REVIEW

Cytochrome P450 enzymes: understanding the biochemical hieroglyphs [version 1; peer review: 3 approved]

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V1 First published: 01 Jul 2015, 4(F1000 Faculty Rev):178
<https://doi.org/10.12688/f1000research.6314.1>

Latest published: 01 Jul 2015, 4(F1000 Faculty Rev):178
<https://doi.org/10.12688/f1000research.6314.1>

Abstract

Cytochrome P450 (CYP) enzymes are the primary proteins of drug metabolism and steroid biosynthesis. These crucial proteins have long been known to harbor a cysteine thiolate bound to the heme iron. Recent advances in the field have illuminated the nature of reactive intermediates in the reaction cycle. Similar intermediates have been observed and characterized in novel heme-thiolate proteins of fungal origin. Insights from these discoveries have begun to solve the riddle of how enzyme biocatalyst design can afford a protein that can transform substrates that are more difficult to oxidize than the surrounding protein architecture.

Keywords

Cytochrome P450, CYP, cytochrome c peroxidase, heme-thiolate proteins, reaction mechanism

Open Peer Review

Approval Status

	1	2	3
version 1 01 Jul 2015			

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Competing interests: The author declares that he has no competing interests.

Grant information: The author(s) declared that no grants were involved in supporting this work.

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How to cite this article: Groves JT. **Cytochrome P450 enzymes: understanding the biochemical hieroglyphs [version 1; peer review: 3 approved]** F1000Research 2015, 4(F1000 Faculty Rev):178 <https://doi.org/10.12688/f1000research.6314.1>

First published: 01 Jul 2015, 4(F1000 Faculty Rev):178 <https://doi.org/10.12688/f1000research.6314.1>

Introduction

The Rosetta Stone of Egyptian antiquity is a trilingual transcription of a Ptolemaic edict of March 27, 196 BCE. The subsequent decoding of the text, rediscovered by Napoleon's army in 1798, opened the writings of the distant past to historians and to the world. A Rosetta Stone—surely that description applies aptly today to the large superfamily of cytochrome P450 (CYP) enzymes, as well as related peroxidases, that have revealed so much about biochemical and chemical biological oxidation. Here, the languages have been the imaginative and interconnected application of structural, mechanistic, and spectroscopic idioms^{1,2}. CYP proteins have long been known to mediate the oxidative processes involved in phase 1 drug metabolism, which occur in the liver. More than 70% of drug compounds are metabolized in this way. It has become increasingly important to identify these drug metabolites and to determine the extent to which they are toxic or actually the active form of the administered drug. For example, the platelet aggregation inhibitor, Plavix, which contains a thiophene ring, is a prodrug, inactive in its administrated form, which is first transformed by liver P450 enzymes to a thiolactone structure. The active form of the drug evolves subsequently in a second, hydrolytic step (Figure 1). By contrast, acetaminophen is transformed by hepatic P450 proteins to a toxic iminoquinone that is the cause of liver failure because of overdoses of this common over-the-counter drug. P450 enzymes of the adrenal cortex orchestrate the extensive tailoring of cholesterol in the intricate biosynthetic pathways that produce steroid hormones. This understanding has led to the development of effective dual drug strategies in oncology, particularly for the treatment of human breast cancer. One of the leading drugs, tamoxifen, functions by initial P450 metabolism to 4-hydroxytamoxifen, which then blocks estrogen receptors in the proliferating tissue. Estrogen, in turn, is biosynthetically produced in a series of P450-mediated oxidative transformations that include removal of the C19 methyl group to form an aromatic A-ring in the steroid estrogen. Blocking this P450-mediated “aromatase” reaction with aromatase inhibitors further reduces the stimulating effect of estrogen on the cancerous cells. P450 proteins are also used by pathogens such as the *Tuberculosis bacillus* to erect its waxy, impermeable cell membrane. In this light, pathogen CYP enzymes are obvious drug targets. Finally, there are numerous microorganisms that can derive food from even the most recalcitrant organic molecules. Here, bacterial P450 enzymes, and other iron proteins, are instrumental in consuming petroleum from environmental oil spills³.

Analysis and discussion of the cytochrome P450 reaction cycle

For all of these reasons, P450 enzymes have received sustained attention for decades. But how do they work? Particularly, what is the role of the unusual coordination of cysteine sulfur to the heme iron center forming the essential heme-thiolate active site (Figure 2)? And why is that sulfur so essential?

It was recognized early in the development of the P450 field that sulfur coordination to the P450 heme was responsible for the red-shifted UV-vis spectrum that displayed a strong (Soret) maximum at approximately 450 nm. Ferrous-CO adducts of typical heme proteins such as myoglobin absorb at approximately 420 nm. The red-shifted porphyrin absorbance band was instrumental in the discovery of P450 enzymes and the reactions they mediate^{4–8}. Indeed, this spectroscopic signature is the origin of the P450 name, the P referring to the fact that these *pigmented* P450 proteins were found in the particulate portion of the cell lysate^{8–10}. Confirmation of the cysteine sulfur ligation came from the first crystal structure of a soluble P450 isolated from *Pseudomonas putida*¹¹. This revelation caused considerable discussion among mechanistic biochemists. Why sulfur?

For the purpose of this analysis, we will use the mechanistic reaction sequence depicted in Figure 2 as a road map^{1,12}. A more detailed description of each step in this scenario is provided in the legend of Figure 2. For further reading, there are a number of very informative and authoritative reviews^{2,13–19}.

It seemed counterintuitive to most coordination chemists and heme protein biochemists that sulfur coordination could be advantageous for an enzyme designed to oxidize even aliphatic hydrocarbons that have very high oxidation potentials and very strong C-H bonds. Thiols themselves are easily oxidized. Furthermore, sulfur coordination generally stabilizes higher metal oxidation states. An important break in the case arrived in 2004 with the announcement that chloroperoxidase, a chloride-oxidizing heme-thiolate protein of fungal origin, had an oxidized form that was an unusual and unique hydroxo-iron(IV) species [Cys-S-Fe^{IV}-OH] (II in Figure 2) and not a ferryl [Cys-S-Fe^{IV}=O]²⁰. Perhaps this curious fact was a clue to the amazing abilities of P450 enzymes to break these strong C-H bonds. Subsequently, both I and II from P450 enzymes were generated and spectroscopically characterized^{21,22}.

Figure 1. P450-mediated biotransformations of the prodrug clopidogrel (Plavix).

Figure 2. Cytochrome P450 hydroxylation mechanism. The reaction cycle proceeds clockwise from the upper left. In the resting form of P450 (R), the iron in the heme-thiolate active site is in the ferric (Fe^{III}) oxidation state. Upon binding of the substrate molecule (S-H), molecular oxygen is bound and reduced to a coordinated hydroperoxide (O). A proton relayed from a glutamic acid through a chain of water molecules facilitates cleavage of the peroxide O-O bond (blue arrows). One role of the cysteine sulfur ligation is thought to be electron donation (push) that weakens the O-O bond. The result of peroxide O-O bond scission is to produce a stable water molecule and a highly reactive and strongly oxidizing intermediate (I). Spectroscopic evidence supports an oxo-iron(IV) porphyrin radical cation formulation for I, which is the species responsible for cleaving even very strong substrate C-H bonds. This C-H bond scission is a one-electron process wherein the substrate proton is transferred to the ferryl oxygen ($\text{Fe}=\text{O}$) of I to produce II and a substrate-derived radical ($\text{S}^\bullet$). Dual roles for the cysteine sulfur in this process are increasing the basicity of the ferryl oxygen that receives the proton while reducing the redox potential of the iron(IV) porphyrin radical cation in I. Finally, collapse of this ensemble [$\text{S}^\bullet \text{HO-Fe}^{\text{IV}}\text{-S-Cys}$] affords the hydroxylated product (S-OH) and the resting enzyme R to complete the cycle.

So why is it significant that the hydroxo-iron(IV) species [$\text{Cys-S-Fe}^{\text{IV}}\text{-OH}$] (II) is protonated? As can be seen, II arises from intermediate I during the substrate C-H bond cleavage event. This C-H activation, a hydrogen atom abstraction, can be dissected into two parts: the scissile proton and an electron that was part of the initial C-H bond. For this reason, this kind of hydrogen atom abstraction has been called a proton-coupled electron transfer²³⁻²⁶. This nomenclature emphasizes the fact that the substrate proton ends up on the ferryl oxygen of I to produce the iron(IV)-hydroxide of II while the electron has filled the radical cation hole in the porphyrin π -system. This approach also makes it apparent that the basicity of the

iron-bound oxygen ($\text{Fe}=\text{O}$) and the redox potential of the porphyrin ring are both important in breaking the C-H bond. An increase of either parameter has the effect of increasing the strength of the FeO-H bond that is formed in II, increasing the driving force for the reaction. In this light, we can understand an important paradigm in hydrocarbon oxidation: Nature breaks strong C-H bonds by making stronger O-H bonds.

If only it were so simple. All of this remarkable catalytic chemistry is being performed within the confines of an enzyme active site that is surrounded by peptide architecture. How does CYP avoid

oxidizing itself? Long-range electron transfer is ubiquitous and essential in biology^{27,28}. If we take inventory of the susceptibility of ordinary amino acid side chains to one-electron oxidation, two of them, tryptophan and tyrosine, stand out in addition to the mysterious cysteine thiolate. Both of these amino acid residues have oxidation potentials near 1 V, as do the π -electron system of porphyrin ring and the heme iron(III). Methionine residues are also potential sites of oxidation, but the oxidation potentials are at least several hundred millivolts higher than those of tyrosine or tryptophan²⁹. The situation for tryptophan is readily illustrated by the fact that in compound I of cytochrome *c* peroxidase (CCP)^{30–35}, the structure corresponding to I in Figure 2 is a histidine-coordinated oxoiron(IV) tryptophan cation radical. This innovation appears to be a strategy to allow CCP to accept successive long-range, one-electron transfers from its substrate cytochrome *c*. The catalytic cycle of CYP is also initiated by two long-range electron transfers from a protein reductase partner^{36–38}.

One solution for CYP might be to isolate the heme center with less easily oxidized amino acid side chains. Indeed, crystal structures do show an abundance of phenylalanine and alkyl chain residues such as leucine and valine nearby¹³. But this local redox insulation, which is probably arranged to facilitate substrate binding, would not be enough. Long-range electron transfer from protein tyrosines to heme centers over distances of 10 Å or more is still facile^{27,28}. Indeed, the turn-on trigger of prostaglandin synthase relies on just such a tyrosine oxidation^{39,40}. Certainly, placing the substrate (S-H) close to the ferryl oxygen as in $[S-H\cdots O=Fe^{IV}]$ would help. Chemists call such an atom transfer between contiguous atoms an inner-sphere process^{41–43}, whereas a long-range electron transfer from some distant amino acid side chain to the heme center would be an outer-sphere process. Generally, inner-sphere processes occur faster than outer-sphere processes even if the two have the same driving force.

So in what ways are CYP proteins engineered through their amino acid sequence to allow long-range electron transfers to the heme-thiolate center in the early steps of oxygen binding and reduction at relatively low potentials, while at the same time preventing long-range electron transfers at the moment the highly oxidizing I is breaking a strong C-H bond? In the end, it is a balance of competitive rates and redox potential modulation by the axial thiolate ligand. Intriguingly, the cysteine thiolate of P450 proteins, as well as those of chloroperoxidase and newly discovered aromatic peroxxygenase (APO) heme-thiolate proteins¹⁷, are held in place by a phalanx of peptide backbone N-H $\cdots$ S hydrogen bonds (Figure 3). Various thiolate electron donor parameters such as coulombic effects, σ - and π -*trans*-axial ligand effects, and field effects of other charges in the active site such as the heme propionate anions all could contribute to the electron push effect⁴⁴. The net result would be a lowering of the redox potential of I and a compensating increase in the basicity of the ferryl oxygen (Figure 2). The extent of this electron donation effect on the ferryl basicity has been dramatically illustrated in two recent cases. For a thermostable CYP, the pK_a of the oxygen-bound proton in II has been measured to be a remarkable value of 11.9²². For the heme-thiolate APO from *Agroclybe aegerita*, that pK_a

Figure 3. Typical active site of cytochrome P450 and aromatic peroxxygenase heme-thiolate proteins.

value for II is 10^{45–47}. By contrast, typical ferryl species, such as compound II of myoglobin, resist protonation even at pH 3⁴⁸. This large difference in ferryl basicity of 7–9 pK_a units corresponds to 400–500 mV in terms of redox potential. Accordingly, sacrificing redox potential to get a more basic oxygen could indeed facilitate C-H bond cleavage while sparing the enzyme from internally generated oxidative stress.

Concluding remarks

Is the story over? By no means! First, there remains considerable uncertainty, over a range as large as 500 mV, as to what the reduction potentials of heme-thiolate compound I intermediates really are²¹. But here it is still early days and there have been only a handful of measurements and estimates. There is, however, a basic difference between the productive cleavage of the substrate C-H bond in S-H by I and a non-productive, long-range electron transfer from an amino acid side chain. In the productive pathway, a substrate proton arrives at the ferryl oxygen during the reaction. By contrast, the long-range electron transfer process would require a proton from some other source. Perhaps that proton is readily available through the water aqueduct leading to the P450 active site, but perhaps not. Perhaps, also, an acidic proton from the water channel can activate the ferryl oxygen for substrate hydrogen abstraction^{49,50}. Another major point of spirited debate has to do with the extent to which energy barriers for C-H bond scission, and thus the rates of these reactions, are affected by the exact electron configurations in oxidants such as I^{51,52}. Although there has been progress recently in the preparation of synthetic ferryl species in both high-spin and intermediate-spin electronic configurations^{53–57}, what matters is the arrangement of spin density at the transition state $[S\cdots H\cdots O-Fe]$.

CYP research continues to be a rich, vibrant, and important field. Determining and understanding the reaction mechanisms of CYP

substrate oxidations over the past several decades have greatly advanced a variety of fields. Numerous spectroscopic techniques and diagnostic reaction probes have been applied to dissecting the mechanism. With this knowledge in hand, drug metabolism pathways can often be anticipated, weeding out poorly performing candidates early in the drug development pipeline. CYP and APO enzymes can now be engineered and evolved for particular purposes^{58–62}. Reaction processes are being developed by using immobilized P450 and APO enzymes. Also, new heme-thiolate proteins are being discovered.

Abbreviations

APO, aromatic peroxygenase; CCP, cytochrome *c* peroxidase; CYP, cytochrome P450.

Competing interests

The author declares that he has no competing interests.

Grant information

This work was supported by the National Institutes of Health (2R37 GM036298).

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgments

The author thanks group members, collaborators, and colleagues in the field for discussion, inspiration, and many insights.

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- 2. Stephen G. Sligar**
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