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Binding Isotope Effects for *para*-Aminobenzoic Acid with Dihydropteroate Synthase from *Staphylococcus aureus* and *Plasmodium falciparum*

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Abstract

Dihydropteroate synthase is a key enzyme in folate biosynthesis and is the target of the sulfonamide class of antimicrobials. Equilibrium binding isotope effects and density functional theory calculations indicate that the substrate binding sites for *para*-aminobenzoic acid on the dihydropteroate synthase enzymes from *Staphylococcus aureus* and *Plasmodium falciparum* present distinct chemical environments. Specifically, we show that *para*-aminobenzoic acid occupies a more sterically constrained vibrational environment when bound to dihydropteroate synthase from *P. falciparum* relative to that of *S. aureus*. Deletion of a nonhomologous, parasite-specific insert from the plasmodial dihydropteroate synthase abrogated the binding of *para*-aminobenzoic acid. The loop specific to *P. falciparum* is important for effective substrate binding and therefore plays a role in modulating the chemical environment at the substrate binding site.

Graphical abstract

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ASSOCIATED CONTENT

Supporting Information

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Supporting Information Figure S1 and Supporting Information Tables S1–S3. Complete experimental procedures for expression and purification of wild-type SaDHPS, wild-type PfHPPK–DHPS, and 628–668PfHPPK–DHPS; measurement of equilibrium BIEs; measurement of V/K KIEs; and all DFT calculations (PDF)

Notes

The authors declare no competing financial interest.

Dihydropteroate synthase (DHPS) is a critical enzyme in the *de novo* folate biosynthetic pathway found in many microorganisms and plants, but not in humans.¹ DHPS catalyzes the formation of dihydropteroate (DHP) through the condensation of 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (DHPP) with *para*-aminobenzoic acid (*p*ABA; Figure 1a). The sulfonamide class of antimicrobials, which function as *p*ABA analogs, are competitive inhibitors of DHPS and act to disrupt folate biosynthesis.¹ Though the efficacy of sulfonamides has been hampered by the development of resistance,^{2,3} therapies combining sulfonamides with the dihydrofolate reductase (DHFR) inhibitors pyrimethamine or trimethoprim are still widely used in the prophylaxis and treatment of malaria.⁴ In addition, recent studies suggest therapies targeting DHPS may be effective alternatives for the treatment of bacterial infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA).^{5,6}

As in most bacteria, DHPS in *S. aureus* (SaDHPS) is expressed as a monofunctional enzyme.⁷ By contrast, DHPS in many lower eukaryotes is often expressed as a domain of a multifunctional polypeptide.^{8–11} For example, DHPS from the malarial parasite *Plasmodium falciparum* (PfDHPS) is expressed as a bifunctional protein with 7,8-dihydro-6-hydroxymethylpterin pyrophosphokinase (PfHPPK), the enzyme preceding DHPS in folate biosynthesis.^{12,13} Due to the lack of crystallographic data for PfHPPK–DHPS, there is currently limited information on the association of the PfHPPK and PfDHPS domains and how this association may impact protein–substrate interactions relative to monofunctional DHPS variants. In the current study, we use binding isotope effects (BIEs) and computational modeling to investigate differences in the *p*ABA binding sites of monofunctional SaDHPS and bifunctional PfHPPK–DHPS.

Equilibrium BIEs report on differences in the bonding environment for enzyme substrates in the free and bound states, preceding catalysis.¹⁴ When measured by the competition of labeled and unlabeled substrates, BIEs manifest due to preferential binding of either the light or heavy isotope. Normal BIEs (>1.0) arise when binding of the light isotope is preferred, which occurs when the bonding environment of the isotopic substitution is weaker in the bound state relative to the free state. By contrast, inverse BIEs (<1.0) arise when binding of the heavy isotope is preferred, which occurs when the bonding environment in the bound state is tighter than the solution state.

Tritium BIEs for [3,5-³H]*p*ABA with SaDHPS and PfHPPK–DHPS were measured via internal competition with [7-¹⁴C]*p*ABA, where the remote ¹⁴C label was used to trace light isotopes at the C3 and C5 positions (Figure 1b). BIE values were obtained for the ternary complex of DHPS with [3,5-³H]*p*ABA and 6-hydroxymethylpterin pyrophosphate (HMPP), a nonreactive substrate analog of the natural DHPP substrate. All BIE measurements were performed using a rapid equilibrium dialysis device (Thermo Fisher Scientific) in an analogous approach to the ultrafiltration method¹⁵ previously used by our lab to measure BIEs for purine nucleoside phosphorylase,¹⁶ uridine phosphorylase,¹⁷ thymidine phosphorylase,¹⁸ and hexokinase.^{19,20}

An equilibrium BIE of 1.014 ± 0.003 (1.4% *normal*) was obtained for [3,5-³H]*p*ABA with SaDHPS (Table 1). The small normal BIE value indicates minimal perturbation of the

vibrational force constants for the C3–H3/C5–H5 bonds in the Michaelis complex. By contrast, the BIE measured for [3,5-³H]*p*ABA with PfHPPK–DHPS was 0.828 ± 0.006 (17.2% *inverse*; Table 1). The large magnitude of this BIE illustrates a significant change in vibrational environment of the isotopically substituted positions upon enzyme binding. The inverse BIE indicates the bonding environment for *p*ABA is tighter in the bound state relative to the free state. Moreover, as compared to the 1.4% *normal* BIE observed for [3,5-³H]*p*ABA with SaDHPS, the 17.2% *inverse* BIE obtained with PfHPPK–DHPS indicates the *p*ABA binding site of the PfDHPS domain presents a more constrained environment.

Interestingly, formation of the SaDHPS-*p*ABA and PfHPPK–DHPS-*p*ABA binary complexes was not observed under the conditions used to measure BIEs on the ternary complexes with HMPP. We conducted additional binding assays to investigate these results and found that *apo*-SaDHPS and *apo*-PfHPPK–DHPS displayed no binding of [7-¹⁴C]*p*ABA (at concentrations up to 100 μ M; Supporting Information Figure S1). Although previous studies on the steady state kinetics of SaDHPS suggest a random order binding mechanism for DHPP and *p*ABA,⁷ our data suggest that both SaDHPS and PfHPPK–DHPS require the binding of DHPP prior to *p*ABA. These results agree with equilibrium binding studies on the DHPS from *Streptococcus pneumoniae* (SpDHPS) that show the binding mechanism for SpDHPS occurs through a strictly ordered mechanism, with DHPP binding to the apoprotein first.²¹

An important consideration for BIE measurements is that protein–ligand interactions occur at a single binding site. The binding of [3,5-³H]*p*ABA at multiple sites on DHPS has the potential to convolute BIE measurements by making the observed values a combination of multiple effects. Observed *V*/*K* kinetic isotope effects (KIEs) report on all steps from substrate binding, up to and including the first irreversible chemical step.²² As isotopic substitutions more than two bonds from the reactive center are generally assumed to have negligible influence on intrinsic KIEs,¹⁴ [3,5-³H]*p*ABA would not be predicted to display a KIE from effects on catalysis and should express the value of the equilibrium BIE. The *V*/*K* KIEs measured for [3,5-³H]*p*ABA with SaDHPS and PfHPPK–DHPS were 1.012 ± 0.002 (1.2% *normal*) and 0.873 ± 0.005 (12.7% *inverse*), respectively (Table 1). These values are similar in type and magnitude to the measured BIE values and support that the BIEs report on the binding of [3,5-³H]*p*ABA at the DHPS active site.

To investigate the pronounced difference in experimental BIEs for [3,5-³H]*p*ABA with SaDHPS and PfHPPK–DHPS, density functional theory (DFT) calculations were used to model environmental and geometric changes experienced by *p*ABA upon enzyme binding. The factors considered in these calculations were (1) changes in dielectric environment, (2) out-of-plane geometric distortion of the C3–H3/C5–H5 bonds, and (3) restriction of the stretching vibrational modes for the C3–H3/C5–H5 bonds. Equilibrium BIEs were calculated (Gaussian 09, RB3LYP/6-31g(d) theory)²³ from the scaled vibrational frequencies of energy-minimized structures of *p*ABA in the “free” and “bound” states. The optimized structure of *p*ABA in the free state was generated using water as an implicit solvent model and was identical for all BIE calculations. Bound states of *p*ABA were

generated by optimizing structures using the solvent models and/or geometric constraints outlined below.

The influence of the dielectric environment was examined by calculating BIEs for [3,5-³H]*p*ABA using bound states with the implicit solvent models (polarizable continuum model, PCM) shown in Figure 2a. These calculations returned normal BIE values (>1.0) that increased in magnitude as the dielectric constant of the solvent in the bound state decreased. The experimental BIE of 1.014 (1.4% *normal*) for [3,5-³H]*p*ABA with SaDHPS could be matched using a solvent model with a dielectric constant between dichloroethane ($\epsilon = 10.1$) and acetone ($\epsilon = 20.5$) in the bound state (Figure 2a and Supporting Information Table S1). These models suggest that changes in dielectric environment upon ligand binding may be sufficient to explain the BIEs observed for [3,5-³H]*p*ABA with SaDHPS.

The effect of ligand distortion on equilibrium BIEs was investigated by imposing geometric constraints on *p*ABA in the bound state. Out-of-plane distortion of the C3–H3 and C5–H5 bonds (with respect to the benzene ring) was modeled by freezing the H3–C3–C4–C5 and H5–C5–C4–C3 dihedral angles (respectively) prior to energy optimization (Figure 2b). Specifically, the H3–C3–C4–C5 and H5–C5–C4–C3 dihedral angles were symmetrically fixed at 1° to 10° (in 1° increments) beyond the equilibrium angle (Supporting Information Table S2). Each structure was then optimized using acetone as an implicit solvent, and BIEs were calculated from the vibrational frequencies relative to the free state of *p*ABA in water. These calculations indicate that out-of-plane restriction of the C3–H3/C5–H5 bonds results in normal BIE values (>1.0) for [3,5-³H]*p*ABA that increase in magnitude with the degree of geometric distortion (Figure 2b). A restriction of approximately 6.5% beyond the equilibrium dihedral angle is predicted to account for the 1.014 (1.4% *normal*) BIE measured for [3,5-³H]*p*ABA with SaDHPS. These models suggest that out-of-plane geometric distortion of the C3–H3/C5–H5 bonds is also consistent with the experimental BIE value measured for [3,5-³H]*p*ABA with SaDHPS.

Importantly, our models suggest that changes in dielectric environment and out-of-plane distortion of the C3–H3/C5–H5 bonds (Figure 2a,b) are inconsistent with the large inverse BIE measured for [3,5-³H]*p*ABA with PfHPPK–DHPS (Table 1). A significant factor contributing to inverse tritium BIEs is steric imposition.²⁰ In constrained bonding environments, steric impingement of approaching groups can cause restriction in the stretching vibrational modes of C–H bonds, thus increasing the vibrational force constant and bond order. As there are currently no crystallographic data for PfHPPK–DHPS from which to identify specific protein–ligand interactions, we modeled a generic representation of steric impingement on *p*ABA in the bound state by restricting the C3–H3/C5–H5 bond lengths prior to optimization. The calculated BIEs indicate that an approximate 5% symmetrical compression of the C3–H3/C5–H5 bonds (relative to equilibrium bond lengths) could account for the BIE of 0.828 ± 0.006 (17.2% *inverse*) measured for [3,5-³H]*p*ABA with PfHPPK–DHPS (Figure 2c and Supporting Information Table S3). These results are consistent with previous studies on the binding of glucose to brain hexokinase that suggest inverse tritium BIEs can originate from steric impingement of protein residues on the C–H bonds of bound substrates.²⁰

The equilibrium BIE measurements presented herein suggest that *p*ABA occupies distinct chemical environments when bound to SaDHPS and PfHPPK–DHPS. These data are particularly interesting as SaDHPS and the PfDHPS domain share high sequence identity for residues predicted by homology to have substrate interactions (Figure 3).^{7,24,25} In addition, the substrate binding domains of DHPS variants are conserved in a classic (β/α)⁸ TIM barrel fold. However, SaDHPS is a monofunctional enzyme that exists as a dimer in solution,⁷ whereas the bifunctional PfHPPK–DHPS has been shown by gel filtration experiments to be a multimer with up to three subunits.¹³ Although the quaternary structure of subunit association for PfHPPK–DHPS is unknown, domain–domain interactions could provide a basis for differences in the SaDHPS and PfDHPS substrate binding sites.

Similar to other enzymes from *P. falciparum*,^{26–29} PfHPPK–DHPS contains several nonhomologous inserts in both the PfHPPK and PfDHPS domains.²⁴ Importantly, recent work has shown deletion of two nonhomologous loops in the PfHPPK domain affects both PfHPPK and PfDHPS activities.²⁴ These and other studies²⁹ on enzymes from *P. falciparum* suggest that plasmodium-specific inserts mediate domain–domain interactions necessary for catalytic activity. The largest insert in the PfDHPS domain is located between residues 628 and 668 of the PfHPPK–DHPS polypeptide (Figure 3). Though its function is unknown, several mutations associated with resistance to sulfonamides, which act as *p*ABA analogs, have been identified in this loop.³⁰

To investigate the influence of residues 628–668 on *p*ABA binding, we expressed a deletion mutant of PfHPPK–DHPS in which this loop was removed (–628–668 PfHPPK–DHPS). Interestingly, –628–668 PfHPPK–DHPS displayed no binding of *p*ABA in either binary or ternary (with HMPP) complexes under conditions used for the equilibrium BIE measurements with the wild-type protein. As such, residues 628–668 of PfHPPK–DHPS appear important for substrate binding and may directly or indirectly contribute to the constrained bonding environment for *p*ABA reported on by the equilibrium BIEs.

In conclusion, we report evidence from equilibrium BIEs to indicate that the *p*ABA binding site of PfHPPK–DHPS presents a more constrained bonding environment relative to that of SaDHPS. DFT calculations indicate that the small, *normal* BIE (1.4%) observed for [3,5-³H]*p*ABA with SaDHPS is consistent with changes in dielectric environment and/or out-of-plane geometric distortion of the C3–H3/C5–H5 bonds upon enzyme binding. By contrast, we found that the large *inverse* BIE (17.2%) measured for [3,5-³H]*p*ABA with PfHPPK–DHPS is consistent with DFT calculations modeling steric impingement on the C3–H3/C5–H5 bonds in the bound state. Deletion of a nonhomologous insert (residues 628–668) from PfHPPK–DHPS abrogated the binding of *p*ABA, indicating that this loop is important for effective substrate binding and may play a role in modulating the chemical environment at the *p*ABA binding site. Though a definitive understanding of the differences in *p*ABA binding sites on SaDHPS and PfHPPK–DHPS awaits crystallographic data, this study highlights the utility of equilibrium BIEs in assessing the chemical environment of protein-bound ligands.

METHODS

Full details for all experimental methods are provided in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The DHPS reaction and isotopically labeled *p*ABA substrates used in the BIE analysis. (a) DHPS catalyzes the formation of DHP via condensation of DHPP with *p*ABA. (b) BIE measurements were performed using [3,5-³H]*p*ABA and [7-¹⁴C]*p*ABA. DHPP, 6-hydroxymethyl-7,8-dihydropterin pyrophosphate; DHPS, dihydropteroate synthase; *p*ABA, *para*-aminobenzoic acid; PP_i, pyrophosphate; DHP, dihydropteroate.

Figure 2.

Calculated effects of changes in dielectric environment and geometric distortion on BIEs for [3,5-³H]pABA. (a) Equilibrium BIEs were calculated for pABA using water ($\epsilon = 78.4$), methanol ($\epsilon = 32.6$), acetone ($\epsilon = 20.5$), dichloroethane ($\epsilon = 10.1$), chlorobenzene ($\epsilon = 5.7$), or carbon tetrachloride ($\epsilon = 2.2$) as an implicit solvent model (PCM) in the bound state. The red line illustrates the experimental BIE value measured for SaDHPS. (b) Calculated effects of out-of-plane distortions of the C3–H3 and C5–H5 bonds. Restraints were imposed on the bound state by fixing the H3–C3–C4–C5 and H5–C5–C4–C3 dihedrals at increasing degrees from the equilibrium angle. The red line illustrates the experimental BIE value measured for SaDHPS. (c) Calculated effects of C3–H3 and C5–H5 bond length compression. The red line illustrates the experimental BIE value measured for PfdHPS.

Figure 3. Comparison of primary structures for the PfDHPS domain and SaDHPS. Sequences of the full length PfHPPK–DHPS and SaDHPS proteins were compared, and the resulting alignment was truncated to show only the DHPS domains. Strictly conserved residues are shown in blue. Red circles identify residues predicted to interact with substrates by homology to other DHPS variants.²⁵ Nonhomologous, plasmodium-specific inserts are shown in green. The insert deleted from PfHPPK–DHPS in this study extends between residues 628 and 668. The sequence alignment was performed using COBALT.³¹

Table 1Binding and V/K Kinetic Isotope Effects for SaDHPS and PfDHPS^a

enzyme	BIE	V/K KIE
SaDHPS	1.014 ± 0.003 (6)	1.012 ± 0.002 (5)
PfHPPK–DHPS	0.828 ± 0.006 (6)	0.873 ± 0.005 (8)

^aBIE measurements for [3,5-³H]pABA with SaDHPS and PfHPPK–DHPS were made using the DHPS-pABA-HMPP ternary complex. BIE and V/K KIE values are presented with $\pm$ standard error. The number of replicates is presented in parentheses. BIE, binding isotope effect; KIE, kinetic isotope effect.