Direct evidence that an extended hydrogen-bonding network influences activation of pyridoxal 5′-phosphate in aspartate aminotransferase

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Pyridoxal 5′-phosphate (PLP) is a fundamental, multifunctional enzyme cofactor used to catalyze a wide variety of chemical reactions involved in amino acid metabolism. PLP-dependent enzymes optimize specific chemical reactions by modulating the electronic states of PLP through distinct active site environments. In aspartate aminotransferase (AAT), an extended hydrogen bond network is coupled to the pyridinyl nitrogen of the PLP, influencing the electrophilicity of the cofactor. This network, which involves residues Asp-222, His-143, Thr-139, His-189, and structural waters, is located at the edge of PLP opposite the reactive Schiff base. We demonstrate that this hydrogen bond network directly influences the protonation state of the pyridine nitrogen of PLP, which affects the rates of catalysis. We analyzed perturbations caused by single- and double-mutant variants using steady-state kinetics, high resolution X-ray crystallography, and quantum chemical calculations. Protonation of the pyridinyl nitrogen to form a pyridinium cation induces electronic delocalization in the PLP, which correlates with the enhancement in catalytic rate in AAT. Thus, PLP activation is controlled by the proximity of the pyridinyl nitrogen to the hydrogen bond microenvironment. Quantum chemical calculations indicate that Asp-222, which is directly coupled to the pyridinyl nitrogen, increases the pKa of the pyridine nitrogen and stabilizes the pyridinium cation. His-143 and His-189 also increase the pKa of the pyridine nitrogen but, more significantly, influence the position of the proton that resides between Asp-222 and the pyridinyl nitrogen. These findings indicate that the second shell residues directly enhance the rate of catalysis in AAT.

Pyridoxal 5′-phosphate (PLP) is one of the most versatile cofactors used in nature (1). PLP-dependent enzymes catalyze diverse reactions, which include transamination, racemization, α-decarboxylation, retro-aldo cleavage, and β- and γ-elimination (1-3). In PLP-dependent enzymes, the transition from the internal aldime, where PLP is covalently bound to a conserved active site Lys residue (Fig. 1A), to the external aldime, where PLP is covalently bound to a substrate, proceeds through a common mechanism that involves a gem-diamine intermediate (4). After the formation of the external aldime, the reaction path differs among each family of PLP-dependent enzymes (2, 3). The interplay between the PLP and the active site environment optimizes the preferred reaction and limits other possible reactions (2). PLP-dependent catalysis has been considered one of the better-understood enzymatic mechanisms. However, the reasons for the diversity of the reaction paths and cofactor activation among different classes of PLP-dependent enzymes remain ambiguous.

The internal and external aldimes contain four ionizable sites: the phenolic oxygen (O3′), phosphate group (PG), pyridine nitrogen (PLP-N1), and the Schiff base (N2) (Fig. 1A). Outside the enzyme active site, in the free form and at physiological pH, PLP is protonated at the O3′ position and deprotonated at the PLP-N1 position. The respective pKa values are ~9 for the O3′ oxygen and ~5.8 for the PLP-N1 nitrogen (5, 6). The respective pKa values of the Schiff base N2 are ~7.0 in the internal aldime and ~10.0 in the external aldime (5). The protonation states of the ionizable groups of PLP play an important role in catalysis. It is well known that in enzymes the local environment of the active site may shift the pKa values of ionizable groups of the substrate(s), active site residues, and cofactors to enhance catalysis (7, 8). It was suggested previously that for different fold-types of PLP-dependent enzymes, PLP...
exhibits different protonation profiles, which influence the catalyzed reaction paths (2).

The homodimeric aspartate aminotransferase (AAT) is a PLP-dependent fold-type I enzyme involved in transamination reactions (9, 10). Each monomer includes a large domain and a small domain, with the active site located at the interface of these two domains (Fig. 1B) (9). The complete catalytic cycle of AAT involves the reversible conversion of L-Asp and α-ketoglutarate to oxaloacetate and L-Glu via a ping-pong bi-bi mechanism (Fig. 1C). In the first half-reaction, PLP of the internal aldimine assists in the conversion of L-Asp to oxaloacetate to generate pyridoxamine 5-phosphate. For the second half-reaction, pyridoxamine 5-phosphate promotes the conversion of α-ketoglutarate to L-Glu, in which the internal aldimine is regenerated. It has been proposed that the Lys residue, freed from the internal aldimine, is involved in the deprotonation of the Cα of L-Asp when the external aldimine is formed (Fig. 2). In *Escherichia coli* AAT, ketimine hydrolysis and Cα deprotonation are partially rate-determining (10, 11). Specifically, the Cα deprotonation is promoted by PLP resonances that stabilize the carbanionic intermediate that is formed (Fig. 2). In AAT, a conserved Asp residue, Asp-222, is hydrogen bonded to the PLP-N1 (10). NMR studies revealed that the PLP-N1 is protonated in wild-type AAT and other model PLP derivatives (6, 14–17). The conserved Asp residue increases the pKₐ of PLP-N1 and stabilizes the pyridinium cation (13, 15). This acidic residue is thought to have a significantly lowered pKₐ to increase the pKₐ of PLP-N1 substantially, from ~5.8 in solution to well above 7.5 in the enzyme. When the PLP-N1 is deprotonated, delocalization of the carbanion charge is negligible due to the destabilization of the long-lived quinonoid resonance form. Formation of a long-lived quinonoid intermediate enhances the rate of catalysis of fold-type I PLP-dependent enzymes (12). Therefore, the protonation state of PLP-N1 is critical for PLP-dependent enzymes that require a longed-lived carbanion (quinonoid) intermediate.

Recent studies performed by Toney and co-workers (18, 19) have shown that reconstituting *E. coli* aspartate aminotransferase with 1-deazapyridoxal-5-phosphate (deaza-PLP), a modified PLP cofactor in which N1 is substituted with carbon, abrogates AAT catalytic activity (>10⁹-fold decrease). Alanine racemase (fold-type III) and O-acetylserine sulfhydrylase (fold-type II), the enzymes with reaction mechanisms that do not involve stabilized carbanion intermediates, reconstituted with the deaza-PLP analog remain active but exhibit activities decreased by 100- and 250-fold, respectively (18, 19). Conserved Arg in alanine racemase and Ser in O-acetylserine sulfhydrylase occupy the position of AAT’s Asp-222 and have significantly higher pKₐ values than aspartate, suggesting that the PLP-N1 is not protonated in these enzymes. In fact, NMR studies showed the PLP-N1 in alanine racemase to be deprotonated (17). These findings provide evidence that an enhanced electron sink effect is catalytically important for fold-type I PLP-dependent enzymes, whereas in fold-type II and III PLP-dependent enzymes this effect is not vital for catalysis (12).

Here, we used site-directed mutagenesis to perturb the extended hydrogen bond network involving PLP-N1 in recombinant *Sus scrofa* (porcine) AAT (Fig. 3). To gain further insight into the local environment effects on PLP-N1 protonation, four mutants were produced as follows: single mutants D222T and H143L, and double mutants H143L/H189L and H143F/H189L.

![Figure 1](attachment:image.png)
High resolution X-ray crystal structures were obtained for the wild-type AAT (AATWT) and three of the four variants (excluding H143F/H189L). The structure of porcine cytosolic AAT was originally determined with the enzyme isolated from pig hearts (9), and the recombinant enzyme has the same crystal form (20). Crystal contacts restrain the small domain of one monomer in the open state (chain A), whereas the small domain in the second monomer is free to open and close (chain B). Despite substituting key residues to disrupt hydrogen bonds to PLP-N1, the structures show that hydrogen-bonding networks are still present but differ between chains and mutant variants. Structural water rearrangements reestablish the network in the D222T mutant in chain B but not in chain A and in the H143L mutant in chain A but not in chain B. Steady-state kinetic analyses of the studied variants show a reduction of catalytic activity with varying effects on $K_m$ and $k_{cat}$. Additionally, quantum chemical cluster calculations with density functional theory (DFT) support the hypothesis that, in addition to Asp-222, the local environment produced by His-143, Thr-139, and His-189 increases the $pK_a$ of the pyridinyl nitrogen, stabilizing the pyridinium form of PLP. The above findings provide evidence that the microenvironment near PLP-N1 controls the acidity of Asp-222 and, in turn, indirectly influences the $pK_a$ of PLP-N1. This study provides insight into how the unique microenvironment around the AAT PLP-N1 regulates PLP activation and ultimately tunes enzymatic activity.

Evidence of second shell residues influencing enzyme activity

The X-ray crystal structures of recombinant AATWT and mutant variants D222T, H143L, and H143L/H189L were solved to 1.2–1.4 Å resolutions. Table 1 contains the data collection and refinement statistics for the four structures. Crystals of H143F/H189L were not suitable for data collection. For the enzyme obtained from pig hearts (9) and for the recombinant enzyme (21), thin plate crystals with an orthorhombic setting were coaxed into forming large crystals by macroseeding (20). For the recombinant enzymes reported here, large hexagonal rod-shaped crystals formed readily under the same crystallization conditions. Interestingly, both forms have the same crystal setting despite the different crystal morphology. In the original work on this enzyme, it was noted that crystal contacts locked the small domain of one monomer in the open position (chain A), whereas the second monomer (chain B) was free to switch from open to closed (9). Crystals soaked with the inhibitor 2-methylaspartate showed that chain A did not bind the ligand, whereas chain B reacted to form the external aldimine (9). As expected, each AAT variant structure contained different conformations of the active site residues near the PLP-N1 in chain A compared with chain B (Fig. 4). The small domain of chain A has a nearby crystal contact (~4–6 Å), whereas in chain B the crystal contact of the small domain is notably farther away (~8–10 Å) (supplemental Fig. S1). The small domain in chain B is more dynamic than the respective domain in chain A, possibly due to the lack of close crystal contacts (Fig. 5). The average B-factors for the small domain in chains A and B are ~13 and ~33 Å², respectively (Fig. 5). No differences were observed between the A and B chain active site residues in AATWT (Figs. 3 and 4, A and B). A hydrogen bond network, present in both monomers, connects the PLP-N1 of the internal aldimine to the bulk solvent. All donor-to-acceptor hydrogen bond distances are in the range of 2.7–2.9 Å with the following linkage: PLP-N1 to Asp-222(O62), Asp-222(O62) to His-143(Ne2), His-143(Nδ1) to Thr-139(Oγ1), Thr-139(Oγ1) to His-189(Nε2), and His-189(Nδ1) to a structural water. The water connected to His-189 is the first of a chain of water molecules leading out to the protein surface (Fig. 3). This extended hydrogen bond network near the active site contributes to the interface stabilization between the large and small domains.

In AATWT, His-143(Ne2) is coupled to PLP-N1 (N–N distance = 4.4 Å) through Asp-222(O62), and the indole ring of Trp-140 and pyridine of PLP are nearly co-planar. The X-ray crystal structure of D222T maintains an extended hydrogen bond network to PLP-N1. Hydrogen bonds connecting His-
143, Thr-139, and His-189 to the surface are preserved, but the active site rearrangements at the site of mutation vary between the two chains (Fig. 4, C and D). In the locked-open chain A, Thr-222 is unable to make a hydrogen bond with PLP-N1. Instead, the PLP and His-143 shift toward each other, and a direct hydrogen bond forms between PLP-N1 and His-143 N/H. The indole ring of Trp-140 tilts away from the pyridine with respect to AATWT, diminishing the - stacking interactions while avoiding unfavorable contacts (Fig. 4C). In the less restricted chain B, a water molecule is inserted between PLP-N1 and the side chains of Thr-222 and His-143, replacing the connection mediated by Asp-222. This structural water displays three short hydrogen bonds (2.5 to 2.6 Å) to Thr-222(O), PLP-N1, and His-143(N). The - interactions between Trp-140 and the pyridine ring of PLP appear unaltered compared with AATWT.

### Table 1

X-ray data collection and refinement statistics

|                | AAT<sub>WT</sub>, 5TOQ | AAT, D222T, 5TOR | AAT, H143L, 5TON | AAT, H143L/H189L, 5TOT |
|----------------|------------------------|------------------|------------------|------------------------|
| Data collection|                        |                  |                  |                        |
| Wavelength (Å) | 0.97872                | 0.97856          | 0.97872          | 0.9786                 |
| Resolution range (Å) | 30.15–1.192 (1.235–1.192) | 27.98–1.35 (1.398–1.35) | 55.34–1.4 (1.45–1.4) | 31.02–1.4 (1.45–1.4) |
| Space group    | P2<sub>1</sub>, γ 2     | P2<sub>1</sub>, γ 2 | P2<sub>1</sub>, γ 2 | P2<sub>1</sub>, γ 2 |
| Total reflections | 1,374,113              | 1,599,763        | 1,428,334        | 1,076,619              |
| Unique reflections | 279,978 (27,884)      | 193,193 (18,798) | 174,668 (17,320) | 174,786 (173,349)     |
| Multiplicity   | 4.9(4.6)               | 8.3(8.1)         | 8.1(7.8)         | 6.2(6.1)               |
| Completeness (%) | 99.1(99.9)             | 98.03(96.21)     | 99.95(100.00)    | 99.77(100.00)         |
| Mean I/σ(I)    | 12.0(4.5)              | 14.64(4.54)      | 16.55(4.70)      | 10.65(3.40)            |
| Wilson B-factor | 0.070(0.394)           | 0.065(0.403)     | 0.077(0.386)     |                        |
| R-merge        | 0.1574 (0.1722)        | 0.1753 (0.2047)  | 0.1844 (0.2018)  | 0.1829 (0.2203)        |
| R-free         | 0.1739 (0.1911)        | 0.1928 (0.2242)  | 0.1973 (0.2229)  | 0.2056 (0.2436)        |
| No. of non-hydrogen atoms | 7399                   | 7436             | 6991             | 7233                   |
| Macromolecules | 6530                   | 6608             | 6517             | 6471                   |
| Water          | 806                    | 827              | 827              | 756                    |
| RMS (bonds)    | 0.005                  | 0.006            | 0.007            | 0.006                  |
| RMS (angles)   | 0.97                    | 0.89             | 1.07             | 0.86                   |
| Ramachandran favored (%) | 97                  | 98               | 98               | 98                     |
| Ramachandran allowed (%) | 2.5              | 2.2              | 2                | 1.7                    |
| Ramachandran outliers (%) | 0.61             | 0                | 0                | 0.12                   |
| Clashscore     | 5.57                    | 2.59             | 2.48             | 4.51                   |
| Average B-factor | 18.62                | 17.64            | 16.36            | 18.27                  |
| Macromolecules | 17.21                  | 16.72            | 16.08            | 17.46                  |
| Solvent        | 29.17                  | 25.00            | 20.22            | 25.21                  |

Figure 4. Atomic coordinates of active site residues in the microenvironment near PLP-N1. A, AAT<sub>WT</sub> chain A; B, AAT<sub>WT</sub> chain B; C, D222T chain A; D, D222T chain B; E, H143L chain A; F, H143L/H189L chain A; G, H143L/H189L chain B; C, E, and G, the black carbon scheme shows resolved alternate conformations of the active site residues in each respective X-ray structure. The blue mesh is the omitted Fo–Fc electron density contoured at 3σ for the newly observed water molecules in D and E.
Evidence of second shell residues influencing enzyme activity

H143L introduced a disruption in the connection between PLP-N1 and His-189. In chain B, it is decoupled, as anticipated, but rearrangements reconnect the network in chain A (Fig. 4, E and F). Similar to D222T chain B, a structural water molecule in H143L chain A occupies the position between His-189 and Asp-222, with His-189 displaying dual conformations. In one conformation, a hydrogen bond exists between Thr-139(Oγ1) and His-189(Nε2) (N...O 3.0 Å), which is equivalent to AATWT. In the second conformation, a new water molecule (modeled at half-occupancy) links His-189 directly to Asp-222, bypassing Thr-139 (Fig. 4E). H143L chain B has no structural water. Here, Asp-222 participates only in a hydrogen bond with PLP-N1 (N...O 2.7 Å), and Thr-139 is hydrogen bonded with His-189, but no bridge between the two residues was found (Fig. 2F). Therefore, the single mutant H143L did not fully disrupt the extended hydrogen bond network.

Substituting both His residues at positions 143 and 189 in the H143L/H189L variant abolishes the network in both chains by isolating Asp-222, and there is no evidence of new structural waters (Fig. 4, G and H). The only hydrogen bond that remains is between the Asp-222(Oε2) and PLP-N1 (N...O 2.7 Å). Only minor structural differences between chains A and B were observed with Asp-222 in chain A displaying minor dual conformations (Fig. 4G) and Asp-222 in chain B showing a single conformation (Fig. 4H).

Kinetic analysis

In a coupled assay, oxaloacetate produced by AAT is reduced by malate dehydrogenase, and NADH oxidation is monitored (21, 23). Native enzyme from porcine heart muscle was used to calibrate the assay, and the Vmax values determined for the native and recombinant wild-type AAT were indistinguishable. The values for kcat and Km for the aspartate to glutamate transamination reaction were measured for the recombinant wild-type enzyme and for the site-directed variants (Table 2). For recombinant AATWT, we obtained kcat = 46.7 ± 3.1 s⁻¹, Km = 384 ± 76 μM, and kcat/Km = 0.12 s⁻¹ μM⁻¹, comparable with previously published values (21), with differences likely due to modest variations in assay conditions. The D222T mutation caused a serious reduction in kcat of ~99%, but l-Asp substrate affinity improved, with Kn for the single mutant H143L and no major change in Km with respect to the wild-type enzyme. Early work by Yano et al. (24) showed that in E. coli AAT an H143N point mutation caused a 60% decrease in the maximum rate, but no structural analysis of the H143N mutant was conducted. The double mutant H143L/H189L, a modification that appears to abolish the hydrogen bond network, exhibited a slightly larger reduction of ~74% in kcat than the single mutant H143L and no significant change in Km values (Table 2). This slight decrease in the kcat of H143L/H189L suggests the possibility that disordered waters could participate in coupling the PLP to the network. Thus, a larger residue was introduced into the double mutant to block this putative channel. The H143F/H189L showed a significant (~98%) reduction in kcat and ~6-fold decrease in Km for l-Asp. Thus, the steric effects in H143F/H189L and removal of the carboxylate in D222T have comparable effects on AAT enzyme activity.

Quantum chemical cluster calculations

PLP activity is dependent on the protonation state of the cofactor and the active site. Because protons are normally not visible in X-ray structures, we used a quantum chemical cluster approach with DFT to analyze potential energies and enthalpies associated with proton transfers in AATWT and mutant variant enzymes (25). Quantum chemical analyses were focused on chain B of the various models because they are not restrained by crystal contacts and are presumably more representative of physiological conformations. Because entropic contributions to free energies are often difficult to compute accurately with cluster quantum mechanical (QM) models of enzymes, we instead computed enthalpies. This approximation is expected to be reasonable for our AAT models on the basis of comparisons with the experimental enzyme kinetics data.

Proton transfer enthalpies were computed to determine which protonation states support transfer of a proton to the PLP-N1 position for activation (Scheme 1). For the AATWT QM model (supplemental Fig. S2), histidines His-143 and His-

![Diagram](image-url)
189 can, in principle, adopt four possible protonation states as follows: His-143+/His-189+ (doubly protonated), His-143-neutral/His-189+, His-143+/His-189-neutral, and His-143-neutral/His-189-neutral (supplemental Fig. S3). These different states can lead to alternative hydrogen bond donor/acceptor pairings. To investigate which protonation states might be functionally relevant, we performed geometry optimizations of the four AATWT models, assuming that the PLP-N1 is protonated, for each of the His-143/His-189 protonation state combinations, and we compared them with the corresponding X-ray structure. Several criteria, including r.m.s.d. values, hydrogen bond distances, and relative residue conformations were considered in the analysis (supplemental Fig. S3).

All optimized AATWT protonation state models yielded relatively small overall r.m.s.d. (0.24–0.26 Å) when the backbone atoms were aligned with the AATWT X-ray structure. To examine the QM models in greater detail, critical residue positions were noted, and differences in the hydrogen bond network were evaluated, specifically PLP-N1 to Asp-222(Oδ2) (2.7 Å), Asp-222(Oδ2) to His-143(Ne2) (2.7 Å), His-143(N81) to Thr-139(Oγ1) (2.7 Å), and Thr-139(Oγ1) to His-189(Ne2) (2.7 Å) (supplemental Fig. S3A). For the His-143+/His-189+ doubly protonated model, the hydrogen bond distances between Thr-139 and His-189 and between His-143 and Thr-139 are elongated (2.9 and 3.1 Å, respectively) compared with the X-ray structure. The hydrogen bond distances between His-143 and Asp-222 and between Asp-222 and PLP-N1 are shortened (both to 2.6 Å) (supplemental Fig. S3B). The His-143-neutral/His-189+ model preserves the hydrogen bond distances appropriately, but the Asp-222 residue reorients significantly, rotating toward His-189, whereas His-189 rotates toward His-143 (supplemental Fig. S3C). The His-143+/His-189-neutral model shortens both the Asp-222 to PLP-N1 and the His-143 to Asp-222 hydrogen bonds (both to 2.6 Å) whereas the His-189 to Thr-139 elongates slightly (2.8 Å) (supplemental Fig. S3D). The His-143-neutral/His-189-neutral model was determined to have the best fit, both in superposition and in maintaining hydrogen bond distances. The distance between Asp-222 and PLP-N1 (2.6 Å) was shortened slightly, and His-189 to Thr-139 was elongated (2.8 Å), but the Asp-222–His-143 and His-143–Thr-139 distances were in good agreement with the X-ray structure (supplemental Fig. S3E).

Using the His-143-neutral/His-189-neutral model, we computed relative enthalpies for proton transfer from Asp-222(Oδ2) to PLP-N1 to determine which protonation state would be expected to be more thermodynamically stable in the wild-type and mutant enzymes (Scheme 1). Calculations for the AATWT QM model identified a single stable minimum in which the proton was located between PLP-N1 (1.08 Å) and Asp-222(Oδ2) (1.54 Å) (Fig. 6A). A relaxed potential energy surface scan shows that the energy increases monotonically when the proton is transferred from PLP-N1 toward Asp-222 (Fig. 6B). In addition, the proton initially placed on Asp-222 transferred spontaneously to PLP-N1 during the geometry optimization. Thus, these calculations demonstrate that there is no stable minimum for the proton located on Asp-222 in AATWT. The interaction between PLN-N1 and Asp-222 can therefore be considered a single-well hydrogen bond, with PLP-N1 as the donor and Asp-222(Oδ2) as the acceptor (26).

Because there is only a single stable minimum in this region of the potential energy surface, relative enthalpies are not meaningful and were therefore not computed.

For the D222T mutant, the model in which the proton is placed on His-143 is more stable than the model containing protonated PLP-N1 by 4.5 kcal/mol (Fig. 7, A and B). In model A-I (His-143+), the minimum for the hydrogen on Ne2 of His-143 is located 1.07 Å from the nitrogen and 1.63 Å from the oxygen of the water molecule (Fig. 7A). For PLP-N1 to become protonated in this arrangement, a thermodynamically unfavorable concerted proton transfer would be necessary (Fig. 7B). The concerted proton transfer pathway was investigated by performing several relaxed potential energy scans in which the position of the proton on His-143 was scanned toward the bridging water molecule and a proton from the bridging water molecule was simultaneously scanned toward PLP-N1. A single transition state (TS), model A-II, corresponding to a concerted dual proton transfer culminating in PLP-N1 protonation, model A-III, was obtained for this reaction path (Fig. 7, A and B).

For the H143L mutant, the relative total energy shows that the proton transfer pathway between Asp-222(Oδ2) and PLP-N1 has two energy minima (Fig. 7, C and D). In one energy minimum, model C-I, the proton is bound to Asp-222(Oδ2) (1.04 Å), and in the other energy minimum, model C-III, the proton is bound to PLP-N1 (1.08 Å). The computed total energies of the two states are essentially equivalent, with a small barrier height of ~2.5 kcal/mol (model C-II). The computed
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\[ \Delta H \] for the proton transfer is \(-0.9\) kcal/mol. The energy contributions of the zero-point energy and the enthalpy flood the potential energy well, making both states energetically equivalent. This feature is indicative of the presence of a low barrier hydrogen bond.

For the H143L/H189L double mutant, the QM model shows two energy minima, similar to that found in H143L, corresponding to protonated Asp-222(O\_2) in model E-I or protonated PLP-N1 in model E-III (Fig. 7, E and F). The potential energy barrier for model E-II is small, with only 2.5 kcal/mol necessary to transfer the proton from Asp-222 to PLP-N1 and 1 kcal/mol required for the reverse transfer. \( \Delta H \) for the proton transfer is 0.1 kcal/mol, making both states, E-I and E-III, essentially equal in population. Thus, similar to the QM calculation of H143L model, a low barrier hydrogen bond is predicted for the double mutant.

Discussion

In \textit{S. scrofa} AAT, an extended hydrogen-bonding network connects the buried side of the active site to bulk solvent (Fig. 3). This network promotes protonation of PLP-N1, resulting in activation and stabilization of the PLP cofactor on the side opposite the substrate-binding site. We have obtained high resolution crystal structures of the recombinant wild-type AAT and several mutant variants of the enzyme to alter and/or disrupt this hydrogen-bonding network. This study provides precise determination of the atomic positions of the active site residues and structural water molecules. The structural details of each variant and DFT calculations provide insights into the local environment requirements for PLP-N1 protonation in AAT.

For the recombinant enzyme and each variant, the overall enzyme fold is conserved, with a dimer (chains A and B) comprising the asymmetric unit and the biological assembly. The active site is positioned at the interface of the large and small domains (supplemental Fig. S1). The large domains within the dimer are essentially identical, but the two active sites display different substrate accessibility. All mutant variants have an r.m.s.d. of \(<0.13\ \text{Å}\) when the main chains are aligned to AAT_{WT}. Crystal packing restrains the small domain in chain A to a mostly open state, whereas the movement of the small domain of chain B is not restricted, allowing access to both the

\[ \text{Figure 7.} \ A, C, \text{ and } E \text{ show the optimized geometries for the D222T, H143L, } \text{and H143L/H189L QM models. The optimized geometries for structures I, II, and III are the reactant, TS, and product, respectively, for each model. Only PLP, four active site residues, and one water molecule are shown for simplicity. The green atoms are the protons of interest. B, D, and F show the relative electronic energy (solid black line) for D222T, H143L, and H143L/H189L, respectively. The dashed red lines are the enthalpies relative to the TS (structure II).} \]
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open and closed states. As a consequence, the overall B-factor of the small domain of chain A (~13 Å²) is significantly lower than that of chain B (~33 Å²). Movements in the small domain alter positions of the active site residues during substrate binding and release. When perturbing the hydrogen bond network by site-directed mutagenesis, the dynamics of the small domain must be taken into consideration. Multiple conformations are observed in the constrained chain A monomers but appear to be limited by crystal contacts and may have a low probability of being populated in solution. The small domain of chain B is highly mobile and arguably more representative of the solution state. With this in mind, chain B was used to model AAT$_{WT}$ and mutant variant enzymes to connect the structural details to kinetic values.

To better understand the role of protons in modulating PLP activity, we performed DFT calculations to estimate the positions of protons near PLP-N1. DFT calculations for AAT$_{WT}$ predict that His-143 and His-189 are neutral (singly protonated) in the internal aldimine. This is in agreement with previous NMR experiments, which determined that the histidines near PLP-N1 are neutral in both E. coli and porcine cytosolic AAT enzymes (27–29). Additionally, DFT predicts that the proton between the carboxylic group of Asp-222 and PLP-N1 is located within a single-well hydrogen bond (Fig. 6). The primary Lewis acid/base residue, Asp-222, influenced by the local environment, significantly increases the pK$_a$ of PLP-N1, thus stabilizing the pyridinium cation. This conclusion is supported by previous reports that used X-ray crystallography and NMR (6, 14–17). When PLP-N1 is protonated, the electronic sink effect of PLP becomes significant. Calculations for the variants show significant disruption in the stability of protonated PLP-N1. D222T (a variant that could participate in hydrogen bonding to PLP-N1 like in fold-type II PLP-dependent enzymes) does not directly hydrogen bond to PLP-N1 but rather Thr-222 is coupled indirectly via a structural water to PLP-N1 (Fig. 4). When a proton was transferred between His-143 and PLP-N1 through the crystallographic water, two stable minima are located. The minimum with His-143 protonated was calculated to be thermodynamically more stable (Fig. 7, A and B). Based on the DFT results for D222T, the population that would contain a protonated PLP-N1 is expected to be minor. Shifting the proton from PLP-N1 to His-143 in D222T changes the electronic structure of PLP, decreasing the electrophilicity of PLP. As a result of the D222T mutation, the k$_{cat}$ is significantly decreased (Table 2). In contrast to the Y225F mutant, the electrophilicity of PLP is perturbed. Therefore, the electronic sink effect of PLP is enhanced when the pyridinium cation is formed. When His-143 and His-189 are mutated to a hydrophobic residue such as Leu, the pK$_a$ of Asp-222 is expected to increase, resulting in a smaller pK$_a$ difference between Asp-222 and PLP-N1. This pK$_a$ shift was demonstrated in our DFT calculations for both H143L and H143L/H189L and is consistent with the kinetic analyses. In both the H143L and H143L/H189L QM models (Fig. 7, C–F), a low barrier hydrogen bond between Asp-222 and PLP-N1 was formed (26, 31). The zero-point energy and enthalpic contributions in H143L and H143L/H189L allow the proton located in the double-well hydrogen bond to reside on both Asp-222 and PLP-N1 (Fig. 7, C–F). This is supported by previous NMR studies, which showed that changing the dielectric of the solvent changes the hydrogen-bonding energetics between the PLP-N1 and Lewis acid/base (14). Both states are energetically accessible explaining the moderate reduction of k$_{cat}$ observed for H143L/H189L (Table 2) and suggests that His-143 and His-189 contribute to the decreased acidity of Asp-222 (31). The DFT calculations are consistent with the kinetics results and provide an explanation for the observations from kinetics measurements.

Once the PLP cofactor binds to the active site of AAT, a single proton transfer must occur to activate PLP in the wild-type enzyme. The hydrogen-bonding network near PLP-N1 extends out to the bulk solvent, and it can provide a path to shuttle a proton through His-189, Thr-139, His-143, and Asp-222 by a Grothhüss-like proton-hopping mechanism to activate PLP. Our current efforts are focused on obtaining a neutron crystal structure of AAT$_{WT}$ to resolve the positions of the hydrogen atoms in the active site and shed additional light on the role of protons in PLP-dependent enzyme activity. It appears that in AAT, PLP cofactor activation via PLP-N1 protonation is influenced not only by Asp-222 but also by the local microenvironment surrounding PLP-N1. Second-shell active site residues often play an important role in enzymatic catalysis. In essence, the electrostatic effects of the second-shell residues change the electronic properties of Asp-222 and PLP-N1, determining the energy profiles for the proton position between these two residues. Specifically, the hydrogen-bonding network near the PLP lowers the pK$_a$ of Asp-222 and at the same time increases the pK$_a$ of PLP-N1, stabilizing the pyridinium cation. This microenvironment can alter the rate of chemical transformation occurring as far as 11 Å away by influencing the position of the proton on PLP-N1, which impacts the delocalization of electrons in the carbamion intermediate.

Experimental procedures

Cloning of AAT and site-directed mutagenesis

The E. coli codon-optimized gene for AAT from S. scrofa was synthesized (GeneScript) with an N-terminal His tag and a TEV protease cleavage site. The gene was introduced into a pDEST-C1 plasmid via gateway cloning technology (33). Site-directed mutagenesis was performed on the wild-type plasmid using complementary primers (IDT) and plasmid replication (Agilent, QuikChange II). The plasmids were verified and then transformed into T7 Express LacIQ E. coli expression host cells.
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**Protein expression, purification, and crystallization**

For recombinant protein expression of wild-type AAT and variants, *E. coli* cells were grown in a bioreactor (BIOFLO 110, New Brunswick Scientific) to an A₆₀₀ of 2.0 at 37 °C, induced with 1 mM isopropyl-β-D-thiogalactopyranoside, and grown for 12 h at 22 °C. The induced cells were collected by centrifugation, resuspended in 5 ml of lysis buffer (50 mM sodium phosphate, pH 7.5, 250 mM NaCl, and 10 mM imidazole) per g of cells, and then lysed by sonication. Lysates, clarified by centrifugation, were loaded onto a 10-ml nickel-Sepharose column (Sepharose 6 Fast Flow, GE Healthcare) equilibrated with buffer A (50 mM sodium phosphate, pH 7.5, 500 mM NaCl, and 10 mM imidazole). AAT was eluted off by using a 50-ml linear gradient of 100% buffer A to 100% buffer B (50 mM sodium phosphate, pH 7.5, 500 mM NaCl, and 500 mM imidazole). The eluted fractions were collected, and 1 mg of TEV protease per 100 mg of target protein was added in buffer dialyzed overnight (50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, and 1 mM DTT). The dialyzed protein fractions were loaded onto a second 10-ml nickel-Sepharose column, and the flow-through containing AAT was collected. The purified AAT was buffer exchanged by dialysis (40 mM NaOAc, pH 5.4, and 2 mM PLP), concentrated to ~24 mg/ml (Amicon Ultra 10K), and crystallized by vapor diffusion using a 1:1 ratio of protein to crystallization solution (40 mM NaOAc, pH 5.4, 2 mM PLP, and 8% w/v polyethylene glycol 6,000) (9).

**X-ray data collection, data reduction, and structure refinement**

Data for each of the AAT crystals were collected using a Rayonix MX300 ccd (100K, LS-CAT ID-F and ID-G, APS, ANL). The data were processed using iMosflm, then scaled, and merged with the Aimless (CCP4 program suite) (34–36). AAT and variants crystallized in an orthorhombic space group (P₂₁₂₁, a = 55.40 Å, b = 124.03 Å, c = 129.26 Å) similar to native porcine AAT (P₂₁₂₁, a = 125.0 Å, b = 130.8 Å, c = 55.8 Å) with axes likely shortened by the cryogenic conditions. The phasing was solved using molecular replacement (Phaser, CCP4) with the native porcine AAT structure (PDB entry 1AJR) as the starting model. Several rounds of XYZ coordinate, real space, occupancy, B-factor, and TLS refinement were carried out using Phenix refine (37, 38). Between each round of refinement the program COOT was used to manually adjust the AAT models (39). The recombinant AAT model was the starting model for the variant enzymes without positional adjustment followed by Phenix refine (without the TLS). For the variant enzymes, omit \( F_o - F_c \) section maps around the active site were generated and then modeled to include the structural features of the mutation(s).

**Kinetics assays**

AAT activity was monitored using a coupled assay with malate dehydrogenase (adapted from the Worthington Enzyme Manual). All kinetics assays were performed in sodium phosphate buffer (50 mM, pH 7.4) and included malate dehydrogenase (1.25 units/ml, Sigma, M2634), NADH (0.25 mM, Sigma, N8129), and 2-oxoglutarate (15 mM, Sigma, K1875). Serial dilutions of the substrate (i.e., Asp, 10 to 0.04 mM, Sigma, A8949) were carried out. Recombinant AAT activity was standardized against native AAT (Sigma, G2751). The native enzyme, obtained in an ammonium sulfate slurry, was desalted and buffer exchanged using a Sephadex G-25 spin column prepared in the assay buffer. Each reaction (100 μl of reaction volume, 25 °C) was initiated by the addition of AAT (75–750 nM) and monitored every 30 s for 15 min using a hybrid microplate reader (Biotek Synergy H4) in 96-well plates (Greiner Bio-One). Reactions were performed in triplicate with background correction and averaged to obtain the initial velocities and standard error. The initial velocities of each reaction were determined using time points 3–10 (1 min 30 s to 5 min). The initial velocities were plotted and fitted to the steady-state equation to obtain the kinetic parameters and standard error. Prism (GraphPad) was used for data fitting. All kinetics assays were performed the same day that the protein was purified.

**Quantum chemical cluster calculations**

A quantum chemical cluster approach (25) was used to estimate the relative stability of the proton(s) that resides between PLP-N1 and the neighboring residue (Asp-222 or His-143) in AATWT and variant mutant enzymes (Scheme 1). The active site models were generated from crystal structures determined in the present work as follows: AATWT (PDB entry 5TOQ), D222T (5TOR), H143L (5TON), and H143L/H189L (5T0). Each model included the internal aldime, the side chains of eight active site residues (Asp-222, His-143, His-189, Thr-139, Trp-140, Ser-257, Arg-266, and Tyr-70), the peptide backbone of five residues (Thr-139, Trp-140, Gly-107, Gly-108, and Thr-109), and two crystallographic water molecules (supplemental Fig. S2). The C and N termini of the peptide backbone were capped with methyl groups. The active site residues of Asp-222, His-143, His-189, and Ser-257 were truncated at Ca. Arg-266 and Tyr-70 were truncated at Cγ and Cβ, respectively (supplemental Fig. S2). At each truncation the Ca, Cβ, or Cγ atom was held fixed at its crystallographic position during the geometry optimization (supplemental Fig. S2). For the backbone atoms, the methyl caps and the Ca of Gly-108 were also constrained (supplemental Fig. S2). All mutant variant models had the same truncations and constraints as the AATWT cluster model. N2 was protonated and O3’ was deprotonated in all models, on the basis of recent work supporting this configuration (17). The active site cluster models consisted of between 187 and 192 atoms. All models were optimized with the B3LYP density functional (40, 41) and the Def2-SVP basis set (42). Empirical D3 dispersion corrections with Becke-Johnson damping (i.e. D3BJ) (43, 44) were included, and all calculations were performed in the presence of polarizable continuum solvation with the SMD solvent model (22) using a dielectric constant (ε) of 4.0. We refer to this level of theory as SMD/B3LYP-D3BJ/Def2-SVP. Tight convergence criteria, as defined in Gaussian 09 (32), were used for both the SCF iterations and geometry optimizations. An ultrafine grid was used in all calculations. Vibrational frequencies were calculated at 298.15 K and 1 atm at the same level of theory as the geometry optimizations. Several small (<300 cm⁻¹) imaginary eigenvalues (frequencies), arising from the use of constraints in the geometry optimizations, were obtained in the vibrational frequency calculations. These imag-
binary frequencies are likely to introduce error in computed entropic contributions (25), which were therefore not included in our analysis. Single-point energies were computed at the SMD/B3LYP-D3BJ/Def2-TZVPP level of theory at the optimized geometries (43, 44). Enthalpies were computed as the sum of the single-point energy ($E_{\text{SVP}}$) and the thermal enthalpic correction (i.e. $H_{\text{corr}}$) (Equation 1).

$$H = E_{\text{SVP}} + H_{\text{corr}} \quad \text{(Eq. 1)}$$

In some cases, relaxed potential energy scans were used to obtain an initial guess of the TS structure for proton transfer reactions. The position of the proton of interest (between PLP-N1 and the neighboring residue) was scanned from either PLP-N1, the protonated O6 of Asp-222, or the protonated Ne2 of His-143 toward the neighboring heavy atom (O or N), using 13–20 steps of 0.05 Å each. For the relaxed potential energy scans only, the nitrogen of Trp-140 and the oxygens of explicit water molecules were frozen to prevent geometric oscillations that hampered convergence. A structure near the TS was then used for a TS optimization. Vibrational frequencies were computed on the final optimized TS structures to confirm the presence of a single imaginary eigenvalue corresponding to the proton transfer of interest. All DFT calculations were performed using the program Gaussian 09 revision E.01 (32). Sample Gaussian 09 input files are provided in the supplemental material.

Author contributions—S. D. and T. C. M. conceived and coordinated the study. S. D. and T. C. M. wrote the paper. K. G. did the initial cloning of AAT$_{WT}$. S. D., T. C. M., and A. Y. K. contributed to the structural and kinetic analysis. S. D., J. M. P., and X. H. contributed to the computational analysis. All authors reviewed the results and approved the final version of the manuscript.

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References

1. Percudani, R., and Peracchi, A. (2003) A genomic overview of pyridoxal-phosphate-dependent enzymes. EMBO Rep. 4, 850–854
2. Toney, M. D. (2005) Reaction specificity in pyridoxal phosphate enzymes. Arch. Biochem. Biophys. 433, 279–287
3. Richard, J. P., Amyes, T. L., Crugerias, J., and Rios, A. (2009) Pyridoxal 5'-phosphate: electrophilic catalyst extraordinaire. Curr. Opin. Chem. Biol. 13, 475–483
4. Oliveira, E. F., Cerqueira, N. M., Fernandes, P. A., and Ramos, M. J. (2011) Mechanism of formation of the internal aldime in pyridoxal 5'-phosphate-dependent enzymes. J. Am. Chem. Soc. 133, 15496–15505
5. Hayashi, H., and Kagamiyama, H. (1997) Transient-state kinetics of the reaction of aspartate aminotransferase with aspartate at low pH reveals dual routes in the enzyme-substrate association process. Biochemistry 36, 13558–13569
6. Sharif, S., Huot, M. C., Tolstoy, P. M., Toney, M. D., Jonsson, K. H., and Limbach, H.-H. (2007) 15N nuclear magnetic resonance studies of acid-base properties of pyridoxal 5'-phosphate aldmines in aqueous solution. J. Phys. Chem. B 111, 3869–3876
7. Dutta Banik, S., and Chandra, A. (2014) A hybrid QM/MM simulation study of intramolecular proton transfer in the pyridoxal 5'-phosphate in the active site of transaminase: influence of active site interaction on proton transfer. J. Phys. Chem. B 118, 11077–11089
8. Limbach, H. H., Chan-Huot, M., Sharif, S., Tolstoy, P. M., Shenderovich, I. G., Denisov, G. S., and Toney, M. D. (2011) Critical hydrogen bonds and protonation states of pyridoxal 5'-phosphate revealed by NMR. Biochim. Biophys. Acta 1814, 1426–1437
9. Rhee, S., Silva, M. M., Hyde, C. C., Rogers, P. H., Metzler, C. M., Metzler, D. E., and Arnone, A. (1997) Refinement and comparisons of the crystal structures of pig cytosolic aspartate aminotransferase and its complex with 2-methylaspartate. J. Biol. Chem. 272, 17293–17302
10. Toney, M. D. (2014) Aspartate aminotransferase: an old dog teaches new tricks. Arch. Biochem. Biophys. 544, 119–127
11. Goldberg, J. M., and Kirsch, J. F. (1996) The reaction catalyzed by Escherichia coli aspartate aminotransferase has multiple partially rate-determining steps, while that catalyzed by the Y225F mutant is dominated by ketimine hydrolysis. Biochemistry 35, 5280–5291
12. Casasnovas, R., Salva, A., Frau, J., Donoso, J., and Muñoz, F. (2009) Theoretical study on the distribution of atomic charges in the Schiff bases of 3-hydroxypridine-4-aldehyde and alanine. The effect of the protonation state of the pyridine and imine nitrogen atoms. Chemical Physics 355, 149–156
13. Yano, T., Kuramitsu, S., Tanase, S., Morino, Y., and Kagamiyama, H. (1992) Role of Asp222 in the catalytic mechanism of Escherichia coli aspartate aminotransferase: the amino acid residue which enhances the function of the enzyme-bound coenzyme pyridoxyl 5'-phosphate. Biochemistry 31, 5878–5887
14. Sharif, S., Denisov, G. S., Toney, M. D., and Limbach, H.-H. (2007) NMR studies of coupled low-and high barrier hydrogen bonds in pyridoxal-5'-phosphate model systems in polar solution. J. Am. Chem. Soc. 129, 6313–6327
15. Sharif, S., Fogle, E., Toney, M. D., Denisov, G. S., Shenderovich, I. G., Buntkowski, G., Tolstoy, P. M., Huot, M. C., and Limbach, H.-H. (2007) NMR localization of protons in critical enzyme hydrogen bonds. J. Am. Chem. Soc. 129, 9558–9559
16. Sharif, S., Schagen, D., Toney, M. D., and Limbach, H.-H. (2007) Coupling of functional hydrogen bonds in pyridoxal-5'-phosphate-enzyme model systems observed by solid-state NMR spectroscopy. J. Am. Chem. Soc. 129, 4440–4455
17. Chan-Huot, M., Dos, A., Zander, R., Sharif, S., Tolstoy, P. M., Compton, S., Fogle, E., Toney, M. D., Shenderovich, I., and Denisov, G. S. (2013) NMR studies of protonation and hydrogen bond states of internal aldmines of pyridoxal 5'-phosphate acid-base in alanine racemase, aspartate aminotransferase, and poly-l-lysine. J. Am. Chem. Soc. 135, 18160–18175
18. Griswold, W. R., Fisher, A. J., and Toney, M. D. (2011) Crystal structures of aspartate aminotransferase reconstituted with 1-deazapyridoxal 5'-phosphate: internal aldime and stable l-aspartate external aldime. Biochemistry 50, 5918–5924
19. Griswold, W. R., and Toney, M. D. (2011) Role of the pyridine nitrogen in pyridoxal 5'-phosphate catalysis: activity of three classes of PLP enzymes reconstituted with deazapyridoxal 5'-phosphate. J. Am. Chem. Soc. 133, 14823–14830
20. Metzler, C. M., Rogers, P. H., Arnone, A., Martin, D. S., and Metzler, D. E. (1979) Investigation of crystalline enzyme-substrate complexes of pyridoxal phosphate-dependent enzymes. Methods Enzymol. 62, 551–558
21. Pan, Q. W., Tanase, S., Fukumoto, Y., Nagashima, F., Rhee, S., Rogers, P. H., Arnone, A., and Morino, Y. (1993) Functional roles of valine 37 and glycine 38 in the mobile loop of porcine cytosolic aspartate aminotransferase. J. Biol. Chem. 268, 24758–24765
22. Marenich, A. V., Cramer, C. J., and Truhlar, D. G. (2009) Universal solvation model based on solute electron density and on a continuum model of the solvent defined by the bulk dielectric constant and atomic surface tensions. J. Phys. Chem. B 113, 6378–6396
Evidence of second shell residues influencing enzyme activity

23. Amador, E., and Wacker, W. E. (1962) Serum glutamic-oxaloacetic transaminase activity a new modification and an analytical assessment of current assay techics. *Clin. Chem.* 8, 343–350

24. Yano, T., Kuramitsu, S., Tanase, S., Morino, Y., Hiromi, K., and Kagaamiyama, H. (1991) The role of His143 in the catalytic mechanism of *Escherichia coli* aspartate aminotransferase. *J. Biol. Chem.* 266, 6079–6085

25. Siegbahn, P. E., and Himo, F. (2011) The quantum chemical cluster approach for modeling enzyme reactions. *Wiley Interdisciplinary Reviews: Computational Molecular Science* 1, 323–336

26. Lankau, T., and Yu, C.-H. (2007) Correlated proton motion in hydrogen bonded systems: tuning proton affinities. *Phys. Chem. Chem. Phys.* 9, 299–310

27. Kintanar, A., Metzler, C. M., Metzler, D. E., and Scott, R. D. (1991) NMR observation of exchangeable protons of pyridoxal phosphate and histidine residues in cytosolic aspartate aminotransferase. *J. Biol. Chem.* 266, 17222–17229

28. Metzler, D. E., Metzler, C. M., Mollova, E. T., Scott, R. D., Tanase, S., Kogo, K., Hiigaki, T., and Morino, Y. (1994) NMR studies of 1H resonances in the 10–18 ppm range for cytosolic aspartate aminotransferase. *J. Biol. Chem.* 269, 28017–28026

29. Mollova, E. T., Metzler, D. E., Kintanar, A., Kagaamiyama, H., Hayashi, H., Hiotsu, K., and Miyahara, I. (1997) Use of 1H-15N heteronuclear multiple-quantum coherence NMR spectroscopy to study the active site of aspartate aminotransferase. *Biochemistry* 36, 615–625

30. Onuffer, J. J., and Kirsch, J. F. (1994) Characterization of the apparent negative co-operativity induced in *Escherichia coli* aspartate aminotransferase by the replacement of Asp222 with alanine. Evidence for an extremely slow conformational change. *Protein Eng.* 7, 413–424

31. Cleland, W. W., Frey, P. A., and Gerlt, J. A. (1998) The low barrier hydrogen bond in enzymatic catalysis. *J. Biol. Chem.* 273, 25529–25532

32. Frisch, M. J., Schlegel, H. B., Scuseria, G. E., Robb, M. A., Cheeseman, J. R., Scalmani, G., Barone, V., Mennucci, B., Petersson, G. A., Nakatsuji, H., Caricato, M., Li, X., Hratchian, H. P., Izmaylov, A. F., Bloino, J., et al. (2010) Gaussian 09, rev. E01. Gaussian Inc., Wallingford, CT

33. Katzen, F. (2007) Gateway® recombinational cloning: a biological operating system. *Expert Opin. Drug Discov.* 2, 571–589

34. Battye, T. G., Kontogiannis, L., Johnson, O., Powell, H. R., and Leslie, A. G. (2011) iMOSFLM: a new graphical interface for diffraction-image processing with MOSFLM. *Acta Crystallogr. D Biol. Crystallogr.* 67, 271–281

35. Winn, M. D., Ballard, C. C., Cowtan, K. D., Dodson, E. J., Emsley, P., Evans, P. R., Keegan, R. M., Krissinel, E. B., Leslie, A. G., McCoy, A., McNicholas, S. J., Murshudov, G. N., Pannu, N. S., Potterson, E. A., Powell, H. R., et al. (2011) Overview of the CCP4 suite and current developments. *Acta Crystallogr. D Biol. Crystallogr.* 67, 235–242

36. Potterson, E., Briggs, P., Turkenburg, M., and Dodson, E. (2003) A graphical user interface to the CCP4 program suite. *Acta Crystallogr. D Biol. Crystallogr.* 59, 1131–1137

37. Adams, P. D., Grosse-Kunstleve, R. W., Hung, L. W., Ioerger, T. R., McCoy, A. J., Moriarty, N. W., Read, R. J., Sacchettini, J. C., Sauter, N. K., and Terwilliger, T. C. (2002) PHENIX: building new software for automated crystallographic structure determination. *Acta Crystallogr. D Biol. Crystallogr.* 58, 1948–1954

38. Winn, M. D., Isupov, M. N., and Murshudov, G. N. (2001) Use of TLS parameters to model anisotropic displacements in macromolecular refinement. *Acta Crystallogr. D Biol. Crystallogr.* 57, 122–133

39. Emsley, P., and Cowtan, K. (2004) Coot: model-building tools for molecular graphics. *Acta Crystallogr. D Biol. Crystallogr.* 60, 2126–2132

40. Becke, A. D. (1988) Density-functional exchange-energy approximation with correct asymptotic behavior. *Phys. Rev. A Gen Phys.* 38, 3098–3100

41. Lee, C., Yang, W., and Parr, R. G. (1988) Development of the Colle-Salvetti correlation-energy formula into a functional of the electron density. *Phys. Rev. B Condens. Matter* 37, 785–789

42. Weigend, F., and Ahlrichs, R. (2005) Balanced basis sets of split valence, triple ξ valence and quadruple ξ valence quality for H to Rn: design and assessment of accuracy. *Phys. Chem. Chem. Phys.* 7, 3297–3305

43. Weigend, F. (2006) Accurate Coulomb-fitting basis sets for H to Rn. *Phys. Chem. Chem. Phys.* 8, 1057–1065

44. Grimme, S., Ehrling, S., and Goerigk, L. (2011) Effect of the damping function in dispersion corrected density functional theory. *J. Comput. Chem.* 32, 1456–1465