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Cytochromes P450: Review on their Basic Principles

PAVEL ANZENBACHER[1*] and EVA ANZENBACHEROVÁ[2]
[1]*Institute of Pharmacology and* [2]*Institute of Medical Chemistry and Biochemistry,*
Faculty of Medicine, Palacky University of Olomouc, Hnevotinska 3, 775 15 Olomouc, Czech Republic

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Cytochromes P450 are heme enzymes present in living species from bacteria to man. Significance of P450s to man is determined by a) their endogenous roles in biosynthesis of various mediators and hormones, often not yet fully elucidated, and b) their participation in many reactions of drug metabolism as well as of many xenobiotics. In this respect, the cytochromes P450 often decide on the final effect of a drug as the majority of drugs are metabolised through pathways involving P450 enzymes. On the other hand, many fundamental questions on their structure and function remain unanswered. It is true for the description of the catalytic cycle, which is still not complete, as well as for modes of interactions of substrates with the enzyme itself (binding of substrates, changes of the heme iron spin state, cooperativity), for mechanism of electron transfer and of oxygen insertion. Structures of the respective active sites are intensively studied to explain substrate specificity of many (mainly liver microsomal) cytochromes P450.

In molecular pharmacology, the implications of drug metabolism by cytochromes P450 for personalized pharmacotherapy are currently evaluated and recommendations for clinical practice to avoid unwanted effects of drugs and drug interactions have started to appear. This only stresses (with development of pharmacogenetics) the importance of cytochromes P450 in all branches of life sciences.

Key Words: Cytochrome P450, Drug metabolism, Oxygen activation, Active site

Introduction

Cytochromes P450 are one of the most extensively studied proteins in biochemistry, biology, biophysics and molecular pharmacology. The number of newly discovered cytochromes P450 or of allelic variants of already described cytochromes P450 still increases and the total number of papers devoted to these interesting enzymes approaches twenty five thousand. The purpose of this review is to contribute to the current discussion on possible directions of further research in this field. For a detailed information, numerous books and reviews are available (Ortiz de Montellano 1995, Anzenbacher & Anzenbacherová 2001, Guengerich 2002).

In the past, there have been some problems with devising a suitable nomenclature for cytochromes P450. It is already the name of this class of enzymes which is not easy to understand for non-specialists in this field. The discovery of an unknown protein component in the spectra of microsomal fraction of rat or pig liver cell homogenate absorbing the visible light at about 450 nm when reduced e.g. with dithionite and subsequently complexed with carbon monoxide (Garfinkel 1958, Klingenberg 1958) led to a suggestion to name this protein after this unique absorption maximum (P for a "pigment", hence P450, originally, P-450). A hemoprotein nature of this protein was confirmed as well and the name "cytochrome P450" seemed to be logical (Omura & Sato 1964). To simplify the name, a term "cytochrome m" (m for monooxygenase) was suggested. Another unfortunate attempt to rename cytochromes P450 was the recommendation made by the Enzyme Commission – "heme-thiolate proteins". The rationale for that was the uniqueness of the mode by which the heme is bound to the apoprotein – by a bond joining the heme iron with a negatively charged sulphur atom of the cysteine residue. However, it was realised soon after that also other hemoproteins as e.g. chloroperoxidase or nitric oxide synthase exhibits the same absorption

*Corresponding author: *E-mail:* anzen@tunw.upol.cz, *Tel:* +420-58-563-2569, *Fax:* +420-58-563-2966

due to the same type of bonding. Lately, an abbreviation CYP has been adopted for proteins which are products of genes coding for cytochromes P450. This abbreviation is by far the most common now and will be used together with abbreviation "P450" also in this review.

The number of CYP enzymes characterised by their primary structure (amino acid sequence or deduced from the sequence of the respective nucleotides) exceeds 2500 (Nelson 2002). This superfamily of proteins (and of the respective genes) is divided into families, subfamilies and finally to individual members according to the similarities in their primary structure (Nelson et al. 1996). Members of a family (labelled by an Arabic numeral, e.g. CYP1) should exhibit at least 40% sequence similarity, those of a subfamily (characterised by an alphabetical character, e.g. CYP1A) should be 55% identical. Individual members of a subfamily are again numbered sequentially, as e.g. CYP1A1 or CYP1A2. To constitute a new entry, in other words, to be a new member of the superfamily of CYP enzymes, the primary structure should differ from a similar CYP protein by more than 3%. The total number of CYP families is now 285. In animals, there are 70 with 191 subfamilies, 86 families are present in lower eukaryotes (with 101 subfamilies). 52 plant families were described (with 127 subfamilies) and 77 CYP families were found in bacteria with 123 subfamilies. The number of individual CYP enzymes in species differ – the highest numbers, approaching two hundred, were found in plants as the plants probably need them to synthesise secondary metabolites including the coloured flower pigments as well as plant toxins to fight the enemies – plants do not have the ability to protect themselves by a specialised shield or by running away like the animals (Nelson 1999). Fifty-seven human CYP genes were sequenced and 47 pseudogenes (Nelson 2002), which is the count typical for an animal species (around sixty). The NO synthases are sometimes taken as CYP enzymes as the catalytic mechanism leading to formation of nitric oxide is in principle the same. A list of known structures of the NO synthase enzymes as well as of other P450-related proteins is posted in the web site of Degtyarenko (2002).

Cytochromes P450: Main Principles, Catalytic Cycle

The presence of an absorption band at about 450 nm in the absorption spectrum of the reduced form complexed with carbon monoxide reflects much more fundamental properties of the heme prosthetic group in this particular class of hemoproteins. This absorption band (named Soret band after its discoverer) usually takes place in the spectra of various forms of hemoproteins at about 380 – 420 nm. A red shift of at least 30 nm means that the distribution of the electron density at the heme is significantly perturbed. It has been documented by theoretical approaches that it is the thiolate sulphur which by means of the direct bond to the heme iron causes this effect (Harris et al.1998). Moreover, the heme iron here can not only bind the molecular oxygen (i.e. dioxygen) but also activate the dioxygen moiety giving (after acceptance of two electrons) one molecule of water and forming a hydroxylated substrate. The overall process describing the most of the reactions catalysed by the P450 enzymes can be expressed by a reaction

$$RH + NAD(P)H + H^+ + O_2 \rightarrow ROH + H_2O + NAD(P)^+$$

where RH is a substrate to be hydroxylated and NAD(P)H is the respective pyridine coenzyme in its reduced or oxidised form. As this reaction is de facto a monooxygenation, CYP enzymes are listed mainly among monooxygenases (EC 1.14., for more information, see Degtyarenko 2002). An older attempt to classify CYP enzymes was to name them collectively as "mixed function oxidases", abbrev. MFO, as the products of the above reaction are both a hydroxylated substrate and a water molecule (which is a characteristic of oxidase reactions). This reaction is, as it has been said above, the most general one describing the most events which take place in presence of CYP enzymes. Often, a monooxygenation (hydroxylation) is the first reaction followed by a next step in which for example a molecule of respective aldehyde is eliminated. This is the case of dealkylations, as N- or O-dealkylations, where the C atom of the alkyl is hydroxylated first and subsequently a respective aldehyde (for example, formaldehyde in the case of methyl radical) is liberated. However, it should be noted here that there are non-monooxygenating activities of cytochromes P450 as e.g. nitric oxide

reductase activity catalysed by $P450_{nor}$ or intramolecular oxidoreductase activities as of the prostacyclin synthase (CYP8A1) or thromboxane synthase (CYP5A1). In this latter case, the P450 enzymes bind the substrate molecule (prostaglandin H_2) which is in fact a peroxide. The substrate then undergoes an intramolecular rearrangement to the respective product, i.e. prostacyclin (CYP8A1) or thromboxane (CYP5A1).

The "special" character of the Fe-S bond has been confirmed by spectroscopic methods (Dawson 1988, Anzenbacher et al. 1989) and the fact of existence of this bond has been documented by crystallographic studies (Poulos 1987, Williams et al. 2000). Exchange of the heme iron bonding cysteine residue for a histidine by site-directed mutagenesis in CYP101 (still often named $P450_{cam}$, camphor hydroxylase from *Pseudomonas putida*) has been shown to result in decreased enzymatic activity thus confirming the role of the proximal heme thiolate ligand in cytochromes P450 in the O-O bond cleavage as well as for correct protein folding, substrate binding and electron transfer (Auclair et al. 2001, Yoshioka et al. 2001). However, and this is one of the most important questions for detailed understanding of the principle of action of all heme enzymes including CYP, there are results from other laboratories indicating that the factors other than the nature of the proximal axial ligand of the heme iron, namely, originating in the protein environment, may be the main determinants of function (Goodin 1996, Harris & Loew 2001). In this respect, the role of the amino acid residues at the distal side is often mentioned as the subtle interactions between the bound dioxygen and the protein may in summary be very important. For example, a distal charge relay system joining dioxygen with charged amino acid residues at the distal side of the heme through a water molecule was proposed in cytochrome $P450_{cam}$ already ten years ago by Gerber and Sligar (1992).

The reaction cycle of the CYP enzymes is rather complicated (figure 1). The classical sequence (White & Coon 1980, Dawson & Sono 1987) involves the binding of a substrate to the active site of the enzyme which should be accompanied by a change of the heme iron coordination. Namely, the heme iron should be, as a result of this interaction, able to

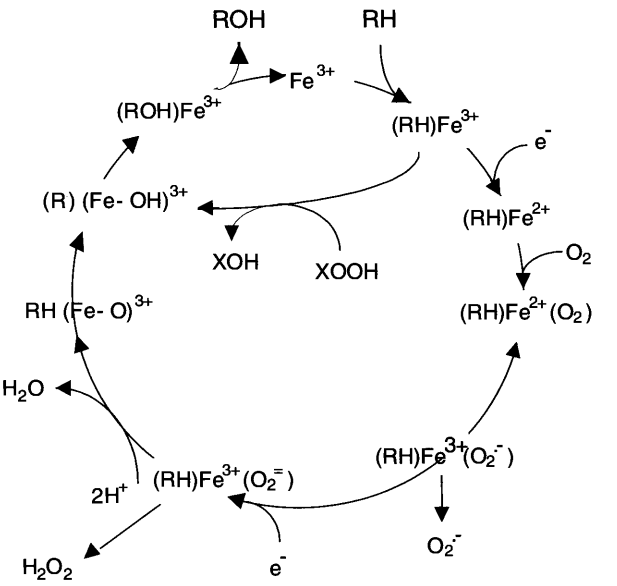

Figure 1 Cytochrome P450 catalytic cycle. RH, substrate, XOOH, a peroxide (X = H or organic radical). The scheme follows the description in the text, i.e. substrate binding, reduction by the first electron and formation of the reduced dioxygen complex (or, oxidized superoxide complex as denoted by double headed arrow). The reduction by the second electron leads to formation of water and of an oxo intermediate with a "reactive oxygen" entering finally the substrate. Peroxide shunt allows for direct formation of a hydroxylated substrate.

bind the dioxygen molecule (molecular oxygen). In other words, the sixth coordination position of the heme iron should be free to accommodate the oxygen molecule. The heme iron atom has four of its coordinations occupied by the heme, the fifth coordination bond is this to the thiolate sulphur and the sixth one can be used to bind another ligand. The sixth ligand (if present) is an oxygen atom from a water molecule present in the active site as the water molecules form a cluster there (Poulos et al. 1987, Poulos 1988). The iron-oxygen bonding determines the low spin state of the heme iron which is reflected in the position of the Soret absorption band – in the resting state, the absorption maximum is at about 417 nm. The fact that the sixth coordination is free for oxygen binding is seen in the spectrum as the position of the Soret maximum is then at about 395 nm. These changes in ligation, coordination and conformation of the active site are reflected in the changes in polarity of the active site facilitating apparently the reduction of the iron atom (Anzenbacher 1978). However, even at the very

beginning of the reaction cycle (figure 1), the situation is not as clear as it has been thought. First of all, the simple scheme beginning with a hexacoordinated low spin heme iron in the resting state which binds a substrate (RH), changing to substrate-bound five-coordinate high spin state with subsequent reduction and oxygen binding may not fully reflect the actual situation. For example, the presence of a (possibly transient?) form with a hexacoordinate but clearly high spin iron has been found (Hildebrandt et al. 1994) both with and without a substrate bound. Then, it has been shown that the sequence of events may not be as indicated – for example, that the reduction may proceed first, in the absence of a substrate bound and that, in general, the kinetics of the reduction cannot be generalized among different CYP enzymes (Guengerich & Johnson 1997).

During the next steps, a reduced (ferrous) dioxygen intermediate is formed (it may however disproportionate to a ferric form and a superoxide radical anion) which is further reduced to the ferrous-hydroperoxide or ferric-peroxide intermediate. Here, the latest stabilized and spectrally characterized intermediate is the reduced dioxygen $Fe(II)O_2$ intermediate (Bonfils et al. 1979). This intermediate has been recently shown to be different between the CYP enzymes and the NO synthase (Bec et al. 1999) indicating significant differences between these two enzyme systems. No more intermediates were characterised, due to their instability. All that is presented is anticipated or expected on the basis of theoretical calculations and on experiments with model systems. For many years,the $Fe{=}O^{4+}$ intermediate was the most serious candidate for the ultimate species responsible for monooxygenation of substrates (Dawson & Sono 1987, Schlichting et al. 2000). More recently, other species such as the hydroperoxo intermediate are considered to be the alternatives (Troy et al. 1998, Newcomb et al. 2000, Suzuki et al. 2002).

The reduction is itself a puzzle. The process involves donation of two electrons, however, their sources may be different. The first electron is donated in microsomal systems from NADPH via the NADPH:cytochrome P450 oxidoreductase (often abbreviated as P450 reductase), which is a flavoprotein with both the FAD and FMN prosthetic group. In mitochondrial and bacterial systems, the electron-transporting chain is longer as it typically involves NADH as the source of electrons, then a flavin-containing reductase with FAD as a cofactor, a Fe-S protein (named after the specific system, e.g. adrenodoxin in adrenals and other steroidogenic tissues, putidaredoxin in *Pseudomonas putida* and the ultimate CYP enzyme (e.g. the CYP11A1, previously named $P450_{scc}$, in adrenal mitochondria or the CYP101, formerly $P450_{cam}$ in *Pseudomonas*) . The second electron, at least in microsomal CYP systems, may be either donated from NADPH through the above mentioned reductase, or through another electron transporting system involving NADH, NADH:cytochrome b_5 oxidoreductase and finally cytochrome b_5 interacting directly with the CYP molecule. The role of cytochrome b_5 is intriguing as it seems to work also as an apoprotein just as an allosteric effector forcing the CYP molecule to adopt an optimal conformation for electron transfer (Yamazaki et al. 1996, 1997).

Some bacterial P450s have the advantage of having both the reductase and the cytochrome P450 linked together being in fact flavohemoproteins. One of the most studied P450s, cytochrome P450 BM3 from *Bacillus megaterium* (CYP102) is a flavohemoprotein with known structure. The P450 part of its molecule shares considerable degree of structural similarity with human liver microsomal P450s. This is also why this enzyme has been used several times to construct models of human microsomal P450s (*see* e.g. papers of Lewis 1997, Lewis et al. 2000). As this is also the case of the nitric oxide synthase synthetising the NO molecule which controls the vascular tone, a question arises, what was the reason for selection and preservation of this particular combination of these two electron transporting systems from bacteria to man. To make the situation with bacterial P450s and their reduction even more complicated, a new bacterial P450 enzyme has been very recently characterized and found to be a new class of P450 enzymes having a fused reductase, Fe-S and P450 domains (Roberts et al. 2002). This protein from *Rhodococcus sp.* utilizes NADH as a source of reducing equivalents.

There are however other factors influencing the effectiveness of the reaction cycle which are studied recently and which certainly will bring important

information on the P450 mechanisms. For example, the lipid-P450 protein interactions were mentioned twenty years ago by Bosterling (1982) and have been re-investigated recently (Yun et al. 1998), or, the presence of allosteric interactions in the CYP systems – mainly with CYP3A4 (Ueng et al. 1997) seems to be the way to modulate the P450 activities. A review on the allosteric regulations in cytochrome P450-catalysed reactions was published recently (Hlavica & Lewis 2001). Here, the cooperativity not only in the metabolism of substrates (in CYP3A4, 2C3 and 1A1), but also allosteric regulations of electron transfer and in the dioxygen binding to the CYP enzymes is discussed. Also, the interactions of cytochrome P450 molecule with other components of electron transporting chain may play a role in modulating the P450 activity. For example, a proof of the influence of interaction of the iron-sulfur protein adrenodoxin on the strength of the heme iron-ligand bond in $P450_{cam}$ complexes with carbon monoxide or nitric oxide was presented recently by Unno et al. 2002. Binding of adrenodoxin apparently induces changes in conformation or electronic structure of the $P450_{cam}$ heme moiety that affect the proximal cysteine-heme iron bond which is then reflected in the distal side of the heme by perturbation of the strength of the heme iron - sixth ligand (NO or CO) bond. Another factor which may decide on the effectiveness of the P450 cycle is the reaction mechanism itself. Various mechanisms were studied involving different sources of oxygen atoms as peroxides, water, molecular oxygen in model systems as well as with CYP enzymes in reconstituted systems (Anzenbacher et al. 1996). The pathway by which the substrate is metabolised seems to depend not only on the structure of the substrate, but also on the particular P450 or, generally, heme enzyme involved in the catalytic process. It should be mentioned here that also other heme enzymes as e.g. peroxidases may activate the O-O bond and take part in reactions previously thought to be solely domain of the P450s (Dorovska-Taran et al. 1998).

Overall Topology and Structure of P450 Active Sites

The overall structure of all CYP enzymes seems to be similar having a "heart-like" shape with a membrane anchor in microsomal CYP enzymes (microsomes are vesicles formed during cell disruption from endoplasmatic reticulum). Soluble, bacterial ones do not possess this hydrophobic N-terminal membrane anchor. Recently, removal of this tail has opened the way for obtaining soluble eukaryotic P450 proteins suitable for crystallographic studies. The first CYP enzyme whose structure was known was CYP101 ($P450_{cam}$) (Poulos et al.1987, Poulos 1988). Other bacterial systems followed but a real breakthrough was recent publication of the structure of rabbit liver microsomal CYP2C5 (Williams et al. 2000) by Johnson's group. The protein is depicted in figure 2 with arrows showing the N terminus (shortened by 22 amino acid residues forming a transmembrane helix) and the C-terminal end of the protein. It is a single chain composed of highly ordered structure with one heme b (bound to a characteristic cysteine residue). Membrane binding is not only achieved by an N-terminal anchor, but also by interactions with other parts of the molecule. These are probably immersed to a certain extent in the membrane and contribute to the association of CYP enzymes with the membrane of the endoplasmic reticulum. Thus, even without the transmembrane helix,

Figure 2 Structure of the rabbit microsomal CYP2C5. Schematic picture based on data from Protein Data bank database of protein structures, accession number 1DT6, entry from Williams et al. 2000. N and C termini labelled by arrows, heme is shown in the center of the molecule.

the CYP2C5 was still bound to the membrane fraction (Williams et al. 2000).

The active sites of CYP enzymes must differ significantly as this should determine the need for so many different CYP species. The most intensively studied are of course those metabolising foreign compounds as drugs. Here, the substrate specificity is rather broad (contrary to other CYP enzymes with specific function – e.g. to the CYP11A1 which starts the utilization of cholesterol by cleaving its side chain, this is also why this enzyme was formerly called P450scc for side chain cleavage). Even the liver microsomal CYP proteins differ by the shape, accessibility and flexibility of their active sites (*see* e.g. Lewis et al. 1999). Molecular modelling of mammalian P450s was reviewed in recent paper of Dai et al. (2000) summarising the major concepts and current approaches. The limits of the molecular modelling lie in their dependence on the chosen "parent", reference molecule with known structure – the results may be more or less templates of it instead of being really new structures. However, this approach has yielded interesting results mainly in explanation of substrate docking in the active sites of CYP proteins (Lewis & Lake 1998).

In the absence of direct experimental methods, structure and properties of active sites of various CYP proteins are studied mostly by site-directed mutagenesis and by spectroscopic methods. An analysis of mammalian CYP structure and function by this approach has been recently reviewed (Domanski & Halpert 2001). Their method of selective replacement of amino acid residues expected to be important for the catalytic activity of the enzymes by alanine has shown that even the structurally conserved residues which have been shown to be determinants of the activity in one CYP enzyme may not be an essential active site building block in another CYP enzyme. For example, though Thr309 and Ile 301 have been shown to play essential role in the catalytic activity of CYP2D6 and CYP2E1 respectively, these amino acid residues do not seem to influence dramatically the steroid oxidations performed by CYP3A4.

As an example of spectroscopic study of properties of CYP active sites, the probing of the flexibility of active sites by UV-VIS absorption and resonance Raman spectroscopies can be presented (Anzenbacher & Hudecek 2001). Spectroscopic methods revealed significant differences in flexibility and stability of CYP forms. Among microsomal CYPs, the most flexible active site has been found in the CYP3A4 enzyme as it is both compressible and the heme vinyl chains may adopt two different conformations. The most rigid active site at least according to spectroscopic studies at high hydrostatic pressure and according to resonance Raman data is present in the CYP1A2. The differences between CYP3A4 and 1A2 apparently reflect their ability to bind various substrates, as CYP3A4 is known to be the least selective P450, metabolising a vast variety of drugs. In similar studies on microsomal P450s using spectroscopy at high pressure, the CYP2B4 was found to be less and CYP2A5 the more stable or flexible P450 enzyme (Bancel et al. 1997).

P450s and Drug Metabolism

Microsomal CYP enzymes are in fact able to perform biotransformations of enormous variety of substrates (Guengerich 1995). Among all drugs metabolised by enzymes in the first phase of biotransformation, CYP proteins are responsible for more than two thirds of these reactions. Human CYP3A4 then metabolises over one half of known substrates being the most important CYP enzyme. It is also the most abundant in the human gastrointestinal tract and liver. Drug substrates, inducers and inhibitors were summarized by Rendic and Di Carlo (1997), useful information is also presented on the Gentest web site (www.gentest.com). The fact that one enzyme may be responsible for several biotransformations, in other words, in the metabolism of more drugs, may often lead to their competition for the same enzyme (e.g. for the CYP3A4) resulting in higher plasma levels of the less successful substrate (with lower affinity to the particular enzyme). This can be the direct cause of even fatal drug interactions described e.g. with antihistaminic agent terfenadine and azole antifungals (Thummel & Wilkinson 1998). A critical review on this subject was given by Bertz and Granneman (1997) and at the web site http://dml.georgetown.edu/depts/pharmacology/davetab.html.

Another factor complicating drug metabolism (and in fact the pharmacokinetics of a drug) based on the activity of CYP enzymes is the presence of various alleles of the CYP enzymes in human genome with different activity or no activity at all. In other words, in the population, there are individuals present which may have their particular CYP enzyme less active than others: a phenomenon which is genetically determined. This may lead to failure of medication in one individual or to the presence of unwanted adverse effects in other case due to elevated levels of unmetabolised substrate.

These effects are studied by pharmacogenetics and pharmacogenomics. Almost all drug-metabolizing human cytochromes P450 are genetically polymorphic, i.e. their genes exist in human population in several variants (alleles). The number of alleles discovered is still growing; for current information, see (www.imm.ki.se/CYPalleles). For example, in the time of preparation of this manuscript, twenty-five alleles of the most important human drug-metabolizing CYP3A4 was known and seventy-seven alleles of another important CYP enzyme, CYP2D6, were found. The most interesting information is however how these alleles are linked to altered drug metabolism. The best known are the alleles of the CYP2D6 enzyme. Here, twenty-five alleles gave no functional protein, five alleles are responsible for proteosynthesis of proteins with decreased activity to CYP2D6 substrates and in three cases the presence of altered CYP2D6 genes in organism is reflected in an increased enzyme activity. The fact that an organism does not possess a functional CYP2D6 enzyme however does not necessarily mean that CYP2D6 substrates are not metabolised at all. Usually, another pathway of metabolism is then utilised, however, with lower effectiveness. According to this, people may be divided to phenotypes of extensive (normal, also named rapid), slow, but also ultrarapid and intermediate metabolizers. Hence, a graph presenting the distribution of values of a certain CYP (e.g. CYP2D6) activity among subjects in tested population may be a curve composed by up to four components. In an ideal case, with sufficiently high number of subjects, it may exhibit four more or less clear maxima: One for the ultrarapid, second for the extensive (or rapid), third for the intermediate and fourth for the slow (named also poor) metabolizers. Another point which should be mentioned here is that the proportion of individuals with given genotype (and phenotype) differs in different populations. For example, the phenotype of poor metabolizers (of CYP2D6 substrates) constitute five to ten per cent in Caucasians, however, there are no poor metabolizers among American Indians or Japanese. Two per cent of poor metabolizers were found in Hindu and 1.2 per cent in Thai population (Llerena et al. 1996).

Although the molecular principles of the pharmacogenetics are known for more than twenty years (Meyer 1994), it is only now when recommendations for corrections of dosing schemes related to different groups in the population are appearing in the literature (Brockmöller et al. 2000, Martin-Facklam et al. 2000). For example, the dose of antiarrythmic drug propafenone (mainly a CYP2D6 substrate) should be lowered to 40% of the average dose in poor metabolizers as well as the dose of beta blocking agent metoprolol. On the contrary, in extensive metabolizers, the dose of propafenone should be 130% and this of metoprolol 140% of the average dose. With omeprazole (antiulcerotic drug, substrate of CYP2C19), the dose for slow metabolizers should be lowered down to 20% of the average dose (Brockmöller et al. 2000). In poor metabolizers, also the unwanted adverse effects are also more pronounced. This is the case of increased cardiotoxicity of tricyclic antidepressants (substrates of CYP2D6) or bleeding after warfarin (in poor metabolizers of CYP2C9 substrates) (Ingelman-Sundberg et al. 1999).

Both the new fields of molecular pharmacology and molecular biology, i.e. pharmacogenetics and pharmacogenomics, are rapidly developing (Guengerich 2000, Brockmoller et al. 2000) and will most probably significantly contribute to this what is called "personalized pharmacotherapy". This is, however, out of the scope of this review, and will deserve another detailed review in the near future.

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