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Examination of Intrinsic Sulfonamide Resistance in *Bacillus anthracis*: A Novel Assay for Dihydropteroate Synthase

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Abstract

Dihydropteroate synthase (DHPS) catalyzes the formation of dihydropteroate and Mg-pyrophosphate from 6-hydroxymethyl-7,8-dihydropterin diphosphate and *para*-aminobenzoic acid. The *Bacillus anthracis* DHPS is intrinsically resistant to sulfonamides. However, using a radioassay that monitors the dihydropteroate product, the enzyme was inhibited by the same sulfonamides. A continuous spectrophotometric assay for measuring the enzymatic activity of DHPS was developed and used to examine the effects of sulfonamides on the enzyme. The new assay couples the production of MgPP_i to the pyrophosphate-dependent phosphofructokinase/aldolase/triose isomerase/ α -glycerophosphate dehydrogenase reactions and monitoring the disappearance of NADH at 340 nm. The coupled enzyme assay demonstrates that resistance of the *B. anthracis* DHPS results in part from the use of the sulfonamides as alternative substrates, resulting in the formation of sulfonamide-pterin adducts, and not necessarily due to an inability to bind them.

Introduction

Dihydropteroate synthase (DHPS), encoded by the *folP* gene in *Bacillus anthracis*, catalyzes the conversion of *para*-aminobenzoic acid (pABA) and Mg-6-hydroxymethyl-7,8-dihydropterin diphosphate (DHP-PP) to Mg-pyrophosphate (MgPP_i) and 7,8-dihydropteroate, an intermediate in the folate biosynthetic pathway of bacteria and other microorganisms. The tetrahydrofolate produced by this pathway is required for one carbon transfer reactions in the biosynthesis of biomolecules including nucleotides and amino acids [1–2]. Humans lack an equivalent to DHPS, making it an attractive drug target. Sulfonamides inhibit DHPS and have been therapeutically successful against a variety of bacterial DHPS enzymes, acting as inhibitors competing with pABA or by acting as functional substrate analogues of pABA [3], which produces a non-functional sulfonamide-pterin adduct. Unable to participate in downstream folate biosynthetic reactions, formation of the adducts results in a depletion of the folate-cofactor pool [3–7].

Bacillus anthracis is naturally resistant to trimethoprim and sulfonamides, which are known to inhibit key enzymes in the folate pathway, namely dihydrofolate reductase (DHFR, EC 1.5.1.3) [8] and DHPS (EC 2.5.1.15). The intrinsic resistance of *B. anthracis* to trimethoprim

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(MIC >2,048 ≤4,096 µg/mL) was attributed to an apparent lack of selectivity of DHFR (IC₅₀ = 77 µM) [8], while the exact mechanism of sulfonamide resistance remains unknown. Recent studies suggest that some xenobiotic-metabolizing enzymes (like arylamine N-acetyltransferase) may also play a role in resistance [9].

Resistance to sulfonamides nearly equivalent to that found in *B. anthracis* can be transferred to an *Escherichia coli folP* mutant, suggesting that the *B. anthracis folP* gene, encoding DHPS, is the source of this organism's sulfonamide resistance [10]. Dihydropteroate synthase from *B. anthracis* Sterne and *E. coli* MG1655 were purified and assayed by the standard radiologic method [11–12], and IC₅₀ values were established for sulfisoxazole, sulfamethoxazole, sulfamethazole, and sulfadiazine [10]. In all cases, IC₅₀ values for the *B. anthracis* DHPS were, on average, 225% higher than those measured for the *E. coli* DHPS. This increase in IC₅₀ is modest compared to other effective competitive enzyme inhibitors, but sulfonamides can also act as substrates of DHPS, forming nonfunctional sulfonamide-pterin adducts. Substrate activity was demonstrated for the *E. coli* DHPS using radio-labeled sulfanilamide, sulfathiazole, and sulfamethoxazole [5–6], but the adducts cannot be detected using the traditional radiometric DHPS enzyme inhibition assay that employs radio-labeled *p*ABA [11]. This makes it difficult to obtain accurate results regarding inhibition of DHPS. The radioassay is utilized by a number of researchers in the field as a screen for possible inhibitors and to understand the mechanisms of sulfonamide resistance. Thus, an assay capable of detecting true DHPS inhibitors as well as compounds that are used as alternative substrates would be a welcome and useful tool.

It would be useful to have a real-time, spectrophotometric enzyme assay capable of detecting DHPS activity and adduct formation that would be suitable to high throughput screening of potential inhibitors. We describe in this report the development of a rapid assay using commercially available reagents that has the potential to be developed into a high-throughput assay. The assay is based on the activity of the unique pyrophosphate-dependent phosphofructokinase (PP_i-PFK) [13]. The MgPP_i produced by DHPS is coupled to PP_i-PFK, aldolase, triosephosphate isomerase [14] and α-glycerophosphate dehydrogenase (α-GPDH) reactions, allowing continuous monitoring of NADH at 340 nm, Figure 1. The new assay was used to probe sulfonamide resistance in *B. anthracis*, and showed the resistance was in part due to the substrate activity of the sulfonamides.

Materials and Methods

Purification of the *B. anthracis* Sterne DHPS

Recombinant DHPS was purified using fast-performance liquid chromatography and dialyzed as previously described [10]. The His-tag was not removed from the protein, since previous studies showed that retaining the His-tag has no effect on enzyme activity [10].

Preparation of 6-hydroxymethyl-7,8-dihydropterin diphosphate

6-Hydroxy-methylpterin diphosphate lithium salt was purchased from Schircks Laboratories and was converted to 6-hydroxymethyl-7,8-dihydropterin diphosphate according to the method described by Aspinall et al. [12] with modifications described by Valderas and Barrow [10].

Continuous spectrophotometric DHPS enzyme assay

Components for the enzymatic coupling reactions were purchased from Sigma-Aldrich and were combined as follows. The reaction mixture in a final volume of 0.25 mL contained the following: 100 mM Hepes, pH 7.5, 0.2 mM β-NADH (disodium salt), 1 mM D-fructose 6-phosphate (potassium salt), 5 mM magnesium chloride, 2 U aldolase type IV from rabbit

muscle, 2.8 U TIM type III-S from rabbit muscle, 3 U α -GPDH type I from rabbit muscle, 1 U PP_i-PFK from *Propionibacterium freudenreichii*, 5 mM dithiothreitol, 1.08 μ g DHPS.

Initial rate experiments

The initial rate was measured as a function of the concentration of *p*ABA or DHP-PP with the other reactant fixed (at saturation) to obtain estimates of K_m values. The DHP-PP concentration was fixed at 20 μ M, while rates (dA/min) were measured for *p*ABA concentrations of 1, 1.43, 2.5, and 10 μ M. Similarly, rates for DHP-PP were determined at 2, 2.86, 5, and 20 μ M, holding the *p*ABA concentration at 100 μ M. Conversion of NADH to NAD⁺ was monitored at 340 nm using a Beckman DU 640 spectrophotometer with temperature maintained at 25°C using a circulating water bath. The reaction was initiated by the addition of DHPS. In the absence of DHPS, no change in absorbance was observed.

Enzyme interaction with sulfonamides

Assays were performed as described above with the exception that 2.15 μ g DHPS was used in a total volume of 500 μ L. DHP-PP was fixed at a concentration of 20 μ M. Sulfadiazine sodium salt (Sigma-Aldrich), sulfamethoxazole (MP Biomedicals), sulfisoxazole (MP Biomedicals), sulfamethazole (Sigma-Aldrich), sulfanilamide (Sigma-Aldrich), and sulfathiazole sodium salt (Sigma-Aldrich) were varied at final concentrations equal to 1, 1.43, 2.5, and 10 μ M, while dapsone (Sigma-Aldrich) was used at 0.1, 0.143, 0.25, and 1 μ M. No *p*ABA was used in these reactions. All compounds were prepared as 50 mg/mL solutions in sterile 100% DMSO, with the exception of sulfadiazine and sulfathiazole which were prepared in sterile water. All compounds were diluted in sterile water to 5 mM prior to use in the DHPS assay.

Inhibition of enzyme activity by 6-hydroxymethylpterin monophosphate

The concentration of 6HMP (Schircks Laboratories) was varied to determine its K_i . Essentially, the assay was performed under the conditions described above with *p*ABA at 100 μ M. The initial rate was measured at pterin concentrations of 10, 15, and 20 μ M with 6HMP fixed at 0, 62.5, and 125 μ M.

Data analysis

Data were plotted in double reciprocal form to assess data quality. Data were fitted using the appropriate rate equations and the algorithm (Marquardt-Levenberg) supplied with the EnzFitter program (version 2.0.16.0) from BIOSOFT, Cambridge, UK. All of the kinetic parameters and their corresponding standard errors were estimated using a simple weighting method.

Initial velocity data for substrate saturation curve were fitted to equation 1:

$$v = \frac{VA}{K_a + A} \quad (1)$$

In this equation, v and V are initial and maximum rates, A is reactant concentration and K_a is the Michaelis constant for A . In the case of alternate substrate (sulfonamide) assays with the concentration of DHP-PP fixed at 20 μ M, all the data were also fitted to equation 1 where A is sulfonamide concentration. Data for competitive inhibition by 6HMP were fitted to equation 2.

$$v = \frac{VB}{K_b(1 + \frac{I}{K_i}) + B} \quad (2)$$

In equation 2, B is DHP-PP concentration, I is the inhibitor concentration, K_i is the inhibition constant for I , and all other terms are defined above.

Results

Continuous spectrophotometric DHPS enzyme assay

The assay, as described in the Methods section provides an accurate reflection of the initial rate of the DHPS reaction. The dependence of initial rate vs. the concentration of DHPS is linear, Figure 2, indicating that over the enzyme concentration used, the assay has been optimized for coupling enzyme concentration and the rate is limited by production of MgPP_i. The error in reproducibility of the assay is within five percent. The slope of the line in Figure 2 is $4.50 \pm 0.08 \text{ min}^{-1}$ ($0.075 \pm 0.001 \text{ s}^{-1}$), which is the turnover number for the *B. anthracis* DHPS.

True Michaelis constants were estimated by measuring the initial rate as a function of one reactant with the other fixed at saturation ($20K_m$) (increasing the concentration of the fixed reactant gave no change in the K_m of the varied reactant). Estimated values of K_{pABA} and K_{DHP-PP} were $2.60 \pm 0.07 \text{ }\mu\text{M}$ and $2.7 \pm 0.7 \text{ }\mu\text{M}$, respectively, (data not shown).

Enzyme interaction with sulfonamides

In the presence of a potential inhibitor that might also function as an alternative substrate, the rate of the enzymatic reaction depends on the kinetic parameters in the presence of the inhibitor (K_m , K_i and k_{cat}). In general, one can observe one of three possibilities: 1) no change in the rate which may result from an inability to bind and inhibit or substrate activity that is equivalent to pABA; 2) an increase in the rate, which likely indicates a substrate activity more efficient than pABA (higher value of V/K , or activation by the analogue); or 3) a decrease in activity which may be a result of inhibition and/or substrate activity that is less efficient than pABA (lower value of V/K). Preliminary experiments, utilizing the coupled spectrophotometric assay and sulfonamide derivatives showed no DHPS inhibition. On the contrary, in the presence of pABA, sulfonamide derivatives behaved as if they were activators (or alternate substrates) of DHPS, giving an increase in the reaction rate (data not shown). In the absence of pABA, enzyme activities comparable to those with pABA alone were observed with the sulfonamide derivatives, suggesting they are efficient alternative substrates. Kinetic parameters for seven sulfonamide derivatives, as well as the natural substrate pABA, are shown in Table 1.

Inhibition of enzyme activity by 6-hydroxymethylpterin monophosphate and determination of K_i

In order to show that inhibition could be detected, 6HMP, an analogue of DHP-PP, was used as a competitive inhibitor. At the highest inhibitor concentration used, the rate is identical when the concentration of coupling enzymes is doubled, and thus the inhibitor had no effect on the rate of the coupling enzymes. The inhibition pattern for 6HMP is shown in Figure 3, giving a K_i of $36 \pm 11 \text{ }\mu\text{M}$.

Discussion

The *B. anthracis* DHPS was used in the development of a coupled spectrophotometric enzyme assay in order to probe its link to the intrinsic sulfonamide resistance of this organism [10]. It has been observed that greater than 95% of field isolates of *B. anthracis* from South Africa's Kruger National Park were resistant to sulphatriad [15–16] and that *B. anthracis* Sterne is also highly resistant to a variety of sulfonamides [10]. All strains of *B. anthracis* possess a single gene, *folP*, with 100% sequence identity encoding DHPS [10]. Near equivalent sulfonamide resistance was transferred to a DHPS mutant strain of *E. coli* with the *B. anthracis folP* gene [10]. No other genes encoding sulfonamide resistance, nor any encoding efflux pumps capable of removing sulfonamide such as OprM and OprJ, were identified by BLAST analysis of all sequenced *B. anthracis* genomes. These findings argue that the single *B. anthracis folP* gene

is likely responsible for the high level of sulfonamide resistance observed in this organism. However, the recently discovered xenobiotic-metabolizing enzymes in *B. anthracis* could represent an additional mechanism for resistance [9]. The amount of their contribution to resistance is not known at this time. Because *B. anthracis* is intrinsically resistant to sulfonamides and because the mechanism of its resistance has not been fully described, its DHPS is particularly suited to examination using the novel DHPS assay. The activity of the *E. coli* DHPS [10] was also studied using the coupled assay; however, detailed studies were not performed.

A range of k_{cat} values from 0.004 s^{-1} to 15.6 s^{-1} has been reported for the synthases from different organisms, while K_{pABA} values are in the range of $0.5 \text{ }\mu\text{M}$ to $3.8 \text{ }\mu\text{M}$, and K_{DHP-PP} values in the range of $1.4 \text{ }\mu\text{M}$ to $30 \text{ }\mu\text{M}$ (BRENDA - The Comprehensive Enzyme Information System – www.brenda-enzymes.info) [17]. Kinetic parameters of the DHPS from *B. anthracis*, which are reported for the first time in this paper, are generally within the range reported for enzymes from other organisms.

Crystallographic studies with the *B. anthracis* DHPS [18] have failed to address whether sulfonamides are able to gain entry to the catalytic site of the enzyme. Of the DHPS crystal structures from *Escherichia coli*, *Mycobacterium tuberculosis*, *Staphylococcus aureus*, and *B. anthracis*, only the *E. coli* enzyme has been crystallized with a bound sulfonamide, specifically sulfanilamide [19]. No crystal structures with *pABA* exist, though the structure of *B. anthracis* DHPS in complex with pteric acid, a product analogue, has been determined [18]. A marked difference in orientation of the ligands in the *pABA* binding site can be observed when comparing the *pABA* moiety of pteric acid in the *B. anthracis* DHPS structure and that with sulfanilamide in the *E. coli* structure. The implication of this observation is that sulfonamides may not bind in a manner similar to *pABA*. Knowledge of how sulfonamides interact with the *B. anthracis* DHPS and of the drug resistance mechanism could impact future drug discovery. If the mechanism of resistance could be resolved, a better understanding would be gained of the nature of the substrate binding site, thereby enabling design of new inhibitors that would be more effective and would not possess the immunologic properties of the sulfonamides.

The DHPS enzyme assay described in this manuscript demonstrates that the intrinsic resistance of *B. anthracis* DHPS is not due to a lack of selectivity for the substrate binding site as previously observed with DHFR and innate trimethoprim resistance [8]. The sulfonamides fit into the substrate binding site and are efficiently converted to sulfonamide-pterin adducts by DHPS instead of acting as competitive inhibitors of the enzyme. In fact, the K_m for some sulfonamide derivatives is lower than that of the natural substrate, *pABA*, indicating a higher steady state affinity of the enzyme for those analogues (Table 1).

All of the sulfonamides contain an aromatic amine group that could substitute for *pABA* and react with DHP-PP. As shown in Table 1, the sulfonamides can be classified into three categories based on their ability to act as alternative substrates as determined by their specificity constants (specificity constant is V/K). In general, less bulky derivatives of *pABA* (sulfadiazine and sulfamethoxazole) with more hydrogen-bonding capability tend to increase the specificity constant. These compounds have K_m lower than that of *pABA* and therefore have a higher steady state affinity for DHPS. Bulkier compounds such as sulfisoxazole, sulfamethizole, sulfanilamide, and sulfathiazole are not as efficient as the natural substrate and fall into the second category. Dapsone, which stands alone in the third group, and possesses the highest specificity constant may be unique in binding to the enzyme as it physically resembles a bi-dentate *pABA* molecule ($K_m = 0.06 \text{ }\mu\text{M}$).

Kinetic studies with 6HMP indicate that this molecule is, in fact, a true competitive inhibitor of DHPS demonstrating linear competitive inhibition, Figure 3. The crystal structure of the DHPS in complex with 6HMP shows an extensive network of hydrogen bonding holding the pterin ring in the active site of the enzyme [18] (Figure 4). According to the structure and the competitive nature of the inhibition, 6HMP binds to the DHP-PP binding site [18]. In the case of the natural substrate (DHP-PP), the β -phosphate of DHP-PP (not shown in Figure 4) binds to Arg254 and His256 [18].

A standard stopped-time radiologic assay, which depends upon the incorporation of radio-labeled *p*ABA into dihydropteroate, has been used for DHPS to now. There are advantages and disadvantages to the radiologic method. The main advantages to the method are sensitivity and cost. However, the disadvantages outweigh the advantages. The radiologic method utilizes time-consuming thin-layer chromatography to separate unreacted radio-labeled *p*ABA from radio-labeled dihydropteroate, which remains at the origin [12]. Additionally, the assay is discontinuous and is therefore not easily adaptable to high throughput screening; one must wait until the TLC is developed to detect errors made in preparing and carrying out the assay. Though the assay has been used with radio-labeled sulfamethoxazole, sulfanilamide, and sulfathiazole to detect adduct formation by alternative DHPS substrates [5–6], radio-labeling of a large number of potential inhibitors simply to detect adduct formation is not practical. The biggest fault of the assay is its inability to detect alternative *p*ABA substrates, which can adversely affect analysis of the efficacy of potential DHPS inhibitors. When a compound is capable of being used as an alternative substrate by DHPS in lieu of *p*ABA, the reaction results in the formation of an adduct and depletes the DHP-PP pool. This makes DHP-PP less available for conversion to radio-labeled dihydropteroate when *p*ABA is also present. The consequence of this DHP-PP unavailability is that more radiolabeled *p*ABA remains unreacted, making it appear as though the *p*ABA analogue is a competitive inhibitor when in actuality it is not. This phenomenon could explain why sulfonamides and monocyclic pteridine analogs show apparent inhibition of the *B. anthracis* and *E. coli* DHPS enzymes respectively in vitro studies, while the actual organism remains resistant to the compounds [10,20]. An alternative method patented by Stachyra and Biton [21] could detect adduct formation because it detects PP_i production, but it too is discontinuous and not suited for high throughput. More recently, an assay of DHPS was described by Fernley et al. [22], which uses spectrophotometric detection making it amenable to high-throughput screening, but it detects the formation of dihydropteroate using the NADP⁺-dependent dihydrofolate reductase (DHFR) as coupling enzyme. Similar to the radiologic method with labeled *p*ABA, it cannot directly account for assay interference by alternative substrates and adduct formation. In the presence of both *p*ABA and sulfa-drugs, the assay seems to be less versatile due to the formation of pterin-sulfa adducts that may also inhibit the next coupling enzyme DHFR. Therefore, in the presence of *p*ABA, the assay cannot distinguish with certainty whether a sulfa drug is an inhibitor or an alternative substrate. Although, the assay can detect alternative substrates (though in the absence of *p*ABA), it requires the separation of the pterin-sulfa adduct by chromatography to test for the possibility of DHFR inhibition. The assay described by Fernley *et al* [22], has a major advantage of using one coupling enzyme (DHFR); however, with the price of introduction of some ambiguities regarding the activity of DHFR in the presence of DHPS products.

Recently, interest in monocyclic pterin compounds has resurfaced as inhibitors of the folate pathway, though no inhibition data has yet been published for the *B. anthracis* DHPS [18, 23]. An interference-free assay capable of detecting true DHPS inhibitors as well as compounds that can be used as alternative substrates is thus a welcome and useful tool.

The novel continuous spectrophotometric method for measuring DHPS activity, described herein, was developed using commercially available reagents, and produces two molecules of triose, which are reduced to glycerol 3-phosphate resulting in a twofold amplification of the

signal (and sensitivity), i.e. for every mole of PP₁ produced, two moles of NADH are oxidized. The coupled assay has been optimized for the concentration of coupling enzymes (PP_i-PFK/aldolase/TIM/ α -GPDH) such that a linear dependence of the rate on the concentration of DHPS from *Bacillus anthracis* is observed up to 8 μ g of enzyme.

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Figure 1. Diagram of the DHPS enzyme assay

6-hydroxymethyl-7,8-dihydropterin diphosphate and pABA are converted to PP_i and dihydropteroate by DHPS. The PP_i-PFK from *P. freundenreichii* converts fructose-6-phosphate to fructose-1,3-bisphosphate. The product is coupled to the aldolase, TIM and α -GPDH reactions. Overall, the disappearance of NADH is monitored at 340 nm.

Figure 2. Linear dependence of the rate on the concentration of *B. anthracis* DHPS for the new method

The assay has been optimized for the concentrations of the coupling enzymes to obtain the linear rate dependence. The slope of the line is equal to the turnover number of the DHPS enzyme (both substrates are at saturation).

Figure 3. DHPS inhibition by 6-hydroxymethylpterin monophosphate

The Lineweaver-Burk plot showing competitive inhibition of the DHPS by 6-hydroxymethylpterin monophosphate with a K_i of $36 \pm 11 \mu\text{M}$. 6HMP concentrations were fixed at $0 \mu\text{M}$ (◆), $62.5 \mu\text{M}$ (■), and $125 \mu\text{M}$ (▲). The points are experimental, while the solid lines are theoretical data based on a fit to the Lineweaver-Burk transformation of equation 2.

Figure 4. A stereo view of the crystal structure of the DHPS enzyme from *B. anthracis* in complex with 6-hydroxymethylpterin monophosphate

The pterin ring makes an extensive network of hydrogen bonds in the enzyme active site. The 6HMP molecule is shown in stick representation with carbon atoms in green. Figure was produced using PyMOL software version 0.99 (DeLano Scientific LLC – www.pymol.org). The PDB code for the structure of the DHPS from *B. anthracis* [17] is 1TWZ (www.rcsb.org).

Table 1
Kinetic parameters for the alternate substrates (*p*ABA analogues).

Substrate	Structure	K_m (μ M)	V_{max} (μ M/min)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
<i>p</i> ABA		2.2 ± 0.1	1.71 ± 0.03	0.109 ± 0.002	$(4.9 \pm 0.2) \times 10^4$
Sulfadiazine		2.89 ± 0.01	1.947 ± 0.002	0.1243 ± 0.0001	$(4.30 \pm 0.02) \times 10^4$
Sulfamethoxazole		2.7 ± 0.3	1.88 ± 0.05	0.120 ± 0.003	$(4.4 \pm 0.5) \times 10^4$
Sulfisoxazole		1.5 ± 0.9	1.8 ± 0.3	0.11 ± 0.02	$(7.3 \pm 4.6) \times 10^4$
Sulfamethizole		0.7 ± 0.1	1.24 ± 0.03	0.079 ± 0.002	$(1.1 \pm 0.1) \times 10^5$
Sulfanilamide		0.6 ± 0.2	1.26 ± 0.07	0.080 ± 0.004	$(1.3 \pm 0.4) \times 10^5$
Sulfathiazole		0.33 ± 0.03	1.39 ± 0.01	0.0887 ± 0.0006	$(2.68 \pm 0.24) \times 10^5$
Dapson		0.058 ± 0.001	0.906 ± 0.002	0.0578 ± 0.0001	$(1.003 \pm 0.012) \times 10^6$