# An unusual yet strongly conserved flavoprotein reductase in bacteria and mammals

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The recent determination of the amino acid sequences of the *Bacillus megaterium* cytochrome P-450 and the flavoprotein component of *Salmonella typhimurium* NADPH-sulfite reductase revealed that these enzymes contain a flavoprotein moiety remarkably similar to mammalian NADPH–cytochrome P-450 reductase. The presence of this oxidoreductase in these very different enzymes suggests that this flavoprotein arose early in evolution and was utilized as an enzymological building block. The multi-domain structure of the reductase further suggests that it arose through a fusion of genes encoding simple flavin electron-transport proteins.

THE TRANSFER of reducing equivalents between enzymes and the electron-carrier cofactors NAD$^+$ and NADP$^+$ is integral to many metabolic reactions. While in some instances electrons are transferred directly between the substrate and the nicotinamide cofactor (as with alcohol dehydrogenase and dihydrofolate reductase), often a bound coenzyme serves as an intermediate in electron transfer. Enzymes utilize a variety of such intermediates, including heme, iron–sulfur clusters, flavins, and more rarely, pteridines. Of this group, flavins such as flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) are the most common.

Flavoproteins can be divided into at least five classes, based upon the reaction catalysed and the electron acceptor[1,2]. Transhydrogenases and electron transferases are two classes of flavoproteins in which the electron acceptor (or donor) is either another redox protein or a nicotinamide cofactor. Members of these two classes are distinguished from other flavoproteins by their limited reactivity with molecular oxygen (O$_2$). Transhydrogenases and electron transferases are often associated with energy-generating electron transport pathways, and the associated redox protein is often a 1-electron acceptor, such as a cytochrome or a non-heme iron-sulfur protein. Well-characterized examples of flavoprotein transhydrogenases include ferredoxin–NADP$^+$ reductase and glutathione reductase; electron transferases are typified by the flavodoxins. The flavoprotein that is the subject of this review is unusual in that it contains both a transhydrogenase domain and an electron transferase domain on a single polypeptide, and thereby represents a fusion of two normally independent flavoproteins. This compound flavoprotein, first characterized as microsomal cytochrome P-450 reductase, has now been shown to be a component of two bacterial enzymes: a *Bacillus megaterium* cytochrome P-450 and sulfite reductase.

## Cytochrome P-450 reductase

NADPH–cytochrome P-450 reductase is a 78 kDa flavoprotein bound to the

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endoplasmic reticulum of most eukaryotic cells. As an essential component of the microsomal cytochrome P-450-dependent monooxygenase system[3,4], it catalyses the transfer of electrons from NADPH to the cytochromes P-450, which are responsible for the oxidation of innumerable foreign and endogenous compounds including drugs, plant metabolites, steroids and prostaglandins (for review see Ref. 5). A most notable aspect of cytochrome P-450 reductase is its flavin content: the single polypeptide chain binds both an FMN and an FAD prosthetic group[6]. As established by Vermilion *et al.*[7], the pathway of electron transfer proceeds from NADPH to FAD to FMN to cytochrome P-450.

The microsomal cytochrome P-450 system differs from known bacterial and adrenal mitochondrial P-450 monooxygenase systems in the pathway of electron transfer. The bacterial and mitochondrial systems use two separate electron transfer components: an FAD-containing reductase, which accepts two reducing equivalents from a nicotinamide cofactor (NADPH or NADH), and a small iron–sulfur protein (a ferredoxin), which catalyses two separate 1-electron transfers from the reductase to cytochrome P-450. Although the evolutionary origin of microsomal cytochrome P-450 reductase appears to be distinct from that of these two-component electron transport chains, it is related in function, and may be derived from a common ancestral pathway.

**Structure.** The sequence of P-450 reductase was first determined for the rat[8], and is now known for a variety of species, including humans[9–14]. The mammalian reductases share about 90% sequence identity, with lesser similarity to the trout (79%)[11] and yeast (33%)[12] enzymes. A detailed analysis of the reductase amino acid sequence, with comparison to flavoproteins of known three-dimensional structure, has facilitated identification of the flavin- and cofactor-binding domains of the enzyme[15]. A cartoon representation of the proposed reductase organization is shown in Fig. 1. The protein is anchored to the endoplasmic reticulum by a short hydrophobic amino-terminal segment; the subsequent catalytic portion of the reductase begins with a 150-residue segment that shows considerable similarity to the bacterial flavodoxins.

Flavodoxins are small (15–23 kDa), acidic, FMN-containing proteins found in bacteria and algae, but not in higher plants or animals. Flavodoxins are strict electron transferases, reacting only with other redox proteins, and are able to replace ferredoxins in many redox pathways (for review see Ref. 16). The three-dimensional structures of several flavodoxins are known. Cytochrome P-450 reductase exhibits several regions of strong sequence similarity with these flavoproteins, most notably with the *Desulfovibrio vulgaris* flavodoxin, which correspond to segments involved in binding the FMN group to these proteins. Moreover, the

predicted secondary structure of this region of the reductase, obtained by the method of Chou and Fasman, is very similar to the secondary structure of the flavodoxins, suggesting that this region of the reductase binds FMN in an overall configuration like that of flavodoxin, despite being incorporated into a much larger protein[15].

Recent studies utilizing site-directed mutagenesis have supported this hypothesis[17]. In flavodoxins there are two residues, which are usually hydrophobic and often aromatic, that are located above and below the FMN isoalloxazine ring. These shielding residues maintain a hydrophobic environment for the flavin, and may participate in pi–pi stacking interactions with the isoalloxazine ring[16]. In P-450 reductase, the proposed flavin-shielding residues are Tyr140 and Tyr178 (Ref. 15). Substitution of aspartate for Tyr140, the residue proposed to be positioned at an angle of 45° over the interior face of the flavin ring, results in a fivefold decrease in reductase activity, although it does not significantly alter FMN binding. In contrast, substitution of aspartate for Tyr178, the proposed exterior ring-shielding residue, coplanar with the flavin ring in flavodoxin, abolishes both reductase activity and FMN binding. Replacement of either, or both, tyrosines with phenylalanine, an obviously conservative replacement, does not affect activity or FMN binding. Interestingly, FAD binding is reduced by the Tyr178→Asp mutation, which may indicate that FAD incorporation is dependent on formation of an intact FMN domain. These results provide strong support for the hypothesis that this region of P-450 reductase binds the FMN prosthetic group. Although the corresponding mutations have not yet been examined in a flavodoxin molecule *per se*, the effects described above are consistent with the FMN being bound in a manner similar to that found in the flavodoxins.

Cytochrome P-450 reductase also exhibits significant sequence similarity to two FAD-containing enzymes, ferredoxin–NADP⁺ reductase and NADH–cytochrome $b_5$ reductase[15]. Both of these enzymes are simple transhydrogenase-type flavoproteins, catalysing electron transfer between a nicotinamide cofactor and a 1-electron acceptor (or donor) protein. Ferredoxin reductase is the terminal electron carrier of the photosynthetic electron transport chain, and cytochrome $b_5$

reductase is a component of the microsomal fatty acid desaturase and elongation pathways, and also a component of the methemoglobin reductase pathway in red blood cells. The similarity between P-450 reductase and these two flavoproteins begins approximately 40 residues beyond the FMN-binding domain, and extends for approximately 60 residues before being disrupted by a 117-amino acid segment in P-450 reductase that appears unrelated to these two flavoproteins. Following this 'insertion', the sequence similarity resumes, and becomes strongest in the carboxy-terminal region of the proteins. By comparison of these sequences to glutathione reductase, an FAD-containing protein whose three-dimensional structure is known, tentative assignments of FAD- and NADPH-binding domains have been made[15].

The initial 60-amino acid segment of similarity has been proposed as the FAD pyrophosphate-binding segment, and the subsequent 117-amino acid insertion in P-450 reductase has been proposed as being involved in orienting the two flavin domains for interflavin electron transport. Because ferredoxin reductase and cytochrome $b_5$ reductase contain only a single flavin, this orienting segment would be unnecessary in these enzymes. The proposed nicotinamide cofactor-binding domain begins at approximately position 480 of P-450 reductase, and extends for approximately 200 residues. The sequence similarity among these proteins in this region is strong, and is supported by a variety of chemical modification studies that have demonstrated that bound cofactor in all three flavoproteins can protect specific residues in this region from modification (for examples see Refs 14, 18 and 19). These results support the assignment of the middle and carboxy-terminal segments of P-450 reductase to binding FAD and NADPH, respectively.

**Evolutionary origins.** The amino-terminal homology of P-450 reductase with flavodoxin, and the carboxy-terminal homology with ferredoxin–NADP⁺ reductase, suggest that P-450 reductase arose through a fusion of the ancestral genes for these two types of flavoproteins. It is noteworthy that flavodoxin can sub-
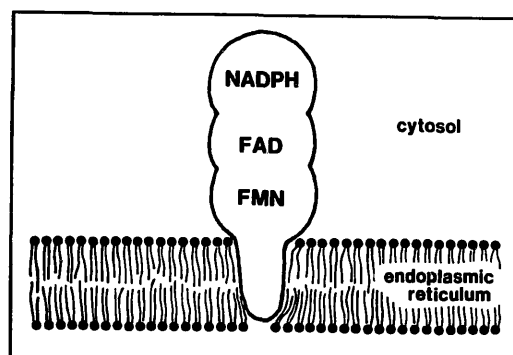


**Figure 1**
Domain organization of cytochrome P-450 reductase. The protein is anchored to the endoplasmic reticulum by its hydrophobic amino-terminal segment, which is followed by the FMN-, FAD- and NADPH-binding domains.

stitute for ferredoxin in a variety of pathways, including the photosynthetic reduction of NADP⁺ with ferredoxin reductase[16]. It has been suggested that the genes for flavodoxin and ferredoxin reductase were arranged in tandem in an operon of an early organism, and at some point fused to give rise to the primordial P-450 reductase gene[15]. That these two proteins could simply fuse in a head-to-tail fashion and yield a functional protein would be surprising, and the additional segment (or insertion) discussed earlier may have been necessary to couple the two proteins effectively. This segment is predicted to be almost entirely α-helical, suggesting that it may be located on the surface of the protein.

The recently determined structure of the rat P-450 reductase gene supports a gene fusion origin for this flavoprotein[20]. The gene is divided into fifteen coding exons, of which three encode the FMN domain and six encode the FAD and NADPH domains. The beginning and end of the FMN domain is clearly coincident with intron positions, as is the start of the FAD domain; this placement of introns precisely between these functional domains suggests that each was originally a separate entity, and that a genetic recombination event brought them together and gave rise to the reductase as a 'fusion protein'.

Cytochrome P-450 arose over two billion years ago, and members of this family of monooxygenases are present in both prokaryotes and eukaryotes (for review see Ref. 21). The earliest P-450s were bacterial, and presumably utilized the two-component electron transport chain (a ferredoxin reductase
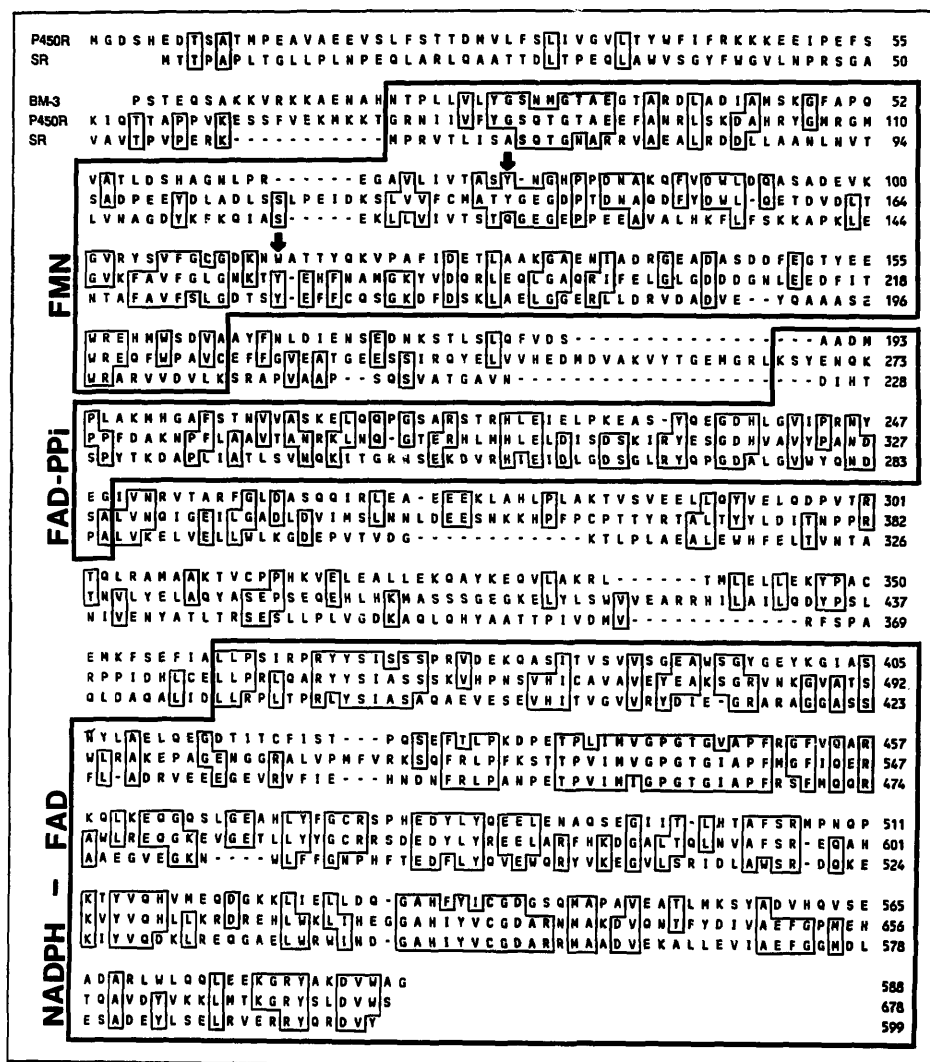
**Figure 2**

Sequence alignment of reductases. The flavoprotein moieties of *B. megaterium* cytochrome P-450$_{BM-3}$ (Ref. 25) (upper line, BM-3) and *S. typhimurium* sulfite reductase[29] (lower line, SR) are aligned with rat cytochrome P-450 reductase[8] (middle line, P450R). Amino acids that match the rat reductase are boxed. Note that the P-450$_{BM-3}$ sequence begins at position 59 of the rat reductase sequence, and corresponds to amino acid 461 of the complete P-450$_{BM-3}$ sequence. The FMN phosphate-binding segment is shaded, and the FMN ring-shielding residues are indicated with arrows. The glycine-rich and Cys–Gly dipeptide-containing segments in the proposed NADPH domain are also indicated by shading. FAD-PPi, proposed FAD pyrophosphate-binding segment.

ferredoxin reductase/ferredoxin electron transport chain; the one exception, *Bacillus megaterium* cytochrome P-450$_{BM-3}$, is unusual in several respects.

Cytochrome P-450$_{BM-3}$ is a 119 kDa fatty acid ω-2 hydroxylase that has been characterized in the laboratory of Armand Fulco at UCLA[23–25]. Like all bacterial P-450s, and in contrast to eukaryotic P-450s, P-450$_{BM-3}$ is a cytosolic rather than membrane-bound enzyme; unlike all other known P-450 monooxygenases, it requires no additional electron transport components for activity. This catalytically self-sufficient enzyme contains 1 mole of heme and 1 mole each of FMN and FAD on a single polypeptide chain. The molecular weight and prosthetic group content suggested that P-450$_{BM-3}$ might represent a fusion of genes corresponding to the mammalian P-450 and its microsomal reductase[23], a supposition that has now been confirmed by sequencing the gene[25].

The protein is divided into two discrete domains, one of which contains the heme group, with the second domain containing FMN and FAD[24]. The two domains are readily separated by limited proteolysis, and each retains its respective prosthetic group(s) when separated. The heme domain of P-450$_{BM-3}$ exhibits about 25% sequence identity with mammalian fatty acid hydroxylases, and the flavin domain shows about 33% identity with mammalian P-450 reductases[25]. The two domains are connected by approximately 30 amino acids, which includes a highly basic segment cleaved during limited proteolysis.

As might be expected, several regions of the *Bacillus* and mammalian reductases contain highly conserved segments, corresponding to regions likely to be involved in flavin binding. An alignment of the *Bacillus* reductase sequence with the rat microsomal reductase is shown in Fig. 2. In the FMN domain, a segment proposed to bind

and a ferredoxin) discussed earlier. The earliest microsomal P-450s arose 1.3 billion years ago, shortly after the prokaryotic–eukaryotic divergence, and presumably utilized microsomal cytochrome P-450 reductase for electron input, as all present-day microsomal P-450s are dependent upon this flavoprotein. Thus the evidence from this system suggests that the reductase was present in early eukaryotes. The evidence presented in the next two sections indicates that this compound flavoprotein almost certainly had evolved well before the emergence of eukaryotes.

## *Bacillus megaterium* P-450 reductase

Although evolutionary studies indicate that cytochrome P-450 should be present in virtually all organisms, including bacteria, its presence in prokaryotes has only been demonstrated in a limited number of species to date (for review see Ref. 22). As noted above, evolutionary studies would also indicate that bacterial P-450 systems should utilize a two-component electron transport chain, rather than the compound flavoprotein, cytochrome P-450 reductase. Indeed, all but one of the bacterial cytochrome P-450 systems so far characterized appear to use a

the FMN phosphate group is strongly conserved (residues 26–34, shaded in Fig. 2), and of the two flavin ring-shielding residues (indicated by arrows), the interior tyrosine (Tyr76) is conserved and the exterior tyrosine is replaced by tryptophan (Trp114), an amino acid commonly found in this position in flavodoxins. The 'insertion' in the FAD domain is maintained in the *Bacillus* reductase, and, as with ferredoxin reductase and cytochrome $b_5$ reductase, the sequence similarity is strongest in the carboxy-terminal region of the protein, the proposed NADPH-binding domain. Two especially conserved segments in this region are noteworthy: a glycine-rich segment, suggestive of a pyrophosphate-binding loop, found in virtually all nicotinamide cofactor-dependent enzymes (residues 436–450, shaded); and a segment containing a Cys–Gly dipeptide present in all P-450 reductases, and also in ferredoxin reductase and cytochrome $b_5$ reductase (residues 633–643, shaded). Although no function has been assigned to this latter segment, a cysteine in cytochrome $b_5$ reductase located nine residues away from this conserved dipeptide has been shown to be protected from chemical modification by NADH[19], supporting the assignment of nicotinamide cofactor binding to this region of these reductases.

The *Bacillus megaterium* P-450$_{BM-3}$ thus contradicts two tenets regarding cytochrome P-450 monooxygenases: (1) that bacterial systems utilize a two component, ferredoxin reductase/ferredoxin electron transport chain; and (2) that cytochrome P-450 reductase is restricted to eukaryotic internal membranes. The presence of the reductase in this bacterium argues that the flavoprotein evolved well before eukaryotes and prokaryotes diverged, an argument supported by the finding of this flavoprotein in sulfite reductase, as discussed below.

## Sulfite reductase

NADPH–sulfite reductase is a multimeric enzyme that catalyses the six-electron reduction of sulfite to sulfide[26], an obligatory step in the biosynthesis of cysteine from sulfate in *Escherichia coli* and *Salmonella typhimurium*. The enzyme, extensively characterized in the laboratory of Lewis Siegel at Duke University, has a subunit structure of $\alpha_8\beta_4$, in which the eight $\alpha$-subunits bind eight flavin molecules, four each of FMN and FAD, and the four $\beta$-subunits each

contain an $Fe_4S_4$ cluster and a siroheme group[27]. As in cytochrome P-450 reductase, each flavin has a distinct role in the electron transfer sequence, with FAD serving as the entry port for electrons from NADPH, and FMN serving as the exit port to the $\beta$-subunit[28]. Indeed, the studies from Siegel's laboratory on the mechanism of electron transfer in sulfite reductase served as a model for the characterization of P-450 reductase by Vermilion *et al.*[7]; the recent determination of the amino acid sequence of the sulfite reductase flavoprotein has revealed the basis for the similarity in the mechanism of these two reductases[29].

The flavoprotein moiety ($\alpha$-subunit) of sulfite reductase from either *S. typhimurium* or *E. coli* shows about 30% sequence identity with rat cytochrome P-450 reductase[29] (see Fig. 2). As with the *Bacillus megaterium* P-450 reductase, a segment corresponding to the flavodoxin FMN phosphate-binding residues is strongly conserved, but, in contrast to the *Bacillus* reductase, only the exterior FMN ring-shielding residue is conserved (Tyr158); the interior tyrosine is replaced with glutamine (Glu119). The 'insertion' in the FAD domain, relative to ferredoxin reductase and cytochrome $b_5$ reductase, is maintained in sulfite reductase, although it is somewhat shorter than in the P-450 reductases. As with the *Bacillus* protein, the greatest sequence similarity is found in the carboxy-terminal portion of the protein, corresponding to the NADPH-binding domain. Notably, the glycine-rich segment present in the *Bacillus* and mammalian reductases is highly conserved in sulfite reductase, as is the segment containing the Cys–Gly dipeptide. Thus, despite the great evolutionary distance, all three reductases are clearly homologous, and all have been strongly conserved through evolution.

Despite the sequence similarity between sulfite reductase and P-450 reductase, the two proteins differ in one important respect: the sulfite reductase flavoprotein component, present as an octamer of identical $\alpha$-subunits, binds only eight flavin molecules rather than the sixteen that would be expected based on its homology with P-450 reductase. As FMN and FAD are present in equal proportions, there are various scenarios for flavin binding in this complex: each subunit may bind one flavin molecule, either FMN or FAD; or half of the subunits may contain a full flavin

complement (FMN plus FAD), while half may lack flavin completely. The actual distribution of the flavin groups is not yet known. Siegel's group has proposed that two $\alpha$-subunits are required for complete electron transfer, with each binding either FMN or FAD, and that for each subunit, the binding of one flavin precludes the binding of a second flavin to that subunit. How this is accomplished is not yet understood.

This arrangement raises two questions: how is the 1:1 ratio of FMN- to FAD-containing subunits established in the octameric complex, and how is intersubunit electron transport accomplished? Clearly there must be subtle differences between sulfite reductase and P-450 reductase that facilitate the octameric assembly and electron transport; one such difference may be the substitution of glutamine for tyrosine at the interior FMN ring-shielding position, as noted earlier. As demonstrated by Shen *et al.*[17], mutations at this position in P-450 reductase inhibit electron transport between the FMN and FAD groups without dramatically altering FMN binding. Similarly, the presence of glutamine at this position in sulfite reductase may inhibit intramolecular electron transport and oblige the enzyme to shuttle electrons between the flavins of complementary subunits.

## Conclusion

The ability to generate, utilize and dispose of reducing equivalents in metabolic reactions is an essential life process, and almost certainly was present in the earliest life forms. The universality of electron transfer coenzymes and cofactors (flavins and nicotinamide dinucleotides) argues that these molecules were utilized in the earliest stages of evolution. It is thus perhaps not surprising that proteins that facilitate the inherent activities of these molecules are also evolutionarily ancient. Cytochrome P-450 reductase represents one such example. As presented here, it is a component of three evolutionarily remote pathways; whether it has been incorporated into other electron transport pathways and enzymes, in other organisms, remains to be seen. The multi-domain structure of this enzyme has revealed its evolutionary origins, and raised a wealth of questions to be addressed by structure–function studies on this unusual flavoprotein. Foremost among these questions must be the orientation of the two flavins, and the mechanism by

which electrons are transferred between these two prosthetic groups.

## Note added in proof

The recently determined crystal structure of ferredoxin reductase[30] strongly supports the predicted domain structure of these flavoprotein reductases.

## References

1 Walsh, C. (1979) *Enzymatic Reaction Mechanisms,* pp. 358–420, W. H. Freeman and Company
2 Massey, V. and Ghisla, S. (1983) in *Biological Oxidations* (Sund, H. and Ullrich, V., eds), pp. 114–139, Springer-Verlag
3 Lu, A. Y. H., Junk, K. W. and Coon, M. J. (1969) *J. Biol. Chem.* 244, 3714–3721
4 Vermilion, J. L. and Coon, M. J. (1974) *Biochem. Biophys. Res. Commun.* 60, 1315–1322
5 Åström, A. and DePierre, J. W. (1986) *Biochem. Biophys. Acta* 853, 1–27
6 Iyanagi, T. and Mason, H. S. (1973) *Biochemistry* 12, 2297–2308
7 Vermilion, J. L., Ballou, D. P., Massey, V. and Coon, M. J. (1981) *J. Biol. Chem.* 256, 266–277
8 Porter, T. D. and Kasper, C. B. (1985) *Proc. Natl Acad. Sci. USA* 82, 973–977
9 Haniu, M., Iyanagi, T., Miller, P., Lee, T. D. and Shively, J. E. (1986) *Biochemistry* 25, 7906–7911
10 Katagiri, M., Murakami, H., Yabusaki, Y., Sugiyama, T., Okamoto, M., Yamano, T. and Ohkawa, H. (1986) *J. Biochem.* 100, 945–954
11 Urenjak, J., Linder, D. and Lumper, L. (1987) *J. Chromatogr.* 397, 123–136
12 Yabusaki, Y., Murakami, H. and Ohkawa, H. (1988) *J. Biochem.* 103, 1004–1010
13 Yamano, S., Aoyama, T., McBride, O. W., Hardwick, J. P., Gelboin, H. V. and Gonzalez, F. J. (1989) *Mol. Pharmacol.* 35, 83–88
14 Haniu, M., McManus, M. E., Birkett, D. J., Lee, T. D. and Shively, J. E. (1989) *Biochemistry* 28, 8639–8645
15 Porter, T. D. and Kasper, C. B. (1986) *Biochemistry* 25, 1682–1687
16 Mayhew, S. G. and Ludwig, M. L. (1975) in *The Enzymes* (Boyer, P. D., ed.), pp. 57–118, Academic Press
17 Shen, A. L., Porter, T. D., Wilson, T. E. and Kasper, C. B. (1989) *J. Biol. Chem.* 264, 7584–7589
18 Chan, R. L., Carrillo, N. and Vallejos, R . H. (1985) *Arch. Biochem. Biophys.* 240, 172–177
19 Hackett, C. S., Novoa, W. B., Ozols, J. and Strittmatter, P. (1986) *J. Biol. Chem.* 261, 9854–9857
20 Porter, T. D., Beck, T. W. and Kasper, C. B. (1990) *Biochemistry* 29, 9814–9818
21 Nelson, D. R. and Strobel, H. W. (1987) *Mol. Biol. Evol.* 4, 572–593
22 Sligar, S. G. and Murray, R. I. (1986) in *Cytochrome P-450 Structure, Mechanism and Biochemistry* (Ortiz de Montellano, P. R., ed.), pp. 429–503, Plenum
23 Narhi, L. O. and Fulco, A. J. (1986) *J. Biol. Chem.* 261, 7160–7169
24 Narhi, L. O. and Fulco, A. J. (1987) *J. Biol. Chem.* 262, 6683–6690
25 Ruettinger, R. T., Wen, L-P. and Fulco, A. J. (1989) *J. Biol. Chem.* 264, 10987–10995
26 Siegel, L. M., Davis, P. S. and Kamin, H. (1974) *J. Biol. Chem.* 249, 1572–1586
27 Siegel, L. M. and Davis, P. S. (1974) *J. Biol. Chem.* 249, 1587–1598
28 Faeder, E. J., Davis, P. S. and Siegel, L. M. (1974) *J. Biol. Chem.* 249, 1599–1609
29 Ostrowski, J., Barber, M. J., Rueger, D. C., Miller, B. E., Siegel, L. M. and Kredich, N. M. (1989) *J. Biol. Chem.* 264, 15796–15808
30 Karplus, P. A., Daniels, M. J. and Herriott, J. R. (1991) *Science* 251, 60–66

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