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*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/150387> since 2016-06-10T10:53:25Z

*Published version:*

DOI:10.1053/j.seminoncol.2013.12.013

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This is the author's final version of the contribution published as:

Gilardi, Gianfranco; Di Nardo, Giovanna. Heme iron centers in cytochrome P450: structure and catalytic activity. *RENDICONTI LINCEI. SCIENZE FISICHE E NATURALI*. None pp: 1-9.  
DOI: 10.1007/s12210-016-0565-z

The publisher's version is available at:

<http://link.springer.com/10.1007/s12210-016-0565-z>

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# **Heme iron centers in Cytochrome P450: structure and catalytic activity**

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**Running title:** Cytochromes P450 as biocatalysts

**Keywords:** Cytochromes P450, heme reactivity, monooxygenases

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## **Abstract**

Heme iron centers are found in a wide range of proteins where they play different roles crucial for many biological processes, including catalysis.

Among heme-containing enzymes, the cytochromes P450 superfamily comprises members distributed in all domains of life where they participate to the metabolism of endogenous and exogenous compounds. These enzymes can perform a series of oxidative reactions on a broad range of chemically different substrates and, for this reason they are optimal candidates for biocatalytic purposes and, in general, technological applications.

In this review, the general features of these enzymes will be discussed with particular emphasis on the structural insights obtained through x-ray crystallography to understand the key steps of their catalytic mechanism where oxygen is activated.

Moreover, one of the finest multi-step reactions catalyzed by cytochromes P450 aromatase (the conversion of androgens into estrogens) will be discussed in detail. In fact, the x-ray structure of this enzyme together with site directed mutagenesis experiments have elucidated the role of key residues involved in substrate binding and catalysis.

This last example shows how function and structure in cytochromes P450 are closely inter-correlated to achieve complex finely tuned catalytic mechanism. It is possible to exploit them for biotechnological applications even if much attention must be paid in not altering their delicate structure.

Enzymes are attractive catalysts able to combine the ability to carry out stereo- and regio-specific reactions at high yield with the capacity to perform in environmentally sustainable mild conditions. Due to the characteristics of the cellular environment in which nature designed them to function, they often function in aqueous solutions at neutral pHs and mild temperatures. Despite these attractive properties, the applicability of enzymes in industrial processes is often limited by their complex and unstable 3D structure that is designed to work in the living environment for a set length of time to ensure degradation and replacement depending on the metabolic needs of the cell. Here protein engineering, with its rational design or random mutagenesis approaches, can help in producing enzymes more amenable to industry. Here durable and robust molecules are needed, able to withstand the test of time often operating in extreme conditions.

In order to perform their activity, enzymes often require cofactors or prosthetic groups inserted in an active site with an architecture defined by amino acids with side chains conferring specific shapes, sizes, charges and reactivity. This forms the basis of their substrate chemo-, regio- and stereo-selectivity as well as the variety of reactions that they can catalyse.

In the orthodox view, enzymes are considered as homogeneous catalysts because they work in the same phase of their substrates and products. However, if necessary they can be easily immobilised on surfaces and therefore be separated, owing to their much larger dimensions. This last point can be an issue when working with homogeneous catalysis. It can therefore be argued that enzymes represent a class of catalysts in their own right, due to their unique and complex nature where a homogeneous catalytic site is included in the protein matrix that play an active role in promoting the formation of specific interactions between the catalytic center and the substrate. Moreover, the protein fold has a crucial role in the selection and recruitment of the substrate from the surface to the catalytic site through specific channels that can be different from those used by the reaction products to be released by the enzyme. The ligands usually required to develop a homogeneous catalyst are already present in enzymes and they are represented by the amino acids that line the catalytic cleft conferring selectivity. In this view, enzymes combine some advantages of both homogeneous and heterogeneous catalysis.

As enzymes are normally very selective, the maintenance of their 3D structure is crucial, and this is achieved in a quite narrow range of conditions. Such conditions are usually mild and therefore the use of high temperatures or strong acids and bases can usually be avoided when using a biocatalyst with an advantage in terms of energy costs and environmental sustainability. On the other hand, if required, enzymes can be extracted from organisms adapted to survive in extreme conditions such as high temperatures or extreme pH. Alternatively, advances in molecular and structural biology techniques have provided the opportunity to manipulate enzymes with the aim to produce biocatalysts optimized for a specific reaction. In particular, protein engineering can be used to change the amino acid identity in specific positions at DNA level with an effect on the structural features of the

enzyme. This approach has been widely used to increase enzyme performance, the chemo-, regio- and stereoselectivity but also the stability of the enzyme (Valetti and Gilardi, 2004) and, nowadays, enzymes are used for industrial applications (Choi et al., 2015).

## **1. Heme centers as catalysts in proteins**

The physico-chemical properties of the iron-porphyrin complex have been exploited by nature to develop a wide range of proteins working in different cells and sub-cellular organelles where they perform a wide range of functions. Depending on the nature of substituents linked to the porphyrin ring and the way the cofactor is bound to the protein matrix, different heme types have been classified and they have been associated to specific functions. The b-type heme (heme-b), is present for example in proteins involved in O<sub>2</sub> storage and transport (myoglobin and hemoglobin) as well as in O<sub>2</sub>-mediated catalysis (peroxidase, catalase, cytochromes P450). Heme-a is present in those enzymes reducing O<sub>2</sub> to H<sub>2</sub>O during catalysis (cytochrome c oxidase) and heme-c is present in electron-transfer proteins such as cytochrome c. From the catalytic point of view, in the presence of heme and O<sub>2</sub>, different physiological phenomena can take place: for example energy can be captured (cytochrome c oxidase) or useful metabolites and hormones can be produced. Heme catalysts are also involved in the detoxification of the organism from xenobiotic compounds as well as reactive oxygen species that can be produced by other heme-containing catalysts.

In this short review, we will focus on a superfamily of heme catalysts involved in many of these processes, depending on the organism and the tissue where they are found but also the conditions where the organism lives.

## **2. Cytochromes P450 as biocatalysts**

Cytochromes P450 are a superfamily of heme-containing enzymes involved in both anabolic and catabolic pathways. They are found in all domains of life, including viruses (Siegel et al., 2007; Lamb et al., 2009) where they play crucial roles in the metabolism of both endogenous and exogenous compounds. In mammals for example, they are involved in steroid hormones, vitamin D and terpenoids biosynthesis as well as detoxifications of xenobiotics including drugs (Ortiz de Montellano, 2015). In plants and insects, they are also involved in chemical defense mechanisms, including herbicide and insecticide resistance (Schuler and Werck, 2003; Mizutani et al., 2013). In bacteria, they are the key players in the adaptation strategies that make bacteria able to use different molecules, including toxic compounds, as fuels for metabolism (Urlacher et al., 2004; Girvan and Munro, 2016;).

The name cytochromes P450 derives from their heme-dependent spectroscopic behavior showing a

typical absorption band at 450 nm when reduced and bound to carbon-monoxide (Omura and Sato, 1964). In 2016 there are 35,166 named P450 sequences in databases, with many organisms having different P450 sequences in their genome, including *Homo sapiens* that has 57 genes coding for these proteins (Nelson, 2009), with the notable exception of *E.coli* that has none.

The chemical diversity of the compounds recognized by these proteins makes them very attractive in the field of biocatalysis. Even more attractive is the fact that these proteins catalyze different reactions. They mainly act as monooxygenases carrying out the following reaction:



Other oxidative reactions carried out by these enzymes include oxidative ester cleavage, dearylation, oxidative ring coupling, ring formation or expansion, aromatic dehalogenation, dearomatization, isomerization (Ortiz de Montellano, 2011; Guengerich, 2001).

In order to perform their reactions, cytochromes P450 require two electrons for each catalytic cycle. The electrons are supplied from NADPH to the cytochrome P450 *via* a reductase that can be a protein domain included in the same polypeptide chain as the catalytic domain or a different and separate protein. According to the organization of electron transfer partners, cytochromes P450 are classified in ten classes (Hannemann et al., 2007). Along with this classification, most of the mammalian cytochromes P450 belong to class II where a cytochrome P450-reductase (CPR) is involved as redox partner. The two proteins are linked to the endoplasmic reticulum membrane through a helix that anchors the protein to the membrane. The CPR carries two cofactors, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Thus, a small redox chain is formed where electrons flow from NADPH to FAD, FMN, heme and O<sub>2</sub> (Figure 1). The electron flow is allowed by a specific protein-protein interaction, based on the complementarity of two faces of CPR and cytochromes P450 carrying opposite charges (Figure 1). Hence, the two proteins behave like dipoles and, once amino acids of opposite charge come close, they can interact forming salt bridges.

### 3. The P450 general fold and catalytic cycle

X-ray crystallography has been widely used to solve the crystal structures of many cytochromes P450 from different sources. The available structures show that these enzymes share a highly conserved fold with a triangular shape, carrying a small domain rich in  $\beta$ -sheets and a larger domain formed mainly by  $\alpha$ -helices. Even if there are small differences in the number and location of  $\alpha$ -helices and  $\beta$ -sheets among different enzymes, most of the secondary structure elements are organized in the same way in the 3D space and heme is located in the core of the protein where the iron atom is bound to a highly conserved cysteine residue. The heme-thiolate ligand motif is

protected and the binding of the cofactor to the protein is also occurring *via* other non-covalent interactions such as hydrogen bonds and electrostatic interactions between the protein arginine residues and the negatively charged heme propionate groups.

The active site of these proteins shows some generally conserved features, such as the presence of important water molecules in the active site of the protein. Figure 2 shows the crystal structure of the heme domain of the bacterial cytochrome P450 BM3, a fatty acid monooxygenase (Nahri and Fulco, 1986). A water molecule is present as sixth ligand for the heme iron that is in the low spin state, as demonstrated by spectroscopic characterization (Lipscomb, 1980; Fisher and Sligar, 1987; Raag and Poulos, 1989). Moreover, this water molecule is part of a network of hydrogen bonds involving both amino acid groups and other water molecules (Figure 2). This network involves also the so-called oxygen activations motif, a highly conserved region where a threonine residue is present and involved in the hydrogen bond with a catalytically important water molecule.

The catalytic cycle of cytochromes P450 is shown in Figure 3 and it has been studied and elucidated through a combination of spectroscopic techniques including x-ray crystallography.

In the first step (step 1), when the substrate access to the active site, in most cases, there is a displacement of the water molecule present as sixth ligand and heme iron becomes pentacoordinated. Also, it moves out of the heme plane of about 0.3 Å and it is in high spin state, as demonstrated by spectroscopic characterization (Figure 3) (Poulos et al., 1985). The change in the iron electronic state upon substrate binding is known to shift the reduction potential toward more positive values, promoting the first electron transfer from the redox partner to the Fe(III) that becomes Fe(II) (step 2 in Figure 3). In the next step (step 3) the molecular oxygen binds the high-spin heme iron, giving a low-spin hexacoordinated iron state, the ferric peroxide ( $\text{Fe}^{3+}\text{-OO}^{\cdot}$ ) (step 3 in Figure 3). In step 4, another electron enters and a negatively charged ferric peroxo anion ( $\text{Fe}^{3+}\text{-OO}^{2-}$ ) forms. The entrance of a first proton leads to the formation of an unstable hydroperoxide intermediate known as “Compound 0” (step 5). The entrance of a second proton leads to the release of a water molecule and a highly reactive oxoferryl species is formed, the so-called “Compound I” ( $\text{Fe}^{4+}=\text{O}$ ) (step 6 in Figure 3). The abstraction of a proton from the substrate generates the so-called “Compound II” ( $\text{Fe}^{4+}\text{-OH}$ ) which hydroxylates the substrate radical according to the radical rebound mechanism (step 7 in Figure 3) (Rittle and Green, 2010). At this point, the substrate is released as product, and the enzyme returns to the resting state with a water molecule in the distal coordination position of the heme iron (step 8 in Figure 3).

X-ray crystallography has largely contributed to the understanding of how the protein scaffold and flexibility contributes to the main steps of the catalytic cycle and to oxygen activation.

First, it is nowadays well known that a reorganization of the active site takes place upon substrate binding and it is generally induced by a conformational change of the protein, mainly involving two helices (F and G) that are connected by a loop (F-G loop) of variable length and flexibility. The

movement of this part of the protein is responsible for opening and closing the substrate access channel through a mouth that is located at the cytosol-membrane interface in those membrane-bound mammalian enzymes that process hydrophobic molecules. Thus, the bioavailability of these compounds is increased in this specific cellular region.

Second, high occupancy reaction intermediates have been generated in the crystalline enzymes at cryogenic temperatures (88-100 K) and rapid diffraction data collection allowed to capture a series of time lapsed pictures of the reaction intermediates at atomic resolution (Schlichting et al., 2000). Moreover, site directed mutagenesis on key residues allowed to understand their role in oxygen activation and catalysis. These studies have been performed on the bacterial cytochrome P450 101 (P450cam), an enzyme able to hydroxylate camphor (Schlichting et al., 2000) and they showed how the enzyme is able to activate molecular oxygen. The crystal structure of the reduced form of cytochrome P450cam, generated by the addition of sodium dithionite to the enzyme crystals, was solved at 1.9 Å resolution (Schlichting et al., 2000) and showed no significant changes compared to the oxidised form of the enzyme.

The crystal structure of the second intermediate of the catalytic cycle (ferrous dioxygen complex), generated by introducing an oxygen flow in the solution containing the protein crystals at cryogenic temperatures (80-100 Kelvin), showed the movement of two residues, Thr252 and Asp251, that creates space to accommodate two new water molecules hydrogen bonded to the oxygen and to the protein backbone (Figure 4A). In particular, the shift of Asp251 allows for a movement in the I helix upon O<sub>2</sub> binding that allows the insertion of the two water molecules involved in H-bonding responsible for O-O bond cleavage. A water molecule is accommodated in the so-called “groove” of helix I and

Few years later, a combination of x-ray crystallography, site directed mutagenesis and activity assays allowed to trap and study the dioxygen complex and to understand how oxygen is activated in order to perform the hydroxylation reaction (Nagano and Poulos, 1995). In these studies, the crystal structure of the dioxygen intermediate shows the importance of Thr252 that promotes the protonation of the oxygen atom (step 5 in Figure 3) with the help of the water molecule present in the groove of helix I. When Thr252 is mutated into alanine (Figure 4B), the enzyme loses its activity. Asp251 is also necessary to have an active enzyme since the mutant Asp251Asn (Figure 4C) is inactive and this can be explained by the fact that, in the mutant, the important water molecule necessary for the formation of Compound I is not present, due to the absence of Asp251 that promotes the movement of helix I that promotes water access into the active site.

In summary, two residues, Thr252 and Asp251, has a concerted role in the active site of the protein. Thr252 accepts a H-bond from the hydroperoxy (FeIII-OOH) intermediate promoting the second protonation on the distal oxygen atom, leading to the O-O bond cleavage and formation of compound I (FeIV=O).

#### **4. Human aromatase: A membrane-bound class II cytochrome P450**

Human aromatase (CYP19A1), also known as estrogen synthase, is a class II cytochrome P450 able to catalyse the conversion of androgens into estrogens in the last step of steroid hormones biosynthesis (Thompson and Siterii, 1974). In particular, the enzyme converts androstenedione, testosterone and 16 $\alpha$ -hydroxytestosterone into estrone, estradiol and estriol, respectively.

It is a membrane bound enzyme present in the endoplasmic reticulum of most tissues and organs of the human body, including ovaries, testis, brain, adipose tissue and bones. Being the enzyme that converts male sexual hormones into females one, the role of the enzyme is crucial in sex dimorphism, development and reproduction. Moreover, the levels of the estrogen products of the enzyme are known to be altered in some pathologies such as breast cancer and endometriosis (Santen et al., 2009). Aromatase inhibitors are currently used for breast cancer treatment in post-menopausal women.

Aromatase is also highly expressed in the brain (Naftolin et al., 1975) and it is part of the so-called neuroendocrine system. High levels of the enzyme are present within different areas of the brain, including hippocampus, amygdala and several other regions of the cerebral cortex (Naftolin et al., 1975; Wagner and Morrell, 1997). Estrogens produced by brain aromatase are known to play important roles for axons growth and migration, regulation of neural plasticity (Davis et al., 1996; Horvath et al., 1999), protection against neurodegenerative pathologies like Alzheimer's and Parkinson's diseases and stroke (Garcia-Segura et al., 2001; Roselli, 2007). Moreover, they influence learning, mood, memory, sexual behaviour and reproductive endocrine functions by acting as neurotransmitters and neuromodulators (Fink et al., 1998; Roepke et al., 2011; Balthazart et al., 2006).

##### **4.1 Crystal structure of human aromatase**

The first crystal structure of full-length placental human aromatase in complex with the substrate androstenedione was published by Ghosh and co-workers in 2009 at a resolution of 2.9 Å and then refined at the resolution of 2.75 Å in 2012 (Ghosh et al., 2009; Ghosh et al., 2012). The 503 amino acids of the polypeptide chain are organised in a fold with a characteristic triangular shape formed by twelve  $\alpha$ -helices (A to L) and ten  $\beta$ -strands (1 to 10), organised in four  $\beta$ -sheets, surrounding a type B heme cofactor (Figure 5A).

The heme cofactor is accommodated in a catalytic pocket of 400 Å<sup>3</sup>, very tight compared to other human cytochromes P450 such as P450 3A4 (530 Å<sup>3</sup>) and 2D6 (540 Å<sup>3</sup>).

The active site of the enzyme (Figure 5B) contains the heme cofactor where the iron atom is bound

to Cys437 and interacts with Arg115, Trp141, Arg375 and Arg435. The peculiarity of the active site of the protein is the presence of a unique proline residue (Pro308) in the helix I that causes a shift in the helix axis necessary to accommodate the androgen substrate (Ghosh et al., 2009).

The 17-keto oxygen of androstenedione forms a hydrogen bond of 2.8 Å with the backbone amide of Met374 and the 3-keto oxygen atom of the substrate forms a hydrogen bond 2.6 Å long with Asp309. In order to do so, this residue was suggested to be protonated at physiological pH.

In 2013 the crystal structure of the amino-terminus truncated recombinant human aromatase (rArom) in complex with the substrate androstenedione was solved at the resolution of 3.29 Å (Lo et al., 2013). The recombinant enzyme shows a compact structure, superimposable to the one of the placental enzyme. However, in order to accommodate substrates as well as inhibitors such as the ones used for breast cancer treatment, some conformational changes must take place at least in those regions involved in ligand access. A combination of methodologies aimed at studying the dynamics and flexibility of proteins in solution was applied to human aromatase to check if the flexibility of the enzyme is affected by ligand binding. In particular, H/D exchange followed by FTIR spectroscopy and time resolved fluorescence were used to measure the kinetics of H/D exchange when aromatase is in the ligand-free form and when complexed with a substrate and an inhibitor (Di Nardo et al., 2013). Our group demonstrated that the flexibility of the protein scaffold is decreased in the presence of a ligand. In particular, the substrate androstenedione and the inhibitor anastrozole induce a significant decrease in the H/D exchange rate constant in  $\alpha$ -helices suggesting a lower flexibility.

#### 4.2 The catalytic mechanism

Among cytochromes P450, human aromatase shows one of the most complicated reaction mechanism involving multiple steps where the enzyme acts as an hydroxylase and a lyase. Aromatase is an example of a very narrow substrate specificity that relies on the architecture of its active site that is complementary to the androgen substrate and justifies the very high affinity for the substrate (nM range) compared to other P450s. Since estrogens are responsible for reproduction and key for life, the reason why this enzyme has a very high substrate specificity and a fine catalytic mechanism can rely on the fact that the enzyme is involved in a vitally important metabolic reaction. The overall reaction requires 3 cycles, each one requiring one mole of molecular oxygen and one mole of NADPH as electrons donor. After a first oxidation at C19 methyl group, aromatase catalyses a second hydroxylation at C19 which removes the 19-*pro*-R hydrogen to yield 19-*gem*-diol. This intermediate spontaneously dehydrates to produce an aldehyde. Then the enzyme acts as a lyase catalysing the oxidative cleavage of C10-C19 bond to produce the aromatic estrogen compound and formic acid (Figure 6).

The mechanism of the final aromatization step is still object of debate and different models have been proposed and reviewed (Di Nardo and Gilardi, 2013). However, the crystal structure shows that the C19 is the closest atom to the heme center and the binding pocket is composed by an oxidation site that includes heme and Thr310 and a protonation site that includes Asp309. This residue is suggested to be involved in an hydrogen bond with the 3-keto moiety of the substrate and also to the proton donor for the final aromatization step.

Thanks to the development of a robust recombinant system of human aromatase and a combination of spectroscopic and site directed mutagenesis studies, our group was able to estimate a pKa of 8.2 for this residue (Di Nardo et al., 2015). Moreover, we demonstrated that the mutant Asp309Asn, where the protonable group of aspartic acid was removed due to the introduction of a non-protonable residue, is inactive indicating a key role in donating the proton to the androgen substrate for the aromatization of the A ring of the steroid (Lo et al., 2013).

One of the question about enzymes catalyzing multi-step reactions is the ability of them to retain and process the substrate and the following reaction intermediates in the active site and only to release the final product (processive reaction) or wether the intermediates, according to their concentration, can freely dissociate and be eventually re-accepted to be further processed (distributive reaction).

This point was addressed for aromatase by pulse chase experiments (Sohl and Guengerich, 2012) as well as bioelectrochemistry (Di Nardo et al., 2015). In the latter case, the enzyme was immobilized on glassy carbon electrodes and electrode-driven catalysis was conducted in the presence of excess of substrate and at a non-saturating substrate concentration. In the first case, the enzyme produced only the first intermediate since it prefers to continue to use this compound rather than processing the first intermediate into the second one. In fact, the affinity for the first substrate is higher than that measured for the first intermediate. When the starting substrate concentration is lowered, the first intermediate formed can compete with it and therefore the second intermediate and the final products are detected. These data showed that the intermediates of aromatase reactions freely dissociate according to the concentrations and therefore aromatase reaction follows a distributive mechanism (Di Nardo et al., 2015).

Another important topic of research in aromatase field is the development of inhibitors for breast cancer treatment. In particular, these drugs are designed to block aromatase activity and, as a consequence, to lower the estrogens levels that are responsible for cancer cells proliferation. Some inhibitors, such as exemastane, are steroidal molecules that compete with the substrate, are processed by the enzyme forming reactive compounds that can covalently bind a nucleophilic site of the enzyme, leading to the irreversible inhibition (Figure 7).

Other non-steroidal inhibitors are currently used but no crystal structure of the enzyme in complex with such inhibitors is available. However, the molecular interaction of aromatase with one of such

molecules (anastrozole) has been studied by Hyperfine Sublevel Correlation (HYSCORE) spectroscopy that show that the N4 atom of the triazole moiety of the drug, directly coordinate the heme iron displacing the water molecule present as sixth ligand (Maurelli et al., 2011). Thus, anastrozole is involved in a quasi-irreversible inhibition of aromatase (Figure 7).

## **5. Concluding remarks.**

Cytochromes P450 are a good example of enzymes with complex finely tuned catalytic mechanism, where function and structure are closely inter-correlated. As a consequence much attention must be paid in not altering their delicate structure when using them for technological exploitation. The wild type enzymes have already been subject of applications in the biosensing, bioanalytical and pharmaceutical areas (Sadeghi et al, 2011; Fantuzzi et al., 2004; Fantuzzi et al., 2011; Panicco et al., 2011).

Their function can also be modulated by mutagenesis changing specific aminoacids by site-directed-mutagenesis and-or by directed-evolution. This allows expansion of the their application in the widest industrial fields.

## References

- Balthazart, J, Ball GF (2006). Is brain estradiol a hormone or a neurotransmitter? *Trends Neurosci*, 29:241-249. doi: 10.1016/j.tins.2006.03. 004
- Choi JM, Han SS, Kim HS (2015) Industrial applications of enzyme biocatalysis: Current status and future aspects. *Biotechnol Adv.* 33:1443-54. doi: 10.1016/j.biotechadv.2015.02.014
- Davis EC, Popper P, Gorski RA (1996) The role of apoptosis in sexual differentiation of the rat sexually dimorphic nucleus of the preoptic area. *Brain Research* 734: 10-18. doi:10.1016/0006-8993(96)00298-3
- Di Nardo G, Breitner M, Bandino A, Ghosh D, Jennings GK, Hackett JC, Gilardi G (2015) Evidence for an elevated aspartate pKa in the active site of human aromatase. *J Biol Chem* 290: 1186-1196. doi: 10.1074/jbc.M114.595108
- Di Nardo G, Castrignanò S, Sadeghi SJ, Baravalle R, Gilardi G (2015) Bioelectrochemistry as a tool for the study of aromatization of steroids by human aromatase. *Electrochem. Comm.* 52:25-28. doi: 10.1016/j.elecom.2015.01.007
- Di Nardo G, Breitner M, Sadeghi S, Castrignanò S, Mei G, Di Venere A, Nicolai E, Allegra P, Gilardi G (2013) Dynamics and flexibility of human aromatase probed by FTIR and time resolved fluorescence spectroscopy. *PLoS ONE* 8: e82118. doi: 10.1371/journal.pone.0082118
- Di Nardo G, Gilardi G (2013) Human aromatase: perspectives in biochemistry and biotechnology. *Biotechnol Appl Biochem* 60:92-101. doi: 10.1002/bab.1088
- Fink G, Sumner BE, McQueen JK, Wilson H, Rosie R (1998) Sex steroid control of mood, mental state and memory *Clin Exp Pharmacol Physiol* 25:764-775. doi:10.1111/j.1440-1681.1998.tb02151.x
- Fantuzzi, A., Fairhead, M. and Gilardi, G. (2004) Direct electrochemistry of immobilised human cytochrome P450 2E1. *J Am Chem Soc* 126:5040 – 5041. doi:10.1021/ja049855s
- Fantuzzi, A., Mak L.H., Capria E., Dodhia, V.R., Panicco, P., Collins. S. and Gilardi, G. (2011) A new standardised electrochemical array for drug metabolic profiling with human cytochromes P450. *Anal Chem* 83:3831–3839. doi:10.1021/ac200309q
- Fisher MT, Scarlata SF, Sligar SG (1985) High-pressure investigations of cytochrome P-450 spin and substrate binding equilibria. *Arch Biochem Biophys* 240:456–463. doi:10.1016/0003-9861(85)90050-5

- Garcia-Segura LM, Azcoitia I, DonCarlos LL (2001) Neuroprotection by estradiol. *Prog Neurobiol.* 63:29-60. doi:10.1016/S0301-0082(00)00025-3
- Ghosh D, Griswold J, Erman M, Pangborn W (2009) Structural basis for androgen specificity and oestrogen synthesis in human aromatase. *Nature* 457:219-23. doi: 10.1038/nature07614.
- Ghosh D, Lo J, Morton D, Valette D, Xi J, Griswold J, Hubbell S, Egbuta C, Jiang W, An J, Davies HML (2012) Novel aromatase inhibitors by structure-guided design. *J Med Chem* 55:8464-8476. doi: 10.1021/jm300930n
- Girvan HM, Munro AW (2016) Applications of microbial cytochrome P450 enzymes in biotechnology and synthetic biology. *Curr Opin Chem Biol.* 31:136-145. doi: 10.1016/j.cbpa.2016.02.018
- Guengerich FP (2001) Uncommon P450-catalyzed reactions. *Curr Drug* 2:93-115. doi: 10.1021/tx0002583
- Hannemann F, Bichet A, Ewen KM, Bernhardt R (2007) Cytochrome P450 systems-biological variations of electron transport chains. *Biochim Biophys Acta* 1770:330-344. doi: 10.1016/j.bbagen.2006.07.017
- Hong Y, Yu B, Sherman M, Yuan YC, Zhou D, Chen S (2007) Molecular basis for the aromatization reaction and exemestane-mediated irreversible inhibition of human aromatase. *Mol Endocrinol* 21:401-414. doi: 10.1210/me.2006-0281
- Horvath TL, Wikler KC (1999) Aromatase in developing sensory systems of the rat brain. *Journal of Neuroendocrinol* 11: 77-84. doi: 10.1046/j.1365-2826.1999.00285.x
- Lamb DC, Lei L, Warrilow AG, Lepesheva GI, Mullins JG, Waterman, MR, Kelly, SL (2009) The first virally encoded cytochrome P450. *J Virol* 83: 8266–8269. doi:10.1128/JVI.00289-09
- Lipscomb JD. (1980) Electron paramagnetic resonance detectable states of cytochrome P-450cam. *Biochemistry* 19:3590–3599. doi: 10.1021/bi00556a027
- Lo J, Di Nardo G, Griswold J, Egbuta C, Jiang W, Gilardi G, Ghosh D (2013) Structural basis for the functional roles of critical residues in human cytochrome P450 aromatase. *Biochemistry* 52:5821-5829. doi: 10.1021/bi400669h
- Lombardi P (1995) The irreversible inhibition of aromatase (oestrogen synthetase) by steroidal compounds. *Curr Pharm Des* 1:23–50. doi:10.1016/S0925-4439(02)00096-0
- Maurelli S, Chiesa M, Giamello E, Di Nardo G, Ferrero VEV, Gilardi G, Von Doorslaer S (2011) Direct spectroscopy evidence for binding of anastrozole to the iron heme of human aromatase.

Peering into mechanism of aromatase inhibition. *Chem Comm* 47:10737-10739. doi: 10.1039/c1cc13872c

Mizutani M, Sato F (2011) Unusual P450 reactions in plant secondary metabolism. *Arch Biochem Biophys* 507:194-203. doi: 10.1016/j.abb.2010.09.026

Naftolin F, Ryan KJ, Davies IJ, Petro Z, Kuhn M (1975) The formation and metabolism of estrogens in brain tissues. *Adv Biosci* 15:105-21.

Nagano S, Poulos TL (2005) Crystallographic study on the dioxygen complex of wild-type and mutant cytochrome P450cam. Implications for the dioxygen activation mechanism. *J Biol Chem* 280:31659-31663. doi:10.1074/jbc.M505261200

Nahri LO, Fulco AJ (1986) Characterization of a catalytically self-sufficient 119,000-dalton cytochrome P-450 monooxygenase induced by barbiturates in *Bacillus megaterium*. *J Biol Chem* 261:7160-7169

Nelson DR (2009) The cytochrome p450 homepage. *Hum Genomics* 4:59-65. doi: 10.1186/1479-7364-4-1-59

Omura T, Sato R (1964) The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification, and properties. *J Biol Chem* 239:2379-2385.

Ortiz de Montellano PR (2015) *Cytochrome P450. Structure, Mechanism, and Biochemistry*. Springer, US.

Ortiz de Montellano PR, Nelson SD (2011) Rearrangement reactions catalyzed by cytochrome P450s. *Arch Biochem Biophys* 507:95-110. doi: 10.1016/j.abb.2010.10.016

Panicco, P., Dodhia, V.R., Fantuzzi, A. and Gilardi, G. (2011) First enzyme-based amperometric platform to determine the polymorphic response in drug metabolism by cytochromes P450. *Anal. Chem.* 83, 2179-2186. doi: 10.1021/ac200119b

Roepke TA, Ronnekleiv OK, Kelly MJ (2011) Physiological consequences of membrane-initiated estrogen signalling in the brain. *Front Biosci* 16:1560-73. doi: 10.2741/3805.

Roselli CE (2007) Brain aromatase: roles in reproduction and neuroprotection. *J Steroid Biochem Mol Biol* 106: 43-150. doi:10.1016/j.jsbmb.2007.05.014

Santen RJ, Brodie H, Simpson ER, Siiteri PK, Brodie A (2009) History of aromatase: saga of an important biological mediator and therapeutic target. *Endocr Rev* 30:343-75. doi: 10.1210/er.2008-0016

- Sadeghi, S.J., Fantuzzi, A. and Gilardi, G. (2011) Breakthrough in P450 bioelectrochemistry and future perspectives. *BBA-Proteins and Proteomics*, 1814, 237-248. doi: 10.1016/j.bbapap.2010.07.010
- Schlichting I, Berendzen J, Chu K, Stock AM, Maves SA, Benson DE, Sweet RM, Ringe D, Petsko GA, Sligar SG (2000) The catalytic pathway of cytochrome P450cam at atomic resolution. *Science* 287:1615-1622. doi:10.1126/science.287.5458.1615
- Schuler Mary A, Werck-Reichhart D (2003) Functional Genomics of P450s. *Ann Rev Plant Biol* 54: 629–667. doi:10.1146/annurev.arplant.54.031902.134840
- Sigel R, Sigel A, Sigel H (2007) *The Ubiquitous Roles of Cytochrome P450 Proteins: Metal Ions in Life Sciences*. Wiley, New York.
- Sohl CD, Guengerich FP (2010) Kinetic analysis of the three-step steroid aromatase reaction of human cytochrome P450 19A1. *J Biol Chem* 285:17734-43. doi: 10.1074/jbc.M110.123711
- Thompson EA Jr, Siiteri PK (1974) The involvement of human placental microsomal cytochrome P-450 in aromatization. *J Biol Chem* 249:5373-8.
- Urlacher VB, Lutz-Wahl S, Schmid RD (2004) Microbial P450 enzymes in biotechnology. *Appl Microbiol Biotechnol* 64:317-325. doi: 10.1007/s00253-003-1514-1
- Valetti F, Gilardi G (2004) Directed evolution of enzymes for product chemistry. *Nat Prod Rep*. 21:490-511. doi:10.1039/b202342n
- Wagner CK, Morrell JI (1997) Neuroanatomical distribution of aromatase mRNA in the rat brain: indications of regional regulation. *J Steroid Biochem Mol Biol* 61:307-314. doi: 10.1016/S0960-0760(96)00237-3