Crystal Structure of a Novel FAD-, FMN-, and ATP-containing L-Proline Dehydrogenase Complex from Pyrococcus horikoshii*§

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Two novel types of dye-linked l-proline dehydrogenase complex (PDH1 and PDH2) were found in a hyperthermophilic archaeon, Pyrococcus horikoshii OT3. Here we report the first crystal structure of PDH1, which is a hetero-octameric complex (αβ)₄ containing three different cofactors: FAD, FMN, and ATP. The structure was determined by x-ray crystallography to a resolution of 2.86 Å. The structure of the β subunit, which is an l-proline dehydrogenase catalytic component containing FAD as a cofactor, was similar to that of monomeric sarcosine oxidase. On the other hand, the α subunit possessed a unique structure composed of a classical dinucleotide fold domain with ATP, a central domain, an N-terminal domain, and a Cys-clustered domain. Serving as a third cofactor, FMN was located at the interface between the α and β subunits in a novel configuration. The observed structure suggests that FAD and FMN are incorporated into an electron transfer system, with electrons passing from the former to the latter. The function of ATP is unknown, but it may play a regulatory role. Although the structure of the α subunit differs from that of the β subunit, except for the presence of an analogous dinucleotide domain with a different cofactor, the structural characteristics of PDH1 suggest that each represents a divergent enzyme that arose from a common ancestral flavoenzyme and that they eventually formed a complex to gain a new function. The structural characteristics described here reveal the PDH1 complex to be a unique diflavin dehydrogenase containing a novel electron transfer system.

Dye-linked dehydrogenases catalyze the oxidation of various amino acids, organic acids, amines, and alcohols in the presence of an artificial electron acceptor such as ferricyanide or 2,6-dichloroindophenol (DCIP). We have identified several novel dye-linked dehydrogenases among the hyperthermophilic archaea, including t-proline dehydrogenase (1) and l-proline dehydrogenase (PDH) (2, 3), which catalyzes the dehydrogenation from l-proline to Δ1-pyrrole-5-carboxylate (P5C). The gene sequence and primary structure of the first PDH isolated from a hyperthermophilic archaeon, Thermococcus profundus DSM9503, have been determined (2, 3). The complete gene is formed by an operon comprised of four genes, pdhA, pdhB, pdhF, and pdhX, in a tandem arrangement in the order pdhA-F-X-B. The purified enzyme has a native molecular mass of 120 kDa and forms an unusual αβγδ heterotetrameric complex (α (54 kDa), β (43 kDa), γ (19 kDa), and δ (11 kDa)) encoded by pdhA, pdhB, pdhF, and pdhX, respectively. In addition, functional analysis of each subunit has shown that the α and β subunits possess NADH and l-proline dehydrogenase activities, respectively (3). Because this multifunctional dye-linked dehydrogenase complex appears to be representative of a new enzyme group (3), we have been seeking others by screening dye-linked dehydrogenases expressed in hyperthermophilic archaea. So far, we have identified two different types of dye-linked PDH in the hyperthermophilic archaeon Pyrococcus horikoshii OT3: PDH1 and PDH2 (4).

Biochemical and gene analyses of PDH1 and PDH2 revealed PDH2 to be similar to the T. profundus PDH. By contrast, the gene encoding PDH1 formed an operon comprised of two consecutive genes, PH1363 and PH1364, and the molecular masses of the encoded α and β subunits were determined to be about 56 and 43 kDa, respectively. The native molecular mass of PDH1 was determined to be 440 kDa by gel filtration study (4), indicating that the enzyme exists as an αβγδ heterotetramer with a subunit structure that is different from that of PDH2. In addition, high performance liquid chromatography (HPLC) analysis recently showed that PDH1 contains three cofactors: FAD, FMN, and ATP (4). The β subunit contains FAD and exhibits proline dehydrogenase activity, while the α subunit contains ATP but exhibits no proline dehydrogenase activity (4). Although both PDH1 and PDH2 genes are annotated as sarcosine oxidase in the P. horikoshii genome, because of their high homology with a monomeric sarcosine oxidase (MSOX), 1

The abbreviations used are: DCIP, 2,6-dichloroindophenol; CAPS, N-cyclohexyl-3-aminopropanesulfonic acid; CPR, NADPH-cytochrome P450 reductase; GR, glutathione reductase; HPLC, high performance liquid chromatography; MSOX, monomeric sarcosine oxidase; OARA, o-aminobenzenedihyde; P5C, Δ1-pyrrole-5-carboxylate; PDH, l-proline dehydrogenase; r.m.s.d., root mean square deviation; TD, trimethylamine dehydrogenase; TSOX, heterotetrameric sarcosine oxidase.

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neither of these enzymes exhibits any sarcosine oxidase activity. The amino acid sequences of the α and β subunits of PDH1 showed 31 and 56% similarity with those of the α and β subunits of PDH2, respectively. Analysis of the genome database revealed that both types of PDH complex are widely distributed among the Thermococcales family of hyperthermophilic archaea, including the species Pyrococcus and Thermococcus.

PDH has also been identified in Escherichia coli and Salmonella typhimurium, and some of the fundamental properties of these enzymes have been investigated (5-8), and the crystal structure of E. coli PutA has been resolved (9). Notably, the primary structure and molecular properties of PDH1 and PDH2 are different from those of PutA. Still, dye-linked dehydrogenases generally have been found to function in the uptake of electrons from a reduced substrate into an electron transfer system. Little is currently known about the physiological function of these enzymes in hyperthermophilic archaea nor is much known about their mechanism catalysis or their electron transfer systems. This makes structural analysis of the archeal PDH complex particularly interesting, as it could shed light on the evolution of energy and amino acid metabolism in hyperthermophilic archaea such as P. horikoshii and T. profundus. Here we describe the unique structure of the PDH1 complex, which is a novel type of archaeal enzyme containing FAD, FMN, and ATP.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification—**PH1363 and PH1364 genes, which, respectively, encode the α and β subunits of PDH1, were amplified and inserted separately into the pET11a expression plasmid. To construct the co-expression plasmid pEPDH1, the region containing PH1363 gene and its T7 promoter was cleaved from pET11a/PH1363 and inserted into pET11a/PH1364 upstream of the region containing the T7 promoter. E. coli strain BL21 CodonPlus (DE3) cells transformed with pEPDH1 were grown at 37 °C in SB medium containing ampicillin. After 6 h, isopropyl β-D-thiogalactopyranoside (1 mM) was added, and cultivation was continued for 3 h at 37 °C. The cells were then collected by centrifugation (10,000 × g, 20 min) and suspended in 10 mM potassium phosphate buffer (pH 7.0). Crude extract was prepared by ultrasonication, and then three purification steps were applied at 90 °C for 10 min, hydrophobic chromatography on Butyl Toyopearl, and gel filtration chromatography on Superdex 200. Active fractions containing (αβ)4 complex were pooled and dialyzed against 10 mM potassium phosphate buffer (pH 7.0).

**Enzyme Assay—**Enzyme activity was spectrophotometrically assayed as described previously using a Shimadzu UV-1200 spectrophotometer. activity was assayed by determining levels of the l-proline dehydrogenation product P5C coupled with OABA was monitored at 440 nm at 50 °C.

**Crystallization—**After concentrating the enzyme to 10 mg/ml, the sample was equilibrated against 200 μl of reservoir solution was equilibrated against 200 μl of reservoir solution at 90 °C. The crystals were grown at 90 °C for 10 min, hydrophobic chromatography on Butyl Toyopearl, and gel filtration chromatography on Superdex 200. Active fractions containing (αβ)4 complex were pooled and dialyzed against 10 mM potassium phosphate buffer (pH 7.0).

**RESULTS AND DISCUSSION**

**Overview of the Structure of the (αβ)4 Octameric Complex—**The crystal structure of a novel l-proline dehydrogenase from Pyrococcus horikoshii and P. horikoshii and E. coli PutA has been resolved (9). Notably, the crystal structure of the αβ heterodimer had a crystalllographic 222 symmetry. A model consisting of 860 residues (α) was refined using REFMAC5 (13). Several cycles of inspection of 2Fo – Fc and Fc – Fo Fourier maps calculated with 2.86-20.0 Å data were carried out, after which the model was rebuilt. FAD, FMN, ATP, Ca2+, Fe3+, SO42−, and 206 water molecules were included in that model. Residues 95-190 in the α subunit had poor electron density and formed a loop to bend the β subunit. Further manual correction and refinement using CNS (14) yielded R and Rwork values of 18.1 and 22.5%, respectively. In both the α and β subunits, there were no residues in the disallowed region of the Ramachandran plot. Molecular graphics figures were created using PyMOL (www.pymol.sourceforge.net).

**The Interface between the α and β Subunits—**As mentioned above, FMN was found between the α and β subunits. The interaction of the subunits from this family appears highly important for the function of the heterodimeric complex; details of the interaction between the PDH1 molecule and the protein will be discussed later. The other interactions between the subunits were mostly hydrophobic: 1) Met168α was buried in a hydrophobic core, formed by Phe288α, Tyr278α, Leu295β, Leu483α, and Ile300αβ; 2) Phe288α and Ile300αβ; 3) hydrophobic residues Leu128β, Ile237α, Thr237α, Ile232α, Leu281β, and Leu281αβ; and 5) a hydrophobic cluster is formed by Leu182α, Phe185α, Leu192α, Pro194α, Leu201α, Val81α, Tyr278β, Leu281β, and Ile300β. In addition, the following protein-protein ionic interactions were observed: Glu203α (G101-Arg285β (NH1) (3.75 Å) and Lys187α (N2-Asp278β (OD2) (2.86 Å).

**Structure of the β Subunit—**The β subunit consists of two domains (supplemental Fig. 1 and Fig. 2A). One is an FAD-binding domain (residues 29–48β, 148β–223β, and 307β–356β) containing the dinucleotide-binding fold observed in enzymes belonging to the glutathione reductase (GR) family (15). The other domain consists of a central five-stranded parallel β
sheet (β1, β2, β5, β9, and β16) flanked by two α helices (α1 and α6) on one side and a three-stranded anti-parallel β sheet (β6, β7, and β8) on the other. The other domain is an interface domain mostly formed by an anti-parallel β sheet having two parts, 496–147β and 223β–306β.

Superposition using DALI (16) showed that the β subunit has similarity with MSOX (r.m.s.d., 2.2 Å for 363 Ca pairs; 22% sequence identity), glycine oxidase (r.m.s.d., 2.1 Å for 344 Ca pairs; 24% sequence identity), N,N-dimethylglycine oxidase (r.m.s.d., 2.3 Å for 350 Ca pairs; 27% sequence identity), and D-amino acid oxidase (r.m.s.d., 3.0 Å for 302 Ca pairs; 16% sequence identity). MSOX is known to belong to the structural class of flavoenzyme originally found in p-hydroxybenzoate hydroxylase and contains only FAD as the cofactor (17). FAD in the β subunit is bound in a manner similar to that seen in MSOX, although we found several key differences. FAD binds...
noncovalently to PDH1β, whereas it binds covalently with Cys315 in MSOX. Moreover, all but one of the residues gathered on the side face of the flavin ring of FAD differ from those in MSOX (Fig. 2B, purple surface). These residues are responsible for creation of a new electron transport pathway from FAD to FMN, which is located at the interface between the α and β subunits (there is no FMN binding in MSOX). Only Arg302 and Trp304 make a π-π interaction with the flavin ring of FMN. By contrast, residues on the re face of the isoalloxazine ring of FAD, which form a substrate-binding site (shown as gray sticks), are moderately conserved: Tyr251, His389, Tyr308, and Arg525 are present in both PDH1β and MSOX.

Finally, a unique FMN-binding site is created between the interface domain of the β subunit and the Cys-clustered domain of the α subunit (Fig. 2, B and C).

Structure of the α Subunit—Analysis of the Pfam (18) protein family database predicts that the α subunit of PDH1 has a dinucleotide domain, although the overall structure is unknown. The crystal structure revealed that the α subunit consists of four domains (supplemental Fig. 2 and Fig. 3A), which include an ATP-binding domain containing residues α101–α217 and α309–α397 with a dinucleotide-binding fold, a central domain containing α218–α308, an N-terminal domain containing α1–α94, and a Cys-clustered domain containing α398–α498. The ATP-binding domain, which contains a FAD-binding motif similar to the one in the β subunit, consists of a five-stranded parallel β sheet (β9, β10, β13, β17, and β29) flanked on one side by two helices (α2 and α4) and by a three-stranded anti-parallel β sheet (β14, β15, and β16) on the other. Analysis using DALI showed this domain to be highly similar to the FAD-binding domain of another flavoenzyme, an alkyl hydroperoxide reductase belonging to the GR family (r.m.s.d., 2.2 Å for 176 Ca pairs; 19% sequence identity) (16). The central domain consists of a four-stranded parallel β sheet (β18, β19, β20, and β24) flanked on one side by two helices (α5 and α6) and by a three-stranded anti-parallel β sheet (β21, β22, and β23) on the other. The topology has some similarity with the NADPH-binding domain of GR, but the domain of PDH1α lacks the ability to bind NADPH. The overall structure of PDH1α is unique because the N-terminal domain and the Cys-clustered domain were fused additionally; the former domain has an α/β structure, and the latter domain consists of four α helices and mediates FMN binding to the α subunit at the interface with the β subunit.

Using HPLC, we detected the presence of ATP in the α subunit of PDH1 (4). The tracing of the electron density of the enzyme clearly shows the binding of an ATP molecule in the α subunit (supplemental Fig. 2), where it serves as a third nucleotide cofactor along with FAD and FMN (Fig. 3A). This is noteworthy, as it is the first report of ATP serving as a cofactor in a flavin-dependent dehydrogenase. ATP is situated within the α subunit in the dinucleotide-binding domain known as the Rossmann fold. Three Gly residues (Gly113–115) lie in parallel with the backbone of the ATP, as is usually observed in FAD-binding sites (Fig. 3B), and O2' and O3' of the ATP ribose make contact with the carboxyl group of the side chain of Glu39–40. In another example of the binding of a nucleotide cofactor, ADP reportedly binds to trimethylamine dehydrogenase (TD) (18), although the physiological function of the ADP is unknown. Looking for similarities in the nucleotide-binding sites in GR, TD, and PDH1α, we found that they all have a GR fold; r.m.s.d. between equivalent Ca positions was 0.928 Å (PDH1α/GR), 0.897 Å (GR/TD), and 0.698 Å (PDH1α/OR). Comparison of the three aligned sequences of the six peptides of the ADP-binding regions (Ile111–Gly118, Ile134–Arg137, Leu140–Met144, Thr179–Leu182, Ile211–Ala215, and Val346–Ala350) showed that 15 (PDH1α/OR), 14 (PDH1α/GR), and 12 (TD/OR) of the 32 residues are identical, thus ADP-binding motifs are moderately conserved. However, a prominent difference was observed in the γ-phosphate-binding site that specifically involves His386 and Asn39 in PDH1α. In TD, His327 and Asp374 disturb ATP binding.

Another binding factor was found from the experimental and $F_o - F_c$ electron density, CAPS, which was included in the crystallization buffer, appeared like a clasp fastened between the ATP-binding domain and the N-terminal domain (Fig. 3A). Interestingly, the site consists of four Trp residues (245α, 268α, 145β, and 139α) and Val142α. This means that four of the five Trp residues in the α subunit are clustered together in this region to form a highly hydrophobic surface.

The four α helices of the Cys-clustered domain include four Cys residues (Cys413α, Cys415α, Cys441α, and Cys452α). The crystal structure revealed that two of them (Cys413α and Cys452α) form a disulfide bridge that is in close proximity to Cys441α (Fig. 3A). Although the Pfam (19) protein family database predicted the existence of a [2Fe-2S]-binding domain within the sequence of PDH1α, we could not confirm the presence of [2Fe-2S]. However, an electron density at 5, near the Cys413α–Cys452α disulfide, was attributed temporarily to the Fe4+ (supplemental Table 2).
FMN-binding Site—The absorption spectrum for PDH1 showed a typical spectrum for flavin compounds, with peaks at 370 and 450 nm; the flavins were confirmed to be FAD and FMN (4). Although it was unclear from the HPLC analysis whether the FMN-binding site is present in the β or α subunit, in the present study we found the electron density for FMN between the Cys-clustered domain in the α subunit and the interface domain in the β subunit, with the isoaflavoxazine ring of FMN sandwiched by two hydrophobic residues, Trp<sup>304</sup>β and Met<sup>444</sup>α (Fig. 2, B and C), and the phosphate group fixed by three basic residues, Arg<sup>46</sup>β, Arg<sup>302</sup>β, and Arg<sup>477</sup>α (Fig. 2C). As far as we know, this manner of binding FMN using the interface of two separate subunits is novel. FAD is known to locate between two domains in enzymes belonging to the ferredoxin-NAD<sup>+</sup> reductase family (20), but the environment of the isoaflavoxazine ring in the FMN-binding site in PDH is quite different. Notably, one of the ferredoxin-NAD<sup>+</sup> reductases, NADPH-cytochrome P450 reductase (CPR), accepts a pair of electrons from NADPH as a hydride ion, with FAD and FMN serving as the port of entry and exit, respectively, and transfers them to a heme of cytochrome P450 (21). But although both PDH1 and CPR are diflavin reductases, they differ in the relative positions of FAD and FMN.

PDH-catalyzed electron transport from l-proline to oxygen or, as in our experiments, to ferricyanide or DCIP cannot be considered physiological. Even the transport to oxygen would be unusual for this enzyme, as Pyrococcus and Thermococcus, the archaeal hyperthermophiles in which it is expressed, grow under anaerobic conditions. In considering the physiological pathway of electron transport, the structural relationship of the two flavins, FAD and FMN, would seem to be very important. The close proximity of their dimethyl benzene portions suggests that an electron pathway from FAD to FMN is reasonable; indeed, a similar pathway can be seen in CPR. Nevertheless, PDH1 is categorized as a different class of diflavin enzyme because of the following differences between the two enzymes: 1) diflavin-binding sites are created by a single polypeptide in CPR, but by two in PDH1; 2) contact between the dimethyl benzene portions are direct in CPR but are indirect via Cys<sup>47</sup>β and Trp<sup>304</sup>β in PDH1; and 3) the relative positions of the diflavin portions differ. In addition, the oxidase activity and dye-linked PDH activity could be detected even if iron were present within the Cys-clustered site, it would be in an excellent position to transfer electrons from FAD via FMN. Then, in a final step, the electron could be transferred via iron to an as yet unknown electron acceptor. In this scenario, the α subunit acts not only to create the FMN-binding site but also to transport electrons to a specific electron acceptor protein via iron. Perhaps the hydrophobic CAPS-binding site plays a role in binding the unknown electron acceptor. Also consistent with this idea is the finding the region is not perturbed by formation of the (αβ)<sub>2</sub> heteroamphatic complex.

So far, four different FAD enzyme folds have been identified based on their sequence-structure analysis (15). In the case of PDH1, both subunits belong to the GR structural family, but each belongs to different subfamily. In the GR family, two separate subfamilies were identified (15): GR<sub>1</sub>, which is represented by GR<sub>23</sub>, and GR<sub>2</sub>, which is represented by d-amino acid oxidase (24, 25). Both subfamilies share a similar overall three-dimensional structure in their FAD-binding domain as well as at least one conserved sequence motif (GxGxGxG). Whereas proteins from the GR<sub>2</sub> subfamily align well with the entire FAD-binding domain, those belonging to GR<sub>1</sub> subfamily align well only in their N-terminal. PDH1<sub>α</sub> clearly belongs to the GR<sub>2</sub> subfamily; however, a BLAST search (26) showed that PDH1<sub>α</sub> has no similarity with the both subfamily except the α subunit of heterotetrameric sarcosine oxidase (TSOX) (27). It is probable that PDH1<sub>α</sub>, PDH2<sub>α</sub>, and TSOX<sub>α</sub> form a different subfamily in GR family. In addition, when we superimposed the structure of the α and β subunits on the common GR fold with different cofactors (r.m.s.d., 0.97 Å for 67 Ca pairs), we found that the overall structure of the α subunit clearly differed from the β subunit (Fig. 3C). The structural characteristics of PDH1 suggest that each represents a divergent enzyme that arose from a common ancestral flavoenzyme and that they eventually formed a complex to gain a new function. We emphasize that our results provide a structural basis for understanding this unique enzyme, which is an FAD-, FMN-, and ATP-containing archaeal l-proline dehydrogenase.

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