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Biocatalysis and substrate chemodiversity: Adaptation of aerobic living organisms to their chemical environment☆

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ABSTRACT

A unique family of proteins, the cytochromes P450, catalyze the oxidation of almost all the compounds of our environment, often called xenobiotics. They play a key role in the adaptation of aerobic living organisms to their always changing chemical environment. How a single family of catalysts can operate in a relatively efficient manner on such extremely diverse substrates? Recent X-ray structures of mammalian xenobiotic-metabolizing P450s (mainly from human liver) that have been published during the 2003–2007 period, allow one to begin to answer this question. These data have shown the great diversity of sizes, shapes, and modes of binding of the substrate binding sites of these mammalian cytochromes P450. They have also shown how conformationally flexible are these active sites, that can adapt themselves to the xenobiotic structure for the best possible efficacy of substrate oxidation catalysis.

1. Introduction

Aerobic living organisms are able to adapt themselves to always changing chemical environments. This adaptation is mainly due to their ability to metabolize and to eliminate the chemicals that are present in their environment (often called xenobiotics). The metabolic pathways that have been selected by aerobic living organisms, as different as mammals, plants or microorganisms, to biotransform and eliminate xenobiotics, are strikingly similar. They all involve two steps (Fig. 1), a functionalization step, that most often results in a monooxygenation of the xenobiotic RH, and a conjugation step that transforms the ROH primary metabolite into a more water-soluble metabolite ROR′ in which R′ is a highly polar residue [1]. The polar and water-soluble final metabolites ROR′ are much more easily eliminated than the parent xenobiotic RH which is very often too hydrophobic to pass the renal barrier in mammals. The functionalization step is most often necessary because many xenobiotics are hydrophobic in nature and sometimes chemically inert. In all aerobic living organisms, this first step is very often catalyzed by cytochrome P450-dependent monooxygenases [2]. These enzymes have the same cofactor that is responsible for O_2 binding and activation, iron protoporphyrin IX, which is bound to the protein via an axial cysteinate ligand (Fig. 2). Moreover, they have in common the mechanism of transfer of an oxygen atom from O_2 to substrates, after consumption of two electrons and two protons, according to the monooxygenase equation (Fig. 2). Cytochrome P450-dependent monooxygenases are widely distributed in aerobic living organisms [2]. Some of them catalyze many oxidative steps involved in the biosynthesis and biodegradation of endogenous molecules that are crucial for the physiology of these organisms. In mammals, they are involved in the biosynthesis of steroid hormones and bile acids from cholesterol, and of some vitamins such as vitamin D. In plants, they play a key role in the biosynthesis of plant mediators such as glucosinolates, flavonoids and alkaloids. In microorganisms, they are involved in the metabolism of terpenes and in the biosynthesis of antibiotics [2]. All the cytochromes P450 responsible for the biosynthesis of important endogenous mediators are substrate specific and highly regulated. At the opposite, the cytochromes P450 involved in the metabolism of xenobiotics (chemicals, such as drugs, not normally found in the body) exhibit a weak substrate specificity (Fig. 2). They play a central role in the adaptation of aerobic living organisms to all the chemicals that are present in their environment. Because of the great number and extreme molecular diversity of these chemicals, those enzymes must have a weak substrate specificity. How can they catalyze the monooxygenation of so diverse substrates in an efficient manner? Some recent data based on the first X-ray structures of mammalian P450-substrate complexes, that have been published between 2003 and 2007, allow one to give a first answer to this question.

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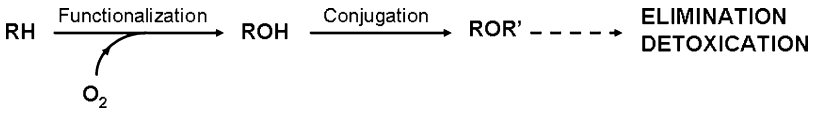

Fig. 1. General pathway used by aerobic living organisms to metabolize xenobiotics in order to eliminate them. R′ is a polar, water-soluble residue introduced into xenobiotic RH in order to facilitate its elimination.

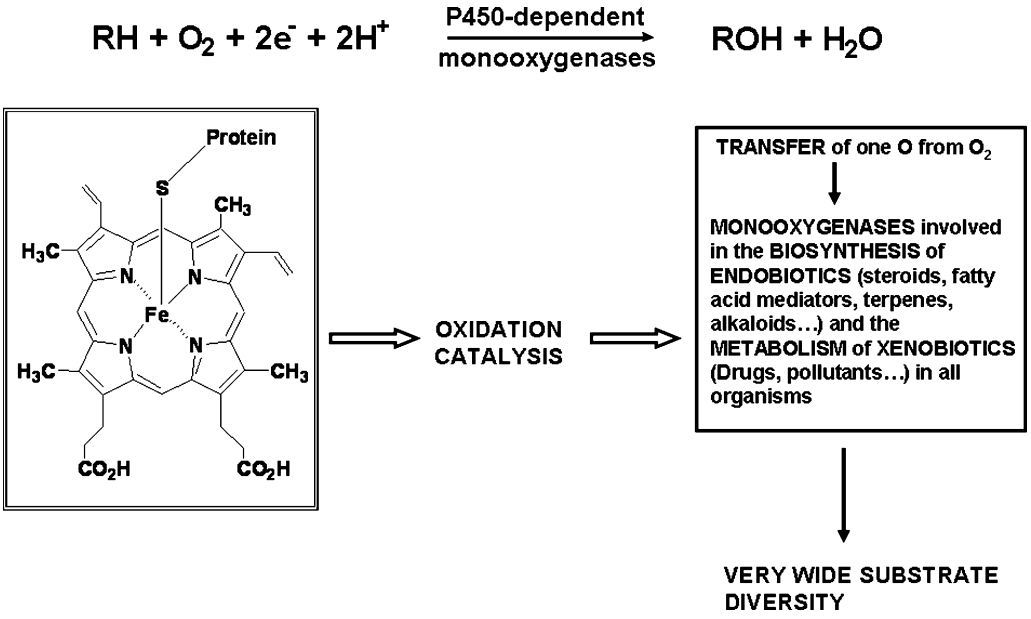

Fig. 2. General presentation of the catalytic site and the diverse reactions catalyzed by cytochrome P450-dependent monooxygenases.

2. Catalytic cycle of dioxygen activation and substrate oxidation by cytochrome P450-dependent monooxygenases

The catalytic cycle of dioxygen activation and substrate oxidation shown in Fig. 3 is common to all cytochrome P450-dependent monooxygenases. It involves three successive intermediates after the binding of O_2 to P450 Fe(II). These intermediates, Fe(III)–O–O^-, Fe(III)–OOH, and a high-valent iron-oxo species (presumably a porphyrin radical cation Fe(IV)=O species), are generated upon successive transfer of one electron and two protons to the Fe(II)O_2 intermediate [3]. The species that is most often responsible for substrate monooxygenation is the high-valent iron-oxo intermediate. It is the only intermediate of the catalytic cycle sufficiently oxidizing to hydroxylate the inert C–H bonds of alkanes.

The stoichiometry of the reactions corresponding to the monooxygenase equation (see Fig. 2), with the consumption of two electrons from NADPH or NADH and 1 mol of O_2 for the formation of 1 mol of hydroxylated metabolite, is only reached for well fitted substrate and monooxygenase couples. Decoupling between electrons consumption and substrate hydroxylation readily occurs (i) by release of O_2^- from the Fe(II)O_2 intermediate if its one-electron reduction is not fast enough, (ii) by release of H_2O_2 from the Fe(III)OOH intermediate if protonation occurs at the oxygen atom bound to the iron, and (iii) by reduction of the iron-oxo intermediate to H_2O if the reaction of this intermediate with the substrate is not fast enough [3] (Fig. 3). Thus, oxidase-type reactions producing O_2^-, H_2O_2 and H_2O compete with the monooxygenation reactions, if the positioning of the substrate in the active site is not optimum for efficient electron, proton and oxygen atom transfer steps of the catalytic cycle [2,3]. The two extreme situations that may occur after interaction between a cytochrome P450 and a chemical are illustrated on Fig. 4. The first one generally occurs in the case of specific cytochromes P450 that are involved in the biosynthesis of important endogenous molecules, in the presence of their specific substrate. The hydroxylation of these substrates only consumes 1 mol of O_2 and two electrons, and occurs with a high efficacy (in terms of kcat/Km). In the second situation, the presence of RH only results in the consumption of O_2 and electrons with a maximum decoupling between electron consumption and substrate hydroxylation. During the oxidation of xenobiotics by xenobiotic-metabolizing cytochromes P450, a partial, sometimes large, decoupling between electron consumption and xenobiotic hydroxylation is occurring.

3. Great substrate diversity of mammalian xenobiotic-metabolizing cytochromes P450

In the human genome, 57 genes are coding for cytochromes P450 [4]. Twenty-seven of them are involved in the biosynthesis or metabolism of endogenous compounds, such as steroid hormones, bile acids, eicosanoids, and vitamins [4]. Fifteen P450s have presently unknown biological functions. The 15 remaining P450s are those that are most often involved in the oxidative metabolism of xenobiotics. They belong to families 1, 2 and 3, and are called CYP (for cytochrome P450) 1A1, 1A2, 2A6, 2A13, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2F1, 3A4, 3A5 and 3A7. Surprisingly, three of them, CYP3A4, CYP2D6 and CYP2C9, are responsible for the oxidative metabolism of about 80% of all drugs currently marketed, and CYP3A4 alone catalyzes the oxidation of 40–50% of these drugs [4]. This implies a very poor substrate specificity of those cytochromes P450, as illustrated in Fig. 5 in which the structures of some substrates metabolized by CYP3A4 are compared. The main sites of oxidation of these substrates by CYP3A4 are also

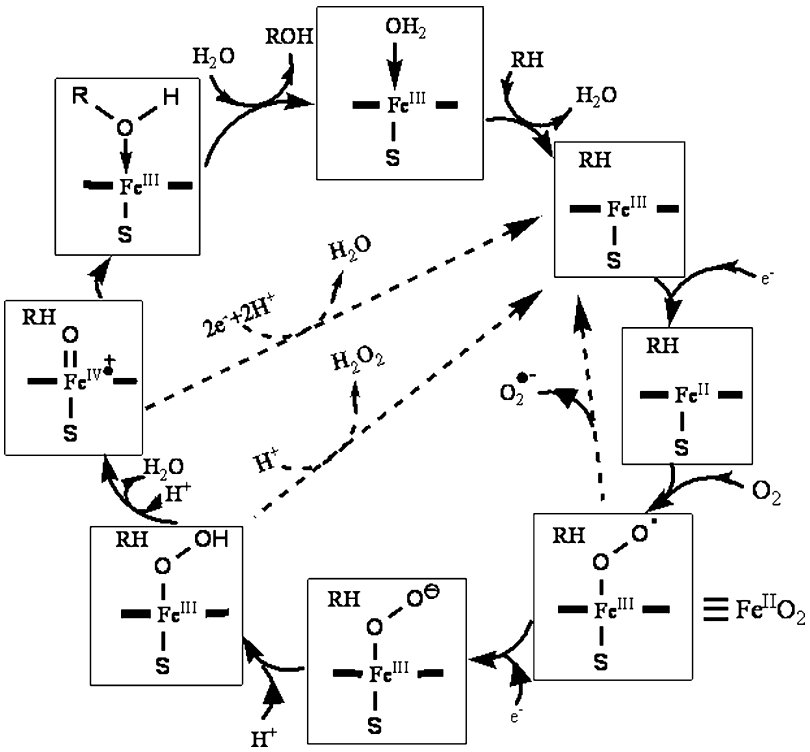

Fig. 3. Catalytic cycle of substrate monooxygenation by cytochromes P450 (—) and oxidase-type coupling pathways (- - -) occurring in competition with the monooxygenase activity [3]. RH = substrate; S = cysteinate ligand from the protein.

indicated in Fig. 5. It clearly appears that CYP3A4 can accept, as substrates, molecules of very different shape, size and polarity. This includes small drugs such as lidocaine, larger compounds such as testosterone or the carcinogen aflatoxin B1, and big and polar drugs such as erythromycin and taxol. The very rich coordination chemistry of cytochromes P450 and their iron porphyrin models, that has been established during these last 30 years [5], with the formation of iron-carbene [6,7], iron-σ-alkyl (or aryl) [8,9], iron-nitrene [10,11], and iron-nitrosoalkane [12–14] complexes, during reactions with a great variety of chemicals, is based on the very poor substrate specificity of xenobiotic-metabolizing P450s.

However, despite their very weak substrate specificity, xenobiotic-metabolizing P450s exhibit relatively high catalytic efficiencies and regioselectivities in the oxidation of most of their substrates. This "xenobiotic-metabolizing P450 paradox" is crucial for the adaptation of aerobic living organisms to their always changing environment – i.e. to all the chemicals that they absorb daily – as it permits a fast enough oxidative metabolism and elimination of those chemicals. It has been difficult to explain this paradox at the molecular level until recently, because of the lack of the necessary tools. In fact, an easy access to catalytically active recombinant mammalian P450s has become possible in the mid 90s, and the first X-ray structure of a mammalian P450, CYP2C5, was only published in 2000 [15].

4. Structures of mammalian xenobiotic-metabolizing cytochromes P450

Many X-ray structures of cytochromes P450 have been published [16] since the structure of a soluble, bacterial P450 (P450cam from *Pseudomonas putida*) that was first reported in 1985 [17]. However, the first X-ray structure of a membrane-bound, mammalian P450, CYP2C5 from rabbit liver, only appeared 15 years later [15]. A comparison of all the P450 structures described so far shows that, despite a very low sequence homology between P450s of various origins, their global 3D structures are strikingly similar [16]. The overall P450 fold is quite conservative and unique to the P450 superfamily [16]. Each P450 seems to be

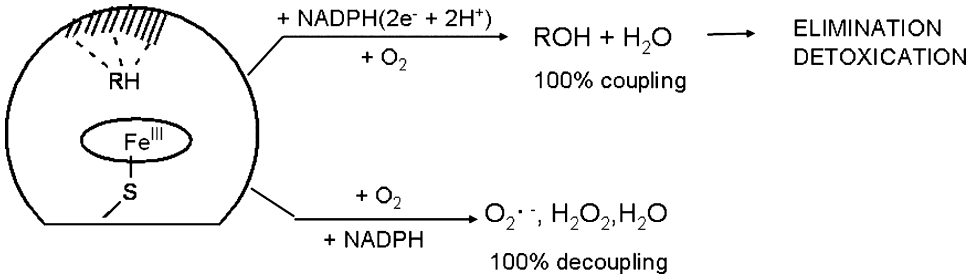

Fig. 4. Two extreme situations possibly occurring upon oxidation of xenobiotics by cytochrome P450-dependent monooxygenases. The first equation corresponds to a complete coupling between consumption of electrons from NADPH and substrate monooxygenation. At the opposite, the second equation corresponds to a complete decoupling between these two reactions.

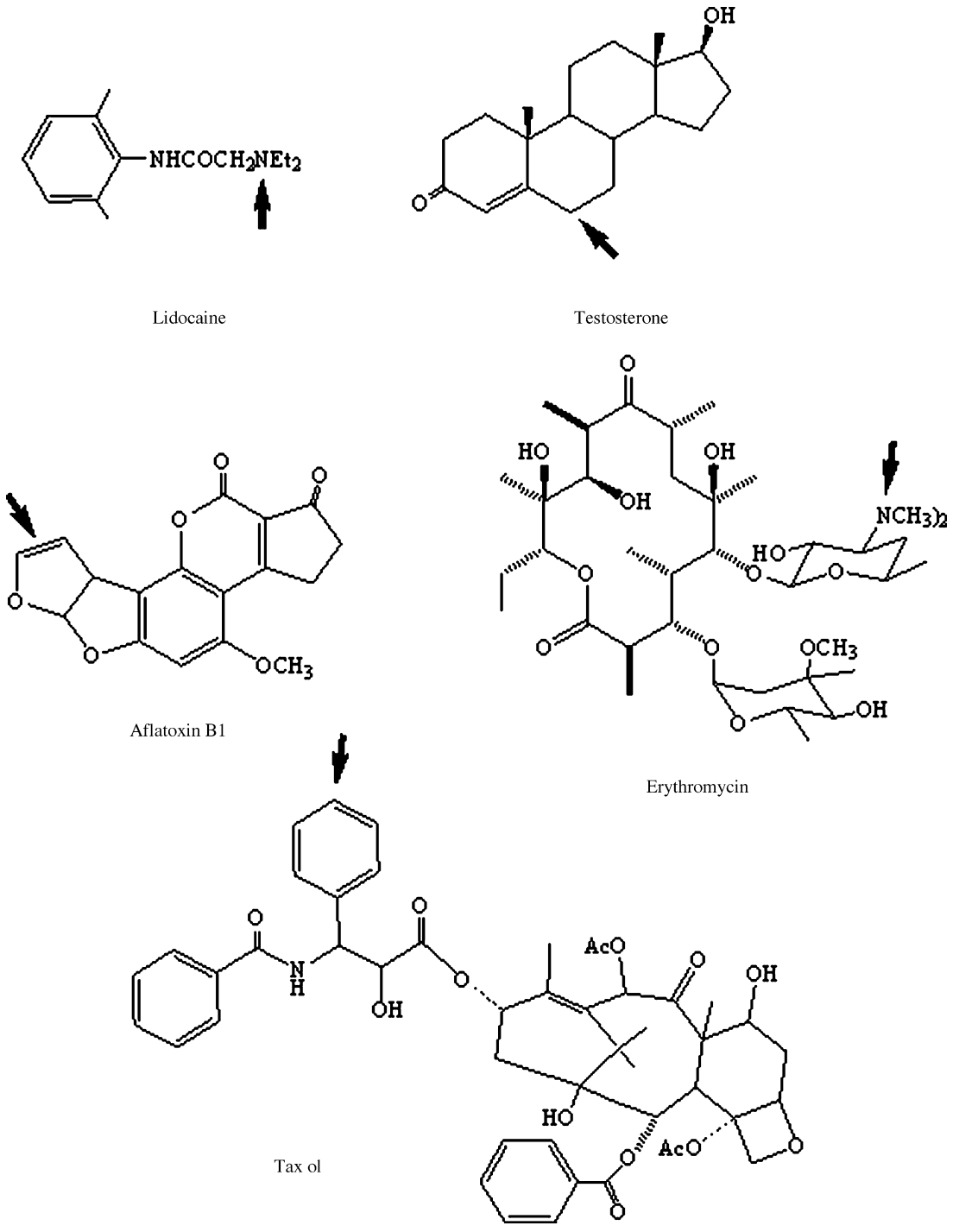

Fig. 5. Illustration of the diversity of the molecular structures of P450 3A4 substrates.

constituted by two domains. The first domain is particularly rich in α-helices, with structural elements such as helices D, E, H, I, J, K and L that are spatially conserved and form the core of the CYP protein. This conserved core is probably necessary to maintain the common structural features that are essential for P450-type catalysis (heme binding, electron transfer from reductases, dioxygen activation and formation of the high-valent iron-oxo species). The second domain contains other regions of the protein, such as the loop between helices B and C, parts of the F and G helices, and the F–G loop, that are hypervariable both in terms of aminoacid sequence and in terms of conformational stability (Fig. 6).

5. Great variability of the substrate binding sites of xenobiotic-metabolizing cytochromesP450

5.1. First X-ray structures of mammalian P450-substrate complexes

The two first X-ray structures of mammalian P450-substrate complexes were published in 2003. They concerned complexes of CYP2C5 from rabbit liver having bound either a derivative of the drug sulfaphenazole, called DMZ [18], or the drug diclofenac [19]. Hydroxylation of DMZ catalyzed by CYP2C5 mainly occurs on the benzylic methyl group of this substrate [20] (Fig. 7). Hydroxyla-

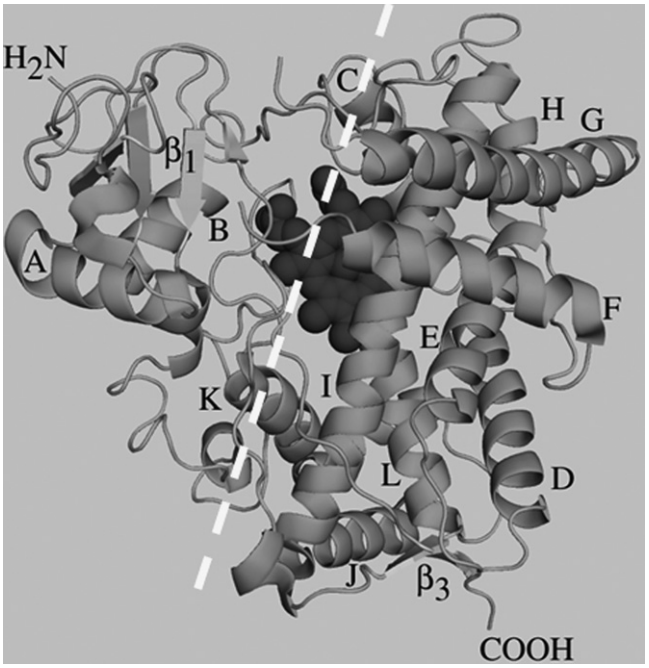

Fig. 6. X-ray structure of rabbit liver CYP2C5 illustrating the two P450 domains. Ribbon diagrams generated using PYMOL (http://www.pymol.org) show the overall fold of CYP2C5 ([15] and PDB code 1DT6). Helices are designated by letters, and β-sheets by numbers, as originally defined for CYP101 [16]. The heme group is rendered as CPK atoms in black.

tion of the other extremity of the molecule (on position *para* of the terminal phenyl group) is a very minor reaction of CYP2C5 (<2%). Hydroxylation of diclofenac by CYP2C5 almost exclusively occurs at position 4′ of the dichlorophenyl ring [19] (Fig. 7). Binding of these two substrates in the CYP2C5 active site leads to very similar dramatic changes in the conformation of this site. These changes result in:

(a) a compaction of the CYP2C5 active site around the substrate,

(b) a structuration of two important components of the substrate binding site, the B–C and F–G loops. The B–C loop appears to be disordered in free CYP2C5 [15] whereas it acquires a partially helical structure with the existence of a small B′ helix in the CYP2C5-DMZ and CYP 2C5-diclofenac complexes [18,19]. Such a structuration is even more spectacular for the F–G loop that could not be modelled from the X-ray structure of free CYP 2C5 because it was too much disordered [15], whereas it was easily modelled from the X-ray structures of the CYP2C5-DMZ and CYP2C5-diclofenac complexes [18,19], and exhibits an α-helical-type, ordered structure,

(c) the closing of the protein access channels for the substrate and for the solvent (H_2O and H_3O^+) [18,19].

These changes should prepare the protein active site and substrate couple for a more efficient catalysis of the monooxygenation of the substrate.

5.2. Comparison of the X-ray structures of human xenobiotic-metabolizing cytochromes P450: great malleability of their substrate binding site

Between 2003 and 2007, the X-ray structures of six of the main human xenobiotic-metabolizing P450s, CYP1A2 [21], CYP2A6 [22], CYP2C8 [23], CYP2C9 [24,25], CYP2D6 [26] and CYP3A4 [27–29], with or without substrates, have been described. Table 1 compares the volumes of some of their substrate binding sites that can be calculated from the Protein Data Bank (PDB). The data show that these volumes greatly differ from one P450 to another one. Thus, the volume of the smallest substrate binding site (of CYP2A6) is roughly six-fold smaller than that of the largest one (of CYP3A4). Moreover, these data confirm the compaction of the P450 active site upon substrate binding that was reported in the case of CYP2C5 and its complexes with DMZ and diclofenac [15,18,19] (*vide supra*). This compaction appears to occur after binding of the drug flurbiprofen to CYP2C9 [25], and of progesterone to CYP3A4 [27] (Table 1). At the opposite, a great increase of the active site volume (by more than 80%) has been recently reported in the case of CYP3A4 after binding of two large drugs, ketoconazole and

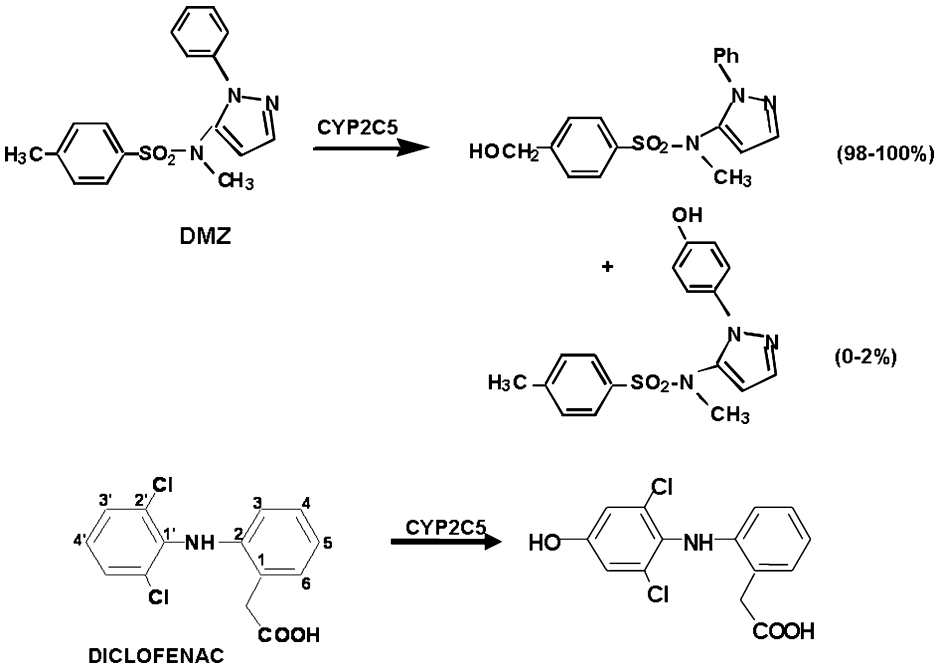

Fig. 7. Formula and sites of hydroxylation by CYP2C5 of the substrates DMZ and diclofenac (from refs. [18,19]).

Table 1
Comparison of the size of the substrate binding active site cavities of some mammalian xenobiotic-metabolizing cytochromes P450[a]

P450	Substrate	Active site volume (Å^3)
1A2	α-Naphthoflavone	375
2A6	Coumarin	280
2D6	–	490
2C5	–	930
2C5	Diclofenac	730
2C9	–	1400
2C9	Flurbiprofen	1100
2C8	–	1570
3A4	–	1580
3A4	Progesterone	1300

[a] Data concerning active site sizes are drawn from ref. [21–27,30] and from the X-ray data available on human P450s in the Protein Data Bank (PDB codes 1Z10, 2F9Q, 1DT6, 1NR6, 1OG2, 1R9O, 1PQ2, 1TQN and 1W0F for CYP2A6, 2D6, 2C5, 2C5-diclofenac, 2C9, 2C9-flurbiprofen, 2C8, 3A4 and 3A4-progesterone, respectively). The solvent-accessible volumes of the active site cavities were calculated using a 1.4 Å probe with the program VOIDOO [31]. The volumes were truncated at the ends of solvent channels in CYP2C8, 2C9 and 3A4 by placing dummy atoms at the exits to block the probe (see for instance ref. [30]). The probe occupied volumes indicated in this table are approximate values, as they depend on the choice of the positioning of the dummy atoms.

erythromycin [29]. CYP3A4 was found to be able to bind two molecules of ketoconazole in its active site. The binding of two molecules of ketoconazole, or of one molecule of erythromycin in the CYP3A4 active site leads to dramatic conformational changes of this active site [29]. These data illustrate the great malleability of the substrate binding site of some xenobiotic-metabolizing cytochromes P450 [16,29,30].

5.3. Different shapes of the substrate binding sites of human, xenobiotic-metabolizing P450s

The substrate binding sites of human, xenobiotic-metabolizing P450s not only exhibit very different sizes but also very different shapes. For instance, the substrate binding cavity of CYP2A6 is small and globally spherical. Even its small, bicyclic substrate, coumarin, is highly constrained by the side chains of the active site aminoacid residues [22]. At the opposite, the CYP3A4 active site is "bowl-like" near the heme iron and involves a wide channel to the exterior of the protein [27–29]. It may bind a large number of substrates of quite different size, above the heme plane in order to be hydroxylated. It may also bind large compounds in other regions of its active site, in a non productive manner for monooxygenation, as this was shown in the case of the drug erythromycin [29]. Finally, as mentioned above, it may bind several substrate molecules at the same time, as in the CYP3A4-ketoconazole complex in which two molecules of ketoconazole are present in the active site [29].

The substrate binding site of CYP2C8 is less spacious in close proximity of the heme iron. It exhibits a Y-shaped cavity that is very well adapted to the Y-shaped drug, montelukast [30]. This explains why montelukast has a very high affinity for CYP2C8, and acts as a strong inhibitor of this P450. The recently published X-ray structure of human CYP1A2 reveals another shape of substrate binding site involving a compact, closed cavity that is highly adapted for the positioning and oxidation of relatively large, planar substrates. In this structure, a flat, polyaromatic substrate, α-naphthoflavone, is present in the CYP1A2 active site [21]. α-Naphthoflavone closely fits the size and shape of the active site cavity. Many Van der Waals interactions with non polar side chains of aminoacids and aromatic interactions between the aromatic rings of α-naphthoflavone and phenylalanine 125 and 226 side chains contribute to the high binding affinity of this substrate for CYP1A2 [21]. The unique architecture of the CYP1A2 substrate binding site is clearly distinct from those already described for members of P450 families 2 and 3. These data illustrate how P450 family 1 proteins have evolved to bind and efficiently oxidize polycyclic aromatic hydrocarbons [21].

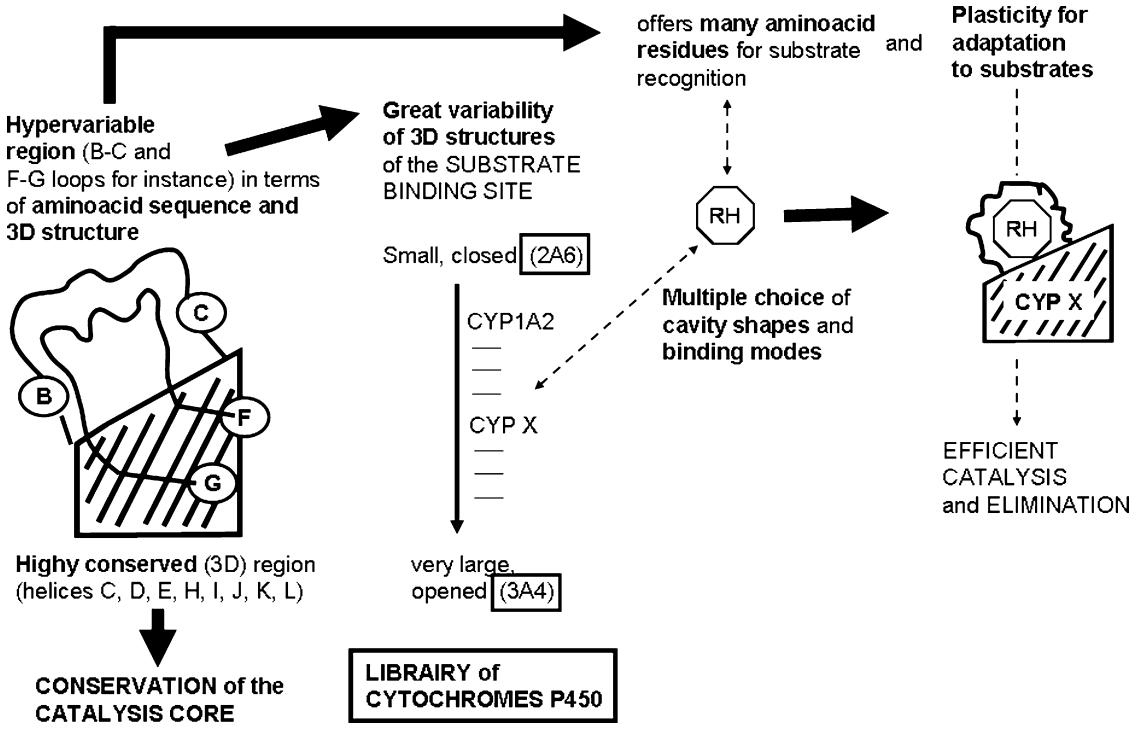

Fig. 8. Schematic illustration of the adaptation of aerobic living organisms to oxidize and eliminate very diverse xenobiotics. The librairy of P450s available for xenobiotic binding and oxidation in a given tissue or organ is shown in the case of human liver. RH is the xenobiotic; each cytochrome P450 is very schematically presented simply to illustrate the flexibility of the domain involving the substrate access channel and substrate binding site.

5.4. Possible explanation for the "xenobiotic- metabolizing P450 paradox": molecular bases for the ability of aerobic organisms to oxidize and eliminate very diverse xenobiotics

The existence, in most mammalian tissues and organs, of a librairy of P450s involving substrate binding sites that so greatly differ in size, shape, and nature of the aminoacids available for diverse binding modes, is an important starting point to understand the adaptability of aerobic organisms to always changing chemical environments. In fact, any xenobiotic having penetrated in a given organ, such as human liver, has the choice between several P450s that exhibit similar global 3D structures, with a well conserved domain containing the catalytic core and a hypervariable domain containing the substrate access channel and the substrate binding site. Because of the great diversity of sizes (Table 1), shapes (Section 5.3), aminoacid sequences, and conformational flexibility (Section 5.1) of those P450 substrate binding sites, any xenobiotic will find one (or several) P450 site(s) that is (or are) most appropriate for its size, shape, and chemical characteristics (Fig. 8). Then, after binding of the xenobiotic to its preferred P450, the conformational flexibility of the active site of this P450 should allow it to adapt its conformation to maximize xenobiotic-P450 active site interactions. This has been shown in the case of CYP2C5 and either DMZ or diclofenac [18,19]. Such an adaptation of the P450 active site to the xenobiotic should lead to a more efficient catalysis of the oxidation of this xenobiotic.

6. Conclusion

Recent data, from the 2003–2007 period, have led to a better understanding of the molecular bases thanks to which aerobic living organisms may oxidize and eliminate very diverse xenobiotics and adapt themselves to always changing chemical environments. Recently published structures of mammalian P450-substrate complexes have shown the great diversity of sizes, shapes, and aminoacid sequences of the substrate binding sites that are offered for the binding and oxidation of xenobiotics by a single superfamily of catalysts, the cytochromes P450. The great diversity of possible binding modes offered by the hypervariable and conformationally flexible domains of the xenobiotic-metabolizing cytochromes P450 explains how they can bind so many chemicals. The conformational flexibility of their substrate binding sites explain how they can optimize protein–xenobiotic interactions and lead to an efficient catalysis of the oxidation of xenobiotics. In other terms, one may say that aerobic organisms have used the possibilities of the biological diversity to cope with the chemical diversity of their environment.

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