Structure-Based Engineering of an Artificially Generated NADP⁺-Dependent D-Amino Acid Dehydrogenase

Junji Hayashi,^a Tomonari Seto,^a Hironaga Akita,^b Masahiro Watanabe,^b
Tamotsu Hoshino,^b Kazunari Yoneda,^c Toshihisa Ohshima,^d Haruhiko Sakuraba^a

Department of Applied Biological Science, Faculty of Agriculture, Kagawa University, Miki-cho, Kita-gun, Kagawa, Japan^a; Research Institute for Sustainable Chemistry, National Institute of Advanced Industrial Science and Technology (AIST), Higashi-Hiroshima, Hiroshima, Japan^b; Department of Bioscience, School of Agriculture, Tokai University, Higashi-ku, Kumamoto-shi, Kumamoto, Japan^c; Department of Biomedical Engineering, Faculty of Engineering, Osaka Institute of Technology, Asahi-ku, Osaka, Japan^d

ABSTRACT A stable NADP⁺-dependent D-amino acid dehydrogenase (DAADH) was recently created from *Ureibacillus thermosphaericus* meso-diaminopimelate dehydrogenase through site-directed mutagenesis. To produce a novel DAADH mutant with different substrate specificity, the crystal structure of apo-DAADH was determined at a resolution of 1.78 Å, and the amino acid residues responsible for the substrate specificity were evaluated using additional site-directed mutagenesis. By introducing a single D94A mutation, the enzyme's substrate specificity was dramatically altered; the mutant utilized D-phenylalanine as the most preferable substrate for oxidative deamination and had a specific activity of 5.33 μmol/min/mg at 50°C, which was 54-fold higher than that of the parent DAADH. In addition, the specific activities of the mutant toward D-leucine, D-norleucine, D-methionine, D-isoleucine, and D-tryptophan were much higher (6 to 25 times) than those of the parent enzyme. For reductive amination, the D94A mutant exhibited extremely high specific activity with phenylpyruvate (16.1 μmol/min/mg at 50°C). The structures of the D94A-Y224F double mutant in complex with NADP⁺ and in complex with both NADPH and 2-keto-6-aminocaproic acid (lysine oxo-analogue) were then determined at resolutions of 1.59 Å and 1.74 Å, respectively. The phenylpyruvate-binding model suggests that the D94A mutation prevents the substrate phenyl group from sterically clashing with the side chain of Asp94. A structural comparison suggests that both the enlarged substrate-binding pocket and enhanced hydrophobicity of the pocket are mainly responsible for the high reactivity of the D94A mutant toward the hydrophobic D-amino acids with bulky side chains.

IMPORTANCE In recent years, the potential uses for D-amino acids as source materials for the industrial production of medicines, seasonings, and agrochemicals have been growing. To date, several methods have been used for the production of D-amino acids, but all include tedious steps. The use of NAD(P)⁺-dependent D-amino acid dehydrogenase (DAADH) makes single-step production of D-amino acids from oxo-acid analogs and ammonia possible. We recently succeeded in creating a stable DAADH and demonstrated that it is applicable for one-step synthesis of D-amino acids, such as D-leucine and D-isoleucine. As the next step, the creation of an enzyme exhibiting different substrate specificity and higher catalytic efficiency is a key to the further development of D-amino acid production. In this study, we succeeded in creating a novel mutant exhibiting extremely high catalytic activity for phenylpyruvate amination. Structural insight into the mutant will be useful for further improvement of DAADHs.

KEYWORDS D-amino acid, D-phenylalanine, NADP, *Ureibacillus thermosphaericus*, dehydrogenases, meso-diaminopimelate, phenylpyruvate

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Address correspondence to Haruhiko Sakuraba, sakuraba@ag.kagawa-u.ac.jp.

The reversible NADP⁺-dependent oxidative deamination of *meso*-diaminopimelate (*meso*-DAP) to produce L-2-amino-6-oxopimelate is catalyzed by *meso*-diaminopimelate dehydrogenase (DAPDH; EC 1.4.1.16) (1). The enzyme acts stereoselectively on the D-center of *meso*-DAP, indicating that it recognizes the difference between D- and L-configuration carbons. It was therefore expected that this enzyme would be useful for one-step production of D-amino acids, which are often utilized as source materials for the industrial production of medicines, seasonings, and agrochemicals (2). However, its high substrate specificity for *meso*-DAP has proven to be a major disadvantage for the practical application of DAPDH. To overcome this limitation, Vedha-Peters et al. (3) created an NADP⁺-dependent D-amino acid dehydrogenase (DAADH) from *Corynebacterium glutamicum* DAPDH using error-prone PCR and site-directed mutagenesis. Although this DAADH has reactivity toward several D-amino acids and can be used for stereoselective synthesis of D-amino acids from the corresponding 2-oxo acids and ammonia in the presence of NADPH, the enzyme is not sufficiently stable for use under the conditions necessary for industrial application.

We recently identified and characterized a highly stable DAPDH from the thermophilic bacterium *Ureibacillus thermosphaericus* (4). By introducing five point mutations, as in the case of the *C. glutamicum* enzyme, we succeeded in creating a stable DAADH from *U. thermosphaericus* DAPDH (5). This DAADH does not act on *meso*-DAP but catalyzes the reversible deamination of D-amino acids, such as D-cyclohexylalanine (relative activity, 100%), D-isoleucine (73%), D-2-aminooctanoate (61%), and D-lysine (53%) (5). Gao et al. (6) subsequently identified another thermostable DAPDH from an uncultivable thermophilic bacterium, *Symbiobacterium thermophilum*, which exhibited even more relaxed substrate specificity. They used site saturation mutagenesis to construct a stable DAADH from *S. thermophilum* DAPDH (7).

To date, the three-dimensional structures of the DAPDH-NADP⁺ binary complex (8), the DAPDH-*meso*-DAP binary complex (9), the DAPDH-NADP⁺-inhibitor ternary complex (9), and the DAPDH-NADPH-inhibitor ternary complex (10) have been solved for the mesophilic *C. glutamicum* enzyme. In addition, the structures of *S. thermophilum* DAPDH in its apo form, in complex with NADP⁺, and in complex with both NADPH and *meso*-DAP have been reported (11). We have also determined the structures of *U. thermosphaericus* DAPDH in the apo form and in complex with NADP⁺ and N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) (12). Furthermore, the structures of a *Clostridium tetani* DAPDH mutant exhibiting catalytic ability for the synthesis of D-amino acids (e.g., D-alanine and D-phenylalanine) have been reported (13). Extensive analysis of these structures has shed light on the structure of the substrate-binding site, the structural features responsible for the high thermostability of thermophilic DAPDHs, and the factors underlying the change in substrate recognition between DAPDH and DAADH caused by introducing mutations. However, the information needed for the production of novel DAADH mutants with different substrate specificities remains limited.

As the first step toward the creation of novel DAADH mutants, we determined the crystal structure of apo-DAADH prepared from *U. thermosphaericus* DAPDH. We compared the active-site architecture of the apo-DAADH (open form) with that of *S. thermophilum* DAPDH in complex with substrate (closed form) and sought the amino acid residues responsible for substrate binding. We then used site-directed mutagenesis to identify the residues responsible for determining the enzyme's substrate specificity. We found that a single mutation (D94A), which has not been tested previously, caused a striking change in the substrate specificity of DAADH. We then succeeded in determining the structures of a D94A-Y224F double mutant in complex with NADP⁺ and in complex with both NADPH and 2-keto-6-aminocaproic acid (KACA), the keto acid analogue of lysine. Based on this structural information, the factors responsible for the difference in substrate specificity between *U. thermosphaericus* DAADH and its mutant were evaluated.

FIG 1 Overall structure of *U. thermosphaericus* DAADH and close-up view of the C-terminal tail. (A) Overall structure of the apo-DAADH dimer. The dinucleotide-binding domain, dimerization domain, and C-terminal domain in one subunit are shown in cyan, green, and magenta, respectively. The C-terminal tail is in red. The adjacent subunit is in white. Residues 1, 155 to 157, 223, and 224 in subunit A and residue 1 in subunit B were disordered and not visible in the electron-density map. (B) C-terminal region of C-terminal His-tagged *U. thermosphaericus* DAPDH. (C) C-terminal region of nontagged *U. thermosphaericus* DAADH. (B and C) The C-terminal tail from the adjacent subunit (subunit A) is in yellow, and the ion-pair interactions around the C-terminal residues are shown as dotted lines.

RESULTS AND DISCUSSION

Catalytic properties of nontagged DAADH. Within the *C. glutamicum* DAPDH dimer, the C-terminal tail reportedly extends from one subunit into the other (8). Moreover, the carboxyl group of the C-terminal residue *Val320 (the asterisk indicates a residue in the neighboring subunit) forms an ion-pair network with surrounding residues (His92, Asp120, and Arg128) nearby the substrate-binding site. This suggests that the interaction around the carboxyl group of the C-terminal residue affects the catalytic activity of the enzyme. In an earlier study, we observed that in *U. thermosphaericus* DAPDH (PDB code 3WYB), the C-terminal amino acid residues *Thr327 and *Arg328 extend with the His tag toward the corresponding region in the other subunit but sterically disrupt the formation of an ion-pair network around *Leu326, which is equivalent to the C-terminal *Val320 in *C. glutamicum* DAPDH (Fig. 1B) (12). In the present study, therefore, we prepared and characterized nontagged *U. thermosphaericus* DAADH.

After transforming *Escherichia coli* with pET11a/DAADH, an expression vector encoding nontagged *U. thermosphaericus* DAADH, the crude extract from the recombi-

TABLE 1 Substrate specificities of nontagged DAADH, His-tagged DAADH, and the D94A and Y224F mutants for D-amino acids^a

Amino acid	Nontagged DAADH		His-tagged DAADH		D94A		Y224F	
	Sp act ^b ($\mu\text{mol}/\text{min}/\text{mg}$)	Relative activity (%)	Sp act ^b ($\mu\text{mol}/\text{min}/\text{mg}$)	Relative activity (%)	Sp act ^b ($\mu\text{mol}/\text{min}/\text{mg}$)	Relative activity (%)	Sp act ^b ($\mu\text{mol}/\text{min}/\text{mg}$)	Relative activity (%)
D-Lysine	10.8 $\pm$ 0.1	100	0.299 $\pm$ 0.001	2.8	1.32 $\pm$ 0.06	12	2.25 $\pm$ 0.02	21
D-Arginine	0.722 $\pm$ 0.024	6.7	0.00738 $\pm$ 0.0002	0.068	0.0815 $\pm$ 0.0027	0.76	0.337 $\pm$ 0.012	3.1
D-Norleucine	0.419 $\pm$ 0.020	3.9	0.107 $\pm$ 0.002	0.99	4.22 $\pm$ 0.10	39	0.315 $\pm$ 0.008	2.9
D-Methionine	0.213 $\pm$ 0.006	2.0	0.0178 $\pm$ 0.0009	0.17	2.60 $\pm$ 0.11	24	0.173 $\pm$ 0.004	1.6
D-Leucine	0.191 $\pm$ 0.002	1.8	0.106 $\pm$ 0.004	0.98	4.80 $\pm$ 0.11	45	0.0895 $\pm$ 0.0015	0.83
D-Valine	0.121 $\pm$ 0.004	1.1	0.0142 $\pm$ 0.0008	0.13	0.185 $\pm$ 0.005	1.7	0.0711 $\pm$ 0.0017	0.66
D-Isoleucine	0.113 $\pm$ 0.002	1.0	0.193 $\pm$ 0.003	1.8	1.70 $\pm$ 0.05	16	0.0763 $\pm$ 0.0016	0.71
D-Phenylalanine	0.099 $\pm$ 0.002	0.92	0.0106 $\pm$ 0.0002	0.098	5.33 $\pm$ 0.14	49	0.0769 $\pm$ 0.0005	0.71
D-Tryptophan	0.0369 $\pm$ 0.0020	0.34			0.244 $\pm$ 0.006	2.3	0.0186 $\pm$ 0.0002	0.17
D-Histidine	0.0168 $\pm$ 0.0003	0.16			0.139 $\pm$ 0.003	1.3	0.0139 $\pm$ 0.0001	0.13

^aThe specific activity of nontagged DAADH with D-lysine was defined as 100% relative activity.^bMeans and standard deviations from three independent experiments.

nant cells exhibited strong D-lysine oxidation activity. Nontagged DAADH was effectively purified through heat treatment, followed by three column chromatography steps, and about 27 mg of purified enzyme was obtained from 0.5 liters of *E. coli* culture. The purified enzyme gave a single band on SDS-PAGE gels. Nontagged DAADH exhibited maximum activity at around pH 10.5 for D-lysine oxidative deamination and pH 9.0 for 2-oxooctanoate reductive amination. The optimum temperature for D-lysine oxidative deamination was 70°C. The activation energy was estimated to be about 71.2 kJ/mol when 30 mM D-leucine was used as the substrate. When incubated for 30 min at various temperatures, the enzyme lost no activity at temperatures below 65°C and retained 30% of its full activity at 70°C. When we assessed the effect of temperature on the activity and stability of C-terminal His-tagged DAADH under the same conditions, the optimum temperature for D-lysine oxidative deamination was 60°C, and activity was completely lost at 70°C (data not shown). Thus, the thermostability of nontagged DAADH is much greater than that of His-tagged DAADH. Interestingly, the nontagged DAADH had a specific activity (V_{max} for D-lysine oxidation) of about 23.2 ± 0.8 $\mu\text{mol}/\text{min}/\text{mg}$ at 50°C (see Table 4), which is about 83 times higher than that reported for C-terminal His-tagged DAADH (0.28 $\mu\text{mol}/\text{min}/\text{mg}$) (5). The K_m values for D-lysine and NADP⁺ were estimated to be 38.1 ± 1.7 mM and 31.9 ± 1.8 μM , respectively.

We next analyzed the substrate specificity of nontagged and His-tagged DAADHs for oxidative deamination under the standard assay conditions (substrate concentration, 30 mM). In both cases, the highest activity was observed with D-lysine (Table 1), but nontagged DAADH exhibited 36 times greater activity than His-tagged DAADH. Although the activities with D-arginine, D-norleucine, D-methionine, D-leucine, D-valine, D-phenylalanine, and D-isoleucine were lower than those with D-lysine, the specific activities of nontagged DAADH with these substrates were 2 to 100 times higher than was observed for His-tagged DAADH, except for D-isoleucine (Table 1). For the reductive amination, nontagged DAADH also exhibited 6 to 10 times higher activity with 2-oxooctanoate (2-amino-octanoate 2-oxo-acid analogue), 2-oxohexanoate (norleucine 2-oxo-acid analogue), 2-oxo-4-methylthiobutanoate (methionine 2-oxo-acid analogue), 2-oxo-4-methylpentanoate (leucine 2-oxo-acid analogue), 2-oxopentanoate (norvaline 2-oxo-acid analogue), 2-oxo-3-methylbutanoate (valine 2-oxo-acid analogue), 2-oxobutanoate (α -aminobutyrate 2-oxo-acid analogue), 2-oxo-3-methylpentanoate (isoleucine 2-oxo-acid analogue), phenylpyruvate (phenylalanine 2-oxo-acid analogue), and pyruvate than did His-tagged DAADH (5 mM 2-oxo-acid analogues were used except for phenylpyruvate [2 mM]) (Table 2). Apparently, the C-terminal His tag has a strongly negative effect on the enzyme's catalytic activity.

Structure of apo-DAADH. The structure of apo-DAADH (PDB code 5GZ1) was determined using molecular replacement and was refined at a resolution of 1.78 Å (Table 3). The asymmetric unit consisted of a single homodimer. The overall fold of the monomer was nearly identical to that of C-terminal His-tagged *U. thermosphaericus*

TABLE 2 Substrate specificity of nontagged DAADH, His-tagged DAADH, and the D94A and Y224F mutants for 2-oxo acids^a

Amino acid	Nontagged DAADH		His-tagged DAADH		D94A		Y224F	
	Sp act ^b ($\mu\text{mol/min/mg}$)	Relative activity (%)	Sp act ^b ($\mu\text{mol/min/mg}$)	Relative activity (%)	Sp act ^b ($\mu\text{mol/min/mg}$)	Relative activity (%)	Sp act ^b ($\mu\text{mol/min/mg}$)	Relative activity (%)
2-Oxooctanoate	13.2 $\pm$ 0.1	100	2.27 $\pm$ 0.06	17	65.5 $\pm$ 0.8	496	11.7 $\pm$ 0.5	89
2-Oxohexanoate	9.21 $\pm$ 0.13	70	1.03 $\pm$ 0.06	7.8	35.0 $\pm$ 0.9	264	7.56 $\pm$ 0.17	57
2-Oxo-4-methylthiobutanoate	6.37 $\pm$ 0.09	48	0.624 $\pm$ 0.012	4.7	25.0 $\pm$ 0.8	189	6.22 $\pm$ 0.12	47
2-Oxo-4-methylpentanoate	4.78 $\pm$ 0.11	36	0.624 $\pm$ 0.012	4.7	15.7 $\pm$ 0.2	119	4.64 $\pm$ 0.06	35
2-Oxopentanoate	4.72 $\pm$ 0.08	36	0.465 $\pm$ 0.015	3.5	7.63 $\pm$ 0.20	58	3.44 $\pm$ 0.10	26
2-Oxo-3-methylbutanoate	2.54 $\pm$ 0.05	19	0.279 $\pm$ 0.004	2.1	1.04 $\pm$ 0.03	7.9	1.89 $\pm$ 0.01	14
2-Oxobutanoate	2.47 $\pm$ 0.03	19	0.201 $\pm$ 0.002	1.5	0.859 $\pm$ 0.052	6.5	0.85 $\pm$ 0.01	6.5
2-Oxo-3-methylpentanoate	2.23 $\pm$ 0.05	17	0.310 $\pm$ 0.007	2.4	3.90 $\pm$ 0.06	30	2.25 $\pm$ 0.07	17
Phenylpyruvate	1.93 $\pm$ 0.02	15	0.235 $\pm$ 0.005	1.8	16.1 $\pm$ 0.3	122	1.67 $\pm$ 0.02	13
Pyruvate	0.470 $\pm$ 0.029	3.6	0.0688 $\pm$ 0.0020	0.52	0.131 $\pm$ 0.004	0.99	0.138 $\pm$ 0.003	1.0

^aThe specific activity of nontagged DAADH with 2-oxooctanoate was defined as 100% relative activity.^bMeans and standard deviations from three independent experiments.

DAPDH (PDB code [3WYB](#)); superposition of DAADH subunit B onto that of DAPDH yields a backbone root mean square deviation (RMSD) of 1.2 Å. As with DAPDH, each monomer in DAADH consisted of three domains, i.e., a dinucleotide-binding domain, a dimerization domain, and a C-terminal domain, and the C-terminal tail extended from

TABLE 3 Data collection and refinement statistics^a

Parameter	apo-DAADH (PDB code 5gz1)	NADP ⁺ -bound D94A-Y224F mutant (PDB code 5gz3)	NADPH-KACA-bound D94A-Y224F mutant (PDB code 5gz6)
Data collection			
Wavelength (Å)	1.0	1.0	1.0
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁	P2 ₁ 2 ₁ 2 ₁
Unit cell parameters			
<i>a</i> (Å)	57.4	63.6	54.8
<i>b</i> (Å)	83.2	78.2	94.8
<i>c</i> (Å)	142.2	69.0	138.8
β (°)		107.3	
Total no. of reflections	461,803	508,421	523,031
No. of unique reflections	65,090	81,038	74,999
Multiplicity	7.1 (7.2)	6.3 (5.6)	7.0 (7.0)
Completeness (%)	98.2 (99.5)	93.7 (83.1)	99.8 (100.0)
$R_{p.i.m.}^b$	0.018 (0.120)	0.035 (0.206)	0.048 (0.658)
$\langle I/\sigma(I) \rangle$	31.7 (9.4)	16.0 (5.6)	9.2 (2.04)
Refinement			
Resolution range (Å)	50–1.78 (1.83–1.78)	50–1.59 (1.62–1.59)	50–1.74 (1.77–1.74)
R/R_{free} (%) ^c	18.1/23.1 (22.5/27.4)	19.1/22.9 (25.2/27.3)	20.8/25.0 (34.4/37.1)
No. of:			
Protein atoms	5,049	4,983	4,931
Water molecules	441	370	397
Ligands		Ethylene glycol, 4; NADP ⁺ , 2	Acetate ion, 1; NADPH, 1; KACA, 1; sulfate ion, 2
B-factor (Å ²)			
Protein	33.4	20.7	35.3
Water	41.2	26.5	39.3
Ethylene glycol		20.0	
NADP ⁺ /NADPH		23.8	35.6
KACA			50.9
Acetate ion			46.8
Sulfate ion			56.7
RMSD			
Bond length (Å)	0.023	0.030	0.028
Bond angle (°)	2.0	2.6	2.4
Ramachandran plot (%)			
Favored regions	99.1	98.1	98.6
Allowed regions	0.9	1.9	1.4
Outliers	0	0	0

^aValues in parentheses represent the highest resolution data shell.^b $R_{p.i.m.} = \sum_{hkl} (1/[r_{hkl} - 1])^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$.^c R_{free} calculated with randomly selected reflections (5%).

FIG 2 Stereographic close-up of the *meso*-DAP-binding site in *S. thermophilum* DAPDH. (A) Hydrogen bonds (dotted lines) around the L-amino acid center of *meso*-DAP. (B) Hydrogen bonds around the D-amino acid center. The structure of NADPH-*meso*-DAP-bound *S. thermophilum* DAPDH (11) (subunit B; white residues and black labels with "/S") is superimposed on that of *U. thermosphaericus* apo-DAADH (subunit B; green residues and red labels with "/U").

one subunit into the other subunit (Fig. 1A). However, the interaction around the carboxyl group of the C-terminal residue distinctly differed between the two enzymes. In the nontagged DAADH, the carboxyl group of C-terminal *Leu326 formed an ion-pair network with the side chains of His96, Asp124, and Arg132 (Fig. 1C). In contrast, the C-terminal residues with the His tag in DAPDH sterically hinder access of the His96 side chain to *Leu326, as mentioned above (Fig. 1B). A similar event would also occur with His-tagged DAADH. From the large difference in the reaction rates between His-tagged and nontagged DAADHs, it appears that formation of the ion-pair interactions around the C-terminal residue is essential for full enzymatic activity.

The crystal structure of the *S. thermophilum* DAPDH-NADPH-*meso*-DAP ternary complex has been reported (11). Superposition of this structure (PDB code 3WBF, subunit B) onto that of *U. thermosphaericus* apo-DAADH (subunit B) enabled an estimation of the amino acid residues involved in substrate binding, as well as the induced-fit movement of these residues. Within the structure of the *S. thermophilum* DAPDH-NADPH-*meso*-DAP complex, the α -carboxylate of the L-amino acid center of *meso*-DAP forms four hydrogen bonds with the side chains of Thr171, Arg181, and His227, and the α -amino group of the L-center forms one hydrogen bond with the side chain of His94 via a water molecule (Fig. 2A). The residues that interact with the L-center

TABLE 4 Kinetic parameters of nontagged DAADH and the D94A mutant for D-amino acids and 2-oxo acids^a

2-Oxo acid	Nontagged DAADH				D94A mutant			
	$V_{\max}$ ($\mu\text{mol}/\text{min}/\text{mg}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1}/mM)	$V_{\max}$ ($\mu\text{mol}/\text{min}/\text{mg}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1}/mM)
2-Oxooctanoate	116 $\pm$ 8	39.0 $\pm$ 3.4	69.4 $\pm$ 4.8	1.78 $\pm$ 0.04	91.9 $\pm$ 3.7	4.32 $\pm$ 0.41	54.9 $\pm$ 2.2	12.7 $\pm$ 0.4
Phenylpyruvate	3.73 $\pm$ 0.15	3.89 $\pm$ 0.15	2.24 $\pm$ 0.09	0.574 $\pm$ 0.09	92.5 $\pm$ 4.1	11.1 $\pm$ 0.5	55.3 $\pm$ 2.4	4.98 $\pm$ 0.02
D-Lysine	23.2 $\pm$ 0.8	38.1 $\pm$ 1.7	13.9 $\pm$ 0.45	0.365 $\pm$ 0.006	1.64 $\pm$ 0.13	14.2 $\pm$ 1.7	0.983 $\pm$ 0.075	0.0696 $\pm$ 0.0033
D-Phenylalanine	0.205 $\pm$ 0.008	23.3 $\pm$ 1.4	0.123 $\pm$ 0.005	0.0053 $\pm$ 0.0001	7.71 $\pm$ 0.37	21.3 $\pm$ 1.7	4.61 $\pm$ 0.22	0.217 $\pm$ 0.012
D-Norleucine	0.647 $\pm$ 0.027	6.76 $\pm$ 0.36	0.387 $\pm$ 0.016	0.0572 $\pm$ 0.0011	4.72 $\pm$ 0.09	3.65 $\pm$ 0.15	2.82 $\pm$ 0.05	0.774 $\pm$ 0.033
D-Methionine	0.454 $\pm$ 0.032	20.9 $\pm$ 2.2	0.271 $\pm$ 0.019	0.0130 $\pm$ 0.0005	3.18 $\pm$ 0.06	10.8 $\pm$ 0.3	1.90 $\pm$ 0.04	0.175 $\pm$ 0.001
D-Leucine	0.206 $\pm$ 0.011	3.11 $\pm$ 0.25	0.123 $\pm$ 0.007	0.0396 $\pm$ 0.0012	5.55 $\pm$ 0.14	9.26 $\pm$ 0.13	3.32 $\pm$ 0.08	0.359 $\pm$ 0.004

^aAll measurements were performed in triplicate ($n = 3$), and the values are shown as means and standard deviations.

of *meso*-DAP in *S. thermophilum* DAPDH (His94, Thr171, Arg181, and His227) were completely conserved in *U. thermosphaericus* DAPDH as His96, Thr173, Arg199, and His249, respectively. For creation of DAADH, however, Thr173, Arg199, and His249 in DAPDH were replaced with Ile, Met, and Asn, respectively (Fig. 2A) (5). In *S. thermophilum* DAPDH, on the other hand, the α -carboxylate of the D-amino acid center of *meso*-DAP forms three hydrogen bonds with the main-chain nitrogens of Met152 and Gly153 and the side chain of Asn253 (Fig. 2B). The α -amino group of the D-center forms two hydrogen bonds with the side chain of Asp92 and main-chain O atom of Asp122. Among the residues that interact with the D-center of *meso*-DAP, Asp92, Asp122, Gly153, and Asn253 were conserved as Asp94, Asp124, Gly155, and Asn276, respectively, in *U. thermosphaericus* DAADH (Fig. 2B). Although Met152 was replaced by Leu154 in *U. thermosphaericus* DAADH, the backbone amide of Leu154 is thought to be situated in a position where it can interact with the D-center carboxyl of the substrate, as Met152 does. In *S. thermophilum* DAPDH, the side chain of Tyr205 forms a hydrogen bond with the side chain of Asp92, and through this interaction, Tyr205 is held at the active-site entrance, where it acts as part of the substrate-binding pocket. These residues were conserved as Tyr224 and Asp94, respectively, in *U. thermosphaericus* DAADH. Thus, interactions among these residues and the substrate may also occur upon substrate binding to DAADH, although the side chain of Tyr224 is situated about 13 Å farther away from that of Asp94 in the apo structure (Fig. 2B).

Substrate specificities of the D94A and Y224F mutants. As mentioned above, Tyr224 and Asp94 in *U. thermosphaericus* DAADH are postulated to be situated around the C α of the substrate D-amino acid during catalysis and to form part of the substrate-binding pocket. Until now, however, there have been no reported mutational analyses of these residues, although both of the residues are strictly conserved among all known DAPDHs and DAADHs and may play a key role in substrate binding. We therefore constructed D94A and Y224F mutants and examined their substrate specificities.

When we compared the substrate specificities for oxidative deamination catalyzed by the parent DAADH (nontagged DAADH) and the D94A and Y224F mutants (Table 1), we found that the substrate specificity of the D94A mutant markedly differed from that of the parent enzyme. The D94A mutant utilized D-phenylalanine as the most preferable substrate and had a specific activity of 5.33 $\mu\text{mol}/\text{min}/\text{mg}$ at 50°C, which is 54 times higher than that of the parent enzyme. Although the relative activities with D-leucine, D-norleucine, D-methionine, D-isoleucine, D-tryptophan, and D-histidine were lower than the activity with D-phenylalanine, the specific activities with all these substrates were 6 to 25 times higher than what was observed with the parent enzyme (Table 1). In contrast, the specific activities toward D-lysine and D-arginine were 12.2% and 11.3%, respectively, of those exhibited by the parent enzyme. The $V_{\max}$ and K_m values for D-lysine, D-phenylalanine, D-norleucine, D-methionine, and D-leucine are summarized in Table 4. For the reductive amination, the D94A mutant also showed significantly higher specific activity toward phenylpyruvate (16.1 $\pm$ 0.3 $\mu\text{mol}/\text{min}/\text{mg}$, 8.3 times), 2-oxooctanoate (65.5 $\pm$ 0.8 $\mu\text{mol}/\text{min}/\text{mg}$, 5.0 times), 2-oxohexanoate (3.8 times), 2-oxo-4-methylthiobutanoate (3.9 times), 2-oxo-4-methylpentanoate (3.3

times), 2-oxopentanoate (1.6 times), and 2-oxo-3-methylpentanoate (1.7 times) than the parent enzyme (Table 2). The $V_{\max}$ and K_m values for 2-oxooctanoate and phenylpyruvate were summarized in Table 4. It was somewhat surprising that the D94A mutant exhibited a much higher catalytic efficiency (k_{cat}/K_m) with 2-oxooctanoate, the most preferable substrate for the parent DAADH. On the other hand, the Y224F mutant exhibited less activity than the parent enzyme with all substrates, especially for oxidative deamination, although the substrate spectrum was comparable (Table 1). This suggests that Tyr224 is essential for proper catalytic activity but is not very important for substrate recognition.

Among the DAADHs created from *S. thermophilum* DAPDH, the H227V mutant reportedly has the highest specific activity with phenylpyruvate (about 2.4 $\mu\text{mol}/\text{min}/\text{mg}$ at 30°C) in the reductive amination (7). The *C. tetani* DAPDH mutant also shows catalytic activity toward the synthesis of D-phenylalanine, but the specific activity with phenylpyruvate is only 0.11 $\mu\text{mol}/\text{min}/\text{mg}$ at 30°C (13). The extremely high activity for reductive amination of phenylpyruvate ($V_{\max}$, 92.5 ± 4.1 $\mu\text{mol}/\text{min}/\text{mg}$ at 50°C) (Table 4) is likely a major advantage of using the D94A mutant for direct synthesis of D-phenylalanine.

Structures of D94A-Y224F mutant with bound NADP⁺ or NADPH-KACA. When the dinucleotide-binding domain and dimerization domain of the NADP⁺-bound D94A-Y224F (subunit A) mutant were superimposed on the equivalent domains of the NADPH-KACA-bound D94A-Y224F (subunit A) mutant (Fig. 3A), a large shift in the positions of two loops (loop 1, Lys219-Thr231; loop 2, Gly149-Gly155) toward the active-site cavity was observed in the NADPH-KACA-bound structure. The movement of Phe224 belonging to loop 1 was estimated to be about 10 Å. Consequently, the residue formed a flap over the active-site cavity as the corresponding Tyr205 does in *S. thermophilum* DAPDH. In contrast, the two loops (loop 3, His244-Asn249; loop 4, Leu273-Pro277) were shifted in the opposite direction against the active-site cavity, and substantial movements of Met247 (7.9 Å) in loop 3 and Asn276 (6.8 Å) in loop 4 were observed. The most striking difference in the NADPH-KACA-bound D94A-Y224F mutant was that Lys150 was flipped toward the active-site cavity, with its side chain lying adjacent to the KACA molecule, while the side chain of Lys150 in the NADP⁺-bound D94A-Y224F mutant was facing in the opposite direction.

Within our model, the N atom of KACA forms a hydrogen bond with the side chain of His96 via a water molecule, W1 (Fig. 3B). The O atom of the KACA carboxyl group is hydrogen bonded with the nicotinamide ribose phosphate of NADPH via a water molecule, W2. The O atom of the C-2 carbonyl group interacts with the side chain of Lys150 and, via another water molecule, W3, with W2. In addition, the carbon skeleton of KACA forms hydrophobic interactions with the side chains of Trp123, Trp148, and Lys150. To remove the hydrogen-bonding interaction between the side-chain N atom of Lys150 and the C-2 carbonyl group of KACA, we constructed a K150M mutant and measured the activity after applying heat treatment to the crude extract. The mutation reduced D-lysine oxidation activity only slightly, to about 90% of that seen with the parent enzyme (data not shown). Because C δ and C ϵ of Lys150 formed a total of eight hydrophobic interactions with C-3, C-4, C-5, and C-6 of KACA, the hydrophobic interaction between Lys150 and KACA, not the hydrogen-bonding interaction between them, is likely important for substrate recognition.

Insight into substrate specificity. As mentioned above, the D94A mutant exhibited extremely high reactivity toward D-phenylalanine in oxidative deamination and toward phenylpyruvate in reductive amination. To examine the factors responsible for the difference in substrate recognition, we modeled the phenylpyruvate molecule into the active site of the NADPH-KACA-bound D94A-Y224F mutant based on the orientation of KACA (Fig. 4) and then minimized the energy of the complex using CNS (14). Within this model, an interaction was formed between the carboxyl group of the substrate and the nicotinamide ribose phosphate, as well as between the C-2 carbonyl group of the substrate and the side chain of Lys150. On the other hand, the phenyl group

FIG 3 Structure around the substrate-binding site in the D94A-Y224F mutant. The final model of the NADP⁺-bound D94A-Y224F mutant (PDB code 5GZ3) was composed of amino acid residues 2 to 326 in each subunit (residues 221 to 226 in subunit B were disordered and not visible in the electron-density map), two NADP⁺ molecules, four ethylene glycol molecules, and 370 water molecules. The final model of the NADPH-KACA-bound D94A-Y224F mutant (PDB code 5GZ6) was composed of amino acid residues 2 to 326 in each subunit (residues 156 to 157 and 259 to 263 in subunit A and 156 to 157, 245 to 246, and 259 to 263 in subunit B were disordered and not visible in the electron-density map), one NADPH molecule, one KACA molecule (see supplemental material), one acetate ion, two sulfate ions, and 397 water molecules. (A) The structure of the NADP⁺-bound D94A-Y224F mutant (subunit A; white) is superimposed on that of the NADPH-KACA-bound D94A-Y224F mutant (subunit A). Loops 1 to 4 in the NADPH-KACA-bound D94A-Y224F mutant are shown in cyan, green, purple, and orange, respectively. Phe224, Lys150, Met247, and Asn276 are shown as stick models. (B) Stereographic close-up of the KACA-binding site in the D94A-Y224F mutant. KACA (yellow) and NADPH (magenta) molecules are shown as stick models. The final σ_A -weighted ($F_o - F_c$) omitted electron-density map for KACA is shown at the 2.1σ level. The hydrogen bonds around KACA are shown as dotted lines.

of the substrate was observed to rotate 53° around C-3 in a clockwise direction relative to the C-3–C-4 bond of KACA. The phenyl group was held at that position via stacking interactions with Trp148 and the nicotinamide ring, in addition to hydrophobic interactions with the side chains of Lys150 (C δ and C ϵ ; 8 interactions), Trp123 (CD2, CE3, and CZ3; 5 interactions), and Ala94 (C β , 2 interactions). This binding mode of the phenylpyruvate would not be allowed in the parent DAADH, because the phenyl group is supposed to make unusual short contacts with the side chain of Asp94. The large substrate-binding pocket formed by Trp148, Lys150, Trp123, and Ala94 may also be related to a high activity of the D94A mutant toward 2-oxooctanoate with a bulky side chain. Taken together, our observations suggest that the D94A substitution enlarged the substrate-binding pocket and enhanced the hydrophobicity of the pocket around the side chain of the substrate. Consequently, the D94A mutant gained strong reactivity

FIG 4 Proposed model for phenylpyruvate binding. The NADPH-KACA-bound D94A-Y224F mutant and phenylpyruvate-bound model are shown in white and cyan, respectively. Phenylpyruvate and NADPH molecules in the phenylpyruvate-bound model are in green and magenta, respectively. The hydrogen bonds around phenylpyruvate are shown as dotted lines.

toward hydrophobic D-amino acids, such as D-phenylalanine, D-leucine, D-norleucine, D-methionine, and D-isoleucine.

Because DAPDH acts stereoselectively on the D-center of *meso*-DAP, studies of DAPDH mutations aimed at changing its substrate spectrum have so far been focused on the amino acid residues around the L-amino acid center of the substrate (7, 13). To enlarge the substrate-binding pocket of *S. thermophilum* DAPDH, for example, the four residues (Phe146, Thr171, Arg181, and His227) interacting with the L-center were chosen for site saturation mutagenesis (7). In the present study, Asp94, whose side chain was predicted to interact with the α -amino group of the D-amino acid, was replaced with Ala, and the resulting mutant was found to obtain unexpected substrate specificity. This indicates that Asp94 may be a particularly useful target for mutational study of *U. thermosphaericus* DAADH.

Through the use of *U. thermosphaericus* DAADH, we have already succeeded in developing novel methods for producing D-branched-chain amino acids (D-leucine, D-isoleucine, and D-valine) (15) for subsequent generation of compounds labeled with stable isotopes (D-[1- ^{13}C , ^{15}N]leucine, D-[1- ^{13}C]leucine, D-[^{15}N]leucine, D-[^{15}N]isoleucine, and D-[^{15}N] valine) (15) and for assaying D-isoleucine without interference from the three other isomers (16). As the next step, the creation of an enzyme exhibiting different specificity and higher catalytic efficiency became a key for the further development of D-amino acid production by the method at the industrial level. In this study, we succeeded in creating a novel mutant with an extremely high catalytic activity for phenylpyruvate amination. Moreover, the much higher catalytic activity of the mutant observed with several 2-oxo acid analogues of D-amino acids would be useful for further development of practical applications. Our results may be informative for the creation of novel DAADH mutants exhibiting different substrate specificities.

MATERIALS AND METHODS

Cloning and protein expression. The expression vector (pET-21a/ubtDAPDHmut) for C-terminal His-tagged *U. thermosphaericus* DAADH was constructed as described previously (5). An expression vector for DAADH without a His tag was constructed by amplifying the DAADH gene fragment using PCR. The oligonucleotide primers used for the amplification were 5'-**CATATG**AGTAAAATTAGAATTGGGATTGTTGGTTAC-3', which contains a unique NdeI restriction site (bold) overlapping the 5'-initiation codon, and 5'-**GGATCC**TTATTATAAAAGTTCTTTCTTAAATCTGGAG-3', which contains a unique BamHI restriction site (bold) proximal to the 3' end of the open reading frame. The expression vector pET-21a/ubtDAPDHmut served as the template. The amplified fragment was digested with NdeI and BamHI and ligated with the expression vector pET11a (Novagen, Madison, WI, USA) previously linearized with NdeI and BamHI to generate pET11a/DAADH, which was then used to transform *Escherichia coli* strain Rosetta(DE3) (Novagen). The transformants were cultivated at 37°C in 0.5 liter of LB medium containing 100 $\mu\text{g}/\text{ml}$ ampicillin until the optical density at 600 nm reached 0.6, after which expression

was induced by adding 1 mM isopropyl- β -D-thiogalactopyranoside to the medium, and cultivation was continued for an additional 3 h at 37°C.

Site-directed mutagenesis. Site-directed mutagenesis was accomplished using a QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA), according to the manufacturer's instructions. The expression vector pET11a/DAADH served as the template, and the following oligonucleotides were used as the mutagenic primers (mutation points are underlined): 5'-CAATTGATAGTTTTCGACACACGACG-3' and 5'-CGTGCCTGTGTCGCAAACTATCAATTG-3' for the D94A mutant, 5'-CAATGCCAAATTTCTTGTGATGATG-3' and 5'-CATATTCATCAAAGAAATTTGGCATTG-3' for the Y224F mutant, and 5'-CATATACGTTCTGGGAATGGGTGTAAGCC-3' and 5'-GGCTTACACCCATTCCCAGAACGTAATG-3' for the K150M mutant. For the construction of the D94A-Y224F double mutant, combinations of the mutations (D94A and Y224F) were employed.

Protein purification. *E. coli* cells were harvested by centrifugation, suspended in 10 mM potassium phosphate buffer (buffer A [pH 7.2]), and disrupted by sonication, after which the cell debris was removed by centrifugation ($15,000 \times g$ for 20 min). The resulting supernatant, which served as the crude extract, was heated at 55°C for 30 min, and the denatured proteins were removed by centrifugation ($15,000 \times g$ for 30 min). The supernatant from that step was loaded onto a Toyopearl SuperQ-650M column (1.5 cm by 4 cm; Tosoh, Tokyo, Japan) previously equilibrated with buffer A. The column was then washed with the same buffer, and the enzyme was eluted with a linear gradient of 0 to 0.5 M NaCl in buffer A. The active fractions were pooled, solid $(\text{NH}_4)_2\text{SO}_4$ was added to 35% saturation, and the resulting solution was placed on a Toyopearl Butyl-650M column (1.5 cm by 4 cm; Tosoh) equilibrated with buffer A supplemented with 35% $(\text{NH}_4)_2\text{SO}_4$. The column was then washed with the same buffer, and the enzyme was eluted with a linear gradient of 35 to 0% $(\text{NH}_4)_2\text{SO}_4$ in that buffer. The active fractions were collected, concentrated, and loaded onto a Superdex 200 26/600 column (2.6 cm by 60 cm; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) previously equilibrated with buffer A. The eluted enzyme solution was desalted and concentrated by ultrafiltration (Amicon Ultra 30K nominal molecular weight limit [NMWL]; Millipore, Tokyo, Japan). Expression and purification of the D94A, Y224F, and D94A-Y224F mutants were accomplished using the same method used for DAADH. C-terminal His-tagged *U. thermosphaericus* DAADH was purified, as described previously (5).

Determination of enzyme activity, protein concentration, and kinetic parameters. Enzyme activity was measured by monitoring the increase in absorbance at 340 nm caused by the formation of NADPH through the oxidative deamination of D-amino acids, or the decrease in absorbance caused by the reductive amination of 2-oxo acid. The reaction mixture for oxidative deamination contained 200 mM glycine-NaOH buffer (pH 10.5), 30 mM D-amino acids, 1.25 mM NADP^+ , and the enzyme in a final volume of 1.0 ml. The reaction mixture for reductive amination contained 200 mM glycine-NaOH buffer (pH 9.0), 200 mM NH_4Cl (pH 9.0), 5 mM 2-oxo acid (2 mM for phenylpyruvate), 0.1 mM NADPH, and the enzyme in a final volume of 1.0 ml. After warming the reaction mixture by incubation for 3 min at 50°C without the cofactor, the reaction was started by addition of the cofactor. The appearance and disappearance of NADPH were monitored from the absorbance at 340 nm (extinction coefficient $\epsilon = 6.22 \cdot \text{mM}^{-1} \cdot \text{cm}^{-1}$). The protein concentration was determined using the Bradford method, with bovine serum albumin serving as the standard (17). To determine the kinetic parameters, initial velocities were measured by varying the concentration of one substrate while keeping the concentrations of the other substrates constant, as previously described (18). All measurements were performed in triplicate ($n = 3$), and the values are shown as means and standard deviations.

Optimal temperature, optimal pH, and thermostability. The optimal temperature for the reaction was determined by performing the standard assay for the oxidative deamination of D-lysine at temperatures ranging from 40 to 75°C. To determine the optimal pH for enzyme activity, D-lysine and 2-oxooctanoate were used as the substrates for oxidative deamination and reductive amination, respectively. The buffers (200 mM) used for the assay were glycine-NaOH (pH 9.0 to 10.5) and disodium hydrogen phosphate-NaOH (pH 10.5 to 11.5) for oxidative deamination and glycylglycine-NaOH (pH 8.0 to 9.0) and glycine-NaOH (pH 9.0 to 10.5) for reductive amination. To determine the effect of temperature on its stability, the enzyme (2.2 mg/ml) was incubated for 30 min at different temperatures in 10 mM potassium phosphate buffer (pH 7.2). After centrifugation ($15,000 \times g$ for 5 min), the residual activity in the supernatant was determined using the standard assay method for oxidative deamination with D-lysine as the substrate.

Crystallization and data collection. For crystallization of the apoenzyme, purified nontagged DAADH in 10 mM potassium phosphate buffer (pH 7.2) was concentrated to 20.0 mg/ml using ultrafiltration (Amicon Ultra 30K NMWL). Crystals were obtained using the sitting-drop vapor diffusion method, in which 1 μl of protein solution was mixed with an equal volume of mother liquor composed of 0.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.5), 0.2 M MgCl_2 , and 23% polyethylene glycol 3350 (PEG 3350). Crystals of NADP^+ -bound D94A-Y224F mutant enzyme were grown in sitting drops, in which 1 μl of enzyme solution (20 mg/ml) containing 1 mM NADP^+ and 5 mM D-lysine was mixed with an equal volume of mother liquor composed of 0.1 M cacodylate buffer (pH 7.0), 0.2 M calcium acetate, and 18% PEG 8000. Crystals of the D94A-Y224F mutant in complex with NADPH and KACA (the lysine oxo-analogue) were grown in sitting drops, in which 1 μl of enzyme solution (20 mg/ml) containing 1 mM NADP^+ and 5 mM D-lysine was mixed with an equal volume of the mother liquor composed of 0.1 M sodium acetate buffer (pH 6.5), 0.2 M ammonium sulfate, and 25% PEG 4000. In all cases, sitting drops were equilibrated against 0.1 ml of mother liquor at 20°C.

Diffraction data for crystals of apo-DAADH and the D94A-Y224F mutant enzyme were collected using an ADSC Quantum charge-coupled-device (CCD) detector system on the BL-5a and AR-NW12 beamlines at the Photon Factory, Tsukuba, Japan. All measurements were carried out on crystals cooled to 100 K

in a stream of liquid nitrogen gas using monochromatized radiation at λ of 1.0 Å. Crystals of apo-DAADH and the NADPH-KACA-bound D94A-Y224F mutant were cryoprotected with Paratone-N mixed with paraffin oil (1:1) (Hampton Research, Aliso Viejo, CA), while crystals of the NADP⁺-bound D94A-Y224F mutant were cryoprotected with 30% ethylene glycol. The data were processed using HKL-2000 (HKL Research, Inc., Charlottesville, VA) (19).

Phasing and refinement. The structure of the apo-DAADH was solved to a resolution of 1.78 Å by molecular replacement using the MOLREP program (20) in the CCP4 program suite (21); the structure of chain A from *U. thermosphaericus* DAPDH (PDB code 3WYB) served as the search model. In the final refined model, $R = 0.181$ ($R_{\text{free}} = 0.231$). The structures of NADP⁺-bound and NADPH-KACA-bound D94A-Y224F mutant enzymes were solved to resolutions of 1.59 Å and 1.74 Å, respectively, by molecular replacement using MOLREP (20), with the apoenzyme structure (chain A) as a search model. In the final models, $R = 0.191$ ($R_{\text{free}} = 0.229$) for the NADP⁺-bound D94A-Y224F mutant, and $R = 0.208$ ($R_{\text{free}} = 0.250$) for the NADPH-KACA-bound D94A-Y224F mutant. In all cases, data in the resolution range of 50 to 3 Å were used in the molecular replacement. The program DM (22) was used for noncrystallographic symmetry (NCS) averaging and solvent flattening of the electron-density map (as implemented in the CCP4 program NCSREF). The model building was performed using the program Coot (23). Maximum likelihood refinement at the maximum resolution was performed using REFMAC5 (24). NCS restraints were imposed during initial refinement. Simulated annealing, energy minimization, and B-factor refinement were performed using CNS (14). Then, after several cycles of inspection of the $(2F_o - F_c)$ and $(F_o - F_c)$ electron-density maps, the model was rebuilt. Water molecules were incorporated using Coot (23), and model geometry was analyzed using MolProbity (25). The data collection and refinement statistics are listed in Table 3.

To determine the number of hydrophobic interactions, the interatomic contacts between atoms from hydrophobic side chains were calculated using the WHAT IF Web server (26). A contact was defined as two atoms for which the distance between the Van der Waals surfaces was less than 1.0 Å. Ion pairs, with a cutoff distance of 4.0 Å, were identified using the WHAT IF Web server (26). Hydrogen bonds were identified using the CCP4mg program (27). Molecular graphics figures were created using PyMOL (<http://www.pymol.org/>).

Accession number(s). The atomic coordinates and structural factors for apo-DAADH (PDB code 5GZ1), the NADP⁺-bound D94A-Y224F mutant (PDB code 5GZ3), and the NADPH-KACA-bound D94A-Y224F mutant (PDB code 5GZ6) have been deposited in the Protein Data Bank (<http://www.rcsb.org>).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.00491-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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