and circular permutation, which dramatically improved pharmacokinetics and protection of mice challenged with Ames spores. This report highlights the potential therapeutic benefit of enzyme-mediated capsule disruption for treating anthrax.

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