Crystal Structures of $\Delta^1$-Piperideine-2-carboxylate/$\Delta^1$-Pyrrylene-2-carboxylate Reductase Belonging to a New Family of NAD(P)H-dependent Oxidoreductases

CONFORMATIONAL CHANGE, SUBSTRATE RECOGNITION, AND STEREOCHEMISTRY OF THE REACTION

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$\Delta^1$-Piperideine-2-carboxylate/$\Delta^1$-pyrrylene-2-carboxylate reductase from Pseudomonas syringae pv. tomato belongs to a novel subclass in a large family of NAD(P)H-dependent oxidoreductases distinct from the conventional MDH/LDH superfamily characterized by the Rossmann fold. We have determined the structures of the following three forms of the enzyme: the unliganded form, the complex with NADPH, and the complex with NADPH and pyrrole-2-carboxylate at 1.55, 1.8, and 1.7 Å resolutions, respectively. The enzyme exists as a dimer, and the subunit consists of three domains; domain I, domain II (NADPH binding domain), and domain III. The core of the NADPH binding domain consists of a seven-stranded predominantly antiparallel $\beta$-sheet fold (which we named SESAS) that is characteristic of the new oxidoreductase family. The enzyme preference for NADPH over NADH is explained by the cofactor binding site architecture. A comparison of the overall structures revealed that the mobile domains I and III change their conformations to produce the catalytic form. This conformational change plays important roles in substrate recognition and the catalytic process. The active site structure of the catalytic form made it possible to identify the catalytic Asp:Ser:His triad and investigate the catalytic mechanism from a stereochemical point of view.

It is well known that the NAD(P)H-dependent malate/lactate dehydrogenase (MDH/LDH), characterized by a six-stranded parallel $\beta$-sheet (Rossmann fold) (1) as the cofactor binding core, constitutes a complex family with multiple enzyme homologs (2). However, MDH from the extremely thermophilic methanogen Methanothermus fervidus and LDH from the Gram-negative bacterium Alcaligenes eutrophus were shown to have no similarity in primary sequence to conventional MDH/LDH family proteins (3, 4). These two enzymes and their homologs have thus been annotated as new MDH/LDHs different from the conventional MDH/LDH. Recent biochemical and genetic studies have revealed that some of the gene products homologous to proteins belonging to the new MDH/LDH family catalyze reactions other than those involved in MDH or LDH activity, as exemplified by L-sulfolactate dehydrogenase (SLDH) (5), ureidoglycolate dehydrogenase (UGDH) (6), 2,3-diketo-1-gulonate reductase (YiaK) (7), and $\Delta^1$-piperideine-2-carboxylate (Pip2C)/$\Delta^1$-pyrrylene-2-carboxylate (Pyr2C) reductase (8). The new MDH/LDH family has now been extended to the new NAD(P)H-dependent oxidoreductase family which contains proteins homologous to, but different in activities from, the new family of MDH/LDH proteins (5, 9). An analysis of the primary sequence similarities and plausible functions of the new family of oxidoreductase proteins allowed the family proteins to be grouped into at least eight clusters (8, 10).

In Pseudomonas strains, the catabolic pathway of d-lysine is different from that of l-lysine although the d- and l-lysines are interconverted by lysine racemase (11–17). l-Lysine is catalyzed via the $\delta$-aminoovalerate pathway containing five carbon fatty acid intermediates to produce glutarate in four steps (11, 12). In contrast, d-lysine is catalyzed via the piperolate pathway containing six carbon cyclic intermediates in which d-lysine is converted to L-$\alpha$-aminoadipate (18). Pip2C reductase has been reported to reduce not only Pip2C, but also its five carbon analog, Pyr2C, to produce L-proline in mammals, plants, and microorganisms (19–21).

A Pseudomonas putida gene, dpkA, encoding a putative protein annotated as a new NAD(P)H-dependent oxidoreductase family distinct from the conventional MDH/LDH superfamily, was cloned and overexpressed in Escherichia coli (8). The gene product (PpDpkA) is the first enzyme in the DpkA clade of the new oxidoreductase family whose function has been identified. The enzyme does not have MDH or LDH activity, but reduces Pip2C and Pyr2C as cyclic imines forming L-pipepcolate and L-proline, respectively (Scheme 1). The gene disruption experiment clarified that the enzyme is essential for the metabolism of d-lysine and d-proline (8). The enzyme preferentially uses NADPH, rather than NADH, as a cofactor in contrast to the enzymes belonging to other clades of the new oxidoreductase family. Except for the enzyme, the action on 2-oxo acids or 2-hydroxy acids is common to proteins...
belonging to other clades. Interestingly, the enzyme has N-methyl-l-amino acid dehydrogenase activity in addition to Pip2C/Pyr2C reductase activity (Scheme 1, and Refs. 22 and 23). The protein catalyzes the high enantioselective synthesis of N-methyl-l-amino acids from methylamine and various α-keto acids. N-Methyl-l-amino acids and l-pipe- colate are constituents of bioactive peptides and polyketides, respectively, and are useful for the synthesis of pharmaceutical products and pesticides.

The dpkA gene product from Pseudomonas syringae pv. tomato, which has been cloned and expressed in E. coli, was found to have a function similar to that from P. putida (8). P. syringae DpkA (PsDpkA) has 343 residues per subunit, with a subunit molecular weight of 36,249. We have determined the structures of the unliganded PsDpkA, PsDpkA in a complex with NADPH, and PsDpkA in a complex with NADPH and the substrate analogue, pyrrole-2-carboxylate (P2CA), where the pyrrole line ring of the substrate Pyr2C is replaced by the pyrrole ring, at 1.55-, 1.8-, and 1.7-Å resolution, respectively. The enzyme exists as a dimer, and are useful for the synthesis of pharmaceutical products and pesticides.

EXPERIMENTAL PROCEDURES

Materials—P. syringae pv. tomato DSM50315 was obtained from DMSZ (Braunschweig, Germany). Pip2C and Pyr2C were synthesized as previously described (20). HisBind Resin was obtained from Novagen (Madison, WI). NADH and NADPH were acquired from Oriental Yeast (Tokyo, Japan). Restriction enzymes and kits for genetic manipulation were purchased from Takara Shuzo (Kyoto, Japan), Toyobo (Osaka, Japan), New England Biolabs (Beverly, MA), and Stratagene. All other reagents were of analytical grade and obtained from Nacalai Tesque (Kyoto, Japan) and Wako Pure Chemical Industries (Osaka, Japan).

Cloning and Expression of DpkA from P. syringae in E. coli—PCR primers were designed based on the nucleotide sequence of an open reading frame, PSPTO2359, located on the 2,613,769–2,614,800-bp region of the P. syringae pv. tomato DC3000 genome. A 1-kbp DNA fragment containing a PsdpkA gene was amplified from the genomic DNA of P. syringae pv. tomato strain DSM50315 by PCR with a PerkinElmer Thermal Cycler 480. PCR was performed in a 50-μl reaction mixture containing 1× Pfu Ultra HF buffer (Strategene), 0.2 mM dNTP, 0.2 mM of each primer (5′-GGGAATTCATATGTCCGCCAGCCACGC-3′ and 5′-GGGAAAGCTTTGCGTCGTCCGATCCGTCT-GCAAAC-3′), 2.5 units of Pfu Ultra HF DNA polymerase, and 180 ng of genomic DNA with preincubation at 95 °C for 2 min followed by 22 cycles of 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 2 min and a final elongation step at 72 °C for 10 min. The PCR product was digested with Ndel and HindIII and ligated into pET21a (+) previously digested with the same restriction enzymes. The resultant plasmid, pPsDPKA, was introduced into E. coli BL21(DE3) to provide the recombinant PsDpkA. The recombinant protein contained eight non-native residues (LEHHHHHH) at the C terminus.

Purification of PsDpkA—E. coli BL21(DE3) carrying pPsDPKA was cultivated in LB medium (3 liters) containing 100 μg/ml ampicillin at 37 °C for 16 h. The culture was supplemented with 1 mm isopropyl-β-d-thiogalactopyranoside and grown for 5 h. The wet cells obtained by centrifugation were suspended in 60 ml of 1× binding buffer containing 5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl (pH 7.9), and 1 mM phenylmethylsulfonyl fluoride. The crude extract obtained by sonication was loaded onto a His-Bind column (30 ml) equilibrated with the 1× binding buffer. The enzyme was eluted according to a His-Bind purification kit protocol. The enzyme fractions were pooled and dialyzed against the 20 mM Tris-HCl buffer (pH 7.0) containing 200 mM l-histidine, and then against the 20 mM Tris-HCl buffer (pH 7.0) 2×. The enzyme solution was concentrated with Centriprep YM-10 (Millipore, Bedford, MA). The final preparation of the enzyme was stored at −80 °C, which did not cause significant inactivation, until use.

Enzyme Assays—N-Methyl-l-amino acid dehydrogenase assays were carried out by measuring the decrease in the amount of NADPH at 340 nm with an MPS-2000 spectrophotometer (Shimadzu, Kyoto, Japan) at 30 °C (24). A standard reaction mixture contained 10 mM pyruvate, 60 mM methylamine-H₂SO₄ (pH 10.0), 0.2 mM NADPH, and the enzyme in a final volume of 1 ml. The Pip2C/Pyr2C reductase was assayed with a decrease in absorbance at 340 nm because of the consumption of NADPH at 30 °C in 1 ml reaction mixture containing 0.062 mM Pip2C (or Pyr2C), 0.15 mM NADPH, and 100 mM Tris-HCl (pH 8.0). The reverse reaction was followed in the same manner except that 20 mM l-pipecolate (or l-proline), 0.25 mM NADP⁺, and 100 mM Bis-Tris propane (pH 10.0) were substituted for the corresponding components.

Crystallization and Data Collection—Crystals of the unliganded PsDpkA were obtained using the vapor diffusion technique in a hanging drop. The initial screening for the crystallization conditions was performed using the sparse matrix screens Crystal Screen I and II from
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Structure Determination and Refinement—The structure of the unliganded enzyme was solved by the multiple isomorphous replacement method, using two isomorphous data sets. The scaling of all the data and map calculations was performed with the CCP4 program suite (28). The program SHARP was used for locating the mercury and platinum sites and for phasing (29). The map was of good quality, and the model of the subunits was gradually built into the 2.0-Å map through several cycles of model building using the program O (30). The structure of the unliganded enzyme was refined by simulated annealing and energy minimization with the program CNS (31). A random sample with 10% of the reflections was set apart for the calculation of the $R_{merge}$. Solvent molecules were picked up on the basis of the peak heights (3.0 $\sigma$) and distance criteria (4.0 Å from protein or solvent) from the $\sigma_A$-weighted $F_{o} - F_{c}$ map. The procedure converged to $R_{factor}$ and $R_{free}$ values of 0.194 and 0.215, respectively.

The same refinement procedure was applied to PsDpkA-NADPH, but using the coordinates of the unliganded enzyme as the initial model. When the $R_{factor}$ value became less than 30%, the simulated annealing $2F_{o} - F_{c}$ map showed the residual electron density corresponding to the bound NADPH. Solvent molecules were picked up from the sigmaA-weighted $F_{o} - F_{c}$ map, and further model building and refinement cycles gave $R_{factor}$ and $R_{free}$ values of 0.188 and 0.209, respectively. The same refinement procedure was applied to PsDpkA-NADPH-P2CA, but using the coordinates of the unliganded enzyme as the initial model. When the $R_{factor}$ value became less than 30%, the simulated annealing $2F_{o} - F_{c}$ map showed the residual electron density corresponding to the bound NADPH and P2CA. Solvent molecules were picked up from the sigma-weighted $F_{o} - F_{c}$ map, and further model building and refinement cycles gave $R_{factor}$ and $R_{free}$ values of 0.178 and 0.194, respectively (TABLE ONE).

Quality of the Structure—The final model of the unliganded enzyme comprises 332 residues (10–341) out of 343 residues for subunit A and 337 residues (7–343) for subunit B with 684 water molecules. The average thermal factors of the main chain atoms (N, Ca, C, and O) in subunits A and B are 20.3 and 19.2 Å$^2$, respectively. The final model of PsDpkA-NADPH comprises 332 residues (10–341) out of 343 residues for subunit A, 337 residues (7–343) for subunit B, and two NADPH with 521 water molecules. The average thermal factors of the main chain atoms (N, Ca, C, and O) in subunits A and B are 21.2 and 20.1 Å$^2$, respectively. The final model of PsDpkA-NADPH-P2CA comprises 332 residues (10–341) out of 343 residues for subunit A, 337 residues (7–343) for subunit B, two NADPH, and two P2CA with 592 water molecules. The average thermal factors of the main chain atoms in subunits A and B are 16.6 and 16.4 Å$^2$, respectively. The differences in the number of residues and the average thermal factors between the subunits A and B in the final models are because of the asymmetry of the intermolecular interactions. The thermal factors for both complexes imply that crystal packing has less of an effect on subunit A than on subunit B.

Hampton Research (25). 2 µl of a protein solution at a concentration of 6 mg/ml were mixed with an equal volume of a reservoir solution containing 0.8 M Na/K tartrate, 20% (v/v) glycerol, and 0.1 M Tris-HCl (pH 7.5) and equilibrated against 400 ml of a reservoir solution at 293 K. Within 3 days, crystals had grown to dimensions of 0.8×1.8×2.0 mm$^3$. Crystals of PsDpkA-NADPH and DpkA-NADPH-P2CA were obtained by soaking unliganded crystals in solutions containing heavy atom reagents. The data sets for heavy atom derivatives were collected to 2.0- and 2.5-Å resolution at 100 K on an R-AXIS IV++ image-plate detector equipped with Osmic Maxflux optics using a wavelength of 1.54 Å (Cu Ka) from a Rigaku rotating anode generator operated at 40 kV and 100 mA. The data were processed using HKL2000 (27) or Crystalclear (Molecular Structure Corporation, a Rigaku company) (TABLE ONE).

| TABLE ONE Data collection and refinement statistics |
|-----------------------------------------------|
| PDB ID | Native | NADPH | NADPH+P2CA |
|--------|--------|-------|------------|
| 1WTJ   | 2CWF   | 2CWH  |

| Diffraction data | Native | NADPH | NADPH+P2CA |
|------------------|--------|-------|------------|
| Resolution (Å)   | 1.55   | 1.80  | 1.70       |
| No. of unique reflections | 119,237 | 77,231 | 92,217 |
| Completeness (%)  | 97.8 (98.3)* | 99.8 (99.1)* | 99.9 (100.0)* |
| $R_{merge}$ (%)   | 4.7 (30.9)* | 5.0 (28.9)* | 7.3 (26.9)* |
| $I/\sigma$        | 39.0 (4.7)* | 33.2 (4.4)* | 35.0 (5.0)* |

| Refinement | Native | NADPH | NADPH+P2CA |
|------------|--------|-------|------------|
| Resolution limits (Å) | 37.5–1.55 | 37.5–1.80 | 37.6–1.70 |
| $R_{factor}$ (%) | 19.39 | 18.82 | 17.81 |
| $R_{free}$ (%) | 21.47 | 20.90 | 19.35 |

| Deviations | Native | NADPH | NADPH+P2CA |
|------------|--------|-------|------------|
| Bond lengths (Å) | 0.004 | 0.005 | 0.005 |
| Bond angles (deg) | 1.28 | 1.28 | 1.31 |

| Mean B factors | Native | NADPH | NADPH+P2CA |
|----------------|--------|-------|------------|
| Main chain atoms (Å$^2$) | 19.74 | 20.68 | 16.51 |
| Side chain atoms (Å$^2$) | 23.05 | 24.13 | 19.51 |
| Hetero atoms (Å$^2$) | – | 27.80 | 15.92 |
| Water atoms (Å$^2$) | 34.36 | 33.59 | 29.93 |

| Ramachandran plot | Native | NADPH | NADPH+P2CA |
|-------------------|--------|-------|------------|
| Favorable | 92.2 | 91.8 | 91.6 |
| Additional allowed | 6.9 | 7.3 | 7.1 |
| Generously allowed | 0.9 | 0.9 | 1.3 |
| Disallowed | 0.0 | 0.0 | 0.0 |

*The values in parentheses are for the highest resolution shells (1.61–1.55, 1.86–1.80, and 1.76–1.70 Å) in the native enzyme, complexed with NADPH, and complexed with NADPH and P2CA, respectively.

**$R_{merge} = \sum_{hkl} \sum_{i} |F_{o,hkl,i} - \langle F_{o,hkl,i} \rangle| / \sum_{hkl} \sum_{i} |F_{o,hkl,i}|$, where $I$ = observed intensity and $\langle I \rangle$ = average intensity for multiple measurements.
RESULTS AND DISCUSSION

Identification and Expression of the dpkA Gene of *P. syringae* and Purification of the Recombinant Enzyme—The complete sequence of the genome of *P. syringae* pv. tomato DC3000 has been reported by Buell et al. (36). A BLAST search of the *P. syringae* genome sequence using the PsDpkA (DpkA from *P. putida* ATCC12633) sequence identified a putative protein, PSPTO2359 (NCBI database accession AA058570), which has been annotated as UGDH encoded by the *allD* gene (36). However, our recent sequence analysis indicated that the annotation for PSPTO2359 is not correct, and we have provided a revised annotation for the gene product as a DpkA protein, which is a multifunctional enzyme with activities of Pip2C/Pyr2C reductase (8) and N-methyl-l-α-amino acid dehydrogenase (24). The amino acid sequence of *P. syringae* DpkA shows 71% sequence identity with that of PpDpkA.

The dpkA gene of *P. syringae* pv. tomato DSM50315 was cloned in *E. coli* by PCR using primers designed on the basis of the PSPTO2359 gene of *P. syringae* pv. tomato DC3000. The nucleotide and deduced amino acid sequences of the cloned gene PsdpkA (DDBJ/EMBL/GenBankTM accession DQ017704) were slightly (eight nucleotides and four amino acid sequences of the cloned gene

The enzyme was purified to homogeneity as a C-terminal histidine-tagged fusion protein. Typically, ~30 mg of the purified enzyme was obtained from 1 liter of *E. coli* culture.

**Characteristics of PsDpkA**—SDS-PAGE analysis of the purified PsDpkA produced only one band with a subunit molecular mass of 36 kDa, which is in good agreement with that calculated from the amino acid sequence (36,249 Da). Purified PsDpkA catalyzed the NADPH-dependent reduction of Pip2C (specific activity, 150 μmol/min·mg⁻¹) and Pyr2C (390 μmol/min·mg⁻¹) to form pipicole and proline, respectively. The enzyme also catalyzed the formation of N-methylalanine from pyruvate and methyamine with a specific activity of 140 μmol/min·mg⁻¹. PsDpkA acted on other α-keto acids, such as α-ketoisovalerate (23%, relative to pyruvate), phenylpyruvate (6.3%), α-ketobutyrate (17%), fluoropyruvate (14%), α-ketoisocaproate (13%), bro-mopyruvate (10%), and α-ketovalerate (8.9%). However, a branch chain α-keto acid, α-ketoisovalerate, was inert. PsDpkA specifically acted on methyamine and not on ammonia. Therefore, the substrate specificities of PsDpkA toward α-keto acids and amines were similar to those of PpDpkA (24). The substrate specificity of the enzyme in the NADP⁺-dependent oxidative reaction was also similar to that of PpDpkA (data not shown) (24).

PsDpkA showed its maximum activity between 30 and 45 °C and was stable between 0 and 35 °C after a 30-min incubation in 20 mM Tris-HCl, pH 7.0. The specific activity of PsDpkA with NADPH is 88× higher than that with NADH for the reductive amination of pyruvate. Thus, NADPH serves as the preferred cofactor for the enzyme. A substrate analog, P2CA, inhibits PsDpkA (8). PsDpkA was also inhibited by P2CA and 2-picolinate. PsDpkA showed 5 and 37% of its original activity in the presence of 1.0 mM P2CA and 1 mM 2-picolinate, respectively.

**Overall Structure**—The overall structure of PsDpkA-NADPH-P2CA is shown in Fig. 1A. The polypeptide chain is folded into a dimeric form with a noncrystallographic 2-fold axis. The subunit structure with secondary structure assignments by the program DSSP (37) is shown in Fig. 1B. Each subunit is divided into three domains based on the rotation of two regions of the enzyme as rigid bodies, which is revealed by the subunit superposition of the unliganded enzyme, PsDpkA-NADPH, and PsDpkA-NADPH-P2CA (see open-closed conformation change below).

Domain I is formed by two parts of the polypeptide chain from the N terminus to Trp-70 and from Arg-314 to the C terminus. Domain II forms the NADPH binding site and consists of two parts of the polypeptide chain from Val-71 to Ala-186 and from His-236 to Asp-313. Domain III comprises the residues from Thr-187 to Gly-235. Domain II occupies the central part of the subunit with domains I and III located at either end.

Domain I has a pseudo four-helix bundle structure (α-helices a1, a2, a3, and a11) carrying the α-helix a10 and the antiparallel β-sheet (b1 and b15) at one end of the bundle. The catalytic His-54 is located on a loop between α-helices a1 and a2. Domain II assumes the open α/β structure with four α-helices (α4, α5, a8, and a9) and eleven β-strands (b2, b3, b4, b5, b6, b7, b8, b9, b10, b13, and b14) as the secondary structures. The core of the NADPH binding domain II is folded into a seven-stranded antiparallel β-sheet (b2, b3, b5, b13, b6, b9, and b10), although the strands b3 and b5 are parallel. The sheet is surrounded by two α-helices (α4 and a5) from the solvent side and four α-helices (a8, a9, a8', and a9') from the protein side, and faces the 2-fold symmetry-related central β-sheet of the other subunit. Domain III is comprised of two α-helices (a6 and a7) and a two-stranded antiparallel β-sheet (b11 and b12).

Domains I and III are mobile, and their conformational change is essential for catalytic activity by restricting the active site cleft size (see open-closed conformation change below). The NADPH binding cavity is formed at the domain interface and at the subunit interface with the nicotinamide-ribose moiety of NADPH directly interacting with one side of the antiparallel β-sheet of domain II.

The program DALI (38) was used to search the Protein Data Bank data base for enzymes possessing three-dimensional structures similar to that of the unliganded PsDpkA. The highest Z scores (strength of structural similarity) were calculated to be 36.1 for SLDH (Ref. 5, PDB ID:1RFM), 34.3 for YiaK (Ref. 9, PDB ID:1NXU and 1S20), and 28.4 for UGDH (PDB ID:1XRH). The NAD(P)H binding domains of these enzymes and PsDpkA are folded into the seven-stranded predominantly antiparallel β-sheet distinct from the Rossmann fold of the six-stranded parallel β-sheet. SLDH, YiaK, UGDH, and PsDpkA are classified into a new family of NAD(P)H-dependent oxidoreductases. We propose that the β-sheet fold characteristic of the new family of oxidoreductases proteins is referred to as the SESAS fold.

The SESAS fold and the α-helices (a8 and a9) of domain II are involved in the formation of the subunit interface around a 2-fold axis. The accessible surface area of PsDpkA and its subunit interface are 24,914 and 5,558 Å², respectively, meaning that a surface area equivalent to 18.2% of one subunit is buried upon dimerization. The interactions observed at the subunit interface are mostly hydrophobic and the hydrophobic area of the subunit interface totals 57.2%. Consistent with the relatively large area utilized at each subunit interface, its high hydrophobicity, and the estimated molecular weight of 67,000–86,000 by a dynamic light scattering experiment at room temperature, we note that the enzyme forms a stable dimer. **Open-Closed Conformation Change**—One of the striking features of YiaK belonging to the new family of NAD(P)H-dependent oxidoreductases is the unusual overall conformational change from the closed to the open form upon binding of the substrates (9). Domains I and III move away from domain II to make the active site cavity large, resulting in the exposure of one side of the bound substrate molecule to the solvent (Fig. 2A). In contrast, the conformational change of PsDpkA induced by the substrate binding is more complex. Unliganded PsDpkA and PsDpkA-NADPH have essentially the same overall structure
because the Cα atoms of subunits A and B in the unliganded form are superimposed onto the corresponding Cα atoms in the NADPH complex with RMS deviations of 0.14 and 0.18 Å. This indicates that the binding of the cofactor NADPH does not induce the molecular conformational change in PsDpkA.

Interestingly, the structure of subunit A in the unliganded form or in the NADPH complex is significantly different from that of subunit B, since the superpositioning of Cα atoms of subunit A onto subunit B in the unliganded form resulted in 332 equivalent Cα atoms with an average RMS deviation of 0.98 Å and a maximum displacement of 4.01 Å. An analysis of the calculated RMS deviations shows that domain II in subunit A has a relatively similar structure to that in subunit B with an average RMS deviation of 0.31 Å for 194 Cα atoms. In contrast to domain II, the average RMS deviations corresponding to domains I and III are 1.25 and 1.81 Å for 89 and 49 Cα atoms, respectively. Thus, there are marked differences in the conformations of domains I and III with respect to domain II between subunits A and B. This may result from the conformational flexibility of domains I and III and the differing packing interactions of subunit A from those of subunit B, although the areas of the contact surfaces to neighboring molecules are comparable with 3,974 and 3,482 Å² for subunits A and B, respectively. Domain I of subunit A in the unliganded form can be superimposed onto domain I of subunit B with an average RMS deviation of 0.40 Å for 89 Cα atoms. Similarly, domain III of subunit A can be superimposed onto domain III of subunit B with an RMS deviation of 0.54 Å for 49 Cα atoms, indicating that domains I and III change their conformations as rigid bodies. Domains I and III of subunit A rotate by 3.4° and 10.8°, respectively, compared with those of subunit B to open the active site cleft. The conformation of subunit A in the unliganded form or in the NADPH complex is thus referred to as the open form and that of subunit B as the closed form.

The crystals of PsDpkA-NADPH-P2CA were obtained by soaking the unliganded crystal in a solution containing NADPH and P2CA. The overall structure of subunit A is relatively similar to that of subunit B in
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FIGURE 2. Superimposed backbone structures. A, subunit structures of the open and closed forms in YiaK, and the catalytic form in PsDpkA are superimposed by fitting the Cα atoms of domain II. Domains I and III in the open and closed forms of YiaK and the catalytic form of PsDpkA are shown in light gray and dark gray, respectively. The cofactors and the substrate analogues (tartrate and P2CA) are represented by stick models. B, subunit structures of the open, closed, and catalytic forms in PsDpkA were superimposed by fitting the Cα atoms of domain II. Domains I and III in the open, closed, and catalytic forms are shown in green, cyan, and red, respectively. Domain II in the open form is shown in gray. The cofactor NADPH and the substrate analogue (P2CA) are represented by stick models.

Comparison of PsDpkA with YiaK, SLDH, and UGDH—X-ray structures of YiaK in the unliganded form (25), YiaK complexed with NAD and tartrate (25), SLDH complexed with NADH (5), and UGDH in the unliganded form have been determined. The primary sequence of PsDpkA is 22.6, 23.0, and 27.1% identical with those of YiaK, SLDH, and UGDH, respectively. The overall fold of the PsDpkA subunit was shown to be similar by DALI calculation to those of YiaK, SLDH, and UGDH (see “Overall Structure”).

The structural observations and light scattering studies suggest that PsDpkA, YiaK, SLDH, and UGDH exist and function as dimers. Domain II of subunit A in the catalytic form is superimposed onto the corresponding subunit in the unliganded PsDpkA. Domain II of subunit B in the catalytic form makes no significant rotation (0.3°) with respect to that in the unliganded form. Thus, PsDpkA does not change its quaternary structure upon binding of the substrate. Similarly, domain II of subunit A in the unliganded YiaK, YiaK-NAD-tartrate, SLDH-NADH, and the unliganded UGDH were superimposed onto the corresponding subunit in the unliganded PsDpkA to produce subunit B (domain II) rotation angles of 4.7, 1.7, 1.5, and 1.2°, respectively, with subunit B (domain II) in the unliganded PsDpkA. Therefore, the quaternary structures of these enzymes are essentially the same, although the unliganded YiaK displays significant rotation of subunit B (25).

Domain II of the catalytic form in PsDpkA is superimposed onto the open and closed forms in YiaK (Fig. 2A). This indicates that domains I and III of YiaK rotate around the axes similar to those of PsDpkA and the orientations of domains I and III of YiaK are significantly different from those of PsDpkA. The domain II superimposition shows that domains I and III of PsDpkA, YiaK, SLDH, and UGDH assume similar but significantly different orientations.
Binding Mode of NADPH—Bound NADPH in the complex with NADPH is shown in Fig. 3 along with the \( \sigma \)-weighted \( 2F_\text{o} - F_\text{c} \) electron density. The hydrogen bonding interactions between the protein residues and NADPH are shown in Fig. 4. Two independent NADPH molecules are unambiguously located on the residual electron density map of the complex with NADPH. The binding mode of the independent NADPH molecules is the same irrespective of where the subunits are in their open or closed forms. The large cavity formed at the domain interface and at the subunit interface accommodates NADPH revealing that dimer formation is essential for enzyme function. NADPH is in the extended conformation similar to that observed in the NADH-dependent SLDH or YiaK (5, 9). The nicotinamide-ribose moiety of the cofactor is located at the domain interface and resides on the SESAS fold. The adenine-ribose moiety is placed at the subunit interface with the pyrophosphate on the boundary region between the domain and the subunit interface.

The adenine moiety is sandwiched by Arg-309 and His-236* from both sides with its amino group exposed to the solvent. The asterisk indicates a residue from another subunit of the dimer unit. His-236*, which makes a parallel stack with the adenine ring, is conserved in the four clades of the new family of oxidoreductases (Fig. 3). The 2′-phosphate of the adenine-ribose is surrounded by the cluster of Arg residues (Arg-309 from the loop between the \( \beta \)-strand b14 and the \( \alpha \)-helix a10, and Arg-314 and Arg-315 located at the C-terminal part of \( \alpha \)-helix a10). The enzyme preference for NADPH over NADH is attributable to the Arg cluster, which is conserved in the DpkA clade of the new oxidoreductase family (39), neutralizing the negative charge of the 2′-phosphate and acting as the 2′-phosphate recognition site. NADH-dependent SLDH (5), YiaK (9), and UGDH, which belong to the same new oxidoreductase family as does PsDpkA, do not have the Arg cluster. The Arg cluster is replaced by Arg, Glu, and Phe in YiaK, Leu, Ile, and Glu in SLDH, and Tyr, Asp, and Gln in UGDH, respectively. In the NADH-dependent enzymes, one acidic and one neutral residue take the places of Arg-314 and Arg-315 in PsDpkA, and the acidic residue occupies the position corresponding to that of the 2′-phosphate of NADPH to interact with the 2′-OH of the adenine ribose of NADH. Arg-309 of PsDpkA acts as the adenine recognition site as well as the 2′-phosphate recognition site. Also, in the NADH-dependent enzymes, the residues corresponding to Arg-309 act as the cofactor adenine recognition site. We
thus propose that the replacement of Arg-314 and Arg-315 by the acidic residue and the neutral residue might allow PsDpkA to function as an NADH-dependent enzyme. The 3'OH group of adenine-ribose interacts with the main chain NH group of Asp-184 on the b10-strand.

The pyrophosphate group is recognized by His-236 and Lys-237* of helix a8 from the other subunit, the main chain NH group of Ala-186 on b10-strand, and Ala-191 and Asp-194 via a water molecule (Figs. 3 and 4). His-236 and Lys-237 are members of the G(G/A)HKGSA consensus sequence characteristics of the DpkA clade of the new family of oxidoreductases (10). Lys-237, which is completely conserved in all members of the family, is the critical residue for the pyrophosphate recognition. The OH groups of the nicotinamide-ribose moiety interact with the side chain carboxylate of Asp-184 on the b10-strand and the main chain NH group of Leu-130 on the loop between b5 and a5. Asp-184 conserved in the new family is essential for the ribose hydroxyl groups. The nicotinamide located at the bottom of the cavity directs its pro-4S hydrogen toward the C2 face side of P2CA. The planar P2CA forms a parallel stack on the nicotinamide plane with the P2CA carboxylate and the amide group of the nicotinamide directing toward the opposite side. The reaction site (C2 atom) of P2CA projects onto the C4 atom of the nicotinamide at a distance of 3.46 Å.

The active site is formed by His-54 and Arg-58 from domain I, Phe-127, Ser-150, Met-151, Thr-166, and Pro-272 from domain II, and His-192 and Gly-193 from domain III. The N3 atom of His-54 interacts with the pyrrole nitrogen atom with the coplanar disposition of imidazole and the pyrrole rings confirming for the first time that His-54, conserved in all members of the new family, is the general acid catalyst. The N1 atom of His-54 is further hydrogen-bonded to the OH group of Ser-53, which is polarized by Asp-194, implying that the Asp:Ser:His triad forms the charge-relay system. A similar charge relay system (Asp:His) has been shown to be essential in malate and lactate dehydrogenases with the Rossmann fold (40). The pyrrole ring of P2CA is approached by the
hydrophobic domain II residues (Phe-127, Met-151, and Pro-272) from the solvent side. The P2CA carboxylate is hydrogen-bonded to the main chain NH groups of His-192 and Gly-193 at the positively charged N-terminal part of the α-helix a6, the guanidino group of Arg-58, and the OH group of Thr-166. The carboxylate is thus tightly fixed to its recognition site formed by Arg-58, Thr-166, His-192, and Gly-193.

The superposition of the active site in the open form onto that in the catalytic form is shown in Fig. 5B. Subunits A and B of the unliganded enzyme or the complex with NADPH were observed to be in the open and closed forms, respectively. Upon binding of P2CA, domains I and III in the open form move to close the active site, while domain III in the closed form moves to open the active site, resulting in the formation of the catalytic form with the encapsulation of P2CA in the active site. Ser-53, His-54, and Arg-58 of domain I in the open form approach P2CA and rearrange their side chains in a direction suitable for catalysis (Figs. 5B and 6). Arg-58 changes its side chain direction to interact with P2CA carboxylate. His-54 switches the hydrogen bond with Arg-58 in the open form to that with the P2CA. Ser-53 makes a hydrogen bond with His-54. Concurrently, Asp-194 of domain III moves to interact with Ser-53 resulting in the formation of the catalytic Asp:Ser:His triad. Met-151 of domain II also changes its side chain conformation to make van der Waals contacts with P2CA. In the closed form of the enzyme, the active site residues except for Met-151 have side chain conformations and mutual interactions similar to those observed in the complex with P2CA.

The Ca atoms of the active site residues shown in Fig. 6 except for Pro-272 were superimposed onto the corresponding Ca atoms of YiaK, SLDH, and UGDH in the unliganded forms with RMS deviations of 1.3, 1.4, and 1.3 Å, respectively. Fig. 2A reveals that the cofactor NADPH and P2CA of PsDpkA are superimposable onto NAD and tartrate of YiaK. The cofactors of PsDpkA and SLDH are overlapped almost completely. The active site fold, and the location of the cofactor and the substrate are thus considered to be conserved among these enzymes.

The active site residues conserved in every member of the new family proteins are Ser-53, His-54, Arg-58, and Thr-166. It is reasonable to assume that the Ser-53:His-54 pair forms a hydrogen bond as the charge relay system, and that Arg-58 and Thr-166 recognize the substrate 2-carboxylate in the new family of oxidoreductases. In PsDpkA, the pyrrole ring of P2CA is recognized by Phe-127, Met-151, and Pro-272, which are not conserved in other members of the new family. The corresponding residues are His, Ile, and Glu in YiaK, Phe, Glu, and Thr in SLDH, and Ser, Asp, and Arg in UGDH. Possibly, these three residues are mainly associated with substrate specificity.

The close similarity of the cofactor orientation in PsDpkA, YiaK, and SLDH belonging to different clades of the new family of oxidoreductases implies that the pro-4S hydrogen of the nicotinamide moiety is directed toward the solvent region in the new family proteins (Fig. 2A). The substrates of the new family are likely to be positioned on the pro-4S hydrogen side of the cofactor based on the active site structures of PsDpkA and YiaK. The conserved Ser:His catalytic pair is juxtaposed to the substrate binding site. The new family of proteins with the SESAS fold is likely to have a common reaction mechanism with pro-4S hydride transfer.

**Mechanism**—The NADH- or NADPH-dependent enzyme requires the active site residue such as histidine, cysteine, or tyrosine, which acts as a proton acceptor or donor to facilitate the hydride transfer between the cofactor and the substrate (41, 42). Alcohol dehydrogenases with the

![FIGURE 6. Schematic diagram showing hydrogen bond and salt bridge interactions of the active site residues. Putative interactions are shown by dotted lines if the acceptor and donor are less than 3.5 Å apart. NADPH is omitted for clarity.](image)

![FIGURE 7. Proposed reaction mechanism of hydride transfer from NADPH to the substrate.](image)
Rossmann fold require a metal ion such as Zn$^{2+}$ bound to the active site to stabilize a transition state such as an alkoxide (43, 44). The catalytic reaction of PsDpkA is not accelerated but inhibited by metal ions (24), and no metal ion bound to the protein was detected on the residual electron density map. On the basis of geometrical considerations, the predicted catalytic mechanism of Pip2C/Pyr2C reductase is shown in Fig. 7. Upon access of the substrate to the active site cleft coordinated by the nicotinamide part of NADPH, the enzyme changes its conformation from the open or the closed form to the catalytic form to enclose the substrate on the nicotinamide plane. The bound substrate stacks on the nicotinamide with a geometrical orientation favorable for hydride transfer from NADPH to the substrate. The pro-$4S$ hydrogen of NADPH is directed toward the C2 atom of the substrate with parallel stacking between the nicotinamide ring and the pyrroline or piperideine ring. The imidazole ring of the catalytic His-54 approaches the pyrroline or piperideine ring of the substrate in the same plane and is hydrogen bonded to the substrate N1 atom as the proton donor. The electron flow from the substrate 2-carboxylate to the substrate ring is suppressed by the full coordination of 2-carboxylate by one guanidino group, two main chain NH groups, and one OH group (Fig. 6). The pro-$4S$ hydrogen of NADPH is considered to be transferred to the C2 $4S$ hydrogen specificity. In concert with the hydride transfer to the mide plane and the substrate, although there is no biochemical evidence NADPH is considered to be transferred to the C2 chain NH groups, and one OH group (Fig. 6). The full coordination of 2-carboxylate by one guanidino group, two main chain NH groups, and one OH group (Fig. 6).

In conclusion, we have determined the three-dimensional structures of the multifunctional enzyme, PsDpkA, in the unliganded form, complexed with NADPH, and complexed with NADPH and P2CA. This is the first structure of proteins belonging to the NADPH-dependent DpkA clade in the new family of NAD(P)H-dependent oxidoreductase distinct from the conventional NAD(P)H-dependent MDH/LDH family characterized by the Rossmann fold. The arrangements of the active site residues, NADPH, and the substrate analogue, and the interactions among them, have been elucidated to provide a putative mechanism for the catalysis of the enzyme. His-54 is identified as the general acid catalyst in the reductive reaction. The enzyme has not only reductase activity, but also N-methyl amino acid dehydrogenase activity. Three reactants (NADPH, N-methylamine, and $\alpha$-keto acid) have to be properly oriented in the active site for N-methyl amino acid synthesis. X-ray studies of the complex with an $\alpha$-keto acid to elucidate the dehydrogenase reaction and mutant work for developing the ability to synthesize a variety of N-methyl amino acids are currently in progress.