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The Relationships Between Cytochromes P450 and H_2O_2: Production, Reaction, and Inhibition

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Abstract

In this review we address the relationship between cytochromes P450 (P450) and H_2O_2. This association can affect biology in three distinct ways. First, P450s produce H_2O_2 as a byproduct either during catalysis or when no substrate is present. This reaction, known as uncoupling, releases reactive oxygen species that may have implications in disease. Second, H_2O_2 is used as an oxygen-donating co-substrate in peroxygenase and peroxidase reactions catalyzed by P450s. This activity has proven to be important mainly in reactions involving prokaryotic P450s, and investigators have harnessed this reaction with the aim of adaptation for industrial use. Third, H_2O_2-dependent inhibition of human P450s has been studied in our laboratory, demonstrating heme destruction and also the inactivating oxidation of the hemethiolate ligand to a sulfenic acid (-SOH). This reversible oxidative modification of P450s may have implications in the prevention of uncoupling and may give new insights into the oxidative regulation of these enzymes. Research has elucidated many of the chemical mechanisms involved in the relationship between P450 and H_2O_2, but the application to biology is difficult to evaluate. Further studies are needed reveal both the harmful and protective natures of reactive oxygen species in an organismal context.

Graphical Abstract

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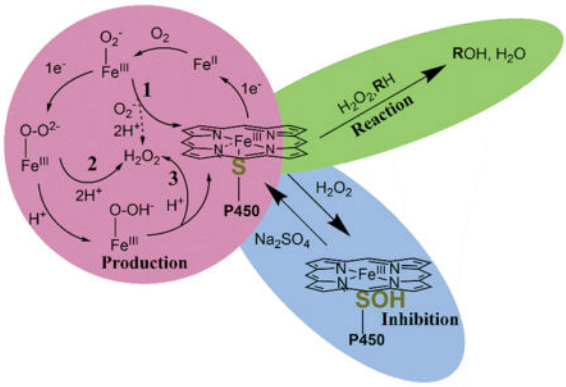

Keywords

Cytochrome P450; reactive oxygen species; thiol; oxidative damage

INTRODUCTION

Gillette et al. [1] first observed the NADPH-dependent production of H_2O_2 in microsomes, and the chemical, biophysical, and biological factors that govern the reactivity of H_2O_2 and oxygen radicals with cytochrome P450 (P450) have been studied extensively since then. However, there are still many unanswered questions regarding the P450 catalytic cycle and its context in biology. Are P450s as inefficient *in vivo* as they are in reconstituted systems? How are P450s regulated when no substrate is present to prevent futile cycling with NADPH and oxygen? These questions become much more difficult to answer in the context of cells, tissues, and organisms.

P450s play two major biological roles: (1) xenobiotic metabolism, with the goal being a decrease in the hydrophobicity of compound for ease of excretion and for further metabolism by enzymes such as sulfotransferases and UDP-glucuronyltransferases, and (2) biosynthesis of bioactive molecules including steroids, vitamins, and oxidized fatty acids [2]. A subset of the latter role is the deactivation and turnover of bioactive molecules, e.g. vitamins A and D [3, 4]. Many diseases are associated with specific P450 variants, and other diseases result from a lack of genes or the substitution of functionally inactive mutants [5–7]. Extensive reviews of endogenous and exogenous substrates and metabolites of P450s have been published elsewhere [2, 8] and this area is beyond the scope of this review.

Iron reacts readily with molecular oxygen and H_2O_2 to produce species capable of performing a diverse array of oxidation reactions. Known broadly as Fenton reactions [9], when this chemistry is uncontrolled it can generate a mixture of nonspecific products with organic reactants and is generally unwanted in most biochemical systems [10]. These are generally controlled *in vitro* by the addition of iron chelating reagents such as EDTA. P450s, as well as some other iron-centered enzymes [11], control the reaction of oxygen with iron in a stereospecific and regiospecific manner. These enzymes have varying efficiencies and are dependent on numerous factors. The iron-oxo reaction also allows for action on a varied

number of substrates, because a general role in much of xenobiotic metabolism is to produce more hydrophilic compounds for facile excretion.

The various P450s are very diverse despite sharing common structural features. P450s are also some of the most promiscuous enzymes, with human P450 3A4 having thousands of reported substrates [12, 13]. Plants have greater numbers of P450-encoded genes than any other kingdom of organisms (e.g., wheat has 1476). These are extensively involved in the synthesis of secondary metabolites and defense molecules [14]. Prokaryotic P450s synthesize important secondary metabolites such as antibiotics and have also been used as model enzymes for the study of all aspects of the general P450 catalytic cycle [15]. The use of prokaryotic P450s to catalyze diverse chemical reactions that are difficult to perform synthetically has proved to be promising as well [16]. This includes the use of H_2O_2 and high-valent oxygen compounds as oxygen surrogates (e.g., peracids, hydroperoxides, iodosylbenzene) for chemical reactions [17]. In this review we discuss the known interactions of P450s with H_2O_2. This oxidative chemistry has implications important to the understanding of P450s in a biological context.

H_2O_2 in Signaling

In recent years H_2O_2 has been recognized as an important secondary signaling molecule, and several laboratories have characterized redox sensitive enzymes [18, 19]. Reactive oxygen species (ROS) have been shown to react specifically with several amino acids, but the sulfur-containing residues cysteine and methionine are the most susceptible to oxidation. The first step of cysteine oxidation by H_2O_2 is formation of a sulfenic acid (-SOH), initially characterized as an anthraquinone-sulfenic acid by Fries [20] and later by Bruice [21]. This oxidation can occur at rates between 10^{-1} M^{-1} s^{-1} (glutathione, GSH) and 10^{8} M^{-1} s^{-1} (peroxiredoxin) [19] (Fig. 1). This large variation in reactivity is due at least in part to the pK_a of the particular oxidized cysteine. Sulfenic acids are reactive species and readily react with free thiols to form disulfide bonds through a dehydration reaction or through a sulfenamide intermediate [22]. This is thought to be the general mechanism of physiological disulfide bond formation (Fig. 1). The reaction can occur in an intra- or intermolecular fashion with free thiols (including GSH) and can be reversed by an NADPH-dependent reaction catalyzed by glutaredoxin [23]. Sulfenic acids can be further oxidized to dioxidation (sulfinic acid, SO_2^-) and trioxidation products (sulfonic acid, SO_3^-), which are mostly irreversible and induce protein degradation and cellular stress responses (Fig. 1) [24].

Oxidative regulation of cysteines in proteins have been known for quite some time. Evidence of oxidative inhibition of glyceraldehyde phosphate dehydrogenase [25] and papain [26] led to an interest in the field [27]. After researchers determined conditions to promote the stability of sulfenic acids, they could be studied in a more systematic fashion [28–30]. Recently mechanisms of stability and function have been elucidated. In the case of epidermal growth factor receptor (EGFR), a sulfenic acid is formed in the kinase domain of the protein (Cys-797) in an H_2O_2-dependent fashion, causing autophosphorylation and activating the EGFR signaling cascade. This sulfenic acid is stabilized by a hydrogen bond with Arg-841, which, when mutated confers resistance to oxidative activation [31]. Additionally, tyrosine phosphoprotein phosphatase 1B (PTP1B) [32], glyceraldehyde

phosphate dehydrogenase (GAPDH) [33], Kelch-like ECH-associated protein-1 (Keap1) [34, 35], P450s [36, 37], and many others [38] have been found to be regulated by sulfenic acid formation. Methods for detecting and analyzing cysteine oxidation have remained challenging, but recent advances in chemical trapping methods and in our understanding of redox biology provide promising new ways to elucidate redox functions of cysteines [39, 40].

H_2O_2 Production Through Pathway Uncoupling

Since the report of Gillette *et al.* [1] on the NADPH-dependent production of H_2O_2 in liver microsomes, there has been interest in this area of study. Several years later, a stoichiometric anomaly observed between NADPH, oxygen consumption, and product formation in liver microsomes [41] was accounted for when H_2O_2 and H_2O production were measured as side products in P450 reactions [42, 43] . H_2O_2 production, plus the generation of superoxide anion by NADPH-P450 reductase [44] and P450 [45], led to the hypothesis that P450 induction may be related to hepatic disfunctions such as ethanol-induced liver damage [46–48]. Uncoupling has been proposed to have potentially damaging circumstances by contributing to ROS production and to accelerating the aging process [49].

ROS production can, at least in principle, occur at three intermediate stages during the normal P450 catalytic cycle (Fig. 2). The first is directly after molecular oxygen binding to the ferrous heme (Fig. 2, Reaction **1**, dashed line). This has been described as the ferric superoxide complex, Fe^{III}-O_2^- [50]. This state is only 1 kcal mol^{-1} above the heme resting state (Fe^{III}), and the oxygen-iron bond can easily be broken to form superoxide anion ($O_2^{\bullet -}$) and iron (III) heme [51]. Superoxide is quickly dismutated (non-enzymatically) to H_2O_2. The rate of this process ($Fe^{II} \rightarrow > Fe^{III} + O_2^{\bullet -}$), termed autoxidation, is related to the stability of the Fe^{II}-O_2 complex and varies among P450s [52], and the rate of autooxidation is dependent on temperature [53, 54]. Structural studies with $P450_{cam}$ have elucidated a coordination sphere surrounding the heme-thiolate ligand, which reduces the sulfur charge and allows for reduction to ferrous heme [55]. This hydrogen bonding network appears to fine-tune the positioning and electron donating ability of cysteine sulfur [56].

The second and third stages at which ROS can be produced, following the second reduction step, are from the P450 peroxo (Fe^{III}-O-O^{2-}) and hydroperoxo (Fe^{III}-O-OH^-) complexes ("Compound 0"). The Fe-O bond of the peroxo complex can be broken, and the oxygen species can be doubly protonated to form H_2O_2 (Fig. 2, Reaction **2**). In a similar fashion, after protonation the hydroperoxo complex can either be further protonated, forming Compound I (Fe^{IV}=O^{3+}) and H_2O, or the Fe-O bond can break, once again forming H_2O_2 (Fig. 2, Reaction **3).**

This uncoupling appears to be dependent on several factors, including pH, substrate positioning in the active site, and a disturbed substrate binding pocket. The heme thiolate allows for the correct amount of "push" and "pull" of electrons for the successful completion of the P450 catalytic cycle [57]. Bacterial enzymes generally have very high coupling efficiencies for native substrates compared to mammalian P450s (Table 1). This difference in coupling efficiency may be due to the number of substrates mammalian P450s can accommodate compared to bacterial enzymes.

ROS generated by P450 from inefficient reaction cycles can, in principle, oxidize cellular proteins, lipids, and DNA. This alteration in cellular redox balance can lead to signaling involved in antioxidant responses, create an oxidatively stressed environment, and potentially lead to disease [72]. Evidence for potential ROS-dependent toxicity of P450s in the *CYP* Subfamilies 1, 2, 3, and 4 has been reviewed recently [49, 73].

ROS-dependent toxicity originating from P450-mediated uncoupling has been difficult to establish *in vivo*. Many *in vitro* reconstituted, microsomal, mitochondrial, and cellular studies have provided evidence that induction of P450s can cause elevated ROS production [67, 74–80]. However, *in vivo* studies in rodents indicate that toxicity may stem from depletion of reducing pools found in cells, such as GSH and reduced pyridine nucleotides (NADPH and NADH) [81, 82]. Conversely, other studies indicate that P450s may have protective effects in the case of the P450 1A subfamily [83, 84]. A major unanswered question in this field is how much do P450s contribute to ROS production. Although much has been written about both topics, there are major issues. One is that much of the experimental work has been done in cell culture, often with the use of inappropriate cellular models (e.g., that do not normally express P450s), or else ROS has been measured using inadequate methods (e.g., several fluorescent dyes [85–87].) A number of papers have touted P450s as a major source of ROS [88–90] although others do not consider this to be as important as mitochondrial leakage, NADPH oxidase, and other sources [91]. *In vivo* work with both rats and mice, using F_2-isoprostane formation [92] (still accepted as the “gold standard” [87]) showed that P450 induction elevated total tissue or urinary ROS only in the case of barbiturate induction, and that was at least in part due to an alteration in levels of pyridine nucleotides due to altered methyl transferase activity [81, 82]. However, other work done has shown that some localized changes (e.g., translocation of P450 2E1 to mitochondria and uncoupling there) may occur and be detrimental [74] (and these ROS changes were confirmed with isoprostane analysis). More *in vivo* studies will be needed to determine the contribution of P450s to proposed ROS-related toxicities [72].

H_2O_2 as a P450 Co-substrate

Reactions with H_2O_2 as the oxygen donor for P450 peroxygenase and peroxidase reactions are known [93, 94]. In these reactions, the ferric heme reacts directly with an oxygen of H_2O_2 or other hydroperoxide species and proceeds with heterolytic cleavage of the oxygen-oxygen bond to form Compound I [95]. Other oxygen donating molecules (“oxygen surrogates”) have been noted to have oxidation activity including iodosylbenzene [96] and sodium chlorite [97]. Additionally, Rittle and Green used *m*-chloroperbenzoic acid as an oxygen surrogate for CYP119 to successfully isolate Compound I [98].

In the case of the peroxidase function of P450s, once Compound I is formed by either H_2O_2 or an organic hydroperoxide, a one-electron oxidation is performed on a substrate, reducing the porphyrin radical of Compound I to form Compound II (Fe^{IV}=O) and a radical product. Compound II performs a subsequent one-electron reduction on another substrate generating ferric heme, water, and a second radical product [17]. Mammalian P450s are known to act on endogenous hydroperoxide species, reducing them to their corresponding alcohols [99,

100]. This may be one of the metabolic mechanisms to reduce the levels of reactive hydroperoxides in cells, although the overall contribution is unknown.

P450s can also perform peroxygenase reactions in which the enzyme can catalyze monooxygenase reactions without the requirement for ferric iron reduction or redox partner proteins. Peroxygenase reactions are thought to react in a chemically similar way to the monooxygenase activity. Various hydroperoxide substrates have been explored in the oxidation of P450 substrates [101–104]. This provides further evidence that P450s may utilize endogenous hydroperoxides as co-substrates *in vivo.* However, there are many technological challenges to study this hypothesis including the need for highly sensitive detection methods and the inherent instability of hydroperoxides [105].

In mammalian P450s the reaction with H_2O_2 is generally very inefficient and is dependent on high concentrations of H_2O_2 that are well above estimated physiological concentrations, suggesting that this reaction does not occur *in vivo* [106]. However, some bacterial enzymes are known to have fast catalytic rates and highly specific hydroxylation products of saturated fatty acids, e.g. $P450_{SP\alpha}$, $P450_{BS\beta}$ [107], and OleT [108–110]. These enzymes and other bacterial P450s are highly stable in the presence of H_2O_2. P450 BM3 (102A1) has been an important enzyme in the study of peroxygenases, especially after engineering an increase in stability [111–113]. Other bacterial enzymes have also shown high stability in the presence H_2O_2 [114, 115].

More recently, scientists have recognized the utility of peroxygenase reactions in the development of P450s as industrial biocatalysts [116, 117]. This industrial role has potential, and ongoing discovery and characterization of novel peroxygenase- and peroxidase-catalyzing P450s may lead to novel enzymes that are useful products, e.g., biofuels and molecules that are difficult or expensive to synthesize.

H_2O_2 as a P450 Inhibitor

It has been known that H_2O_2 and other peroxides can inhibit P450 activity as well through heme degradation [118, 119]. Furthermore, it has been shown that incubation with H_2O_2 can also inhibit P450 by oxidizing the heme thiolate ligand to a sulfenic acid, thus inhibiting P450 catalysis [36, 37]. This phenomenon, first identified in human recombinant P450 4A11 [37], can affect other human P450 enzymes, as well as other drug metabolizing enzymes. Spectral studies indicated that loss of the proximal heme ligand inhibited carbon monoxide binding and/or ferric heme iron reduction by NADPH-P450 reductase but can be reversed using a reducing agent, e.g., dithiothreitol, *tris*-carboxyethylphosphine (TCEP), or sodium dithionite ($Na_2S_2O_4$). In these studies, human P450s 2D6, 2C8, 3A4, and 4A11 exhibited redox sensitivity and P450 1A2 was redox insensitive, suggesting that there is differential redox regulation among P450s [36]. P450 1A2 was found to undergo extensive oxidation of one ancillary cysteine (Cys-159) that had no effect on catalysis. This was contrary to the case of P450 3A4, which showed an irreversible inhibition related to hyperoxidation of ancillary Cys-468. This may be reasonable, as Sevrioukova has recently reported a cysteine-depleted P450 3A4 enzyme with higher catalytic activity [120].

P450s 2D6, 2C8, and 4A11 seemed to behave similarly both in inhibitory and spectral aspects [37]. In anaerobic spectral studies, P450s in the presence of CO, NADPH-P450 reductase, and NADPH exhibited a maximal absorbance of 420 nm, indicating a 5-coordinate heme center. After the addition of dithionite, the typical 450 nm absorbance was observed. This change was interpreted as H_2O_2-dependent oxidation of the heme-thiolate ligand to a sulfenic acid which lost iron coordination. Dithionite was then able to reduce the sulfenic acid, allowing for re-liganding of the thiolate to the iron (Figure 3) [37 16183].

There have been multiple reports of variation in pharmacokinetics (PK) of drugs due to disease and/or inflammation [121]. PK differences have been observed in celiac patients (which were reversed with treatment [122]) in which ROS levels are elevated, in untreated rheumatoid arthritis patients with extended verapamil half-lives compared to treated patients [123], and in P450 1A2 activity in patients with heart failure [124]. Other reports highlighting a two- to fivefold change increase in the area under the curve (AUC) in P450 substrates has been reviewed in detail recently by Coutant and Hall [125]. There is a strong link between autoimmune and inflammatory diseases to increased ROS production and also transcriptional downregulation of P450s [125, 126]. The redox sensitivity observed with some P450s may explain this variability, or at least contribute to it. Further testing in cellular and animal models is needed to confirm this, in that an alternate explanation is that the inflammation lowers overall levels of P450s at a pre-translational level or through other phenomena [127, 128].

This inhibition may function as a sensor where P450s are switched off when there is a high oxidizing environment and a low amount of NADPH may be present. The reducing equivalents of NADPH and/or NADH may be required to perform functions critical for life such as reversing glutathionylated GAPDH [33] or maintaining general redox homeostasis [129–131]. This may also be a negative feedback loop in place to limit uncoupling and further H_2O_2 production. Interestingly, in 1971 Hrycay and O'Brien hypothesized that heme-thiolate sulfenylation could occur and that modified P450s would preferentially catalyze peroxidase reactions over monooxygenase reactions [119]. This hypothesis requires further testing.

Conclusions

In summary, P450s and H_2O_2 have an intricate intertwined relationship which has both potentially harmful and beneficial results. P450 uncoupling seems to have the potential to be harmful and cause toxicity, but this uncoupling inefficiency may be a cost paid by P450s to allow for reactivity with many substrates. Hence, more *in vivo* studies are needed to fully understand the contributions P450s have to ROS-dependent toxicities and to determine if prevention of uncoupling is a reasonable defense against these diseases. Peroxidase and peroxygenase activities of P450s most likely do not occur in mammals, due to the amount of H_2O_2 required for catalysis, but are important for some prokaryotes in metabolism of specific substrates. Discovery and characterization of novel P450s that catalyze reactions with H_2O_2 as a co-substrate is a promising field and should be pursued further. Reversible H_2O_2-dependent inhibition of P450s is also an interesting interaction that needs further evaluation. Both *in vivo* and *in vitro* studies are required to fully understand if the

sulfenylated heme-thiolate ligand have implications regarding uncoupling, peroxidase activity, and protein stability.

Acknowledgments

This article is dedicated to the memory of Prof. Dr. Klaus Ruckpaul, who passed away recently. He worked in the P450 field for many years, often under difficult conditions. Prof. Ruckpaul worked on some aspects of ROS and P450. He is fondly remembered for starting the International Conferences on Cytochrome P450 in 1976, which continues today. The poster awards are named in his honor.

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HIGHLIGHTS (Bullet Points)

- P450s produce H_2O_2 during the reaction cycle, a process known as uncoupling, thus releasing reactive oxygen species that may have implications in disease.
- H_2O_2 is used as an oxygen-donating co-substrate in peroxygenase and peroxidase reactions catalyzed by P450 and has implications in industrial chemical synthesis.
- H_2O_2-dependent inhibition of human P450s occurs through the inactivating oxidation of the heme-thiolate ligand to a sulfenic acid (-SOH), which may have physiological implications.

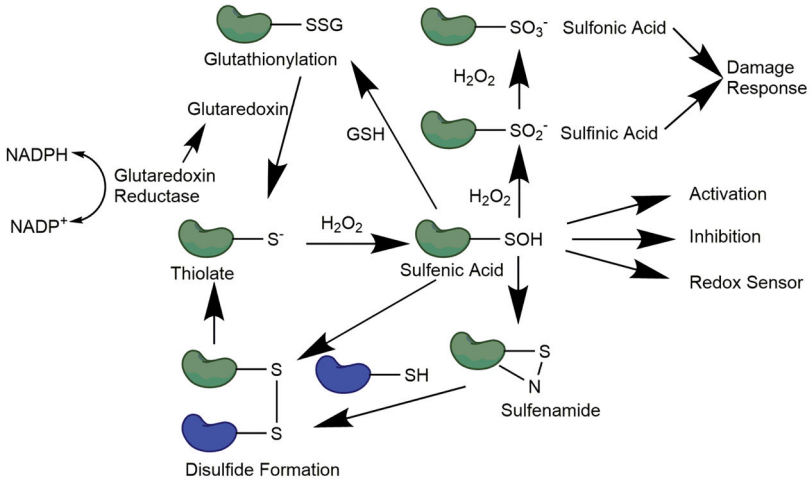

FIG. 1. General redox cycle of protein thiols

In the presence of H_2O_2, thiols can be oxidized to sulfenic acids, which may elicit a physiological response. Sulfenic acids can then form disulfide bonds, which can be reduced to free thiols. Sulfenic acids can also be further oxidized to sulfinic or sulfonic acids, which can trigger cellular damage responses.

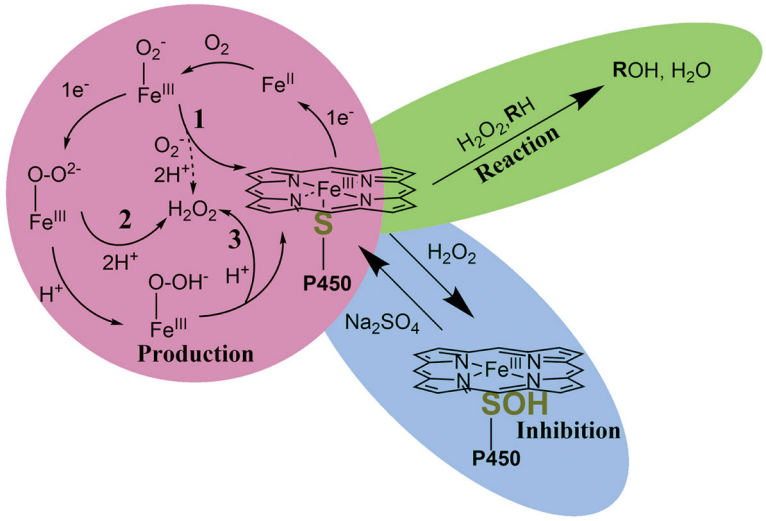

FIG. 2. P450 Production, Reaction, and Inhibition with H_2O_2

P450s can produce H_2O_2 in three separate reactions after molecular oxygen binds to the ferrous heme (red circle, numbered reactions). H_2O_2 can also be used as a co-substrate and oxygen donor in peroxygenase and peroxidase reactions (green oval). Additionally, H_2O_2 can inhibit catalysis through sulfenylation of the heme-thiolate ligand (blue oval).

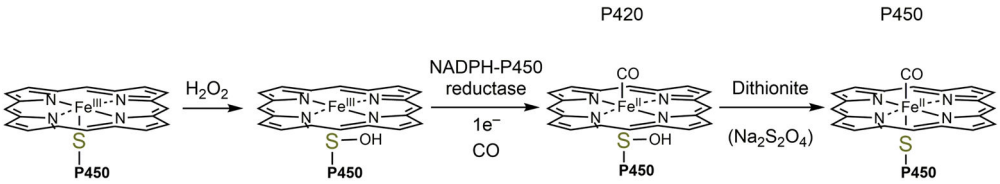

FIG. 3. Mechanism of loss of activity due to sulfenylation

In the presence of H_2O_2, the heme-thiolate ligand becomes oxidized and loses its coordination with the heme iron. The sulfenic acid is not reduced by NADPH-P450 reductase but can be reduced by dithionite, re-forming the heme coordination.

Table 1

Human P450: Coupling efficiency (Product/NADPH ratio)

P450	Substrate	% coupling efficiency	Reference
1A1	Phenacetin	2.5	[58]
1A2	Methanol	7.5	[58, 59]
	7-Ethoxycoumarin	1.2	
	Phenacetin	5.1	
2A6	Coumarin	25	[60]
2B6	17-α-ethynylestradiol	48	[61]
	Efavirenz	42	
2C9	*(S)*-flurbiprofen	21	[62]
	(S)-Warfarin	4	
2D6	Bufuralol	39	[63, 64]
	3-Methoxyphenylethylamine	43	
	4-Methoxyphenylethylamine	42	
2E1	*N*-Nitrosodimethylamine	5.6 and 59 (± b_5)	[65]
2J2	Ebastine	2–17	[66]
3A4	Testosterone	10–16	[67]
4A11	Lauric acid	31	[68]
17A1	Progesterone	22, 41	[69, 70]
	17α-Hydroxyprogesterone	1.3, 10	
	Pregnenolone	97, 61	
	17α-Hydroxypregnenolone	4, 44	
19A1	Androstenedione	5	[71]
	19-Hydroxy androstenedione	34	
	19-Aldehyde androstenedione	33	