Putidaredoxin Reductase, a New Function for an Old Protein*

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Properties of recombinant wild type (WT) and six-histidine tag-fused (His₆) putidaredoxin reductase (Pdr), a FAD-containing component of the soluble cytochrome P450cam monooxygenase system from Pseudomonas putida, have been studied. Both WT and His₆ Pdr were found to undergo a monomer-dimer association-dissociation and were partially present as an NAD⁺-bound form. Although molecular, spectral, and electron transferring properties of recombinant His₆ Pdr to artificial and native electron acceptors were similar to those of the WT protein, the presence of eight additional C-terminal amino acid residues, Pro-Arg-His-His-His-His-His-His-His, had a crucial effect on the enzyme interaction with oxidized pyridine nucleotide. Under anaerobic conditions, NAD⁺ induced in His₆ Pdr spectral changes indicative of flavin reduction and formation of the charge transfer complex between the reduced FAD and NAD⁺. The reaction proceeded considerably faster in the presence of free histidine and thiol-reducing agents, such as dithiothreitol and reduced glutathione. In the presence of excess NAD⁺ as a reductant, the midpoint potential of Pdr was calculated to be −285 mV (2). The reduction of Pdr by NADH is a stoichiometric process that results in the formation of a long-wavelength charge transfer complex without the appearance of a semiquinone intermediate (3). The reduction of Pdr with NADPH is approximately 3 orders of magnitude slower than with NADH (4). No charge transfer band could be detected when the flavoprotein was reduced with either NADPH or sodium dithionite. Using NADPH as a reductant, the midpoint potential of Pdr was calculated to be −315 mV (4). Titration of Pdr with NADPH in the presence of excess NAD⁺ resulted in a stoichiometric reduction. This was explained as a result of the very tight association of NAD⁺ with the reduced FAD and formation of the charge transfer complex that was able to shift the equilibrium of the reduction reaction toward product.

In P450cam monooxygenase, the reactions of NADH oxidation and Pdx reduction catalyzed by Pdr are tightly coupled. Investigation of the role of NAD⁺/NADH in the catalytic reduction of Pdx has demonstrated that the pyridine nucleotide complex of Pdr is important in the enhancement of the reduction of Pdx (5). NAD⁺ was shown to be essential for the kinetically favored oxidation of Pdr from the two-electron reduced form to the semiquinone by oxidized Pdx. When Pdr was photoreduced in the presence of NAD⁺, it transferred the first electron to Pdx at a rate that could account for catalytic turnover. In contrast, the rate of Pdx reduction by Pdr that was photoreduced in the absence of NAD⁺ was 3 orders of magnitude lower.

Due to instability, a semiquinone form of Pdr could not be detected and characterized by conventional spectrophotometric techniques. We have recently utilized laser flash photolysis techniques to produce and investigate the electron transferring properties of one-electron reduced species of the flavoprotein (6). Upon flash-induced reduction by 5-deazariboflavin semiquinone, Pdr was found to form a blue neutral FAD semiquinone (FADH⁻). FADH⁻ was unstable and partially disproportionated into fully oxidized and fully reduced flavin. The rate of FAD semiquinone disproportionation was enhanced by NAD⁺. It was established that FADH⁻ was capable of transferring an electron to Pdx with the rate constant that can account for catalytic turnover (6).

Quite unexpectedly, our laser flash photolysis experiments revealed that under anaerobic conditions and in the presence of NAD⁺, the flavin in the recombinant Pdr molecule, expressed with the C-terminal six-histidine tag (His₆ Pdr), became reduced without any external source of electrons. This indicates that, in addition to FAD, there must be another redox center(s) transferred to the terminal oxygenase cytochrome P450cam (CYP101) that catalyzes hydroxylation of camphor consuming two electrons and molecular oxygen per reaction cycle.

Pdr has a molecular mass of 45.6 kDa and contains one tightly bound FAD per single polypeptide. Redox measurements, where sodium dithionite was used as a reductant, demonstrated that the midpoint potential of Pdr is −285 mV (2).

† The abbreviations used are: Pdr, putidaredoxin reductase; His₆ Pdr, recombinant Pdr with the C-terminal six-histidine tag; Pdx, putidaredoxin; FADH⁻, blue neutral FAD semiquinone; DCF, 5-deazariboflavin; MALDI-TOF, matrix-assisted laser desorption-ionization-time-of-flight mass spectrometry; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); TMB, 2-nitro-5-thiobenzamide; DTT, dithiothreitol; DAlP, 2,5-dichlorophenylindophenol; PCMB, para-chloromercuribenzoic acid; NEM, N-ethylmaleimide; NADase, NAD glycohydrolase; WT, wild type; GR, glutathione reductase.

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A New Function of Putidaredoxin Reductase

in Pdr. The fact that Pdr belongs to a class of bacterial oxygene-nase-coupled NADH-dependent ferredoxin reductases that have a structural fold similar to that of the enzymes of the glutathione reductase (GR) family (7) suggests that the second redox center in Pdr is likely to involve sulfhydryl groups. Although Pdr has six cysteine residues, it does not have putative disulfide redox centers encoded by CXXC or CXXXC motifs, characteristic features of pyridine nucleotide-disulfide oxi-doreductases. Despite the low amino acid sequence similarity between oxygene-nase-coupled NADH-dependent ferredoxin reductases and GR-family enzymes, these proteins are thought to have evolved from a common ancestor but, in the course of evolution, have lost (or GR family enzymes have acquired) the cysteine residues essential for the catalysis (7). In the present work, we demonstrate that Pdr is a bifunctional enzyme that, in addition to catalyzing electron transfer to Pdx, can function as NAD(H)/dihithiol/disulfide oxidoreductase. Lacking traditional disulfide redox centers, Pdr seems to be unique in this class of enzymes.

**EXPERIMENTAL PROCEDURES**

**Materials—**Proteocatechuate, protocatechuate-3,4-dioxigenase, 5,5′-dithiothreitol, (DTNB), dithiothreitol (DTT), GSH, and Sephacryl S-100-HR were from Sigma. The Ni²⁺/H₁₁₀₃₂₀ and Sephacryl S-100-HR were from Sigma. The Ni²⁺/H₁₁₀₃₂₀ phosphate buffer, pH 7.5, at 30°C for 30 min, and any excess of reducing agent was removed from the proteins by chromatography on Sephadex G-25 in the same buffer. The number of thiols in Pdr was quantitated by the addition of a large excess of DTNB to the protein solutions and following the oxidation of 2-nitro-5-thiobenzoic aniline (TNB) at 412 nm. Extinction coefficients of TNB under native and denaturing conditions are 14.2 mm⁻¹ cm⁻¹, respectively (10, 11). Thiol modification was performed by incubation of 25 μM Pdr with 500 μM N-ethylmaleimide, para-chloromercuribenzoic acid, or CdCl₂ for 30 min under anaerobic conditions in the presence or absence of 1 mM NADH. After the reaction, excesses of the thiol-modifying agent and NADH were removed by gel filtration on Sephadex G-25.

**Analytical Procedures—**A F₆₈₅₆ value of His₆-Pdr (Pdr⁺) was determined from a Lineweaver-Burk plot, where initial rate constants for NAD⁻-induced flavin reduction were calculated from the linear intervals of the kinetic traces of the absorbance change at 455 nm versus time recorded under anaerobic conditions at different concentrations of oxidized pyridine nucleotide. NADH:DTNB oxidoreductase assays were performed in the reaction mixtures containing 4.5 mM DTNB in 150 mM potassium phosphate buffer, pH 7.0, at 25°C, in the absence and presence of different concentrations of histidine. During anaerobic and aerobic measurements, final enzyme concentrations were 0.1 and 3 μM, respectively. The reactions were started by the addition of NADH, and the change of absorbance was recorded at 412 nm (ε = 13.6 mM⁻¹ cm⁻¹ (11)). The initial rate constants for the reaction of DTNB reduction catalyzed by Pdr were determined from the slopes of linear intervals of the kinetic traces. For the reactions carried out under aerobic conditions, fast phase of the kinetic traces was used for calculations. A low concentration of DTNB to TNB occurred in the absence of enzyme, and this was subtracted from the rate in the presence of enzyme.

Pdr reduction activity was measured at 455 nm using an extinction coefficient of 2.5 mm⁻¹ cm⁻¹ as previously described (12). Specific reductase activities were calculated employing molar extinction coefficients of 6.22 mm⁻¹ cm⁻¹ at 340 nm, 1.02 mm⁻¹ cm⁻¹ at 420 nm, and 21 mm⁻¹ cm⁻¹ at 600 nm for NADH, ferricyanide, and DCIP, respectively. Flavin fluorescence intensity was measured using a Hitachi F-4500 fluorescence spectrophotometer (λₐₒ = 455 nm; λₑₓ = 522 nm). Laser flash photolysis experiments were carried out as described previously (6).

**RESULTS**

**Spectral and Molecular Properties of Recombinant WT and His₆-Pdr**—To facilitate purification and to obtain large quantities of Pdr for mechanistic and structural studies, we have cloned and expressed in *Escherichia coli* a six-histidine tag fused enzyme. Incorporation of a sequence of six histidine residues and an α-thrombin cleavage site to the amino terminus of Pdr resulted in no expression of an active flavoprotein, most likely due to interference of the peptide with the FAD binding site located in the N-terminal part of the protein. In contrast, the presence of the His₆ tag at the C terminus did not affect binding of the flavin cofactor, and, as a result, high levels of expression of the properly folded enzyme were achieved (up to 70 nmol/l g of cells).

During purification procedures, no difference in chromatographic behavior of WT and His₆-Pdr was found. It was noticeable that both proteins eluted from the gel filtration column in two bands (Fig. 1). A slower moving band corresponded to a molecular mass of ~46 kDa and contained 95% of the enzyme. A band of greater mobility containing the rest of the enzyme corresponded to a molecular mass of ~93 kDa. SDS-PAGE analysis showed that under nonreducing conditions, both bands consisted of a mixture of 46- and 93-kDa proteins, whereas only one band corresponding to the 46-kDa protein could be seen on the gel in the presence of DTT (Fig. 1, inset). This suggests that Pdr undergoes a monomer-dimer association-dissociation in solution. Further evidence for such a reversible association was obtained from mass spectrometry analysis that required considerably lower enzyme concentrations. Two peaks were present in the MALDI-TOF mass spectrum of Pdr corresponding to the monomeric and dimeric protein (Fig. 2). As seen in inset A, peak 1 had a shoulder corresponding to a protein that was ~670 Da heavier than Pdr. We hypothesized that this fraction may

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represent NAD$^+$/NADH-bound Pdr. Indeed, when His$_6$ Pdr was reduced with NADH and quickly reoxidized prior to the mass spectral analysis, the portion of the fraction with the mass of 47,304 Da was significantly increased (Fig. 2, inset B).

The absorbance spectra of WT and His$_6$ Pdr were similar and had absorption maxima at 379 and 455 nm, a pronounced shoulder at 480 nm, and a small broad absorption peak in the long wavelength area (Fig. 3). The long wavelength absorption has not been reported for the native Pdr from *P. putida* (3). The extinction coefficient of His$_6$ Pdr at 455 nm was calculated to be $10.9 \pm 0.3 \text{M}^{-1} \text{cm}^{-1}$. Fluorescent yield upon flavin excitation in His$_6$ Pdr was low and similar to that of the WT protein, indicating that the flavin fluorescence in both proteins is quenched (data are not shown). The ferricyanide, DCIP, and Pdx reductase activities of His$_6$ Pdr were similar to those of the WT enzyme (Table I).

**Redox Properties of WT and His$_6$ Pdr**—The difference between WT and His$_6$ Pdr was first revealed during laser flash photolysis experiments. Compared with the WT enzyme, reduction of His$_6$ Pdr by 5-deazariboflavin (dRF) semiquinone proceeded with a rate constant 2 times smaller. Hyperbolic fits to the plots of $k_{obs}$ versus enzyme concentration for the reactions of Pdr with dRF semiquinone give limiting $k_{obs}$ values of $1.3 \times 10^4$ and $6.2 \times 10^3 \text{s}^{-1}$ and dissociation constants ($K_d$) of 10 and 11 $\mu$M for WT and His$_6$ Pdr, respectively (Fig. 4A). Like the WT enzyme (6), His$_6$ Pdr produced the blue neutral FAD semiquinone after one-electron reduction by dRF radical. However, FADH$_2$ of His$_6$ Pdr was less stable than that produced by the WT protein. Within the studied time interval, 50–60% of the FAD semiquinone produced by His$_6$ Pdr, versus 20–25% in the WT flavoprotein, disproportionated to form the fully oxidized and...
show absorbance difference spectra between the oxidized and distinct phases and isosbestic points appearing during the reaction. Flavin and NADH oxidoreductase (Pdr) was reduced. During the second phase, flavin reduction, charge transfer complex formation, and production of NADH were continuous. With WT enzyme, an initial decrease in absorbance at 455 nm was followed by an increase, indicating that some of the flavin was getting reduced and then partially oxidized Flavin Reduction in Pdr— Although there are no CXXC or CXXXXC motifs characteristic for disulfide redox centers (13) in the amino acid sequence of Pdr, the results described above suggest that thiols provide the source of electrons for flavin reduction. Indeed, if in addition to NADH an excess of thiol-reducing agent such as DTT or GSH was present in the anaerobic cell, the flavin reduction in His6 Pdr proceeded faster, was complete, and resulted in the formation of significant amounts of NADH (Fig. 6). It should be noted that DTT and GSH did not promote the FAD reduction itself but required the presence of NADH. The initial rate constants of the flavin reduction in His6 Pdr measured from kinetic traces of the absorbance change at 455 nm versus time were equal to 0.3, 0.4, and 0.5 nmol of FAD reduced min⁻¹ in the absence and presence of DTT or GSH, respectively (Fig. 6, inset A).

In the presence of thiol-reducing agents, NADH also induced flavin reduction in WT Pdr. In the presence of an excess of DTT, reduction of FAD was slow, such that ~85% of the flavin was reduced over a period of 2 weeks, and practically no NADH formed (Fig. 7, spectrum b). In contrast, reaction of WT Pdr with NADH in the presence of GSH was considerably faster, was completed within 36 h, and resulted in full flavin reduction and formation of considerable amounts of NADH (Fig. 7, spectrum c). Since the reduction of WT Pdr required long periods of time, particular care was taken to ensure that the cuvette was anaerobic throughout the experiment. The amount of protocatechual acid in the experiments involving WT Pdr was increased from 100 to 200 μM. The ability of Pdr to accept electrons from thiol-reducing reagents and use them for NADH reduction (e.g. functioning as a thiol:NADH oxidoreductase) proves that the redox-active groups in the protein are free thiols.

Significantly longer reaction times with thiol-reducing agents in the experiments described above was not the only difference between WT and His6 Pdr. Upon the addition of either GSH or DTT to His6 Pdr, reduction of the flavin, formation of the charge transfer complex, and production of NADH were continuous. With WT enzyme, an initial decrease in absorbance at 455 nm was followed by an increase, indicating that some of the flavin was getting reduced and then partially reoxidized (Fig. 7, inset). It took ~25 h before irreversible flavin reduction, charge transfer complex formation, and NADH production began. In His6 Pdr, therefore, the C-terminal peptide containing a six-histidine tag appears to greatly facilitate thiol oxidation and flavin reduction.

**Effect of Thiol-reducing Agents and Histidine on NADH-induced Flavin Reduction in Pdr**— Although there are no CXXC or CXXXXC motifs characteristic for disulfide redox centers (13) in the amino acid sequence of Pdr, the results described above suggest that thiols provide the source of electrons for flavin reduction. Indeed, if in addition to NADH an excess of thiol-reducing agent such as DTT or GSH was present in the anaerobic cell, the flavin reduction in His6 Pdr proceeded faster, was complete, and resulted in the formation of significant amounts of NADH (Fig. 6). It should be noted that DTT and GSH did not promote the FAD reduction itself but required the presence of NADH. The initial rate constants of the flavin reduction in His6 Pdr measured from kinetic traces of the absorbance change at 455 nm versus time were equal to 0.3, 0.4, and 0.5 nmol of FAD reduced min⁻¹ in the absence and presence of DTT or GSH, respectively (Fig. 6, inset A).

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We next clarified if free histidine could affect redox properties of Pdr. It appeared that histidine significantly stimulated NAD\(^+\)-induced flavin reduction in His\(_6\) Pdr (Fig. 6, inset B). The initial rate constant of the reaction approached 0.7 nmol of FAD reduced min\(^{-1}\) at 200 mM histidine. Free histidine was also found to promote NAD\(^+\)-dependent electron transfer to FAD in WT Pdr. Although this reaction was not nearly as fast as that observed in His\(_6\) Pdr, it was complete in 72 h and did not require the presence of sulfhydryl reducing agents (data not shown).

**NADH-dependent DTNB Reductase Activity of Pdr**—In order to elucidate whether Pdr can catalyze the reverse reaction, NADH:disulfide oxidoreduction, we measured NADH-dependent DTNB reductase activity of the enzyme. Both WT and His\(_6\) Pdr were capable of reducing DTNB. The reaction was highly affected by oxygen. Under aerobic conditions, there was a lag period of a few minutes in the absorbance change at 412 nm (Fig. 8A). The continuation of the lag phase was dependent on DTNB, NADH, and protein concentrations. Under strict anaerobic conditions and saturating concentrations of the reaction components, no lag phase in the DTNB reduction was observed (Fig. 8A, inset). Moreover, the values of \(k_{cat}\) for the reaction of disulfide reduction carried out under anaerobic conditions were 1 order of magnitude higher than those measured in the presence of oxygen (Table II). WT and His\(_6\) Pdr had similar turn-over numbers and \(K_m\) values for both substrates. It should be mentioned that the \(K_m\) values for NADH measured under anaerobic conditions were 2 orders of magnitude lower than those determined in the presence of oxygen.

There was no effect of *Neurospora* NADase on either the continuation of the lag phase or kinetic parameters of the reaction. In contrast, salts, such as ammonium and lithium sulfates, had a pronounced stimulating effect on DTNB reduction by both WT and His\(_6\) Pdr. Histidine had a dual effect on the reaction (Fig. 8B). It stimulated DTNB reduction only by His\(_6\) Pdr under aerobic conditions. In the presence of 1 mM histidine, the continuation of the lag phase was 2 times shorter, and the rate constant of the reaction was maximal and 50% larger than corresponding parameters measured in the absence of the amino acid (Fig. 8B, inset). In contrast, the DTNB reductase activities of WT and His\(_6\) Pdr measured under anaerobic conditions were inhibited by histidine. The reason for this dual effect of histidine on the kinetics of DTNB reduction is not clear at the moment. Only under anaerobic conditions and at pH below 6.5, both WT and His\(_6\) Pdr were capable of catalyzing NADH-dependent reduction of oxidized glutathione. The reaction was slow (< 0.2 mol of NADH oxidized min\(^{-1}\) mol\(^{-1}\) Pdr) with complex kinetics. Elucidation of the detailed mechanism of the disulfide reduction catalyzed by Pdr requires further studies.

**Effect of SH Group Modification on Redox Properties of Pdr**—In order to further investigate the role of thiols in Pdr catalysis, we have studied the effect of SH group modification on redox properties of the enzyme. Under native and reducing conditions, only 3 of 6 cysteine residues in WT and His\(_6\) Pdr

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**FIG. 6.** Effect of thiol-reducing agents and histidine on the interaction of His\(_6\) Pdr with NAD\(^+\). Spectra were recorded before (solid line) and over a period of 3 h after the addition of 5 mM DTT (dotted lines) to the anaerobic solution of 7 \(\mu\)M His\(_6\) Pdr in 100 mM potassium phosphate, pH 8.0, containing 1 mM NAD\(^+\). Selected spectra are shown. The spectral changes observed in the presence of 5 mM GSH were identical to those measured in the presence of DTT, except they occurred faster. Inset A, an absorbance change at 455 nm versus time in anaerobic solutions containing 15 \(\mu\)M His\(_6\) Pdr and 2 mM NAD\(^+\) in the absence and presence of 5 mM DTT or 5 mM GSH (curves a, b, and c, respectively). Inset B represents the kinetic traces of the absorbance change at 455 nm versus time recorded in anaerobic solutions of 17 \(\mu\)M His\(_2\) Pdr and 2 mM NAD\(^+\) in the absence and presence of 2, 20, 50, and 200 mM histidine (curves a, b, c, d, and e, respectively).

**FIG. 7.** NAD\(^+\)-induced flavin reduction in WT Pdr in the presence of thiol-reducing agents. Spectra of WT Pdr (20 \(\mu\)M) were taken before (a) and either 2 weeks after the addition of 5 mM DTT (b) or 2 days after the addition of 5 mM GSH (c). Anaerobic solutions contained 100 mM potassium phosphate, pH 8.0, and 2 mM NAD\(^+\). Inset, a plot of absorbance changes at 340 \(\times\) 0.3 (A), 455 (B), and 750 nm (C) during NAD\(^+\)-induced flavin reduction in WT Pdr in the presence of GSH as a function of time.
The absence and presence of 1 mM histidine, respectively. The anaerobic conditions (growth) by His6 (Fig. 8) is a plot of the initial rate constants for the reaction of DTNB reduction to be nonreactive or inaccessible under these conditions. Cd2+ reactions of Pdr with thiol-modifying agents were carried out in the presence and absence of NADH. When an excess of CdCl2 was added to the solution of Pdr, the protein irreversibly precipitated, presumably due to the formation of multiple intermolecular bridges via the cadmium ion and aggregation of the protein. No protein precipitation occurred during Pdr modification with PCMB or NEM, and absorbance spectra of modified proteins were similar to those of unmodified enzymes (Fig. 9).

However, the thiol-modifying agents affected the interaction between Pdr and NADH. The recombinant flavoprotein could not be completely reduced even by a large excess of NADH. Approximately 13 and 27% of WT and His6 Pdr, respectively, remains oxidized under anaerobic conditions in the presence of a 40-fold excess of reduced pyridine nucleotide. In contrast, the addition of NEM to the partially reduced enzyme resulted in completion of flavin reduction and in a slight increase of a charge transfer complex between the reduced flavin and oxidized pyridine nucleotide (Fig. 9, spectra b and c). This result can best be explained if Pdr and NAD+ form a tight complex. As mentioned previously, this complex was detected by mass spectrometry analysis. In addition, complete reduction of Pdr by NADH in the presence of Neurospora NADase also demonstrates that part of the flavoprotein is tightly bound to oxidized pyridine nucleotide. By hydrolyzing oxidized pyridine nucleotide produced during reaction of the flavoprotein with NADH, NADase permits the reaction to proceed to completion to yield the fully reduced enzyme (14). When this enzyme was present in the reaction mixture, the addition of an excess of NADH to Pdr resulted in complete FAD reduction followed by disappearance of the charge transfer complex between the reduced flavin and oxidized pyridine nucleotide (Fig. 10). If cysteine residues are involved in or promote Pdr-NAD+ complex formation, then reaction of thiol groups with the sulphydryl-modifying reagents would result in a release of NAD+ and its replacement with NADH. The fact that NAD+ does not induce any changes in the absorbance spectra of either PCMB- or NEM-modified His6 Pdr supports this view.

Although SH group modification completely eliminated an inducing effect of NAD+ on the electron flow from thiols to FAD, it did not affect the ability of Pdr to transfer electrons to K3Fe(CN)6, DCIP, and Pdx, indicating that interaction sites of the flavoprotein with these electron acceptors and NADH were not perturbed by the modifying agents. Furthermore, there was no inhibitory effect of thiol modification on the DTNB reductase activity of Pdr. It is possible that either modification was reversed upon reoxidation of the enzyme or sulphydryl groups involved in the thiol-disulfide exchange were not affected by NEM and PCMB.

**DISCUSSION**

**Properties of Recombinant Pdr**—Although Pdr was discovered more than 3 decades ago (1), it has not been studied very

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**FIG. 8. Reduction of DTNB by WT Pdr under aerobic conditions.** A, the effect of NADH and DTNB concentrations on the rate of DTNB reduction and continuation of the lag phase. The reaction mixtures contained 150 mM potassium phosphate buffer, pH 8.0, 3 μM WT Pdr, and 4.3 mM DTNB. Measurements were performed at 412 nm at 25°C. The arrows indicate the time when NADH was added. Traces a, b, c, d, and e were recorded in the presence of 1, 2, 4, 8, and 12 mM NADH, respectively. Trace f was obtained in the presence of 7 mM DTNB and 10 mM NADH. The inset to A shows a kinetic trace analogous to a but recorded in the presence of 0.1 μM WT Pdr under anaerobic conditions at 20°C. B demonstrates an effect of histidine on DTNB reduction by His6 Pdr under aerobic conditions. The reaction mixture contained 4 μM His6 Pdr, 4.3 mM DTNB, and 6 mM NADH in 150 mM potassium phosphate buffer, pH 8.0. Traces a and b were recorded in the absence and presence of 1 mM histidine, respectively. The inset to B is a plot of the initial rate constants for the reaction of DTNB reduction by His6 Pdr (a, c, d) and anaerobic conditions (b, e) versus histidine concentration. The reaction mixtures contained 7 mM DTNB, 10 mM NADH, and 0–50 mM histidine in 150 mM phosphate buffer, pH 8.0. Final enzyme concentrations were similar to those of unmodified enzymes (Fig. 9).

**TABLE II**

Kinetic parameters for the DTNB reductase activity of Pdr

| Condition | Pdr | kcat | Km forNADH | Kd forDTNB |
|-----------|-----|------|------------|------------|
| Aerobic   | WT  | 30   | 3.6        | 4.7        |
|           | His6 | 27   | 3.7        | 4.2        |
| Anaerobic | WT  | 260  | 0.012      | 3.6        |
|           | His6| 240  | 0.015      | 3.7        |

* Measured at 7 mM DTNB and 1 mM NADH.
* Measured at 4.3 mM DTNB and various concentrations of NADH.
* Measured at 1 mM NADH and various concentrations of DTNB.

**TABLE III**

Determination of free SH groups in Pdr

| WT Pdr | His6 Pdr |
|--------|----------|
| 2.6 ± 0.2 | 3.0 ± 0.3 |
| 3.0 ± 0.3 | 2.9 ± 0.2 |
| 4.9 ± 0.2 | 5.0 ± 0.2 |

* Incubated for 30 min at 37°C.
* Incubated for 30 min at 42°C.
Although molecular, spectral, and electron transferring properties of recombinant WT and His₆ Pdr are similar, the presence of eight additional C-terminal amino acid residues has a dramatic effect on the enzyme. In our laser flash photolysis experiments, the C-terminal peptide hindered interaction of His₆ Pdr with the 5-deazariboflavin radical and significantly destabilized the FAD semiquinone (Fig. 4). Finally, and most importantly, engineering of the six-histidine tag fusion protein showed that Pdr can function as a pyridine nucleotide:disulfide oxidoreductase.

Similarity of Pdr with the Enzymes of the GR Family—In order to understand what processes occur upon binding of oxidized pyridine nucleotide to His₆ Pdr, it would be helpful to compare Pdr and lipoamide dehydrogenase, a member of the glutathione reductase family. The spectral changes observed upon NAD⁺ binding to the two-electron reduced form (EH₂) of lipoamide dehydrogenase, in which both flavin and redox active disulfide groups are present in the active site (13, 17), were similar to those induced by oxidized pyridine nucleotide in His₆ Pdr and were explained as follows. In uncomplexed EH₂, the oxidation-reduction potential of the active center dithiol/disulfide is considerably more positive than that of FAD (18), and electrons are located principally in the dithiols, whereas the flavin is largely oxidized. The long wavelength absorbance in the absorbance spectrum of EH₂ arises from charge transfer between thiolate anion and oxidized flavin and is responsible for the red color of this intermediate (13, 19). The binding of NAD⁺ to EH₂ is thought to influence the equilibrium distribution of electrons between the flavin and the disulfide redox couple by increasing the amount of reduced flavin with resultant charge transfer from reduced flavin to bound oxidized pyridine nucleotide (17). The oxidation of EH₂ by NAD⁺ is very rapid and is over within milliseconds. It is accompanied by proton release and becomes thermodynamically unfavorable at low pH.

Similarity between the spectral changes induced by NAD⁺ in His₆ Pdr and lipoamide dehydrogenase led us to consider that Pdr might have redox-active dithiols. This was later confirmed when we found that Pdr can function as a pyridine nucleotide: dithiol/disulfide oxidoreductase catalyzing both forward and reverse reactions, NAD⁺-dependent oxidation of thiols, and NAD⁺-dependent reduction of disulfides. The lack of putative disulfide redox centers and the complexity of the thiol-disulfide exchange reactions catalyzed by Pdr indicate that there are some significant differences between the mechanism of function of His₆ Pdr and lipoamide dehydrogenase. The absence of a shoulder at 530 nm in the absorbance spectrum of uncomplexed Pdr, characteristic for thiolate to flavin charge transfer complex (13, 19), and a considerably slower reaction of NAD⁺ with Pdr than with the EH₂ form of lipoamide dehydrogenase indicate that there must be a thermodynamic or/and conformational barrier(s) for migration of electrons from the dithiols to the flavin in Pdr. The GR family of enzymes have conserved nascent thiols encoded by CXXC or CXXXXC motifs (13). In the amino acid sequence of Pdr, the two closest cysteine residues are 15 amino acid groups apart. Our computer modeling studies using an oxygenase-coupled NAD⁺-dependent ferredoxin reductase component in biphenyl dioxygenase from P. putida as a molecular model (7) show that potential dithiol couples in Pdr can function as a pyridine nucleotide:disulfide oxidoreductase.

First, we have found that recombinant Pdr is capable of forming dimers in solution. The dimer formation, not reported for native Pdr, was detected by gel filtration, electrophoresis, and mass spectrometry methods and occurred in the wide range of protein concentration (0.4–40 mg/ml). The fact that Pdr dimers were sensitive to the sulfhydryl-reducing agent treatment suggests that cysteine residues may be involved in the dimerization process. Elucidation of whether or not this reversible association-dissociation is specific and important for Pdr catalysis requires further investigations.

Second, mass spectrometry analysis revealed that oxidized Pdr has a high affinity for NAD⁺ and, even after long purification procedures, is partially present in solution in the NAD⁺-bound form. This may explain why Pdr does not bind to affinity resins, such as 5'-AMP-Sepharose, used for purification of NAD⁺-dependent enzymes. Existence of an NAD⁺-bound Pdr also explains the inability of a large excess of NADH to fully reduce complex (13, 19), and a considerably slower reaction of NAD⁺ with Pdr than with the EH₂ form of lipoamide dehydrogenase indicate that there must be a thermodynamic or/and conformational barrier(s) for migration of electrons from the dithiols to the flavin in Pdr. The GR family of enzymes have conserved nascent thiols encoded by CXXC or CXXXXC motifs (13). In the amino acid sequence of Pdr, the two closest cysteine residues are 15 amino acid groups apart. Our computer modeling studies using an oxygenase-coupled NAD⁺-dependent ferredoxin reductase component in biphenyl dioxygenase from P. putida as a molecular model (7) show that potential dithiol couples in Pdr can be placed no closer than 10 Å from the isoalloxazine ring of FAD. The remote location of redox active sulfhydryl groups from the flavin and/or from each other might be one of the obstacles for the fast electron migration to the flavin. Binding of NAD⁺ to the protein, therefore, might influence flavin-dithiol interaction by bringing thiol groups together close enough so they could form disulfide or/and by bringing the
Another aspect that should be addressed is the mechanism of thiol-disulfide exchange reactions catalyzed by traditional dithiol oxidoreductases where the thiolate anion is the reactive nucleophile. The $pK_a$ values of protein sulfhydryl groups can be very different from the typical value of 8.5. In most cases, the low $pK_a$ has been attributed to the presence of a nearby positively charged center that stabilizes the thiolate anion electrostatically. There is at least one base, usually a histidine, close to one sulfur of disulfide in the active sites of disulfide oxidoreductases, such as lipoamide dehydrogenase, glutathione reductase, and thioredoxin reductase. In addition to unfavorably positioned dithiols, another reason for the considerably longer time of the reaction of NAD$^+$ with Pdr than with lipoamide dehydrogenase, glutathione reductase, and thioredoxin reductase. This is due to the fact that the thiol-disulfide exchange catalyzed by these enzymes is a very slow process. In contrast, the thiol-disulfide exchange catalyzed by NAD-dependent dithiol oxidoreductases is much faster. The mechanism of NAD$^+$-induced FAD reduction in His$_6$ Pdr is summarized in Scheme 1.

Finally, an important property of the GR family is that the electron transferring and dithiol/disulfide oxidoreductase activities of Pdr can be distinguished. Pdr can be considered as a bifunctional enzyme that, under oxygen- and NADH-saturating conditions, acts as NADH-dependent ferredoxin reductase, supplying electrons for the P450cam monoxygenase, whereas in the oxygen-depleted cells it can function as dithiol/disulfide oxidoreductase, catalyzing various reactions depending on what pyridine nucleotide and dithiol/disulfide is available. Considering structural homology between Pdr and the GR family enzymes and some resemblance between mech-
A New Function of Putidaredoxin Reductase

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REFERENCES

1. Katagiri, M., Ganguli, B. N., and Gunsalus, I. C. (1968) J. Biol. Chem. 243, 3543–3546
2. Gunsalus, I. C., and Wagner, G. C. (1978) Methods Enzymol. 52, 166–188
3. Roome, P. W., Jr., Philley, J. C., and Peterson, J. A. (1983) J. Biol. Chem. 258, 2593–2598
4. Roome, P. W., and Peterson, J. A. (1988) Arch. Biochem. Biophys. 266, 32–40
5. Roome, P. W., and Peterson, J. A. (1988) Arch. Biochem. Biophys. 266, 41–50
6. Sevrioukova, I. F., Hazzard, J. T., Tollin, G., and Poulos, T. L. (2001) Biochemistry 40, 10592–10600
7. Senda, T., Yamada, T., Sakurai, N., Kubota, M., Nashizaki, T., Masai, T., Fukuda, M., and Mitsui, Y. (2000) J. Mol. Biol. 304, 397–410
8. Patil, P. V., and Ballou, D. P. (2000) Anal. Biochem. 286, 187–192
9. Prosgay, A. J., Engelke, D. R., and Williams, C. H., Jr. (1989) J. Biol. Chem. 264, 2856–2864
10. Riddles, P. W., Blakley, R. L., and Zerner, B. (1979) Anal. Biochem. 94, 75–81
11. Ellman, G. L. (1959) Arch. Biochem. Biophys. 82, 70–77
12. Holden, M., Mayhew, M., Bunk, D., Roitberg, A., and Vilker, V. (1997) J. Biol. Chem. 272, 21720–21725
13. Williams, C. H. J. (1991) in Chemistry and Biochemistry of Flavoenzymes (Müller, F., ed) Vol. III, pp. 121–211, CRC Press, Inc., Boca Raton, FL
14. Massey, V., and Veeger, C. (1961) Biochim. Biophys. Acta 48, 33–47
15. Peterson, J. A., Lorence, M. C., and Amarnah, B. (1990) J. Biol. Chem. 265, 6066–6073
16. Aoki, M., Ishimori, K., and Morishima, I. (1998) Biochim. Biophys. Acta 1386, 157–167
17. Matthews, R. G., Ballou, D. P., and Williams, C. H., Jr. (1979) J. Biol. Chem. 254, 4974–4981
18. Matthews, R. G., and Williams, C. H., Jr. (1976) J. Biol. Chem. 251, 3956–3964
19. Massey, V. (1963) Enzymes pp. 275–306, Academic Press, New York
20. Theime, R., Pai, E. F., Schirmer, R. H., and Schulz, G. E. (1981) J. Mol. Biol. 152, 763–782
21. Mattevi, A., Obmolova, G., Sokatch, J. R., Betzel, C., and Hol, W. G. J. (1992) Protein Struct. 13, 336–351
22. Thorpe, C., and Williams, C. H., Jr. (1981) Biochemistry 20, 1507–1513
23. Muiswinkel-Voetberg, H. (1973) Eur. J. Biochem. 33, 285–291
24. Miller, S. M., Massey, V., Williams, C. H., Ballou, D. P., and Walsh, C. T. (1991) Biochemistry 30, 2690–2612
25. Bild, G. S., Janson, C. A., and Boyer, P. D. (1980) J. Biol. Chem. 255, 8109–8115
