Evolution of bioinorganic motifs in P450-containing systems

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Abstract
The choice of bioinorganic motifs by Nature results in a spectacular variety of active-site structures even within the same protein family. Here, we use the concept of the bioinorganic motif to discuss the function and evolution of P450-containing and other related systems. Apart from P450, these systems include a FAD flavoprotein or domain, a FMN domain, ferredoxins and cytochrome b$_5$. Analysis of available complete genomes can shed light on what an ancestral P450-containing system could be.

Introduction
The P450 enzymes constitute a large superfamily of haem-thiolate proteins involved in the metabolism of diverse chemical compounds. Usually, they act as terminal oxidase in multicomponent electron-transfer (ET) chains, called here P450-containing mono-oxygenase systems, although self-sufficient non-mono-oxygenase P450s are also described. In the previous review [1], we postulated that all P450-containing mono-oxygenase systems share common structural and functional domain architecture. Apart from P450 itself, these systems can comprise several fundamental redox domains: FAD flavoprotein or domain, FMN domain, ferredoxin (Fd) and cytochrome b$_5$. These ubiquitous redox domains, in various combinations, are widely distributed in biological systems. FMN domain, Fd or cytochrome b$_5$ transfer electrons between the FAD domain and P450. While P450-containing systems are found throughout all kingdoms of life, some organisms lack one or more of these redox domains completely. Here, we review the current state of the affairs in the light of data that became available as a result of the genomic revolution and the progress in structural biology.

In 1995, the first complete genome sequence of a free-living organism, the bacterium Haemophilus influenzae, was published [2]. Since that, complete genomes of 36 species, including three eukaryotes, have been sequenced (see http://www.ebi.ac.uk/genomes/; when this paper is published, the number is likely to have increased). The analyses of the complete genome data provide unprecedented insight into molecular evolution of biochemical systems.

In the same time, we have witnessed an extraordinary increase in quantity and quality of protein structural information. In the last 5 years, the structures of key components of P450-containing systems were determined, viz. NADPH:cytochrome P450 reductase (CPR), NADPH:adenodoxin reductase (AdR) and adrenodoxin (Adx), together with structures of new P450s from different kingdoms of life and other haem-thiolate proteins.

On the other hand, our knowledge of function of P450 and P450-containing systems does not catch up with the relevant sequence and structural information. For example, of 83 Drosophila melanogaster P450s, CYP6A2 is the only functionally

Key words: cytochrome b$_5$, electron transfer, ferredoxin, flavoprotein, genome.

Abbreviations used: 3D, three-dimensional; CPR, NADPH:cytochrome P450 reductase; AdR, NADPH:adenodoxin reductase; Adx, adrenodoxin; bSR, NADH:cytochrome b$_5$ reductase; BIM, bioinorganic motif; ET, electron transfer; FADPNR, FAD-dependent pyridine nucleotide reductase; Fe$_3$, Fe$_5$, bacterial-type ferredoxin; Fd, ferredoxin; Fd, flavodoxin; FNR, ferredoxin:NADP$^+$ reductase; NOS, nitric oxide synthase; Pdr, putidaredoxin reductase; Pdx, putidaredoxin; Rdx, rhodocoxin; RdR, rhodocoxin reductase; SIR, sulphite reductase; SIR-FP, SIR flavoprotein subunit; SPT, SWISS-PROT/TrEMBL; Tdx, terpredoxin reductase; TdR, terpredoxin.

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characterized enzyme [3]. For the majority of prokaryotic P450s, the physiological electron donors are unknown. The availability of complete genomes makes it possible to find potential redox partners for P450s and to predict what an ancestral P450-containing system could be.

Bioinorganic motif (BIM) is defined as a common structural feature shared by functionally related complex proteins [4]. We employed here the concept of BIM for discussion of function and evolution of P450-containing and other related systems. In an attempt to control the number of literature references without sacrificing the information content, in this paper we have used the cross-references to the biological databases in the form database name:accession number, for the SWISS-PROT/TrEMBL (SPTR) [5], InterPro (http://www.ebi.ac.uk/interpro/) and PDB [6] databases. The referenced database entries themselves contain comprehensive literature and database references.

What is P450?

'Haem-thiolate proteins' [7] is the recommended collective name for a class of haemproteins in which the haem-iron fifth ligand is a cysteine thiolate group (Figure 1a). Apart from P450s, haem-thiolate proteins include: cystathionine \( \beta \)-synthase (EC 4.2.1.22; haemoprotein H450; serine sulphhydrase; \( \beta \)-thionase), fungal chloroperoxidase (EC 1.11.1.10; InterPro:IPR000028) and nitric oxide synthase (NOS; EC 1.14.13.39).

The proximal haem-iron ligand mutants of myoglobin (His \( \rightarrow \) Cys) were constructed as 'model' haem-thiolate proteins [8,9].

According to the above definition, sirohaem-containing enzymes (InterPro:IPR000660), which include sulphite reductases (SiRs) and nitrite reductases [10], also should be classified as haem-thiolate proteins because the sirohaem-iron fifth ligand is provided by a cysteine. The difference is that the same cysteine serves as a ligand to an Fe\( _3 \)S\( _4 \) cluster (Figure 1b). The sirohaem and the Fe\( _3 \)S\( _4 \) cluster are strongly exchange-coupled [11] and exhibit spectral features quite different from those of haem-thiolate proteins.

The more we learn about P450 enzymes, the more difficult it becomes to give a good definition to this protein group. In contrast to non-haem iron oxygenases, which demonstrate variety of BIMs in their active sites [12], all P450s share the same BIM. Paradoxically, of all known haem-thiolate proteins (and haem proteins in general), the P450s

![Figure 1](https://via.placeholder.com/150)

**Figure 1**

BIMs in P450-containing and related systems

BIMs (e–h) are found in intermediate redox domains. Although each of these BIMs occurs in several non-homologous protein families, the known P450-containing systems use only four protein families as intermediate domains. (a) Haem-iron co-ordination in haem-thiolate proteins, [Fe\( (\mu^3\)-porphyrin)S\( _4 \)]\( _{Cys} \); (b) polynuclear haem-iron-sulphur centre of sirohaem-containing enzymes, [Fe\( (\mu^3\)-porphyrin)[Fe\( (\mu^3\)-porphyrin)]\( _{Cys} \); (c) zinc atom at the dimer interface of CYPI 19, [Zn\( (\mu^3\)-porphyrin)\( (\mu^3\)-porphyrin)\( ]_{Cys} \); (d) zinc atom at the dimer interface of NOS oxygenase domain, [Zn\( (\mu^3\)-porphyrin)\( (\mu^3\)-porphyrin)\( ]_{Cys} \); (e) haem-iron co-ordination in cytochrome b\( _{6} \), [Fe\( (\mu^3\)-porphyrin)\( (\mu^3\)-porphyrin)\( ]_{Cys} \); (f) dinuclear iron-sulphur centre of Adx-type and plant-type Fds, [Fe\( _3 \)S\( _4 \)]\( _{Cys} \); (g) tetranuclear iron-sulphur centre of bacterial-type Fds, [Fe\( _4 \)S\( _4 \)]\( _{Cys} \); (h) trinuclear iron-sulphur centre of bacterial-type Fds, [Fe\( _3 \)S\( _4 \)]\( _{Cys} \).
Table I

Homologues of the components of P450-containing systems in complete genomes

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*Excluding CPR homologues.
*Including NADPH+glutamate synthase (EC 1.4.1.13) small subunit homologues.
*Including dicluster Fe₃S₄+Fe₄S₄ Fds.
*Including two CYP102 (P450+CPR fusion) proteins.

are by far the most functionally diverse. It seems that the most appropriate definition should be a structural one, based either on sequence or threedimensional (3D) structure similarity. For example, P450s may be defined as sequences having statistically significant matches with Inter-
Pro: IPR001128. Still, we humans are also interested in a functional definition.

The existing nomenclature for P450 genes and their products based on the use of root symbol CYP (CYP[family][subfamily][gene]) is used throughout the text [13]. As at 2 October 2000, all known CYP genes are classified into 215 different families (http://drnelson.utmem.edu/CytochromeP450.html). Between superfamily and family, the intermediate levels such as B-class and E-class [15] and clans (http://drnelson.utmem.edu/CytochromeP450.html) also have been introduced.

Just 5 years ago, the only structurally characterized P450s were those of bacteria. By now, the 3D structures of a fungal (Fusarium oxysporum CYP55; PDB: 1ROM, PDB: 2ROM), animal (rabbit CYP2C5; PDB: 1DT6) and archaeal (Sulfolobus solfataricus CYP119; PDB: 1F4T, PDB: 1F4U) P450s have been determined. The co-ordinates of a Mycobacterium tuberculosis CYP51 (SPTR: P77901), the member of a unique P450 family occurring in bacteria, animals, fungi and plants [16], have been deposited in the PDB. The long-awaited structure of a mammalian microsomal P450 (PDB: 1DT6) reveals the familiar triangular prism structure; however, the unique features of the enzyme, most importantly the details of its association with the membrane, remain to be clarified [17].

Surprisingly little is known about quaternary structure of P450s. A hexameric organization of microsomal P450s has been suggested by Myasoedova ([18] and references therein). Bacillus megaterium CYP102 was shown to exist as a mixture of monomeric and oligomeric species [19]. The plant fatty acid hydroperoxide lyase appears to be a tetramer in vivo [20] while CYP119 exists as a dimer. The crystal structure of CYP119 (PDB: 1F4T) revealed a bound zinc atom at the dimer interface (Figure 1c). Interestingly, the unrelated haem-thiolate protein NOS also contains zinc at the interface of the oxygenase domain dimer (Figure 1d).

What are the P450-containing systems?
The simplest and most universal, if not too helpful, definition would be 'the enzyme system that includes P450 protein/domain'. Indeed, the functional diversity of P450 proteins is also reflected in the diversity of enzyme systems containing P450. The classification of P450-containing mono-oxygenase systems into two main types, bacterial/mitochondrial (type I) and microsomal (type II), clearly fails to describe this diversity. Some P450s do not require any other protein components for their function while most of P450s act as terminal oxidases in multi-component ET chains called P450-containing mono-oxygenase systems. Apparently, the P450-containing mono-oxygenase systems evolved by recruiting ready redox proteins/domains with conserved BIMs (Figures 1c–1h). These redox domains can be classified into two groups: FAD domains and intermediate domains. Within each group, the domains are functionally interchangeable. The general electron flow scheme for the P450-containing mono-oxygenase systems is:

\[
\text{NAD(P)H} \rightarrow \text{FAD domain} \rightarrow \text{intermediate domain} \rightarrow \text{P450 domain} \rightarrow \text{O}_2 \quad (1)
\]

A search of the complete genomes for potential components of P450-containing systems reveals several patterns in an otherwise very diverse distribution (Table 1).

**P450 systems without other protein components**
Nitric oxide reductase (CYP55, P450nor) is a B-class P450 involved in denitrification in several fungal species. In contrast to most of the P450s, this enzyme does not possess mono-oxygenase activity but is able to reduce nitric oxide (\('\text{NO}'\)) to form N₂O directly using NAD(P)H as electron donor [21,22]:

\[
\text{NAD(P)H} \rightarrow \text{CYP55} \rightarrow \text{\textquoteright}\text{NO} \quad (2)
\]

In *Fusarium oxysporum*, the two distinct products of the single gene *CYP55*, P450norA and P450norB, are located in the mitochondria and the cytosol, respectively [23]. The 3D structure of a soluble CYP55 from *F. oxysporum* has been determined (PDB: 1ROM; PDB: 2ROM). Allene oxide synthase (CYP74A; EC 4.2.1.92) [24], fatty acid hydroperoxide lyase (CYP74B) [25], prostacyclin synthase (CYP8; EC 5.3.99.4) [26] and thromboxane synthase (CYP5; EC 5.3.99.5) [27] are examples of E-class P450s which do not require a reductase or molecular oxygen for their catalytic activity. Substrates for all these enzymes are fatty acid derivatives containing partially reduced dioxygen (either hydroperoxy or epidioxy groups). These highly specific
enzymes may be 'emancipated' P450s that lost the need for redox partners as a result of substrate choice. On the other hand, CYP55 is rather a descendant of a self-sufficient P450 from the pre-oxygen era [1].

**P450 reductase**

Eukaryotic microsomal P450s and some bacterial E-class P450s receive electrons from a FAD- and FMN-containing enzyme, CPR (EC 1.6.2.4) [28]. Microsomal CPR is membrane-bound protein that interacts with different P450s. In *B. megaterium* (SPTR:P14779) and *Bacillus subtilis* (SPTR:O08394; SPTR:O08336), CPR is a C-terminal domain of CYP102, a single polypeptide self-sufficient soluble P450 system (P450 is an N-terminal domain). The general scheme of electron flow in the CPR/P450 system is:

$$\text{NADPH} \rightarrow \text{FAD} \rightarrow \text{FMN} \rightarrow \text{haem} \rightarrow O_2 \quad (3)$$

The reduction of P450 is not the sole physiological function of CPR. The final step of haem oxidation by mammalian haem oxygenase (EC 1.14.99.3) requires CPR and O_2 [29]. In yeast, CPR affects the ferrireductase activity [30], probably transferring electrons to the flavocytochrome ferric reductase (InterPro: IPR002916).

The 3D structure of the hydrophilic domain of rat microsomal CPR has been solved (PDB:1AMO). The crystal structure confirmed that CPR contains domains structurally similar to FMN-containing protein flavodoxin (Fld; InterPro:IPR001094) and the FAD-containing enzyme ferredoxin:NADP+ reductase (FNR; InterPro:IPR001709). The crystal structure of *B. megaterium* P450 domain–FMN domain complex (PDB:1BVY) revealed some differences between the Fld-like domains in CYP102 and microsomal CPR around the FMN–FAD interaction sites.

The other enzyme systems containing homologues of CPR are NADPH: sulphite reductase (SiR; EC 1.8.1.13), NOS and methionine synthase reductase (EC 2.1.1.13). *Escherichia coli* SiR is a hetero-oligomer consisting of the \(\alpha\) subunit (flavo-protein subunit or SiR-FP) homologous with CPR and the \(\beta\) subunit (haemoprotein subunit) containing an Fe\(_{2}\)S\(_{2}\) cluster and a sirohaem bridged by a shared cysteine thiolate ligand (Figure 1b). The electron flow in the SiR is:

$$\text{NADPH} \rightarrow \text{FAD} \rightarrow \text{FMN} \rightarrow \text{Fe}_{2}\text{S}_{2} \rightarrow \text{sirohaem} \rightarrow \text{HSO}_{4}^{-} \quad (4)$$

The long accepted quaternary structure of SiR has been \(\alpha_{2}\beta_{2}\) [11]; the recent experiments, however, strongly suggest the \(\alpha_{2}\beta_{4}\) structure [31]. The notable structural differences of SiR-FP include a more compact spatial arrangement of FMN, FAD and NADPH than in CPR [32] and disordered Fld domain [33]. The purified SiR-FP can act as CPR in vitro, supporting the CYP17-catalysed 17\(\alpha\)-hydroxylation of pregnenolone [34].

NOS exists as a homodimer, each monomer consisting of two major domains: an N-terminal oxygenase domain, which is a haem-thiolate protein, and a C-terminal reductase domain homologous to CPR. The interdomain linker between the oxygenase and reductase domains contains a calmodulin-binding sequence. The monomers are catalytically inactive; it was shown that the ET from flavin to haem occurs exclusively between adjacent subunits in the dimer [35]:

$$\text{NADPH} \rightarrow (\text{FAD} \rightarrow \text{FMN})_{\text{chain A}} \rightarrow (\text{haem})_{\text{chain A}}$$

$$\text{NADPH} \rightarrow (\text{FAD} \rightarrow \text{FMN})_{\text{chain B}} \rightarrow (\text{haem})_{\text{chain B}}$$

Methionine synthase reductase (SPTR:O60471) catalyses the regeneration of methylcobalamin, a cofactor of methionine synthase (EC 2.1.1.13), in a reductive methylation reaction in which S-adenosylmethionine serves as a methyl donor [36].

$$\text{NADPH} \rightarrow \text{FAD} \rightarrow \text{FMN} \rightarrow \text{cobalamin} \quad (6)$$

Although the naturally occurring P450 system containing separate FNR and Fld domains is not found, it was shown that FNR and Fld from *E. coli* can serve as an ET system for microsomal P450s [37]. Kinetic studies indicate a ping–pong mechanism in which the Fld acts as a single-electron shuttle between FNR and P450 [38].

**Adrenodoxin reductase (AdR)**

AdR (InterPro:IPR000759) is a monomeric flavoenzyme that is a part of mitochondrial P450-containing mono-oxygenase systems. The IUBMB Enzyme Nomenclature Committee classifies both AdR and FNR as EC 1.18.1.2 because they apparently catalyse the same reaction. However, the direction of the reaction catalysed by FNR under physiological conditions is opposite to that catalysed by AdR:

$$\text{FNR: } [\text{Fe}_{2}\text{S}_{2}]^{2+} + \text{NADP}^+ \rightarrow [\text{Fe}_{2}\text{S}_{2}]^{3+} + \text{NADPH}$$

$$\text{AdR: } [\text{Fe}_{2}\text{S}_{2}]^{3+} + \text{NADPH} \rightarrow [\text{Fe}_{2}\text{S}_{2}]^{2+} + \text{NADP}^+$$
Also the nature of Fe₃S₆ Fds involved in the two reactions is different: with a plant-type Fd and Adx, respectively. The use of the same EC number for FNR and AdR accounts for the misnaming of AdR entries in the databanks [39].

The crystal structure of AdR has been solved (PDB: 1CJC). The enzyme consists of two domains with similar folds; one domain binds FAD and the other binds NADPH/NADP⁺. According to SCOP (Structural Classification of Proteins) [40], the two-domain structure of AdR is quite similar to the structure formed by the middle and C-domains of trimethylamine dehydrogenase (EC 1.5.99.7). This similarity suggests a distant relationship, probably on a superfamily level (A. G. Murzin, personal communication).

The physiological function of non-animal AdR homologues is enigmatic. For example, of more than 135 P450 sequences from Arabidopsis thaliana [41], none appears to be of the mitochondrial type. Nevertheless, A. thaliana contains both AdR (SPTR: O49356) and Adx (SPTR: O49551). Other organisms such as M. tuberculosis have AdR but do not have Adx. AdR homologues from Mycobacterium leprae (SPTR: O33064) and M. tuberculosis (SPTR: Q10547) contain a bacterial-type Fd domain at the N-terminus.

**Putidaredoxin reductase (PdR)**

Putidaredoxin reductase (PdR; SPTR: P16640), together with terpredoxin reductase (TdR; SPTR: P33009) and rhodocoxin reductase (RdR; SPTR: P43494), belong to the large and diverse superfamily of FAD-dependent pyridine nucleotide reductases (FADPNRs; InterPro: IPR001327). Of the three reductases, only PdR has been characterized functionally. Members of FADPNR are well presented in the archaea, bacteria and eukarya. The crystal structures available for this superfamily outnumber those known for CPR and Adx. However, given the great structural variety of the FADPNRs, e.g. in relative orientations of subdomains and locations of their active sites, one may expect quite a few surprises when the 3D structure of PdR is solved.

**Cytochrome b₅**

Cytochromes b₅ are ubiquitous electron-transport proteins/domains involved in a variety of biochemical processes (InterPro: IPR001199). The redox centre is the bis-histidinyl co-ordinated haem (Figure 1f). Until the recent discovery and crystallization of cytochrome b₅ homologue from bacterium Ectothiorhodospira vacuolata (PDB: 1CXY), the cytochrome b₅ proteins/domain were found only in the eukarya. The only other prokaryotic cytochrome b₅ domain-containing protein is M. tuberculosis hypothetical fatty acid desaturase (SPTR: P71799).

It has been long known that cytochrome b₅ can serve as an effector (activator or inhibitor) of P450s although the mechanisms of its action remained unknown. It was hypothesized that cytochrome b₅ is involved in the transfer of the second electron to P450, either from CPR or NADH:cytochrome b₅ reductase (b5R; EC 1.6.2.2) [42]:

\[
\text{NADPH} \rightarrow \text{CPR} \rightarrow \text{cytochrome } b₅ \rightarrow P450 \rightarrow O₂ \tag{7}
\]

\[
\text{NADH} \rightarrow b5R \rightarrow \text{cytochrome } b₅ \rightarrow P450 \rightarrow O₂ \tag{8}
\]

More recently, evidence appeared that holo- and apo-cytochrome b₅ effect the transfer of the first electron to CYP3A4 [42]. The full ability of the b5R/cytochrome b₅ ET system to support P450 catalysis has been demonstrated in vitro using purified Saccharomyces cerevisiae b5R and cytochrome b₅ and Candida albicans CYP51 [43]. In this system, both the first and second electrons are donated by b5R:

\[
\text{NADH} \rightarrow b5R \rightarrow \text{cytochrome } b₅ \rightarrow \text{CYP51} \rightarrow O₂ \tag{8.1}
\]

**Adrenodoxin (Adx)-type ferredoxins**

This family (InterPro: IPR001055) includes small soluble Fe₃S₆ cluster-binding (Figure 1f) proteins found in bacteria and eukarya. In mitochondrial mono-oxygenase systems, Adx functions as a soluble electron carrier between the AdR and several membrane-bound P450s [44]:

\[
\text{NADPH} \rightarrow \text{AdR} \rightarrow \text{Adx} \rightarrow \text{CYP11A1} \rightarrow O₂ \tag{9.1}
\]

\[
\text{NADPH} \rightarrow \text{AdR} \rightarrow \text{Adx} \rightarrow \text{CYP11B1} \rightarrow O₂ \tag{9.2}
\]

\[
\text{NADPH} \rightarrow \text{AdR} \rightarrow \text{Adx} \rightarrow \text{CYP11B2} \rightarrow O₂ \tag{9.3}
\]

\[
\text{NADPH} \rightarrow \text{AdR} \rightarrow \text{Adx} \rightarrow \text{CYP27} \rightarrow O₂ \tag{9.4}
\]

In bacteria, putidaredoxin (Pdx; SPTR: P00259), terpredoxin (TdX; SPTR: P33007) and rhodocoxin (Rdx; SPTR: P43493) serve as electron donors.
carriers between corresponding NADH-dependent Fd reductases and soluble P450s:

\[
\text{NADH} \rightarrow \text{PdR} \rightarrow \text{Pdx} \rightarrow \text{CYP101} \rightarrow O_2 \quad (10.1)
\]

\[
\text{NADH} \rightarrow \text{TdR} \rightarrow \text{Tdx} \rightarrow \text{CYP108} \rightarrow O_2 \quad (10.2)
\]

\[
\text{NADH} \rightarrow \text{RdR} \rightarrow \text{Rdx} \rightarrow \text{CYP116} \rightarrow O_2 \quad (10.3)
\]

The general scheme of electron flow in the P450 systems containing Adx-type Fds is:

\[
\text{NAD(P)H} \rightarrow \text{FAD} \rightarrow \text{Fe}_2\text{S}_2 \rightarrow \text{haem} \rightarrow O_2 \quad (11)
\]

The 3D structures of Adx (PDB:1AYF), Pdx (PDB:1PUT; PDB:1PDX) and Tdx (PDB:1B9R) have been solved. Despite low sequence similarity between Adx-type and plant-type Fds (InterPro: IPR000564), the two classes have similar folding topologies. The crystal structure of full-length oxidized bovine Adx suggests that Adx exists as a dimer \textit{in vivo} [45]. Computer modelling was used to predict an ET pathway between two Fe$_2$S$_2$ clusters in the dimer. If there is sufficient coupling between clusters, the electron flow in the Adx-containing system will be:

\[
\text{NADPH} \rightarrow \text{FAD} \rightarrow \text{Fe}_2\text{S}_2 \rightarrow \text{Fe}_2\text{S}_2 \rightarrow \text{haem} \rightarrow O_2 \quad (12)
\]

In the recently determined crystal structure of the chemically cross-linked AdR-Adx complex, the Fe$_2$S$_2$ cluster and the isoalloxazine ring of FAD are 10 Å apart, which suggests electron tunneling between the redox centres [46].

**Bacterial-type ferredoxins**

Bacterial-type Fds (InterPro: IPR001450) are Fe$_2$S$_2$/Fe$_3$S$_4$ cluster-binding proteins (Figures 1g and 1h) widely distributed in bacteria and archaea. They function as electron donors in a variety of enzyme systems and indeed are the most probable components of the 'primitive' P450 systems. Typical bacterial-type Fds are either mono- or dicluster, but some organisms, such as \textit{Methanococcus jannaschii}, have several polyferredoxin genes; e.g. SPTR:Q58593 may contain up to 10 Fe$_2$S$_2$ clusters.

Fe$_3$S$_4$ Fds (Fe$_3$s; InterPro: IPR001080) constitute the second family of iron-sulphur proteins presumably involved in ET from the FAD domain to P450. Of these proteins, \textit{Streptomyces griseolus} Fe$_3$s (SPTR:P18324 and SPTR:P18325) were isolated and spectroscopically characterized while Fe$^3$ from \textit{Streptomyces clavuligerus} (SPTR:Q9KJ92) was shown to be required for clavulanic acid biosynthesis. \textit{B. subtilis} genome contains the single Fd gene, \textit{fer} (SPTR:P50727). Its physiological role is unknown but it was shown that Fer protein can pass electrons from \textit{E. coli} FNR (SPTR:P28861) to the \textit{B. subtilis} CYP107H (SPTR:P53554) and has an EPR spectrum consistent with Fe$_3$S$_4$ cluster structure (A. W. Munro, personal communication). Other Fe$_3$s associated with P450 operons (SPTR:P26910, SPTR:P46374, SPTR:Q45218, SPTR:Q9XDA9 and SPTR:P71820) were never purified or characterized in any way so we should be careful in assigning their function.

**Early P450 systems?**

There exists a widely accepted opinion that the archaea, at least so-called extremophiles, are the most ancient organisms on Earth [47,48]. The assumption that the archaea have changed very little since pre-oxygen conditions, together with the availability of several complete genomes, makes the archaea attractive for speculations on the origin and early evolution of ET chains [49].

To date, there are only two known archaeal P450s: \textit{Sulfolobus solfataricus} CYP119 (SPTR:Q55080), which is a typical B-class P450, and \textit{Halobacterium} sp. CYP174A1 (SPTR:Q9HS37), more similar to E-class P450s. This suggests at least two different P450 systems in the archaea. CYP119 is very slowly reduced by Pdx and PdR [50], which may indicate that it has very different physiological partners.

No archaeal CPR homologues have been found. As for the structural domains of CPR, the only FNR homologue is the \textit{g}-subunit of cytochrome $c_6$ hydrogenase from \textit{Pyrococcus abyssi} (SPTR:Q9V045) and the only Fld homologues are the hypothetical proteins from \textit{Methanococcus thermoautotrophicum} (SPTR:O26935; SPTR:O27827) and \textit{M. jannaschii} (SPTR:Q57954). The FADPNR-like proteins and bacterial-type Fds are found in all archaeal genomes, while \textit{Aeropyrum pernix}, \textit{Pyrococcus horikoshii} and \textit{Pyrococcus abyssi} contain AdR homologues. Therefore, the most plausible ET chain for archael P450 system is:

\[
\text{NAD(P)H} \rightarrow \text{FAD} \rightarrow \text{Fe}_3\text{S}_4 \rightarrow \text{haem} \rightarrow \text{acceptor} \quad (13)
\]

\[
\text{NAD(P)H} \rightarrow \text{FAD} \rightarrow \text{Fe}_3\text{S}_4 \rightarrow \text{haem} \rightarrow \text{acceptor} \quad (14)
\]
Adx-type Fds were not found in archaea. Alternatively, other Fe₃S₄ Fds, viz. plant-type and Rieske-type (InterPro: IPR001281), although not known as physiological components of P450 systems, may serve as redox domains in archaeal P450 systems.

Conclusion

The evolution of P450-containing mono-oxygenase systems can be described as the recruitment of various non-homologous redox proteins/domains typical for a given organism. The proteins/domains that directly interact with P450 (intermediate domains) exhibit the diversity of BIMs while their reductases all contain FAD as a redox centre. The early (non-mono-oxygenase) P450s must have been self-sufficient oxidoreductases; later in their evolution, some P450s may have lost the need for redox partners.

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14. Reference deleted
Control of electron transfer in neuronal NO synthase


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Abstract

The nitric oxide synthases (NOSs) are dimeric flavocytochromes consisting of an oxygenase domain with cytochrome P450-like Cys-ligated haem, coupled to a diflavin reductase domain, which is related to cytochrome P450 reductase. The NOSs catalyse the sequential mono-oxygenation of arginine to N-hydroxyarginine and then to citrulline and NO. The constitutive NOS isoforms (cNOSs) are regulated by calmodulin (CaM), which binds at elevated concentrations of free Ca\textsuperscript{2+}, whereas the inducible isoform binds CaM irreversibly. One of the main structural differences between the constitutive and inducible isoforms is an insert of 40–50 amino acids in the FMN-binding domain of the cNOSs. Deletion of the insert in rat neuronal NOS (nNOS) led to a mutant enzyme which binds CaM at lower Ca\textsuperscript{2+} concentrations and which retains activity in the absence of CaM. In order to resolve the mechanism of action of CaM activation we determined reduction potentials for the FMN and FAD cofactors of rat nNOS in the presence and absence of CaM using a recombinant form of the reductase domain. The results indicate that CaM binding does not modulate the reduction potentials of the flavins, but appears to control electron transfer primarily via a large structural rearrangement. We also report the creation of chimaeric enzymes in which the reductase domains of nNOS and flavocytochrome P450 BM3 (Bacillus megaterium III) have been exchanged. Despite its very different flavin redox potentials, the BM3 reductase domain was able to support low levels of CaM-dependent NO synthesis, whereas the NOS reductase domain did not effectively substitute for that of cytochrome P450 BM3.

Introduction

Nitric oxide synthases (NOSs) are dimeric flavocytochromes which catalyse the synthesis of NO and citrulline from arginine in two consecutive mono-oxygenation steps [1,2]. There are three main mammalian isoforms which are expressed in specific cell types: neuronal (nNOS), endothelial (eNOS) and macrophage or inducible (iNOS). The NO generated in these cells is involved, respectively, in neurotransmission, regulation of blood flow/pressure and immune response. nNOS and eNOS generate NO to act as a signalling molecule, targeting guanylate cyclase, whereas iNOS produces large amounts of NO to attack proteins and DNA either directly or as peroxynitrite, following combination with superoxide. The three isoforms can also be categorized according to their amino acid sequences and regulation mechanisms [3]. The constitutive NOSs (nNOS and eNOS) are regulated by the reversible binding of calmodulin (CaM) through changes in the intracellular Ca\textsuperscript{2+} concentration. iNOS, however, is expressed with CaM bound permanently and remains active in the absence of free Ca\textsuperscript{2+} [4]. The CaM-binding site lies within a linker region between the N-terminal oxygenase and