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Minireview

Molecular evolution of P450 superfamily and P450-containing monooxygenase systems

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This paper reviews the classification of the P450 superfamily which is mainly based on sequence homology. The widely accepted classification by Nebert et al. [(1991) DNA Cell Biol. 10, 1–14] as well as the results of a 'two-step' multiple sequence alignment technique show that the molecular evolution of P450s, in contrast to that of many protein families, does not follow phylogeny. The data suggest that during the evolution of P450s, gene duplications and gene fusions, horizontal gene transfer and intron loss events have occurred. 'Weak' and 'strong' hierarchies in the clustering of P450 sequences were revealed. A novel evolutionary tree of the P450 superfamily has been constructed using a multiple alignment of consensus sequences. The simple classification of known P450-containing monooxygenase systems into three-, two- and one-component systems is further discussed. Particularly, the multidomain enzyme, nitric oxide synthase (NOS), should be classified as an example of a eukaryotic one-component P450 system since its N-terminal (haem) domain exhibits similarity with microsomal P450s.

Multiple sequence alignment; Hierarchy; Consensus sequence; Evolutionary tree; Nitric oxide synthase; P450-containing monooxygenase system

1. INTRODUCTION

The P450 enzymes are a large superfamily of haemoprotein monooxygenases in prokaryotes and eukaryotes. These enzymes play an important role in the oxidative metabolism of a wide variety of both exogenous and endogenous substrates. At present, more than 230 distinct P450 genes and pseudogenes have been identified. The nomenclature system, based on evolution of the P450 superfamily, was proposed and developed by Nebert and co-workers [1–4]. According to the last update [4], this superfamily comprises 36 gene families, of which 10 include subfamilies, and 26 are represented by only one gene. In addition to the amino acid sequence homology, gene and genome structure data were used to model their evolution. The subdivision of P450 into families, subfamilies, orthologues and allelic variants is arbitrary if it is based only on sequence homology [3,5].

The existing definitions of sequences similarity are ambiguous. Sequence *identity* has been used as a quantitative criterion for P450 classification [3,6] but a significant spread in the cluster boundary values (up to 10%) [4] disqualifies this parameter. Moreover, for such a comparison, sequences should be previously aligned; none of the existing algorithms for multiple alignment is guaranteed to give an optimal solution. Therefore, different alignments for the same set of sequences could give different identity values. Sequence similarity can be increased by using different substitution matrices. *Evolutionary* or *structural* similarity [7] may contain considerably more information than identity. One could choose a scoring matrix both for performing multiple alignments and sequence clustering, that in its turn results in different values of similarity.

2. MULTIPLE ALIGNMENTS AND CLASSIFICATION OF P450 SEQUENCES

Multiple sequence alignments of the P450 proteins have been constructed using various methods [5,6,8–13]. The comparison of dendrograms for representative members of P450 superfamily obtained by different authors [5,11,14] shows that their branching topologies are similar. Almost all prokaryotic P450s and fungal CYP55 form one major class, 'B-class', and all eukaryotic P450s and bacterial CYP102 belong to the other major class, 'E-class' (we have adopted these terms from [5]). The eukaryotic P450 families always form such stable clusters as {CYP1, CYP2, CYP17, CYP21}, {CYP3, CYP4, CYP6, CYP52, CYP102}, and {CYP11, CYP27}. CYP7 and CYP19 are the most distant gene families in the E-class. CYP2, the most expansive P450 family, can, in all cases, be divided into five clusters of subfamilies: {CYP2A, CYP2B, CYP2G}, {CYP2C, CYP2E, CYP2H}, {CYP2D}, {CYP2F}, and

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Abbreviations: CYP, P450 gene symbol; CYP, corresponding P450 protein symbol; NOS, nitric oxide synthase.

{CYP2J}. CYP2D and CYP2J are the most distant subfamilies in CYP2 family. The trout CYP1A1 always forms the most outlying branch in its family. Among B-class P450s, CYP104 is the most remote sequence in all cases. P450s from *Streptomyces* (CYP105 and CYP107) and fungal CYP55 are always clustered. Nevertheless, there are some differences in branching topologies of the trees, but these details need not be described here. We will only dwell on two observations with regard to possible causes of these differences.

The first one concerns the traditionally used multiple alignment techniques. In a progressive sequence alignment method [15], the correct tree is not only an aim but also a tool for the construction of multiple alignment. Hence, different tree topologies lead to different alignments. Thus, the problem of choosing the 'best' scoring matrix can be divided into two different problems of uncertain priority: the choice of a scoring matrix for the construction of the optimal alignment and the one for obtaining the most correct clustering.

The other observation concerns the division of hierarchies in P450 superfamily into 'weak' and 'strong' [16]. Indeed, the composition of stable clusters described above is largely insensitive to any changes in the sequence arrangement, as well as to the multiple alignment method and replacement of one substitution matrix by another. This is evidently a case of strong hierarchy, when the intersection of any two clusters either coincides with one of them or is empty. On the other hand, weak hierarchy allows the interaction of any three clusters to fit an intersection of any two of them [17]. Small differences in lengths of ancient branches can lead to different hierarchies on the same sequence set. Indeed, the relative positions of five major branches in the E-class, together with those of most families in the B-class, are extremely variable, depending on the algorithm used for multiple alignment and tree building [5,11,14]. In other words, these clusters form a weak hierarchy. Another case of a weak hierarchy can be observed *within* any stable P450 group where the order of sequence clustering also varies significantly.

3. 'TWO-STEP' MULTIPLE ALIGNMENTS

The strong hierarchy of clustering into stable groups and the weak hierarchy of clustering patterns within these groups suggest that all distances between any member of (sub)family P and any member of (sub)family Q are approximately the same and should correspond to the distance between the putative ancestors of P and Q. Although the structure of these ancestral sequences is unknown, it is possible to construct consensus sequences for each cluster [18] and calculate distances between them. According to the neutral theory of molecular evolution [19], only a few amino acid residues in the key regions of a protein molecule are highly conserved because they are functionally important. Thus, the rate of protein evolution is limited by the number of conserved regions of a molecule, i.e. it can be determined as the rate of consensus sequence evolution. That is why a two-step multiple alignment procedure can be performed after preliminary clustering. The result of such a two-step technique combines the equal representation of each P450 subfamily in the final multiple alignment with the preferential comparison of conserved regions and the reduction of random matches between subfamilies. The choice of a scoring matrix or a gap penalty does not dramatically affect the alignment within a single subfamily.

Gotoh [5] has performed a multiple sequence alignment within each family or group of related families as the first step. As the second step, the groups of sequences were aligned. In addition, predicted secondary structure and hydropathy indices were used for obtaining a similarity score between the groups.

Analogously, we have used a two-step multiple alignment procedure [20]. First, the sequences were aligned within each subfamily (family) using a standard progressive multiple alignment method [21]. The corresponding consensus sequences were built up for each of these multiple alignments. Second, the obtained consensus sequences and those sequences which are the only representatives of their subfamilies (families) were then aligned together.

The use of consensus sequences both speeds up the multiple alignment procedure and facilitates the perception of results. Indeed, instead of 178 full-length P450 sequences only 26 consensus sequences and 30 unique sequences were aligned.

The general topology of the obtained dendrogram

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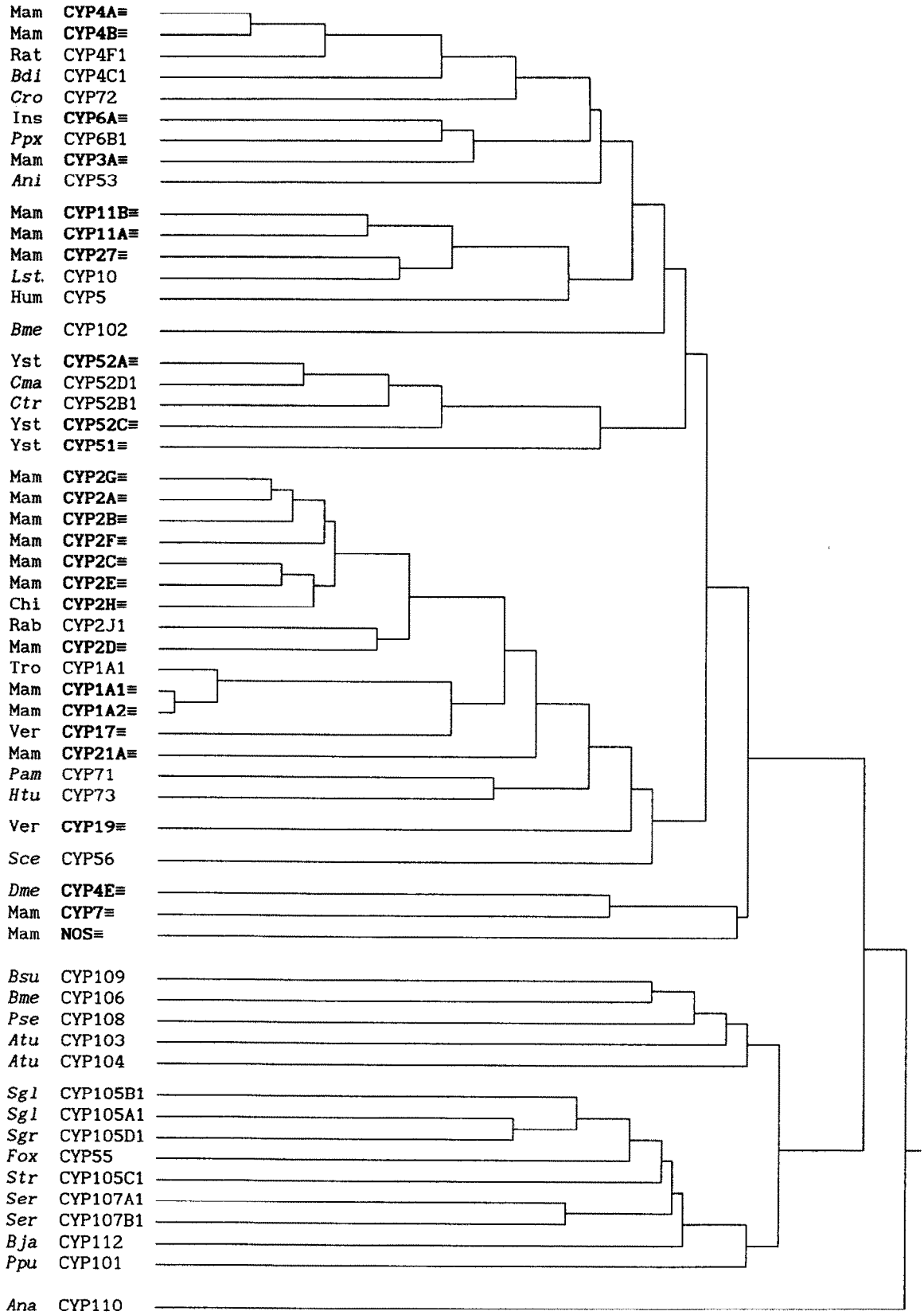
Fig. 1. Dendrogram for the P450 superfamily obtained with a multiple alignment of 26 consensus sequences and 30 unique sequences by the MULTALIN program [14]. Consensus sequences are indicated by ≡. Systematic names of P450s correspond to the last nomenclature [4]. NOS≡ identifies the consensus sequence for nitric oxide synthases. Ins, insects; Mam, Mammalia; Ver, Vertebrata; Yst, yeasts; Chi, chicken; Hum, human; Rab, rabbit; Rat, rat; Tro, trout; *Bdi, Blaberus discoidalis* (cockroach); *Dme, Drosophila melanogaster* (fruit fly); *Ppx, Papilio polyxenes* (butterfly); *Lst, Lymnea stagnalis* (pond snail); *Cro, Catharanthus roseus* (Madagascar periwinkle); *Htu, Helianthus tuberosus* (Jerusalem artichoke); *Pam, Persea americana* (avocado); *Ani, Aspergillus niger*; *Cma, Candida maltosa*; *Ctr, Candida tropicalis*; *Fox, Fusarium oxysporum*; *Sce, Saccharomyces cerevisiae*; *Ana, Anabaena* spp.; *Atu, Agrobacterium tumefaciens*; *Bja, Bradyrhizobium japonicum*; *Bme, Bacillus megaterium*; *Bsu, Bacillus subtilis*; *Ppu, Pseudomonas putida*; *Pse, Pseudomonas* spp.; *Ser, Saccharopolyspora erythraea*; *Sgl, Streptomyces griseolus*; *Sgr, Streptomyces griseus*; *Str, Streptomyces* spp.


Mam CYP4A≡
Mam CYP4B≡
Rat CYP4F1
Bdi CYP4C1
Cro CYP72
Ins CYP6A≡
Ppx CYP6B1
Mam CYP3A≡
Ani CYP53
Mam CYP11B≡
Mam CYP11A≡
Mam CYP27≡
Lst. CYP10
Hum CYP5
Bme CYP102
Yst CYP52A≡
Cma CYP52D1
Ctr CYP52B1
Yst CYP52C≡
Yst CYP51≡
Mam CYP2G≡
Mam CYP2A≡
Mam CYP2B≡
Mam CYP2F≡
Mam CYP2C≡
Mam CYP2E≡
Chi CYP2H≡
Rab CYP2J1
Mam CYP2D≡
Tro CYP1A1
Mam CYP1A1≡
Mam CYP1A2≡
Ver CYP17≡
Mam CYP21A≡
Pam CYP71
Htu CYP73
Ver CYP19≡
Sce CYP56
Dme CYP4E≡
Mam CYP7≡
Mam NOS≡
Bsu CYP109
Bme CYP106
Pse CYP108
Atu CYP103
Atu CYP104
Sgl CYP105B1
Sgl CYP105A1
Sgr CYP105D1
Fox CYP55
Str CYP105C1
Ser CYP107A1
Ser CYP107B1
Bja CYP112
Ppu CYP101
Ana CYP110

(Fig. 1) strongly differs from the other published trees of the P450 superfamily [3,5,11,14]. Surprisingly, in this case two yeast families, CYP51 and CYP52, were clustered. It is confusing that the consensus of three *Drosophila* sequences classified previously in the CYP4E subfamily [4] does not form a cluster with the other representatives of the CYP4 family but is most similar to CYP7. In contrast to [5], cyanobacterial CYP110 is not included in the E-class but appears to be the most distant sequence in the present P450 superfamily. Two plant P450s, CYP71 and CYP73, are related to the {CYP1, CYP2, CYP17, CYP21} cluster, whereas CYP72 is close to the CYP4 family cluster.

A loss of information inevitably happens during the first step, i.e. the formation of consensus sequences or profiles for groups. However, such a loss of information might also take place in an evolutionary process. It is known that secondary and tertiary structures are more conserved than the primary structure, reflecting a certain degeneracy of the protein structural code [22]. Indeed, comparative studies of the predicted secondary structures of P450 proteins have shown strong conservation of several structurally important patterns of $P450_{cam}$ (CYP101) in all P450s [5,10,12,23]. Hence, fragments of the extant sequences also correspond to the fragments of the ancestral sequences, and it is possible to use the consensus sequence as a putative ancestor for evolutionary modelling. More than half of the sequences used for the second phase of the two-step alignment are unique P450s. It is not possible to consider only conserved fragments of these sequences. Obviously, new sequence information can result in a significant change in our notion of molecular evolution of P450s.

4. COMPLEXITY OF EVOLUTION OF P450 GENES

The evolution of P450s is not restricted to phylogeny, although phylogenetic analysis can be applied to several groups of orthologous genes, such as *CYP1A1, 1A2, 2E1, 7, 11A1, 11B1, 17, 19, 21A1,* and *27*. Some P450 genes, for example the mammalian *CYP1A1* and *CYP1A2*, or the rat *CYP2D1, 2D2, 2D3, 2D4*, and *2D5* genes, seemingly have appeared via gene duplication events [3] and should be considered paralogous. In such subfamilies as *CYP2A, 2B, 2C, 2D, 3A*, and *4A*, which presumably have arisen through numerous species-specific gene duplication and conversion events, the orthologue assignments are impossible [3]. This kind of evolution can be concerned with intra-specific specialization. Kizawa et al. [24] hypothesize that a *Fusarium oxysporum* $P450_{dNIR}$ gene (*CYP55*) is a xenologous gene of prokaryotic origin. 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. Finally, convergent evolution cannot be ignored either, e.g. for membrane-binding anchors or for substrate-binding sites of enzymes from the different families but with a similar substrate specificity. That is why *any* dendrogram of the whole P450 superfamily cannot be considered a phylogenetic tree.

The global phylogenetic tree of living organisms [25] indicates that the major prokaryotic groups are almost as diverged from each other as they are from eukaryotes. In most bacteria the function of P450 is the oxidative utilization of carbon sources. Some mycoplasma genera do not contain cytochromes at all [26] yet contain P450 [27]. Thus, the P450 system can also be considered as one of the most ancient respiratory systems. This indicates that P450s are, in many respects, universal molecules. To date, however, P450s have been isolated only from a few prokaryotic genera. For instance, the most excessively studied bacterial genome, *Escherichia coli*, is not known to contain any P450 gene. Moreover, P450 sequences are known only for 6 of 23 major groups of organisms [25], namely flavobacteria (*Agrobacterium, Pseudomonas*), Gram-positive bacteria (*Bacillus, Streptomyces*), cyanobacteria (*Anabaena*), fungi (yeasts, *Aspergillus, Fusarium*), plants, and animals. Nothing is known about the P450 genes in archaea, although this kingdom is likely to contain P450 systems [28]. It is certain that, without further information about P450s in different prokaryotes, the evolution of the P450 gene superfamily will remain largely unclear.

It is interesting that the cluster {CYP3, CYP4, CYP6, CYP53, CYP72} (Fig. 1) includes P450s from various origins, such as mammals, insects, fungi, and plants. (According to [5], this cluster should comprise yeast CYP52, bacterial CYP102 and cyanobacterial CYP110 families as well.) Conversely, {CYP19} and {CYP7} clusters are represented only by vertebrate sequences. Cluster {CYP1, CYP2, CYP17, CYP21} also contains only vertebrate P450s, although plant CYP71 and CYP73 border with this cluster. The {CYP51, CYP52} cluster contains only yeast P450s. In addition, *CYP4* genes have the maximal known number of introns among the eukaryotic P450 genes: from 10 in *CYP4B1* [29] to 12 in *CYP4A1* [30]. A selective loss of introns during evolution may be a common occurrence [28,31]. The ancestors of prokaryotes and eukaryotes have evidently had introns which were lost by most of the prokaryotes and lower eukaryotes [32]. Therefore, we suppose that the *CYP4* genes preserve the most 'archaic' structure among known eukaryotic P450 genes, and that the putative ancestor of the group {CYP3, CYP4, CYP6, CYP53, CYP72} should resemble the ancestor of all P450s of the E-class as well. In contrast, the *CYP1A* genes have retained only six introns and therefore appear to be the most recent family among the known vertebrate P450s.

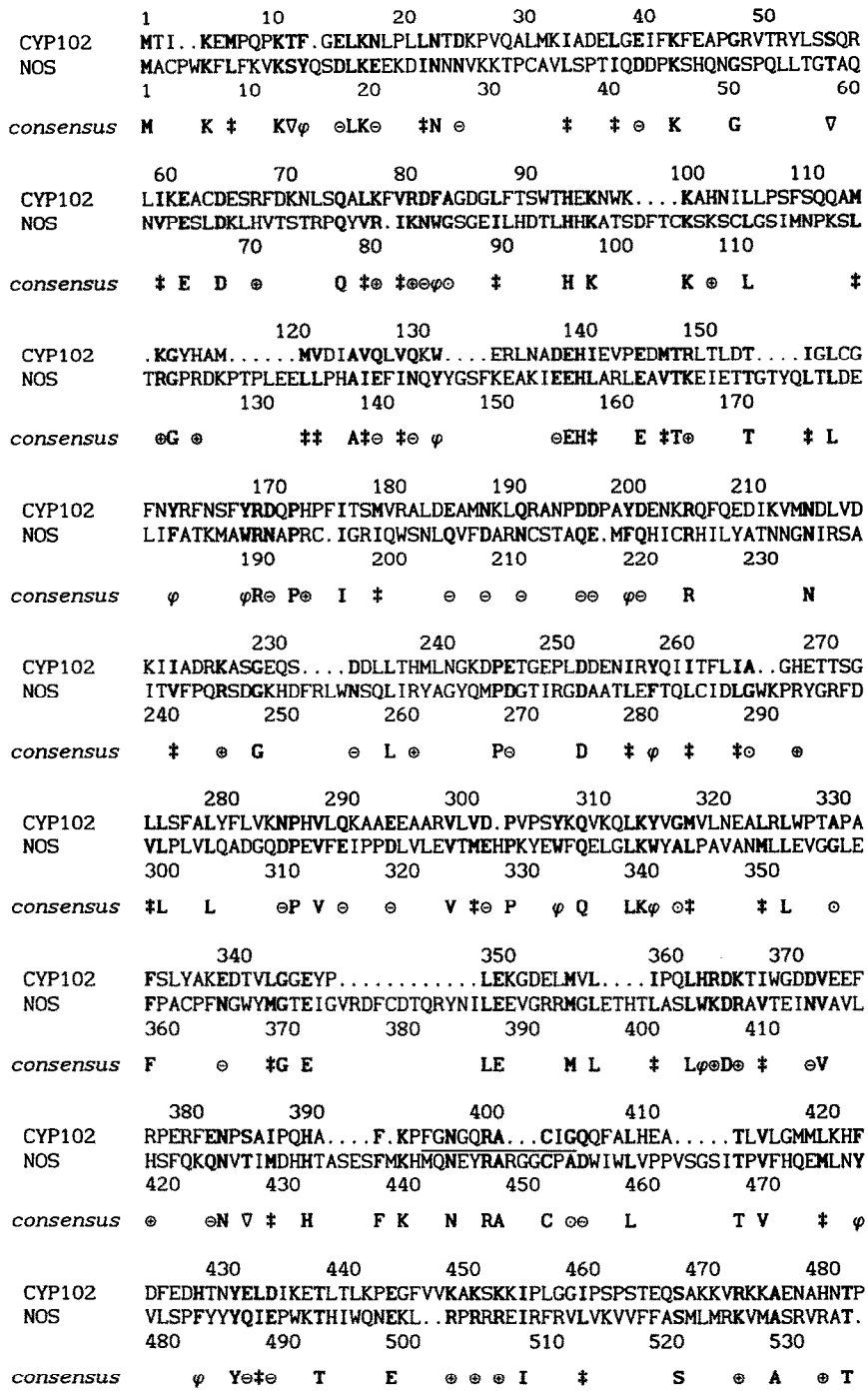


Fig. 2. Comparison of N-terminal domains of *Bacillus megaterium* $P450_{BM-3}$ (CYP102) and murine nitric oxide synthase (NOS). The alignment and consensus sequence were constructed using the program, MULTALIN [14]. The special characters designating groups of similar amino acids were used: ⊖ (E,D,N,Q); ⊕ (H,K,R); φ (F,H,W,Y); ‡ (I,L,M,V); ⊙ (A,G); ∇ (S,T). The haem-binding motif sequence of CYP102 is underlined. CYP102 and NOS are 13.9% identical.

5. EVOLUTION OF P450-CONTAINING MONOOXYGENASE SYSTEMS

Recent studies show that an inducible form of murine nitric oxide synthase (NOS) contains FAD, FMN and haem, and suggest that this is the first catalytically self-sufficient mammalian P450 system [33]. The C-terminal domain of NOS is clearly homologous to NADPH-P450 reductase [34], whereas its N-terminal domain has only a weak homology with P450 sequences. Nelson et al. [4] have aligned the N-terminal domain of NOS and 57 P450s from different subfamilies and concluded that

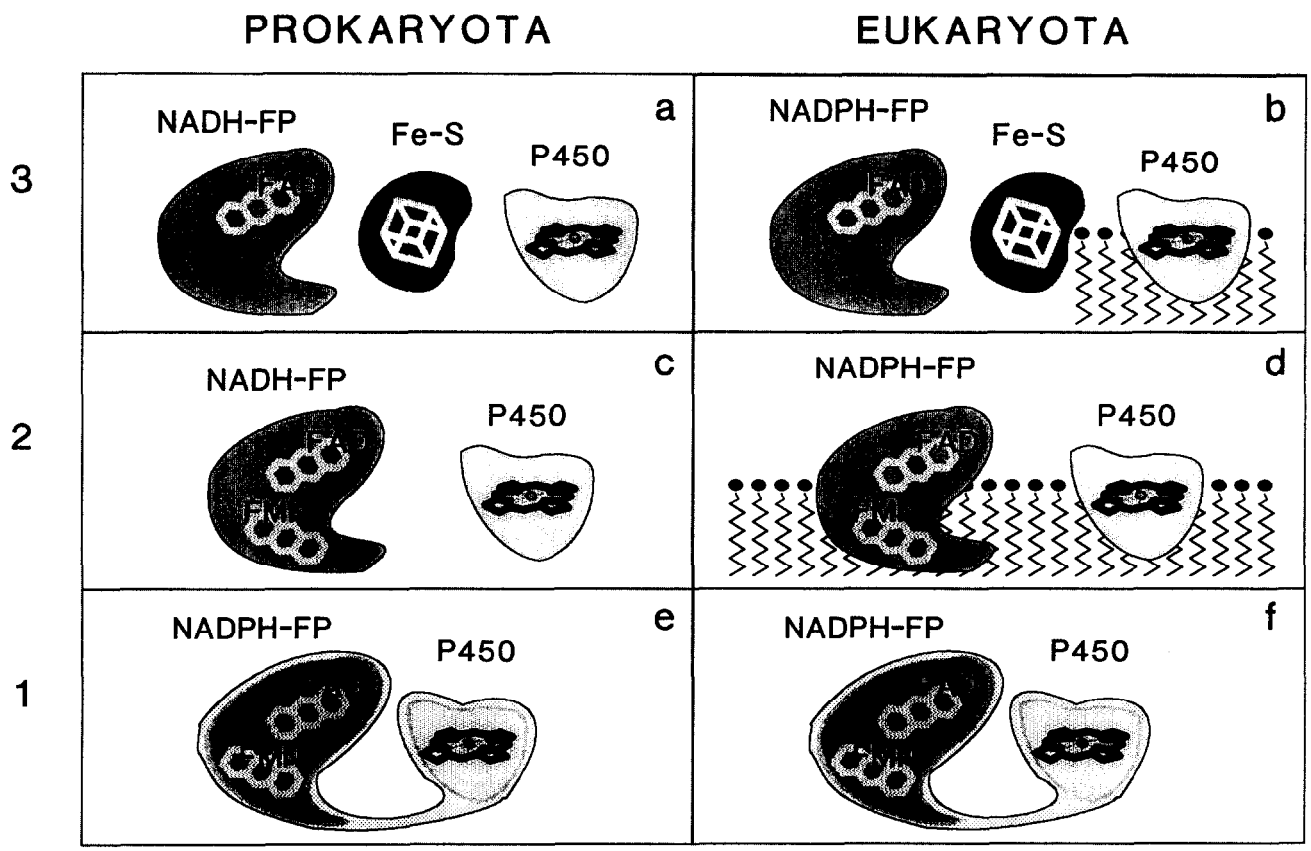


Fig. 3. Classification of P450-containing monooxygenase systems. (a) Bacterial three-component system (*Pseudomonas putida*); (b) mitochondrial three-component system; (c) bacterial two-component P450 monooxygenase system (*Streptomyces carbophilus*); (d) microsomal two-component P450 monooxygenase system; (e) bacterial one-component P450 monooxygenase system (*Bacillus megaterium* $P450_{BM-3}$); (f) soluble one-component P450-like system (nitric oxide synthase). NADH-FP and NADPH-FP, NADH- and NADPH-dependent flavoproteins, respectively; Fe-S, an iron–sulfur protein.

NOS is an example of convergent evolution. On the other hand, the local similarity between the N-terminal domains of NOS and $P450_{BM-3}$ can be revealed in spite of the absence of the characteristic haem-binding motif in the NOS sequence (Fig. 2).

There exist a widespread division of P450-containing monooxygenase systems into two main types, microsomal and bacterial/mitochondrial [35]. On the other hand, a principal classification of all the P450-containing systems known can be made using the number of their protein components where the NOS system falls into a corresponding place (Fig. 3).

Mitochondrial and most of the bacterial P450 systems have three components, an FAD-containing flavoprotein (NADPH or NADH-dependent reductase), an iron–sulfur protein, and P450. The eukaryotic microsomal P450 system contains only two components, a flavoprotein, containing both FAD and FMN (NADPH-dependent P450 reductase), and P450. A prokaryotic two-component P450 monooxygenase system from *Streptomyces carbophilus* has been recently described [36]. This system is composed of $P450_{sca}$ haemoprotein and NADH-dependent P450 reductase containing both FAD and FMN. Finally, a soluble monooxygenase $P450_{BM-3}$ (CYP102) from *Bacillus megaterium* exists as a single polypeptide chain with two functional parts, the haem and flavin domains. It represents a unique bacterial one-component system [37]. However, the 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 [38]. In the context of this classification, NOS could be viewed as a eukaryotic one-component P450 system.

The present scheme does not cover cytochrome b_5-containing redox pathways, although it has been known that cytochrome b_5 can serve as an effector or electron donor for P450s [39].

Thus, all these diverse systems share a common 'redox domain' architecture. It has been suggested that the multidomain enzyme, NADPH-P450 reductase, arose via a fusion of the ancestral genes of flavodoxin, which is homologous to the FMN domain, and ferredoxin-$NADP^+$ reductase, which is homologous to the NADPH and FAD domains [40]. The known P450 sys-

tems always contain a NADH- or NADPH-binding domain, a FAD-binding domain, an iron–sulfur protein or a FMN-binding domain, and a P450 protein (haem domain). All the P450s are obviously homologous whereas the Fe-S (ferredoxin-like) and FMN-binding domains (flavodoxin-like) are examples of functional analogy. Thus, an ancestor of the two-component P450 monooxygenase system might contain at least three different proteins. Similarly, fusion of the two ancestral genes, encoding P450 and NADPH-P450 reductase, could have resulted in the appearance of one-component system. Hence, $P450_{BM\text{-}3}$-like enzymes should be considered as the evolutionarily most 'advanced' P450 monooxygenase system. It is easy to imagine the mechanism of gene fusion when the ancestral genes are adjacent [41]. For instance, in *Pseudomonas* spp., the genes encoding P450, putidaredoxin reductase and putidaredoxin are adjacent [42,43].

Fusion of adjacent genes for monofunctional proteins is analogous to the loss of introns. Nebert et al. [28] have proposed that an early ancestral P450 gene could have contained more than 100 mini-exons which were then fused. Their study has provided evidence for at least 33 exons in an ancestor of eukaryotic P450 genes. Porter et al. [44] have revealed the correlation between exons and structural domains of NADPH-P450 reductase and hypothesized that a selective loss of introns has occurred in this gene as well.

Similarly, the evolution of multicomponent systems resembles the evolution of multidomain proteins. For instance, within a certain type of P450 monooxygenase systems, the reductases represent relatively conserved components whereas P450s are highly variable components. It is clear that the evolutionary rates for reductase and haem domains in one-component systems should be studied independently.

Unfortunately, the P450 sequences from prokaryotic two-component systems are not available, and only a single sequence of a prokaryotic one component-type P450 system has been published. The proposed evolutionary relationship of P450 and NOS systems is questionable. Comparative study of the known P450 sequences shows that there are also major diverged groups within each of remaining three types, especially within eukaryotic two-component P450 systems. Obviously, this latter case is an example of evolution of the P450 component alone, since different microsomal P450s interact with a universal reductase.

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