Interaction and electron transfer between putidaredoxin reductase (Pdr) and putidaredoxin (Pdx) from *Pseudomonas putida* was studied by molecular modeling, mutagenesis, and stopped flow techniques. Based on the crystal structures of Pdr and Pdx, a complex between the proteins was generated using computer graphics methods. In the model, Pdx is docked above the isoalloxazine ring of FAD of Pdr with the distance between the flavin and [2Fe-2S] of 14.6 Å. This mode of interaction allows Pdx to easily adjust and optimize orientation of its cofactor relative to Pdr. The key residues of Pdx located at the center, Asp38 and Trp106, and at the edge of the protein-protein interface, Tyr33 and Arg66, were mutated to test the Pdr-Pdx computer model. The Y33F, Y33A, D38N, D38A, R66A, R66E, W106F, W106A, and Δ106 mutations did not affect assembly of the [2Fe-2S] cluster and resulted in a marginal change in the redox potential of Pdx. Electron accepting ability of Δ106 Pdx was similar to that of the wild type protein, whereas electron transfer rates from Pdr to other mutants were diminished to various degrees with the smallest and largest effects on the kinetic parameters of the Pdr-to-Pdx electron transfer reaction caused by the Trp106 and Tyr33/Arg66 substitutions, respectively. Compared to WT Pdx, the binding affinity of all studied mutants to Pdr was significantly higher. Experimental results were in agreement with theoretical predictions and suggest that (i) Pdr-Pdx complex formation is mainly driven by steric complementarity, (ii) bulky side chains of Tyr33, Arg66, and Trp106 prevent tight binding of oxidized Pdx and facilitate dissociation of the reduced iron-sulfur protein from Pdr, and (iii) transfer of an electron from FAD to [2Fe-2S] can occur with various orientations between the cofactors through multiple electron transfer pathways that do not involve Trp106 but are likely to include Asp38 and Cys39.

In the three-component camphor hydroxylase system from *Pseudomonas putida*, a FAD-containing NADH-putidaredoxin reductase (Pdr) receives two electrons as hydride from NADH and delivers these as single reducing equivalents to two molecules of a [2Fe-2S] ferredoxin, putidaredoxin (Pdx). Two molecules of Pdx, in turn, donate electrons to one molecule of P450cam that oxidizes d-camphor to 5-exo-hydroxycamphor using molecular oxygen (1). Reactions of NADH oxidation/Pdx reduction and Pdx oxidation/camphor hydroxylation are highly coupled and proceed with the turnover numbers of 16,000 and 2,000 min⁻¹, respectively (2,3). To provide efficient catalytic turnover of P450cam monooxygenase, Pdx must form productive transient or long-lived electron transfer complexes with its redox partners. To date,
there is more supporting evidence for the electron transfer shuttle mechanism in the P450cam monoxygenase, according to which Pdx acts as a freely diffusible shuttle between Pdr and P450cam (4-7), as opposed to the mechanism that requires formation of a ternary complex between the three redox partners (8,9).

Determination of the X-ray and NMR structures of P450cam (10) and Pdx (11), respectively, has enabled a better understanding structure-function relations in these proteins. The lack of structural information on Pdr, however, was the primary reason that the Pdr-Pdx redox couple was the least studied in this system. The reported data are contradictory and suggest that steric, electrostatic, or hydrophobic components are involved in the association between the flavo- and iron-sulfur proteins (5,12-15).

Investigation of the Pdr-Pdx interaction is necessary for unraveling the mechanism of the P450cam and other homologous three-component monooxygenase systems. The long-range interprotein electron transfer is a fundamental process and, thus, elucidation of specific interactions that assist and stabilize Pdr-Pdx complex and mediate FAD-to-[2Fe-2S] electron transfer is of fundamental importance.

Recently determined X-ray structures of oxidized Pdr and oxidized and reduced Pdx have provided a structural base for studying electron transfer and molecular recognition in the Pdr-Pdx redox couple (16-18). In this study, we utilized computer graphics techniques and crystal structures of the proteins to develop three-dimensional models for the Pdr-Pdx complex. To test the validity of the proposed models and to further investigate structure-function relations in Pdx, four residues of the iron-sulfur protein located at the protein-protein interface in the model complexes were mutated and the redox properties of the Pdx mutants and their ability to accept electrons from Pdr were studied. Agreement between experimental and theoretical results and the similarity between the hypothetical Pdr-Pdx complex and crystallographic complexes of analogous redox pairs suggest that the manner of Pdr-Pdx interaction in the model and solution complexes is most likely to be similar.

Materials and Methods

Materials – The bacterial growth media were obtained from Difco. Glucose, glucose oxidase, catalase, poly-L-lysine, MOPS, and bovine serum albumin were from Sigma. All other reagents used in the study were of the highest purity grade available.

Cloning of Pdx mutants – The pET plasmid with the previously cloned wild type gene of Pdx (16) was used as a template to introduce mutations into the coding region of the iron-sulfur protein. The 5'-oligonucleotide primers CCCGCCGCAACAGGGCGGAATCGG CATGCTG (R66A) and CCCGCCGCAACAGGGCGGAATCGG CATGCTG (R66E) together with the 3'-nucleotide primer corresponding to the C-terminus of Pdx, and the 3'-nucleotide primers ATCACCGACAATATCGGAGACATCATT GGAGACTGC (Y33A), ATCACCGACAATATCGGAGACATCATT GGAGAC (Y33F), GGCGCTGCCACAAGCGACCGCAAT ATCGTA (D38A), GGCGCTGCCACAAGCGACCGCAAT ATCGTA (D38N), GCGAATTCTCAGAATTGCTATCGGGA ACATC (W106F), GCGAATTCTCAGAATTGCTATCGGGA ACATC (W106A), and GCGAATTCTCAGAATTGCTATCGGGA ACATC (Δ106) together with the 5'-nucleotide primer corresponding to the N-terminus of Pdx were used in the first PCR amplification. The synthesized 120-150 bp fragments containing the Y33A, Y33F, D38A, D38N, R66A, and R66E mutations served as 3'- or 5'-nucleotide primers for the second PCR. The 330 bp fragments of DNA encoding the Pdx mutants were digested with NdeI and EcoRI, and ligated into unique NdeI/EcoRI sites of the pET vector and the sequence was confirmed.

Protein Expression and Purification - Wild type (WT) and mutants of Pdx were expressed in E. coli and purified as described elsewhere (16). In the final preparations of WT, Y33A,
Y33F, D38A, D38N, R66A, and R66E Pdx the 
$A_{412}/A_{278}$ ratio was 0.49-0.51, whereas in the preparations of the W106A, W106F, and Δ106 mutants the corresponding ratio was 0.64. Protein concentration was measured by Lowry method (19) with bovine serum albumin used as a standard. The extinction coefficient of Pdx determined spectrophotometrically for the purest protein fractions was estimated to be $5.9 \pm 0.3 \text{ mM}^{-1} \text{ cm}^{-1}$ at 455 nm. Expression and purification of the 6-histidine tagged Pdr was carried out as previously described with $\varepsilon_{454\text{nm}}=10.9 \text{ mM}^{-1} \text{ cm}^{-1}$ used for protein concentration calculations (2).

Spectroscopic and Stopped Flow Assays – All UV-visible spectroscopy was performed using a Cary 3 spectrophotometer. Stopped flow experiments were carried out on SX.18MV instrument (Applied Photophysics, UK). Solutions were made anaerobic by multiple evacuating and flushing with pre-purified argon and included oxygen scrubbing system consisting of 1 mM glucose, 1 Unit/ml glucose oxidase, and 1 Unit/ml catalase. Reduction of WT and mutants of Pdx by Pdr was followed at 22°C at 507 nm, an isosbestic point for the two-electron reduced and oxidized flavoprotein, in 50 mM phosphate buffer, pH 7.5. Pdr (30 µM) was reduced with a slight excess of NADH before reacting with Pdx. The kinetic data were analyzed using IgorPro software (WaveMetrics, Inc.)

EPR measurements of the dithionite-reduced Pdx were performed on Bruker EPR spectrometer (Bruker BioSpin GmbH) under the following conditions: microwave power, 1 mW; modulation frequency, 100 kHz; modulation amplitude, 3 G; temperature, 77 K.

Electrochemical measurements – Cyclic voltammetry (CV) was carried out using a BAS 100W potentiostat (Bioanalytical Systems, West Lafayette, IN) according to a procedure developed by Avila et al. (20) with some modifications. A single-compartment electrochemical cell (0.4 ml) contained a platinum wire counter electrode, a 3 M Ag/AgCl reference electrode in a Luggin capillary (+205 mV vs. SHE), and an indium-doped tin oxide quartz plate (Quantum Coating) as the working electrode. The electrode was cleaned by 20 min sonication in 10%alconox solution followed by 20 min sonication in water. The sample solutions contained 80-100 µM Pdx and polylysine in a molar ratio of 1:2 in 100 mM MOPS buffer, pH 7.0. For all studied proteins, the observed peak current during voltammetry increased linearly with the square root of the scan rate, indicative of diffusion-controlled electron transfer. Halfwave potentials determined by CV were close to those measured by Osteryoung square wave voltammetry (21) and reported in the Supplemental Materials. Chemical reversibility was judged by the ratio of anodic and cathodic peak currents designated as $i_{pa}/i_{pc}$, whereas kinetic reversibility was judged by the separation between cathodic and anodic peak potentials, as denoted by $\Delta E_p$ (Table 3).

Identification of the Docking Regions in Pdr and Pdx – A surface complementary algorithm for protein docking, embodied in the program GRAMM (22), was used to identify binding sites in Pdr and Pdx. The method performs an exhaustive computational search of all possible configurations of the protein complexes to find those with the highest protein-protein surface structure complementarity. The lowest energy docking positions were analyzed to determine preferred binding regions on Pdr and Pdx and to select a plausible model where the distance between the isoalloxazine ring of FAD and the [2Fe-2S] cluster was the shortest. The starting model was optimized by adjusting positions of Pdx and the side chains of interacting amino acid residues of both proteins using the program O (23). The structures of model complexes were
energy-minimized using the program CNS (24).

**Other calculations** - Electron transfer coupling and possible electron transfer pathways between the redox centers of Pdr and Pdx were analyzed using the program HARLEM (25) based on either the pathway tunneling (26) or direct distance approaches (27). All calculations were performed using default settings. To initiate the pathway analysis, the flavin and iron-sulfur moieties were defined as electron donor and acceptor groups, respectively.

The surface area of the interface in the Pdr-Pdx complex was calculated using GRASP (28). The figures were prepared with MOLSCRIPT (29), RASTER3D (30), and PYMOL (31).

**RESULTS**

**Molecular Properties of Pdr and Pdx** – During turnover of P450cam monooxygenase, electron flow is directed from reduced Pdr to oxidized Pdx. Being able to donate two electrons, the reduced flavoprotein molecule must bind and dissociate from two molecules of Pdx during the catalytic cycle. To date, only the crystal structure of oxidized Pdr has been solved (17). Based on analogy with other NAD(P)H-dependent electron transferring flavoenzymes, where binding of the reduced pyridine nucleotides and hydride transfer occur on the re-side of the isoalloxazine ring of the flavin and the surface surrounding the opposite, si-side of the flavin cofactor serves as an electron acceptor/redox partner binding site, the hydrophobic groove on the si-side of Pdr formed by the side chains of Pro46, Val302, and Trp328 was proposed to be a docking site for Pdx. These residues are closest to the flavin and have the highest electronic coupling values (Fig. 1a,c). According to the X-ray structures of the oxidized and reduced glutathione reductase (32,33) and the NADH-dependent ferredoxin reductase component of biphenyl dioxygenase (34), structural homologues of Pdr, residues shielding the si-side of the flavin do not undergo conformational changes during redox transition. This suggests that the surface properties of the oxidized and reduced molecules of Pdr are likely to be similar. The structure of oxidized Pdr, therefore, can be used as a model of the reduced flavoprotein.

The structural data on the [2Fe-2S] ferredoxins suggest that the iron atom located closer to the molecular surface accepts and donates electrons during redox cycling (35). In Pdx, amino acid residues comprising the solvent accessible Asp38-Thr47 metal cluster binding loop have the highest electronic coupling values and are most likely to be involved in interprotein electron transfer (Fig. 1c,d). Taken together, analysis of the surface properties of Pdr and Pdx gives a clear indication on what parts of the molecules might interact in solution.

**Models of the Pdr-Pdx Complex** – To identify contact regions and generate the starting model for the Pdr-Pdx electron transfer complex, the X-ray structures of the proteins (PDB codes 1Q1R and 1XLP (17,18)) were used as input to the program GRAMM (22). The resulting docking positions between Pdr and Pdx were analyzed to select models where the distance between the isoalloxazine ring of FAD and the [2Fe-2S] cluster were less than 15-20 Å, which many consider to be the maximum possible distance for electron transfer to occur at physiologically relevant rates (27). Among the lowest energy docking positions there was a complex, designated as Model 1, where the edge-to-edge distance between
the flavin and iron-sulfur cofactors was approximately 15 Å (Fig. 2a-c). In this model, the 1,378 Å² protein-protein interface includes 2 salt bridges, 4 hydrogen bonds, and multiple van der Waals contacts (Table 1). The aromatic ring of the C-terminal Trp106 of Pdx is positioned at the center of the groove close to Trp330 of Pdr, whereas Tyr33 and Arg66 flank the protein-protein interface (Fig. 2b). The best route for electron flow from FAD to [2Fe-2S] is predicted to pass through Lys50 and Ala51 of Pdr and Asp38 and Cys39 of Pdx (Fig. 2c, Table 2). For this particular pathway, the direct distance analysis predicts a maximum electron transfer rate of 6.3 x 10² s⁻¹, an average atomic packing density of 0.55, and a corresponding β-decay factor of 1.76 Å⁻¹.

Notably, bulky Tyr33 and Arg66 of Pdx form van der Waals contacts with Thr66-Arg65 and a salt bridge with Glu335 of Pdr, respectively, at the edge of the complex interface. In the model complex, Pdx can reorient without disrupting these interactions and change the angle and the distance between the [2Fe-2S] cluster and FAD by making seesaw-like movements. One of these possible orientations of Pdx with the edge-to-edge distance between the flavin and iron-sulfur cofactors of 12.3 Å is shown in Figure 2d-f. Specific Pdr-Pdx interactions formed in this hypothetical complex, designated as Model 2, are listed in Table 1. Trp106 of Pdx shifts toward the side of the interface and its carboxyl group is in position to form a salt bridge with Arg310 of Pdr. The best electron transfer route from FAD to the [2Fe-2S] cluster is predicted to proceed through Trp330 of Pdr and Asp38 and Cys39 of Pdx with the estimated maximum electron transfer rate being two orders of magnitude higher than that predicted for Model 1 (Table 2). The optimal orientation for the Pdr-Pdx docking, therefore, is not optimal for electron transfer.

Rationale for the Mutation of Specific Amino Acids of Pdx – In order to test whether the model Pdr-Pdx complexes resemble that formed in solution, four residues of Pdx were selected for mutation. Tryptophan 106 was chosen because it has substantial conformational freedom in Pdx (16,36,37) and can occupy multiple positions at the interface in the Pdr-Pdx complex. Theoretical calculations predict that elimination of the indole ring or its reorientation could either slow down or facilitate interprotein electron transfer process (Table 2). Being positioned at the center of the protein-protein interface, the aromatic ring of Trp106 may serve as hydrophobic “lining” and regulate FAD-to-[2Fe-2S] electron transfer by improving electronic coupling between the active sites of the interacting proteins or/and by preventing Pdx from approaching Pdr too close. Thus, according to the models, removal or replacement of the indole ring of Trp106 with less bulky or less hydrophobic amino acid residues should affect Pdx-Pdr association and/or electron transfer events. To investigate the role of the C-terminal residue of Pdx in the redox partner interaction and interprotein electron transfer, W106F, W106A, and Δ106 mutants were prepared.

Strategically important positions at the interface and sensitivity to redox state change (18) were the reasons why Tyr33 and Arg66 in Pdx were selected for mutagenesis studies. In reduced Pdx, Tyr33 shifts by more than 2.0 Å and the tyrosine ring rotates by nearly 130º to form a hydrogen bond with Asp9. The side chain of Arg66, on the other side, swings toward the metal cluster binding loop to form hydrogen bond(s) with the Ser42-Ser44 peptide. According to the model
complexes, reduction driven movements of the side chains of Tyr33 and Arg66 should cause steric hindrance and disrupt specific interactions with the structural elements of Pdr and, thus, assist in dissociation of the reduced form of Pdx from the flavoprotein. Elimination of the hydroxyl moiety of Tyr33 or replacement of the positively charged Arg66 with the shorter or acidic amino acid residue must affect redox dependent reorganization and, as a consequence, slow down the Pdx-Pdr association/dissociation process and interprotein electron transfer. To test this hypothesis and to further investigate structure-function relations in Pdx, Y33F, Y33A, R66A, and R66E substitutions were made.

Finally, in the hypothetical Pdr-Pdx complexes, Asp38 is located at the center of the protein-protein interface next to the [2Fe-2S] ligand Cys39 and either forms hydrogen bonding interactions with the amide nitrogen of Ala51 of Pdr or mediates electron transfer to [2Fe-2S] (Tables 1 and 2). Moreover, Pdx can be positioned in such way that the carboxyl group of Asp38 could approach and establish a hydrogen bond with the imidazole ring of His44 of Pdr. The electron transfer pathway in this case is predicted to lead from FAD through Pro46, Leu45, and His44 of Pdr to Asp38 and Cys39 of Pdx (not shown). Asparagine 38, therefore, might be the central residue that assists binding and electronic coupling between Pdx and Pdr. To study the functional role of this amino acid residue, D38A and D38N mutants were prepared. The latter substitution was also made to solve the controversy of the previously reported results (38,39).

Characterization of Pdx Mutants – Similar to WT Pdx, all mutants were highly expressed in E. coli and purified as holoproteins. Absorbance spectra of the mutants in the visible region were similar to that of WT Pdx with the maxima observed at 329-333, 410-414, and 455-457 nm (Fig. 3). Owing to removal or replacement of tryptophan, W106F, W106A, and Δ106 Pdx had lower absorption in the near UV-region and higher A412/278 ratios (0.64 versus 0.51 in WT Pdx). As seen from the spectral overlay, all purified proteins had identical extinction coefficient at 455 nm estimated to be 5.9±0.3 mM\(^{-1}\) cm\(^{-1}\). This value is considerably lower than that measured previously for native Pdx, 10.4 mM\(^{-1}\) cm\(^{-1}\) (40), but close to those determined for the [2Fe-2S] ferredoxins from *Pseudomonas sp.* and *Azotobacter vinelandii* ($\varepsilon_{460}\text{nm}=6.3$ mM\(^{-1}\) cm\(^{-1}\)) (41) and $\varepsilon_{458}\text{nm}=7.0$ mM\(^{-1}\) cm\(^{-1}\) (42), respectively). No significant difference between the EPR spectra of reduced WT and mutants of Pdx was detected (data not shown). Deviation between the $g_{\perp}$ and $g_{\|}$ values of the reduced proteins did not exceed 0.0025 and 0.0008, respectively. Spectral analysis, thus, indicates that neither of introduced mutations affects assembling and environment of the [2Fe-2S] cluster.

Effect of Mutations on Redox Potential of Pdx – Reduction potentials for the [2Fe-2S]\(^{2+/1+}\) couple of the various Pdx mutants were measured by cyclic (CV, Fig. 4a) and Osteryoung square wave voltammetry in the range of 0 to -750 mV vs. Ag/AgCl. The $E_{1/2}$ values determined by CV for WT and mutants of Pdx were shown graphically in Figure 4b and those relative to standard hydrogen electrode (+205 mV vs. 3 M Ag/AgCl) are given in Table 3. According to the voltammetric measurements, deletion of Trp106 slightly increased the reduction potential of Pdx, whereas other mutations resulted in a marginal decrease of $E_{1/2}$. The W106A substitution caused the largest electronegative shift, -27 mV (Table 3).
Direct electron transfer with the electrode was observed for all studied iron-sulfur proteins. The reduction/oxidation of WT and mutants of Pdx was chemically reversible as judged by the \(i_{pa}/i_{pc}\) ratio (Table 3); an \(i_{pa}/i_{pc}\) value close to unity indicates a simple one-electron reduction without influence of following reactions or decomposition for either redox species. In contrast, as denoted by the \(\Delta E_p\) values (Table 3), the kinetic reversibility of reduction/oxidation of Pdx mutants varied significantly. Under the conditions of our experiments, a facile single-electron reduction would be characterized by diffusion-limited \(\Delta E_p\) of ca. 59 mV. Larger values of potential difference between cathodic and anodic peaks demonstrate additional kinetic limitations on the timescale of the measurement, and are suggestive of structural reorganizations coupled to the electron transfer event.

Kinetics of Pdr-to-Pdx Electron Transfer – Electron transfer rates from two-electron reduced Pdr to WT and mutants of Pdx were measured by stopped-flow spectrophotometry under anaerobic conditions. Reduction of the [2Fe-2S] cluster was monitored at 507 nm, an isosbestic point for the two-electron reduced and oxidized forms of Pdr. Plots of \(k_{obs}\) vs. Pdx concentration were nonlinear (Fig. 5). The limiting values of \(k_{obs}\) (\(V_{max}\)) and apparent dissociation constants, \(K_d\), calculated from the hyperbolic fits are given in Table 3. Different amino acid substitutions affected kinetics of Pdr-to-Pdx electron transfer to a different degree. Based on the kinetic parameters, Pdx mutants can be divided into three groups. Electron transfer rates from Pdr to \(\Delta 106\) and W106F Pdx were the highest and closest to that of the wild type iron-sulfur protein, whereas the \(K_d\) values measured for the mutants were 1.5-2 times lower than the corresponding parameter calculated for WT Pdx.

The second group, W106A and D38A Pdx, is characterized by the intermediate \(V_{max}\) and \(K_d\) values, ca. 30-50% of those of WT. Notably, within the 0-30 \(\mu\)M concentration range, where the Pdx:Pdr ratio is less than or equal to 1:1, the dependence of \(k_{obs}\) on [Pdx] for WT, all three Trp106 mutants, and D38A was highly similar (Fig. 5, Inset). An apparent bimolecular rate constant determined from the linear plot was found to be \(2.9 \times 10^6\) M\(^{-1}\) s\(^{-1}\). It appears that during single turnover these mutant proteins interact with Pdr similar to WT Pdx but are less efficient at higher concentrations, when multiple turnovers take place. One of the reasons for this effect might be decreased ability of the reduced mutants to dissociate from Pdr.

The rates of electron transfer from Pdr to the third group of Pdx mutants, D38N, Y33A, Y33F, R66A, and R66E, were decreased by 70-80% relative to that of WT Pdx. Except R66E Pdx, saturation of the \(k_{obs}\) vs. [Pdx] plots occurred early, at low concentrations of the iron-sulfur proteins. As a result, the apparent \(K_d\) values calculated for this group of mutants were considerably lower than that determined for WT Pdx (Table 3). Diminished electron accepting ability of these proteins, therefore, is not due to weaker binding to Pdr but might be because of inability of the mutants to optimally orient and/or dissociate from the flavoprotein. Taken together, the kinetic results suggest that dissociation of the reduced iron-sulfur protein is likely to be the rate-limiting step in the Pdr-to-Pdx electron transfer reaction.
DISCUSSION

The most direct way to identify interacting surfaces, orientation of cofactors, and possible pathways for the electron flow from Pdr to Pdx would be crystallization of the complex. Unfortunately, crystallization of multi-protein complexes is challenging and may be limited by relatively weak binding and heterogeneity. Computer modeling is an alternative approach to investigate mechanisms of protein-protein interaction and molecular recognition (43-46). We utilized this approach and produced several Pdr-Pdx electron transfer complexes that provided a structural basis for designing experiments to test the models. A specific manner of Pdr-Pdx interaction in the computer-generated models allowed us to select four key residues of the iron-sulfur protein, Tyr33, Asp38, Arg66, and Trp106, for site-directed mutagenesis and test the validity of the models.

Introduced mutations did not alter spectral properties of Pdx but caused small shifts (+2 to -28 mV) in reduction potential of the [2Fe-2S]^{2+/1+} couple. Such deviations in redox potential can affect the Pdr-to-Pdx electron transfer reaction and might be, in part, responsible for the perturbed kinetics of Pdx reduction. However, since there is no direct correlation between the shift in the redox potentials of Pdx mutants and the rates of their reduction (Table 3), it can be concluded that significant changes in the kinetics of electron transfer from Pdr to Tyr33, Asp38, Arg66, and Trp106, for site-directed mutagenesis and test the validity of the models.

According to the experimental results, the C-terminal Trp106 is not required for Pdr-to-Pdx electron transfer but the aromatic nature of its side chain and the C-terminal carboxyl group itself can affect this process to some extent by influencing redox properties and binding affinity of Pdx. This is in contrast to the Pdx-P450cam redox couple where Δ106 Pdx was shown to bind to the hemoprotein 50 times less tightly than the wild type iron-sulfur protein (47,48). Comparison of the experimentally measured and theoretically calculated electron transfer rates for Pdr-WT Pdx and Pdr-Δ106 Pdx complexes indicates that Model 1 is the closest to the solution Pdr-WT Pdx complex, whereas Model 3 better predicts the effects of Trp106 deletion (Table 2).

Role of Tyr33 and Arg66 – Significant changes in the kinetics of electron transfer from Pdr to Tyr33 and Arg66 Pdx mutants, redox potentials of which are only 3-21 mV lower than that of the wild type iron-sulfur protein, agree well with the theoretical predictions. The fact that the iron-sulfur proteins with the smaller side chains at positions 33, 66, and 106 have higher affinity to Pdr (Table 3) supports the hypothesis that Tyr33, Arg66, and Trp106 regulate Pdr-Pdx binding and electron transfer by controlling how close Pdx can approach Pdr. Limiting the interaction may be necessary to give bound Pdx freedom to reorient for efficient electron transfer and to facilitate dissociation of the reduced protein.

The most dramatic effect on the kinetics of FAD-to-[2Fe-2S] electron transfer was caused by the R66E mutation in Pdx. In accord with the molecular modeling results, the kinetic data demonstrate the importance of the positively charged guanidinium group of Arg66 in Pdx-Pdr association. By establishing electrostatic or hydrogen...
bonding interactions with the structural elements of Pdr. Arg66 might assist in initial docking or/and stabilization of the transient complex between the redox proteins. It should not be ruled out, however, that a decrease in electron accepting ability of the Tyr33 and Arg66 mutants might be, in part, due to destabilization of the reduced form of Pdx (18). As electrochemical experiments show, the kinetic reversibility of the oxidation/reduction transition, defined by $\Delta E_p$ (Table 3), in these iron-sulfur proteins and in D38N Pdx significantly deviates from that of WT and other mutants, suggesting that larger structural reorganization is required upon electron transfer to these mutants.

**Role of Asp38** — Our kinetic results on D38N Pdx are in accord with the data of Aoki et al. (39) but contradict those of Holden et al. (38) who showed a 60% decrease and 20% increase in activity of the mutant toward Pdr, respectively. We also found that the D38N substitution has a more profound effect on the kinetic reversibility of redox transition in Pdx than the D38A mutation. Owing to a significant effect of D38N mutation on the redox properties of Pdx, it is not possible at the moment to define the precise role of Asp38 in structure and function of Pdx. Based on the currently available data, we speculate that the negative charge rather than the length of the side chain of the residue at position 38 might be the major factor that helps to orient the Pdx molecule in the active site of Pdr and establish a productive electron transfer complex. According to the Pdr-Pdx complex models, electrostatic repulsive interactions between Asp38 and Glu18, the closest negatively charged residue of Pdr, can direct redox partner docking to achieve optimal orientation between the redox centers.

**Model Complexes Support Previously Reported Results** — In agreement with the NMR and site-directed mutagenesis experiments (14,38,39), our modeling studies identified Val28, Glu72, and Cys73 of Pdx as potential residues that might interact with Pdr. In the models, Glu72 can form either a salt bridge or water-mediated hydrogen bond with Lys409 of Pdr (Table 1) and elimination of its negative charge should weaken interaction between the redox proteins. This prediction supports data reported by Aoki et al. who observed 40% decrease in the electron transfer from Pdr to Pdx when the E72Q mutation was introduced in the iron-sulfur protein (39). Valine 28 and Cys73, on the other side, are positioned at the interface in the model complexes and contact Pdr. The modeling data suggest that replacement of these residues with the large side chain amino acids could cause steric hindrance and disrupt Pdr-Pdx complex formation. This suggestion explains results of Holden et al. who showed that reduction of the C73R mutant by Pdr was 85% less efficient than electron transfer to the wild type protein (38), but contradicts the conclusion that the 30-40% decrease in activities of the C73G and C73S Pdx mutants is a result of perturbed Pdr-Pdx interaction. According to our models, small side chain amino acids at position 73, such as serine or glycine, should not affect association of the proteins. Electrochemical measurements performed in this study showed that the C73S mutation causes the largest negative shift in the redox potential of Pdx (-28 mV) and has the largest effect on the kinetic reversibility of the $[2Fe-2S]^{2+/1}$ couple (Table 3), which might be responsible for the lower electron accepting ability of the mutant. Thus, the small side chain amino acid substitutions of Cys73 are likely to affect Pdr-Pdx interaction.
interaction indirectly by influencing the redox properties of the iron-sulfur protein (18).

Finally, our modeling results are in accord with the NMR, mutagenesis, isothermal calorimetry, and laser flash photolysis studies that demonstrated involvement of steric (39), hydrophobic (6,15) or both, hydrophobic and electrostatic factors (49) in the association between Pdr and Pdx. As computer models suggest, association between Pdr and Pdx is likely to be guided by the surface complementarity with the majority of binding energy provided by hydrophobic interactions between the residues surrounding the redox centers (Trp320, Trp330, Pro46, Pro47, Val302, Pro303, Leu306, and Leu306 in Pdr and Trp106 and Met70 in Pdx) with a lesser input from polar and charge-charge interactions formed on the periphery of the protein-protein interface (Arg66-Pdx/Glu335-Pdr, Glu72-Pdx/Lys409-Pdr, Asp103-Pdx/Arg310-Pdr or Trp106-Pdx/Arg310-Pdr).

Structural Similarity between the Pdr-Pdx Models and Crystallographic Complexes of Analogous Redox Pairs – In order to further analyze the physiological relevance of the proposed Pdr-Pdx complex, we compared the computer generated Pdr-Pdx models with the crystal structures of the complexes between analogous redox couples. In the complex between ferredoxin-NADP+ reductase and ferredoxin from the cyanobacterium Anabena (Fig. 6a), where the [2Fe-2S] cluster positioned 7.4 Å away from the exposed C8-methyl of the isoalloxazine ring of FAD, the redox partners are oriented along their molecular dipoles and form multiple hydrogen bonds and van der Waals contacts at the 1600 Å² interface (50). In the covalently linked bovine adrenodoxin reductase-adrenodoxin complex (Fig. 6b), mainly polar interactions are established between the proteins at the 580 Å² protein-protein interface with the shortest distance between the FAD-O3 and the metal center of 9.7 Å (51). Finally, in fumarate reductase from E. coli (Fig. 6c), the [2Fe-2S] ferredoxin-like subunit interacts extensively with the FAD-containing subunit forming multiple hydrogen bonds and four salt bridges at the 1450 Å² interface (52). In this electron transfer complex, the closest iron is 12.0 Å away from the C7-methyl group of FAD.

Evidently, despite differences in protein folds, there is striking resemblance between the crystallographic complexes and the Pdr-Pdx model (Fig. 2 and 6). The manner of interaction, relative orientation between cofactors, complementarity of the active sites, and weak, mainly non-ionic interaction between the redox proteins are the common features of the compared structures. This structural similarity, as well as agreement between the theoretical and experimental results on Pdr-Pdx interaction strongly suggests that the overall geometry of our models is most likely correct. Although none of the three computer-generated Pdr-Pdx complexes can be considered “the best” in terms of ability to precisely define interacting residues and predict effects of mutations, the models can serve as a plausible structural base for future studies on the mechanism of this redox couple.

REFERENCES
FOOTNOTES

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1 The abbreviations used are: Pdr, putidaredoxin reductase; Pdx, putidaredoxin; CV, cyclic voltammetry.

FIGURE LEGENDS

Fig. 1. Molecular surfaces of Pdr (a) and Pdx (b) showing charge distribution and positions of the cofactors and surrounding residues. Acidic and basic functional groups are in red and blue, respectively. (c) and (d) Electronic coupling between the FAD and [2Fe-2S] cofactors and the surface residues of Pdr and Pdx, respectively. Electronic coupling maps were calculated using the program HARLEM (25). Atoms with the highest and lowest coupling values are colored in red and blue, respectively.

Fig. 2. Computer generated models of the complex between Pdr (pink) and Pdx (light blue). (a)-(c) Model with the highest surface structure complementarity predicted by GRAMM (22). (d)-(f) An optimized model where position of Pdx was changed to bring the [2Fe-2S] cluster closer to FAD. The cofactors and the key interacting residues are in ball-and-stick or cpk representation. (c) and (f) Electron transfer pathways from FAD to the metal center calculated using the program HARLEM (25). Dotted lines indicate through-space jumps.

Fig. 3. Absorbance spectra of 90 µM WT and mutants of Pdx in 50 mM phosphate buffer, pH 7.5.

Fig. 4. (a) Cyclic voltammograms obtained at an indium-doped tin oxide electrode from a solution of 80 µM WT (solid line) or W106A mutant of Pdx (dotted line) and 160 µM polylysine in 100 mM MOPS, pH 7.0, at sweep rate of 2 mV/s. (b) Redox potentials of WT and mutants of Pdx measured by CV and given vs. the Ag/AgCl electrode.

Fig. 5. Plots of $k_{obs}$ vs. [Pdx] for the reaction of electron transfer from Pdr to Pdx. The iron-sulfur cluster reduction in WT (filled circles), Δ106 (open circles), W106F (filled squares), W106A (open squares), D38N (filled triangles), D38A (open triangles), Y33F (filled diamonds), Y33A (open diamonds), R66A (+), and R66E (*) was monitored under anaerobic conditions at 507 nm and 22°C. The reaction buffer contained 50 mM phosphate buffer, pH 7.5, 30 µM Pdr, 1 mM glucose, 1 U/ml of glucose oxidase, 1 U/ml catalase, and different concentrations of Pdx. Solid, dashed, and dotted lines represent hyperbolic fits to the data. Inset shows linear dependence of $k_{obs}$ versus [Pdx] for the reduction of WT, Δ106, W106F, and W106A Pdx by Pdr when the molar ration between Pdr and Pdx is less or equal to 1:1.
Fig. 6. Crystal structures and electron transfer pathways in the complexes between ferredoxin:NAD$^+$ reductase and ferredoxin from the cyanobacterium Anabena ((a), PDB code 1EWY (50)), bovine adrenodoxin reductase and adrenodoxin ((b), PDB code 1E6E (51)), and the FAD- and [2Fe-2S]-containing subunits of fumarate reductase from E. coli ((c), PDB code 1KF6 (52)). The flavo- and iron-sulfur proteins are shown in green and blue, respectively. Electron transfer pathways between FAD (yellow) and the metal cofactors (pink) were predicted by HARLEM (25). Dotted lines represent through-space jumps.
<table>
<thead>
<tr>
<th>Characteristics of the model complexes between Pdr and Pdx</th>
<th>Model 1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Model 2&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Model3&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>$E_{total}$ (kcal/mol)</strong>&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-10,904</td>
<td>-10,932</td>
<td>-10,934</td>
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<tr>
<td>Cofactor inter-planar angle (º)</td>
<td>83</td>
<td>48</td>
<td>55</td>
</tr>
<tr>
<td>Area of interface ($\text{Å}^2$)</td>
<td>1,378</td>
<td>1,285</td>
<td>1,362</td>
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<tr>
<td>Salt bridge interactions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pdr</td>
<td>Pdx</td>
<td>Pdr</td>
<td>Pdx</td>
</tr>
<tr>
<td>Arg310</td>
<td>Asp103</td>
<td>Arg310</td>
<td>Trp106&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glu335</td>
<td>Arg66</td>
<td>Lys409</td>
<td>Glu72</td>
</tr>
<tr>
<td>Hydrogen bonding interactions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lys56-N&lt;sub&gt;ζ&lt;/sub&gt;</td>
<td>Ser42-Oγ</td>
<td>Arg65-N&lt;sub&gt;ζ&lt;/sub&gt;</td>
<td>Tyr33-O</td>
</tr>
<tr>
<td>Ala51-N</td>
<td>Asp38-Oδ</td>
<td>Asn384-N&lt;sub&gt;δ&lt;/sub&gt;</td>
<td>Arg66-N&lt;sub&gt;ζ&lt;/sub&gt;</td>
</tr>
<tr>
<td>Glu60-Oε</td>
<td>Val28-O</td>
<td>Lys339-N&lt;sub&gt;ζ&lt;/sub&gt;</td>
<td>Ser44-Oγ</td>
</tr>
<tr>
<td>Lys339-N&lt;sub&gt;ζ&lt;/sub&gt;</td>
<td>Val74-O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>van der Waals contacts (bond length 2.8-4.0 Å)</td>
<td>Ala25</td>
<td>Asp9</td>
<td>Asn38</td>
</tr>
<tr>
<td>Ser61</td>
<td>Val28, Val36</td>
<td>Leu64</td>
<td>Glu72, Cys73</td>
</tr>
<tr>
<td>Arg65, Thr66</td>
<td>Tyr33</td>
<td>Thr66</td>
<td>Asn30, Gly31</td>
</tr>
<tr>
<td>Pro303, Leu306</td>
<td>Arg104</td>
<td>Val302</td>
<td>Arg65</td>
</tr>
<tr>
<td>Trp330</td>
<td>Arg104, Thr75</td>
<td>Trp330</td>
<td>Ser44, Asp39</td>
</tr>
<tr>
<td>Glu335</td>
<td>Met70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro380</td>
<td>Glu72</td>
<td></td>
<td></td>
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<tr>
<td>Hydrophobic interactions</td>
<td>His44, Pro46,</td>
<td>Trp106</td>
<td>Pro303, Leu306,</td>
</tr>
<tr>
<td></td>
<td>Pro47, Ala65,</td>
<td></td>
<td>Trp106</td>
</tr>
<tr>
<td></td>
<td>Val302, Trp330</td>
<td></td>
<td>Trp106</td>
</tr>
</tbody>
</table>

<sup>a</sup> Model with the highest surface structure complementarity predicted by GRAMM (22).

<sup>b</sup> Optimized Model 1.

<sup>c</sup> Similar to Model 2 but contains a different rotamer of Trp106 in Pdx.

<sup>d</sup> Total energy of the minimized models.

<sup>e</sup> The C-terminal carboxyl group of Trp106.

<sup>f</sup> Potential water bridged interaction.
Table 2. **Electronic coupling, pathways, and electron transfer rates calculated for the model Pdr-Pdx complexes using the program HARLEM** (25).

To initiate analysis, the default settings in HARLEM were used and the FAD and [2Fe-2S] moieties were defined as electron donor and acceptor groups, respectively.

<table>
<thead>
<tr>
<th></th>
<th>Model 1</th>
<th>Model 1- Δ106&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Model 2</th>
<th>Model 2 - Δ106&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Model 3</th>
<th>Model 3-Δ106&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAD-[2Fe-2S] distance (Å)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14.6</td>
<td>14.3</td>
<td>12.3</td>
<td>12.0</td>
<td>12.1</td>
<td>11.9</td>
</tr>
<tr>
<td>Electronic coupling (H&lt;sub&gt;AB&lt;/sub&gt;)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.5 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>1.2 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>4.9 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>3.5 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>2.8 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>3.6 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>Atom packing density (ρ)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.55</td>
<td>0.47</td>
<td>0.62</td>
<td>0.57</td>
<td>0.57</td>
<td>0.57</td>
</tr>
<tr>
<td>Average decay exponential (β) (Å&lt;sup&gt;-1&lt;/sup&gt;)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.76</td>
<td>1.91</td>
<td>1.61</td>
<td>1.71</td>
<td>1.73</td>
<td>1.72</td>
</tr>
<tr>
<td>Electron pathway&lt;sup&gt;f&lt;/sup&gt;</td>
<td>FAD(O4)-K50&lt;sub&gt;Pdr&lt;/sub&gt;-A51&lt;sub&gt;Pdr&lt;/sub&gt; same FAD(O4)-W330&lt;sub&gt;Pdr&lt;/sub&gt; same FAD(O4)-W330&lt;sub&gt;Pdr&lt;/sub&gt; same</td>
<td>FAD(O4)-W330&lt;sub&gt;Pdr&lt;/sub&gt; same FAD(O4)-W330&lt;sub&gt;Pdr&lt;/sub&gt; same FAD(O4)-W330&lt;sub&gt;Pdr&lt;/sub&gt; same</td>
<td>FAD(O4)-W330&lt;sub&gt;Pdr&lt;/sub&gt; same FAD(O4)-W330&lt;sub&gt;Pdr&lt;/sub&gt; same FAD(O4)-W330&lt;sub&gt;Pdr&lt;/sub&gt; same</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>→D38&lt;sub&gt;Pdx&lt;/sub&gt;-C39&lt;sub&gt;Pdx&lt;/sub&gt;-Fe1</td>
<td>→D38&lt;sub&gt;Pdx&lt;/sub&gt;-C39&lt;sub&gt;Pdx&lt;/sub&gt;-Fe1</td>
<td>→D38&lt;sub&gt;Pdx&lt;/sub&gt;-C39&lt;sub&gt;Pdx&lt;/sub&gt;-Fe1</td>
<td>→C39&lt;sub&gt;Pdx&lt;/sub&gt;-Fe1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum electron transfer rate (s&lt;sup&gt;-1&lt;/sup&gt;)&lt;sup&gt;g&lt;/sup&gt;</td>
<td>6.3 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1.3 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>2.4 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>1.3 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>7.9 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.3 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> The C-terminal residue Trp106 of Pdx was deleted from the coordinate files and the models were minimized.

<sup>b</sup> Distance between the closest edges of the cofactors.

<sup>c</sup> H<sub>AB</sub> is the coupling matrix element that describes the degree of wave function overlap occurring between donor and acceptor.

<sup>d</sup> ρ is the fraction of sampled space between atoms of redox cofactors that fall inside the van der Waals radii of the protein atoms.

<sup>e</sup> β is the matrix element decay factor that represents the exponential fall-off of the electronic tunneling rate with distance.

<sup>f</sup> Atoms and residues identified by HARLEM to provide the most efficient electron transfer path from FAD to the [2Fe-2S] cluster.

<sup>g</sup> Maximum electron transfer rate predicted for this particular electron pathway.
Table 3. *Kinetic Parameters for the [2Fe-2S] Cluster Reduction Reaction and Reduction Potentials of Pdx mutants*

<table>
<thead>
<tr>
<th>Pdx</th>
<th>$V_{\text{max}}$ (s$^{-1}$)</th>
<th>$K_d$ (µM)</th>
<th>$E_{1/2}^a$ (mV vs SHE)</th>
<th>$i_{pc}/i_{pa}^b$</th>
<th>$\Delta E_p^c$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>235 (100%)</td>
<td>66</td>
<td>-162</td>
<td>1.00</td>
<td>70</td>
</tr>
<tr>
<td>Δ106</td>
<td>240 (102%)</td>
<td>48</td>
<td>-160</td>
<td>1.03</td>
<td>67</td>
</tr>
<tr>
<td>W106F</td>
<td>196 (83%)</td>
<td>34</td>
<td>-166</td>
<td>0.99</td>
<td>69</td>
</tr>
<tr>
<td>W106A</td>
<td>127 (54%)</td>
<td>22</td>
<td>-189</td>
<td>1.00</td>
<td>72</td>
</tr>
<tr>
<td>D38A</td>
<td>106 (45%)</td>
<td>19</td>
<td>-184</td>
<td>1.10</td>
<td>75</td>
</tr>
<tr>
<td>D38N</td>
<td>77 (33%)</td>
<td>11</td>
<td>-187</td>
<td>1.03</td>
<td>110</td>
</tr>
<tr>
<td>Y33F</td>
<td>50 (21%)</td>
<td>10</td>
<td>-183</td>
<td>1.07</td>
<td>80</td>
</tr>
<tr>
<td>Y33A</td>
<td>61 (26%)</td>
<td>6</td>
<td>-177</td>
<td>1.00</td>
<td>94</td>
</tr>
<tr>
<td>R66A</td>
<td>59 (25%)</td>
<td>8</td>
<td>-169</td>
<td>1.03</td>
<td>81</td>
</tr>
<tr>
<td>R66E</td>
<td>50 (21%)</td>
<td>30</td>
<td>-169</td>
<td>1.07</td>
<td>96</td>
</tr>
<tr>
<td>C73S</td>
<td></td>
<td></td>
<td>-190</td>
<td>0.98</td>
<td>115</td>
</tr>
</tbody>
</table>

$^a$ Determined by cyclic voltammetry.
$^b$ Ratio of cathodic to anodic peak currents.
$^c$ Potential difference between cathodic and anodic peaks.
Figure 1
Figure 2
Figure 3
Figure 4

(a) Current (A) x 10^8 vs. Potential (mV)

(b) Potential (mV) for various mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Potential (mV)</th>
</tr>
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<tbody>
<tr>
<td>Δ106</td>
<td>-360</td>
</tr>
<tr>
<td>WT</td>
<td>-370</td>
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<tr>
<td>W106F</td>
<td>-380</td>
</tr>
<tr>
<td>R66A</td>
<td>-390</td>
</tr>
<tr>
<td>R66E</td>
<td>-400</td>
</tr>
<tr>
<td>Y33A</td>
<td>-360</td>
</tr>
<tr>
<td>Y33F</td>
<td>-370</td>
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<tr>
<td>D38A</td>
<td>-390</td>
</tr>
<tr>
<td>D38N</td>
<td>-400</td>
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<td>W106A</td>
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<tr>
<td>C73S</td>
<td>-370</td>
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</table>
Figure 5
Figure 1. Osteryoung square wave voltammograms (a) obtained at an indium-doped tin oxide electrode from a solution of 80 µM WT (solid line) or W106A mutant of Pdx (dotted line) and 160 µM polylysine in 100 mM MOPS, pH 7.0, at sweep rate of 2 mV/s. (b) Comparison of redox potentials of WT and mutants of Pdx determined by CV (•) and OSWV (?) techniques. The potential scale is given vs the Ag/AgCl electrode.
The putidaredxin reductase-putidaredxin electron transfer complex: Theoretical and experimental study
Vadim Yu. Kuznetsov, Emek Blair, Patrick J. Farmer, Thomas L. Poulos, Amanda Pifferitti and Irina F. Sevrioukova

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