Two-step Ligand Binding in a (βα)_8 Barrel Enzyme

SUBSTRATE-BOUND STRUCTURES SHED NEW LIGHT ON THE CATALYTIC CYCLE OF HisA

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Background: HisA catalyzes a ring-opening isomerization reaction in histidine biosynthesis.

Results: Catalytic residues and conformational changes upon substrate binding are clarified by structures, kinetics, and mutagenesis.

Conclusion: Closing of active site loops in HisA brings the substrate into a product-like conformation before catalysis.

Significance: This exemplifies coupled conformational changes in a (βα)_8 barrel enzyme and its substrate and clarifies the mechanistic cycle of HisA.

HisA is a (βα)_8 barrel enzyme that catalyzes the Amadori rearrangement of N^+-(5'-phosphoribosyl)formimino-5-aminoimidazole-4-carboxamide ribonucleotide (ProFAR) to N^+-(5'-phosphoribosyl)formimino-5-aminoimidazole-4-carboxamide-ribonucleotide (PRFAR) in the histidine biosynthesis pathway, and it is a paradigm for the study of enzyme evolution. Still, its exact catalytic mechanism has remained unclear. Here, we present crystal structures of wild type Salmonella enterica HisA (SeHisA) in its apo-state and of mutants D7N and D7N/D176A in complex with two different conformations of the labile substrate ProFAR, which was structurally visualized for the first time. Site-directed mutagenesis and kinetics demonstrated that Asp-7 acts as the catalytic base, and Asp-176 acts as the catalytic acid. The SeHisA structures with ProFAR display two different states of the long loops on the catalytic face of the structure and demonstrate that initial binding of ProFAR to the active site is independent of loop interactions. When the long loops enclose the substrate, ProFAR adopts an extended conformation where its non-reacting half is in a product-like conformation. This change is associated with shifts in a hydrogen bond network including His-47, Asp-129, Thr-171, and Ser-202, all shown to be functionally important. The closed conformation structure is highly similar to the bifunctional HisA homologue PriA in complex with PRFAR, thus proving that structure and mechanism are conserved between HisA and PriA. This study clarifies the mechanistic cycle of HisA and provides a striking example of how an enzyme and its substrate can undergo coordinated conformational changes before catalysis.

The (βα)_8 barrel enzyme HisA, or N^+-(5'-phosphoribosyl)formimino-5-aminoimidazole-4-carboxamide-ribonucleotide (ProFAR)5 isomerase, catalyzes the fourth step in the histidine biosynthesis pathway. The reaction is an Amadori rearrangement, in which the aminoldose ProFAR is converted to the corresponding ketose PRFAR (Fig. 1). HisA has attracted considerable attention as a model both for the evolution of catalytic function and for evolution of the (βα)_8 barrel architecture itself. Another (βα)_8 barrel in tryptophan biosynthesis, TrpF, catalyzes an equivalent Amadori rearrangement on a chemically related substrate (5'-phosphoribosylanthranilate (PRA)). TrpF activity has been imparted on HisA by directed evolution (1) and by serial passaging a Salmonella enterica strain that lacked the trpF gene (2). Further, some Actinobacteria possess a HisA-like enzyme named PriA (phosphoribosyl isomerase), which catalyzes both the HisA and TrpF reactions (3). In some members of the genus Corynebacterium, PriA has evolved to become a newly respecialized HisA enzyme, termed subHisA, when trpF was regained through horizontal gene transfer (4).

The most well characterized HisA is the enzyme from Thermotoga maritima (TmHisA) (5–7). It possesses striking 2-fold symmetry and a significant level of sequence identity between its N- and C-terminal half-barrels, providing evidence that HisA evolved by duplication and fusion of an ancestral half-

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The abbreviations used are: ProFAR, N^+-(5'-phosphoribosyl)formimino-5-aminoimidazole-4-carboxamide ribonucleotide; PRFAR, N^+-(5'-phosphoribosyl)formimino-5-aminoimidazole-4-carboxamide-ribonucleotide; PRA, 5'-phosphoribosylanthranilate; rCdRP, reduced 1-(2-carboxyphenyl) amino)-1-deoxyribulose 5-phosphate; PRPP, phosphoribosyl pyrophosphate; IPTG, isopropyl 1-thio-galactopyranoside; AICAR, 5-aminoimidazole-4-carboxamido-1-beta-D-ribofuranosyl 5'-monophosphate; Bicine, N,N-bis(2-hydroxyethyl)glycine; PDB, Protein Data Bank; r.m.s., root mean square.

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This article contains supplemental Movie 1.

The atomic coordinates and structure factors (codes SAHE, SAHF, and SAWJ) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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Barrel (5). This 2-fold symmetry has also been observed in the solved structures of the PriA enzymes from Streptomyces coelicolor (ScPriA) and Mycobacterium tuberculosis (MtPriA), the C. efficiens subHisA, and also TmAHisF, the homologous (βα)8 barrel that catalyzes the subsequent step in histidine biosynthesis (4, 5, 8–10). However, TrpF does not possess the same symmetrical architecture (11, 12).

To date, there has been no structure available of any HisA in complex with its substrate or product. For this reason, mechanistically important residues have been inferred through comparisons with related enzymes. The structure of T. maritima TrpF (TmTrpF) in complex with its product analogue, reduced 1-[2-carboxyphenyl]amino]-1-deoxyribulose 5-phosphate (rCDRP), was used to infer a common catalytic mechanism for TmAHisA and TmATrpF (7). In this mechanism, isomerization of each substrate (ProFAR and PRA) involves protonation of the furanose ring oxygen by a general acid, a Schiff base intermediate, proton abstraction at the C2' position by a general base, and a spontaneous enol-keto tautomerization (Fig. 1). Based on mutagenesis of structurally equivalent residues in TmAHisA and TmATrpF, Asp-8 (TmAHisA numbering) was proven to be essential for activity and subsequently suggested to be the general base (7). In the same study, mutagenesis of Asp-127 suggested that it was the most likely candidate for the catalytic acid in TmAHisA.

In contrast to the results with TmAHisA, structures of MtPriA in complex with both rCDRP (the TrpF product analogue) and PRFAR (the product of the HisA reaction) showed that Asp-130 in MtPriA, the equivalent of Asp-127 in TmAHisA, was too distant from the furanose ring oxygen to be responsible for catalysis. Instead, Asp-175 was proposed to be the catalytic acid, and this was supported by mutagenesis showing that the MtPriA(D175A) mutant had lost activity toward both of its substrates, ProFAR and PRA (10).

The discrepancies between the identities of the proposed catalytic residues in HisA and PriA and the lack of ligand-bound HisA structures inspired us to study this enzyme further. Moreover, T. maritima is a deep-branching anaerobic hyperthermophile that is predicted to resemble the last common ancestor of all bacteria (13) and has acquired ~24% of its genetic material from archaeal species through horizontal gene transfer (14). Given this unusual evolutionary history of T. maritima, we sought a new representative of “archetypal” HisA enzymes. S. enterica HisA (SeHisA) is both a canonical example (15) and a model for enzyme evolution (2). Here we present structures of SeHisA in the apo-state and in complex with its substrate, ProFAR, at two distinct stages of the catalytic cycle and clarify the mechanism for ligand binding and catalysis of HisA based on structures, kinetics, and mutational data.

Experimental Procedures

Cloning—The hisA coding sequence from S. enterica was amplified from a colony suspension using PCR with Phusion High-Fidelity DNA polymerase (Thermo Scientific, Waltham, MA) and the primers SeHisA.for and SeHisA.rev (Table 1) before being cloned into pEXP5-CT/TOPO (Life Technologies, Inc.) according to the manufacturer’s instructions. This vector encoded a C-terminal hexahistidine (His6) tag for hisA. DNA sequencing confirmed the correctness of the resulting plasmid, pEXP5-CT-hisA.

Enzymatic synthesis of the HisA substrate, ProFAR, required phosphoribosyl pyrophosphate (PRPP) as the starting material. To produce this from ribose 5-phosphate, we cloned the S. enterica prsA gene, which encodes PRPP synthetase. The prsA gene was amplified directly from S. enterica cells with Phusion polymerase and the primers SePRSPr.sor and SePRPPS.rev (Table 1). The primers introduced restriction sites for BamHI (5’ end) and EcoRI (3’ end), allowing the PCR product to be cloned into the expression vector pJEX401 (DNA2.0 Inc., Menlo Park, CA). The vector encoded an N-terminal His6 tag for prsA. The construction of pJEX401-prsA was confirmed by DNA sequencing.

HisF and HisH, which form the heterodimer imidazole glycerol phosphate synthase, were required for the HisA-coupled enzyme assay. Vectors for expressing the Escherichia coli genes (pCA24N-hisF and pCA24N-hisH) were taken from the ASKA

![FIGURE 1. Mechanism for the isomerization of ProFAR to PRFAR catalyzed by HisA (7).](http://www.jbc.org/)

TABLE 1
Oligonucleotides used in this study

| Primer name | Primer sequence (5’→3’) |
|-------------|------------------------|
| SeHisA.for  | ATGATTATTCCGGCATTAGATTTAATTG |
| SeHisA.rev  | TAAGATTATTCCGGCATTAGATTTAATTG |
| SePRPPS.for | CGCTTGAATTCAATGCTCGAACATGGCGG |
| SePRPPS.rev | GCCAATACCGCCGGCGGATTGAAAGGCGAT |
| D7N.for    | GCCAATACCGCCGGCGGATTGAAAGGCGAT |
| D7N.rev    | GCCAATACCGCCGGCGGATTGAAAGGCGAT |
| D129N.for  | CGCTTGAATTCAATGCTCGAACATGGCGG |
| D129N.rev  | GCCAATACCGCCGGCGGATTGAAAGGCGAT |
| D176N.for  | GCCAATACCGCCGGCGGATTGAAAGGCGAT |
| D176N.rev  | GCCAATACCGCCGGCGGATTGAAAGGCGAT |
| D176A.for  | GCCAATACCGCCGGCGGATTGAAAGGCGAT |
| D176A.rev  | GCCAATACCGCCGGCGGATTGAAAGGCGAT |
| S202A.for  | GCCGATATTTCTGGCCTGGTTGGTGGAGG |
| S202A.rev  | GCCGATATTTCTGGCCTGGTTGGTGGAGG |

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library (16). The sequences encoding C-terminal green fluorescent protein tags were removed from each vector by digestion with NotI (New England Biolabs, Ipswich, MA), followed by religation of the plasmid.

Site-directed Mutagenesis—Site-directed mutagenesis was carried out according to the QuikChange II protocol. The primers for introducing each hisA mutation (encoding the D7N, D129N, D176N, D176A, and S202A amino acid substitutions) into pEXP5-CT-hisA are listed in Table 1. A double mutant D7N/D176A was made by introduction of the D176A mutation into pEXP5-CT-hisA(D7N). Each mutated plasmid was used to transform either E. coli XL10-Gold or E. coli BL21-Gold(DE3) cells. Transformed cells were spread on LB agar plates containing 100 μg/ml ampicillin. Single colonies were used to inoculate cultures, from which plasmid DNA was prepared using the QIAprep Miniprep kit (Qiagen, Hilden, Germany). The presence of each desired mutation was confirmed by sequencing.

Protein Expression and Purification—All proteins were expressed in E. coli BL21(DE3) or E. coli BL21-Gold(DE3) cells, apart from PRPP synthetase, which was expressed in E. coli MC1061. Single colonies were used to inoculate 10-ml aliquots of LB medium containing the appropriate antibiotic: ampicillin (50 or 100 μg/ml) for pEXP5-CT-hisA; kanamycin (30 μg/ml) for pJEX401-prsA; and chloramphenicol (34 μg/ml) for pCA24N-hisF and pCA24N-hisH. After growth to saturation (overnight at 37 °C), each culture was used to inoculate 1 L LB medium in a shake flask, which was incubated at 37 °C until the A660 reached ~0.5. Cultures were moved to room temperature, and after 30 min, expression was induced by the addition of 0.5 mM IPTG. IPTG-induced protein expression was carried out for 20 min, before extensive washing with lysis buffer supplemented with 25 mM imidazole. His6-tagged proteins were eluted with lysis buffer supplemented with 500 mM imidazole. For kinetics, the HisA assay; each preparation was typically 15–25% pure.

Protein-containing fractions were pooled. For ProFAR, the presence of ProFAR in peak fractions was tested in HisA activity assays (see below) and confirmed with liquid chromatography mass spectrometry (LC-MS), using a Poroshell 120 EC-C18 3 × 50-mm column. Pooled fractions were lyophilized to remove residual ammonium bicarbonate and stored at −80 °C. The yield and purity of ProFAR were quantified using the HisA assay; each preparation was typically 15–25% pure.

Crystallization, Data Collection, and Refinement—Crystallization was done in sitting drop vapor diffusion experiments. For wild type SeHisA, crystals were obtained with 1.5 μl of 15 mg/ml HisA protein and 1.5 μl of reservoir solution containing 0.1 M HEPES, pH 7.5, 0.8 μM NaH2PO4, and 0.8 μM KH2PO4 (ICSG+ screen, Hampton Research, Aliso Viejo, CA) at 20 °C. Diamond-shaped crystals grew to a size of ~0.2 × 0.2 × 0.2 mm within 24 h. Crystals were cryoprotected in reservoir solution supplemented with 20% glycerol. For SeHisA(D7N), similar crystals were obtained with 20 mg/ml protein and a reservoir solution containing 0.2 M ammonium acetate, 0.1 M sodium acetate, and 20% PEG 4000 (pH set to 5.15). These crystals were soaked for 2 min in a cryosolution containing 50 mM sodium HEPES, pH 7, 150 mM NaCl, 30% PEG 4000, and 15% glycerol, to which ProFAR had been added as a lyophilized powder. SeHisA(D7N/D176A) cocryrstallized with ProFAR at 8 °C in a drop containing 0.075 μl of 23 mg/ml protein solution, to which ProFAR had been added as a lyophilized powder, and 0.125 μl of reservoir solution containing 0.1 M Bicine, pH 9.0, and 20% PEG6K (ICSG+ screen, Hampton Research). One rod-shaped crystal with the approximate dimensions 0.3 × 0.04 × 0.04 mm appeared after 2 days. The crystal was cryoprotected in reservoir solution supplemented with 15% glycerol. All crystals were vitrified in liquid nitrogen.

Data were collected at ESRF (Grenoble, France) and Diamond Light Source (Didcot, UK) and processed using XDS (19). Data statistics are summarized in Table 2. The wild type SeHisA structure was solved by molecular replacement with the pro-

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Preparation of ProFAR—The HisA substrate, ProFAR, was prepared according to methods modified from Ref. 17. E. coli strain FB1, which lacks the his operon (18), was transformed with plisGIE-tac (for IPTG-inducible overexpression of HisG and HisI). A saturated 10-ml culture of the transformed strain was used to inoculate LB (1 liter) containing 100 μg/ml ampicillin and was grown with shaking at 37 °C. At A660 ~0.6, protein expression was induced with IPTG (1 mM), and the culture was incubated for a further 16 h at 28 °C. The cells were harvested by centrifugation (3,000 × g, 15 min) and washed in 100 mM Tris-HCl, pH 7.5, before being stored at −80 °C.

At the time of ProFAR synthesis, cell pellets were thawed, resuspended, and lysed in 50 mM Tris-HCl and 300 mM KCl, pH 7.5, with lysozyme (0.5 mg/ml) and Benzonase nuclease (50 units) added, at room temperature for 20 min. Debris was removed by centrifugation (17,000 × g for 1 min), and the supernatant was used for ProFAR synthesis, as described previously (17). ProFAR was purified from the lysate by anion exchange chromatography with a HiPrep Q FF 6/10 column (GE Healthcare, Little Chalfont, UK). The column was equilibrated with 60 mM ammonium bicarbonate, and ProFAR was eluted with a gradient of 60–250 mM ammonium bicarbonate. The presence of ProFAR in peak fractions was tested in HisA activity assays (see below) and confirmed with liquid chromatography mass spectrometry (LC-MS), using a Poroshell 120 EC-C18 3 × 50-mm column. Pooled fractions were lyophilized to remove residual ammonium bicarbonate and stored at −80 °C. The yield and purity of ProFAR were quantified using the HisA assay; each preparation was typically 15–25% pure.
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**Structure Determination—**SeHisA, SeHisA(D7N) and SeHisA(D7N/D176A) in Complex with ProFAR—To gain further insight into the mechanism of substrate binding and catalysis, we set out to determine substrate-bound structures of SeHisA. The HisA substrate, ProFAR (Fig. 1) was synthesized according to previously published methods (17). After many fruitless attempts, a 1.6 Å complex structure in space group *P*3,2,1 with clear electron density was obtained using SeHisA(D7N/D176A) as the search model, HisA (PDB entry 4GJ1) as the search model, which in turn had been solved using the *Campylobacter jejuni* HisA (PDB entry 4GJ1) as the search model. The SeHisA structures were rebuilt using Coot (21) and refined in phenix.refine (22). Refinement statistics are presented in Table 2. Structure figures were prepared using PyMOL version 1.7 (Schrödinger, LLC). Detailed structure comparisons were done using the LSQ commands in O (23, 24).

**Table 2**

| PDB accession number | SeHisA | SeHisA(D7N)-ProFAR | SeHisA(D7N/D176A)-ProFAR |
|----------------------|--------|--------------------|--------------------------|
| Data collection      |        |                    |                          |
| Beam line            | ESRF ID23–2 | ESRF ID23–2 | Diamond 103 |
| Detector             | CADCHEX | Pilatus            | Pilatus                  |
| Space group          | P6, 22 | P6, 22             | P3, 21                   |
| Unit cell parameters |        |                    |                          |
| a, b, c (Å)          | 86.93, 86.93, 121.84 | 86.68, 86.68, 121.84 | 46.61, 46.61, 197.95 |
| a, b, c (degrees)    | 90, 90, 120 | 90, 90, 120 | 90, 90, 120 |
| Molecules/asymmetric unit | 1 | 1 | 1 |
| Matthews coefficient (Å³/Da) | 2.38 | 2.38 | 1.53 |
| Resolution range (Å) | 50–1.7 (1.75–1.70) | 50–2.2 (2.33–2.20) | 40.4–1.6 (1.70–1.60) |
| Wavelength (Å)       | 0.8726 | 0.8729 | 0.9762 |
| Total reflections†    | 590,799 (47,084) | 260,524 (40,601) | 202,180 (32,377) |
| Unique reflections‡   | 30,376 (2,463) | 25,987 (4,201) | 63,157 (10,139) |
| Completeness (%)§     | 100 (100) | 99.97 (100) | 99.5 (99.7) |
| Redundancy*           | 19.1 (19.1) | 10.0 (9.7) | 3.1 (3.2) |
| B-factor (Å²)         | 86.93, 86.93, 121.84 | 86.68, 86.68, 121.84 | 46.61, 46.61, 197.95 |
| Ramachandran plot     | 90, 90, 120 | 90, 90, 120 | 90, 90, 120 |
| Data collection       |        |                    |                          |
| Beam line            | ESRF ID23–2 | ESRF ID23–2 | Diamond 103 |
| Details              |        |                    |                          |
| Resolution range (Å) | 47.2–1.7 | 47.3–2.2 | 40.4–1.6 |
| Reflection (test set) | 30,376 (1,520) | 25,987 (1,310) | 34,069 (1,632) |
| Total no. of atoms   | 1,983 | 1,855 | 2,135 |
| Protein atoms        | 1,820 | 1,751 | 1,885 |
| Water atoms          | 115 | 55 | 213 |
| Other atoms          | 48 | 49 | 37 |
| R<sub>work</sub>|1/R<sub>free</sub> (%) | 17.42/20.62 | 16.97/21.80 | 16.62/20.22 |
| Average B-factor (overall) (Å²) | 25.9 | 44.7 | 33.8 |
| Average B-factor (protein, ligands) (Å²) | 25.5 | 45.0 | 32.9 |
| Average B-factor (water) (Å²) | 32.5 | 42.0 | 42.4 |
| r.m.s. deviation      | From ideal bond length (Å) | 0.008 | 0.008 | 0.007 |
| From ideal bond angle (degrees) | 1.105 | 1.141 | 1.198 |
| Model                | Amino acids | 1–16, 24–174, 181–244 | 1–245 |

a Values in parentheses refer to the highest resolution shell.

b Correlation coefficient between intensities from random half-data sets.

c Correlation coefficient between intensities from random whole-data sets.
d Correlation coefficient between intensities from random half-data sets.

**Results**

**Structure Determination—**SeHisA, SeHisA(D7N) and SeHisA(D7N/D176A) were expressed in *E. coli* with C-terminal His<sub>6</sub> tags. The mutation of Asp-7 was assumed (and then shown; see below) to make HisA inactive. Apo-crystals were obtained under several conditions with commercial crystallization screens, most of which contained phosphate or sulfate. Both SeHisA and SeHisA(D7N) crystallized in space group *P*6,22. A 1.7 Å SeHisA structure was solved by molecular replacement and showed a single protein molecule in the asymmetric unit. The enzyme has two phosphate ions from the crystallization solution bound to the substrate-binding site (Fig. 2A), and two loops are disordered.

**Enzyme Kinetics—**The HisA activity assay was adapted from one described previously (25). Assay mixtures contained 50 mM Tris-HCl (pH 7.5), 5 mM 2-mercaptoethanol, 25 mM L-glutamine, 2 μM purified HisF, and 2 μM purified HisH. The ProFAR concentration was varied from 1 μM to 1 mM, and each reaction was initiated by the addition of a HisA protein (either SeHisA or one of the mutated variants) to a final concentration of 0.1 μM (increased to 10 μM when activity was poor). Reactions were performed at 25 °C, and a decrease in absorbance at 300 nm was detected with a Cary 100 spectrophotometer (Agilent Technologies). The extinction coefficient difference for the substrate and product of the coupled reaction (ε<sub>ProFAR-AICAR</sub> = 5,637 M⁻¹ cm⁻¹) was determined previously (25). The initial reaction rates were plotted and fitted to the Michaelis-Menten model using GraphPad Prism. Each enzyme variant was assayed in biological duplicate and technical triplicate.

gram Phaser (20) using a mutant SeHisA<sup>6</sup> as the search model, which in turn had been solved using the *Campylobacter jejuni* HisA (PDB entry 4GJ1) as the search model. The SeHisA structure was used as the search model for SeHisA(D7N) and SeHisA(D7N/D176A). The structures were rebuilt using Coot (21) and refined in phenix.refine (22). Refinement statistics are presented in Table 2. Structure figures were prepared using PyMOL version 1.7 (Schrödinger, LLC). Detailed structure comparisons were done using the LSQ commands in O (23, 24).

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<sup>6</sup> A. Söderholm, X. Guo, and M. Selmer, unpublished data.
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FIGURE 2. Overall structure of SeHisA. The $\beta\alpha$ loops on the catalytic side of the $\beta(\alpha)\alpha$ barrel are colored by symmetry, and ligands are shown as sticks. A, apo-SeHisA with two phosphate ions. B, SeHisA(D7N) in complex with ProFAR. C, SeHisA(D7N/D176A) in complex with ProFAR. The reacting ribose is oriented to the left in all figures.

tron density for all loops and the complete ligand was obtained by co-crystallizing SeHisA(D7N/D176A) with ProFAR. In addition, a 2.2 Å complex of the structure with ProFAR (albeit with partially disordered loops) was obtained by briefly soaking SeHisA(D7N) crystals with ProFAR in cryosolution at pH 7.0.

The structures of SeHisA(D7N) and SeHisA(D7N/D176A) in complex with ProFAR are the first protein structures in complex with this labile metabolite and the first structures of a HisA enzyme with a native ligand (Fig. 2, A and B).

Overall Structure—SeHisA adopts a classical ($\beta\alpha$)$_4$ barrel fold (Fig. 2, A–C). As expected for enzymes with this fold (26), the $\alpha\beta$ loops (connecting each $\alpha$-helix to the subsequent $\beta$-strand) on the “back side” of the enzyme, are short; in contrast, the catalytic face of the protein exposes the longer and flexible $\beta\alpha$ loops 1–8. $\beta\alpha$ loops 1 and 6 are partially unstructured in the apo-SeHisA and SeHisA(D7N)-ProFAR structures. To test whether the extensive 2-fold symmetry observed in TmHisA (5) was also present in the S. enterica enzyme, the N-terminal and C-terminal halves (residues 1–123 and 124–240) of the fully ordered SeHisA(D7N/D176A)-ProFAR structure were superimposed. The r.m.s. deviation between the half-barrels was only 1.7 Å for 100 $C_n$ atoms, and the sequence identity was 19%, similar to the 2.1 Å r.m.s. deviation and 23% sequence identity observed for the two halves of TmHisA. The loop lengths follow this 2-fold symmetric pattern, and the longest loops, 1 and 5, form $\beta$-hairpins stabilized by interactions with the end of the loop and the preceding $\beta$-strand.

Structure of the SeHisA(D7N/D176A)-ProFAR Complex—ProFAR binds to SeHisA(D7N/D176A) in an extended conformation across the binding cleft, with both ribose entities in the C2′-endo-conformation (Fig. 3A). The complete ligand makes extensive interactions with surrounding residues in the C-terminal parts of the $\beta$ strands and in the eight $\beta\alpha$ loops that shape and cover the active site (Fig. 3). Loops 1, 2, 5, and 6 are covering the ligand and sealing the active site, for which reason we will refer to this as the closed structure (Fig. 2C). The closed loop structures are stabilized by interactions with ProFAR as well as by direct and water-mediated interloop hydrogen bonds. Following the 2-fold symmetry of both the enzyme and the substrate, the phosphate groups at the two ends of ProFAR are coordinated by the N termini of loops 4 and 8, together with the backbones of loops 3 and 7. At the reacting end of the ligand, the phosphate (phosphate 1) of ProFAR interacts with the backbone amide hydrogens of Gly-177 in loop 6, Gly-204, Gly-225, and Arg-226 and with four structured waters. The phosphate adjoining the non-reacting ribose (phosphate 2) forms hydrogen bonds with the backbone amide hydrogens of Gly-81, Gly-102, and Ser-103 and with the side chains of Ser-103 and Arg-83. Four ordered water molecules surround this phosphate, two of which are strongly coordinated by backbone atoms of residues Val-82, Thr-104, Gly-80, and Val-100 and two that are coordinated by loops 5 and 2.

The reacting ribose of ProFAR is kept in place by hydrogen bond interactions between both the 2′ hydroxyl and its neighboring secondary amine to Asn-7 (replacing Asp-7) and between the 3′-hydroxyl and Ser-202. The non-reacting ribose is positioned by a hydrogen bond between the 2′-hydroxyl group and Asp-129. The identical sugar puckers of the two riboses are stabilized by a bridging water molecule coordinated by His-47 and from the opposite side by a hydrogen bond network between Ser-202, Thr-171, and Asp-129 (Fig. 3, A and B). Because Asp-176 is mutated to Ala in this structure, it cannot form a hydrogen bond to the ligand; however, the reacting ring oxygen is only 4.1 Å away from the Ala side chain, suggesting that it would be at appropriate hydrogen bonding distance to Asp-176 in the wild type enzyme (see below).

The carboxamide group of ProFAR forms direct hydrogen bonds with the carbonyl oxygens of Gly-19 on loop 1 and Gly-144 on loop 5. The carboxamide aminoimidazole of ProFAR is sandwiched between a hydrophobic surface formed by Val-49 and Leu-51 and Trp-145 in loop 5, which makes a $\pi$-stacking interaction with its opposite side (Fig. 3A).

Comparison with the Apo- and SeHisA(D7N)-ProFAR Complex Structures—Superimposing the apo-SeHisA and SeHisA(D7N)-ProFAR structures (with partly disordered loops 1 and 6) onto the SeHisA(D7N/D176A)-ProFAR structure gives r.m.s. deviation values of 1.27 Å over 222 $C_n$ atoms and 1.22 Å over 223 $C_n$ atoms, respectively. The main structural deviations are around loops 2 and 5, which are 12 Å apart in the apo- and SeHisA(D7N)-ProFAR structures, compared with 5 Å in the SeHisA(D7N/D176A)-ProFAR structure (Fig. 2). We therefore define SeHisA(D7N)-ProFAR as the open liganded structure. All ProFAR interactions with loops 1, 2, 5, and 6 are absent in this open structure (Fig. 4), demonstrating that loop ordering and loop closure are not required for substrate binding.

Comparison of ProFAR in the open and closed structures shows that phosphate 1 and the reacting ribose superpose well (Fig. 5A). Apart from loop interactions, they form identical interactions with SeHisA with a native ligand (Fig. 2). The closed loop structures are stabilized by interactions with ProFAR as well as by direct and water-mediated interloop hydrogen bonds. Following the 2-fold symmetry of both the enzyme and the substrate, the phosphate groups at the two ends of ProFAR are coordinated by the N termini of loops 4 and 8, together with the backbones of loops 3 and 7. At the reacting end of the ligand, the phosphate (phosphate 1) of ProFAR interacts with the backbone amide hydrogens of Gly-177 in loop 6, Gly-204, Gly-225, and Arg-226 and with four structured waters. The phosphate adjoining the non-reacting ribose (phosphate 2) forms hydrogen bonds with the backbone amide hydrogens of Gly-81, Gly-102, and Ser-103 and with the side chains of Ser-103 and Arg-83. Four ordered water molecules surround this phosphate, two of which are strongly coordinated by backbone atoms of residues Val-82, Thr-104, Gly-80, and Val-100 and two that are coordinated by loops 5 and 2.

The reacting ribose of ProFAR is kept in place by hydrogen bond interactions between both the 2′ hydroxyl and its neighboring secondary amine to Asn-7 (replacing Asp-7) and between the 3′-hydroxyl and Ser-202. The non-reacting ribose is positioned by a hydrogen bond between the 2′-hydroxyl group and Asp-129. The identical sugar puckers of the two riboses are stabilized by a bridging water molecule coordinated by His-47 and from the opposite side by a hydrogen bond network between Ser-202, Thr-171, and Asp-129 (Fig. 3, A and B). Because Asp-176 is mutated to Ala in this structure, it cannot form a hydrogen bond to the ligand; however, the reacting ring oxygen is only 4.1 Å away from the Ala side chain, suggesting that it would be at appropriate hydrogen bonding distance to Asp-176 in the wild type enzyme (see below).

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interactions with the enzyme (Figs. 3 and 4). The rest of the ligand adopts two different conformations (Fig. 5A). In the open structure, the distance between the two phosphate groups is 1.5 Å shorter, and the part of ProFAR comprising the non-reacting ribose plus carboxamide aminomimidazole is rotated relative to the fixed part of the ligand. This results in a shift of 2.5 Å of the 3'-hydroxyl of this ribose toward the C-terminal half of the barrel and of 1.5 Å of the carboxamide toward the N-terminal half of the barrel (Fig. 5A).

These differences can be explained by conformational changes in the surrounding region going from the open to the closed structure. In the open structure, the side chain carboxylate of Asp-129 is 3.5 Å from the 2'-hydroxyl of the non-reacting ribose, making water-mediated interactions with the 3'-hydroxyl as well as with Thr-171 (Figs. 4 and 5B). Upon loop closure, it moves 4 Å to form the interactions observed in the closed structure (Fig. 5C). In addition, crystal packing interactions involving loop 5 in the open structure position Trp-145 far from the carboxamide aminomimidazole moiety, with which it stacks in the closed structure. The shorter distance between the phosphate groups in the open structure results in phosphate 2 being in a position closer to the center of the enzyme, hence lacking the interactions with the side chains of Arg-83 and Ser-103 that are observed in the closed structure (Fig. 5D).

The phosphate ions in the apo-SeHisA structure bind in positions that are similar to the phosphates of the substrate (Fig. 2). Phosphate 1 in the apo-structure resides in two alternative positions about 2 Å apart, interacting in one position with loop 8 and in the other with both loops 7 and 8 (Fig. 5E). The position of the corresponding phosphate in the closed ligand structure is between these positions, and in the open structure it is close to loop 8, lacking the amide interactions that occur upon closure of loop 6 (Fig. 5D). Phosphate 2 in the apo-structure is positioned at a similar distance from the center as in the closed structure and stabilized by the same interactions with Arg-83 and Ser-103 (Fig. 5E). Thus, in the closed structure, both phosphates form additional interactions with the enzyme.

Comparison with Previous Structures—The Dali server (27) was used to search the PDB for structures most similar to SeHisA(D7N/D176A)-ProFAR because this was the only one of
our structures in which all of the loops were ordered. The closest structural homologue was the MtPriA(D11N)-PRFAR complex (PDB code 2Y88, Z-score 37.0). This was followed by the other PriA complex structures (Streptomyces splicus HisAp with degraded ProFAR (PDB code 4TX9, Z-score 36.7) and MtPriA-PRFAR (PDB code 3ZS4, Z-score 36.6)) and then by several apo-PriA structures. The closest structural homologue annotated as a HisA was the enzyme from C. jejuni (PDB code 4GJ1, Z-score 33.6), which was the top hit when performing the Dali search using apo-SeHisA. Despite its lower structural similarity with SeHisA(D7N/D176A)-ProFAR, the C. jejuni enzyme (CjHisA) has a substantially higher degree of sequence identity (51%) than MtPriA (33%). This result emphasizes that with the significant structural rearrangements upon ligand binding, the conformational state has a larger impact on overall structural similarity than sequence identity. Notably, the SeHisA structure was less similar to TmHisA (PDB code 1Q02, Z-score 27.8) than it was to many HisF structures.

SeHisA(D7N/D176A) was carefully compared with TmHisA (5) and with MtPriA in complex with the reaction product PRFAR (10) because both proteins have been biochemically characterized, and the structures have fully ordered loops. The apo-TmHisA structure superimposes on SeHisA with an r.m.s. deviation of 1.84 Å over 208 Cα atoms. SeHisA and MtPriA superimpose with an r.m.s. deviation of 1.39 Å over 242 Cα atoms, and the structures are highly similar; notably, the conformations of all loops are almost identical.

A previously overlooked aspect of HisA catalysis is that ring opening (Fig. 1) should increase the overall length of the product, PRFAR, relative to the substrate, ProFAR. Comparison of the product PRFAR in the MtPriA structure with ProFAR in the two structures of SeHisA shows that PRFAR adopts a conformation that, besides the reacting ribose, is very similar to the extended conformation of ProFAR observed in the closed SeHisA(D7N/D176A)-ProFAR and MtPriA-PRFAR structures, including the hydrogen bond network of Asp-129 around the non-reacting ribose and the stacking of Trp-145 with the carboxamide aminoimidazole moiety.

ConSurf analysis (28) shows that 39 residues display more than 96% conservation among sequences with >25% sequence identity to SeHisA. This conservation was mapped on a structure-based sequence alignment of sequences from previously
characterized HisA and PriA enzymes (Fig. 7). Mapping the surface conservation on SeHisA confirmed that the entire substrate-binding pocket displays high conservation (data not shown). The suggested catalytic residues in TmHisA and MtPriA, corresponding to Asp-7, Asp-129, and Asp-176 in SeHisA, were all completely conserved. Of these, Asp-7 in β1 and Asp-176 in loop 6 are embedded in conserved regions. The phosphate-binding sites and a motif spanning β2 and loop 2 also display high conservation. The alignment showed that all side chains involved in direct or water-mediated hydrogen bonds with ProFAR are conserved except for Ser-103 (sometimes conservatively replaced with threonine). Of the 39 highly conserved residues, 10 are not conserved in TmHisA, demonstrating that TmHisA is an outlier. Most interestingly, the substrate-binding residue Ser-202 is 100% conserved according to ConSurf analysis but is replaced by alanine (Ala-194) in TmHisA. Only two of the highly conserved residues are different in the PriA sequences. One is Ser-81 in MtPriA, corresponding to Gly-79 in SeHisA, which has been shown to be critical for the TrpF activity of PriA (9).

Enzyme Kinetics—The steady state kinetic parameters of SeHisA were determined using a coupled assay (25). In brief, the conversion of ProFAR to PRFAR by SeHisA (Fig. 1) was coupled to the conversion of PRFAR to AICAR, which in turn is catalyzed by the HisF-HisH heterodimer, imidazole
glycerol phosphate synthase. Using this assay, we determined that SeHisA had a $k_{\text{cat}}$ of 7.8 ± 2.4 s$^{-1}$ and a $K_m$ of 17.0 ± 0.1 μM; therefore, the catalytic efficiency ($k_{\text{cat}}/K_m$) was 4.5 × 10$^3$ s$^{-1}$ μM$^{-1}$ (Table 3).

**Identification of Catalytic Residues—SeHisA isomerizes ProFAR using acid-base catalysis (Fig. 1) (7, 29). Our structures showed that Asp-7 is well positioned to act as the catalytic base at pH 5.15) in ligand solution at physiological pH. In this crystal form, loop 5 is involved in crystal packing, preventing loop closure.

The βαβ$_4$ barrels of SeHisA and TmHisA both show strong 2-fold symmetry, confirming that this is a general feature of HisA enzymes. The symmetry of HisA and its bis-phosphorylated substrate, ProFAR (Fig. 1), raises the question of how the enzyme recognizes the correct orientation of the substrate. The open SeHisA(D7N)-ProFAR complex structure shows that ligand-loop interactions are not involved in the initial direction-specific recognition of the substrate. Con- served but subtle deviations from perfect 2-fold symmetry seem sufficient to dictate the correct binding of ProFAR to the open state of HisA. At the end of β2, the conserved residues Val-49 and Leu-51 make hydrophobic interactions with the aminoimidazole carboxamide moiety (Fig. 4A). In contrast, at the end of symmetry-equivalent strand 6, the conserved residue Thr-171 (symmetry-related to Val-49) is involved in a hydrogen bond network and water-mediated ligand binding (Figs. 4 and 5B). Docking of ProFAR in the

**Discussion**

Here we have presented the structure of SeHisA, in addition to kinetic parameters and an in depth examination of the active site. Our two substrate-bound structures (SeHisA(D7N)-ProFAR, which represents initial substrate binding, and SeHisA(D7N/D176A)-ProFAR, where the substrate adopts a product-like conformation) provide greater insight into the HisA active site and reaction mechanism than was previously possible. These are the first ever protein structures to be co-crystallized with the labile metabolite ProFAR, which has a short half-life compared with crystallization time scales but is slightly more stable at neutral to alkaline pH (17). With this in mind, a closed ProFAR complex structure where the ordered loops cover the ligand was achieved with the double-mutant SeHisA(D7N/D176A) crystallized in an alkaline condition (pH 9) and at low temperature (8 °C). A second, open, ProFAR complex structure with disordered loops was obtained by brief soaking of a SeHisA(D7N) crystal (obtained at pH 5.15) in ligand solution at physiological pH. In this crystal form, loop 5 is involved in crystal packing, preventing loop closure.

The βαβ$_4$ barrels of SeHisA and TmHisA both show strong 2-fold symmetry, confirming that this is a general feature of HisA enzymes. The symmetry of HisA and its bis-phosphorylated substrate, ProFAR (Fig. 1), raises the question of how the enzyme recognizes the correct orientation of the substrate. The open SeHisA(D7N)-ProFAR complex structure shows that ligand-loop interactions are not involved in the initial direction-specific recognition of the substrate. Conserved but subtle deviations from perfect 2-fold symmetry seem sufficient to dictate the correct binding of ProFAR to the open state of HisA. At the end of β2, the conserved residues Val-49 and Leu-51 make hydrophobic interactions with the aminoimidazole carboxamide moiety (Fig. 4A). In contrast, at the end of symmetry-equivalent strand 6, the conserved residue Thr-171 (symmetry-related to Val-49) is engaged in a hydrogen bond network and water-mediated ligand binding (Figs. 4 and 5B). Docking of ProFAR in the

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**Table 3**

| Enzyme               | $k_{\text{cat}}$ | $K_m$ | $k_{\text{cat}}/K_m$ | % SeHisA |
|----------------------|------------------|-------|----------------------|----------|
| SeHisA               | 7.8 ± 2.4        | 17.0 ± 0.1 | 4.5 × 10$^3$ s$^{-1}$ μM$^{-1}$ | 100      |
| SeHisA(D7N)          | ND               | ND    | ND                   | ND       |
| SeHisA(D129N)        | 0.52 ± 0.04      | 6.3 ± 3.2 | 8.3 × 10$^4$ | 18.5     |
| SeHisA(D176N)        | 0.019 ± 0.001    | 17 ± 1  | 1.1 × 10$^3$ | 0.3      |
| SeHisA(S202A)        | 0.8 ± 0.2        | 40 ± 2 | 1.9 × 10$^4$ | 4.1      |

* ND, not detected.
opposite orientation suggests that the aminoimidazole carboxamide moiety may also clash slightly with loop 2. Upon loop ordering, additional interactions are formed with the asymmetric central part of the ligand (Fig. 3).

Our ProFAR-bound structures illuminate key details of the acid-base catalysis that is carried out by HisA. The general base is Asp-7, which is essential for activity (Table 3) and well positioned to abstract a proton from C2' according to the structures of SeHisA(D7N/D176A) and SeHisA(D7N) with ProFAR (Fig. 3). In the MtPriA-PRFAR structure, showing a state after ring opening, the equivalent Asn-11 forms hydrogen bonds to the C2' and C3' hydroxyls of the product. The structural details of the intermediate on which the general base acts (Fig. 1) are unknown. However, our structures suggest that Asp-7 could stabilize the Schiff base through a salt bridge, potentially taking on an additional function in catalysis. Asp-7 occupies a nearly identical position in the wild type apo-structure, and PROPKA3 (30) predicts that the pKₐ of its carboxylate is elevated to ~5.5 due to its relatively hydrophobic chemical environment. This indicates that it will be deprotonated at physiological pH, albeit with increased basicity as required to accept the C2' proton.

Our data confirm unequivocally that Asp-176 has the role of general acid. This residue is located on the flexible loop 6, which is only ordered in the closed SeHisA(D7N/D176A)-ProFAR structure. Despite being mutated to alanine in this structure, the amino acid overlays perfectly with the equivalent residue (Asp-175) in TmpPriA-PRFAR (Fig. 6). In silico mutagenesis of Ala back to Asp showed that the Asp side chain is positioned to donate a proton to the furanose ring oxygen. During this work, we also solved a SeHisA(D7N) structure with partial electron density for ProFAR and loop 6, where the Asp-176 side chain was positioned for catalysis (data not shown). Further evidence for the role of Asp-176 was provided by the introduction of the D176N mutation, which reduced kₐ by 400-fold but did not alter Kₐ at all (Table 3). The equivalent residue in TmpHisA, Asp-169, would need to move 4 Å upon substrate binding in order to act in the same fashion. Such a movement is entirely consistent with the pattern of loop closures we have observed for SeHisA. Thus, the catalytic residues, Asp-7 and Asp-176 in SeHisA, are strictly conserved in SeHisA, TmpHisA, and MtPriA (10).

In TmpHisA, the equivalent of the D129N mutation was shown to reduce turnover ~2,500-fold (7), albeit in assays conducted 55 °C below the temperature optimum of the enzyme. This suggested to the authors that Asp-129 might be the catalytic acid. Our ProFAR complex structures show that Asp-129 is too far from the furanose ring oxygen to play such a direct role in catalysis. Nevertheless, the D129N mutation in SeHisA did reduce kₐ (by 15-fold) while also lowering Kₐ (Table 3). This indicates a role for this aspartate in catalysis rather than binding (Table 3). Upon loop closure, Asp-129 moves 4 Å, making changes in its hydrogen bonding network to stabilize the extended product-like conformation of ProFAR together with Thr-171, Ser-202, and His-47 (Fig. 5C). In the open structure, only one of the side chain oxygens of Asp-129 is involved in a hydrogen bond (Fig. 4B), whereas in the closed structure, both side chain oxygens should function as hydrogen bond acceptors (Fig. 3B). Thus, the introduction of Asn in place of Asp-129 appears to be favorable for substrate binding to the open enzyme conformation, but the inability of Asn to accept a second hydrogen bond disfavors the transition to the product-like ligand conformation in the closed enzyme. These observations explain the effects of the D129N mutation on Kₐ and kₐ. The same logic is also likely to explain why mutation of the equivalent residue in MtPriA (D130A) leads to a 20-fold decrease in kₐ while not affecting Kₐ (10).

The functional importance of His-47 and Thr-171 has previously been demonstrated in HisA and/or PriA (7, 9, 10); here we have demonstrated that Ser-202 also has an important role. Ser-202 makes hydrogen bond interactions with the 3' hydroxyl of the reacting ribose in both the open and closed enzyme conformations (Figs. 3, 4, and 5B and C). Therefore, the negative impact of the S202A mutation on both kₐ and Kₐ (Table 3) is consistent with a role in initial binding of the substrate as well as in stabilization of the product-like conformation. The absence of an equivalent to Ser-202 in TmpHisA (in which it is replaced by Ala) might explain why the D129N mutation has a more detrimental effect on this enzyme.

The phosphates in the apo-SeHisA structure most likely bind to the enzyme’s preferred phosphate binding sites. Thus, electrostatic attraction by the conserved Arg-83 on the second phosphate, bringing it into the preferred binding site, may contribute to extending the substrate while phosphate 1 is kept tightly in place (Fig. 5, D and E). Preliminary density functional theory calculations indicate that there is
no significant energy difference between the two observed conformations of ProFAR in gas phase (data not shown). The movement of Gly-204 to make an additional interaction with phosphate 1 in the closed structure (Fig. 5D) is necessary sterically to allow closure of loop 6. In the MtPriA(D11N)-PRFAR structure (10), a salt bridge between Arg-19 and Asp-175 was suggested to be essential for recruiting loop 6. In contrast, the equivalent salt bridge (between Arg-15 and Asp-176) is not needed in SeHisA because the closed structure was obtained with a D176A mutant.

Sequence analysis showed that SeHisA, rather than TmHisA, is an archetypal representative of the HisA family (Fig. 7). Further, SeHisA is more similar to the PriA enzymes in sequence and structure than it is to TmHisA. The structural similarity is particularly close between SeHisA(D7N/D176A)-ProFAR and MtPriA(D11N)-PrFAR, in which all ligand-binding interactions are conserved. Thus, we have shown that neither structure nor the mechanism of ProFAR isomerization has diverged in HisA and PriA, despite selection for bifunctionality in the latter enzyme. When the sequence/structure distance from SeHisA to TmHisA and the uneven phylogenetic distribution of PriA enzymes are both considered, the most parsimonious explanation is that PriA enzymes have evolved from HisA ancestors in a handful of lineages (31).

Based on our crystal structures and mutagenesis of SeHisA, we can propose a general mechanism of substrate binding and catalysis in HisA (Fig. 8). The apo-SeHisA structure and the SeHisA(D7N)-ProFAR structure show that active site loops 1 and 6 are inherently flexible and that the catalytic cycle begins with a discrete, loop-independent, substrate-binding step (Fig. 8, A and B). Hydrophobic residues at the end of β2 (Val-49 and Leu-51) and the symmetry-related hydrophilic β6 (Thr-171) guide the substrate to bind in the correct orientation. Next, as observed in the SeHisA(D7N/D176A)-ProFAR structure, loops 1 and 6 become ordered, and loops 2 and 5 adopt a closed conformation resulting in complete sequestration of the substrate. Coupled with loop closure, the substrate adopts its extended conformation. The non-reacting half of the substrate adopts a product-like conformation, as observed in MtPriA(D11N)-PrFAR, with phosphate 2 located in a similar position as it is in the apo-SeHisA structure (Fig. 8C and supplemental Movie 1). The interactions stabilizing this conformation of ProFAR involve Asp-129 and the hydrogen-bonding network to Thr-171 and Ser-202, an electrostatic attraction on phosphate 2 by Arg-83, and additional interactions with loops 1, 2, 5, and 6. The functional importance of this transition is supported by the effect of the D129N mutation. In the closed state, the substrate is buried from the surrounding water, and the catalytic residues Asp-7 and Asp-176 are positioned for acid-base catalysis (Fig. 8D). The interactions of the enzyme with the product stay the same apart from around the reacting ribose, as shown in the MtPriA(D11N)-PRFAR structure (10). The catalytic cycle is completed when the loops open to release the product PRFAR. In conclusion, our two substrate-bound structures illuminate a two-step mechanism for ProFAR binding to HisA and provide an example of coupled conformational changes of enzyme and substrate in enzymatic catalysis.

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Author Contributions—M. S. and W. M. P. conceived and coordinated the study. M. S., W. M. P., A. S., X. G., and M. S. N. designed and analyzed the experiments. A. S. and X. G. performed crystallographic experiments. M. S. N. performed biochemical experiments. J. N. constructed expression vector pEXPS-CT-hisA. G. B. E. synthesized and purified ProFAR, together with M. S. N. A. S., X. G., M. S. N., W. M. P., and M. S. analyzed data and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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