Substrate-mediated Stabilization of a Tetrameric Drug Target Reveals Achilles Heel in Anthrax

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Bacillus anthracis is a Gram-positive spore-forming bacterium that causes anthrax. With the increased threat of anthrax in biowarfare, there is an urgent need to characterize new antimicrobial targets from B. anthracis. One such target is dihydrodipicolinate synthase (DHDPS), which catalyzes the committed step in the pathway yielding meso-diaminopimelate and lysine. In this study, we employed CD spectroscopy to demonstrate that the thermostability of DHDPS from B. anthracis (Ba-DHDPS) is significantly enhanced in the presence of the substrate, pyruvate. Analytical ultracentrifugation studies show that the tetramer-dimer dissociation constant of the enzyme is 3-fold tighter in the presence of pyruvate compared with the apo form. To examine the significance of this substrate-mediated stabilization phenomenon, a dimeric mutant of Ba-DHDPS (L170E/G191E) was generated and shown to have markedly reduced activity compared with the wild-type tetramer. This demonstrates that the substrate, pyruvate, stabilizes the active form of the enzyme. We next determined the high resolution (2.15 Å) crystal structure of Ba-DHDPS in complex with pyruvate (3HIJ) and compared this to the apo structure (1X19). Structural analyses show that there is a significant (91 Å2) increase in buried surface area at the tetramerization interface of the pyruvate-bound structure. This study describes a new mechanism for stabilization of the active oligomeric form of an antibiotic target from B. anthracis and reveals an “Achilles heel” that can be exploited in structure-based drug design.

The etiology of anthrax was first described by Robert Koch in 1876 (1). His work was the first to demonstrate unequivocally that Bacillus anthracis is the causative agent of the disease. Given the tolerance of its spores to extreme physical conditions and their ease of dissemination, the use of B. anthracis in bioterrorism and biological warfare is a threat to developed countries worldwide. For example, in 2001 a number of letters containing B. anthracis endospores were distributed via the U.S. postal system, which resulted in 11 cases of respiratory anthrax. Since then, there has been heightened interest in the development of anti-anthrax agents (2), especially considering the limited range of current effective therapeutics and the ability of B. anthracis to develop antibiotic resistance (3). One potential therapeutic target is dihydrodipicolinate synthase (DHDPS), which catalyzes the first committed step in the lysine biosynthetic pathway (Fig. 1A).

The biosynthetic pathway leading to lysine and its immediate precursor, meso-diaminopimelate (meso-DAP), is known as the DAP pathway (4) (Fig. 1A). The first committed reaction in the pathway is the condensation of pyruvate and (5)-aspartate semi-aldehyde (ASA) to form hydroxypyruvatehydroxypicolinic acid (5), which is catalyzed by DHDPS (Fig. 1, A and B). The DAP pathway in bacteria leads to the de novo synthesis of lysine, essential for protein synthesis, and meso-DAP, which is a vital constituent of the peptidoglycan layer in the bacterial cell wall (6) (Fig. 1A). Thus, targeting the DAP pathway through disrupting the first committed step, DHDPS, will yield a novel class of therapeutic agents to treat anthrax. This notion is supported by recent work (7) that identified dapA, the gene encoding DHDPS from B. subtilis, as one of only 271 genes essential for cell viability from a total of >4,100 genes encoded by its genome. Additionally, mammals do not synthesize lysine and therefore do not possess the DHDPS enzyme. This suggests that specific inhibitors of DHDPS would have selective antibacterial activity with low toxicity in a mammalian host.

The structure of DHDPS in the absence of substrate has been determined for a number of species of bacteria, including that of B. anthracis (8) (Fig. 1B), Thermotoga maritima (9), Thermoaerobacter tengcongensis (10), Escherichia coli (11), Mycobacterium tuberculosis (12), Corynebacterium glutamicum (13), and Staphylococcus aureus (14, 15). The enzyme usually...
was amplified by PCR and cloned into the pET11a expression vector as described elsewhere (22). Briefly, recombinant protein was produced in the host strain E. coli BL21-DE3 as follows. Cells harboring vector were cultured at 37 °C in Luria broth containing 100 µg ml⁻¹ ampicillin to an A₆₀₀ of 0.6. Expression of recombinant Ba-DHDPS was induced by addition of isopropyl 1-thio-β-d-galactopyranoside to a final concentration of 1.0 mM. Cells were harvested 3 h post-induction and resuspended in 20 mM Tris-HCl, pH 8.0, before lysis by sonication. Ba-DHDPS was subsequently isolated by anion-exchange and hydrophobic interaction liquid chromatography as described elsewhere (22).

Generation of Dimeric Ba-DHDPS (L170E/G191E)—The dimeric form of Ba-DHDPS was generated using the QuikChange II XL site-directed mutagenesis kit (Stratagene). The mutagenic oligonucleotide primer sets, 5'-GATGCAGGC-GGCCGTGTGGAACCAATGACAGAATCTATTG-3' and 5'-CAATGATTTCGTCATCGTATTTTCCACATCGCCGCTGCA-TC-3', and 5'-GTATACTACGCGGTGATGACGAAATACCGCTACACGATTTG-3' and 5'-CCATAGCTGTTAGCGTTAACATCAGCACCGCTGTATA-CTAC-3', were designed to introduce amino acid substitutions L170E and G191E, respectively. Mutagenesis was performed according to the manufacturer's instructions. Briefly, 125 ng of primer and 10 ng double-stranded DNA template utilized per reaction. Second strand synthesis was achieved through 18 cycles of amplification. Following a 1-h DpnI digestion, plasmid was transformed into E. coli XL10-Gold UltraCompetent cells. The introduction of point mutations was verified by DNA sequencing.

DHDP-DHDP Coupled Enzyme Kinetic Assay—Enzyme kinetic analyses of Ba-DHDPS and Ba-DHDPS (L170E/G191E) were performed using the DHDP-DHDP coupld assay as described in a previous study (23). Assays were routinely performed in duplicate at a constant temperature of 30 °C with reaction mixtures allowed to equilibrate in a temperature-controlled Cary 4000 UV-visible spectrophotometer for 12 min before initiating the reaction with 60 nM DHDP. Prior to the experiment, pyruvate and ASA concentrations were routinely quantified by the addition of limiting amounts of substrate by measuring the consumption of NADPH at 340 nm in the Cary 4000 UV-visible spectrophotometer. Initial velocity data were best fitted to a Ping Pong Model using ENZFITTER.4 Rate versus enzyme concentration assays were also conducted with Ba-DHDPS concentrations ranging from 2.5 to 120 nm.

CD Spectroscopy—CD spectra were recorded using an AVIV 410-SF CD spectrometer. Wavelength scans were performed between 190 and 250 nm in 20 mM Tris, 150 mM NaCl with 0.15 mg ml⁻¹ Ba-DHDPS and Ba-DHDPS (L170E/G191E) in 1-mm quartz cuvettes. Data were analyzed using the CONTINLL algorithm from the CDPro software package (25) and the SP29 protein data base. For thermal denaturation scans, ellipticity at 222 nm was monitored between 20 and 90 °C in 1 °C steps.

Analytical Ultracentrifugation—Absorbance-based sedimentation velocity and equilibrium experiments were performed in a Beckman model XL-I analytical ultracentrifuge

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using a 4-hole An-60 Ti or an 8-hole An-50 Ti rotor. Double-sector quartz cells were loaded with 380 µl of sample and 400 µl of reference (20 mM Tris, 150 mM NaCl, pH 8.0) for sedimentation velocity, or a 100-µl sample and a 120-µl reference for sedimentation equilibrium. Experiments were conducted at 4 °C using a rotor speed of 40,000 rpm (sedimentation velocity) or 12,000 and 18,000 rpm (sedimentation equilibrium) with absorbance measured at 227 nm (1.6 µM enzyme). Solvent density, solvent viscosity, and estimates of the partial specific volume of B. anthracis DHDPS alone and in the presence of 2.0 mM pyruvate or 2.0 mM ASA were fitted to various self-associating equilibrium models using SEDPHAT (32). Sedimentation velocity data were fitted to a single discrete species or a continuous sedimentation coefficient [s] model (27–29) using the program SEDFIT. Additionally, van Holde-Weischet analysis (30) was performed using the ULTRASCAN software package (31), whereas sedimentation equilibrium data were fitted to various self-associating equilibrium models using the program SEDPHAT (32).

**RESULTS**

**Effect of Pyruvate on the Secondary Structure Stability of Ba-DHDPS**—We recently reported that recombinant Ba-DHDPS are significantly increased when the enzyme is purified in the presence of its substrate, pyruvate (22). To examine the effect of pyruvate on the stability of Ba-DHDPS in aqueous solution, thermal denaturation experiments monitored by CD spectroscopy were conducted in the presence and absence of the substrates pyruvate or ASA over the temperature range of 20–90 °C. Initially, wavelength scans were performed at 20 °C to monitor global secondary structure in the presence of substrates, which revealed no change in response to either pyruvate or ASA (Fig. 2A). Ba-DHDPS appears to follow a three-state mechanism for thermal unfolding in the absence of pyruvate and in the presence of ASA, with a potential intermediate persisting at temperatures of ~50–60 °C (Fig. 2B). However, in the presence of 2.0 mM pyruvate, the thermal denaturation of Ba-DHDPS is delayed with respect to temperature and unfolding appears to occur via a two-state mechanism without the propagation of an intermediate, indicating that pyruvate stabilizes the folded state of the enzyme. Accordingly, the effect of pyruvate on the quaternary and tertiary structure of Ba-DHDPS was sought.

**Effect of Pyruvate on the Quaternary Structure of Ba-DHDPS**—To characterize the quaternary structure of Ba-DHDPS in solution in the absence and presence of pyruvate, absorbance-detected sedimentation velocity and equilibrium analyses were conducted in the analytical ultracentrifuge. The absorbance versus radial position profiles of Ba-DHDPS (1.6 µM) during sedimentation velocity in the absence of pyruvate are shown in Fig. 3A, whereas Fig. 3B shows the equivalent data sets in the presence of pyruvate. Two predominant boundaries were observed for Ba-DHDPS in the absence of substrate (Fig. 3A). By contrast, the radial absorbance profiles showed a single predominant boundary in the presence of pyruvate (Fig. 3B). These data were analyzed initially using the enhanced van Holde-Weischet method (30), which is a model-independent approach to analyzing sedimentation velocity data. The resulting integral distribution for Ba-DHDPS in the absence of pyruvate (Fig. 3C, white circles) suggests it exists in a reversible self-association with sedimentation coefficients ranging from 2 S through to 6 S. This is consistent with previous reports of DHDPS from E. coli, which was determined to exist in equilibrium between a dimer and tetramer (38). In the presence of
pyruvate (Fig. 3C, black circles), the equilibrium appears to be shifted greatly in favor of the larger species. To define the oligomeric species of Ba-DHDPS in solution, the data were subsequently fitted to a continuous sedimentation coefficient (c(s)) distribution model (27–29) (Fig. 3D). In the absence of pyruvate, the c(s) distribution shows two non-baseline resolved species, in approximately equal proportions, with standardized sedimentation coefficients (s_{20,w}) of 4.0 and 6.5 S (Fig. 3D and Table 1). These values correspond to the DHDPS dimer and tetramer (38), respectively (Table 1). By contrast, in the presence of pyruvate, a significantly greater proportion of the tetrameric enzyme (Fig. 4B, Table 2). Moreover, wild-type Ba-DHDPS had a maximum catalytic turnover (kcatal) of 92 s^{-1}, Ka_{NAD} of 0.25 mM, and K_{VPR} of 1.2 mM compared with 1.7 s^{-1}, K_{NAD} of 4.0 μM, and K_{VPR} of 1.0 mM.

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**TABLE 1**

Summary of hydrodynamic properties of wild-type Ba-DHDPS

| Species | Mass (kDa) | s_{20,w} (Svedberg) | βf_w | K_{D-D} minus pyruvate (μM) | K_{D-D} plus pyruvate (μM) |
|---------|------------|---------------------|------|----------------------------|----------------------------|
| Dimer   | 62         | 4.0                 | 1.17 | 0.66                       | 0.66                       |
| Tetramer| 124        | 6.5                 | 1.27 | 1.9                        | 1.9                        |

a Relative molecular mass calculated from amino acid sequence.

b Standardized sedimentation coefficient taken from the ordinate maximum of the c(s) distribution (Fig. 3D).

c Frictional ratio calculated using the v method from SEDNTERP (26).

d Dissociation constant for tetramer to dimer. Note that the rate of dissociation (k_{off}) is 10^{-2} s^{-1} in the absence of pyruvate (supplemental Fig. 4A) and 10^{-5.1} s^{-1} in the presence of pyruvate (supplemental Fig. 4B).

Ba-DHDPS in solution, the data were subsequently fitted to a continuous sedimentation coefficient (c(s)) distribution model (27–29). In the absence of pyruvate, the c(s) distribution overlaid with the non-linear least squares best-fit (solid line) to a continuous sedimentation coefficient distribution model (c(s)) (27–29). C. van Holde-Weichert integral distribution plot from extrapolation of raw data in A and B. The corrected sedimentation coefficient is plotted against the boundary fraction for the samples in the absence (black circles) and presence (white circles) of pyruvate. The raw data are presented as open symbols (C) plotted at time intervals of 10 min overlaid with the non-linear least squares best-fit (solid line) to a continuous sedimentation coefficient distribution model (c(s)) (27–29). A, B. Absorbance at 227 nm measured as a function of radial position from the axis of rotation (cm) for Ba-DHDPS (1.6 μM) centrifuged at 40,000 rpm in the absence (A) and presence (B) of pyruvate. The raw data are presented as open symbols (C) plotted at time intervals of 10 min overlaid with the non-linear least squares best-fit (solid line) to a continuous sedimentation coefficient distribution model (c(s)) (27–29). C. van Holde-Weichert integral distribution plot from extrapolation of raw data in A and B. The corrected sedimentation coefficient is plotted against the boundary fraction for the samples in the absence (black circles) and presence (white circles) of pyruvate. D, c(s) plotted as a function of s_{20,w} (S) for Ba-DHDPS in the absence (dashed line) and presence (solid line) of pyruvate.

**FIGURE 3. Analytical ultracentrifugation analyses.** Absorbance at 227 nm measured as a function of radial position from the axis of rotation (cm) for Ba-DHDPS (1.6 μM) centrifuged at 40,000 rpm in the absence (A) and presence (B) of pyruvate. The raw data are presented as open symbols (C) plotted at time intervals of 10 min overlaid with the non-linear least squares best-fit (solid line) to a continuous sedimentation coefficient distribution model (c(s)) (27–29). C. van Holde-Weichert integral distribution plot from extrapolation of raw data in A and B. The corrected sedimentation coefficient is plotted against the boundary fraction for the samples in the absence (black circles) and presence (white circles) of pyruvate. The raw data are presented as open symbols (C) plotted at time intervals of 10 min overlaid with the non-linear least squares best-fit (solid line) to a continuous sedimentation coefficient distribution model (c(s)) (27–29). C. van Holde-Weichert integral distribution plot from extrapolation of raw data in A and B. The corrected sedimentation coefficient is plotted against the boundary fraction for the samples in the absence (black circles) and presence (white circles) of pyruvate. D, c(s) plotted as a function of s_{20,w} (S) for Ba-DHDPS in the absence (dashed line) and presence (solid line) of pyruvate.

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0.05 mM, and 3.7 mM for Ba-DHDPS (L170E/G191E) (Table 2). These results show that the Ba-DHDPS tetramer is significantly more active than the dimeric form of the enzyme, which highlights the significance of the substrate-mediated stabilization phenomenon described above. To support this we show that the rate versus enzyme concentration profile of wild-type Ba-DHDPS was nonlinear at low enzyme concentrations, which indicates that the native dimer was significantly less active than the tetrameric species (supplemental Fig. 2).

Crystal Structure of Ba-DHDPS Bound to Pyruvate—To elucidate the structural mechanism behind the substrate-mediated stabilization phenomenon observed in solution, we solved the crystal structure of Ba-DHDPS in complex with pyruvate. Interestingly, the newly solved pyruvate-bound crystal structure (PDB ID: 3HIJ) shared the same space group, unit cell parameters, and a similar resolution to the structure of substrate unbound Ba-DHDPS (PDB ID: 1XL9). Crystallization and preliminary diffraction analysis were reported recently (22). The initial structure revealed four monomers in the asymmetric unit, arranged in the biologically relevant tetramer (Fig. 5A). The first round of refinement gave an R cryst of 21.4% (R free of 23.2%), which also revealed electron density associated with Lys163 in the active site of all four monomers that wasn’t accounted for by the search model. This is the site where pyruvate binds DHDPS (39). The pyruvate-bound molecule was modeled manually at Lys163 (Fig. 5B), and later rounds of structural refinement were performed using REFMAC5. The iterative model-building tool, WINCOOT, was used to model in waters, Na+/H11001 ions, and glycerol molecules. To make a direct comparison to the Ba-DHDPS substrate-unbound structure (PDB ID: 1XL9), a round of simulated annealing was performed on the substrate-bound structure (PDB ID: 3HIJ) with a starting temperature of 5000 K to ensure both structures possessed the same R free set of reflections. The final molecule had an R cryst of 15.3% (R free of 21.0%) to 2.15-Å resolution. The resulting model was examined using PROCHECK, which revealed that 99.2% of residues in Ba-DHDPS bound to pyruvate were in the favored regions of a Ramachandran plot. The 8 residues in the “disallowed” regions were Tyr109 and Ile142 from chains A,
B, C, and D, which is consistent with the crystal structure of substrate-unbound \textit{Ba}-DHDPS (8). Tyr^{109} is an imperative catalytic site residue, and Ile^{142} interacts with its equivalent neighbor at the tight dimer interface providing structural stability. A full table of statistics is provided (Table 3). Both the pyruvate-bound and the previously reported ligand-free structures are very similar (Fig. 5). The two tetramers align at alpha carbon with a root mean square deviation of 0.50 Å, and little difference could be observed in active site residues (Fig. 5B). However, the “weak” dimer interface is more extensive in terms of buried surface area in the presence of pyruvate (Table 4). This is the interface where the two tight dimers dock to form the tetramer (Fig. 1A). At this interface the buried surface area increases from 1735 Å² in the absence of pyruvate to 1826 Å² when bound to pyruvate. By comparison, the buried surface area at the “tight” dimer interface has only changed from 2780 Å² in the apo structure to 2782 Å² in the presence of pyruvate. Additionally, the number of interactions is significantly greater at the weak dimer interfaces as calculated by STING MILLENIUM (40) (Table 4). There is also an increase in hydrogen bonds at the tight dimer interface in the pyruvate-bound structure (Table 4). In total, there were twelve more hydrogen bond interactions at both interfaces in the crystal structure of pyruvate-bound \textit{Ba}-DHDPS (PDB ID: 3HIJ) relative to the apo structure (PDB ID: 1XL9).

**DISCUSSION**

The recently described DHDPS structure from methicillin-resistant \textit{S. aureus}, the first reported native dimeric DHDPS enzyme, was observed to have greater solution stability in the presence of pyruvate (14). It is thus of interest to probe the role of this substrate in stabilizing the active quaternary structure of DHDPS. Disruption of quaternary structure offers a new approach for inhibitor design of active oligomeric enzymes, revealing an “Achilles heel” to target in pathogenic bacteria such as \textit{B. anthracis}. Therefore, we examined the effect of pyruvate in stabilizing the \textit{Ba}-DHDPS tetramer.

The solution stability of \textit{Ba}-DHDPS was initially investigated at the secondary structure level using CD spectroscopy. Neither substrate of DHDPS, pyruvate nor ASA, had an effect on the overall secondary structure of \textit{Ba}-DHDPS at 20 °C (Fig. 2A). This agrees with previous x-ray crystallographic studies of apo- and substrate-bound intermediate states of \textit{E. coli} DHDPS (41). However, the thermostability of \textit{Ba}-DHDPS was significantly stabilized in the presence of pyruvate compared with the unliganded enzyme (Fig. 2B). By contrast, the presence of ASA, which is the second substrate to bind, showed no effect on the stabilization of the enzyme (Fig. 2B). Interestingly, the unfolding of ligand-free \textit{Ba}-DHDPS revealed the presence of an intermediate state that persisted over a temperature range of 50–60 °C (Fig. 2B). The intermediate could potentially represent a collapsed molten globule state of \textit{Ba}-DHDPS that has native-like secondary structure, but a more dynamic tertiary structure, a phenomenon that has been previously observed in other bacterial enzymes (42). This intermediate state was not propagated during thermal denaturation in the presence of pyruvate (Fig. 2B). In fact, the presence of pyruvate significantly stabilized the folded state of the recombinant enzyme for a further 20 °C relative to the thermal denaturation profile in the absence of pyruvate (Fig. 2B). Conversely, ASA had no effect on the thermostability of the enzyme with respect to secondary structure (Fig. 2B), which demonstrates that the stabilization phenomenon is specific to pyruvate and thus associated with Schiff-base formation at the active site lysine (Lys^{163}).

Given the observed secondary structure stabilization phenomenon induced by pyruvate, the propensity for the substrate to stabilize the quaternary structure in solution was therefore investigated. Absorbance-based analytical ultracentrifugation experiments revealed that \textit{Ba}-DHDPS existed in an apparent equilibrium between two oligomeric states, the dimer and tetramer, at low micromolar concentrations at 4 °C (Fig. 3). Sedimentation velocity analysis showed that pyruvate significantly shifted the apparent equilibrium in favor of the tetramer in solution (Fig. 3, C and D), thereby enhancing tetramerization in solution. The stabilization of quaternary structure was quantified using sedimentation equilibrium analysis (supplemental Fig. 1), showing that the tetramer was 3-fold tighter in the presence of pyruvate (Table 1). The importance of the tetrameric quaternary structure of \textit{Ba}-DHDPS is highlighted by kinetic

**TABLE 3**

| Structure and refinement statistics |  |
|------------------------------------|---|
| $R_{cryst}$ ($R_{free}$)           | 0.153 (0.170)$^a$ |
| Number of atoms                    | 10,075 |
| Protein                            | 8,894 |
| Na$^+$ ions                        | 4 |
| Glycerol                           | 24 |
| Water                              | 1,153 |
| Root mean square deviation$^a$     | 0.024 |
| Bonds (Å)                          | 1.87$^b$ |
| Average B factors                  | 17.8 |
| Protein                            | 22.1 |
| Neutral                             | 53.8 |
| Water                              | 28.8 |

$^a$ $R_{cryst}$ ($R_{free}$) = \sum |F_{o}-F_{c}|/\sum |F_{o}|, where $F_{o}$ and $F_{c}$ are the observed and calculated structure factor amplitudes, respectively.

$^b$ Values in parentheses are for the highest resolution shell.

$^c$ $R_{free}$ is the $R$-factor calculated with 5% of the reflections chosen at random and omitted from refinement.

$^d$ The root mean square deviation of bond lengths or bond angles from ideal geometry.

**TABLE 4**

| Interface analysis of self association interfaces |  |
|--------------------------------------------------|---|
| Interface                                        | H-bonds$^a$ | Hydrophobic interactions$^a$ | Electrostatic interactions$^a$ | Buried surface area$^b$ | $\Delta^c$ |
| Pyruvate-unbound                                 | 2 | 28 | 0 | 2780 |
| Pyruvate-bound                                   | 8 | 28 | 0 | 2782 |

$^a$ Analysis was performed using STING MILLENIUM (40).

$^b$ Analysis was performed using PISA (44).

$^c$ pdb ID: 1XL9.

$^d$ Crystal structure of \textit{Ba}-DHDPS was solved bound to pyruvate.

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and solution studies of the dimeric mutant, \( \text{Ba-DHDPS} \) (L170E/G191E). Our results show that the dimeric mutant was significantly less active than the wild-type tetramer (Table 2 and Fig. 4B). This is consistent with recent studies demonstrating that the enzymatic activity of dimeric mutants of \( \text{E. coli} \) DHDPS possess <2.5% of the catalytic activity of the wild-type tetramer (43).

To probe the structural mechanism governing the pyruvate-mediated stabilization of the active tetrameric form, x-ray diffraction studies of \( \text{Ba-DHDPS} \) in the presence of pyruvate were undertaken. Recombinant native \( \text{Ba-DHDPS} \), purified as described previously (22), crystallized in identical conditions to those published for the unliganded enzyme (PDB ID: 1XL9) (8). The final structure of \( \text{Ba-DHDPS} \) in complex with pyruvate was solved to a resolution of 2.15 Å, and initially no significant changes could be observed in comparison to the pyruvate-unbound structure. However, upon close inspection of the weak dimer interface using PISA analysis (44), significant differences were observed. Moreover, an increase in buried surface area of 91 Å\(^2\) resulted when the enzyme was bound to pyruvate relative to the apo structure (Table 4). This agreed with the STING MILLENIUM (40) analysis, which predicted greater inter-residue contacts at the weak dimer (and also the tight dimer) interface with a total of twelve more hydrogen bonds for \( \text{Ba-DHDPS} \) when bound to pyruvate (Fig. 5C and Table 4). This is significant given that the pyruvate-bound structure solved in this study (3HJ1) shares the same resolution, unit cell parameters, and space group with the pyruvate-unbound structure (1XL9). Furthermore, this explains the increased thermostability (Fig. 2B) and the tetramerization propensity of \( \text{Ba-DHDPS} \) (Fig. 3) in the presence of pyruvate given the greater number of noncovalent interactions formed at the tetramerization interface upon substrate binding.

In addition, temperature-dependent dynamic disorder of atoms in the crystal, represented by crystallographic temperature factors, can be used to predict potential conformational dynamics within the structure. Interestingly, the crystal structures of pyruvate-bound and -unbound \( \text{Ba-DHDPS} \) share a very similar trend across all residues with respect to average main-chain temperature factor, except for a region of 15 residues from position 220 to 235 (supplemental Fig. 3). In this region the temperature factors for the pyruvate unbound structure are much higher than in other positions in the enzyme. Not surprisingly, most of these residues are located at the weak dimer interface, where the greatest structural changes were observed upon substrate binding (Fig. 5C). This suggests that pyruvate reduces conformational dynamics at the weak dimer interface and thus enhances tetramerization and thermostability of the enzyme.

Finally, substrate-mediated stabilization of quaternary structure and enhanced thermostability have been previously reported for another enzyme, namely l-tryptophan 2,3-dioxygenase, from \( \text{Bacillus brevis} \) (45, 46). Thus, this phenomenon could be of much broader utility and biological relevance to oligomeric enzymes.

In conclusion, this study describes the structural basis for substrate-mediated stabilization of the active oligomeric form of \( \text{Ba-DHDPS} \). Bacterial DHDPS has long been considered a potential antibiotic target (16, 24), which has recently been validated by knock-out studies demonstrating that DHDPS is the product of one of only 271 essential genes in \( \text{Bacillus species} \) (7). Accordingly, our study provides significant structure-function knowledge that can be applied to rationale drug design strategies in the pipeline to generating novel anti-anthrax agents. This study has thus identified an “Achilles heel” in an essential enzyme and antibiotic target from a significant human pathogen.

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REFERENCES

1. Koch, R. (1876) \textit{Beitr. Biol. Pflanz.} \textbf{2}, 277–310
2. Brey, R. N. (2005) \textit{Adv. Drug Deliv. Rev.} \textbf{57}, 1266–1292
3. Athanna, A., Massalha, M., Athanna, M., Nura, A., Medlej, B., Ofek, I., Bast, D., and Rubinstein, E. (2004) \textit{J. Antimicrob. Chemother.} \textbf{53}, 247–251
4. Scapin, G., and Blanchard, J. S. (1998) \textit{Adv. Enzymol. Relat. Areas Mol. Biol.} \textbf{72}, 279–324
5. Yugari, Y., and Gilvarg, C. (1965) \textit{J. Biol. Chem.} \textbf{240}, 4710–4716
6. Smith, H., Strange, R. E., and Zwarttouw, H. T. (1956) \textit{Nature} \textbf{178}, 865–866
7. Kobayashi, K., Ehrlich, S. D., Albertini, A., Amati, G., Andersen, K. K., Arnaud, M., Asai, K., Ashikaga, S., Aymeric, S., Bessieres, P., Boland, F., Brignell, S. C., Bron, S., Bunai, K., Chapuis, J., Christiansen, L. C., Danchin, A., Debabarouille, M., Deryvn, E., Deuerling, E., Devine, K., Devine, S. K., Dreesen, O., Errington, J., Fillingler, S., Foster, S. J., Fujita, Y., Galizzi, A., Gardan, R., Eschevins, C., Fukushima, T., Haga, K., Harwood, C. R., Hecker, M., Hosoya, D., Hullo, M. F., Kakeshita, H., Karamata, D., Kasahara, Y., Kawamura, F., Koga, K., Koski, P., Kuwana, R., Imamura, D., Ishimaru, M., Ishikawa, S., Ishio, I., Le Coq, D., Masson, A., Maule, C., Meima, R., Mellado, R. P., Moir, A., Moriya, S., Nagakawa, E., Nanamiya, H., Nakai, S., Nygaard, P., Ogura, M., Ohanan, T., O’Reilly, M., O’Rourke, M., Pragai, Z., Pooley, H. M., Rapoport, G., Rawlins, J. P., Rivas, L. A., Rivolta, C., Sadaie, A., Sadaie, Y., Sarvas, M., Sato, T., Saxid, H. H., Scanlan, E., Schumann, W., Seegers, J. F., Sekiguchi, J., Sekowska, A., Séro, S. J., Simon, M., Strager, P., Studer, R., Takamatsu, H., Tanaka, T., Takeuchi, M., Thomaides, H. B., Vagner, V., van Dijl, J. M., Watabe, K., Wipat, A., Yamamoto, H., Yamamoto, M., Yamamoto, Y., Yamane, K., Yata, K., Yoshida, K., Yoshikawa, K., Zubert, U., and Ogasawara, N. (2003) \textit{Proc. Natl. Acad. Sci. U.S.A.} \textbf{100}, 4678–4683
8. Blagova, E., Ledlikov, V., Milloti, N., Fogg, M. J., Kallioma, A. K., Branigan, J. A., Wilson, K. S., and Wilkinson, A. J. (2006) \textit{Proteins} \textbf{62}, 297–301
9. Pearce, F. G., Perugini, M. A., McKechar, H. J., and Gerrard, J. A. (2006) \textit{Biochem. J.} \textbf{400}, 359–366
10. Wolterink-van Loos, L., Sevissen, M., Cabiéres, M. C., Fransen, M. C., and van der Oost, J. (2008) \textit{Extremophiles} \textbf{12}, 461–469
11. Dobson, R. C., Griffin, M. D., Jameson, G. B., and Gerrard, J. A. (2005) \textit{Acta Crystallogr. D Biol. Crystallogr.} \textbf{61}, 1116–1124
12. Kefala, G., Evans, G. L., Griffin, M. D., Devenish, S. R., Pearce, F. G., Perugini, M. A., Gerrard, J. A., Weiss, M. S., and Dobson, R. C. (2008) \textit{Biochem. J.} \textbf{411}, 351–360
13. Rice, E. A., Bannon, G. A., Glenn, K. C., Jeong, S. S., Sturman, E. J., and Rydel, T. J. (2008) \textit{Arch. Biochem. Biophys.} \textbf{480}, 111–121
14. Burgess, B. R., Dobson, R. C., Bailey, M. F., Atkinson, S. C., Griffin, M. D., Jameson, G. B., Parker, M. W., Gerrard, J. A., and Perugini, M. A. (2008) \textit{J. Biol. Chem.} \textbf{283}, 27598–27603
15. Girish, T. S., Sharma, E., and Gopal, B. (2008) \textit{FEBS Lett.} \textbf{582}, 2923–2930
16. Dogovski, C., Atkinson, S. C., Dommaraju, S. R., Hor, L., Dobson, R. C., Hutton C. A., Gerrard, J. A., and Perugini, M. A. (2009) in Encyclopedia of Life Support Systems (Doelle, H. W., and Rokem, S., eds) Vol. 11, pp. 116–136, Eolss Publishers Co. Ltd., Isle of Man, UK

17. Frisch, D. A., Gengenbach, B. G., Tommey, A. M., Sellner, J. M., Somers, D. A., and Myers, D. E. (1991) Plant Physiol. 96, 444–452

18. Dereppe, C., Bold, G., Ghisalba, O., Ebert, E., and Schär, H. P. (1992) Plant Physiol. 98, 813–821

19. Laber, B., Gomis-Ruth, F. X., Romaõ, M. J., and Huber, R. (1992) Biochem. J. 288, 691–695

20. Cremer, J., Treptow, C., Eggeling, L., and Sahm, H. (1988) J. Gen. Microbiol. 134, 3221–3229

21. Domigan, L. J., Scally, S. W., Fogg, M. J., Hutton, C. A., Perugini, M. A., Dobson, R. C., Muscroft-Taylor, A. C., Gerrard, J. A., and Devenish, S. R. (2009) Biochim. Biophys. Acta 1794, 1510–1516

22. Voss, J. E., Scally, S. W., Taylor, N. L., Dogovski, C., Alderton, M. R., Hutton, C. A., Gerrard, J. A., Parkers, M. W., Dobson, R. C., and Perugini, M. A. (2009) Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. 65, 188–191

23. Dobson, R. C., Valegård, K., and Gerrard, J. A. (2004) J. Mol. Biol. 338, 329–339

24. Hutton, C. A., Perugini, M. A., and Gerrard, J. A. (2007) Mol. Biosyst. 3, 458–465

25. Sreerama, N., and Woody, R. W. (2000) Anal. Biochem. 287, 252–260

26. Laue, T. M., Shah, B. D., Ridgeway, T. M., and Pelletier, S. L. (1992) in Analytical Ultracentrifugation in Biochemistry and Polymer Science (Harding, S. E., Rowe, A. J., and Horton, J. C., eds) pp. 90–125, Royal Society of Chemistry, Cambridge, UK

27. Schuck, P. (2000) Biophys. J. 78, 1606–1619

28. Perugini, M. A., Schuck, P., and Howlett, G. J. (2000) J. Biol. Chem. 275, 36758–36765

29. Schuck, P., Perugini, M. A., Gonzales, N. R., Howlett, G. J., and Schubert, D. (2002) Biophys. J. 82, 1096–1111

30. Demeler, B., and van Holde, K. E. (2004) Anal. Biochem. 335, 279–288

31. Demeler, B. (2005) in Analytical Ultracentrifugation: Techniques and Methods (Scott, D. J., Harding, S. E., and Rowe, A. J., eds) pp. 210–229, Royal Society of Chemistry, Cambridge, UK

32. Vistica, J., Dam, J., Balbo, A., Yikilmaz, E., Mariuzza, R. A., Routela, T. A., and Schuck, P. (2004) Anal. Biochem. 326, 234–256

33. Leslie, A. G. (1991) in Crystallographic Computing 5: From Chemistry to Biology (Moras, D., Podjarny, A. D., and Therry, J. C., eds.) pp. 50–61, Oxford University Press, Oxford, UK

34. Evans, P. R. (2006) Acta Crystallogr. D Biol. Crystallogr. 62, 72–82

35. Winn, M. D., Isupov, M. N., and Murshudov, G. N. (2001) Acta Crystallogr. D Biol. Crystallogr. 57, 122–133

36. Emsley, P., and Cowtan, K. (2004) Acta Crystallogr. D Biol. Crystallogr. 60, 2126–2132

37. Laskowski, R. A., Moss, D. S., and Thornton, J. M. (1993) J. Mol. Biol. 231, 1049–1067

38. Perugini, M. A., Griffin, M. D., Smith, B. J., Webb, L. E., Davis, A. J., Handman, E., and Gerrard, J. A. (2005) Eur. Biophys. J. 34, 469–476

39. Blickling, S., Beisel, H. G., Bozic, D., Knäblein, J., Laber, B., and Huber, R. (1997) J. Mol. Biol. 274, 608–621

40. Melo, R. C., Ribeiro, C., Murray, C. S., Veloso, C. J., da Silveira, C. H., Neshich, G., Meira, W., Jr., Carceroni, R. L., and Santoro, M. M. (2007) Genet. Mol. Res. 6, 946–963

41. Blickling, S., Renner, C., Laber, B., Pohlenz, H. D., Holak, T. A., and Huber, R. (1997) Biochemistry 36, 24–33

42. Pedone, E., Bartolucci, S., Rossi, M., Pierfederici, F. M., Cacciamani, T., and Tanfani, F. (2003) Biochem. J. 373, 875–883

43. Griffin, M. D., Dobson, R. C., Pearce, F. G., Antonio, L., Whitten, A. E., Liew, C. K., Mackay, J. P., Trewella, J., Jameson, G. B., Perugini, M. A., and Gerrard, J. A. (2008) J. Mol. Biol. 380, 691–703

44. Krissinel, E., and Henrick, K. (2007) J. Mol. Biol. 372, 774–797

45. Matsumura, M., Osada, K., and Aiba, S. (1984) Biochim. Biophys. Acta 786, 9–17

46. Forouhar, F., Anderson, J. L., Mowat, C. G., Vorobiev, S. M., Hussain, A., Abashudze, M., Bruckmann, C., Thackray, S. J., Seetharaman, J., Tucker, T., Xiao, R., Ma, L. C., Zhao, L., Acton, T. B., Montelione, G. T., Chapman, S. K., and Tong, L. (2007) Proc. Natl. Acad. Sci. U.S.A. 104, 473–478