Cytochrome P450 (*cyp*)

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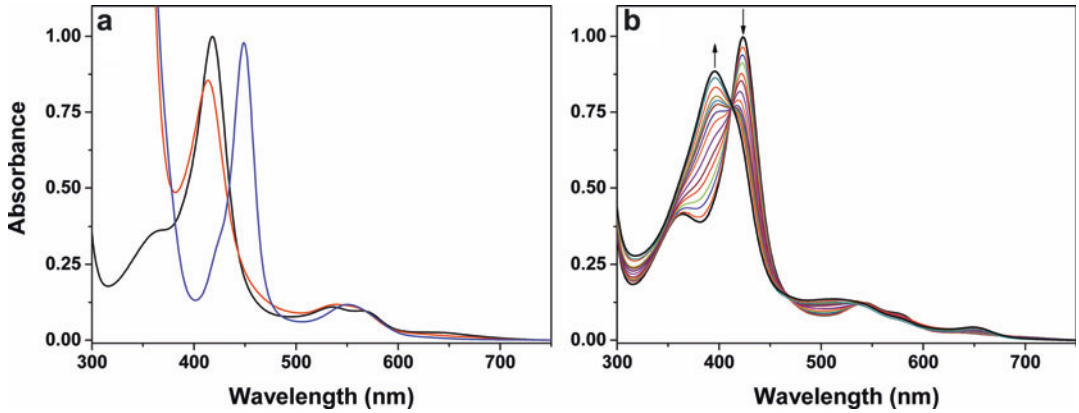
Synonyms

CYP; Cytochrome P450; Mixed function oxidase; Monooxygenase; P450

Historical Background

The cytochromes P450 (P450s or CYPs) form a superfamily of enzymes found in organisms from archaea and bacteria through to man (Munro et al. 2007). P450s were discovered as a consequence of their unusual UV-visible absorbance properties, originating from their heme cofactor, which is bound to the protein through a cysteine sulfur in its thiolate form (Denisov et al. 2005). This heme iron coordination state gives rise to an absorption band at ~450 nm when the P450 heme iron is in the reduced (ferrous) state and bound to the inhibitor carbon monoxide (CO). This absorbance spectrum explains the title P450 (or pigment at 450 nm). Early studies were done independently by Martin Klingenberg and by David Garfinkel (Klingenberg 1958; Garfinkel 1958). This P450 spectrum was first reported by Klingenberg, who prepared rat liver microsomes and then reduced the sample with NADPH (or dithionite) and bubbled it with CO. The P450 spectrum was revealed as an absorption peak at 450 nm in an absorbance difference spectrum produced by subtraction of the reduced (Fe^{II}) spectrum from the CO-bound (Fe^{II}-CO) spectrum (Klingenberg 1958). Subsequent studies by Tsuneo Omura and Ryo Sato demonstrated that the P450s bind heme and that the retention of cysteine thiolate coordination in their Fe^{II}-CO state typically leads to a shift of the heme Soret maximum to ~450 nm (Omura and Sato 1964; Fig. 1a). Moreover, through studies to solubilize membrane-associated P450 from microsomal membranes, Omura and Sato identified a new form of the protein with its Fe^{II}-CO Soret band at ~420 nm (hence P420). The P420 Fe^{II}-CO spectral state is common to P450s that are structurally disrupted or otherwise denatured and is likely due to protonation of the cysteine thiolate to the thiol form (Perera et al. 2003). The process was shown to be reversible in selected P450 enzymes following addition of a P450 substrate or by changing solution pH (Ogura et al. 2004; Dunford et al. 2007; Driscoll et al. 2010). P450s also typically show other characteristic changes on binding (i) substrates and (ii) heme iron-coordinating inhibitors. In case (i), P450 substrate binding often displaces a weakly bound "distal" water ligand that ligates the heme iron on the opposite face to the cysteine thiolate. This induces electronic reorganization in the ferric heme iron 3d orbitals and a switch from a low-spin to a high-spin state that is accompanied by a heme Soret shift from ~418 to ~390 nm. In case (ii), inhibitors such as azole drugs (e.g., ketoconazole, econazole) and nitric oxide displace the distal water and bind tightly to inhibit catalysis, while inducing a Soret band shift to longer wavelength (~425 nm and 434 nm, respectively) (Quaroni et al. 2004; Driscoll et al. 2010).

In early studies, P450s were shown to be monooxygenases involved in the insertion of an oxygen atom from dioxygen (O_2) into substrates including 17-hydroxy-progesterone (by bovine P450c21, now known as CYP21A1) and *D*-camphor (by *Pseudomonas putida* P450cam, CYP101A1 (Cooper et al. 1963; Katagiri et al. 1968). Subsequent research has revealed that the P450s are found in almost all life forms, including mammals (with 57 *CYP* genes in humans), amphibians, fish, plants, algae, insects, bacteria, and archaea (Crespi et al. 1991; Heilmann et al. 1988; Fujita et al. 2004; Rodgers et al. 1993; Ramaswamy et al. 2007; Mayer et al. 1978; Belcher et al. 2014; Yano et al. 2000). CYP genes have also been identified in viruses, presumably obtained from infected host organisms (Lamb et al. 2009). The number of *CYP* genes identified was >35,000 as of April 2016 (Nelson 2016), and numbers continue to



Cytochrome P450 (*cyp*), Fig. 1 UV-visible spectral features of cytochrome P450. Panel (**a**) UV-visible spectra for the heme (P450) domain of the *Bacillus megaterium* P450 BM3 enzyme (CYP102A1, ~10 μM) in its low-spin, oxidized (Fe^{III}) form with Soret maximum at 418 nm (*black*); its dithionite-reduced (Fe^{II}) state with Soret maximum at 409 nm (*red*); and its reduced/CO-bound (Fe^{II}-CO) form with a diagnostic "P450" peak at 449 nm (*blue*). Panel (**b**) Typical substrate binding-induced heme spectral changes observed during a substrate (arachidonic acid)-binding titration of the BM3 heme domain (~10 μM). As substrate binds (in the range from 0 to 25 μM), the ferric P450 undergoes a conversion from the low-spin form (~418 nm, *down arrow*) to the near-fully substrate-bound, high-spin form (~393 nm, *up arrow*). Intermediate spectra at different substrate concentrations are shown, revealing isosbestic points at approximately 406 nm and 465 nm, as well as the development of a small peak at ~650 nm that is characteristic of a cysteine thiolate-to-ferric high-spin heme iron charge transfer transition

grow at a pace with new data emanating from genome sequences. The P450s are now known to have numerous roles across different life forms, including metabolism and interconversion of steroids (Yoshimoto and Auchus 2015), oxidation of fatty acids and eicosanoids (Johnson et al. 2015), drug and xenobiotic metabolism (Guengerich 2006), synthesis of antibiotics (Haslinger et al. 2014), catabolism of unusual carbon sources in microbes (Hedegaard and Gunsalus 1965), and synthesis of alkenes, diterpenoids, and alkaloids (Rude et al. 2011; Matsuba et al. 2015; Farrow et al. 2015).

Classification and Diverse Functions of Cytochromes P450

The advent of genome sequencing resulted in the identification of large numbers of new cytochrome P450 (*CYP*) genes in organisms from all the domains of life. Notable examples include cow (*Bos taurus*) with at least 62 *CYP* genes (Nelson 2015); *Mycobacterium tuberculosis* H37Rv with 20 *CYP* genes, compared to *Mycobacterium leprae* (which has a single functional *CYP* gene and which has undergone extensive gene deletion and decay, resulting in less than half of the genome containing functional genes) (Cole et al. 1998, 2001); the malaria mosquito *Anopheles gambiae* with ~104 *CYP* genes (Holt et al. 2002), tomato (*Solanum lycopersicum*) with ~457 *CYP* genes (including pseudogenes) (The Tomato Genome Consortium (TGC) 2012; Nelson 2012); and ~111 CYP genes in *Aspergillus nidulans* (Nelson 2007). Eukaryotes typically have larger numbers of CYP genes than prokaryotes. However, this is not always true, and the genomes of the bacteria *Streptomyces avermitilis* and *Mycobacterium smegmatis* have 33 and 39 *CYP* genes, respectively (Ikeda et al. 2003, UCSC Microbial Genome Browser), while the eukaryotic protozoan parasite *Leishmania donovani* has only three P450s and the parasite *Trypanosoma brucei* (and other *Trypanosoma* species) may only have two CYP genes based on current data (Nelson 2004; Verma et al. 2011). *S. avermitilis* produces the antiparasitic compounds the avermectins, and it was proposed that around one third of its *CYP* genes are involved in

synthesis of secondary metabolites (Ikeda et al. 2003). The bacterium *Escherichia coli* is devoid of CYP genes but has proven to be an excellent expression system for the production of P450 enzymes from both prokaryotes and eukaryotes (Green et al. 2001; Gillam et al. 1993).

The burgeoning numbers of P450 enzymes identified from gene sequences led to the comparative analysis of the enzymes and their gene sequences and to their classification into the P450 superfamily – the first such attempt to classify related proteins according to their levels of amino acid sequence similarity (Nebert et al. 1987, 1991). P450s are classified into the same family if they share ≥40% amino acid sequence identity with other members of that family, or into the same subfamily if they have ≥55% amino acid sequence identity. Typically, the substrate selectivity is similar for members of the same family, with substrate specificity and reactivity even more similar among subfamily members. Using this system, the 57 human P450s are classified into 18 different families (CYPs 1–5,7,8,11,17–21,24,26,27,39,46 and 51) and then into subfamilies (denoted A,B *etc*), with further numbering indicating the specific identity of the subfamily member. For example, CYP1A1 and 1A2 catalyze typical reactions including benzo[a]pyrene 3-hydroxylation and caffeine N3-demethylation, respectively. CYPs 4B1, 4F12 and 4F22 are lipid hydroxylases, catalyzing lauric acid ω-hydroxylation, arachidonic acid ω- and ω-1 hydroxylation, and vitamin K ω-hydroxylation; while CYP51A1 is a member of the sterol 14-alpha demethylases found across eukaryotes, and which catalyze lanosterol 14a–demethylation *en route* to cholesterol biosynthesis (Guengerich 2015). Plant P450s are currently classified in the CYP51,71–99 and 701–722 families, including P450s with functions including linalool hydroxylation and epoxidation (CYP71B31) in a monoterpene pathway in *Arabidopsis thaliana*, multi-step oxidation of *ent*-kaurenic acid in *A. thaliana* gibberellin synthesis by CYP88A3 and A4, and CYP71AV1 in the medicinal plant *Artemisia annua*, that converts amorphadiene to artemisinic acid in the pathway for synthesis of the sesquiterpene lactone and leading antimalarial drug artemisinin (Schuler 2015). Bacterial and archaeal P450s are classified in the CYP51, 101–299 and 1001–1050 families, with prominent members being the *Pseudomonas putida D*-camphor hydroxylase P450cam (CYP101A1) and the *Bacillus megaterium* fatty acid hydroxylase P450 BM3 (CYP102A1) (McLean et al. 2015; Nelson 2009).

This classification system continues to be used, although the large numbers of *CYP* genes being identified present significant challenges in their accurate assignments. However, other features of P450s lead to simpler routes to different forms of classification. A major schism occurs between eukaryotic P450s and those from bacteria and archaea. The eukaryotic P450s are almost exclusively membrane associated, attached to either the matrix side of the mitochondrial inner membranes in animal P450s (mitochondrial P450s are not a feature of plants and fungi) (Feyereisen 2006) or to endoplasmic reticulum (ER) membranes (Poulos and Johnson 2015). The 57 functional human P450s (not including pseudogenes) consist of 50 "microsomal" P450s located primarily in the ER of liver and other tissues and 7 P450s in the mitochondria of adrenal glands, gonads, liver, kidney, and other sites. The adrenal mitochondrial P450s are involved in the transformation of cholesterol into pregnenolone (CYP11A1) and other steroid oxidations (CYPs 11B1 and 11B2), with CYPs located mainly in liver and kidney mitochondria having roles in 24-hydroxylation of vitamin D3 and 27-hydroxylation of cholesterol in formation of bile acids (CYP27A1), 24-hydroxylation of 25-hydroxyvitamin D3 (CYP24A1), and 1α-hydroxylation of 25-hydroxyvitamin D3 (CYP27B1) (Omura 2006). While the physiological function of the final human mitochondrial P450 (CYP27C1) remains uncertain, recent studies on zebra fish and bullfrog CYP27C1 revealed that the enzyme is a dehydrogenase that converts vitamin A_1 (a rhodopsin and the precursor of 11-*cis* retinal) into vitamin A_2 (a porphyropsin and precursor of 11-*cis* 3,4-didehydroretinal). This causes a red-shift in photoreceptor sensitivity and improves the organisms' ability to see and respond to near-infrared light. The authors suggested one potential

role for the human CYP27C1 ortholog may be in retinoid biosynthesis in skin (Enright et al. 2015).

In contrast, the bacterial/archaeal P450s are almost invariably soluble, cytoplasmic enzymes that lack a membrane-spanning helix (McLean et al. 2015). The eukaryotic P450s have an extended N-terminal region (~30 to 50 amino acids) that crosses the membrane with an ~20 hydrophobic amino acid helical segment that corresponds approximately to the ~3 Å span of the hydrophobic core of the membrane (Andersen and Koeppe 2007). This membrane "tethering" portion precedes the catalytic core structure of the P450 itself, as shown recently by the first crystal structure of a membrane-bound P450 (the *Saccharomyces cerevisiae* sterol 14α-demethylase CYP51A1) with its transmembrane helix intact (Monk et al. 2014). P450s are directed to the mitochondrion by a specific targeting pre-sequence, which is removed by the matrix processing protease, followed by integration of the mature form of the peptide into the mitochondrial inner membrane and exposure of the catalytic core of the P450 to the matrix (Omura 2006). The microsomal and mitochondrial P450s also use distinct redox partner proteins, as explained in the *Cytochrome P450 Redox Partner Systems* section below.

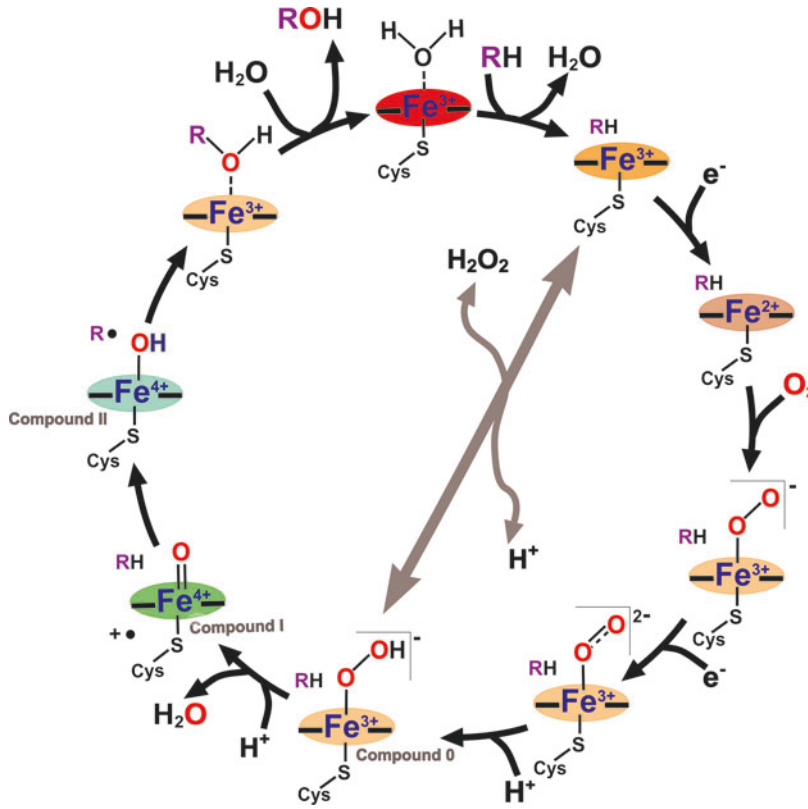
Although P450s are typically classified as monooxygenases, they possess several different catalytic activities dependent on the particular P450 isoform and the substrate involved. A typical P450 catalytic cycle shows how reduction of the P450 ferric heme iron to the ferrous state allows dioxygen (O_2) to bind the iron, followed by a further reduction event and protonation steps that generate the highly reactive compound I (heme Fe^{IV}=O) species that ultimately leads to oxygen insertion into the substrate (Munro et al. 2013; Fig. 2). This is usually shown as substrate hydroxylation, but in reality, the P450s exhibit a much greater range of reactivities and have been described as "Nature's most versatile catalyst" (Coon 2005). Among the numerous reaction types known to be catalyzed by natural and engineered P450s are reduction, decarboxylation, demethylation and dealkylation (including N-, O-, and S-dealkylation), dehydrogenation, epoxidation, N- and S-oxidation, C-C bond cleavage and C-C and C-O bond coupling, nitration, polycyclic aromatic hydroxylation and epoxidation, cyclopropanation, intramolecular amination, oxidative dehalogenation, deformylation of aldehydes, *cis-trans* bond isomerization and molecular rearrangements, aryl dehalogenation and O- and N-dearylation, phosphatidyl choline hydrolysis, and aldoxime dehydration. These and other P450 reactions are used in both catabolic and synthetic reactions in P450s from a range of different organisms (Guengerich 2001; Bernhardt and Urlacher 2014; Guengerich and Munro 2013; Munro et al. 2007). P450s are crucial to mammalian xenobiotic metabolism and steroid biosynthesis, to the production of terpenoids (e.g., limonene and pinene) and alkaloids (e.g., morphine, nicotine, and caffeine) in plants, and for the bacterial catabolism of unusual compounds (e.g., camphor, terpineol, and morpholine) as well as for oxidative processes in the synthesis of several antibiotics (e.g., teicoplanin, vancomycin, erythromycin, and pikromycin). Examples of biomedically and biotechnologically important P450 reactions are described in the *Biotechnological and Biomedical Aspects of P450s* section below.

Structure and Mechanism of Cytochromes P450

The vast majority of P450s perform monooxygenase reactions, catalyzing the reductive scission of molecular oxygen (O_2) and the insertion of a single oxygen atom into a substrate bound in the P450 active site, with the second oxygen atom reduced to water according to the scheme:

$$RH + O_2 + 2e^- + 2H^+ \rightarrow ROH + H_2O$$

In this scheme, RH is the substrate and ROH is the hydroxylated product. However, as described in the *Structure and Mechanism of Cytochromes P450* section above, there are many other catalytic outcomes possible in a P450 reaction. In addition, the apparent simplicity of this scheme belies the

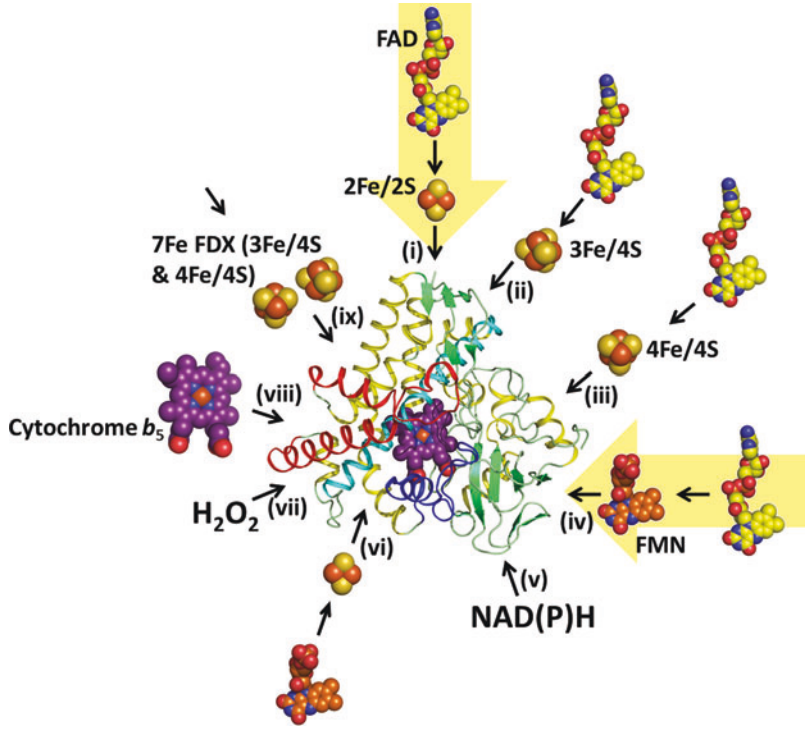


Cytochrome P450 (*cyp*), Fig. 2 The P450 catalytic cycle. The intermediates in the P450 catalytic cycle are shown with heme iron colored to approximate the color of the particular species. In *red* at the *top* is the resting (Fe^{III}) low-spin form of the P450, axially coordinated by cysteine thiolate and the oxygen of a water molecule. Binding of the substrate (RH) displaces the distal water ligand, usually converting the heme iron to the high-spin form. This species is reduced by a redox partner to the Fe^{II} form, which binds dioxygen, forming the ferric-superoxo $(Fe^{III}\text{-}O_2)^-$ complex. A further single electron reduction forms the ferric-peroxo complex $(Fe^{III}\text{-}O_2)^{2-}$, which is then protonated twice in processes involving conserved active site amino acids as proton donors. The first protonation produces the ferric-hydroperoxo $(Fe^{III}\text{-}OOH)^-$ compound 0 form. This species is further protonated, resulting in dehydration of an unstable intermediate to produce the highly reactive (ferryl-oxo, porphyrin radical cation) compound I (Fe^{IV}=O). Compound I abstracts a proton from the substrate, forming compound II (ferryl-hydroxo) and a substrate radical ($R^{\bullet}$). The substrate radical reacts with compound II and is oxidized (hydroxylated in this case). In the final step, the product (RH) dissociates, and a water molecule returns as the sixth ligand to the heme iron. The brown double-headed arrow across the cycle shows how hydrogen peroxide (H_2O_2) can be used to convert substrate-bound P450 directly to compound 0. This peroxide shunt mechanism is used naturally by CYP152 family P450s, including the alkene-producing $OleT_{JE}$ (Rittle and Green 2010; Belcher et al. 2014)

complexity of the P450 reaction cycle, as illustrated in Fig. 2. This capacity of P450s to perform *regio*- and *stereo*-specific oxygenation reactions has led to great interest in the exploitation of P450s for synthetic applications (e.g., in making drug metabolites and steroids) (Girvan and Munro 2016). As a result, their mechanistic, structural, and catalytic properties have been intensively studied. Recent advances include the definitive characterization of short-lived catalytic cycle intermediate species, in particular the high-valent iron-oxo compound I (Rittle and Green 2010). Crucial to P450 reactivity is that the heme prosthetic group (heme *b*) is ligated by a conserved proximal cysteine-thiolate. This facilitates catalysis through its electron-donating character, assisting dioxygen cleavage during the cycle. Cysteine thiolate coordination also contributes to

the distinctive UV-visible spectral properties of P450s (Fig. 1). In the resting form, the ferric (Fe^{III}) P450 heme iron typically has water bound as the sixth (axial) ligand at the distal face. Substrate binding usually displaces the distal water, resulting in an electronic reorganization of the heme iron d-orbitals from a low-spin ($S = {}^{1}/_{2}$) to a high-spin ($S = {}^{5}/_{2}$) form. This is usually accompanied by an increase in the heme iron redox potential, which favors heme iron reduction. Two single electrons are supplied consecutively by redox partners. These reduce the heme iron firstly to the ferrous (Fe^{II}) form (facilitating binding of dioxygen to form a ferric-superoxo species) and then to the ferric-peroxo species. Two successive protonation events produce first a ferric hydroperoxo species (Compound 0) and then the highly reactive ferryl-oxo (Fe^{IV}=O) porphyrin radical cation (Compound I) species following the loss of a water molecule. Compound I abstracts a proton from the substrate, forming a substrate radical and the ferryl-hydroxy (Fe^{IV}=OH) species (compound II). The substrate radical attacks compound II by the radical rebound mechanism (Groves 2006), resulting in the formation of a transiently heme-bound oxygenated product (shown as hydroxylation in Fig. 2) that rapidly diffuses out from the active site and is replaced by a water molecule that coordinates the heme iron to complete the cycle (Denisov et al. 2005). An alternative mechanism to the canonical P450 catalytic cycle is the peroxide shunt pathway that bypasses the need for NAD(P)H-derived electrons by using H_2O_2 (or organic oxidants, such as iodosylbenzene or cumene hydroperoxide) (Fig. 3). This mechanism is used naturally by some P450s, most notably by the "peroxygenases" in the bacterial CYP152 family, where substrate-bound, ferric P450 is converted to compound I (via compound 0) by H_2O_2 in enzymes such as the fatty acid decarboxylase $OleT_{JE}$ (CYP152L1) (Belcher et al. 2014).

The structural features of P450 enzymes are intensively studied in order to understand, e.g., how substrates are bound and oxidized, how inhibitors can be designed, and how P450s can be engineered for novel activities. There are currently over 100 unique P450 crystal structures deposited in the Protein Data Bank (PDB, www.rcsb.org), and recent years have seen large increases in the numbers of structures solved for mammalian and other eukaryotic P450 enzymes. The first structurally characterized P450 was the camphor hydroxylase P450cam (CYP101A1) from *Pseudomonas putida.* This is possibly the most extensively characterized P450 and has provided many important insights into P450 structure/folding and catalytic mechanism (Poulos et al. 1987; Raag and Poulos 1991; Raag et al. 1991). The second P450 structure solved was that of the P450 (heme) domain of the natural P450-cytochrome P450 reductase fusion fatty acid hydroxylase P450 BM3 (CYP102A1, BM3) from *Bacillus megaterium* (Ravichandran et al. 1993). The P450 BM3 heme domain is structurally related to mammalian CYP4 family fatty acid hydroxylases and also catalyzes fatty acid hydroxylation (Miura and Fulco 1974). These prokaryotic enzymes continue to be important model systems in the P450 superfamily, with BM3 (in particular) proving to be a popular system for protein engineering studies aimed at the introduction of novel catalytic activities (Butler et al. 2013). The P450s share a common structural fold that is well conserved regardless of differences in amino acid sequences, with a triangular prism shape and the heme buried in the center of the molecule (Fig. 3, central image). P450s are predominantly α-helical proteins with a smaller amount of β-sheets. The active site is a substrate-binding pocket of variable size and chemical character (suited to the relevant substrate(s)) above the heme prosthetic group on the opposite face from the thiolate ligand. Conserved secondary structural elements such as the substrate-binding and heme-binding regions, the BC- and FG-helix regions, and the I-helix have subtle variations in their amino acid composition, positions, flexibility, and dynamics that contribute to the diversity of P450 substrate types recognized and the enzymatic reactions catalyzed, despite the overall similarities in P450 structural fold (Poulos and Johnson 2015). Unlike their soluble prokaryotic counterparts, eukaryotic P450s are integral membrane proteins with a single N-terminal transmembrane helical region that tethers them to



Cytochrome P450 (*cyp*), Fig. 3 Diversity of P450 redox partner systems. The central panel shows a cartoon representation of the secondary structure of a typical P450 enzyme (the cholesterol oxidizing CYP125A1 from *M. tuberculosis*), with alpha-helices in *yellow*, beta-sheets in *green* and the heme prosthetic group shown with carbons as purple spheres, pyrrole nitrogens in *blue*, and oxygens of heme propionates in *red*. The central iron atom of the heme is shown in *orange*. The I-helix is shown in *blue*, and the FG region (important to substrate recognition) is in *red*. The P450s can obtain electrons from a variety of proteins and chemicals. (i) A class I redox system with electrons donated by NAD(P)H and passed through an FAD-containing reductase and a 2Fe-2S-ferredoxin, as typified by P450cam (CYP101A1) and its putidaredoxin reductase and putidaredoxin partners (Sevrioukova et al. 2010). (ii) A different class I-type system using a 3Fe-4S ferredoxin, as seen in the case of the *M. tuberculosis* sterol demethylase CYP51B1 and its ferredoxin partner (Rv0764c, Fer) (McLean et al. 2006). (iii) Another class I-type system using a 4Fe-4S ferredoxin, as seen for fatty acid hydroxylation by *B. subtilis* P450 BioI (CYP107H1) driven by a NAD(P)H-dependent, FAD-binding reductase (Green et al. 2003). (iv) The class II redox system, with electrons donated by NADPH and passed through FAD and then FMN cofactors in cytochrome P450 reductase (CPR or POR) to the P450. A similar system is seen in the cineole oxidizing P450cin (CYP176A1) from *Citrobacter braakii*, where NADPH-dependent electron transfer to the P450 occurs through a FAD-binding flavodoxin reductase and a FMN-containing flavodoxin (Kimmich et al. 2007). (v) The direct reduction of two molecules of nitric oxide (NO, one of which is bound to the P450 heme iron) by NAD(P)H to form N_2O, as exemplified by the CYP55A1 nitric oxide reductase from *Fusarium oxysporum* (Shoun et al. 2012). (vi) P450 heme iron reduction by NAD(P)H via FMN and 2Fe-2S cofactors contained in the phthalate dioxygenase reductase-like (PDOR) module of CYP116B subfamily P450-PDOR fusion proteins. An example is the thiocarbamate herbicide oxidizing CYP116B1 from *Cupriavidus metallidurans* (Warman et al. 2012). (vii) Direct conversion of substrate-bound P450 ferric heme iron to the compound 0 (ferric-hydroperoxo) form by H_2O_2, as seen naturally in P450 peroxygenases such as the fatty acid decarboxylase $OleT_{JE}$ (CYP152L1) (Belcher et al. 2014). (viii) P450 reduction by cytochrome b_5 (itself reduced by a NADH-dependent cytochrome b_5 reductase) in eukaryotic P450 systems, as exemplified by hydroxylation of 4-propylybenzoic acid by fungal CYP5150A2. Due to its positive potential, it is likely that b_5 delivers only the second electron required for oxidative catalysis, with the other electron likely derived from NADH through the FAD-binding b_5 reductase (Ichinose and Wariishi 2012). (ix) A non-NAD(P)H-dependent archaeal redox partner for *S. acidocaldarius* CYP119A1 that uses pyruvic acid as an electron donor with a CoA-dependent reductase and a 7Fe (4Fe-4S and 3Fe-4S cluster-containing) ferredoxin delivering electrons to facilitate lauric acid hydroxylation (Puchkaev et al. 2002)

endoplasmic reticulum or mitochondrial membranes. This presents problems for expression and purification of intact eukaryotic P450s in heterologous hosts and particularly with respect to isolating P450 samples that can be crystallized for structural analysis. However, these issues have been overcome by the deletion of the N-terminal transmembrane regions in a number of eukaryotic P450s, usually together with introduction of additional amino acid mutations that further improve solubility and/or expression of the truncated P450s. The first eukaryotic P450 crystal structure solved was for the *Oryctolagus cuniculus* (rabbit) progesterone 21-hydroxylase CYP2C5 (Williams et al. 2000), and a number of other eukaryotic P450s have been solved using similar strategies, e.g., rabbit CYP2B4 (Scott et al. 2003). A major breakthrough in eukaryotic P450 crystallography came recently with the determination of the first full-length (membrane-bound) P450 structure of the sterol 14α-demethylase (CYP51F1, also known as *ScErg11p*) from *Saccharomyces cerevisiae* that catalyzes the first step in ergosterol biosynthesis. This structure was obtained from CYP51F1 endogenously overexpressed in *S. cerevisiae* with an engineered His-tag to enable efficient purification. The CYP51F1 structure shows a N-terminal amphipathic helix at the luminal side of the membrane, connected to a transmembrane helix that exposes the P450 catalytic unit on the cytoplasmic side of the lipid bilayer. The P450 substrate-binding region is orientated to face the membrane, from which it likely accesses its natural hydrophobic substrate (lanosterol) (Monk et al. 2014).

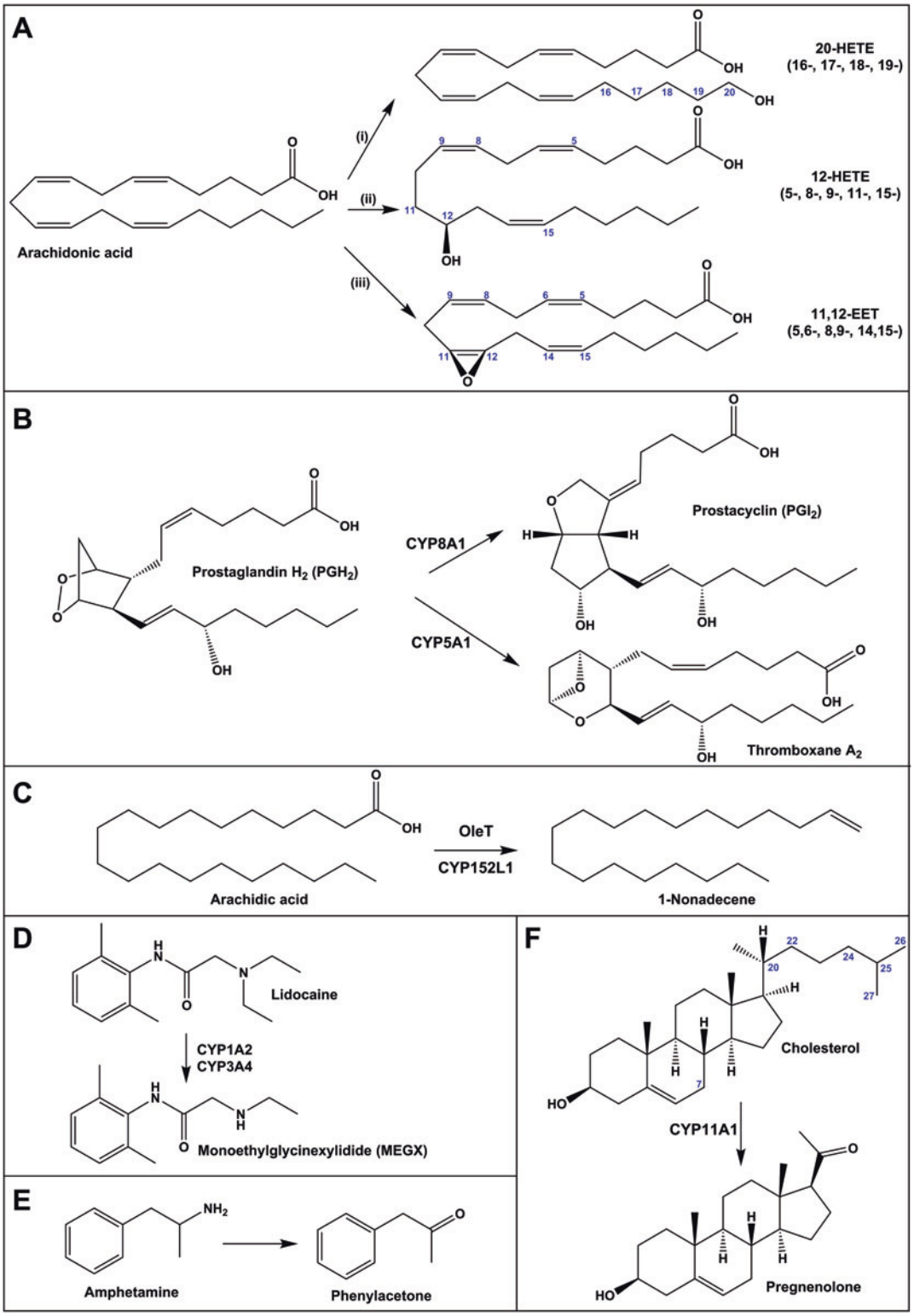
Cytochrome P450 Redox Partner Systems

The majority of P450 enzymes require redox partner proteins for functionality. The redox partners transfer electrons derived from NAD(P)H to the substrate-bound P450 at two different steps in the catalytic cycle (Fig. 2). In a productive reaction, the first electron is transferred from the redox partner to the ferric, substrate-bound form reducing it to the ferrous state. In various P450s, this step may be facilitated by an increase in the heme iron Fe^{III}/Fe^{II} redox potential that occurs due to a substrate-dependent electronic reorganization in the heme iron d-orbitals, converting the ferric low-spin state to a high-spin state (Daff et al. 1997). The ferrous heme iron can then bind dioxygen and form a ferric-superoxo state, which is then further reduced by the redox partner to form the ferric-peroxo form. Subsequent protonation and dehydration steps lead to the production of compound I, the major species responsible for catalyzing P450 substrate oxidation reactions (Rittle and Green 2010). In eukaryotic P450 systems, the redox partner to the microsomal P450s is NADPH-cytochrome P450 oxidoreductase (abbreviated as CPR or POR), which is a diflavin enzyme (containing FAD and FMN cofactors) that is also bound to the ER membrane by an N-terminal transmembrane anchor region (Waskell and Kim 2015; Fig. 3). The FAD and FMN cofactors reside on two distinct domains of the CPR, with the FMN located in a smaller flavodoxin-like N-terminal domain and FAD bound to the larger C-terminal ferredoxin reductase (FDR)-like domain. NADPH binds to the FDR domain, and two electrons are donated to the CPR FAD as a hydride ion (H^-) to reduce this cofactor to its hydroquinone (HQ) state. However, in the cell, it is considered that the CPR FMN cofactor is predominantly in its one-electron reduced semiquinone (SQ) state as a consequence of its high redox potential. The main mechanism for electron transfer from CPR to P450 likely involves a 1-3-2-1 cycle in which the digits reflect the number of electrons on the flavins and in which the FAD HQ passes its two electrons one at a time to the FMN SQ, forming FMN HQ species that donate single electrons to the ferric-superoxo and then the ferric-peroxo species in the catalytic cycle. The FMN HQ has a much lower potential than the FMN SQ, making these reactions feasible, and the CPR inter-domain "hinge" region enables the FMN domain to rotate away from its partner FAD/NADPH-binding domain in order for electron transfers to the P450 (Munro et al. 2013). A similar type of reaction occurs in some soluble, bacterial P450 enzymes, most notably in flavocytochrome P450 BM3 (CYP102A1)

in which a soluble CPR (devoid of a membrane anchor region) is covalently attached to a soluble fatty acid hydroxylase P450. This is a biotechnologically important P450 enzyme that has the highest catalytic rate reported for a monooxygenase P450, primarily due to the very fast electron transfer mediated by its fused CPR partner (Noble et al. 1999). However, the BM3 CPR undergoes a 0-2-1-0 cycle in which the CPR is fully oxidized in the resting state and where electrons are delivered to the P450 by an FMN SQ (Hanley et al. 2004). In animals, the mitochondrial P450 redox partner system has similarities to the apparatus used widely in bacteria and archaea, likely consistent with the proposed endosymbiotic origin of mitochondria (Sagan 1967). The partners are the NADPH-dependent, FAD-binding adrenodoxin reductase (ADR) and the 2Fe-2S iron-sulfur cluster-binding protein adrenodoxin (AD). NADPH reduces the ADR FAD to its HQ state, and two single electron transfers from the ADR reduce two oxidized AD clusters from the $[2Fe\text{-}2S]^{2+}$ to the $[2Fe\text{-}2S]^{+}$ state. AD molecules then reduce substrate-bound mitochondrial P450s (primarily involved in steroidogenesis) at the same catalytic cycle stages as described for CPR above (Grinberg et al. 2000).

The CPR (class II) and ADR/AD (class I) redox partner systems represent the major pathways of P450 electron transfer in higher organisms. However, studies in recent years have unveiled a greater diversity of P450 redox partner systems in microbes. These include bacterial/archaeal systems that use FDRs along with different types of ferredoxins (containing 3Fe-4S and 4Fe-4S clusters). For example, an unusual P450 redox partner system was characterized in the thermophilic archaeon *Sulfolobus acidocaldarius* (formerly *S. solfataricus*), involving the P450 (CYP119A1), a 2-oxo-acid ferredoxin reductase, and a 7Fe (3Fe-4S and 4Fe-4S)-containing ferredoxin. Hydroxylation of lauric acid at 70 °C was demonstrated using pyruvic acid as an electron source (Puchkaev and Ortiz de Montellano 2005). Another example is the cineole degrading P450cin (CYP176A1) from *Citrobacter braakii*, in which an FMN-binding flavodoxin (cindoxin) replaces the ferredoxin in a novel class I system and where the P450 catalzyes enantioselective hydroxylation of the monoterpenoid 1,8-cineole (eucalyptol) to form (1*R*)-6β-hydroxycineole in the pathway to its degradation and use as a carbon source by *C. braakii* (Stok et al. 2015; Fig. 3).

Other bacterial P450s operate without redox partners, including the fatty acid hydroxylase $P450SP_{\alpha}$ (CYP152B1) from *Sphingomonas paucimobilis* and the alkene-generating fatty acid decarboxylase $OleT_{JE}$ (CYP152L1) from *Jeotgalicoccus* sp. 8456, which use hydrogen peroxide directly to form the reactive catalytic cycle intermediate compound 0 (ferric-hydroperoxo) and to bypass requirement for redox partner-mediated electron transfers (Fujishiro et al. 2011; Belcher et al. 2014; Fig. 4). In the case of P450nor (CYP55A1) from the pathogenic fungus *Fusarium oxysporum*, the enzyme binds NADH in the P450 active site and reduces two molecules of nitric oxide (nitrogen monoxide, NO) to form nitrous oxide (dinitrogen monoxide, N_2O) as part of an anaerobic respiratory process for conversion of nitrite to N_2O (Shoun et al. 2012). Other novel systems include the bacterial CYP116B family, in which the P450s are naturally fused to a phthalate dioxygenase reductase-like FMN- and 2Fe-2S cluster-containing reductase system. These enzyme favor NADPH over NADH as the electron donor, with examples including the *Cupriavidus metallidurans* CYP116B1 that was shown to hydroxylate thiocarbamate herbicides (Warman et al. 2012) and *Acinetobacter radioresistens* CYP116B5 that oxidizes alkanes and enables the bacterium to grow on medium-to-long chain alkanes as a sole carbon source (Minerdi et al. 2015). The white-rot basidiomycete fungus *Phanerochaete chrysosporium* was also reported to hydroxylate 4-propylbenzoic acid using the P450 CYP5150A2 and cytochrome b_5 (b_5) and b_5 reductase redox partners with NADH reductant. Similar data were presented for b_5 reductase and b_5 in supporting activity of hepatic microsomal P450s. Given the positive redox potential of b_5 proteins (ca 0 mV vs. the normal hydrogen electrode, NHE), it appears likely that they deliver only the second electron (to the high potential P450 ferric-peroxo catalytic cycle intermediate),



Cytochrome P450 (*cyp*), Fig. 4 (continued)

with the first electron coming from the b_5 reductase (Ichinose and Wariishi 2012; Henderson et al. 2013; Fig. 3). Other unusual P450s include fusions to other potential redox partners and to non-redox partner proteins (McLean et al. 2015).

Biomedical and Biotechnological Aspects of P450s

The P450s catalyze numerous important chemical reactions in humans and other organisms. They are crucial to xenobiotic detoxification and steroid metabolism in mammals and are also key to the production of valuable compounds such as flavors and fragrances (e.g., the sequiterpene (+)-nootkatone), the anti-cancer monoterpene-indole alkaloid drug vinblastine in plants (Cankar et al. 2011; Butler 2008), and antibiotics including the anthelmintic drug avermectin in microbes (Ikeda et al. 1999). As knowledge develops on structural and catalytic properties of P450s, interest has also increased in the engineering of P450s and their applications for producing valuable molecules.

Several mammalian P450 enzymes have pivotal roles in steroid metabolism, with steroid synthesis beginning in the mitochondrion with a three-step oxidation reaction catalyzed by the cholesterol side chain cleaving enzyme CYP11A1 (P450scc) that transforms cholesterol into pregnenolone as the committed step (Fig. 4). Human CYP11A1 deficiencies are very rare but effectively abolish steroid production in gonads and the adrenal cortex (Tajima et al. 2001). Mitochondrial CYP11B1 catalyzes 11β-hydroxylation of deoxycorticosterone to form corticosterone, as does CYP11B2 (aldosterone synthase). Mutations in CYP11B1 result in a form of congenital adrenal hyperplasia called 11β-hydroxylase deficiency, in which the mineralocorticoid deoxycorticosterone accumulates. This results in the masculinization of females due to rerouting of cortisol precursors to androgens. Males and females develop hypertension and low serum potassium later in life and are also prone to adrenal crisis in which low blood

Cytochrome P450 (*cyp*), Fig. 4 Typical P450 reactions. Panel (**a**) Arachidonic acid (released from membrane glycerophospholipids by the action of phospholipase A2 enzymes) is the substrate for P450s that either epoxidize or hydroxylate the substrate to create epoxyeicosatrienoic acids (*EETs*) or hydroxyeicosatetranoic acids (*HETEs*), respectively. Arachidonic acid is metabolized to different products by various eukaryotic P450 subfamily members. Scheme (i) shows formation of 20-HETE (CYP2U1, CYP4A, CYP4F) through a ω,ω-1 hydroxylase reaction. Scheme (ii) shows formation of the mid-chain HETE 12(*R*)-HETE in a lipoxygenase-like P450 reaction (CYP2C9, CYP1B1) .Scheme (iii) shows formation of the epoxyeicosatrienoic acid 11,12-EET (CYP2C8, CYP2J2) in an epoxygenase P450 reaction. Other products formed in these different types of reactions are indicated in parentheses (Edin et al. 2015). Panel (**b**) Arachidonic acid is also metabolized by cyclooxygenase to form prostaglandin G_2 (PGG_2), which rearranges to produce prostaglandin H_2 (PGH_2). PGH_2 is the substrate for the prostacyclin synthase (CYP8A1) and thromboxane synthase (CYP5A1) P450s that catalyze molecular rearrangement reactions to cleave the epidioxy bond of PGH_2 to form prostacyclin (PGI_2) and thromboxane A_2, respectively (Hecker et al. 1987; Hecker and Ullrich 1989). The reaction requires neither electrons from NAD(P)H/redox partners or dioxygen. PGI_2 has vasodilatory and platelet anti-aggregation effects, whereas thromboxane A_2 induces vasoconstriction and platelet aggregation. The balance between these reactions is thus important in human health (Munro et al. 2007). Panel (**c**). The oxidative decarboxylation of arachidic acid (C20:0) to 1-nonadecene catalyzed by the P450 peroxygenase $OleT_{JE}$ (CYP152L1) from *Jeotgalicoccus* sp. ATCC 8456 (Belcher et al. 2014). Panel (**d**) The N-deethylation of the antiarrhythmic drug lidocaine by CYP1A2 and CYP3A4, producing the metabolite monoethylglycinexylidide (*MEGX*) (Wang et al. 2000). Panel (**e**) The deamination of amphetamine to phenylacetone catalyzed by rabbit CYP2C3 (Yamada et al. 1997). Panel (**f**) The conversion of cholesterol to the steroid hormone pregnenolone by the side-chain cleavage enzyme CYP11A1 (P450scc). CYP11A1 performs two successive hydroxylations at the C20 and C22 positions, followed by a carbon-carbon bond cleavage reaction between C20-C22 to generate pregnenolone and the side product 4-methylpentanal. In other P450-dependent reactions, cholesterol is hydroxylated at positions 7a, 24(s), 25, and 27 by CYP7A1, CYP46A1, and CYP3A4, respectively, in the production of bile acids. The C26 hydroxylation of cholesterol and its ketone derivative 4-cholesten-3-one is also catalyzed by *Mycobacterium tuberculosis* P450 enzymes (CYP125A1 and CYP142A1) in the first step of host cholesterol catabolism that is important for infection by *M. tuberculosis* and for its survival in the host macrophage (McLean et al. 2012)

pressure results from cortisol deficiency (Auchus and Miller 2015). CYP17A1 (a steroid 17-hydroxylase/17,20-lyase) is a microsomal P450 that catalyzes both the 17-hydroxylation of pregnenolone and progesterone and the 17,20-lyase reaction with the hydroxylated steroids to form dehydroepiandrosterone (DHEA) and androstenedione, respectively. Mutations cause effects that range in severity from partial loss of 17-hydroxylase or 17,20-lyase activity through to complete, combined activity loss. The loss of 17,20-lyase activity prevents synthesis of androgens and estrogens, with catastrophic effects on male and female sexual development (Auchus and Miller 2015). Hepatic P450s are also crucial to oxidative transformations of drugs and other xenobiotics, usually leading to their inactivation and excretion. However, a number of these P450s are also implicated in the activation of chemical carcinogens, including polycyclic hydrocarbons (CYP1A1 and 1B1) and nitrosamines (CYP2A6 and 2E1) (Guengerich 2015). For example, CYP1A1 catalyzes successive hydroxylation and epoxidation reactions on benzo[a]pyrene that transform this cigarette smoke component to carcinogenic and genotoxic (+) and (−) benzo[a] pyrene-7,8-diol-9,10-epoxide products (Shimada and Fujii-Kuriyama 2004). Defects in other human *CYP* genes are associated with diseases such as rickets (*CYP2R1*, *CYP27B1*) and hypercholesterolemia (*CYP7A1*) (Pikuleva and Waterman 2013).

Important biotechnological applications of P450s include the engineering of these enzymes, using either directed evolution (DE) approaches (i.e., random or semi-random mutagenesis of the relevant P450 combined with screening of mutants for desired properties) or structure-guided mutagenesis to produce novel variants with desirable catalytic properties. The high activity mid- to long-chain fatty acid hydroxylase P450-CPR fusion enzyme P450 BM3 has proven an excellent template for such programs. Notable successes include BM3's conversion to a hydroxylase of short chain alkanes and to an enantioselective epoxidase of terminal alkenes using DE approaches (Peters et al. 2003; Kubo et al. 2006). Other DE and rational mutagenesis studies have produced BM3 mutants that generate human drug metabolites that replicate those produced by the relevant human P450s. Examples include metabolites of the gastric proton pump inhibitor (PPI) omeprazole and of other PPI drugs (Butler et al. 2013, 2014) and of diverse drugs such as chlorzoxazone, lidocaine, and diclofenac (Ren et al. 2015). The effective production of bona fide human drug metabolites has become increasingly important due to FDA requirements for toxicity testing of major human drug metabolites as well as their parent drugs (Guengerich 2015). In other work, Frances Arnold's group demonstrated that BM3 could be used to catalyze the asymmetric cyclopropanation of styrenes, with the efficiency of the reaction enhanced considerably in the T286A mutant in which a residue crucial to efficient monooxygenation chemistry is substituted (Coelho et al. 2013a). Cyclopropanation of olefins is widely used in fine chemical synthesis, and further studies showed more efficient cyclopropanation of stytrenes both in vivo (*E. coli* cells) and in vitro, using the diazoester ethyl diazoacetate for carbene transfer in a BM3 variant in which the cysteine thiolate ligand was mutated to a serine, resulting in a large increase in potential of the heme iron and abolition of monooxygenase activity (Coelho et al. 2013b). A quadruple mutant of BM3 was also used for the conversion of amorpha-4,11-diene to its epoxide in a semisynthetic pathway for the production of the antimalarial artemisinin (Dietrich et al. 2009). Eukaryotic P450s also provided a test case for Stephen Sligar and colleagues in the development of nanodisc technology. Nanodiscs are discoidal membrane bilayers that are stabilized and made soluble in aqueous solution through their being encircled by two amphipathic helical protein belts known as membrane scaffold proteins. Purified, detergent solubilized P450s and other membrane proteins are typically assembled into nanodiscs by mixing with scaffold protein and phospholipids at appropriate stoichiometry, followed by detergent removal using hydrophobic beads (Denisov and Sligar 2016). Examples of the use of this novel technology on P450 redox systems include its application for the analysis of the influence of

the nanodisc membrane bilayer and its lipid composition on the redox potentials of the CPR flavin cofactors and to explore the complex catalytic mechanism of the CYP17A1 P450 that converts pregnenolone to dehydroepiandrosterone (Das and Sligar 2009; Gregory et al. 2013). Other recent studies have demonstrated how light can be used to drive P450 function directly though covalent attachment of a Ru^{II}-diimine complex to the BM3 heme domain. The ruthenium "photosensitizer" is continuously reduced photocatalytically to the Ru^{I} state, which passes electrons to the heme iron and enables fatty acid hydroxylation (Tran et al. 2013). The cyanobacterium *Synechocystis* sp. PCC6803 was also engineered by introduction of a vector encoding two plant P450s (CYP79A1 and CYP71E1) and a glycosyltransferase that transform *L*-tyrosine into the cyanogenic glucoside and insect antifeedant dhurrin. The P450s were successfully incorporated into the cyanobacterial thylakoid membranes, close to photosynthetic complexes, and catalysis was driven by light though electron transfer via photosystem I and ferredoxin to the P450s, producing both dhurrin and the pathway precursor *p*-hydroxyphenylacetaldoxime (Wlodarczyk et al. 2016).

Summary

The P450s are remarkable catalysts that are able to perform a myriad of different chemical reactions. They span all the major domains of life and were the first protein group classified into an enzyme "superfamily". P450s have pivotal roles in animal health, particularly with respect to the metabolism of steroids, other lipids, and xenobiotics. P450 deficiencies are implicated in several human diseases, and the activities of mammalian xenobiotic metabolizing P450 enzymes with compounds such as arylamines and polycyclic aromatic hydrocarbons can result in the activation of these chemical carcinogens. In plants and microbes, P450s have diverse roles in the synthesis of molecules such as terpenoids, alkaloids, macrolide antibiotics, and mycotoxins. Large numbers of P450s are found in *Streptomyces* and other bacteria, where they are involved in the production of industrially important molecules such as the antibiotics erythromycin and vancomycin and the anthelmintic avermectin. Numerous P450s are also encoded in the genome of the pathogen *Mycobacterium tuberculosis*, where they are implicated in bacterial secondary metabolism, in regulation of respiration through modification of respiratory menaquinone, and in bacterial infection and survival in the macrophage through their role in catabolism of host cholesterol. The astonishing array of oxidative and other activities associated with P450s has been further expanded through characterization of the properties of engineered variants of biotechnologically important P450s, and these enzymes are increasingly utilized in the industrial scale production of high-value compounds, such as the antimalarial artemisinin and the cholesterol-lowering drug pravastatin (Dietrich et al. 2009; McLean et al. 2014). The diverse roles of the P450s in areas such as human and animal health, biotechnology, and synthetic biology reveal "Nature's most versatile catalyst" to be of economic importance, with several potential applications in areas such as chemical synthesis, bioremediation, and human medicine (Coon 2005; Rylott et al. 2011).

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Cytokine Responsive 6 (CR6)

▶ GADD45

Cytosolic NADP-Isocitrate Dehydrogenase

▶ IDH1 (Isocitrate Dehydrogenase 1)

Cytosolic Phospholipase A2 (pla2G4A)

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Synonyms

cPLA2; Group IV phospholipase A2

Historical Background

The phospholipase A2 was the first of the phopsholipases to be identified when, in 1877, Bokay observed that the lecithin was degraded by a ferment obtained from pancreatic juice. Subsequently, in 1902, this enzyme, known as lecithinase, was detected also in cobra venom where it was observed to induce hemolytic activity through the lysis of erythrocytes membrane. The enzyme was then isolated from human pancreatic tissue by Gronchi and colleagues in 1936 (Glaser and Vadas 1995; Vance and Vance 2002). Since then, an increasing number of PLA2 have now been identified and grouped according to their biochemical features. However, the first evidence that mammalian cells contain a cytosolic calcium-dependent PLA2 able to specifically cleave arachidonic acid was reported only 50 years later by Flesch in 1985 (Flesch et al. 1985) and Alonso in 1986 (Alonso et al. 1986). Subsequently, important information regarding the role of cPLA2s in physiological processes and disease was provided by the knockout mouse model that revealed that cPLA2α knockout mice have normal growth and lifespan but exhibit an age-related renal dysfunction, ulcerative lesions of the small intestine, enlarged heart, and female reproduction defects demonstrating that metabolites generated by cPLA2s catalytic activity regulate several normal physiological processes (Bonventre et al. 1997; Downey et al. 2001; Uozumi et al. 1997; Takaku et al. 2000).

Introduction

Phospholipases are a ubiquitous group of enzymes that hydrolyze phospholipids to generate molecules that may have potent biological activity. Phospholipids are dynamic molecules localized within lateral phases of membrane bilayers and in subcellular organelles. Phospholipids breakdown mediated by phospholipases generates both hydrophobic and hydrophilic molecules that can act at the site of production, at distal site within the cell or can be secreted and act outside the cell. Many of them exert their cellular function

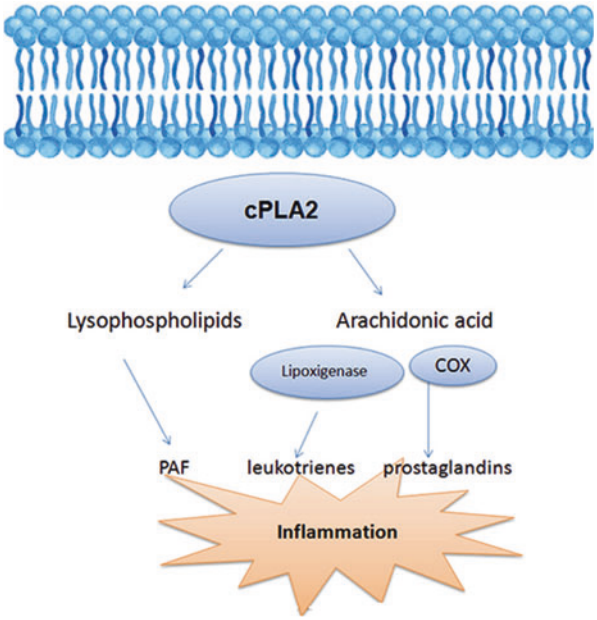
through extracellular or intracellular receptors. The classification of phospholipases is based on the site of attack. Phospholipases that catalyze the hydrolysis of the acyl-ester group are classified as phospholipase A (PLA): PLA1 hydrolizes the 1-acyl ester bond of phospholipids and PLA2 the 2-acyl ester bond. Some phospholipases hydrolize both acyl ester group and are known as phospholipase B. Phospholipase C (PLC) and phospholipase D (PLD) are both phosphodiesterases and catalyze the cleavage of glycerophosphate bond and the removal of the base group, respectively (Vance and Vance 2002). PLA2 that releases fatty acids from the second carbon group of glycerol specifically recognizes the sn-2 acyl bond of phospholipids and catalytically hydrolyzes the bond releasing arachidonic acid and lysophospholipids. In particular, arachidonic acid is converted in inflammatory mediators such as prostaglandins and leukotrienes which are categorized as potent inflammatory mediators implicated in many disorders such as asthma and arthritis. However, it can also directly modulate cellular function by altering membrane fluidity, activating protein kinases, and regulating gene transcription (Katsuki and Okuda 1995). On the other hand, lysophospholipids are involved in the control of phospholipid remodeling and membrane perturbation. Thus, PLA2 activity is tightly regulated in order to maintain levels of arachidonic acid and lysophospholipids necessary for the correct cellular homeostasis (Katsuki and Okuda 1995; Vance and Vance 2002). To date in humans 17 genes and 25 PLA2 isoforms have been identified. PLA2s can be distinct in groups on the base of their specific features such as sequence, molecular weight, disulfide bonding patters, and Ca^{2+} dependency. They are: (1) the secreted *small* molecular weight PLA2s (sPLA2s), (2) the Ca^{2+}-independent PLA2s (iPLA2s), (3) the larger cytosolic Ca^{2+}-dependent PLA2s (cPLA2s), (4) the platelet-activating factor-acetylhydrolases (PAF-AH), and (5) the lysosomal PLA2s. Differently from other PLA2 isoforms, considerable information is available about cPLA2 structure, function, and mechanisms of regulation (Gentile et al. 2012).

Nomenclature and Structure

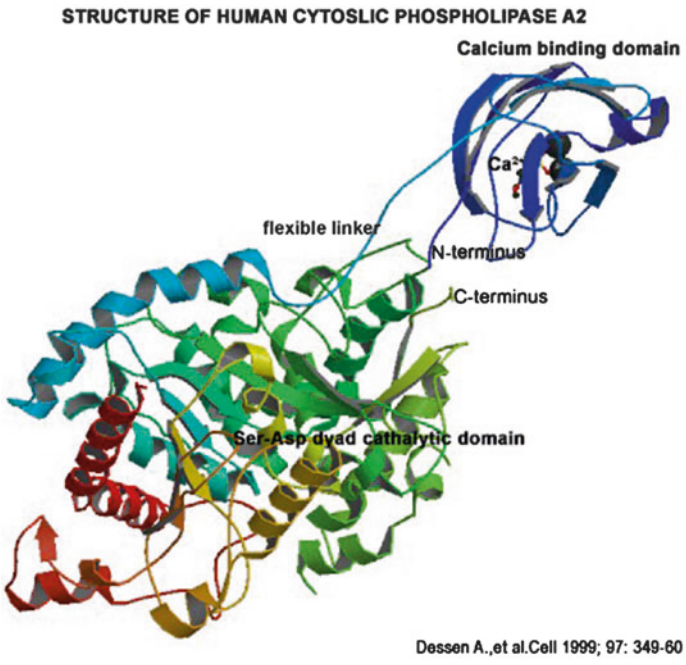
The cytoplasmic-calcium dependent PLA2 (cPLA2), also referred to as group IV PLA2, is a family of enzymes containing six members: IVA (cPLA2α), IVB (cPLA2β), IVC (cPLA2γ), IVD (cPLA2δ), IVE (cPLA2ε), and IVF (cPLA2ζ). The groups IVB, C, E, and F are encoded by a gene cluster on human chromosome 15, whereas the cPLA2α is encoded by a gene on human chromosome 1 and cPLA2γ on chromosome 19. Among the members of the group IV PLA2, the cPLA2α is the most studied since it specifically recognizes the sn-2 acyl bond of arachidonic acid to generate prostaglandins and leukotrienes, important lipid inflammatory mediators implicated in many disorders such as asthma and arthritis (Fig. 1). It is highly conserved throughout evolution since human and mouse homologues share more than 95% of aminoacid identity and more that 80% with zebrafish and *Xenopus laevis* (Ghosh et al. 2006). X-ray crystal structure of cPLA2s revealed that they contain an N-terminal calcium-dependent lipid binding/C2 domain that promotes interaction of the protein with membranes and a catalytic domain (Fig. 2) (Dessen et al. 1999).

Catalytic Domain

Classical lipase activity is exerted by a catalytic domain containing a Ser-Asp/Gln-His triad; however, the group IV PLA2 family catalytic domain lacks of the His residue, thus it is composed by an unusual Ser-Asp dyad located in a deep cleft lined by hydrophobic residues. This funnel is covered by a flexible lid that moves to allow the access of the substrate to the active site (Dessen et al. 1999). This Ser-Asp dyad catalytic domain is highly conserved throughout evolution since it has been described also in the plant lipid acylhydrolase patatin and in phospholipase from *Pseudomonas aeruginosa* (Ghosh et al. 2006; Rydel et al. 2003). The serine (Ser228 in cPLA2α) is present in the pentapeptide sequence G-L-S-G-S that is similar to the lipase consensus sequence G-X-S-X-G. A conserved arginine (Arg200 in cPLA2α) is also required for catalysis (Dessen et al. 1999). Comparative structural analysis of the catalytic domain



Cytosolic Phospholipase A2 (pla2G4A),
Fig. 1 Cytosolic PLA_2 function. $cPLA_2$ catalyze the hydrolysis of fatty acids from the sn-2 acyl bond of phospholipids releasing arachidonic acid and lysophospholipids. In particular, arachidonic acid is converted in inflammatory mediators such as prostaglandins and leukotrienes which are categorized as potent inflammatory mediators implicated in many disorders such as asthma and arthritis



Cytosolic Phospholipase A2 (pla2G4A),
Fig. 2 Structure of the human cytosolic PLA_2. The diagram has been obtained from the protein data bank (http://www.rcsb.org/pdb/)

of the enzymes belonging to the group IV PLA2s revealed that it is essential for the arachidonoyl selectivity. In fact, the only differences into the catalytic domain are in two residues between cPLA2α and cPLA2γ. These differences may be responsible for the lack in specificity towards arachidonic acid and in sensitivity to inhibitors such as pyrrolidine-2 (Ghosh et al. 2006).

Calcium-Binding Domain

It is necessary the mobilization of intracellular calcium to obtain the maximal cathalytic activity of the enzyme. Calcium mobilization is mediated by an N-terminal calcium-dependent lipid-binding domain that colocalizes the catalytic domain with its membrane substrate (Ghosh et al. 2006). The calcium-binding domain of the cPLA2s is a classical C2 domain, present in a variety of mammals' proteins, whose function is primarily to promote the interaction between protein and membrane. A C2 domain is composed of about 120 aminoacids that share a common fold of eight antiparallel β-sheet. Structural and functional analysis of the C2 domain of cPLA2α revealed that it contains three calcium-binding loops (CBL) that bind two calcium ions each through two acidic residues such as Asp and Asn. In the unbound state, the membrane-binding face of the C2 domain is electronegative due to the presence of these two residues, and do not interact with membranes. The binding with calcium ions determines the so-called electrostatic switch of the C2 domain that can bind anionic phospholipids in membranes. In particular, calcium binding to the C2 domain of cPLA2s promotes the interaction with phosphorylcholine rather than to anionic phospholipids. Alignment of the C2 domains of the members of the group IV of phospholipases revealed that cPLA2α contains seven calcium-binding residues, four of which are conserved in the other members of the group. The conserved residues are present in CBL1 and CBL 2 and are crucial for the binding to the membrane phospholipids. Another important structural difference between cPLA2α and the other members of the IV group is represented by the length of the linker that connects the catalytic domain to the C2 domain: in cPLA2α the two domains are connected by a 5-residues flexible linker that may undergo rotational changes affecting the interaction of the catalytic domain with the membrane, whereas in other cPLA2s the C2 and catalytic domain are connected by an approximately 120-residues linker that can influence both membrane-binding properties and enzyme tridimensional conformation (Dessen et al. 1999; Ghosh et al. 2006).

Regulation

Expression

cPLA2α is encoded by a gene on human chromosome 1 next to the gene encoding COX2; it is ubiquitous and constitutively expressed in human cells. Its expression is enhanced by proinflammatory cytokines and growth factors and is inhibited by glucocorticoids as indicated by the presence of the INF-γ and glucocorticoid responsive elements on its promoter (Miyashita et al. 1995). Studies demonstrated that in smooth muscle cells cPLA2α expression is regulated by STAT-3 (Ghosh et al. 2006) and, moreover, that in several types of cancer it is overexpressed and upregulated by the oncogene *ras* through the phosphorylation of the kinases JNK and ERK (Van Putten et al. 2001).

cPLA2β gene is on human chromosome 15 near a gene cluster that encodes cPLA2γ, ε, and ζ and separated from this cluster by the genes Sptbn5 and Ehd4. These cPLA2s are highly homologous sharing 45–50% residues in the catalytic domain suggesting that they are arisen from an ancestral gene by duplication (Ohto et al. 2005). cPLA2β mRNA is widely expressed in human pancreas, liver, heart, and cerebellum, and its gene is immediately downstream of a complete JmjC domain which is a metalloenzyme present in nuclear protein with the ability to bind DNA. This implies that cPLA2β mRNA may undergo complex transcriptional and splicing regulation resulting in the production of diverse proteins. In fact, when the JmjC domain is completely transcribed, cPLA2β lacks C2 domain and is not able to bind membranes (Ghosh et al. 2006). cPLA2δ was found to be expressed in stratified squamous epithelium of the fetal skin and is significantly increased in the upper epidermis of psoriatic lesions. cPLA2δ gene encodes a 90 kDa protein which contains both C2 and catalytic domains (Chiba et al. 2004). cPLA2γ gene is

on chromosome 19, and its mRNA is present in skeletal and cardiac muscle and in brain (Ghosh et al. 2006).

Localization

cPLA2s exert their activity mainly on membranes where they access the substrate. Upon stimulation, intracellular calcium concentration increases and induces the enzyme translocation from cytosol to membrane. cPLA2α has been shown to translocate primary to the perinuclear envelope, to Golgi, and to the endoplasmic reticulum. In particular, it has been shown that short-duration and low-concentration calcium transients induce translocation to Golgi, whereas high-concentration calcium transients induce translocation to ER. Moreover, CBL1 and CBL3 are critical for the specific targeting of cPLA2α to the Golgi apparatus. The localization to Golgi regulates Golgi architecture and membrane-trafficking events (Dessen et al. 1999). cPLA2α can localize also inside the nucleus as demonstrated by studies on endothelial cells. In particular, it has been shown that endothelial cells cycle entry is associated with release of high levels of arachidonic acid, which has been implicated in regulating cell proliferation (Herbert et al. 2005). Other sites of cPLA2α localization are represented by the membranes of the forming phagosomes in macrophages and by neutrophils membranes (Ghosh et al. 2006). Association of PLA2α with membranes is enhanced by several binding proteins such as vimentin, p11, annexin-1, caveolin-1, and cPLA2α-interacting protein PLIP (Ghosh et al. 2006). The localization of other members of the group IV PLA2s has been investigated by GFP-tagged proteins. This strategy demonstrated that cPLA2δ localizes to nuclear envelope in response to calcium ionophore and that cPLA2ε localizes to punctate structures in resting cells whereas neither cPLA2ε nor cPLA2ζ localize to membrane after calcium stimulation. Finally, cPLA2γ is constitutively bound to membrane of Golgi and ER since it contains a CAAX sequence in the C-terminus that is farnesylated (Ghosh et al. 2006). Moreover, studies in which cPLA2γ is overexpressed in COS cells revealed that it is bound to mitochondria suggesting that it can play a role in mitochondrial function such as initiating apoptosis (Duan et al. 2001).

Phosphorylation

cPLA2s activity is also regulated by phosphorylation. This molecular event is well studied for cPLA2α which contains several phosphorylation sites recognized by MAPKs, MNK-1, and CAMKII. Cellular studies demonstrated that phosphorylation at these sites only modestly increases arachidonic acid release after calcium transient increase, but induces a conformational change of the enzyme that enhances the interaction of the catalytic domain with its membrane substrate. However, phosphorylation is not sufficient to promote membrane binding in the absence of calcium increase (Ghosh et al. 2006).

Summary

Phospholipases are a ubiquitous group of enzymes that hydrolyze phospholipids to generate molecules that may have potent biological activity. Among phospholipases, phospholipase A2 releases fatty acids from the second carbon group of glycerol, specifically recognizes the sn-2-acyl bond of phospholipids, and catalytically hydrolyzes the bond releasing arachidonic acid and lysophospholipids. Arachidonic acid is then converted in inflammatory mediators such as prostaglandin and leukotrienes that are potent inflammatory mediators implicated in many disorders such as asthma and arthritis. PLA2s can be distinct in groups on the base of their specific features such as sequence, molecular weight, disulfide bonding patterns, and calcium dependency. The group of which considerable information are available about structure, function, and mechanisms of regulation are the large cytosolic calcium-dependent PLA2 (cPLA2). cPLA2s, also referred to as group IV PLA2, is a family of enzymes containing six members: cPLA2α,

cPLA2β, cPLA2γ, cPLA2ε, and cPLA2ζ. X-ray crystal structure of cPLA2s revealed that they all contain an N-terminal calcium-dependent lipid binding/C2 domain that promotes interaction of the enzyme with membrane and a catalytic domain. The two domains are connected by a linker that has a variable length from 5 residues in the cPLA2α to 120 residues in the other members of the group. The C2 domains contain calcium-binding loops that bind calcium ions through the acidic residues Asp and Asn. The binding of these two residues with calcium ions determines the so-called electrostatic switch of the C2 domain that becomes electropositive and can bind anionic phospholipids in the membrane. The catalytic domain is represented by a Ser-Asp dyad located in a deep funnel lined by hydrophobic residues and covered by a flexible lid that moves to allow the access of the substrate to the active site. The regulation of cPLA2s activity is complex involving transcriptional and posttranscriptional processes, localization, phosphorylation, and intracellular calcium concentration increase. cPLA2s exert their activity mainly on membranes where they access the substrate. Upon stimulation, intracellular calcium concentration increases and induces the enzyme translocation from cytosol to membranes. These latter may be represented by nuclear envelope, Golgi and ER membranes, phagosomes, and plasma membranes. Recent studies revealed that cPLA2s are implicated also in regulating intracellular membrane trafficking being involved in the formation of carriers from donor membranes (Leslie 2015). cPLA2s activity is also regulated by phosphorylation even if phosphorylation alone is not sufficient to promote membrane binding in absence of calcium increase.

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Cytotactin

► Tenascin-C (TNC, Tnc)

Cytotoxic Cell Protease-1 (CCP1)

► Granzyme B

Cytotoxic T Lymphocyte Associated Serine Esterase 1 (CTLA1)

► Granzyme B

Cytovillin

► Ezrin

cyt-PTPe

► PTPe (RPTPe and Cyt-PTPe)