Dihydropteroate synthase from Streptococcus pneumoniae: structure, ligand recognition and mechanism of sulfonamide resistance

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DHPs (dihydropteroate synthase) catalyses an essential step in the biosynthesis of folic acid and is the target for the sulfonamide group of antimicrobial drugs. In the present paper we report two crystal structures of DHPs from the respiratory pathogen Streptococcus pneumoniae: the apoenzyme at 1.8 Å (1 Å = 0.1 nm) resolution and a complex with DHPPP (6-hydroxymethyl-7,8-dihydropterin monophosphate) at 2.4 Å resolution. The enzyme forms a α/β barrel structure, with a highly conserved binding pocket for recognition of the pterin substrate, DHPPP (6-hydroxymethyl-7,8-dihydropterin pyrophosphate). There is a fixed order of substrate binding: DHPPP binds first, followed by the second substrate, pABA (p-aminobenzoic acid). Binding of PP, also allows the enzyme to recognize pABA or sulfonamide drugs, which act as pABA analogues. Using equilibrium and pre-steady state kinetic fluorescence measurements, we show that the on-rate for DHPPP binding to the enzyme is relatively low (2.6 × 10^7 M⁻¹·s⁻¹) and propose that binding of this substrate induces a large scale movement of the second loop in the enzyme structure to participate in the formation of the pABA-binding site. Two mutations which confer resistance to sulfonamide drugs do not affect DHPPP binding, but have a substantial effect on pABA and sulfonamide recognition. The results show that binding of DHPPP and pABA are separate distinguishable events in the reaction cycle, and that mutations which confer resistance to sulfonamide drugs act exclusively on the second step in the binding process.

Key words: antimicrobial drug, crystal structure, dihydropteroate synthase, fluorescence, folic acid, sulfonamide.

INTRODUCTION

Folic acid is an essential component of the diet for higher organisms, including humans. Bacteria, plants and some disease-causing parasites have the ability to synthesize folic acid de novo: a sequence of six chemical steps transforms GTP into dihydrofolate (reviewed in [1]). Several enzymes from the folate pathway have no equivalent in humans, making them ideal candidates as targets for antimicrobial drugs. Consequently, there has been continuing interest in the structures and mechanisms of the folate biosynthesis enzymes [2–6]. DHPs (dihydropteroate synthase; EC 2.5.1.15) catalyses an essential step within the folic acid biosynthetic pathway: nucleophilic attack by the amino group of pABA (p-aminobenzoic acid) displaces PP, from DHPPP (6-hydroxymethyl-7,8-dihydropterin pyrophosphate), leading to the formation of dihydropteroate (Figure 1). This chemical step constitutes a pivotal point in the folate pathway: pABA is synthesized by the chorismate pathway [7] and feeds into the folic acid biosynthesis pathway at this point. The sulfonamides, which were essentially the first group of synthetic antibiotics to be widely used, are potent inhibitors of DHPs [8]. They inhibit the reaction by acting as alternative substrates [9], leading to a ‘dead end’ sulfapterin product. Resistance to sulfonamides is widespread in bacteria and parasites, and generally correlates with mutations to the DHPs gene [10]. Steady-state kinetic analysis has shown that sulfonamide resistance mutations lead to raised K, values for the drug [10–12].

The crystal structures of DHPs have been determined from a variety of bacterial sources: Escherichia coli [13], Staphylococcus aureus [14], Mycobacterium tuberculosis [15] and Bacillus anthracis [16]. In addition, the structure of a bifunctional complex of DHPs fused to the preceding enzyme in the pathway, HPPK (6-hydroxymethyl-7,8-dihydroprotein pyrophosphokinase), has been reported from Saccharomyces cerevisiae [4]. DHPs adopts an α/β (TIM) barrel fold, with the active site identified at one end of the barrel by co-crystallization with a variety of pterin ligands [1]. The DHPs enzymes are structurally related to methyltetrahydrofolate-dependent methyltransferase, and conservation of the pterin-binding sites between the two enzymes has been noted [17]. Complexes of several DHPs enzymes bound to the oxidized form of the substrate, 6-hydroxymethylpterin pyrophosphate or 6-hydroxymethylpterin phosphate, which presumably acts as an inhibitor of the enzyme, show the same orientation of the bound pterin ring [4,14–16]. Comparisons between different DHPs structures do show, however, that there are significant variations in the conformations of some of the loop regions in the vicinity of the active site. Loop regions 1 and 2, which are likely to be of importance to catalysis, show a variety of conformations or else are absent from electron-density maps, suggesting a high degree of conformational flexibility. This has complicated the identification of the binding site for the second substrate, pABA. Achari et al. [13] reported the structure of a ternary complex of E. coli DHPs with dihydropterin and sulfanilamide, a sulfonamide which acts as an analogue...
of pABA. Although E. coli DHPS is able to use sulfonamides as substrates [9], this structure did not allow an unambiguous reconstruction of the transition state within the active site, as the amino group within sulfanilamide was not aligned for optimal S_n-2 attack of the DHPPP substrate. An alternative orientation of the pABA aromatic ring was suggested from a more recent report of the co-crystallization of B. anthracis DHPS with pteroic acid, an analogue of dihydropteroate [16]. These authors proposed a different location for the pABA-binding site, with an orientation more suited to the S_n-2 attack of the methylene carbon attached to the C-6 position on the DHPPP pterin ring. To date, a crystal structure of DHPS with pABA bound has not been fully described, and the precise location of the second substrate during the reaction cycle remains an open question.

Streptococcus pneumoniae is a respiratory pathogen and a major cause of community-acquired pneumonia; resistance of streptococcal clinical isolates to antibiotics is now common [18]. Sequencing of the DHPS gene from S. pneumoniae has shown that one or two residue insertions, within the predicted loop 2 of the enzyme, are responsible for sulfonamide resistance [19,20]. A more general survey of the distribution of sulfonamide resistance mutations established that they tended to cluster to sites within loops 1 and 2 [15]. Interestingly, alignments of DHPS gene sequences show regions of relatively high sequence conservation within these same regions [15,16], suggesting that they may be involved in pABA recognition. Although sulfonamides are no longer in extensive clinical use, there are compelling reasons to try to understand the origin of sulfonamide resistance. First, DHPS is one of the few biosynthetic enzymes which has been successfully exploited as a target for antimicrobial drugs. Knowledge of the structure and mechanism of the enzyme could be employed to develop a more informed approach to drug design, perhaps by exploitation of the conserved DHPPP-binding site [16,21].

Secondly, the structural similarity between sulfonamides and pABA raises an intriguing question: how can a single site mutation permit DHPS to discriminate between them? To date, relatively few enzymological studies on DHPS have been reported, and are mainly confined to steady-state kinetics [10,11,22]. Here, we have exploited fluorescence-based equilibrium and pre-steady-state kinetic binding studies, in combination with crystallography, to examine the structural changes which occur in DHPS upon binding substrates. S. pneumoniae DHPS is an ideal enzyme for this work, as it lends itself to binding measurements using the intrinsic fluorescence of the protein, as well as the fluorescence of DHPPP. The results suggest that extensive structural changes occur within the second loop region on binding the first substrate (DHPPP) and that sulfonamide-resistance mutations in this loop have a drastic effect on pABA binding.

**EXPERIMENTAL**

**Materials**

The 6-hydroxymethylpterin pyrophosphate was obtained from Schirks Laboratories and was reduced to DHPPP using sodium dithionite as described previously [23]. Selenomethionine, pABA and sulfamethoxazole were obtained from Sigma.

**Protein expression and purification**

The cloning and expression in E. coli of DHPS from S. pneumoniae has been described previously [22]. A similar method was employed to that described by Vinicombe and Derrick [22] for purification of the enzyme from E. coli, with some modifications. Competent XL2-Blue E. coli cells (Stratagene) were transformed with the expression plasmid and grown in LB (Luria–Bertani) broth [24] supplemented with 100 μg/ml ampicillin at 37°C. Expression of DHPS was induced by addition of IPTG (isopropyl β-D-thiogalactopyranoside) at a final concentration of 1 mM; cells were allowed to grow for a further 3 h after induction before being harvested by centrifugation for 20 min at 5000 g at 4°C. For production of DHPS with selenomethionine incorporation, growth was conducted in a defined medium designed to suppress methionine biosynthesis [25]. The cell pellet was washed twice with 10 ml of lysis buffer [100 mM Tris/HCl (pH 8.0) and 50 mM NaCl, using one Complete™ protease-inhibitor tablet (Roche) per 50 ml] plus 1 mM DTT (dithiothreitol) and 1 mM EDTA, and then resuspended in 50 ml of lysis buffer. Cells were lysed by sonication, and the cell debris was sedimented by centrifugation at 20000 g, for 30 min at 4°C. The supernatant was retained, ammonium sulfate was added to 50% saturation, and the solution was left on ice for 1 h. The resulting precipitate, containing DHPS, was retrieved by centrifugation at 20000 g for 15 min at 4°C. The precipitate was resuspended in 50 ml of resuspension buffer (20 mM Tris/HCl, pH 8.0) and dialysed overnight against 5 litres of the same buffer. The enzyme was then subjected to ion-exchange chromatography using a Resource Q column (GE Healthcare Biosciences), equilibrated in 20 mM Tris/HCl (pH 8.0). DHPS was eluted by application of a linear gradient from 0 to 1 M NaCl in the same buffer; appropriate fractions were identified by SDS/PAGE, pooled and dialysed overnight against resuspension buffer. The protein preparation was then subjected to a second ion exchange step, using a Mono Q column (GE Healthcare Biosciences), equilibrated in 20 mM Tris/HCl (pH 8.0). DHPS was eluted by application of a linear gradient from 0 to 1 M NaCl in the same buffer. At this stage the DHPS preparation was sufficiently pure for use in kinetics or binding studies. However, for crystalization, a final purification step was found to assist in crystal growth. The appropriate fractions from the Mono Q column were pooled, concentrated to a final volume of 2-ml and applied to a Superdex 200 gel-filtration column (GE Healthcare Biosciences), equilibrated in 20 mM Tris/HCl (pH 8.0) with 150 mM NaCl. The enzyme was eluted in a single peak, and the appropriate fractions were pooled and dialysed overnight against 20 mM Tris/HCl (pH 8.0).

**Site-directed mutagenesis**

Site-specific mutations were introduced by using the QuickChange mutagenesis kit (Stratagene) following the manufacturer's instructions.

**Crystallization and data collection**

Purified DHPS was concentrated by ultrafiltration to a protein concentration of 13 mg/ml. Rod-shaped crystals were grown...
by the hanging-drop method, by mixing 2 μl of the protein solution with 2 μl of the well solution, consisting of 0.2 M ammonium iodide and 20 % (w/v) poly(ethylene glycol) 3350. The crystals generally appeared within 7–14 days. Crystals of selenomethionine-incorporated DHPS were grown in the same way. To obtain the complex with DHPP (6-hydroxymethyl-7,8-dihydropterin monophosphate), hanging-drop crystallization trials were set up as described for native and selenomethione DHPS; crystals from this preparation formed a different crystal form. Again, refinement was carried out using COOT [29] and the CCP4 program REFMAC5 [30], as implemented within the CCP4 suite [31]. The structure comprised a dimer in the asymmetric unit, and no non-crystallographic symmetry restraints were applied during refinement. The final structure was lacking residues 1–6 (chain A) and 1–5 (chain B) from the N-terminus, and residues 304–314 (chain A) and 303–314 (chain B) from the C-terminus.

**Table 1 X-ray data processing and refinement statistics**

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Structure determination and refinement

Attempts to solve the structure of *S. pneumoniae* DHPS from the native 1.8 Å (1 Å = 0.1 nm) resolution dataset by molecular replacement, using DHPS structures from *E. coli*, *Staph. aureus*, *M. tuberculosis* and *B. anthracis*, gave ambiguous results. *S. pneumoniae* DHPS was therefore prepared with selenomethionine incorporation; crystals from this preparation formed a different spacegroup from the native dataset (Table 1). A dataset was collected at a single wavelength, and subjected to SAD (single-wavelength anomalous diffraction) phasing and automated model building using the SOLVE/RESOLVE package [27]. The resulting partial model was taken as the input for molecular replacement of the native 1.8 Å resolution dataset using PHASER [28]. Iterative rounds of rebuilding and maximum likelihood refinement were carried out using COOT [29] and the CCP4 program REFMAC5 [30], as implemented within the CCP4 suite [31]. The structure comprised a dimer in the asymmetric unit, and no non-crystallographic symmetry restraints were applied during refinement. The final structure was lacking residues 1–6 (chain A) and 1–5 (chain B) from the N-terminus, and residues 304–314 (chain A) and 303–314 (chain B) from the C-terminus.

Fluorimetric titration assays

Equilibrium fluorescence titrations were recorded using a Varian Cary Eclipse fluorescence spectrophotometer. To determine the equilibrium binding constant (K_d) for PP, binding to the sulfonamide-sensitive form of DHPS, an excitation wavelength...
of 290 nm (2.5 nm slit) and an emission wavelength of 340 nm (10 nm slit) were employed. Titration was carried out in a fluorescence cuvette with a volume of 3 ml, containing 50 mM Tris/HCl (pH 8.0), 5 mM MgCl2 (the same buffer as employed by Vinnicombe and Derrick [22]) and a protein concentration of 50 μg/ml. For determination of the binding of pABA to the sulfonamide-sensitive form of DHPS, an excitation wavelength of 265 nm (2.5 nm slit) and an emission wavelength of 340 nm (10 nm slit) were employed. Typically, four separate readings were averaged at each point in the titration. The pABA titration required correction for the inner filter effect arising from pABA absorption: data from a control titration, with no enzyme, were used to establish the correction parameters as described by Birdsall et al. [33]. Determination of the $K_d$ for binding of pABA to the Y63 (insertion of a tyrosine residue at position 63) and GS60 (insertion of a Gly-Ser dipeptide beginning at position 60) sulfonamide resistance mutants required a different approach, as their binding affinity for pABA was reduced. Titrations of PPi, into a solution of 150 μg/ml DHPS in Tris/MgCl2 buffer was carried out in the presence of various concentrations of pABA. Fluorimeter settings and excitation and emission wavelengths were the same as those used for the titration of PPi, into the sulfonamide-sensitive form of DHPS. All fluorescence equilibrium binding data were fitted to the binding models given in the text using the software package DYNAFIT [34].

**Stopped-flow transient kinetics**

Stopped-flow fluorescence measurements were carried out using an SX.18MV spectrometer (Applied Photophysics). For measurement of the binding of PPi, using the intrinsic tryptophan fluorescence of the enzyme, an excitation wavelength of 290 nm was used, and an emission wavelength of 340 nm, through the use of a second monochromator. For measurement of pterin fluorescence, an excitation wavelength of 330 nm was used, in combination with a cut-off filter of 400 nm in line with the emission photomultiplier. The temperature of the observation chamber and drive syringes was maintained by water circulation at 25°C. The mixing ratio was 1:1 for all experiments. Solutions of ligands and enzyme were prepared by dilution into 50 mM Tris/HCl (pH 8.0) and 5 mM MgCl2, and precautions to maintain an anaerobic environment were implemented as described previously [23]. Data acquisition and processing were controlled with a 32-bit RISC processor workstation.

**RESULTS**

In order to investigate the mechanism of substrate binding and sulfonamide resistance in *S. pneumoniae* DHPS, it was valuable to determine the crystal structure of the enzyme. The structure of the *S. pneumoniae* DHPS apoenzyme was refined to 1.8 Å resolution and used to solve the structure of a complex obtained from a DHPS crystal grown in the presence of the pterin-containing substrate DHPPP, to a resolution of 2.4 Å (Table 1). Density for the ligand was observed in only one of the two potential binding sites within the enzyme dimer (Figures 2A and 2B); a similar phenomenon has been observed in the DHPS structure from *Staph. aureus* [14]. Furthermore, although density was well defined for the pterin ring and ω-phosphate, there was no evidence for presence of the β-phosphate (Figure 2B). Our interpretation of this observation is that the β-phosphate has probably been removed by hydrolysis from the DHPPP ligand during crystallization. For this reason, we will refer to this structure as a complex with DHPP, i.e. the monophosphorylated form of DHPPP. Determination of the structure of the DHPS–DHPPP binary complex enabled identification of the pterin-binding site within the enzyme and placed some constraints on the probable location of the PPi moiety in the DHPS–DHPPP structure.

Although there was a dimer in the asymmetric unit, the structures of the two subunits in the apoenzyme structure were essentially identical, with an RMSD (root mean square deviation) of 0.73 Å for Cα atoms. This was also the case for the DHPP complex, where the equivalent RMSD was 0.51 Å. Perhaps surprisingly, there was also little difference between the apoenzyme structure and the DHPP complex (RMD 0.27 Å). The pterin ring of DHPP binds within a well-defined cavity on the enzyme surface, with the phosphate group pointing outwards. A mixture of polar and apolar residues line the cavity: Asp56, Asn110, Asp201 and Lys382 form hydrogen bonds with the pterin ring (Figure 2C), and Asn17, Arg262 and His384 are involved in recognition of the phosphate moiety within DHPP. These residues are highly conserved in other bacterial enzymes, and have similar functions in pterin and phosphate recognition [13–16]. There was little change in the conformations of the side chains of these residues on binding of DHPP, and no evidence for occupancy of the pterin-binding site by a residue from loop 2, as is the case for *B. anthracis* DHPS [16].

*S. pneumoniae* DHPS adopts a TIM barrel-type fold, in common with the structures of DHPS enzymes which have been determined from other sources [4,13–16]. Even after successive rounds of model building and refinement, electron density for some parts of the polypeptide chains in the native and DHPP complex structures was either weak or absent. In particular, loops 1 and 2, which contain a number of highly conserved residues and are the sites for sulfonamide-resistance mutations, were poorly defined (Figure 3A). This observation indicates that these loops are highly mobile and that their mobility may be linked to catalysis. The principal core secondary-structure elements that define the TIM barrel overlay well with those from other structures, but significant deviations were observed in some loop regions. An example of this variation is presented in Figure 3(B), which shows an overlay of DHPS structures from *S. pneumoniae* and *Staph. aureus*. The L5 loop incorporates three short α-helices in the *S. pneumoniae* structure, but is much shorter in the *Staph. aureus* enzyme. Other minor differences were also apparent: the loop from the end of the 6loop7b helix to α7 is also longer in *S. pneumoniae* DHPS and adopts a different conformation from those of other bacterial DHPS structures. This modification could be associated with dimer formation, as some residues within this loop region (e.g. Phe248) form direct contacts with residues in the adjacent subunit.

Numerous attempts were made to obtain structures of *S. pneumoniae* DHPS in complex with PPi, pABA or several sulfonamide drugs by co-crystallization or ligand soaking. Where crystallographic data were collected and analysed, the resulting structures did not result in any plausible density being obtained for the ligands, although it was noted that soaking with PPi, and pABA or sulfonamide led to cracking of the crystals. These observations are consistent with the conclusion that recognition of pABA or sulfonamide requires a specific conformational change from the apoenzyme structure. We therefore sought to examine alternative ways in which pABA and sulfonamide binding could be studied. It has been shown previously, through the use of a radiochemical binding assay, that the *S. pneumoniae* DHPS apoenzyme has a very low affinity for pABA or sulfonamides [22]. The binding of PPi, to the enzyme dramatically increases the affinity of the enzyme for pABA, presumably because PPi, is capable of imitating the substrate DHPPP. An analysis of the binding of PPi, to the enzyme will therefore provide some clues to the structural changes which occur on forming the pABA-binding site.
S. pneumoniae DHPS contains a single tryptophan residue, Trp93; a change in the environment of the side chain of this residue on ligand binding could be reflected in a change in the intrinsic fluorescence of the enzyme. The results of an equilibrium titration of PPi into the enzyme are shown in Figure 4: binding of the ligand produces a 65% enhancement in fluorescence intensity. Data fitting showed that the response was consistent with a single saturable binding site and gave a $K_d$ of $350 \pm 20$ μM for the sulfonamide-sensitive form of the enzyme. Trp93 is situated in a short loop between the third β-strand and α-helix in the TIM barrel fold and lies approx. 15 Å from the α-phosphate in the DHPP ligand (Figure 2D). Solvent-accessibility calculations, using the method of Lee and Richards [35] and implemented using AREAIMOL from the CCP4 suite [31], established an average area of 28 Å² for Trp93 across both chains in the apoenzyme dimer. A structural rearrangement on binding of PPi could lead to the indole ring becoming more buried in the DHPS–PPi binary complex, and hence explain the fluorescence enhancement.

It has been established that sulfonamide resistance in S. pneumoniae DHPS can be caused by mutations to residues which lie within the second loop region [19,20]. To examine the effects of sulfonamide resistance mutations on the ligand-binding behaviour of the enzyme, two resistance mutations were introduced into the coding sequence for the sulfonamide-sensitive form of DHPS, and the mutant enzymes were expressed and purified. Mutations known to cause sulfonamide resistance frequently involve insertion of one or more residues into the loop regions of the enzyme. We selected two which have previously been shown to confer sulfonamide resistance on S. pneumoniae DHPS [19]: Y63 (which denotes insertion of a tyrosine residue at position 63) and GS60 (similarly, insertion of a Gly-Ser dipeptide beginning at position 60). The effect of PPi binding to both these sulfonamide-resistant enzymes on the fluorescence of Trp93 was studied (Figure 4). Although the fluorescence yield of each mutant was comparable with the sulfonamide-sensitive form of the enzyme, titration of PPi up to a concentration of 2 mM had only a minimal effect on the intrinsic fluorescence: a quench of less than 3%. This value was too small to accurately measure a $K_d$ value for PPi, binding. This observation suggested that either the Y63 and GS60 mutations had substantially reduced affinity for PPi, or they had indirectly affected the response of Trp93.
Figure 3  Location of selected loop regions and comparison of the structures of *S. pneumoniae* and *Staph. aureus* DHPS

(A) Stereo view cartoon of *S. pneumoniae* DHPS monomer, showing the approximate locations of the loop regions with missing electron density (shown with broken lines). Loops 1, 2, and 5 are designated L1, L2 and L5 respectively. A space-filling model of the DHPP ligand indicates the position of the active site. (B) Stereo view cartoon comparison of the structures of *S. pneumoniae* DHPS (light grey) with *Staph. aureus* DHPS (dark grey). Figures were created using PyMOL (http://pymol.sourceforge.net/).

Figure 4  Equilibrium binding of PPᵢ to sulfonamide-sensitive *S. pneumoniae* DHPS measured from intrinsic protein fluorescence

Enzymes used are as follows: sulfonamide-sensitive DHPS (squares), Y63 mutant (circles) and GS60 mutant (triangles). Data points are the means of four readings. The data were fitted to a single saturable binding site model with a *K*₅₀ of 350 ± 20 μM and a fluorescence enhancement of 65%.

To extract the rate constant for the binding of PPᵢ to the sulfonamide-sensitive form of DHPS, the change in intrinsic fluorescence after rapid mixing of enzyme and PPᵢ was recorded by stopped flow. The results showed a rapid rise in fluorescence on binding of PPᵢ, over approx. 100 ms, which could be fitted to a single exponential curve (Figure 5A). The apparent rate constant, *k*ₐₚ, was recorded over a range of concentrations of PPᵢ, and *k*ₐ and *k*ᵦ for the binding were deduced as 5.6 ± 0.3 × 10⁴ M⁻¹·s⁻¹ and 21 ± 1 s⁻¹ respectively (Figure 5B). It is interesting to note that the on-rate for PPᵢ binding to the enzyme is relatively slow. It is not possible, from these data, to distinguish between a slow rate of association between PPᵢ and the enzyme, or a rapid binding, followed by a slow isomerization step. The calculated equilibrium *K*₅ᵢ (*k*ᵦ/*k*ₐ) is 375 μM, which agrees well with the value obtained by equilibrium fluorescence titration shown in Figure 4.

Changes in the fluorescence of the pterin ring have been used to measure binding of 6-hydroxymethyl-7,8-dihydropterin to the
apparent rate constant ($k_{\text{app}}$) of 22.6 s$^{-1}$. The results are the means for four independent experiments. The lower panel shows the difference for binding of DHPPP to the enzyme, or afterwards in a slow isomerization step.

Figure 5, it was not possible to determine whether the change in pterin fluorescence occurs immediately upon DHPPP binding to the enzyme, or afterwards in a slow isomerization step.

The experiment was repeated for the Y63 and GS60 sulfonamide-resistant variants, and the results are summarized in Table 2. It is noteworthy that the calculated equilibrium binding constant for DHPPP is only about one order of magnitude lower, at 33 $\mu$M, than that for PP$i$ (350 $\mu$M). In thermodynamic terms, this means that addition of the pterin moiety to the PP$i$, ligand only contributes approx. 6 kJ/mol to the free energy for binding. This is a surprising observation, given that the pterin-binding pocket is apparently well adapted to recognition of the pterin ring. It is also clear that the sulfonamide-resistance mutations have little effect on the binding of DHPPP, suggesting that both sulfonamide-resistant mutants are also capable of binding to PP$i$, with the same affinity as the wild-type enzyme. The fluorescence from DHPPP in the enzyme:DHPPP binary complex was similar for the sulfonamide-sensitive and -resistant forms of the enzyme. The failure to observe any change in intrinsic enzyme fluorescence on binding of PP$i$, noted above, is therefore more likely to be due to a difference in the environment of Trp$^{95}$ in the Y63 and GS60 mutants in the PP$i$-bound state. Interestingly, determination of the crystal structure of the GS60 mutant apoenzyme, in the same crystal form as the sulfonamide-sensitive enzyme form, revealed no significant differences between the two structures (results not shown). Repetition of the stopped-flow experiment shown in Figure 6 using DHPP, the ligand identified in the crystal structure, showed no appreciable change in pterin fluorescence on rapid mixing with either the sulfonamide-sensitive or -resistant forms of the enzyme (results not shown). These observations lead us to conclude that it is primarily the binding of the PP$i$, moiety within the DHPPP substrate which induces a wide-scale rearrangement of the DHPS active site.

We have shown previously that there is no measurable affinity for pABA binding to the $S. pneumoniae$ DHPS apoenzyme and have suggested that the enzyme (E) adopts a compulsory substrate binding order, with DHPPP binding first, followed by pABA [22]:

$$E + \text{DHPPP} + \text{pABA} \leftrightarrow E + \text{DHPPP} + \text{pABA}$$

$$\leftrightarrow E + \text{DHPPP} \rightarrow \text{pABA}$$

It was also shown that pABA and sulfonamide drugs bind to the E-PP$i$-binary complex [22]. These observations suggest that binding of DHPPP or PP$i$, induces a structural change which forms the pABA-binding site. With this binding model in mind, we set out to use a fluorescence method to measure pABA binding to DHPS. The results of a titration of pABA into a solution of the sulfonamide-sensitive form of $S. pneumoniae$ DHPS and PP$i$, are shown in Figure 7(A). pABA is weakly fluorescent, with excitation and emission wavelength maxima of 265 nm and 340 nm respectively. In the absence of enzyme, titration with pABA produces a linear response in fluorescence. In the presence of enzyme, a quench phase in the titration profile is observed (Figure 7A). The data were fitted to a compulsory order binding model, where pABA is capable of binding only to the DHPS–PP$i$, binary complex:

$$E + \text{PP$i$} + \text{pABA} \leftrightarrow E + \text{PP$i$} + \text{pABA}$$

$$\leftrightarrow E + \text{PP$i$} \rightarrow \text{pABA}$$

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Rate and equilibrium constants for the binding of DHPPP to sulfonamide-sensitive and -resistant $S. pneumoniae$ DHPS</th>
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</thead>
<tbody>
<tr>
<td>$K_{\text{d}}$</td>
<td>$k_{\text{on}}$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)</td>
</tr>
<tr>
<td>Sulfonamide-sensitive</td>
<td>(2.6 ± 0.2) × 10$^5$</td>
</tr>
<tr>
<td>Y63</td>
<td>(2.4 ± 0.1) × 10$^5$</td>
</tr>
<tr>
<td>GS60</td>
<td>(2.0 ± 0.2) × 10$^5$</td>
</tr>
</tbody>
</table>

Dihydropteroate synthase from $S. pneumoniae$ 385

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change in fluorescence was monitored (Figure 7B). In the case of the sulfonamide-resistant enzymes, binding of PP, had little effect on the intrinsic fluorescence (Figure 4), hence the decrease in fluorescence in this experiment is attributable to pABA binding.

The experiment was carried out at different concentrations of pABA, and the results were fitted to the same compulsory order binding model given above. The fluorescence of the ternary complex was 10–20% of that for the apoenzyme and DHPS–PP, binary complexes. The $K_d$ for pABA binding to the Y63 variant was determined to 50 ± 6 μM (Figure 7B). Repetition of the same experiment with the GS60 mutant gave a $K_d$ of 16 ± 6 μM for pABA binding (results not shown). The results show that the $K_d$ for pABA binding to the resistant mutant enzymes is around two orders of magnitude higher relative to that for the sulfonamide-sensitive form of the enzyme. The experiment shown in Figure 7(B) was also adapted to measure binding of a sulfonamide drug, sulfamethoxazole, to the sulfonamide-sensitive form of DHPS; this gave a $K_d$ of 2.3 ± 0.1 μM. By contrast, repeating the same experiment with the Y63 and GS60 sulfonamide-resistant enzymes produced no detectable binding response. The impairment in binding affinity of pABA caused by the Y63 and GS60 mutations is therefore also reflected in the binding of a sulfonamide drug.

**DISCUSSION**

The present study has shown, through a combination of structural and ligand-binding studies, that the binding of DHPPP and pABA to DHPS can be considered as two discrete events and that sulfonamide-resistance mutations exclusively affect the second, pABA-binding, step. To date, enzymological studies of DHPS have largely been confined to steady-state kinetics [10,11], and our current knowledge of the DHPS catalytic cycle is rudimentary by comparison with the preceding enzyme in the folate pathway, HPPK [3,6,23,36]. Earlier studies on the effects of sulfonamide-resistance mutations have shown changes in steady-state kinetic parameters in the DHPS from Neisseria meningitidis [11]. $K_m$ values, however, consist of a composite of individual rate constants and are dependent on the kinetic model for the enzyme concerned. Changes in $K_m$, which occur as a result of sulfonamide-resistance mutations, are difficult to interpret in the absence of such a model. There are therefore obvious strengths in an approach which breaks down the reaction pathway into individual binding steps.

The structure of S. pneumoniae DHPS, reported in the present paper, shows a highly-conserved arrangement of the pterin-binding site; a pocket which appears well adapted for recognition of the pterin ring. In the light of this observation, the fact that the binding affinity for DHPPP is only about one order of magnitude higher than that for PP, is surprising. We also find that the on-rates for both DHPPP and PP, binding to the enzyme are relatively low. Given that binding of these ligands is necessary for pABA/sulfonamide recognition, we infer that association of DHPPP or PP, induces a significant structural change in the enzyme, probably involving several loop regions. This would explain the slow associated fluorescence changes which occur on the binding of DHPPP or PP, The large enhancement in the fluorescence of Trp$^{56}$ suggests that PP, binding induces a change in the environment of this residue. The Y63 and GS60 mutations, which are situated in loop 2 (L2 in Figure 3A), do not appear to affect DHPPP binding, but do alter the enhancement in Trp$^{56}$ fluorescence by PP, (Figure 4). Tyr$^{65}$ lies at the N-terminal end of α-helix 2 at the end of loop 2 and approx. 23 Å from the α-phosphate of DHPP; a large

**Figure 7** Equilibrium fluorescence titrations to determine pABA-binding affinity

(A) Titration of pABA into 500 μM PP, with various concentrations of sulfonamide-sensitive S. pneumoniae DHPS: squares, 50 μg/ml; diamonds, 100 μg/ml; triangles, 150 μg/ml. Data points are the means of four separate measurements. The data were fitted to a compulsory order binding model, as outlined in the text. The $K_d$ for pABA was determined to be 0.13 ± 0.02 μM (pABA), pABA concentration. (B) Titration of PP, into Y63 DHPS (150 μg/ml) in the presence of 40 μM (triangles) and 80 μM (squares) pABA. The $K_d$ for pABA was determined to be 50 ± 6 μM (pyrophosphate), PP, concentration.

fluorescence to PP, binding shown in Figure 4. The results of the data fitting established that the fluorescence of the E–PP,–pABA ternary complex was substantially quenched relative to the binary complex. The $K_d$ for pABA was determined to be 0.13 ± 0.02 μM. If the fluorescence of DHPS is arbitrarily set at 100%, the relative fluorescence of the binary complex is 173%, and that of the ternary complex (with pABA bound) is 96%. Stopped-flow experiments, in which pABA was rapidly mixed with the DHPS:PP, binary complex, indicated that pABA addition was too fast to be measured reliably (less than 10 ms; results not shown).

A repetition of the experiment shown in Figure 7(A) with the Y63 and GS60 sulfonamide-resistance mutants showed a much reduced response with increasing pABA concentration, suggesting that the pABA-binding affinity of these enzymes was weaker than the sulfonamide-sensitive form (results not shown). It was not possible to determine an accurate $K_d$ for pABA binding to the sulfonamide-resistance mutants using this approach, so an alternative method was devised: PP, was titrated into a solution containing pABA and DHPS, and the
conformational change would be required to place this residue close to the probable position of DHPPP. Interestingly, Trp93 lies roughly equidistant between Tyr40 and the DHPP ligand, and is partially exposed to solvent. A restructuring of loop 2 on binding of DHPPP or PPi, such that it packs against Trp93 and contributes to a binding site for pABA or sulfonamide, would be consistent with the results presented here. Presumably sulfonamide-resistance mutations would disrupt the conformation of loop 2, altering the interaction with Trp93 and changing its fluorescence properties. It is interesting to note that the structurally equivalent residue to Trp93 in E. coli DHPS, Ser48, packs against loop 2 in that structure.

We have also shown that the sulfonamide-resistance mutations have a drastic effect on pABA binding affinity for the DHPS–PPi binary complex, increasing the $K_d$ approx. 100-fold. The simplest explanation for this observation is that the structure of loop 2 in the DHPS–PPi binary complex is altered by the Y63 or GS60 mutations in such a way as to impair pABA or sulfonamide recognition. In our analysis of the effects of sulfonamide-resistance mutations on the $K_d$ for pABA binding to DHPS–PPi, we are making the tacit assumption that the same differences would also be reflected in binding to the DHPS–DHPPP complex. We regard this as a reasonable assumption for the following reasons: we have shown previously that differences in $K_d$ values for sulfonamides binding to DHPS–PPi are reflected in $K_d$ values determined from steady-state kinetics [22]. In addition, our observations that PP, and DHPPP binding induce pABA/sulfonamide binding and that both ligands bind with similar affinities to the apoenzyme support this proposition.

Despite its established importance as a drug target, the mechanism of DHPS remains poorly understood. Although crystal structures of the enzyme have been reported from a variety of organisms, it is not always clear how each structure is related to the reaction pathway. Both of the crystal structures reported in the present paper, of the apoenzyme and DHPP complex, seem to relate to a form of the enzyme at the beginning of the reaction cycle. We were unable to obtain structures of complexes with DHPPP, or ternary complexes with PP and pABA. Nevertheless, the results provide evidence for a subtle and dynamic response of the loop regions around the DHPPP-binding site, perhaps explaining why intermediate states in the reaction cycle are difficult to crystallize. The precise structure of the pABA-binding site remains poorly defined in DHPS from any organism. Our results have shown that the origin of an explanation for the molecular basis for sulfonamide resistance probably lies in changes to the behaviour of the loop regions surrounding the active site. Further work will require characterization of these changes in more detail.

We thank the Wellcome Trust for grant funding to support this work.

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26. The Authors Journal compilation © 2008 Biochemical Society

Received 26 November 2007/22 February 2008; accepted 5 March 2008
Published as BJ Immediate Publication 5 March 2008, doi:10.1042/BJ20071598