Biochemical and Structural Studies of Uncharacterized Protein PA0743 from *Pseudomonas aeruginosa* Revealed NAD⁺-dependent l-Serine Dehydrogenase*  

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**Anatoli Tchigvintsev†, Alexander Singer‡, Greg Brown‡, Robert Flick‡, Elena Evdokimova§, Kemin Tan§, Claudio F. Gonzalez‡, Alexei Savchenko‡, and Alexander F. Yakunin‡†**

From the †Department of Chemical Engineering and Applied Chemistry, Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontario M5S 1L6, Canada, ‡Biosciences Division, Argonne National Laboratory, Midwest Center for Structural Genomics and Structural Biology Center, Argonne, Illinois 60439, and §Department of Microbiology and Cell Science, Genetics Institute, University of Florida, Gainesville, Florida 32611-0700

**Background:** β-Hydroxyacid dehydrogenases are ubiquitous enzymes, most of which remain uncharacterized.

**Results:** Biochemical, crystallographic, and mutational analyses identified uncharacterized *Pseudomonas aeruginosa* protein PA0743 as a l-serine dehydrogenase and characterized the molecular details of its active site.

**Conclusion:** PA0743 is the first NAD⁺-dependent l-serine dehydrogenase potentially involved in serine catabolism.

**Significance:** Our study provides molecular insights into the mechanism of β-hydroxyacid dehydrogenases.

The β-hydroxyacid dehydrogenases form a large family of ubiquitous enzymes that catalyze oxidation of various β-hydroxy acid substrates to corresponding semialdehydes. Several known enzymes include β-hydroxyisobutyrate dehydrogenase, 6-phosphogluconate dehydrogenase, 2-(hydroxymethyl)glutarate dehydrogenase, and phenylserine dehydrogenase, but the vast majority of β-hydroxyacid dehydrogenases remain uncharacterized. Here, we demonstrate that the predicted β-hydroxyisobutyrate dehydrogenase PA0743 from *Pseudomonas aeruginosa* catalyzes an NAD⁺-dependent oxidation of l-serine and methyl-l-serine but exhibits low activity against β-hydroxyisobutyrate. Two crystal structures of PA0743 were solved at 2.2–2.3 Å resolution and revealed an N-terminal Rossmann fold domain connected by a long α-helix to the C-terminal all-α domain. The PA0743 apostructure showed the presence of additional density modeled as HEPES bound in the interdomain cleft close to the predicted catalytic Lys-171, revealing the molecular details of the PA0743 substrate-binding site. The structure of the PA0743-NAD⁺ complex demonstrated that the opposite side of the enzyme active site accommodates the cofactor, which is also bound near Lys-171. Site-directed mutagenesis of PA0743 emphasized the critical role of four amino acid residues in catalysis including the primary catalytic residue Lys-171. Our results provide further insight into the molecular mechanisms of substrate selectivity and activity of β-hydroxyacid dehydrogenases.

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† To whom correspondence should be addressed. Tel.: 416-978-4013; Fax: 416-978-8528; E-mail: a.tchigvintsev@utoronto.ca.

‡ The abbreviation used is: HIBA, 3-hydroxyisobutyric acid.
The first β-hydroxyacid dehydrogenase was purified from pig kidney and biochemically characterized in 1957 (15). This enzyme has been shown to be highly specific to β-HIBA and catalyzed a reversible reaction of NAD-dependent oxidation of this substrate to methylmalonate semialdehyde. The HIBA dehydrogenases purified from Candida rugosa and rabbit liver were also found to be specific to HIBA and were active against both R- and S-HIBA isomers (12, 16). However, the HIBA dehydrogenases purified from Pseudomonas putida E23, rat, Escherichia coli, and Haemophilus influenzae were found to be active toward several substrates (in addition to HIBA) including L- or D-glycerate, 2-methyl-DL-serine, L-serine, phenylserine, D-threonine, hydroxybutyrate, and 3-hydroxypropionate (1, 17–19). Recent works have also revealed that some HIBA dehydrogenases can use NADP+ as a cofactor and identified novel substrates for these enzymes (succinate semialdehyde, 3-hydroxypropane sulfonate, methylglyoxal, and 3-hydroxypropionate) (11, 20). The structurally and biochemically characterized β-hydroxyacid dehydrogenases include the HIBA dehydrogenase TTHA0237 from Thermus thermophilus, 2-(hydroxymethyl)glutarate dehydrogenase Hgd from Eubacterium barkeri, and the sheep 6-phosphogluconate dehydrogenase (2, 4, 5). TTHA0237 is an NADP+-dependent dehydrogenase that produces methylmalonate semialdehyde as a product, whereas the E. barkeri Hgd catalyzes the NAD+ +NADP+ -dependent conversion between (S)-2-(hydroxymethyl)glutarate and (S)-2-formylglutarate. Most of the 6-phosphogluconate dehydrogenases are NADP+-dependent enzymes, which oxidatively decarboxylate 6-phosphogluconate to give ribulose 5-phosphate. Crystal structures have revealed that the first two enzymes have tetrameric oligomerization, whereas the sheep 6-phosphogluconate dehydrogenase is a dimer (2, 4, 5). The protomers of these enzymes have two domains: the N-terminal α/β domain with the nucleotide binding region and the C-terminal helical catalytic domain. These domains are connected by one characteristic long α-helix (α7 in TTHA0237). The inhibitor, mutational, and structural studies with the sheep 6-phosphogluconate dehydrogenase and rat HIBA dehydrogenase demonstrated that a conserved Lys residue (Lys-183 and Lys-173, respectively) plays a critical role in catalysis (1, 3, 5). However, our knowledge of β-hydroxyacid dehydrogenases remains limited, and the vast majority of these enzymes are still uncharacterized.

The genome of the opportunistic pathogen Pseudomonas aeruginosa encodes six members of the β-hydroxyacid dehydrogenase superfamily: PA0743 (annotated as a probable HIBA dehydrogenase), PA1500 (a probable oxidoreductase), PA1576 (a probable HIBA dehydrogenase), PA2199 (a probable dehydrogenase), PA3312 (a probable HIBA dehydrogenase), and PA3569 (a HIBA dehydrogenase), which share 32–62% sequence identity with each other and remain biochemically uncharacterized. Based on the increased 3-hydroxyisobutyrate dehydrogenase activity of the E. coli cell-free extracts containing the PA3569 gene (mmsB) cloned into a pUC18 plasmid, it has been proposed that PA3569 is indeed a HIBA dehydrogenase (21). This is supported by the demonstration of this activity in the purified homologous protein PP4666 (MmsB) from P. putida (54.3% sequence identity) (17). Here, we present the results of biochemical and structural characterization of the P. aeruginosa β-hydroxyacid dehydrogenase PA0743. We have demonstrated that this protein is an NADP+-dependent l-serine dehydrogenase and determined the crystal structure of this protein both in the apoform and in complex with NADP+. The structure and site-directed mutagenesis of PA0743 suggest that several conserved residues including Lys-171 are critical for its enzymatic activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals were purchased from Sigma. R-HIBA and S-HIBA was a kind gift of Dr. Naoki Kunishima (RIKEN Spring-8 Center, Harima Institute, Japan).

**Gene Cloning, Overexpression, and Purification of PA0743**—The PA0743 open reading frame was PCR-amplified using chromosomal DNA of the P. aeruginosa PA01 strain and the cloning primers containing the restriction sites for BamHI and NdeI and was cloned into the modified pET15b vector (Novagen) in which the tobacco etch virus protease cleavage site replaced the thrombin cleavage site and a double stop codon was introduced downstream from the BamHI site (22). The overexpression plasmid was transformed into the E. coli BL21(DE3) Gold strain (Stratagene). PA0743 was overexpressed in E. coli and purified using metal chelate affinity chromatography on nickel affinity resin (Qiagen) with a high yield (>50 mg/liter of culture) and homogeneity (>95%) as described previously (22). Purified proteins were concentrated using a centrifugal membrane concentrator (Millipore), frozen as drops in liquid nitrogen, and stored at −80 °C.

Gel filtration analysis of the oligomeric state of PA0743 was performed with a Superdex 75 16/60 column (Amersham Biosciences) equilibrated with 10 mM HEPES-K (pH 7.5) and 0.2 mM NaCl using ÄKTA FPLC (Amersham Biosciences). The column was calibrated with ribonuclease A (13.7 kDa), chymotrypsinogen (25 kDa), ovalbumin (43 kDa), albumin (67 kDa), and aldolase (158 kDa).

**Enzymatic Assays**—Dehydrogenase activity of PA0743 against various substrates was measured spectrophotometrically by following the increase of absorbance at 340 nm. The assays were performed at 37 °C in a reaction mixture (1 ml) containing 50 mM diethanolamine buffer (pH 11.0), 5 mM NAD+ (or NADP+), 5–15 mM substrate, and 1–10 µg of PA0743. The pH dependence of dehydrogenase activity of PA0743 with L-serine (5 mM) was determined using a polybuffer system described by Heering et al. (23). The formation of aldehydes as the products of the PA0743 reaction was analyzed using a colorimetric assay with 3-methyl-2-benzothiazolinone hydrazone as described previously (24), whereas the production of ammonia was tested using the phenol-hypochlorite method (25). For determination of the K_m and V_max of the wild-type and mutant PA0743, the assays contained substrates at concentrations of 0.025–50.0 mM. Kinetic parameters were determined by nonlinear curve fitting from the Lineweaver-Burk plot using GraphPad Prism (version 4.00 for Windows, GraphPad Software, San Diego, CA).

**Site-directed Mutagenesis of PA0743**—Site-directed mutagenesis was performed using the QuickChange™ site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions.
The amino acids selected to be mutated were all changed to alanine. DNA encoding wild-type PA0743 cloned into the modified pET15b was used as a template for mutagenesis. The standard PCR mixture contained 50–100 ng of template DNA and 150–250 ng of each mutagenizing primer. The methylated plasmid was digested with DpnI, and 4 μl of each reaction were used to transform competent DH5α cells. Plasmid was purified from the resulting ampicillin-resistant colonies using the Qiaprep Spin Mini Prep kit (Qiagen), and all mutations were verified by DNA sequencing. Plasmids containing the desired mutations were transformed into the E. coli BL21(DE3) strain, and the mutant PA0743 proteins were overexpressed and purified in the same manner as the wild-type PA0743.

Growth Experiments—The P. aeruginosa wild-type (PAO1) and PA0743 deletion (PW2350) strains were obtained from the P. aeruginosa PAO1 transposon mutant library (University of Washington Genome Center) (26). The strains were grown aerobically at 37°C (250 rpm) on MOPS minimal medium supplemented with glucose (0.2%) and L-serine or other substrates as nitrogen sources (leucine, valine, uracil, and thymine; 1 g/liter). In other growth experiments, the MOPS medium was supplemented with NH4Cl as a nitrogen source (9.5 mM; no glucose), and L-serine or the above mentioned nitrogen compounds were added as carbon sources. The growth culture was followed by measuring the absorbance at 600 nm.

Data Collection and Structure Determination—Crystals of selenomethionine-enriched PA0743 protein (both alone and in complex with NAD+) were used to collect diffraction data at a wavelength 0.9792 Å at the APS beamline 19-ID of the Structural Biology Center.

FIGURE 1. Structure-based sequence alignment of PA0743 and several β-hydroxyacid dehydrogenases. The secondary structure elements of PA0743 and human HIBA dehydrogenase are shown above and below the alignment, respectively. Residues conserved in all aligned β-hydroxyacid dehydrogenases are boxed. The residues comprising the four characteristic β-hydroxyacid dehydrogenase sequence motifs (3) are highlighted in different colors and labeled below the alignment. The PA0743 residues mutated to Ala in this work are marked with an asterisk above the alignment and numbered below the alignment. The proteins compared are PA0743 (UniProtKB Q9I5I6), PA3569 (P28811), rat HIBA dehydrogenase (P29266), the HIBA dehydrogenase TTHA0237 from T. thermophilus (Q5SLQ6), 2-(hydroxymethyl)glutarate dehydrogenase Hgd from E. barkeri (Ebar) (Q0QLF5), and the predicted human HIBA dehydrogenase (HIBADH) (P31937).
RESULTS AND DISCUSSION

Dehydrogenase Activity of PA0743—Sequence analysis of PA0743 revealed the presence of four conserved sequence motifs of $\beta$-hydroxycarboxy dehydrogenases (3) involved in dinucleotide cofactor binding (GLGXGMG; motif-1), substrate binding (DAPVSGG; motif-2), catalysis (GXGGXXGXXXX; motif-3) with a catalytic Lys (Lys-171 in PA0743), and cofactor-binding (KDL; motif-4) (Fig. 1). This level of sequence similarity between PA0743 and PA3569 suggests that PA0743 shares 32.9–38.2% sequence identity with the biochemically characterized HIBA dehydrogenases from rat and T. thermophilus (Fig. 1). In addition, PA0743 shows rather high similarity (62% sequence identity) to PA3569, which has been proposed to be a HIBA dehydrogenase in P. aeruginosa (Fig. 1) (21). This level of sequence similarity between PA0743 and PA3569 suggests that PA0743 might be another HIBA dehydrogenase in P. aeruginosa.

To determine the substrate specificity of PA0743, its gene was overexpressed in E. coli, and recombinant PA0743 was affinity-purified with a high yield to over 95% homogeneity as assessed by SDS-PAGE gels (not shown). Purified PA0743 was tested for the presence of dehydrogenase activity against both S-HIBA and R-HIBA using NAD$^+$ as the electron acceptor and the reaction mixture described for the HIBA dehydrogenase TTHA0237 (4). PA0743 showed low NAD$^+$-dependent activity with both substrates (Fig. 2), and therefore it was tested for dehydrogenase activity against a broader range of substrates described in previous works on $\beta$-hydroxycarboxy dehydrogenases. With these substrates, PA0743 demonstrated significant NAD$^+$-dependent dehydrogenase activity against l-serine, methyl-DL-serine, methyl-(S)-(+-)-3-hydroxy-2-methylpropionate, D-glycerate, tert-butyl-3-hydroxypropionate, and methyl-2,2-dimethyl-3-hydroxypropionate as well as detectable activity toward several other substrates (Fig. 2). Previously, a number of HIBA dehydrogenases have been shown to be active against several substrates including l-serine, but their activity or affinity toward l-serine was lower than those with HIBA (1, 2, 4, 17).

Like the $\beta$-hydroxycarboxy dehydrogenases from rat, rabbit liver, and T. thermophilus (1, 4, 16), PA0743 exhibited maximal activity at alkaline pH (pH 10–11). PA0743 can also use NADP$^+$ as a cofactor for the oxidation of l-serine, but this activity was significantly lower than that with NAD$^+$ (4–6%). This is consistent with the presence of an Asp (Asp-31) downstream of the motif-1 in the PA0743 sequence, which is known to determine the preference of HIBA dehydrogenases for NAD$^+$, whereas the NADP$^+$-dependent enzymes usually contain an Arg at this position (as in TTHA0237) (Fig. 1). The biochemical analysis of the PA0743 reaction mixture after incubation with l-serine revealed no presence of free ammonium, suggesting that PA0743 is not a deaminating dehydrogenase and, like other $\beta$-hydroxycarboxy dehydrogenases (3), this enzyme catalyzes a cofactor-dependent oxidation of a hydroxy acid substrate to a corresponding semialdehyde. This was confirmed by the detection of an aldehyde-containing product in the PA0743 reaction mixture using a hydrazone-based spectrophotometric assay for aldehydes (data not shown). Thus, with l-serine or methyl-l-serine as substrates, the proposed reaction product of PA0743 will be 2-aminomalonate semialdehyde or 2-aminomethylmalonate semialdehyde (Fig. 3). With all substrates, PA0743 exhibited classical, hyperbolic saturation kinetics with the lowest $K_m$ to l-serine and methyl-DL-serine (Table 1). The catalytic efficiency of PA0743 with l-serine as substrate is comparable with that reported for other $\beta$-hydroxycarboxy dehydrogenases (4, 17).
characterized NADP⁺-dependent l-serine dehydrogenases from the short-chain dehydrogenase/reductase superfamily (Agrobacterium tumefaciens ICR1600 and E. coli YdfG) (42–44), PA0743 has a lower $K_m$ and higher catalytic efficiency toward l-serine (Table 1).

Crystal Structure of PA0743—PA0743 was crystallized using the hanging drop vapor diffusion protocol, and its crystal structure was solved to 2.2-Å resolution using the single wavelength anomalous diffraction method (Table 2). The structure revealed a two-domain protein with the N-terminal Rossmann fold domain ($\alpha$1–$\alpha$8 and $\beta$1–$\beta$9; residues 1–162) and the C-terminal all-$\alpha$ domain ($\alpha$10–$\alpha$15; residues 198–296) connected by the long $\alpha$9 helix (residues 166–195) (Fig. 4A). The N-terminal domain interacts with the short fragment of the long $\alpha$9 helix, whereas the C-terminal domain is wrapped around this helix, covering almost half of its length at three sides and creating a large cleft between the two domains (Fig. 4A). Analysis of the crystal contacts in the final model using the quaternary prediction server PISA suggests that PA0743 forms a tetramer with four protomers arranged as a dimer of dimers (Fig. 4). This is consistent with the results of gel filtration experiments that

![Proposed reactions catalyzed by PA0743.](image)

**FIGURE 3.** Proposed reactions catalyzed by PA0743. The enzyme catalyzes the NAD⁺-dependent dehydrogenation of l-serine (A) or methyl-l-serine (B) at C3, producing 2-aminomalonate semialdehyde or 2-aminomethylmalonate semialdehyde, respectively, and NADH.

| Kinetic parameters of PA0743 with various substrates | $K_m$ (mM) | $V_{max}$ (μmol/min/mg protein) | $k_{cat}$ (s⁻¹) | $k_{cat}/K_m$ (s⁻¹ M⁻¹) |
|------------------------------------------------------|------------|---------------------------------|----------------|-------------------------|
| Wild type                                            |            |                                 |                |                         |
| l-Serine                                             | 2.5 ± 0.1  | 18.7 ± 0.3                      | 10.4 ± 0.2     | 0.4 × 10⁴               |
| Methyl-l-Serine                                      | 2.4 ± 0.2  | 17.2 ± 0.4                      | 9.6 ± 0.2      | 0.4 × 10⁴               |
| DL-Glyceric acid                                     | 19.8 ± 0.4 | 10.4 ± 0.1                      | 5.8 ± 0.1      | 0.3 × 10³               |
| MDHP⁺                                                | 17.4 ± 1.2 | 20.9 ± 0.7                      | 11.6 ± 0.4     | 0.7 × 10³               |
| NAD⁺                                                 | 3.4 ± 0.2  | 2.8 ± 0.1                       | 1.6 ± 0.1      | 0.5 × 10³               |
| T96A                                                 | 12.3 ± 1.8 | 2.6 ± 0.2                       | 1.4 ± 0.1      | 0.1 × 10³               |
| S122A                                                | 10.6 ± 0.5 | 0.8 ± 0.02                      | 0.4 ± 0.01     | 0.4 × 10²               |
| N175A                                                | 4.2 ± 0.2  | 4.9 ± 0.1                       | 2.7 ± 0.1      | 0.6 × 10³               |
| Y219A                                                | 11.0 ± 2.4 | 1.4 ± 0.1                       | 0.8 ± 0.1      | 0.7 × 10²               |

* Methyl-2,2-dimethyl-3-hydroxypropionate.

**TABLE 2**

Crystallographic data collection and model refinement statistics

| Data collection                          | PA0743 (apo) (Protein Data Bank code 3OBB) | PA0743 (+ NAD⁺) (Protein Data Bank code 3Q3C) |
|------------------------------------------|-------------------------------------------|------------------------------------------|
| Space group                             | P6,22                                      | P6,22                                    |
| Cell dimensions (Å) (a, c)               | 92.6, 124.9                                 | 92.7, 126.5                              |
| Wavelength                              | 0.97921                                    | 0.97921                                  |
| Resolution (Å)                          | 30.32-2.2 (2.28-2.2)                       | 37.39-2.3 (2.38-2.3)                     |
| $R_{merge}$                             | 0.116 (0.747)                              | 0.096 (0.743)                            |
| $I/σ(I)$                                | 32.6 (3.6)                                 | 48.7 (2.2)                               |
| Completeness (%)                        | 99.7 (100.0)                               | 99.2 (94.0)                              |
| Redundancy                              | 10.5 (10.7)                                | 15.2 (6.7)                               |
| Refinement                              |                                           |                                          |
| Resolution (Å)                          | 30.32-2.20                                 | 37.39-2.3                                |
| Number of reflections*                  | 15,695/837                                 | 14,766/750                               |
| $R_{merge}/R_{free}$                     | 0.197/0.243                                | 0.191/0.262                              |
| Number of atoms                         | 2,251                                      | 2,200                                    |
| Protein                                 | 2,142                                      | 2,125                                    |
| Major ligand (NAD)                      | 44                                         | 31                                       |
| Solvent                                 | 70                                         | 31                                       |
| Other                                   | 31                                         |                                          |
| R.m.s. deviations                       | 0.015                                      | 0.008                                    |
| Bond lengths (Å)                        | 1.65                                       | 1.33                                     |
| Ramachandran plot                       | Most favored (%)                           | 91.6                                      |
|                                         | Additionally allowed (%)                   | 7.9                                       |
|                                         | Disallowed (%)                             | 0.0                                       |

* Number of reflections overall and in the test set.
showed that PA0743 exists as a tetramer in solution (120.2 ± 7 kDa; predicted monomer molecular mass, 30.8 kDa). In the PA0743 dimer, two protomers are connected by the extensive non-polar and polar interactions between the side chains of residues located mainly on the long α9 helix as well as on the α13, α14, α15, α10 (Arg-208), and α11 helices (Fig. 4B). Two dimers of PA0743 are connected by polar and non-polar interactions between the α-helices (α13, α14, and α15 of the C-terminal domain creating a butterfly-like tetrameric structure (Fig. 4C)). A Dali search (45) for the PA0743 structural homologs identified several β-hydroxycarboxyl dehydrogenases including the predicted human HIBA dehydrogenase (Protein Data Bank codes 2GF2 and 219P; Z-score, 40.8 – 41.4; root mean square deviation, 1.2 – 1.4 Å), tartronate semialdehyde reductase GarR from Salmonella typhimurium (Protein Data Bank codes 1VPD and 1TEA; Z-score, 37.1; root mean square deviation, 2.1 Å), and the 2-(hydroxymethyl)glutarate Hgd from E. barkeri (Protein Data Bank code 3CKY; Z-score, 35.8; root mean square deviation, 1.9 Å). The high Z-scores with relatively low sequence similarity (30 – 40% sequence identity) suggest strong evolutionary conservation of this structural fold.

The apoprotein of PA0743 also revealed an additional weaker electron density located in the interdomain cleft. We have modeled this density as being partially occupied (occupancy of 0.5) by a weakly bound HEPES molecule based on its shape and presence in the crystallization solution (Fig. 5, A and C). In the HIBA dehydrogenase TTHA0237, the interdomain cleft accommodates the protein active site with the NADP cofactor and sulfate ion bound near the catalytic Lys-165 (4). In the structure of the E. coli 6-phosphogluconate dehydrogenase complex with 6-phosphogluconate (Protein Data Bank code 3FWN), the substrate is also bound near the catalytic Lys-183, and the long axis of the substrate is oriented almost perpendicular to the long α8 helix (46). In the PA0743 structure (Fig. 6A), the modeled HEPES molecule is bound deeply in the interdomain cleft with its sulfonic group located near the predicted catalytic Lys-171 (3.5–3.9 Å) and conserved Asn-175 (also motif-3; 2.4 – 3.2 Å) and Asp-247 (motif-4; 3.1 Å), whereas its hydroxyl group is close to the conserved Tyr-219 (3.4 Å). The modeled HEPES molecule likely mimics the position of a substrate (l-serine or methyl-l-serine) bound in the PA0743 substrate-binding site, which is formed by the residues from two protomers forming a dimer (Figs. 5A and 6A). With l-serine or methyl-l-serine bound in the PA0743 active site, the substrate amino, carboxyl, and methyl groups can be easily accommodated in the substrate-binding site and can potentially interact with side chains of conserved Ser-122 (motif-2), Ser-210 (from another subunit), Lys-246 (motif-4), or Phe-239 or with the Gly-123 and Gly-124 main chain groups (motif-2) (2.9 – 5.7 Å to the HEPES sulfonic group). The conserved Phe-239 restricts binding of larger substrates on one side, and the motif-2 residues (Ser-122, Gly-123, and Gly-124) confine the active site on another side. The structure of the PA0743-HEPES complex suggests that homologous dehydrogenases (HIBA-like) bind their substrates differently from 6-phosphogluconate dehydrogenases with the substrate long axes positioned parallel to the long α7 helix and oriented toward the Tyr-219 side chain. Thus, our results together with the structure of TTHA0327 (4) have revealed the location of the substrate-binding site and suggest a possible orientation of a bound substrate in HIBA-like dehydrogenases. The relatively large size of the substrate-binding site is in line with the observed substrate promiscuity of β-hydroxycarboxyl dehydrogenases.

**Crystal Structure of PA0743 in Complex with NAD**

To obtain a structure of a PA0743-NAD **+** complex, the wild-type protein crystals were soaked in the reservoir solution containing 10 mM NAD **+**, and the structure was solved using the single wavelength anomalous diffraction method. The structure revealed the presence of the cofactor in the active site located in the interdomain cleft (Fig. 5, B and D, and Table 2). The NAD **+** molecule is coordinated by the interactions with side chains and main chain groups of conserved residues located in both protein domains of one protomer. Its adenosine moiety is located in the shallow pocket of the N-terminal domain and interacts mainly with the hydrophobic side chains of Ile-7 (3.5 Å), Phe-30 (3.7 Å), Leu-32 (3.3 Å), and Leu-65 (3.7 Å) (Fig. 6B). The ribose 2’- and 3’-hydroxyls of adenosine interact with the side chain carboxyl oxygens of Asp-31 (2.4 and 2.7 Å), explain-
L-Serine Dehydrogenase PA0743

FIGURE 5. Close-up view of PA0743 structure with bound HEPES or NAD⁺. A and B, surface presentation of the PA0743 active site with the modeled HEPES or bound NAD⁺. The substrate-binding site (A) is formed by the residues from two protomers (from one dimer) shown in different colors (gray and tan), whereas the cofactor-binding site (B) is made of the residues from one protomer. Both ligands are almost completely buried in the PA0743 active site. The location of the cofactor- and substrate-binding sites is labeled (NAD site and Substrate site with an arrow, respectively). C and D, electron density maps showing the positions of bound HEPES (C) or NAD⁺ (D) and selected residues (labeled). The omit map was generated by omitting the HEPES (C) and NAD⁺ (D) from the model. Cyan-colored density represents the resulting Fo − Fe map contoured at 3.0 σ. Ligand atoms and selected PA0743 residues are labeled and shown as a stick representation with magenta carbon atoms representing the ligand and green carbon atoms representing the selected PA0743 residues.

In several dehydrogenases including the *E. coli* 6-phosphogluconate dehydrogenase and ketopantoate reductase, binding of a redox cofactor has been shown to induce a large conformational change producing a closed conformation of the active site (46, 48). The superposition of the PA0743 apostructure with the structure of the NAD⁺ complex (generated using the SSM server) revealed no significant structural rearrangements with a pairwise root mean square deviation value of the main chain Cα atoms of 0.43 Å for the superposition of residues 2–296. No considerable cofactor-induced changes have been found in the structure of the HIBA dehydrogenase TTHA0237 either (4). In Fig. 6C, the superimposed PA0743 structures show the active site with the bound NAD⁺ and HEPES molecules illustrating the relative arrangement of the cofactor and substrate-like ligand near the catalytic Lys-171. In the superimposed model of the PA0743 active site, the C2 atom of HEPES is located 3.8 Å away from the C4 atom of the nicotinamide ring and likely mimics the location of the L-serine C3 atom positioned 3.8 Å away from the side chain of the conserved Gly-10 main chain amide (3.5 Å). The oxygen atoms of the pyrophosphate moiety of NAD⁺ are coordinated by interactions with the main chain amido groups of Gly-10 (3.0 Å), His-11 (2.9 Å), and Met-12 (2.7 Å) (Fig. 6B), which are located on the β1-α1 loop containing HIBA dehydrogenase motif-1 GXGXMG (Fig. 1).

The nicotinamide moiety of NAD⁺ is mostly buried in the interdomain cleft of the PA0743 protomer (Figs. 5B and 6B). Two ribose hydroxyl groups of the nicotinamide mononucleotide interact with the side chain of the conserved Lys-246 (2.5 and 3.0 Å). The e-amino group of Lys-246 is coordinated by the interaction with the side chain carboxyl oxygen of the conserved Asp-247 (3.0 Å) (Fig. 6B). These two conserved residues comprise sequence motif-4 of β-hydroxyacid dehydrogenases (Fig. 1). It is known that carboxylate-lysine interactions stabilize the protonated form of lysine (by increasing their pKₐ), which can be required to bind a substrate (47). Another carboxyl oxygen of Asp-247 is close to the side chain of the conserved Asn-175 from motif-3 (2.6 Å) (Fig. 6B). The nicotinamide ring of NAD⁺ is coordinated by van der Waals stacking of the side chains of the conserved Met-12 (3.7 Å) and Phe-239 (3.9 Å) (Fig. 6B). Because the nicotinamide ring is in the syn conformation (its amido group is close to the pyrophosphate moiety), the hydride ion transfer can occur only on the si-face of the nicotinamide ring where the predicted catalytic Lys-171 is located. This suggests that like the sheep 6-phosphogluconate dehydrogenase and TTHA0237 (4, 5) PA0743 is a pro-S (B) dehydrogenase.

The positions of bound HEPES (C) or NAD⁺ (D) and selected residues (labeled). The omit map was generated by omitting the HEPES (C) and NAD⁺ (D) from the model. Cyan-colored density represents the resulting Fo − Fe map contoured at 3.0 σ. Ligand atoms and selected PA0743 residues are labeled and shown as a stick representation with magenta carbon atoms representing the ligand and green carbon atoms representing the selected PA0743 residues.
Mutational Studies of PA0743 and Potential Catalytic Mechanism—To identify the residues of PA0743 important for its catalytic activity, we mutated eight conserved and semiconserved residues to Ala and determined the catalytic activity of purified mutant proteins against l-serine. Site-directed mutagenesis revealed an important role of the conserved Asn-175 of PA0743 motif-3 (4.4–5.0 Å to Lys-171) (Fig. 7 and Table 1). In the apostructure of PA0743, the Asn-175 side chain is involved in the coordination of the modeled HEPES sulfonic group, suggesting that it can potentially coordinate the substrate (l-serine or methyl-l-serine) hydroxyl group (Fig. 6A). Alanine replacement of conserved residues from PA0743 motif-2 and motif-4 (Ser-122, Lys-246, and Asp-247) also resulted in proteins with very low activity (Fig. 7). The structure of PA0743 shows that Lys-246 is directly involved in the cofactor coordination (nicotinamide ribose oxygens), suggesting that the side chains of Ser-122 and Asp-247...
might contribute to substrate binding (Fig. 6B). The substrate-binding site of PA0743 accommodates two semiconserved aromatic residues, Trp-214 and Tyr-219, whose replacement to Ala also had a strong negative effect on PA0743 activity (Fig. 7). We propose that the Tyr-219 side chain hydroxyl might contribute to substrate coordination (interaction with the serine carboxyl group), whereas the role of Trp-214 in the activity of PA0743 remains unclear.

As expected, the replacement of the predicted catalytic Lys-171 by Ala produced essentially inactive protein, confirming its critical role in PA0743 activity (Fig. 7). We propose that, as in TTHA0237 and E. barkeri Hgd, the PA0743 Lys-171 functions as a catalytic base, accepting the proton from the substrate hydroxyl. After binding of L-serine to the PA0743 active site, the substrate C3 hydride is transferred to the C4 of the NAD\(^+\) nicotinamide ring, whereas its hydroxyl proton is transferred to the unprotonated side chain of Lys-171. The developing negative charge on the carbonyl group of malonate semialdehyde can be stabilized by the side chain of the conserved Asn-175. The Lys-171 side chain is positioned close to the side chain of the semiconserved Thr-96 (2.8 Å; replaced by a Ser in some HIBA dehydrogenases), and the T96A mutant protein also exhibited a greatly reduced activity (Fig. 7 and Table 1). This suggests that the interaction of the catalytic Lys-171 with a hydroxyl side chain (Thr or Ser) is important for the activity of PA0743 (probably through the maintenance of the proper orientation and/or unprotonated state of Lys-171). Overall, the results of site-directed mutagenesis of PA0743 support a catalytic role of Lys-171 and identify other residues important for activity.

Implications for Potential role of PA0743 in P. aeruginosa—Thus, biochemical characterization of PA0743 has revealed that this protein exhibits an NAD\(^+\)-dependent dehydrogenase activity with substrate preference for L-serine and methyl-L-serine. An NADP\(^+\)-dependent serine dehydrogenase has been purified from A. tumefaciens and is proposed to function in an unknown biosynthetic pathway (42). NAD\(^+\)-dependent dehydrogenases appear to function primarily in catabolic reactions (51), suggesting that PA0743 might be involved in serine/methionine degradation. A role in serine metabolism has been proposed previously for the HIBA dehydrogenase from P. putida E23 (17). In addition, several Pseudomonas species have been shown to be able to grow on L-serine as a sole nitrogen source (17). In addition, several Pseudomonas species have been shown to be able to grow on L-serine as a sole nitrogen source. P. aeruginosa wild-type and PA0743 deletion strains, suggesting that this protein might also be active against malonate semialdehyde (55). One hypothesis is that in vivo the expected reaction products of PA0743 (2-aminomalonate semialdehyde and 2-aminomethylmalonate semialdehyde; Fig. 3) are deaminated by a presently unknown deaminase, producing methylmalonate or malonate semialdehydes, which are substrates for a methylmalonate-semialdehyde dehydrogenase. Thus, the activity of the L-serine dehydrogenase PA0743 might provide a link between serine degradation and the distal pathway of valine catabolism. Other, presently uncharacterized \(\beta\)-hydroxyacid dehydrogenases from P. aeruginosa are also expected to produce semialdehydes as reaction products. Biochemical and structural characterization of these proteins will reveal novel biochemical reactions and provide a greater understanding of the molecular mechanisms of substrate specificity and catalysis by \(\beta\)-hydroxyacid dehydrogenases.

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