REVIEW

Structural domains of P450-containing monooxygenase systems

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All known P450-containing monooxygenase systems share common structural and functional domain architecture. Apart from P450 itself, these systems can comprise several fundamentally different protein components or domains, all of which are shared by other multicomponent/multidomain enzyme systems with various functions: FAD flavoprotein or domain, FMN domain, FeS2 ferredoxin, FeS4 ferredoxin, and cytochrome b5. Either FMN domain, ferredoxins or cytochrome b5 serve as the electron transport intermediate between the FAD domain and P450. The molecular evolution of both P450-containing systems and of each particular component does not follow phylogeny in general. Gene fusion and horizontal gene transfer events can lead to the appearance of novel redox chains in the same manner that artificial chimeric proteins can be constructed by humans. Recent studies using genetic and protein engineering techniques to investigate the separate domains and their interaction are described.

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Introduction

The P450 enzymes constitute a large superfamily of haem-thiolate proteins (NC-IUB, 1991) involved in the metabolism of a wide variety of both exogenous and endogenous compounds (Nebert and Gonzalez, 1987). Usually, they act as terminal oxidase in multicomponent electron transfer chains, called here P450-containing monooxygenase systems.

There exists a widespread division of P450-containing monooxygenase systems into two main types, bacterial/mitochondrial (type I) and microsomal (type II). On the other hand, a principal classification of P450-containing systems was undertaken using the number of their protein components (Degtyarenko and Archakov, 1993; Hanukoglu, 1995). However, the proposed schemes did not cover cytochrome b5-containing redox pathways, although it has been known that cytochrome b5 can serve as an effector or electron donor for P450s (Ingelman-Sundberg and Johansson, 1980; Hlavica, 1984; Aoyama et al., 1990; Truan et al., 1993).

Mitochondrial and most of the bacterial P450 systems have three components: an FAD-containing flavoprotein (NADPH or NADH-dependent reductase), an iron–sulphur protein and P450. The eukaryotic microsomal P450 system contains two components: NADPH:P450 reductase (CPR), a flavoprotein containing both FAD and FMN, and P450. CPR appears to be a fusion protein consisting of domains which are homologous to ferredoxin:NADP+ reductases (FAD domain) and flavodoxin (FMN domain) (Porter and Kasper, 1986; Smith et al., 1994). A unique prokaryotic two-component P450 monooxygenase system from Streptomyces carbophilus has been described (Serizawa and Matsuoka, 1991). This system is composed of P450sca haemoprotein and NADH-dependent P450 reductase containing both FAD and FMN. Finally, a soluble monooxygenase P450b0M,3 (CYP102) from Bacillus megaterium exists as a single polypeptide chain with two functional parts, the haem and flavin domains (Narhi and Fulco, 1986). It represents a unique bacterial one-component system. However, sequence and functional comparisons show that these domains are more similar to P450 and the flavoprotein of the microsomal two-component P450 monooxygenase system than to the relevant proteins of the three-component system (Ruettinger et al., 1989).

Mammalian nitric oxide synthase (NOS) contains FAD, FMN and haem and was the first eukaryotic catalytically self-sufficient P450-like system described (White and Marletta, 1992). The C-terminal domain of NOS is clearly homologous to CPR, whereas its N-terminal domain is of a low similarity to P450 sequences. The bacterial NOS system, functionally similar to the mammalian one, was recently discovered in Nocardioid spp.
(Chen and Rosazza, 1994). Analogously, newly described fatty acid hydroxylase from *Fusarium oxysporum* seems to be a fusion protein of P450 and reductase domains similar to P450<sub>BM-3</sub> (Nakayama and Shoun, 1994). In the context of the proposed classification, mammalian NOS and *F. oxysporum* monooxygenase could be viewed as eukaryotic one-component P450 systems.

On the other hand, all P450-containing monooxygenase systems described so far share common structural and functional domain architecture (Table I). Here, we mean by ‘domain’ (a segment of) polypeptide existing as an independently folding unit and possessing a certain function. Hence there are no fundamental differences between the protein domain and the individual protein component and all the P450 systems can be considered as three-domain systems (Figure 1):

(i) NADH- or NADPH-dependent FAD-containing reductase (FAD domain), (ii) an iron–sulphur protein (in a three-component system) or FMN-binding domain (in a two- and one-component system) homologous to bacterial flavodoxin, and (iii) P450 protein (haem domain). All the P450s are obviously homologous. In contrast, FAD domains may be categorised into three major families: members of the flavoprotein pyridine nucleotide cytochrome reductases family (a term proposed by Hyde *et al.*, 1991), flavoproteins similar to pyridine nucleotide–disulphide oxidoreductases, and adrenodoxin reductases. Similarly, Fe–S (ferredoxin-like) and FMN-binding domains (flavodoxin-like) are examples of functional analogy. In our opinion, cytochrome *b<sub>s</sub>* could be categorised as another analogue of this ‘intermediate’ domain. It is worth noting that while these domains possess the same function, they have different specificity. In three-component systems reductase interacts with specific ferredoxin, which, in turn, interacts with the corresponding P450 protein (putidaredoxin reductase → putidaredoxin → P450<sub>cam</sub>, terpredoxin reductase → terpredoxin → P450<sub>terp</sub>, adrenodoxin reductase → adrenodoxin → few mitochondrial P450s); it is natural that in one-component systems, at least *in vivo*, reductase can interact only with the relevant P450 domain of the same polypeptide chain. In a microsomal two-component system, different microsomal P450s interact with a ‘universal’ P450 reductase. Finally, cytochrome *b<sub>s</sub>* is a non-specific electron donor, so it can interact with P450, microsomal acyl-CoA and sterol desaturases, haemoglobin, cytochrome *c*, cytochrome *b<sub>s</sub>*, etc. However, it was shown recently that specificity of the interaction of different microsomal P450 proteins from the same organism with cytochrome *b<sub>s</sub>* exists (Omata *et al.*, 1994a).

Thus, an ancestor of any known P450 monooxygenase system should contain at least three different proteins. An intermediate electron donor component might be presented by one of the functionally interchangeable proteins: flavodoxin-like, ferredoxin or cytochrome *b<sub>s</sub>*. In the case of a flavodoxin-like protein containing system, the fusion of its ancestral gene with the gene encoding the FAD component led to the appearance of new protein family, P450 reductase. Similarly, the fusion of the two ancestral genes, encoding P450 and CPR, resulted in the origin of the natural chimeric P450 system. Hence, P450<sub>BM-3</sub>-like enzymes should be considered as the most evolutionarily ‘advanced’ P450 monooxygenase system. Indeed, P450<sub>BM-3</sub> represents the most effective P450 monooxygenase known (Narhi and Fulco, 1986). At the same time, this fusion could result in some lack of flexibility when multiple drug-metabolising enzymes are required. A eukaryotic metabolic system usually employs multiple P450 enzymes of different specificity and one ‘universal’ P450 reductase (for a review, see Hanukoglu, 1992).

**Families of structural domains of P450-containing monooxygenase systems**

Tables I and II summarise the information on structural domain families featured in P450-containing systems, and a number of other enzyme systems, respectively.

**FAD domain**

On the basis of sequence similarity, FAD flavoproteins/domains can be categorised into three major families:

(i) FAD flavoprotein/domain shared by members of the ferredoxin:NADP<sup>+</sup> reductase (FNR) family, also referred to as the flavoprotein pyridine nucleotide cytochrome reductases family (Hyde *et al.*, 1991). This family contains ferredoxin:NADP<sup>+</sup> reductases, NADH:cytochrome *b<sub>s</sub>* reductase, NADPH:P450 reductase, NADPH: sulphite reductase (Ostrowski *et al.*, 1989a, b), NADH: and NADPH:nitrate reductases (Campbell and Kinghorn, 1990; Hyde *et al.*, 1991), yeast flavohaemoglobin (Zhu and Riggs, 1992), β-subunit of phagocyte flavocytochrome *b* (cytochrome *b<sub>-245</sub>* (Segal *et al.*, 1992), and phthalate dioxygenase reductase (Correll *et al.*, 1992). In turn, all members of this family seem to share the same structural framework (‘FNR-like module’): the N-terminal subdomain, which binds the flavin, and the C-terminal subdomain, which binds pyridine nucleotide (Correll *et al.*, 1993). To date, three-dimensional structures of three members of the family have been solved (see *P450 systems in three dimensions*). In all of them, the flavin subdomain represents an antiparallel β-barrel structure, while the NAD(P) subdomain has the βαβ topology, typical of pyridine dinucleotide-binding folds.
(Schulz, 1992). In spite of such structural similarities, the level of amino acid identity between family members is at, or below, the limit of significance (for instance, nitrate reductase is only 15% identical with FNR). It has been suggested that the developed interface between the FAD- and NADPH-binding subdomains in FNR would not allow them to be functionally separated (Karpplus et al., 1991). Indeed, experiments on the expression of the FAD-binding subdomain showed that it does not fold correctly in the absence of the NADPH subdomain (Smith et al., 1994).

(ii) Iron–sulphur protein reductases, involved in oxidative metabolism of a variety of hydrocarbons (putidaredoxin reductase, terredoxin reductase, rubredoxin reductase, ferredoxin:NAD\(^+\) reductase components of benzene 1,2-dioxygenase, toluene 1,2-dioxygenase, chlorobenzene dioxygenase, biphenyl dioxygenase), share sequence similarity with a number of other flavoprotein oxidoreductases, in particular with the family of pyridine nucleotide–disulphide oxidoreductases (glutathione reductase, trypanothione reductase, lipoyamide dehydrogenase, mercuciric reductase, thioredoxin reductase, alkyl hydroperoxide reductase), NADH oxidase, NADH peroxidase and flavoprotein subunit of flavocytochrome c sulphide dehydrogenase (Eggink et al., 1990; Kuriyan et al., 1991; Mason and Cammack, 1992). Comparison of the crystal structures of human glutathione reductase and Escherichia coli thioredoxin reductase reveals different locations of their active sites, suggesting that the enzymes diverged from an ancestral FAD/NAD(P)H reductase and acquired their disulphide reductase activities independently (Kuriyan et al., 1991). Since glutathione reductase (GR) represents the structural prototype of the family, we shall refer to this large group of proteins as the GR family. All crystal structures resolved so far for the members of this family show similar topology, but the relative orientations of their FAD- and NAD(P)H-binding subdomains may vary significantly (see P450 systems in three dimensions). By contrast with the FNR family, the FAD- and NAD(P)H-binding subdomains in the GR family share both the sequence and spatial similarity (ββββ fold), suggesting that these proteins evolved by gene duplication (Schulz, 1980).

(iii) NADPH:adrenodoxin reductase, which shows no sequence similarity with other known ferredoxin reductases over the entire length of sequence (Hanukoglu and Gutfinger, 1989). However, the FAD and NAD(P)-binding sites appear in both putidaredoxin reductase and adrenodoxin reductase at nearly identical positions (Hanukoglu, 1995). Furthermore, adrenodoxin reductase shares local similarity with glutamate synthases and NADH peroxidase.

**Ferredoxins**

Iron–sulphur proteins involved in electron transfer from FNR (FAD domain) to P450 could be categorised into two families: adrenodoxin-like Fe\(_2\)S\(_4\) proteins (adrenodoxin, putidaredoxin, terredoxin) and Fe\(_3\)S\(_4\) ferredoxins (Streptomyces griseolus Fd-1 and Fd-2, Streptomyces griseus ferredoxin soy, Rhodococcus fascians and Bradyrhizobium japonicum ferredoxins). Comparative analysis showed that Fe\(_2\)S\(_4\) ferredoxins of Streptomyces are highly homologous to Fe\(_3\)S\(_4\) ferredoxins but that they lacked a fourth cysteine conserved in Fe\(_3\)S\(_4\) proteins (O’Keefe et al., 1991). Note that Fe\(_2\)S\(_2\) proteins from the adrenodoxin family appear to be evolutionary unrelated to the family of chloroplast-type Fe\(_2\)S\(_2\) ferredoxins (Harayama et al., 1991).

**Flavodoxins**

Flavodoxins act in various electron-transport systems as functional analogues of ferredoxins (Wakabayashi et al., 1989). Although a number of flavodoxins have been found only in some bacteria and algae, these proteins share a similarity with the number of protein domains of both prokaryotic and eukaryotic origin. Except for the FMN binding domain in CPRs and NO\(_x\)es, flavodoxin-like domains were found in Penicillium vitale catalase (Melik-Adamyan et al., 1986), human erythrocyte NADPH:flavin reductase (Chikuba et al., 1994) and Desulfovibrio gigas nickel–iron hydrogenase (Volbeda et al., 1995). The three-dimensional (3-D) structures of a number of flavodoxins have been determined (Rao et al., 1992). The protein adopts the familiar ββββ conformation found in the nucleotide-binding domains of the pyridine-nucleotide dehydrogenases. There is a pronounced asymmetry in the localisation of basic and acidic residues in the 3-D structure of flavodoxin, which results in the occurrence of a dipole which may be important for protein–protein interaction (Fukuyama et al., 1990).

**Cytochromes b\(_5\)**

Cytochromes b\(_5\) are ubiquitous electron transport proteins found in animals, plants and yeasts. In contrast to microsomal and mitochondrial (Lederer et al., 1983) membrane-bound cytochrome b\(_5\) proteins, cytochromes b\(_5\) from erythrocytes are water-soluble cytosolic proteins (Abe et al., 1985). It was shown recently that mRNA, corresponding to the soluble form of cytochrome b\(_5\), is also distributed in other animal tissues (Giordano and Steggles, 1993).
Cytochrome \( b_5 \) is homologous to the haem-containing redox domain of a number of oxidoreductases, such as plant and fungal nitrate reductases, chicken sulphite oxidase and yeast flavocytochrome \( b_5 \) (\( l \)-lactate dehydrogenase). On the basis of sequence comparison, it was also hypothesised that the globin family was evolved from an ancestral cytochrome \( b_5 \)-like protein (Runnegar, 1984). It is notable that all cytochromes \( b_5 \) and their homologues described so far were found only in eukaryota.

**P450 domain**

P450s represent the most variable domain of the system. The existing P450 nomenclature, based on the divergent evolution of the P450 superfamily, was proposed and developed by Nebert and co-workers (Nelson et al., 1993). However, the evolution of P450s is not restricted to phylogeny. Phylogenetic analysis can only be applied to several groups of orthologous genes, such as CYP1A1, 1A2, 2E1, 7, 11A1, 11B1, 17, 19, 21A1, 27 and 51. Some P450 gene clusters, for example the mammalian CYP1A1 and CYP1A2, rat CYP2D cluster (Nelson et al., 1993), house fly CYP6A and CYP6C (Cohen and Feyereisen, 1995) gene clusters, have seemingly appeared via gene duplication events and should be considered paralogous. In P450 gene subfamilies such as CYP2A, 2B, 2C, 2D, 3A, 4A, and 52A, which, presumably, have arisen through numerous species-specific gene duplication and conversion events, the orthologue assignments are impossible. This kind of evolution can be concerned with intraspecific specialisation. The aforementioned gene fusion events can lead to the appearance of a one-component P450 system. It has been hypothesised that the F. oxysporum P450nor (CYP55) gene is a xenologous gene of prokaryotic origin (Kizawa et al., 1991). Another example of a much more ancient horizontal gene transfer event could be the integration of former mitochondrial genes (families CYP11 and CYP27) into the nuclear genome. The evolution of eukaryotic P450 genes also includes the loss of introns (Nebert et al., 1988). Finally, convergent evolution cannot be ignored either, such as in the membrane-binding anchors or in the substrate-binding sites of enzymes from the different families but with a similar substrate specificity. That is why any dendrogram constructed for the whole P450 superfamily cannot be considered a phylogenetic tree.

The same is true for other domains of the system; the molecular evolution of each component does not follow phylogeny in general, although phylogenetic analysis can be applied to several groups of orthologous proteins (most eukaryotic NADPH:P450 reductases, microsomal cytochromes \( b_5 \) and NADH:cytochrome \( b_5 \) reductases).

Taking into account that horizontal gene transfer events may occur, the subdivision of P450-containing monooxygenase systems into prokaryotic and eukaryotic seems to be quite formal. Furthermore, there are no fundamental differences between P450 systems of various cellular localisation. For instance, mitochondrial P450 could be easily converted into a microsomal system solely by altering the targeting signal sequence. Experiments with modified mitochondrial P450c27 (CYP27), whose mitochondrial targeting signal sequence was replaced by the microsomal targeting signal of microsomal P450, proved that this protein, when expressed in yeast, is localised on microsomes and shows monooxygenase activity in the presence of adrenodoxin and NADPH:adrenodoxin reductase (Sakaki et al., 1992).

Despite the apparent simplicity of the proposed model, the evolutionary relationships of prokaryotic and eukaryotic one-, two-, and three-component P450-containing systems are not obvious. Indeed, it is known that, on the basis of sequence similarity, all P450s can be categorised into two main classes: B-class and E-class (Gotoh, 1992). Only P450 proteins of prokaryotic three-component systems and fungal P450nor (CYP55) belong to B-class P450s, whereas all other known P450s from distinct systems are of E-class. Moreover, the data suggest that the divergence of the P450 superfamily into B- and E-classes, and within E-class, further divergence into stable P450 groups should be very ancient and occurred before the appearance of eukaryotes. Therefore, the origin of membrane anchors should be an independent event, at least in each major P450 group. A very weak similarity between the N-terminal membranous segments could be explained as a result of the ‘evolutionary game’ with the absence of charged residues (or, at least, of uncompensated charges). On the other hand, the acquisition of membrane-binding properties by one of the protein components of the microsomal monooxygenase system resulted in anchoring other components to the membrane, although this can occur in different ways. Microsomal P450 proteins and CPRs are anchored by the N-terminal transmembrane segment, whereas microsomal cytochromes \( b_5 \) have a C-terminal hydrophobic segment. Other components can possess lipid anchors such as NADH:cytochrome \( b_5 \) reductase (Ozols et al., 1984) and endothelial NOS (Busconi and Michel, 1993), which have an N-terminal myristoylated residue.
P450 systems in three dimensions

To date, three-dimensional structures of the following proteins/domains have been reported: (i) P450s: P450cam (Poulos et al., 1987), P450 domain of P450BM3 (Ravichandran et al., 1993), P450terp (Hasemann et al., 1994), P450eryF (Cupp-Vickery and Poulos, 1995); (ii) FNR family: ferredoxin:NADP+ reductase (FNR) (Kuriyan et al., 1991), phthalate dioxygenase reductase (PDR) (Correll et al., 1992), FAD domain of nitrate reductase (Lu et al., 1994); (iii) GR family: glutathione reductase (Karpus and Schulz, 1987), trypanothione reductase (Bailey et al., 1993), lipoamide dehydrogenase (Mattevi et al., 1991), mercuric reductase (Schiering et al., 1991), thioredoxin reductase (Kuriyan et al., 1991), NADH peroxidase (Stehle et al., 1993), flavoprotein subunit of flavocytochrome c sulphide dehydrogenase (Chen et al., 1994); (iv) adrenodoxin-like Fe2S2 ferredoxins family: putidaredoxin (Pochapsky et al., 1994); (v) bacterial Fe3S4 ferredoxins (Stout, 1982; Duée et al., 1994; Sery et al., 1994); (vi) flavodoxins (Rao et al., 1992; Volbeda et al., 1995); (vii) cytochrome b5 family: cytochrome b5 (Argos and Mathews, 1975; Guelis et al., 1990) and flavocytochrome b5 (Xia and Mathews, 1990). Thus, each family of structural domains except the adrenodoxin reductase is represented by at least one resolved spatial structure which could be used for homology modelling. Figure 2 shows some 3-D structures of interest. The combination of the structure and homology information provides insights into protein–protein interactions and the mechanism of electron transfer between the components of the system.

The P450 molecule represents an α/β protein which is shaped like a triangular prism; the overall structure might be roughly divided into an ‘α-rich domain’ (‘right side’) and a ‘β-rich domain’ (‘left side’) (Cupp-Vickery and Poulos, 1995). However, this division appears to be artificial since ‘α-rich’ and ‘β-rich domains’ comprise the discontinuous assemblies of secondary structure segments and do not constitute independent folding units. Although the sequence identity between any two P450s with known 3-D structures reaches only 20% or less, the overall topology of these proteins is similar (Figure 2a and b). However, P450s differ in the orientation of several helices. The root mean square (r.m.s.) deviation of Cα atoms in helical segments of P450 is 2.8 Å (for comparison, FNR and FAD share 15% identity and the r.m.s. deviation of Cα atoms for FNR-like core is only 1.5 Å). The most dramatic variations between the P450 structures are found in regions responsible for substrate binding and access (Li and Poulos, 1994). In the crystal structure of P450BM3, the access channel for the substrate, a long chain fatty acid, is open, whereas in P450cam it is closed (Li and Poulos, 1995). The B′ helix, which is responsible for substrate binding and hydroxylation specificity in P450cam, is positioned differently in other P450s. Although the crystal structure of P450cam has been extensively used to predict 3-D structures for eukaryotic P450 (Ferency and Morris, 1989; Graham-Lorence et al., 1991; Poulos, 1991; Zvelebil et al., 1991; Vijayakumar and Salerno, 1992; Boscott and Grant, 1994), it is worth noting that even the prediction of 3-D structure for other bacterial enzymes using P450cam as a template apparently cannot be successful with current homology modelling techniques. The recent studies of Ruan et al. (1994) revealed that P450BM3 provides a more suitable template for homology modelling of thromboxane synthase (CYP5) and possibly a number of other P450 families. However, keeping in mind the known structural differences between B-class P450s, the models (especially of substrate-binding sites) based on the sole structure of the E-class P450 representative, should be treated with caution. Information on other 3-D structures of this large group would obviously be of primary interest for homology modelling.

FNR-like proteins consist of β-barrel (FAD) and pyridine nucleotide-binding (NADPH) subdomains with well conserved topology (Figure 2g). However, they also show some important structural differences. Minor differences in the structure of the β-barrel subdomain of PDR and FNR result in selectivity between FMN and FAD (Correll et al., 1993). Comparison of FAD complexes of FNR and nitrate reductase revealed that even the same cofactor differs in orientation of its adenine-ribose part (Lu et al., 1994). The specificity of PDR and nitrate reductase for NAD(H) and FNR for NADP(H) is presumed to be determined by the presence of either an acidic or basic residue, respectively, close to the 2′-phosphate binding site. The relative orientation of two subdomains in these flavoproteins also varies substantially, reflecting different rotations around a common axis.

The members of the glutathione reductase family, although having a similar overall topology, show a variety of structural differences concerning packing of subdomains, mode of pyridine nucleotide binding and localisation of redox-active cysteine residues. A lack of the interface subdomain in the case of thioredoxin reductase (Kuriyan et al., 1991) and the appearance of an extra N-terminal domain in the case of mercuric reductase (Schiering et al., 1991) are the most impressive examples of such differences. Thus, in spite of an apparent abundance of structural information (seven crystal structures for this family), the 3-D database is still very restricted for modelling reductase components of bacterial P450-containing systems.

To date, no X-ray or NMR structure is known for complexes of the redox domains reviewed here. The only resolved example of an interface between plant-type Fe2S2 ferredoxin and FNR-like domains is PDR, where this interface is formed within a single polypeptide. The contacts between these two domains include eight direct
hydrogen bonds and six solvent bridges (Correll et al., 1993). It is tempting to suggest PDR as a guide for construction of the models for complexes of FNR with other electron carriers; however, the model FNR-ferredoxin complex that was built in that way was not consistent with the results of experiments. Furthermore, plant-type FeS$_2$ ferredoxins do not share homology with any domain group of known P450-containing systems. In this connection, the recent crystallisation and preliminary X-ray studies of CPR (Djordjevic et al., 1995) cannot be overrated. Among other things, this long-awaited structure will allow an understanding of spatial relationships between redox domains.

Although 3-D structures are available for two interacting in vivo proteins, P450cam and putidaredoxin, it is still unknown which structures form the interface between them. Experiments by Stayton et al. (1989) have suggested a putative binding site for putidaredoxin based on competition studies between putidaredoxin and cytochrome $b_5$ in their association with P450cam. Computer-aided docking of two 3-D structures has been used to build a model of a cytochrome $b_5$–P450cam complex, where the interface is formed by carboxyl groups of cytochrome $b_5$ and basic residues of P450cam (Stayton et al., 1989; Stayton and Sligar, 1990; Omata et al., 1994a, b). By analogy, it was hypothesised that putidaredoxin contains a cluster of acidic residues which interact with P450cam. On the other hand, it has been proposed that solvent-exposed side chains of several hydrophobic residues of putidaredoxin (Trp106, Val4, Val6 and Val98) are involved in the binding of P450cam (Pochapsky et al., 1994).

A model of the cytochrome $b$ domain of nitrate reductase, built on the basis of bovine cytochrome $b_5$, was docked to the FAD (FNR-like) domain of nitrate reductase (Lu et al., 1995). It was shown that the surface of cytochrome $b_5$ can be docked on that of reductase in a number of different orientations, one of which is energetically most favourable.

Nitric oxide reductase: homologous but not analogous

Nitric oxide reductase P450 (CYP55, P450nor) is a soluble haemoprotein involved in denitrification by the fungus Fusarium oxysporum (Kizawa et al., 1991). In contrast to other P450s, this enzyme does not possess monooxygenase activity but is able to reduce nitric oxide (NO) in the presence of NADH to form nitrous oxide (N$_2$O). Moreover, the reaction of NO reduction by P450nor does not require other protein components (Nakahara et al., 1993). This suggests that P450 should receive electrons directly from NADH. A mechanism for the NO reduction catalysed by P450nor has been proposed (Shiro et al., 1995) that differs fundamentally from mechanisms proposed earlier for other NO reductases. The amino acid sequence of P450nor shows a greater similarity to bacterial P450s, in particular to the CYP105 family (up to 40% identity), than to any eukaryotic P450. Recent promising studies on crystallisation and preliminary X-ray diffraction of P450nor (Nakahara et al., 1994) are expected to provide the opportunity to understand the mechanism of this unique P450 reaction.

Interestingly, a functional analogue of P450nor, the cytochrome $b$ subunit of bacterial NO reductase (NorB), is homologous to subunit I of cytochrome oxidase. It was proposed that cytochrome oxidase developed from NO reductase (Saraste and Castresana, 1994), particularly because NO was present in the earth’s atmosphere before the appearance of molecular oxygen. Despite the fact that P450 and cytochrome oxidase are not homologous, both represent part of redox chains that should evolve from an anaerobic to an aerobic reactions catalysis. We hypothesised earlier that P450 might be one of the most ancient respiratory enzymes (Degtyarenko and Archakov, 1993). Perhaps P450nor, which has retained a nitrate respiration function, can serve as evidence of this early period of P450 evolution.

It was originally proposed that a fungal genome acquired CYP55 gene via horizontal gene transfer from bacteria (e.g. streptomycetes), and then divergent evolution led to a novel function of P450, distinct from monooxygenase (Kizawa et al., 1991). However, the scheme of P450nor evolution should be different, assuming that monooxygenase activity is not a primary function of P450s! A redox chain as complex as a three-component (or three-domain) P450 system could not arise from scratch; obviously, there must be some self-sufficient P450-like haemoprotein somewhere among its ancestors. Thus, it is more plausible that both Streptomyces and Fusarium obtained P450 via horizontal gene transfer from some denitrifying bacterium. To activate the P450 monooxygenase ability, it was necessary to use two additional protein components (note that none of these are unique to P450 systems only). It is of further interest that F. oxysporum also possesses P450 monooxygenase, microsomal fatty acid hydroxylase resembling P450$_{BM-3}$ (Nakayama and Shoun, 1994). Thus, anaerobic and aerobic P450 redox chains can coexist within one cell.
Nitric oxide synthase: analogous or homologous?

During the last five years, the growing interest in the biological role of nitric oxide (NO) and nitric oxide synthase (NOS) has led to a huge number of publications in this field (see Knowles and Moncada, 1994; Sessa, 1994 and references cited therein). We would like to draw attention only to the evolutionary relationships of the NOS family and the P450-containing systems.

The pronounced sequence similarity of NOS with CPR (Bredt et al., 1991) and spectral properties typical for P450 haemoproteins suggested that NOS is a eukaryotic one-component P450 system. Reactions catalysed by NOS, such as L-arginine N\textsuperscript{\text{\textgamma}}-hydroxylase, N\textsuperscript{\text{\textgamma}}-hydroxy-L-arginine mono-oxygenase and NADPH:cytochrome c reductase, prove the functional identity of haem and reductase domains of NOS with P450 and of CPR, respectively. On the other hand, if homology of NOS and reductase is more or less obvious, there are different views on the origin of the similarity between the NOS haem domain and P450s. Nelson et al. (1993) classified NOS as an example of convergent evolution. Renaud et al. (1993) showed the functional identity of NOS and P450 3A as hydroxy-L-arginine mono-oxygenases and proposed that a nine amino acid long peptide, highly conserved among all NOS proteins and similar to the haem-binding consensus sequence of P450s, should be responsible for haem binding in NOSs. It should be noted that the relative location of these segments in protein sequences differs substantially, which causes serious problems in the alignment of P450 and NOS sequences. Alternatively, in our previous work (Degtyarenko and Archakov, 1993), employing alignment of N-terminal domains of P450\textsubscript{BM-3} and NOS, it was demonstrated that the similarity between these sequences can be observed without introducing long gaps. However, the segment containing conserved cysteine residue of NOS, which may correspond to the haem ligand of P450, was found to be completely different from the well known P450 ‘haem cystein’ signature.

Besides the NOS family, only P450\textsubscript{BM-3} represents a one-component P450 system with a known sequence. Even assuming common ancestry of their haem domains, this does not mean that these systems are descended from a common P450/CPR fusion protein. Taking into account that the reductase domain is much more conserved than the haem domain, it is more likely that corresponding gene fusion events occurred independently in NOS and CYP102 families.

Intron loss or intron acquisition?

The fusion of adjacent genes resulting in the appearance of multidomain protein is analogous to the intron loss events during the evolution of genes. From the ‘introns early’ theory point of view, exons correspond to primordial mini-genes and introns are the descendants of the spacers. In contrast, the ‘introns late’ theory assumes that introns were inserted into ancestors of contemporary split genes and, therefore, there is no correspondence between protein modules and exons (see recent discussion in Stoltzfus et al., 1994). It should be noted that each of these theories might appear true for a particular gene/protein family. Nebert et al. (1988) proposed that an early ancestral P450 gene could have contained at least 33 exons which were then fused. The number of exons in known eukaryotic P450 genes varies from 3 to 13 (Table III). Previously, it was speculated that those groups of P450s which contain the maximum number of introns are more ancient (Degtyarenko and Archakov, 1993). This statement needs to be corrected, because at least those eukaryotic P450 genes which are seemingly xenologues of bacterial or organellar origin have acquired introns (CYP55, CYP11s). It would be of interest to establish whether there is any correspondence between exons and structural units of CYP55 once its crystal structure is resolved. In any case, the only obvious thing is that from known P450s none seems to have arisen through exon shuffling. It is enigmatic that such a large molecule (400–530 amino acids) exists as a monodomain protein. The average length of a typical protein ‘module’ is 125–150 amino acids (Trifonov, 1994), hence P450 might comprise a polypeptide three or four modules in length, but no ‘half-P450’ or ‘quarter-P450’ homologue has ever been found. In the case of reductases and NOS, there are enough exons to attribute some functions to several groups of these (Porter et al., 1990; Miyahara et al., 1994); nevertheless there is no single exon corresponding to any particular structural–functional domain. The experiments on the CPR gene showed that expression only of those groups of exons which correspond to complete flavodoxin-like and FNR-like protein yields functionally active protein domains, whereas smaller polypeptides seemingly are unable to fold into native conformation (Smith et al., 1994).
Protein surgery and artificial P450 systems

Modern methods of genetic and protein engineering could be considered as an ‘alternative’ molecular evolution, directed and accelerated by humans. From this viewpoint, there are no differences between ‘naturally occurring’ horizontal gene transfer and heterologous expression, ‘natural’ and artificial fusion molecules, processing of proteins in vivo and limited proteolysis in vitro. The discovery of the ‘natural’ chimera enzyme P450BM3 (Narhi and Fulco, 1986) inspired several groups to investigate the structure and function of multidomain/multicomponent P450 systems using two complementary experimental approaches: dissection of multidomain proteins into distinct domains and construction of artificial fusion proteins from different redox domains.

Separated haem (P450) and flavin (CPR) domains obtained by proteolysis (Narhi and Fulco, 1986) or genetic engineering (Li et al., 1991; Munro et al., 1994) were used in studies of kinetics of electron transfer and catalysis. Each domain was shown to retain characteristic catalytic and spectral properties, whereas the rate of electron transfer between the isolated domains is considerably slower than for the native enzyme. It was proposed that the proper mutual orientation of P450 and reductase domains in the intact P450BM3 protein is maintained by the linker region (Munro et al., 1994). Analogously, experiments on limited proteolysis revealed that the haem and flavin domains of neuronal NOS can be isolated in functionally intact forms (Sheta et al., 1994).

Smith et al. (1994) demonstrated that the FAD and FMN domains of human CPR, expressed in E. coli as independent polypeptides, could be reconstituted to form a functional complex possessing P450 and cytochrome c reductase activity. Thus, an artificial system simulating a possible ancestral three-component P450 system was constructed. The catalytically active FAD domain of corn leaf NADH:nitrate reductase was expressed in E. coli and was found to exhibit spectral properties identical with NADH:cytochrome b5 reductase (Hyde and Campbell, 1990). Similarly, genetically engineered cytochrome b5-like domains of Chlorella vulgaris NADH:nitrate reductase (Cannons et al., 1991) and yeast flavocytochrome b2 (Brunt et al., 1992), showing spectra virtually identical with those of microsomal cytochrome b5, were expressed independently in E. coli.

Reciprocally, the possibility of obtaining a functional one-component P450 system from a two-component system has been shown in gene fusion experiments. To date, the following eukaryotic analogues of P450BM3 have been constructed: bovine CYP17/yeast CPR (Shibata et al., 1990), rat CYP1A1/yeast CPR (Sakaki et al., 1994) and human CYP1A1/human CPR (Wittekindt et al., 1995) expressed in Saccharomyces cerevisiae; bovine CYP17/rat CPR, rat CYP4A1/rat CPR (Fisher et al., 1992; Shet et al., 1994) and human CYP3A4/human or rat CPR (Shet et al., 1993) expressed in E. coli.

Two other domains considered in this review were recently used by Quinn et al. (1994), who constructed and expressed a functional chimera protein comprising the soluble domain of rat cytochrome b5 as N-terminus and the FAD domain of spinach assimilatory NADH:nitrate reductase as C-terminus. This chimera was termed flavocytochrome b5 by analogy with the yeast flavocytochrome b5; note, however, that the flavin domain of flavocytochrome b5 is not homologous with the FAD domain of the FNR family, but represents an eightfold βαβααβαβ-barrel (Lindqvist et al., 1991) and is shared by a large number of different enzymes (for a review, see Farber and Petsko, 1990).

Pueyo et al. (1989) obtained a covalent complex between Azotobacter vinelandii flavodoxin and Anabaena ferredoxin:NADPH reductase; similarly, Pirola et al. (1994) constructed a cross-linked Desulfovibrio vulgaris flavodoxin–spinach FNR complex. Both systems possess NADPH:cytochrome c reductase activity. In fact, these functional heterologous complexes represent novel CPRs.

Finally, P450 systems without other protein components can be briefly discussed. We have already mentioned the self-sufficient system P450nor from F. oxysporum catalysing NADH-dependent NO reduction. Recently, the monoxygenase activity of ‘monodomain’ P450 systems has also been shown. Gonnindaraj et al. (1994) found that, in the presence of FMN and NADPH, the haem domain of P450BM3 can catalyse, at a slow rate, the hydroxylation of a fatty acid substrate (myristic acid) without the reductase domain. A similar reaction has been shown for microsomal P450 (rabbit CYP2B4)-catalysed p-hydroxylation of aniline, in the presence of 1000-fold excess of FMN over P450 (Uvarov et al., 1994). At the same time, it was shown that the covalent binding of FMN with CYP2B4 using carbodiimide yielded a conjugate able to catalyse NADPH-dependent N-demethylation of aminopyrine and dimethylnitrosamine and p-hydroxylation of aniline, at rates which are only 30–40% lower than those of the microsomal system.
The P450 Systems on WorldWide Web

To facilitate access to electronic resources for all researchers working in the field of P450 proteins and P450 containing systems, we have established an experimental www-server called The Directory of P450-containing Systems (K.N.Degtyarenko and P.Fábián, in preparation), available at <http://www.icgeb.trieste.it/p450/>.

Currently it contains the most up-to-date list of accession numbers for sequences of both the P450 superfamily and proteins mediating electron transfer to P450 [NADPH:P450 reductases, specific Fe–S proteins and NAD(P)H:ferredoxin reductases, cytochrome b₅ reductases and cytochromes b₅₁] and their homologues. All the referenced sequences are provided with cross-links to sequence databanks EMBL/GenBank, SWISS-PROT and PRF. Special pages are dedicated to genes with a known exon/intron structure and a selected bibliography on the subject.

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References


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Note added in proof
The crystal structure of one more FNR-like protein, NADH:cytochrome b5 reductase, has recently been reported [Nishida, H., Inaka, K. and Miki, K. (1995) FEBS Lett., 361, 97-100].
Legends to figures

Fig. 1. Schematic representation of the domain structure of P450 systems (see text).

Fig. 2. Three-dimensional structures of redox domain representatives. (a) B-class P450: *Pseudomonas putida* P450cam (CYP101), 2CPP (Poulos et al., 1987); (b) E-class P450: haem domain of *Bacillus megaterium* P450BM-3 (CYP102), 2HPD (Ravichandran et al., 1993); (c) Fe₂S₂ ferredoxins: *P. putida* putidaredoxin, 1PUT (Pochapsky et al., 1994); (d) Fe₄S₄ ferredoxins: *Desulfovibrio africanus* ferredoxin, 1FXR (Sery et al., 1994); (e) cytochromes b₅: bovine cytochrome b₅, 3B5C (Argos and Mathews, 1975); (f) FMN domain: *D. vulgaris* flavodoxin, 2FX2 (Watt et al., 1991); (g) FAD domain (FNR-like): spinach ferredoxin:NADP⁺ reductase, 1FNR (Karplus et al., 1991); (h) FAD domain (GR-like): *Escherichia coli* thioredoxin reductase, 1TRB (Kuriyan et al., 1991). All drawings were produced using the program MOLSCRIPT (Kraulis, 1991).