# $^1$H NMR Identification of a β-Sheet Structure and Description of Folding Topology in Putidaredoxin†

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ABSTRACT: Putidaredoxin (Pdx), a 106-residue globular protein consisting of a single polypeptide chain and a [2Fe-2S] cluster, is the physiological reductant of P-450$_{cam}$, which in turn catalyzes the monohydroxylation of camphor by molecular oxygen. No crystal structure has been obtained for Pdx or for any closely homologous protein. The application of two-dimensional $^1$H NMR methods to the problem of structure determination in Pdx is reported. A β-sheet consisting of five short strands and one β-turn has been identified from distinctive nuclear Overhauser effect patterns. All of the backbone resonances and a majority of the side-chain resonances corresponding to protons in the β-sheet have been assigned sequence specifically. The sheet contains one parallel and three antiparallel strand orientations. Hydrophobic side chains in the β-sheet face primarily toward the protein interior, except for a group of three valine side chains that are apparently solvent exposed. The potential significance of this "hydrophobic patch" in terms of biological activity is discussed. The folding topology, as determined by the constraints of the β-sheet, is compared with that of other [2Fe-2S] proteins for which folding topologies are known.

Putidaredoxin (Pdx)[1] is a 106-residue globular protein consisting of a single polypeptide chain and a [2Fe-2S] cluster that is bound to the protein by cysteinyl sulfide linkages (Tanaka et al., 1974). First isolated from extracts of *Pseudomonas putida* grown on media containing camphor as the sole carbon source (Cushman et al., 1967), Pdx was identified as the biological reductant of P-450$_{cam}$. P-450$_{cam}$ catalyzes the hydroxylation of camphor by molecular oxygen as the first step in camphor catabolism by *P. putida*. Two electrons are required for each molecule of substrate turned over, one electron being transferred in each of two discrete steps from Pdx to P-450$_{cam}$. Pdx also acts as an effector for monooxygenase activity by P-450$_{cam}$, the fully reduced ternary (enzyme–substrate–$O_2$) complex being incompetent for turnover unless Pdx is present (Lipscomb et al., 1976; Shiro et al., 1989). Pdx is in turn reduced by the NADH-dependent flavoprotein Pdx reductase [for a review of the *Pseudomonas* camphor monooxygenase system, see Murray et al. (1985)]. The operon containing the genes for these proteins, located on the naturally occurring *Pseudomonas*-carried CAM plasmid, has been cloned, and the genes that encode P-450$_{cam}$, Pdx, and Pdx reductase have been sequenced and expressed in *Escherichia coli* (Unger et al., 1986; Koga et al., 1989).

Specificity in biological electron transfer is a topic of considerable importance; the prevention of "short circuits", that is, nonspecific or unregulated electron transfer between redox centers, is crucial for maintaining biological systems in a nonequilibrium (i.e., living) state. However, relatively little is known of the factors that control specificity for nonmembrane electron-transfer partners. Such partners must distinguish each other from among other potential redox partners, achieve the proper relative orientation, dock, transfer electrons, and then dissociate. Few nonmembrane redox complexes have been characterized in any detail, with the nonphysiological cytochrome $b_5$–cytochrome *c* complex being perhaps the best characterized (Rodgers et al., 1988; Wendeloski et al., 1987). Recent work has begun to investigate the complexes formed by cytochrome P-450$_{cam}$ with various redox partners (Stayton et al., 1988, 1989; Stayton & Sligar, 1990). Some structural rationale can be made for the experimental observations made in these studies, as a high-resolution X-ray crystal structure of P-450$_{cam}$ is available (Poulos et al., 1985, 1986, 1987). P-450$_{cam}$ is the only member of this extensive and important class of enzymes for which a three-dimensional structure is available. In light of this, the determination of the tertiary structure of Pdx takes on added significance, although it is as yet unknown owing to the lack of progress in obtaining crystals of Pdx suitable for X-ray analysis. None of the series of analogous [2Fe-2S] proteins that act as reductants of P-450s [e.g., adrenodoxin, megaredoxin, or linredoxin (Tanaka et al., 1973; Berg et al., 1976; Ullah et al., 1983)] have been characterized structurally. Recent work by Markley and coworkers has shown that some plant [2Fe-2S] ferredoxins are amenable to NMR structural methods (Oh et al., 1988, 1990; Oh & Markley, 1990a,b). However, a significant obstacle to the complete structural characterization of these proteins by NMR is the paramagnetism of the [Fe-S] cluster in both accessible oxidation states [Fe(II)-Fe(III) and Fe(III)-Fe(III)]. At low temperatures, antiferromagnetic coupling between formally unpaired electronic spins on each of the two Fe(III) atoms renders the oxidized protein diamagnetic, as evidenced by the lack of an EPR signal (Moleski et al., 1970; Tsibris et al., 1968; Munck et al., 1972). At ambient temperatures, however, thermal population of a paramagnetic state gives rise to hyperfine interactions, which result in line broadening and shortened relaxation times for nuclei near the [Fe-S] cluster. Nevertheless, a large fraction of the oxidized Pdx $^1$H NMR spectrum is unaffected by hyperfine interactions, making structural characterization of the oxidized protein by $^1$H NMR

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[1] Abbreviations: Adx, adrenodoxin; COSY, correlated spectroscopy; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; Fdx, ferredoxin; fid, free induction decay; HOHAHA, homonuclear Hartmann–Hahn spectroscopy; JR, jump–return; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, two-dimensional NOE spectroscopy; Pdx, putidaredoxin; RELAY, relayed coherence transfer spectroscopy; 3Q, triple quantum; 2Q, double quantum.

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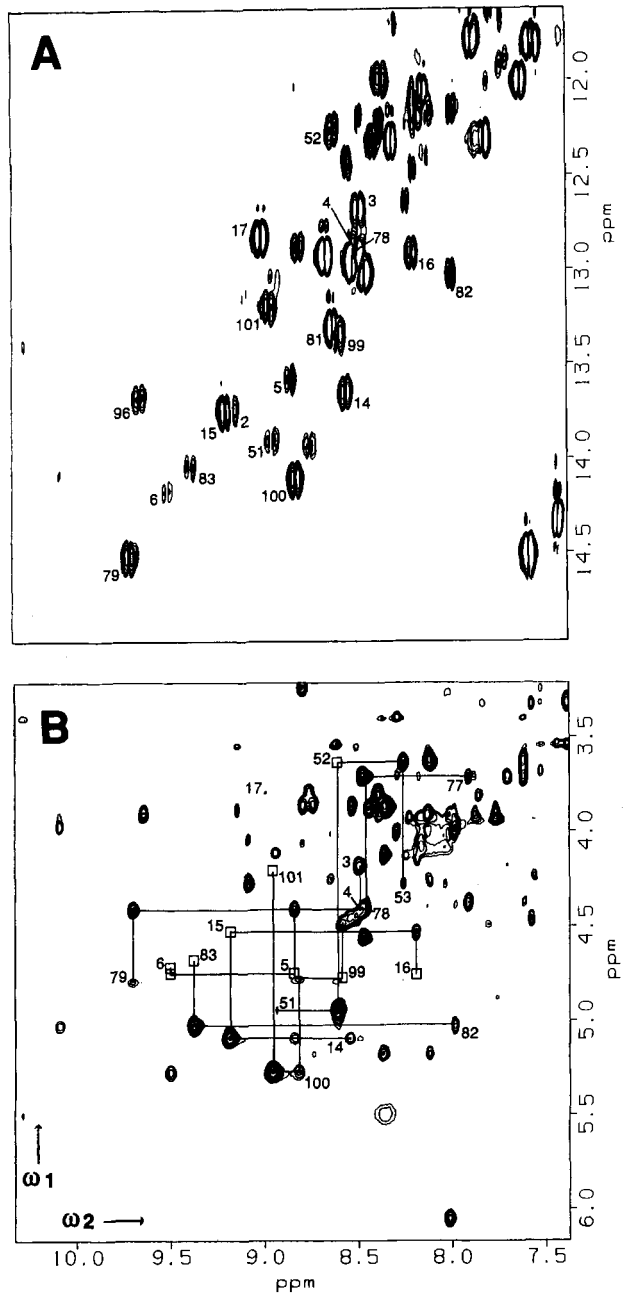


FIGURE 1: (A) NH, $C_\alpha H$ region of the 2Q correlation spectrum (500 MHz, pH 7.4 in 90% $H_2O$/10% $D_2O$, 5 mM oxidized Pdx) showing a direct correlation between NH and $C_\alpha H$ resonances ($\omega_2 = \omega_{NH}$, $\omega_1 = \omega_{NH} + \omega_{C\alpha H}$). Cross peaks are labeled indicating the amino acid residue to which they have been assigned; the numbers correspond to the amino acid sequence number, as noted in Figure 2. (B) NH ($\omega_2$), $C_\alpha H$ ($\omega_1$) region of the NOESY spectrum (500 MHz, pH 7.4 in 90% $H_2O$/10% $D_2O$, 5 mM oxidized Pdx, $\tau_m$ = 100 ms) showing sequential connectivity within the $\beta$-sheet. Cross peaks are labeled indicating the assignment of the NH proton ($\omega_2$).

methods feasible (see Figure 1). Recent work by La Mar and co-workers indicates that it may be possible to characterize those regions affected by hyperfine interactions in the reduced protein by using driven NOE experiments with short recycle times (Dugad et al., 1990).

## Materials and Methods

Oxidized Pdx was isolated from bacterial cultures as described previously (Gunsalus & Wagner, 1978). The protein was purified by using $N_2$-saturated buffers containing 0.1% 2-hydroxyethanethiol (HET). Purification consisted of cell lysis and centrifugation, concentration of the cell-free extracts, and subsequent elution of Pdx-containing fractions from a (diethylaminoethyl)cellulose column (0–0.6 M KCl gradient, 0.1 M Tris, pH 8.0, 0.1% HET). Brown-colored fractions were combined and concentrated by using a membrane concentrator (Amicon YM-10) and further purified by size-exclusion chromatography on Bio-Rad P-30 size-exclusion gel (0.05 M Tris, pH 7.4, 0.1% HET). Fractions were checked by optical spectroscopy, with an absorbance ratio of $A_{325}/A_{280} = 0.68$ being the criterion for sufficient purity.

Samples were prepared for NMR spectroscopy by concentration to ca. 6 mM with a centrifugal membrane concentrator. Buffer exchange was accomplished by using a spin column packed with Bio-Rad P-2 gel preequilibrated with the appropriate buffer [$N_2$-saturated 90% $H_2O/D_2O$ or 100% $D_2O$, buffered with Tris-$d_6$ (MSD Isotopes), pH 7.4, 0.01 M]. Final concentrations of NMR samples were typically 5 mM as determined by optical spectra. Samples were sealed in NMR tubes under argon in order to minimize sample degradation during long runs. No preservatives were added. This preparation method often yielded samples that showed little or no degradation as judged by one-dimensional $^1H$ NMR and optical spectroscopy after several days at 17 °C.

All NMR spectroscopy was performed at 17 °C, pH 7.4 (uncorrected for isotope effects). One- and two-dimensional NMR experiments were performed either on the LDB-500 500-MHz NMR spectrometer at Brandeis University built by A. Redfield and S. Kunz, or on a Bruker AM-500 500-MHz NMR spectrometer at the Research Institute of Scripps Clinic in La Jolla, CA. A spectral width of 8000 Hz in $\omega_2$ was used on the LDB-500 for all experiments, with a digital resolution of 7.81 Hz/point. Data sets were zero-filled to 2048 complex points. Typically, 240 or 480 increments of $t_1$ were used. Phase sensitivity in the $\omega_1$ dimension was obtained on the LDB-500 for all experiments by using the method of States (States et al., 1982). Two-dimensional experiments described here that were performed on the LDB-500 include the NOESY, NOESY-JR, HOHAHA, and HOHAHA-JR experiments. The NOESY experiment was performed by using a standard pulse sequence and phase cycling, with presaturation for water suppression when appropriate (Kumar et al., 1980; Billeter et al., 1982). The NOESY-JR experiment was performed in a similar manner (without presaturation of the water resonance), except the observe $\pi/2$ pulse was replaced with a $\pi/2_x$–$\tau$–$\pi/2_{-x}$ ("jump–return") semiselective pulse with the carrier placed on water in order to avoid excitation of the water resonance (Plateau & Gueron, 1982). Typically, 100–200-ms mixing times were used in the NOESY and NOESY-JR experiments. The HOHAHA experiment was performed as described by Bax and Davis (1985) with presaturation of the water signal. A 32-ms spin-lock period was used, with 1-ms trim pulses. The HOHAHA-JR experiment was performed in a similar manner, but without presaturation of the water, and the mixing period was followed by a Z-filter (Rance, 1988) in which the first hard pulse was followed by a homogeneity spoil pulse and the last pulse was a $\pi/2_x$–$\tau$–$\pi/2_{-x}$ semiselective pulse.

Experiments described here that were performed on the AM-500 include 2QF- and 3QF-COSY, 2Q, RELAY, and NOESY. A spectral width of 7462 Hz in $\omega_2$ was used on the AM-500 for all experiments, with a digital resolution of 3.64 Hz/point. The spectral width in $\omega_1$ was also 7462 Hz for all experiments except for the 2Q experiment, in which case a spectral width of 14 924 Hz in $\omega_1$ was used. Phase sensitivity in the $\omega_1$ dimension was obtained for all experiments on the AM-500 by using time-proportional phase incrementation (Redfield & Kunz, 1975; Bodenhausen et al., 1977). A total

of 400 increments of $t_1$ were used for the NOESY experiment, while 500 increments of $t_1$ were used for all coherence transfer experiments. Water suppression in all cases was performed by presaturation of the water resonance. 2QF-COSY, 3QF-COSY, and RELAY experiments were performed by using standard sequences (Rance et al., 1983; Piatini et al., 1982; Eich et al., 1982). The RELAY experiment was performed by using a mixing time of 40 ms. Typically, a mixing time of 100–200 ms was used in the NOESY experiment.

Data processing for all experiments was performed on a VAX 11/780 computer with D. Hare's FTNMR program. Data sets were zero-filled to 2048 complex points in $t_2$ prior to transformation, if required. An unshifted sine bell over the appropriate time domain was used prior to transformation of coherence-transfer fids in which antiphase multiplets are observed as cross peaks (2QF- and 3QF-COSY, RELAY, and 2Q), while a Gaussian weighting function was used for apodization of NOESY and HOHAHA data. NOESY-JR data were treated with a 10-Hz convolution difference weighting function and a cosine bell prior to $t_2$ transformation. Transformation in $t_1$ was performed after zero-filling to 2048 complex points and application of a $\pi/4$ shifted sine bell over the nonzero data points. The first data set ($t_1 = 0$) of all cosine-modulated experiments was multiplied by 0.5 in order to reduce $t_1$ noise (Otting et al., 1986), and a cubic spline base-line straightening function was applied to the data sets prior to $t_1$ transformation.

## Results

*Identification of β-Sheet Structures.* Although complete sequential assignment of the $^1$H NMR spectrum of oxidized Pdx is still in progress, analysis of the data thus far allows us to demonstrate the existence of a five-strand β-sheet involving residues 2–6, 14–17, 50–53, 81–83, and 96–101. Also identified is a β-turn associated with one edge of the sheet involving residues 79–82. These features are readily identified in two-dimensional $^1$H spectra owing to the observation of typical NOE connectivities and $J$-couplings resulting from β-sheet structure (see Figure 2). Strong $C_\alpha H_i/NH_{i+1}$ NOEs are typically seen, with weak or nonexistent $NH_i/NH_{i+1}$ sequential NOEs and often weak intraresidue $C_\alpha H$–NH NOEs as well. Typical $^3J_{C_\alpha H\text{-}NH}$ couplings are >8 Hz, owing to the 120° dihedral angle between the $C_\alpha H$ and NH protons (Pardi et al., 1984). Significant downfield NH chemical shifts and slow NH proton exchange with the solvent are also common features of β-sheets, due to the networked hydrogen-bonding patterns found therein. NOESY data obtained in $D_2O$ identify 20 slowly exchanging amide protons in oxidized Pdx among the amide resonances unaffected by hyperfine interactions, 18 of which have been assigned to NH protons in the β-sheet. These are noted in Figure 2.

The strand pairings in the β-sheet of Pdx can be characterized by the observed intra- and interstrand NOEs. Strong interstrand $C_\alpha H_i/C_\alpha H_j$ NOEs are diagnostic for antiparallel strand arrangements and are observed in a number of cases, as noted in Figure 2. Also associated with antiparallel strands are strong $C_\alpha H_i/NH_{j+1}$, $NH_{i+1}/C_\alpha H_j$, and $NH_{i+1}/NH_{j-1}$ NOEs. Parallel strand associations tend to give interstrand NOEs from $C_\alpha H_i/NH_j$, $NH_{i+1}/NH_j$, $NH_{i+1}/NH_{j+2}$, and $NH_{i+1}/C\alpha H_{j+1}$ interactions.

*Sequence-Specific Assignment of the β-Sheet-Associated Spin Systems.* Sequence-specific resonance assignments in Pdx were made by using the sequential assignment methodology described by Wüthrich (1986). The first step in this process is the identification of as many complete or nearly complete spin systems as possible. This simplifies sequential assignment considerably, since often only relatively few spin systems need to be identified by amino acid type in order to assign them unambiguously to a unique position in the sequence. The spin-system idenfications described here in brief were done in the context of assignment of the entire proton spectrum of oxidized Pdx. Only those pertinent to the β-sheet will be discussed here. The relevant assignments are summarized in Table I.

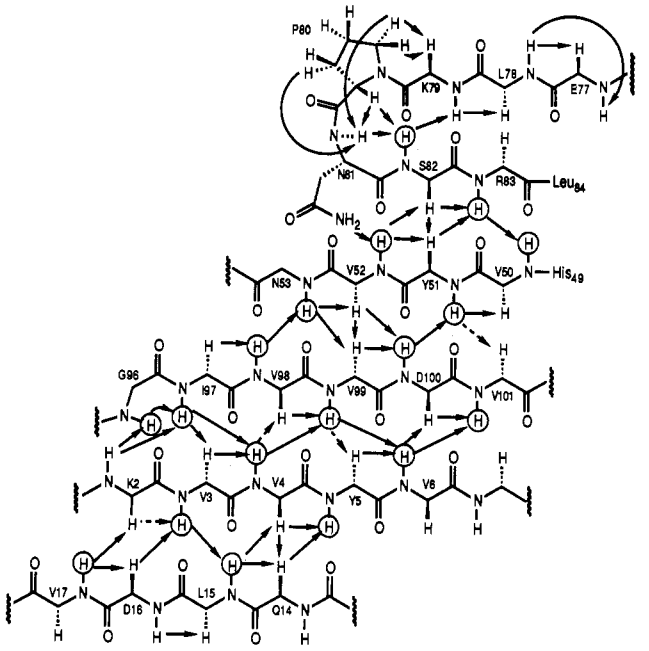


FIGURE 2: Schematic representation of the β-sheet and associated amino acid residues in Pdx, as detailed by $^1$H NMR. Residues are labeled with the standard one-letter code for amino acids. Dark arrows indicate observed NOEs, while dotted arrows indicate NOEs that are expected but unobserved due to spectral overlap or due to bleaching caused by water suppression. Circled amide protons are those observed to be at slow exchange with solvent in NOESY spectra obtained in 100% $D_2O$.

*Aliphatic Spin Systems.* The β-sheet in Pdx contains 9 of the 14 valine residues in Pdx (Vals 3, 4, 6, 17, 50, 52, 98, 99, and 101), which is not unexpected in terms of valine's preference for this type of secondary structure (Chou & Fasman, 1978). Valine spin-system identification was accomplished by using the RELAY connectivity between the NH protons and the $C_\beta H$ protons, which were in turn connected to the $C_\gamma H_3$ protons in 2QF-COSY. Identification was in most cases buttressed by a HOHAHA and/or RELAY connectivity between the $C_\gamma H_3$ protons and $C_\alpha H$ protons.

Two leucine residues were assigned in the course of this work. Leu 78, although not part of the β-sheet, is part of a connecting loop between the β-turn and the region sequentially preceding it, making its identification crucial for the verification of sequential assignments (Ye and Pochapsky, unpublished results). Leu 15 is found in the antiparallel edge strand of the sheet, Gln 14–Val 17. Spin-system identifications were made by linking the $C_\delta H_3$ and $C_\gamma H$ resonances in 2QF-COSY spectra, which in turn could be connected via RELAY and HOHAHA experiments to the $C_\beta H_2$, $C_\alpha H$, and NH resonances.

A single isoleucine spin system is found in the β-sheet, Ile 97. The 3QF-COSY spectrum allows ready identification of the $C_{\gamma 2}H_2$–$C_{\delta 2}H_3$ spin system, by virtue of this being the only methyl-containing spin system able to pass through a three-quantum filter (Muller et al., 1986). Strong NOEs connect the $C_{\gamma 2}H_2$–$C_{\delta 2}H_3$ spin system to the NH resonance, which was assigned by sequential connectivity to Gly 96 and Val 98. The

Table I: Oxidized Putidaredoxin $^1H$ Resonance Assignments for $\beta$-Sheet and Related Spin Systems[a]

| residue | $^1H$ resonance assignments |
|---|---|
| Ser 1 | $C_\alpha H$ 3.88; $C_\beta H_2$ 3.40, 3.57 |
| Lys 2 | NH 9.15; $C_\alpha H$ 4.58; $C_\beta H_2$ 1.29, 1.55; $C_\gamma H_2$ 1.12, 1.00; $C_\delta H_2$ 1.25, 1.38; $C_\epsilon H_2$ 2.65, 2.82 |
| Val 3 | NH 8.48; $C_\alpha H$ 4.18; $C_\beta H$ 1.38; $C_\gamma H_3$ 0.04, 0.45 |
| Val 4 | NH 8.50; $C_\alpha H$ 4.40; $C_\beta H$ 1.78; $C_\gamma H_3$ 0.495, 0.53 |
| Tyr 5 | NH 8.84; $C_\alpha H$ 4.72; $C_\beta H_2$ 2.23, 2.72; $C_\delta H$ 6.48; $C_\epsilon H$ 6.05; OH 8.02 |
| Val 6 | NH 9.50; $C_\alpha H$ 4.68; $C_\beta H$ 2.18; $C_\gamma H_3$ 0.56, 0.715 |
| Gln 14 | NH 8.55; $C_\alpha H$ 5.095; $C_\beta H_2$ 1.68, 1.68; $C_\gamma H_2$ 1.82, 1.97 |
| Leu 15 | NH 9.19; $C_\alpha H$ 4.54; $C_\beta H_2$ 1.31, 1.23; $C_\gamma H$ 1.31; $C_\delta H_3$ 0.52, 0.60 |
| Asp 16 | NH 8.19; $C_\alpha H$ 4.71; $C_\beta H_2$ 2.14, 2.42 |
| Val 17 | NH 9.00; $C_\alpha H$ 3.81; $C_\beta H$ 1.58; $C_\gamma H_3$ 0.66, 0.58 |
| Ala 18 | NH 8.40; $C_\alpha H$ 3.92; $C_\beta H_3$ 1.10 |
| Val 50 | NH 8.19; $C_\alpha H$ 4.115; $C_\beta H$ (1.78); $C_\gamma H_3$ (0.36), (0.385) |
| Tyr 51 | NH 8.945; $C_\alpha H$ 4.96; $C_\beta H_2$ 2.62, 2.69; $C_\delta H$ 6.63; $C_\epsilon H$ 6.65 |
| Val 52 | NH 8.615; $C_\alpha H$ 3.63; $C_\beta H$ 1.89; $C_\gamma H_3$ 0.66, 0.81 |
| Asn 53 | NH 8.26; $C_\alpha H$ 4.28; $C_\beta H_2$ 2.57, 2.79; $N_\gamma H_2$ 8.04 |
| Phe 56 | NH 8.66; $C_\alpha H$ 4.26; $C_\beta H_2$ 2.57, 2.98; $C_\delta H$ 6.96; $C_\epsilon H$ 7.10; $C_\zeta H$ 7.00 |
| Ala 76 | NH 7.62; $C_\alpha H$ 4.38; $C_\beta H_3$ 1.41 |
| Glu 77 | NH 7.91; $C_\alpha H$ 3.71; $C_\beta H_2$ 1.58, 1.81; $C_\gamma H_2$ 2.09, 2.26 |
| Leu 78 | NH 8.49; $C_\alpha H$ 4.41; $C_\beta H_2$ 1.58, 1.59; $C_\gamma H$ 1.17; $C_\delta H_3$ 0.67, 0.82 |
| Lys 79 | NH 9.71; $C_\alpha H$ 4.80; $C_\beta H_2$ 1.58, 1.73; $C_\gamma H_2$ 0.93, 1.07; $C_\delta H_2$ 1.81, 1.95; $C_\epsilon H_2$ 2.28, 2.28 |
| Pro 80 | $C_\alpha H$ 4.24; $C_\beta H_2$ 2.28, 1.76; $C_\gamma H_2$ 2.14, 1.83; $C_\delta H_2$ 3.52, 3.70 |
| Asn 81 | NH 8.62; $C_\alpha H$ 4.68; $C_\beta H_2$ 2.58, 2.71; $N_\gamma H_2$ 6.545, 7.45 |
| Ser 82 | NH 7.985; $C_\alpha H$ 5.03; $C_\beta H_2$ 3.95, 4.00; OH 10.095 |
| Arg 83 | NH 9.375; $C_\alpha H$ 4.65; $C_\beta H_2$ 1.02, 1.05; $C_\gamma H_2$ 1.56, 1.22; $C_\delta H_2$ 3.08, 3.39; $N_\epsilon H$ 10.29 |
| Gly 96 | NH 9.64; $C_\alpha H_2$ 2.82, 4.02 |
| Ile 97 | NH 7.77; $C_\alpha H$ 1.03; $C_\beta H$ 0.46; $C_{\gamma 1} H_3$ 1.10; $C_{\gamma 2} H_2$ 0.64, 1.34; $C_{\delta 2} H_3$ 0.36 |
| Val 98 | NH 4.88; $C_\alpha H$ 4.48; $C_\beta H$ 1.39; $C_\gamma H_3$ 0.61, 0.75 |
| Val 99 | NH 8.59; $C_\alpha H$ 4.75; $C_\beta H$ 1.48; $C_\gamma H_3$ 0.30, 0.36 |
| Asp 100 | NH 8.825; $C_\alpha H$ 5.28; $C_\beta H_2$ 2.50, 2.50 |
| Val 101 | NH 8.96; $C_\alpha H$ 4.23; $C_\beta H$ 1.88; $C_\gamma H_3$ 0.58, 0.72 |

[a] Assignments are not stereospecific. All chemical shifts are in ppm relative to external standard DSS. Assignments shown in parentheses are tentative owing to paramagnetic broadening, which prevents observation of coherence transfer.

remainder of the spin system is identified by a combination of 2QF-COSY and NOESY data. The significant upfield shift experienced by the $C_\alpha H$ proton of Ile 97 is attributable to ring-current shielding of this proton by the benzyl side chain of Phe 56, with which this proton also shows strong NOE cross peaks.

The only proline in the $\beta$-sheet, Pro 80, occupies position II of the $\beta$-turn, which is not surprising since 30% of all $\beta$-turns in known protein structures have a proline at this position (Chou & Fasman, 1979). The Pro 80 spin system can most easily be identified in the 2Q spectrum, since the absence of a diagonal permits the identification of correlations between the $C_\beta H_2$ and $C_\gamma H_2$ protons, which occur in the crowded 1.8–2.5 ppm region.

*Aromatic Spin Systems.* Three tyrosine residues are found in Pdx, two of which, Tyr 5 and Tyr 51, are part of the $\beta$-sheet. Identification of the aromatic spin system of Tyr 5 was straightforward: strong 2QF-COSY cross peaks connect the $C_\delta H$ and $C_\epsilon H$ resonances; NOESY connectivity from the aromatic resonances identifies the $C_\beta H_2$ protons, which are in turn connected to the remainder of the spin system in RELAY spectra. The identification of the Tyr 51 side-chain spin system was not as simple. Sequential assignment of an X–Val–X–Val fragment by connectivity with the sequence Asp 53–Ala 76 clearly identifies the NH, $C_\alpha H$, and $C_\beta H_2$ protons of the Tyr 51 spin system, all of which are connected by RELAY and HOHAHA experiments, with confirmatory direct and remote connectivities observed in the 2Q spectrum (Ye and Pochapsky, unpublished results). The NH, $C_\alpha H$, and $C_\beta H_2$ protons of residue 51 are tightly connected by an NOE to a broadened pair of nearly degenerate resonances at 6.63 and 6.65 ppm, which in turn show a large number of NOE connectivities to resonances assigned to nearby side chains. The number of NOE connectivities from these broadened resonances to structural features that are at some distance from each other in the $\beta$-sheet implies that the protons responsible for these resonances are likely to be symmetry related and interchanging between two sites. NOE connectivity between the broad resonances and a number of upfield-shifted methyl and alkyl resonances (including the spin system of Lys 79, vide infra) that do not appear to be ring current shifted by any identified aromatic residue prompts the identification of these resonances to the symmetry-related pairs of $C_\delta H$ and $C_\epsilon H$ aromatic protons of Tyr 51. A weak direct connectivity between these two resonances in the 2Q spectrum confirms these assignments. Differentiation between the $C_\delta H$ and $C_\epsilon H$ resonances is based on relative NOEs observed to the $C_\beta H_2$ protons.

Pdx contains only one phenylalanine residue, Phe 56, the aromatic spin system of which is readily identified in 2Q and 2QF-COSY spectra. As with the Tyr spin systems, NOESY connectivity from the aromatic resonances identifies the $C_\beta H_2$ protons, which are in turn connected to the remainder of the spin system by RELAY and HOHAHA.

*Hydrophilic and Charged Amino Acid Spin Systems.* Only one glycine identification is reported here, Gly 96. The Gly 96 spin system is readily identified in 2Q spectra by virtue of the distinctive remote connectivity ($\omega_1 = C_\alpha H + C_{\alpha'} H$, $\omega_2$ = NH). The identification of two of the seven serine residues in Pdx, Ser 1 and Ser 82, is described here. Connectivity between the $C_\beta H_2$ and NH of Ser 82 is obtained from HOHAHA spectra, as is the connectivity between the $C_\beta H_2$ and $C_\alpha H$. The $C_\beta H_2$ also gives rise to a remote peak ($\omega_1 = C_\beta H_a + C_\beta H_b$, $\omega_2 = C_\alpha H$) in the 2Q spectrum at the position of the $C_\alpha H$ proton. Interestingly, a remote connectivity from the $C_\beta H_2$ is also observed to an exchangeable proton resonance (10.1 ppm) identifying the OH proton resonance of this residue. This identification is substantiated by extensive NOE connectivities.

The $C_\alpha H$ and $C_\beta H_2$ resonances of Ser 1 are identified via connectivities found in the 2QF-COSY spectra and remote and direct connectivities in 2Q spectra. NOEs from the spin system of Ser 1 to the NH proton of Lys 2 permit sequential connectivity.

Two aspartate residues are found in the sheet out of a total of nine in Pdx, Asp 16 and Asp 100. These residues are straightforward to assign by using RELAY and HOHAHA data to connect the $C_\beta H_2$, $C_\alpha H$, and NH resonances. The spin system of the single asparagine residue in the sheet, Asn 81, residue III of the $\beta$-turn, is identified in a similar manner. NOE connectivity between the $C_\beta H_2$ protons and a pair of exchangeable proton resonances at 6.54 and 7.45 ppm identifies the $N_\delta H_2$ proton resonances of Asn 81. It is of interest to note that Asn is the residue most commonly found at position III of a $\beta$-turn, accounting for 19% of the residues in that position in known structures (Chou & Fasman, 1979).

Although no glutamate residues are involved in sheet formation, one is found in the region sequentially adjacent to the $\beta$-turn, Glu 77. This residue provides an important link for verifying the sequential assignment of the turn and is therefore

included here. RELAY and HOHAHA connectivity provide spin-system identification, with characteristic remote connectivities in the 2Q spectra providing verification. The same procedure provides the identification of the Gln 14 spin system, a member of the edge antiparallel strand Gln 14–Val 17. The side-chain amide protons of Gln 14 were not identified, presumably owing to fast exchange with solvent protons.

The identification of two lysine spin systems is described here: Lys 2, which is found in the strand Ser 1–Val 6, and Lys 79, which is residue I of the β-turn. Identifications were made by using a combination of NOESY, 2QF-COSY, and HOHAHA data. Only a single arginine residue is found in the sheet, Arg 83. The resonances assigned to this spin system are somewhat broadened relative to other resonances in the sheet, making it likely that the side chain at least is relatively close to the iron–sulfur cluster. The identification of the $N_\epsilon H$ proton was accomplished via an NOE connectivity to the rest of the spin system as well as a 2Q correlation with the $C_\delta H_2$ protons.

*Sequential Assignments.* Strand Gln 14–Val 17 was first assigned as X-Leu-Asp-Val, with the valine adjacent to an alanine, which fits only the series Gln 14–Leu 15–Asp 16–Val 17–Ala 18. The second strand has the sequence Ser-X-Val-Val-Tyr-Val, which is unique for Ser 1–Lys 2–Val 3–Val 4–Tyr 5–Val 6. The adjacent parallel paired strand was assigned by identifying the sequence Gly-X-Val-Val-Asp-Val, which can only be satisfied by the sequence of Gly 96–Val 101. Val 50–Tyr 51–Val 52 (originally identified as Val-X-Val) was identified by sequential connectivity with the long sequence Asn 53–Ala 76 (Ye and Pochapsky, unpublished results). The β-turn, assigned as X-Pro-Asn-Ser-Arg, could only be fit to the sequence of Lys 79–Arg 83. These assignments are confirmed by sequential connectivity with residues Ala 76–Glu 77–Leu 78.

The edge strand of the β-sheet, Gln 14–Leu 15–Asp 16–Val 17, shows the characteristics of antiparallel orientation with respect to the second strand, Lys 2–Val 3–Val 4–Tyr 5–Val 6. Two other antiparallel strand pairings (Ile 97–Val 98–Val 99–Asp 100–Val 101 with Val 50–Tyr 51–Val 52 and Val 50–Tyr 51–Val 52 with Ser 82–Arg 83) are identified by similar NOE patterns. Arg 83 and Val 50 are apparently both reasonably close to the [Fe-S] cluster, as indicated by the broadening observed to their assigned resonances. The adjacent amino acid residues, Leu 84 and His 49, have not been assigned, and it cannot yet be established whether these residues are also involved in the formation of the β-sheet.

The parallel strand pairing of Lys 2–Val 3–Val 4–Tyr 5–Val 6 and Ile 97–Val 98–Val 99–Asp 100–Val 101 is longer than the other pairings in the sheet; and, as expected for parallel strands, the pairing occurs in the interior of the sheet (Richardson & Richardson, 1990). The β-turn (Lys 79–Pro 80–Asn 81–Ser 82) is readily recognized as a type I turn by virtue of the characteristically strong Lys 79 $C_\alpha H$–Pro 80 $C_{\delta,\delta'}H$, Pro 80–Asn 81 $C_\beta H$/NH, and Lys 79–Pro 80 $C_\alpha H/C_\delta H$ NOEs, and a weak effect between the Pro 80 $C_\alpha H$ and Ser 82 NH. Pro 80, as expected, occupies position II of the four-residue turn sequence. Interestingly, the side-chain $N_\delta H_2$ protons of Asn 81, which are nondegenerate, show significant NOEs to resonances assigned to the adjacent strand (Val 52 NH). This may indicate that one of the $N_\delta H_2$ protons is involved in a hydrogen bond with the carbonyl oxygen of Val 52, providing further stabilization of the secondary structure at this point.

Residues Glu 77 and Leu 78 appear not to be involved in the formation of the β-sheet, despite the strong NOE observed between the $C_\alpha H$ of Leu 78 and the NH proton of Lys 79, which is indicative of a relatively extended conformation. This lack of involvement is deduced from the absence of a characteristic $C_\alpha H/C_\alpha H$ NOE between Leu 78 and Arg 83. Residues 77 and 78 evidently form an extended conformation loop that connects the β-turn to an α-helix that ends at Ala 76 (Pochapsky and Ye, unpublished results).

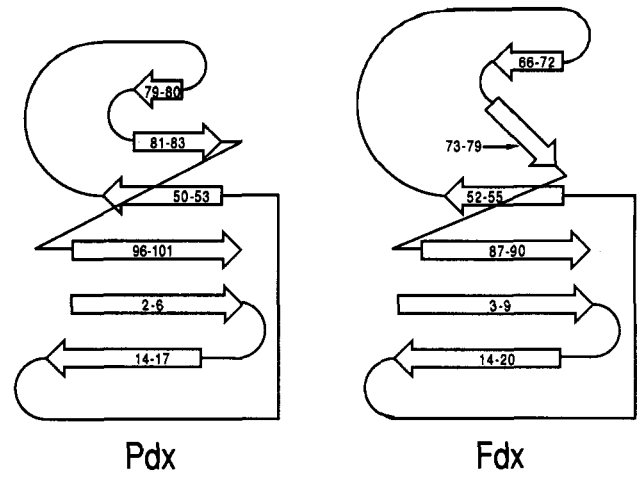


FIGURE 3: Comparison of the folding topology of the ferredoxin from *Spirulina platensis* (Tsukihara et al., 1981) and Pdx. No tertiary structural information is implied other than for the β-sheet and β-turn in Pdx. β-Sheet residues for *Spirulina* are based on hydrogen-bonding patterns shown in Figure 10 of Tsukihara et al. (1981), while the two strands that are not involved in the β-sheet in *Spirulina* are those shown in the schematic representation of the global fold; see Figure 11 of Tsukihara et al. (1981).

## DISCUSSION

*Folding Topology.* The presence of an extensive β-sheet in Pdx as described in this work places this protein well in line with what has been observed for the other [2Fe-2S] proteins for which structural information is available (Tsukihara et al., 1982; Oh et al., 1990; Oh & Markley, 1990a,b). The sheet contains the N-terminal polypeptide chain and a fragment near the C-terminus, as well as representative intermediate regions of the protein, and hence is sufficient to define a folding topology. This topology is shown in Figure 3, along with the topology of the *Spirulina* ferredoxin. The most striking feature of this topology is that despite the relatively low sequence homology (see Figure 4), there is little difference between the overall topology of Pdx and that of the *Spirulina* ferredoxin. This is not to imply that the global fold is identical; the β-sheet of Pdx is "wider" than are the corresponding sheets in either the *Spirulina* or the *Anabaena* ferredoxins, containing five strands instead of four as well as the turn (Tsukihara, 1981; Oh et al., 1990; Oh & Markley, 1990a,b). The major differences are in fact found in the C-terminal half of the protein, where the sequence homology is lowest (Figure 4). This region in *Spirulina* consists primarily of β-strands connected by loops, while in Pdx there is a significant amount of compact structure, including the tight turn, which makes up the edge of the β-sheet, and a helical region, which will be described in detail in a later publication.

*The "Hydrophobic Patch", Val 4–Val 6–Val 98.* The β-sheet described in this work for the most part has a clearly defined "hydrophobic" and "hydrophilic" side, with side chains facing the interior of the protein being for the most part hydrophobic in nature, while those facing solvent are hydrophilic. This breaks down for three residues, Val 4, Val 6, and Val 98. On the basis of sheet geometry and the lack of NOEs with the side chains to other interior residues, these valines appear in close association and on the surface of the protein. It is of

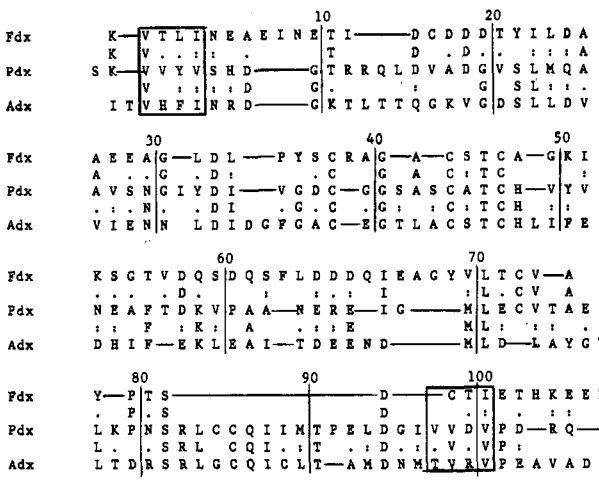

FIGURE 4: Sequence homology between *Spirulina platensis* ferredoxin (Fdx), Pdx, and porcine adrenodoxin (Adx). Regions corresponding to the "hydrophobic patch" in Pdx are highlighted. Numbering is for the Pdx sequence. For clarity, the first three residues of Fdx (Ala-Thr-Tyr) are not shown. The Fdx sequence is that of Wada et al. (1974), the Pdx sequence is that of Koga et al. (1989), and the Adx sequence is that of Akhrem et al. (1978).

interest to note that although the sequences of the homologous regions of adrenodoxin suggest that these regions are also involved in β-sheet formation, a similar hydrophobic patch is unlikely to be present (see Figure 4). This observation gains further significance when the observations of a number of researchers are noted. Lambeth and co-workers found that the interaction of Adx with Adx reductase was strongly modulated by ionic strength, while Roome noted that the interaction of Pdx with Pdx reductase was more affected by the chaotropicity of the added salt, with activity following the Hoffmeister series (Lambeth et al., 1979; Roome et al., 1983). The implication of these data is that hydrophobic interactions are important in the recognition and binding of Pdx by Pdx reductase but may not be as important in the interaction between Adx and Adx reductase. If so, the hydrophobic patch provides a potential site for recognition and binding of Pdx reductase. It is not clear whether Pdx reductase and P-450$_{cam}$ bind to the same region of Pdx, although it seems conservative to use the same electron-transfer pathway in both cases. Stayton and Sligar (1990) note that mutations resulting in the loss of net positive charge density (Lys or Arg to Gln or Glu) from the P-450$_{cam}$ surface thought to be involved in binding redox partners cause relatively small changes in the Pdx/P-450$_{cam}$ binding equilibrium, consistent with a binding interaction dominated by hydrophobic and van der Waals contributions.

*Tertiary Structural Considerations.* Although there are insufficient data as yet to completely describe the tertiary structure of Pdx, some interesting observations can be made on the basis of the present study. In particular we note the relatively slow exchange of the Ser 82 OH proton with the solvent as well as significant NOEs between the Ser 82 OH and Tyr 51 aromatic protons and the side-chain protons of Leu 78 and Lys 79. Clearly, the Ser 82 hydroxyl group, though on the nominally hydrophilic side of the β-sheet, interacts strongly with a number of other residues and is probably not significantly solvent exposed. The side chain of Lys 79 shows significant NOEs with protons on the Tyr 51 aromatic side chain. A significant ring current shift, presumably from the Tyr 51 ring, is observed to the Lys 79 $C_\gamma H_2$ protons as well. In light of these observations, it appears that the β-turn is performing a sheet-breaking function by introducing a significant twist to the polypeptide chain, thus allowing the side chains of residues 78 and 79 to interact with the "back" face of the sheet. The geometry of the turn, based on the observed NOEs, is shown in Figure 5.

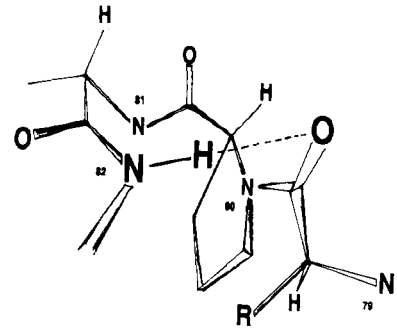

FIGURE 5: Geometry of the type I β-turn formed by Lys 79, Pro 80, Asn 81, and Ser 82 in Pdx as deduced from the observed NOEs. For clarity, only the amide proton of Ser 82, which is involved in turn stabilization, is shown. The proline residue introduces a significant twist to the polypeptide chain, permitting the side chain of Lys 79 (indicated by R) to interact with the side chains of Ser 82 and Tyr 51 (not shown).

*Conclusions.* Specificity in biological electron transfer is crucial to efficient energy utilization and storage. As yet, relatively few examples of electron-transfer complexes have been studied in any detail from a structural point of view. Hence, the structural characterization of [Fe-S] proteins in general and Pdx in particular takes on added importance, since the structure of its redox partner, P-450$_{cam}$, has been determined crystallographically. Pdx is shown to contain a significant amount of β-sheet secondary structure, as do other [2Fe-2S] proteins of similar size for which structural information is available. The present results indicate that NMR methods are suitable for structural determination of Pdx. Further structural information concerning Pdx in both redox states will be forthcoming in future publications.

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