ELECTRON TRANSFER PROTEINS OF CYTOCHROME P450 SYSTEMS

Israel Hanukoglu

I. INTRODUCTION

The reactions catalyzed by diverse P450 systems generally involve hydroxylations of substrates. These reactions are named monooxygenations because P450 cata-

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lyzes incorporation of only one atom of molecular O_2 into substrate while reducing the second into H_2O with the following stoichiometry:

$$RH + O_2 + NAD(P)H + H_+ \rightarrow ROH + H_2O + NAD(P)^+$$

Thus, each cycle of monooxygenation requires two electrons that originate from pyridine nucleotides, NADH or NADPH. The function of the electron transport proteins of P450 systems is to accept the two electrons from NAD(P)H and to transfer them one at a time to the P450 during the monooxygenase reaction sequence (White and Coon, 1980; Black and Coon, 1987; Archakov and Bachmanova, 1990; Takemori et al., 1993; for a review of the history of the field see Omura, 1993a). The known P450 systems can be grouped into four categories based on their electron transport components:

1. Mitochondrial systems include two proteins, NADPH specific adrenodoxin reductase with FAD cofactor, and adrenodoxin[1], a [2Fe-2S] ferredoxin type iron-sulfur protein (previous reviews: Lambeth et al., 1982; Orme-Johnson, 1990; Lambeth, 1991; Hanukoglu, 1992). FAD can be reduced by two electrons from NADPH, which are transferred one at a time to adrenodoxin which is a one electron carrier. Both proteins are located on the matrix side of the inner mitochondrial membrane (Mitani, 1979; Hanukoglu, 1992). Whereas the P450s are anchored to the membrane, the electron transfer proteins are soluble and may have greater mobility in the matrix. They are encoded as larger precursors, and proteolytically processed to their mature sizes after transfer into mitochondria (Omura, 1993b).

2. Microsomal systems depend on a single NADPH specific P450 reductase that contains both FAD and FMN as cofactors (previous reviews: Black and Coon, 1987; Archakov and Bachmanova, 1990; Schenkman and Greim, 1993). FAD can accept two electrons from NADPH and FMN functions as the single electron carrier. FAD and FMN have midpoint potentials of –328 mV, and –190 mV. Thus, the route of electron transfer is NADPH → FAD → FMN → P450 (Vermillion et al., 1981; Oprian and Coon, 1982). Some microsomal P450s may receive the second electron from NADH through cytochrome b_5 reductase and cytochrome b_5 (previous reviews: Arinc, 1991; Borgese et al., 1993; Schenkman, 1993).

P450 reductase can reduce cytochrome c in addition to cytochromes P450 and b_5. Hence, in early work it was named "cytochrome c reductase," despite cytochrome c's being an artificial acceptor, normally located in the inter-membrane space of the mitochondria. The reductase and the P450s are located on the cytoplasmic side of the endoplasmic reticulum membrane, anchored to the membrane by their hydrophobic amino termini which are inserted into the membrane during translation. The amino termini include both the signal and membrane anchor sequences and are not cleaved after membrane insertion (Haniu et al., 1989; Pernecky et al., 1993; Sakaguchi and Omura, 1993; Tashiro et al., 1993).

3. Bacterial P450cam (from *Pseudomonas putida*, "cam" for camphor substrate) type systems include a ferredoxin reductase and a ferredoxin (named putidare-

doxin) that are functionally similar to the mitochondrial electron transfer proteins (Gunsalus and Sligar, 1978). P450cam and the electron transfer proteins are all soluble and do not appear to be membrane associated. The proteins are encoded by an operon in the CAM plasmid (Koga et al., 1989; Peterson et al., 1990).

4. Bacterial P450meg (from *Bacillus megaterium*) type system composed of one large soluble protein with a P450 reductase domain and a cytochrome P450 domain each of which are homologous to the microsomal P450 system components (Ruettinger et al., 1989; Oster et al., 1991; Boddupalli et al., 1992; Ravichandran et al., 1993).

In contrast to the multiplicity of cytochromes P450 (Nelson et al., 1993), there appears to be only one form of each of mitochondrial adrenodoxin reductase, adrenodoxin, and the microsomal P450 reductase, encoded by one or two similar nuclear genes in all animal species (for review see Hanukoglu, 1992). Thus, the electron transfer proteins are not specific to individual P450s and serve as electron donors for different cytochromes P450 in different tissues. While in some plant species there is a single gene for P450 reductase (Meijer et al., 1993), in others there are several related forms of P450 reductase (e.g., Lesot et al., 1992). The function and specificities of these remain to be determined.

This chapter presents a review of the structural and functional aspects of the flavoenzyme and ferredoxin type electron transfer proteins of the mitochondrial, microsomal and bacterial P450 systems. The studies summarized below established several general principles for P450 system electron transport chains: (1) The reductases are generally expressed at much lower levels than P450s, there being only one molecule of reductase per about 10 or more molecules of P450, (2) the protein components are independently mobile and do not form static multicomponent complexes, (3) proteins that are redox partners form transient high affinity 1:1 complexes during their random diffusions, in accordance with the principles of mass action. Dissociation constants of these protein-protein complexes are strongly influenced by the redox states of the proteins and other molecules in the environment, such as P450 substrate, ions, and phospholipids, and (4) the transfer of an electron between two redox partners depends on the formation of a specific high affinity 1:1 complex between the two proteins. In P450 systems electron transfer is not always coupled to substrate monooxygenation. P450s and their electron transfer proteins may transfer electrons to other acceptors, such as O_2. This type of "uncoupling" or "leaky electron transport" is observed in both mitochondrial and microsomal systems (Hornsby, 1989; Archakov and Bachmanova, 1990; Hanukoglu et al., 1993). The regulation of protein–protein complex formation generally enhances productive associations for monooxygenase activities and helps to minimize uncoupled reactions that produce harmful free radicals.

II. SEQUENCE AND STRUCTURAL RELATIONSHIPS

Although the reductases of the mitochondrial and microsomal P450 systems are all flavoenzymes that bind NADP, as summarized below, their sequences represent different superfamilies of enzymes with no apparent evolutionary relationship.

A. Adrenodoxin Reductase and Putidaredoxin Reductase

Adrenodoxin reductase (~50 kD) and putidaredoxin reductase (~45 kD) are both soluble proteins that contain FAD as a cofactor (Chu and Kimura, 1973; Hiwatashi et al., 1976; Gunsalus and Sligar, 1978). In immunoelectron microscopy of adrenal cells, adrenodoxin reductase appears as membrane associated (Mitani, 1979). However, its sequence does not have a hydrophobic membrane spanning segment (Fig. 1). Thus, it probably functions as a peripheral membrane protein associated with ionic interactions.

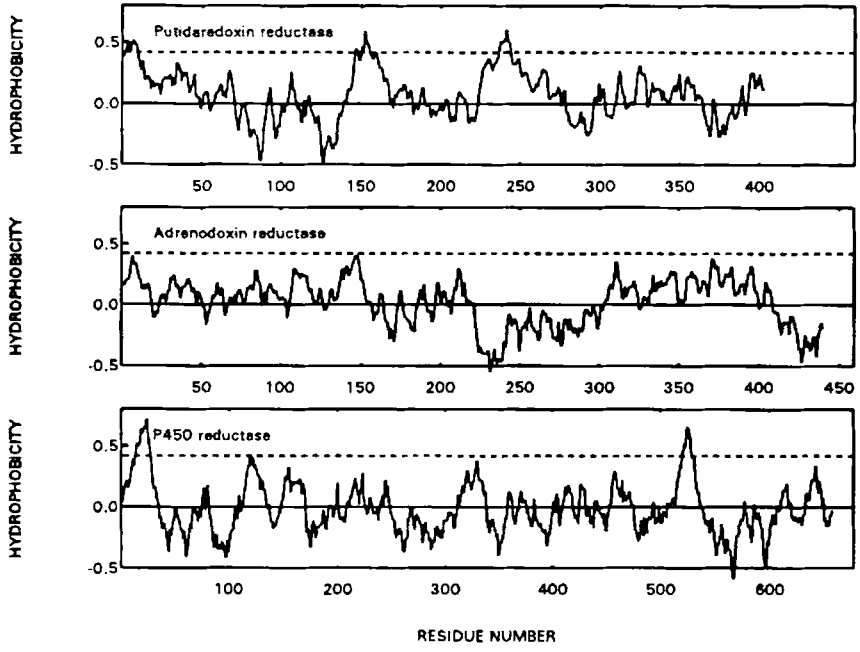

Figure 1. Hydrophobicity profiles of putidaredoxin reductase (Koga et al., 1989; Peterson et al., 1990), human adrenodoxin reductase (Solish et al., 1988), and human P450 reductase (Haniu et al., 1989). Each point is the average hydrophobicity of a 20 residue segment (Eisenberg et al., 1984). In the hydrophobicity scale, the cutoff for the prediction of a transmembrane segment is 0.42 (*dashed line*) (Eisenberg et al., 1984).

Despite functional similarities, adrenodoxin reductase shows no sequence homology with putidaredoxin reductase or other types of oxidoreductases (Hanukoglu and Gutfinger, 1989) (Fig. 2). The hydrophobicity profiles of these two enzymes are also highly different (Fig. 1). However, putidaredoxin reductase shows unequivocal sequence homology with rubredoxin reductase (Eggink et al., 1990), and the ferredoxin-NAD reductase component of benzene and toluene dioxygenases from P. putida (Irie et al., 1987; Zylstra et al., 1989), over the entire lengths of these enzymes (Fig. 2).

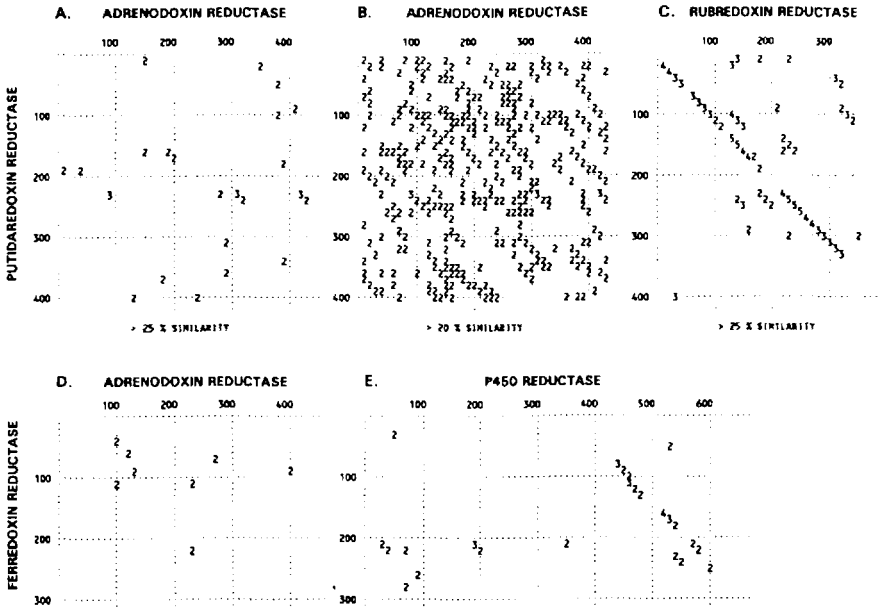

Figure 2. Matrix plot sequence comparisons: (*A, B, C*) Putidaredoxin reductase (Koga et al., 1989; Peterson et al., 1990) versus human adrenodoxin reductase (Solish et al., 1988), and (C) rubredoxin reductase (Eggink et al., 1990). (*D, E*) Spinach ferredoxin reductase (Karplus et al., 1984) versus (D) adrenodoxin reductase and (*E*) human P450 reductase (Haniu et al., 1989). The numbers on the axes correspond to the residue numbers of the mature enzyme. The parameters used in the analysis: Range = 30, compression factor = 10, cutoff point = 25% (20% in B). Thus, each digit on the plots indicates that within a 30 residue long segment to the right of the marked position at least 25% of the residues are identical in both sequences. The numbers indicate the percentile range of identical residues in the 30 residue segment, e.g., 3 = 30–39%, 4 = 40–49%. The diagonal lines indicate homologous sequences. If the cutoff for sequence similarity is lowered, instead of a diagonal line a random distribution of points appears.

The FAD and NAD(P) binding sites of both adrenodoxin reductase and putidaredoxin reductase were identified using an ADP dinucleotide binding site consensus motif (Hanukoglu and Gutfinger, 1989; Peterson et al., 1990). FAD and NAD(P) both have ADP as a common part of their structures. In most FAD or NAD(P) binding enzymes, the sites that bind this ADP portion also share a similar conformation of a βαβ-fold. The most highly conserved sequence in this fold is Gly-X-Gly-X-X-Gly/Ala which forms a tight turn between the first β-strand and the α-helix (Rossman et al., 1975; Wierenga et al., 1985; Hanukoglu and Gutfinger, 1989). Analyses of adrenodoxin reductase sequence led to the discovery that in NADP binding sites of this type, there is an Ala instead of the third Gly residue, and it was proposed that this is a major determinant of NADP vs. NAD specificity of enzymes (Hanukoglu and Gutfinger, 1989). This hypothesis was verified for glutathione reductase (Scrutton et al., 1990; Mittl et al., 1993). Consistent with the predicted difference between NAD and NADP binding sites, the sequences of putidaredoxin reductase and the other homologous ferredoxin reductases match the NAD motif (Fig. 3).

Although the entire sequences of putidaredoxin and adrenodoxin reductases share no similarity, the FAD and NAD(P) motifs appear in both enzymes at nearly identical positions: the FAD site at the amino terminus, and the NAD(P) site at 146–151 residues from the amino terminus (Figures 3 and 4). Similar spacing of the FAD and NADP sites is also observed in many other flavoenzymes (Hanukoglu and Gutfinger, 1989). The sequence of the FAD binding amino terminus is highly conserved across species (Yamazaki and Ichikawa, 1990). A bovine adrenodoxin

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                                        FAD binding site

Adrenodoxin R (Bovine)      1   STQEQTPQICVVGSGPAGFYTAQHLLKHHSRAHVDIYEKQLVPFGL
Adrenodoxin R (Human)       1   STQEKTPQICVVGSGPAGFYTAQHLLKHP QAHVDIYEKQPVPFGL
Putidaredoxin R             1     MNANDNVVIVGTGLAGVEVAFGLRASGWEGNIRLVGDATVIPHH
FAD consensus motif                 ±••••G  G  •G•   A  +••±  G  ±•  ••
Secondary structure                 TββββββTαααααααααααααααTTββββββT

                                        NAD(P) binding site

Adrenodoxin R (Bovine)    137 RELAPDLSCDTAVILGQGNVALDVARILLTPPDHLEKTDITEAALGALR
Adrenodoxin R (Human)     136 QELEPDLSCDTAVILGQGNVALDVARILLTPPEHLERTDITKAALGVLR
Putidaredoxin R           141 CIRRQLIADNRLVVIGGGYIGLEVAATAIKANMHVTLLDTAARVLERVT
NADP consensus motif                  +••••G  G  •A•-•A   ••  •G  +•   •
NAD  consensus motif                  +•  •  G  G   G     •   •        •  •  -
Secondary structure                   TββββββTαααααααααααααααTTββββββT
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Figure 3. Predicted FAD and NAD(P) binding sites of adrenodoxin reductase (Solish et al., 1988; Hanukoglu & Gutfinger, 1989; Sagara et al., 1987) and putidaredoxin reductase (Koga et al., 1989; Peterson et al., 1990). The residues are numbered starting with the amino terminus of the mature protein. The FAD and NADP motifs are from Hanukoglu & Gutfinger (1989), and the NAD motif from Wierenga et al. (1986). (·) indicates a hydrophobic residue. (+), (–), and (±) indicate charged and hydrophilic residues respectively.

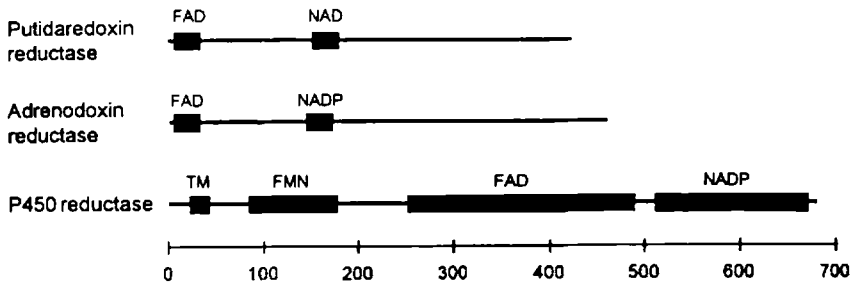

Figure 4. The localization of the FAD and NAD(P) binding sites of putidaredoxin and adrenodoxin reductases and the structural domains of P450 reductase. The FAD and NAD(P) binding sites of putidaredoxin and adrenodoxin reductases refer to the ADP portion of these coenzymes. The domains of P450 reductase is based on the prototype structure of ferredoxin reductase (Karplus et al., 1991), and on the model of Porter et al. (1990). *TM*: transmembrane segment.

reductase cDNA expressed in yeast with an extra segment encoding four additional residues at the N-terminus did not yield an active enzyme, suggesting that the four residues disrupted the incorporation of FAD into the apoprotein (Akiyoshi-Shibata, 1991).

The evolution of the enzymes with the FAD and NAD(P) binding domains noted above remains an enigma because of the lack of sequence homology outside of these very short domains. One explanation may be found in the observation that in the adrenodoxin reductase gene, the FAD and NADP binding sites are encoded by separate small exons (Lin et al., 1990). In this gene, exon 7 starts at the end of the α-helix of the $\beta\alpha\beta$ fold. One alternative splicing product encodes six extra residues at this position in both bovine and human genes (Sagara et al., 1987; Solish et al., 1988). However, this form represents ~1% of the total reductase mRNA population (Brentano et al., 1992), and expression of its cDNA in *E. coli* did not yield active enzyme, suggesting that the extra residues disrupt the structure of the enzyme (Brandt and Vickery, 1992).

Whereas the bovine adrenodoxin reductase was reported to be glycosylated (Hiwatashi et al., 1976; Suhara et al., 1982), a recent study could not corroborate previous evidence for functional glycosylation of the bovine enzyme (Warburton and Seybert, 1994). The porcine enzyme is free of carbohydrate (Hiwatashi and Ichikawa, 1978). Adrenodoxin reductase expressed in *E. coli* functioned as well as the native enzyme in a reconstituted mitochondrial P450scc system. Thus, the apoprotein may be assembled to active holoenzyme without eukaryotic posttranslational modifications (Sagara et al., 1993).

Structure-function studies using chemical modification methods implicated the following roles for specific residues of adrenodoxin reductase: (a) FAD binding: a

histidine residue (Yamazaki et al., 1992), (b) NADP binding: a histidine and a cysteine (Hiwatashi et al., 1976; Hiwatashi and Ichikawa, 1978), (c) adrenodoxin binding: a tryptophan (Sarkissova et al., 1990), Lys-243 (Hamamoto et al., 1988), and a cluster of basic residues in the amino terminal portion (Hara and Miyata, 1991). Site directed mutations suggest that Arg-239 and Arg-243 of human adrenodoxin reductase interact with adrenodoxin (Brandt and Vickery, 1993). Limited proteolysis cleaves the enzyme to yield two large fragments that may represent distinct domains (Warburton and Seybert, 1988).

Adrenodoxin reductase has been crystallized in several laboratories (Nonaka et al., 1985; Hanukoglu et al., 1992; Kuban et al., 1993). The elucidation of its crystal structure is necessary to increase our understanding beyond the sequence analyses, to identify in detail the cofactor pockets, and the electron acceptor ferredoxin binding sites, and to elucidate the routes of electron transfer.

B. Adrenodoxin and Putidaredoxin

Adrenodoxin (~14 kD) and putidaredoxin (~11.5 kD) are soluble [2Fe-2S] ferredoxin type iron-sulfur proteins that function as a single electron (for previous reviews see Estabrook et al., 1973; Gunsalus and Sligar, 1978). The sequences of adrenodoxin and putidaredoxin can be aligned over their entire lengths with only a few gaps (Cupp and Vickery, 1988). A recently cloned [2Fe-2S] ferredoxin from *E. coli* also shares 36% identity with adrenodoxin and putidaredoxin (Ta and Vickery, 1992). Waki and associates (1986) reported the purification of a [2Fe-2S] protein from bovine liver mitochondria that can support C-25 and C-27 hydroxylations of steroids, but with an amino terminus sequence completely different from adrenodoxin. The functional and structural relationship of this protein to adrenodoxin may be elucidated after cloning of its cDNA.

Bovine adrenodoxin is translated from multiple species of mRNA encoded by a single gene (Sagara et al., 1990). The protein sequences encoded by these mRNAs differ only in the C-terminus of the signal peptide and the first two residues of the mature sequence (Okamura et al., 1987). In the human genome there are two genes, but both encode the same protein product (Chang et al., 1990). In polyacrylamide gel electrophoresis of adrenodoxin purified from different tissues two major bands or a broad band may be observed around ~12–14 kD (Driscoll and Omdahl, 1986; Bhasker et al., 1987). This heterogeneity was considered to represent a multiplicity of tissue specific forms of ferredoxin. However, it apparently results from proteolytic cleavage of up to 14 residues from the carboxy terminus of the mature adrenodoxin during the purification process (Hiwatashi et al., 1986; Sakihama et al., 1988). Trypsin treatment of purified adrenodoxin produces a truncated form of adrenodoxin (des 116–128) which shows a lower K_m (higher affinity) in supporting P450 activity (Cupp and Vickery, 1989).

Putidaredoxin sequence is shorter than the adrenodoxin sequence at the carboxy terminus. The deletion or alteration of the terminal tryptophan residue adversely affects its interaction with P450cam (Davies and Sligar, 1992).

In the [2Fe-2S] center two iron atoms are coordinated to four cysteines and two labile sulfur atoms. Mature bovine adrenodoxin sequence includes five cysteines. Chemical modification and site-directed mutagenesis studies indicated that *Cys*-46, 52, 55, and 92 are involved in iron-sulfur coordination, whereas *Cys*-95 is free (Cupp and Vickery, 1988; Uhlmann et al., 1992). In putidaredoxin sequence, the corresponding cysteines 39, 45, 48, and 86 are conserved and function as the ligands to the iron atoms (Gerber et al., 1990). Putidaredoxin sequence includes two additional cysteines that are apparently free.

A cluster of negatively charged residues of bovine adrenodoxin have been implicated in complex formation with both adrenodoxin reductase and mitochondrial P450s in studies employing different approaches (Lambeth et al., 1984; Tuls et al., 1987; Hara and Miyata, 1991; Coghlan and Vickery, 1992). Site directed mutation of *Asp*-76 and *Asp*-79 showed that these residues are essential for adrenodoxin binding to both the reductase and P450scc (Coghlan and Vickery, 1992). In contrast, modification of all lysine or arginine residues did not affect adrenodoxin interactions with either of its redox partners, suggesting that these are not located at the binding site for either protein (Tuls et al., 1987). These findings support the conclusions based on kinetic studies (see below) that the binding sites of these two enzymes on adrenodoxin overlap. Yet, the differential effects of carboxy-terminal truncation at *Arg*-115 on interactions with adrenodoxin reductase and P-450 suggest that the sites are not identical (Cupp and Vickery, 1989). A mutated form of adrenodoxin missing six amino terminal residues supported only 60% of the activity of P450scc suggesting that the amino terminal residues may also play a role in P450scc binding or electron transfer (Sagara et al., 1992). Modification of *His*-56 reduced the binding of adrenodoxin to its redox partners (Miura et al., 1991), and *Tyr*-82 mutation affected binding of adrenodoxin to mitochondrial P450s without affecting cytochrome c reduction (Beckert et al., 1994). Proton NMR studies indicate that the reduction of adrenodoxin changes the conformation of the residues in the putative binding site for its redox partners (Miura and Ichikawa, 1991).

Bovine and chick adrenodoxins can be phosphorylated. This may affect their interaction with P450 and hence the activity of P450 (Monnier et al., 1987; Mandel et al., 1990). Adrenodoxin cDNAs expressed in *E. coli* encode proteins functionally as active as the native protein, indicating that the [2Fe-2S] centers of these proteins can be properly assembled in bacteria, and that eukaryote specific posttranslational modifications are not necessary for activity (Tang and Henry, 1993; Coghlan and Vickery, 1992; Uhlmann et al., 1992).

The structure of adrenodoxin crystals currently available cannot be solved because of their complexity (Marg et al., 1992). The elucidation of the complete structure of adrenodoxin awaits formation of different crystals suited for crystal-

lographic analysis. Proton NMR studies indicate that the structure of adrenodoxin is similar to that of Spirulina platensis ferredoxin (Miura and Ichikawa, 1991).

C. P450 Reductase

P450 reductase (~77 kD) is the only known enzyme with both a FAD and a FMN cofactor. The enzyme is anchored to the endoplasmic reticulum membrane by a highly hydrophobic transmembrane segment at its amino terminus (Fig. 1). The expression of a P450 reductase cDNA with a deleted anchor sequence yields a cytosolic enzyme in the yeast (Urban et al., 1990). This hydrophobic domain can be cleaved with hydrolytic enzymes producing a large soluble peptide (~71 kD) that retains the FAD and FMN spectra of the native enzyme, can reduce cytochrome c as an artificial acceptor, but not P450 (Iyanagi et al., 1978; Strobel et al., 1980; Black and Coon, 1982; Lu, 1991). The expression of rat P450 reductase cDNA in *E. coli* yielded truncated forms in addition to the full size protein with FAD and FMN cofactors. These findings show that the holoenzyme can be assembled in bacteria and that the hydrophobic termini may be degraded inside the bacteria (Porter et al., 1987). Functional reductase was also obtained in large yields in a baculovirus expression system (Tamura et al., 1992).

The sequences of P450 reductases from mammalian species are highly conserved, yet the yeast reductase shares only 30–35% similarity with the mammalian enzymes (Yabusaki et al., 1988; Haniu et al., 1989; Ohgiya et al., 1992). The transmembrane segment, and FAD, FMN, NADPH, and P450 binding sites of the enzyme have been tentatively identified by sequence analyses and structure-function studies summarized below (Fig. 4). FMN binding domain of P450 reductase shares a very short segment of similarity with flavodoxins (Porter and Kasper, 1986). Although P450 reductase does not interact with a ferredoxin, its FAD and NADP domains are homologous to plant ferredoxin-NADP reductase (Porter and Kasper, 1986) (Fig. 2). These observations led to the suggestion that P450 reductase evolved by fusion of two genes encoding a flavodoxin and a ferredoxin reductase type enzyme (Porter and Kasper, 1986). The gene sequence of the rat reductase revealed a general correspondence between the exons and the predicted structural domains of the protein (Porter et al., 1990).

P450 reductase and plant ferredoxin reductase sequences are also homologous to NADPH-sulfite reductase, NADH cytochrome b_5 reductase, NADH nitrate reductase, and nitric oxide synthase (Karplus et al., 1991; Bredt et al., 1991). These enzymes constitute an evolutionarily related superfamily. P450 reductase was claimed to be homologous also to glutathione reductase (Haniu et al., 1986; Porter and Kasper, 1986; Porter et al., 1990). However, homology matrix plot analysis does not reveal any significant similarity between these two enzymes. The prototype tertiary structure of spinach ferredoxin-NADP reductase is completely different from that of glutathione reductase which includes the βαβ NADP binding motif noted above (Karplus et al., 1991).

The Type I FAD and NAD(P) binding site motifs noted above for adrenodoxin reductase do not appear in P450 reductase and other related enzymes. The residues involved in binding FAD and NADP in the prototype structure of ferredoxin reductase are conserved in other members of this family and present a consensus sequence entirely different from the Type I FAD and NAD(P) motifs (Karplus et al., 1991). The relative orientations of the isoalloxazine ring of FAD and of nicotinamide ring of NADP are also suggested to be different in these families of flavoenzymes (Sem and Kasper, 1992).

By sequence alignment with a flavodoxin, *Tyr*-178 of rat P450 reductase was suspected to be involved in FMN binding, and its replacement, but not of *Tyr*-140, indeed abolished FMN binding (Shen et al., 1989). *Cys*-471, *Cys*-565, and *Lys*-601 of P450 reductase (aligned with *Lys*-244 of ferredoxin reductase) are probably located in the NADP binding site as these residues are protected against chemical modification in the presence of $NADP^+$ (Haniu et al., 1986; 1989; Vogel and Lumper, 1986; Slepneva and Weiner, 1988; Muller et al., 1990). However, the mutation of *Cys*-566 of rat reductase (corresponding to *Cys*-565 of human and porcine enzymes) to Ala or Ser did not abolish catalytic activity and NADPH binding. Moreover, the position that corresponds to *Cys*-565 has a Ser residue in yeast reductase (Yabusaki et al., 1988), indicating that this *Cys* is not essential for activity (Shen et al., 1991).

Kinetic analysis and site-directed mutagenesis have been used by Kasper and co-workers to further refine the mechanisms of electron transfer by P450 reductase. Kinetic analysis of reduction of cytochrome c indicates a mechanism that is very dependent on ionic strength (Sem and Kasper, 1995). NADPH and cytochrome c bind at independent sites. Isotope effects indicate that hydride transfer from NADPH is rate limiting only at high ionic strengths (0.2–0.75M). At lower, more physiological pH's, $NADP^+$ release becomes limiting. At very high ionic strength, a conformational change that intervenes between NADPH binding and hydride transfer may be rate determining. Site directed mutagenesis of two acidic clusters ^{207}Asp.Asp.Asp^{209} and ^{213}Glu.Glu.Asp^{215} demonstrates that the first cluster is critical for P450 reaction but not for cytochrome c reduction, while the reverse selectivity occurs for the second cluster (Shen and Kasper, 1995). Site directed mutagenesis has also been used to show that the positive charge of arginine 597 is a major site for binding of the 2′-phosphate of NADPH (Sem and Kasper, 1993). Serine 457 has also been implicated in the hydride transfer from NADPH to FAD. The S457A mutant shifts to hydride transfer as the slow step instead of NADP+ release (Shen and Kasper, 1996).

The binding of reductase to various P450s has been suggested to be mediated by both hydrophobic and complementary charge interactions (Schenkman, 1993; Shen and Strobel, 1993; Voznesensky and Schenkman, 1994). The amino terminal region of the reductase was implicated in binding to P450 (Black and Coon, 1982). Chemical modification studies showed that carboxyl residues in the range of 109–130, within the FMN binding domain, are involved in interaction with P450

(Nadler and Strobel, 1991). Quantitative analyses of ionic dependence of reactions indicated that different numbers of charged residues are involved in interactions between various P450s and the P450 reductase (Voznesensky and Schenkman, 1994). In the binding of P450 reductase to cytochrome b_5 only charge-pair interactions appear to be involved (Dailey and Strittmatter, 1980; Schenkman, 1993).

III. LEVELS OF EXPRESSION

A. Mitochondrial and Bacterial P450cam Systems

The mitochondrial electron transfer proteins adrenodoxin reductase and adrenodoxin are expressed in all human tissues examined (Brentano et al., 1992; Hanukoglu, 1992). Their highest levels of expression are observed in steroidogenic cells especially in adrenal cortex and ovarian corpus luteum (Hanukoglu, 1992). In these two tissues the molar ratios of adrenodoxin reductase, adrenodoxin and P450 were estimated as 1:(3–10):8, using specific antibodies against each component (Ohashi and Omura, 1978; Hanukoglu and Hanukoglu, 1986). The levels of these proteins show no significant sex, or interindividual variation in bovine adrenal cortex (Hanukoglu and Hanukoglu, 1986). Earlier spectroscopic measurements also showed that adrenodoxin and mitochondrial P450 are present at equal molar concentrations (Estabrook et al., 1973). These independent estimations contradict the findings of another report that mitochondrial P450 levels are similar to that of the adrenodoxin reductase and much lower than that of adrenodoxin (Hamamoto et al., 1986). Similar to mitochondrial systems, in *Pseudomonas putida* grown on camphor, putidaredoxin reductase, putidaredoxin and P450cam were found at a molar ratio of about 1:8:8 (Roome et al., 1983).

Consistent with the level of the protein, the level of adrenodoxin reductase mRNA is also about 10-fold lower than that of P450scc (Hanukoglu et al., 1987). This correlation between the relative levels of the enzymes and their mRNAs indicates that the low level of adrenodoxin reductase reflects its mRNA level, and does not result from lower translational efficiency of its mRNA. In contrast, the low level of putidaredoxin reductase apparently results from the low translatability of the mRNA that has an unusual GTG initiation codon; mutation of this to the normal ATG codon enhanced its expression to levels comparable to those of putidaredoxin and P450cam (Davies et al., 1990).

The levels of mitochondrial P450 system proteins in steroidogenic tissues are regulated by specific trophic hormones as part of the physiological mechanisms regulating the steroid output of these tissues (for review see Hanukoglu, 1992; and chapter by Waterman in this volume). The level of adrenodoxin reductase is correlated with the level of adrenodoxin and P450scc in both adrenal cortex and corpus luteum *in vivo* (Hanukoglu, 1986). In bovine adrenal cortex cells in primary culture grown to confluence without ACTH, the levels of the enzymes drastically decrease. ACTH increases the levels of all three enzymes and their mRNAs, but

with some differences in their time courses (Hanukoglu et al., 1990a). In analogy to the regulation observed in steroidogenic tissues, the levels of some mitochondrial P450 system enzymes in other tissues may also be regulated by the relevant physiological factors (chapter by Armbrecht in this volume).

B. Microsomal Systems

The expression of microsomal electron transfer proteins, P450 reductase, and cytochrome b_5 has been detected in various tissues by Western blot analysis using specific antibodies and RNase protection assays using cDNA probes (Hamamoto et al., 1986; Katagiri et al., 1989; Arinc, 1991; Shephard et al., 1992). The highest level of P450 reductase is observed in the liver (Katagiri et al., 1989). The levels of P450 reductase mRNA show several fold differences among tissues and at most 3-fold difference among adult human liver samples (Shephard et al., 1992). The average ratio of P450 reductase to total cytochromes P450 in rat and human livers was estimated as 1:15 and 1:7 respectively (Shephard et al., 1983; McManus et al., 1987). In the bovine adrenal cortex the molar ratio of P450 reductase to cytochrome b_5 was estimated to vary between 1:0.6 and 1:4 in different zones (Hamamoto et al., 1986). The level of the cytochrome b_5 reductase is at least ten fold lower than that of cytochrome b_5 (Hamamoto et al., 1986; Archakov and Bachmanova, 1990; Borgese et al., 1993).

Some hormone and drug treatments that increase the expression of specific P450s, show little effect on reductase levels; thus the ratio of P450 reductase to various P450s in liver microsomes may vary from 1:15 to about 1:100 under different treatments (Shephard et al., 1983; Shiraki and Guengerich, 1984). The expression of P450 reductase in rat liver is strongly dependent on thyroxine stimulation (Waxman, 1992). In adrenocortical cells ACTH stimulation enhances P450 reductase expression similar to its effects on other steroidogenic enzymes. However, phenobarbital which induces P450 reductase in liver, shows no similar effect in adrenocortical cells (Dee et al., 1985). Thus, P450 reductase gene apparently possesses a repertoire of regulatory elements that can respond to a variety of stimuli in a cell specific manner and in concert with the responses of its redox partners.

IV. PROTEIN-PROTEIN INTERACTIONS

A. Mitochondrial and Bacterial P450cam Systems

The stoichiometry of about one reductase molecule per ten P450 precludes the structural organization of these enzymes in rigid complexes or arrays of electron transport chains. The findings summarized below indicate that during catalytic turnover adrenodoxin functions as a mobile electron carrier: its oxidized form binds

to reductase, accepts one electron from it and dissociates, and then binds to P450 and unloads its electron.

The ferredoxin of both mitochondrial and P450cam systems can form a tight 1:1 complex ($K_d < 10^{-7}$) with either the reductase or P450 (Chu and Kimura, 1973; Gunsalus and Sligar, 1978; Hanukoglu and Jefcoate, 1980; Hanukoglu et al., 1981; Sakamoto et al., 1981; Lambeth et al., 1982; Lambeth, 1991). As noted above, the binding sites on adrenodoxin for its different electron transfer partners overlap; thus, the concurrent specific association of the reductase and the P450 with the ferredoxin is sterically impossible. In adrenal cortex there are two mitochondrial P450s, P450scc (CYP11A) and P450c11 (CYP11B), at nearly equal levels and distributed evenly across mitochondria (Hanukoglu, 1992). Studies in rat adrenal mitochondrial preparations suggest that adrenodoxin may interact preferentially with P450scc (Yamazaki et al., 1993).

Kinetic studies also show that a 1:1:1 ternary complex of adrenodoxin reductase, adrenodoxin, and P450 does not form under equilibrium or steady state turnover conditions (Hanukoglu and Jefcoate, 1980; Hanukoglu et al., 1981; Lambeth et al., 1982; Lambeth, 1991). Kido and Kimura (1979) reported that a ternary complex can form under equilibrium conditions; however, their results can be interpreted differently, refuting their conclusion of a ternary complex (see discussion by Lambeth et al., 1982). In contrast to interactions with P450, adrenodoxin can reduce the artificial acceptor cytochrome c while in a 1:1 complex with adrenodoxin reductase, showing that its binding sites for adrenodoxin reductase and cytochrome c are not overlapping (Lambeth et al., 1982). A covalently crosslinked complex of adrenodoxin reductase-adrenodoxin can reduce cytochrome c at maximal rates in absence of added free adrenodoxin; while the same complex requires excess free adrenodoxin for P450scc reduction (Hara and Kimura, 1989). These findings further indicate that the sites of intermolecular electron transfer between adrenodoxin and cytochromes c and P450 are different.

Turko et al. (1989) obtained a ternary complex by chemical crosslinking, yet it is difficult to ascertain that in such complexes the proteins are crosslinked at the high affinity binding sites identified in equilibrium binding studies. Harikrishna et al. (1993) designed and expressed in COS cells fused proteins including two or three components of the mitochondrial P450 system at different orientations. Cells expressing some of these constructs showed greater activity than cells expressing each component from separate cotransfected vectors. It should be noted however, that each construct may not necessarily function on its own transferring electrons within the fused multi-protein complex, as its individual components may interact with other fused proteins in the mitochondria.

The association of adrenodoxin with reductase and P450 is dependent on ionic interactions and can be affected strongly by changes in pH and ionic composition (Hanukoglu et al., 1981; Jefcoate, 1982; Lambeth and Kriengsiri, 1985; Hamamoto et al., 1993). These findings suggest that complementary charged residues are involved in the specific complex formations. Indeed, site directed mutations of

human adrenodoxin reductase and adrenodoxin suggest that *Arg*-239 and *Arg*-243 of adrenodoxin reductase interact directly with *Asp*-76 and *Asp*-79 of adrenodoxin (Brandt and Vickery, 1993). Hydrophobic interactions do not appear to play a role in adrenodoxin-P450 complex formation as the K_d of the complex was unaffected by an uncharged detergent (Hanukoglu et al., 1981).

Adrenodoxin reductase-adrenodoxin couple can oxidize NADPH at a high steady rate even in absence of P450scc, and transfer electrons directly to O_2 to produce superoxide radical (Hanukoglu et al., 1993). This leakage of electrons to O_2 is enhanced by the addition of P450, showing that adrenodoxin can bind to substrate-free P450 and reduce it. However, electron leakage is reduced in the presence of P450 substrate. These findings suggest that to minimize the production of harmful superoxide in the cell, the supply of NADPH to the mitochondrial P450 systems may be regulated together with substrate availability (Hanukoglu et al., 1993).

The binding of adrenodoxin to P450 is strongly influenced by the binding of substrate and the redox states of the proteins. Cholesterol and adrenodoxin mutually enhance each other's affinity for P450scc (Lambeth et al., 1980; Hanukoglu et al., 1981; Jefcoate, 1982). Reduced adrenodoxin binds more strongly to oxidized rather than reduced P450scc (Lambeth, 1991). Similar effects have been also observed in the P450cam system (Davies and Sligar, 1992). These effects enhance the binding of adrenodoxin to P450-substrate complex and reduce the propensity of the system to function as a superoxide producing NADPH oxidase (Lambeth, 1991; Hanukoglu et al., 1993).

The monooxygenase activity of mitochondrial P450s shows Michaelis-Menten dependence on free reduced adrenodoxin (Hanukoglu and Jefcoate, 1980; Hanukoglu et al., 1981; Lambeth, 1991). Reductase competes with P450 for binding to the same site on adrenodoxin, and oxidized adrenodoxin can bind to P450 in competition with reduced adrenodoxin and consequently inhibit the catalytic activity of the P450 (Hanukoglu and Jefcoate, 1980; Hanukoglu et al., 1981; Lambeth, 1991). Thus, during monooxygenation the electron transport system would function most efficiently if most of the adrenodoxin molecules are maintained in reduced form and unbound to reductase. These biological design specifications are apparently met by the following characteristics of the system: (1) a relatively low concentration of reductase to minimize the competition of reductase with P450 for binding to adrenodoxin, (2) a much faster turnover rate of adrenodoxin reduction by reductase than the rate of adrenodoxin oxidation by P450 so that the low concentrations of reductase suffice for the system, and (3) redox equilibria that favor dissociation of adrenodoxin from reductase after reduction, and binding of adrenodoxin to P450-substrate complex.

Some of the characteristics of mitochondrial P450 systems noted above are similar to those of the mitochondrial oxidative phosphorylation system which includes four multienzyme complexes (I–IV) embedded in the inner mitochondrial membrane, and a small soluble protein (cytochrome c) located in the intermembrane space (Gupte et al., 1984; Hackenbrock et al., 1986). The stoichiometry of

these five components (1:2:3:7:9) precludes rigid structural organization of enzymes on the membrane. Electron transfer between complexes I, II, and III is mediated by ubiquinone, and between complexes III and IV by cytochrome c. A functional 1:1:1 aggregate of complex III, cytochrome c, and cytochrome c oxidase apparently cannot form as the same domain on cytochrome c is involved in binding both to its reductase (complex III) and oxidase (complex IV) (Capaldi, 1982). Thus, like adrenodoxin, cytochrome c functions as an electron shuttle between its reductase and oxidase, and not as an "electron bridge" between the two enzymes. Studies with fluorescently labeled cytochrome c in intact mitochondria indicate that the protein diffuses in three dimensions and not only along the inner membrane surface (Cortese and Hackenbrock, 1993). Immuno-electron microscopy shows adrenodoxin molecules in association with the inner membrane, and in the matrix space (Hatano et al., 1989; Hanukoglu et al., 1990) suggesting that adrenodoxin may similarly diffuse in the mitochondrial matrix.

It is significant that both the mitochondrial P450 and oxidative phosphorylation systems include small soluble proteins as electron transporters for enzymes embedded in the inner mitochondrial membrane. A major reason for this may be that proteins may diffuse faster on the surface than in the plane of the inner mitochondrial membrane which has a particularly high protein content. Indeed the diffusion rate of cytochrome c is over ten-fold faster than those of the membrane embedded complexes (Gupte et al., 1984). Similarly, adrenodoxin may have greater mobility. Rotational mobility measurements in a mitochondrial preparation showed that only about 30% of P450 are mobile and that the proportion of mobile P450 increases after addition of adrenodoxin (Ohta et al., 1991). Yet, these findings should be interpreted with the caveat that the studies were carried out in viscous 60% sucrose or 80% glycerol. In artificial phospholipid vesicles, P450scc shows high mobility as assessed by different approaches (Dhariwal et al., 1991).

B. Microsomal Systems

The functions of microsomal P450 systems involve complex interactions among many different forms of P450, and the electron transfer proteins P450 reductase and cytochrome b_5 and its reductase, all of which are anchored to the endoplasmic reticulum membrane. The lopsided stoichiometry of these proteins, noted above, sterically would not permit their organization in rigid clusters in the membrane. Many different lines of evidence indicate that these proteins can move laterally in the membrane and transfer electrons after formation of high affinity 1:1 complexes. The biphasic kinetics of P450 reduction by reductase was interpreted as evidence for the organization of these proteins in clusters; however, alternative explanations suggest that these observations are consistent with non-rigid random distribution of the proteins (Peterson et al., 1976; Yang, 1977; Taniguchi et al., 1979; Archakov and Bachmanova, 1990; Kanaeva et al., 1992). In immunogold labeled sections of hepatocytes a uniform random distribution of microsomal P450s is observed. In

microsomes ferritin labeled P450 antibodies occasionally appear in clusters, yet these may result from aggregation of molecules during microsome preparation (Tashiro et al., 1993). Studies with double immunogold labeling of both the reductase and the P450s are needed to further examine the relative organization of these proteins in the endoplasmic reticulum.

The reduction of P450 by the reductase is dependent on the formation of a specific high affinity 1:1 complex ($K_d < 10^{-7}$), between the two enzymes, though individual P450s differ in their affinity for the reductase (Miwa et al., 1979; Archakov and Bachmanova, 1990). Reconstitution studies using purified components indicated that the strength of this association is dependent on the phospholipid environment (Taniguchi et al., 1979; French et al., 1980; Ingelman-Sundberg et al., 1983). The binding of reductase to P450 is also modulated by P450 substrates which can decrease the K_d for the reductase-P450 complex, enhancing the affinity of the reductase to P450-substrate complex rather than substrate free P450 (French et al., 1980). Some substrates can increase P450 reduction potential, thus facilitating reduction by the reductase (Archakov and Bachmanova, 1990).

In addition to P450s, P450 reductase can also bind with high affinity to cytochrome b_5 and reduce it. This pathway of electron transport has been implicated in both P450 and non-P450 mediated reactions supported by cytochrome b_5 (Noshiro et al., 1980; Ilan et al., 1981; Fisher and Gaylor, 1982; Schenkman, 1993). As noted above, the binding of the reductase to different P450s and cytochrome b_5 may be mediated by electrostatic and hydrophobic interactions (Dailey and Strittmatter, 1980; Schenkman, 1993). Complementing these findings, chemical modification and site-directed mutation of P450s identified positively charged residues (Lys and Arg) on $P450_d$ (CYP1A2) that are involved in complex formation with the P450-reductase by charge pairing (Shimizu et al., 1991; Shen and Strobel, 1993). Some microsomal P450s may also form a tight 1:1 complex with cytochrome b_5 and receive the second electron from it during the usual catalytic cycle of monooxygenation (Ingelman-Sundberg and Johansson, 1980; Kuwahara and Omura, 1980; Bonfils et al., 1981; Hlavica, 1984; Schenkman, 1993).

The relative concentrations of P450 reductase and cytochrome b_5 can play major roles in determining reaction rates and specificities. One good example for this effect is P450c17 which is expressed in the adrenal cortex and some gonadal cells catalyzing steroid C17-hydroxylation and lyase (C21 side chain cleavage) reactions at rates characteristic of each cell type. *In vitro* studies showed that cytochrome b_5 can stimulate the lyase activity, and increasing P450 reductase concentration increased the lyase activity relative to hydroxylase. In testicular microsomes where the lyase activity predominates, the reductase activity is higher than in the adrenal. Hence, the *in vitro* findings are considered to reflect physiological regulatory mechanisms based on relative levels of reductase vs. cytochrome b_5 expression (Hall, 1991; Takemori and Kominami, 1991; Kominami et al., 1993).

Co-expression of P450 reductase, and various microsomal P450s in yeast cells and mammalian cell lines generally enhanced the activities of the P450s many fold,

as the endogenous levels of the reductase may be limiting in these cells (Murakami et al., 1990; Peyronneau et al., 1992; Lin et al., 1993; Sawada et al., 1993; Truan et al., 1993). Fused enzymes expressed from constructs including P450 reductase and a microsomal P450 cDNAs also showed high P450 activity in yeast cells. Yet, the stability of the mRNAs and proteins encoded by the constructs may be lower than their individual components and depends on the length and sequence of the hinge region between the two enzymes (Sakaki et al., 1990; Shibata et al., 1990). The N-terminal region of the yeast reductase is highly divergent from that of mammalian reductases (Yabusaki et al., 1988). Hybrid constructs of yeast-rat P450 reductase with the N-terminal sequence of the yeast enzyme appeared to have higher stability in yeast cells (Bligh et al., 1992).

Chemical modification studies indicated that the interaction of cytochromes P450 and b_5 is dependent on carboxyl groups of the b_5 which are considered to be paired with complementary charges on the P450 (Tamburini et al., 1985; Schenkman, 1993). The binding sites on cytochrome b_5, for the b_5 reductase and P450 are overlapping as its reduction by NADH-cytochrome b_5 reductase is inhibited in the presence of P450. In contrast, the P4502B4 sites for binding P450 reductase and cytochrome b_5, appear to be different because addition of b_5 does not affect the K_m for the reductase (Tamburini and Schenkman, 1987; Schenkman, 1993). Both P450 reductase and cytochrome b_5 can reduce cytochromes c and P450, and it was suggested that their binding sites for these cytochromes share a short stretch of similar structure (Davydov et al., 1992). The mapping of the sites of interactions among these proteins awaits elucidation of the crystal structure of P450 reductase.

NOTE

1. Adrenodoxin was named reflecting its first isolation from the adrenal cortex (Kimura and Suzuki, 1967). In some reports it is referred to as "ferredoxin." In this review the term "adrenodoxin" is used because the term "ferredoxin" is also employed as a class name that does not distinguish among various ferredoxin type proteins (Beinert, 1990; Nomenclature Committee, 1979).

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