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Spectroscopic studies of the cytochrome P450 reaction mechanisms

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Abstract

The cytochrome P450 monooxygenases (P450s) are thiolate heme proteins that can, often under physiological conditions, catalyze many distinct oxidative transformations on a wide variety of molecules, including relatively simple alkanes or fatty acids, as well as more complex compounds such as steroids and exogenous pollutants. They perform such impressive chemistry utilizing a sophisticated catalytic cycle that involves a series of consecutive chemical transformations of heme prosthetic group. Each of these steps provides a unique spectral signature that reflects changes in oxidation or spin states, deformation of the porphyrin ring or alteration of dioxygen moieties. For a long time, the focus of cytochrome P450 research was to understand the underlying reaction mechanism of each enzymatic step, with the biggest challenge being identification and characterization of the powerful oxidizing intermediates. Spectroscopic methods, such as electronic absorption (UV-Vis), electron paramagnetic resonance (EPR), nuclear magnetic resonance (NMR), electron nuclear double resonance (ENDOR), Mossbauer, X-ray absorption (XAS), and resonance Raman (rR), have been useful tools in providing multifaceted and detailed mechanistic insights into the biophysics and biochemistry of these fascinating enzymes. The combination of spectroscopic techniques with novel approaches, such as cryoreduction and Nanodisc technology, allowed for generation, trapping and characterizing long sought transient intermediates, a task that has been difficult to achieve using other methods. Results obtained from the UV-Vis, rR and EPR spectroscopies are the main focus of this review, while the remaining spectroscopic techniques are briefly summarized.

Graphical abstract

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1. Introduction

Cytochromes P450 constitute a very broad and diverse class of enzymes and are present in the vast majority of organisms from all three domains of life. [1,2] Recent progress in genomics and bioinformatics brings thousands of new sequences of P450 enzymes every year, so that at the time of writing this review the total number of identified cytochromes P450 exceeds 40 thousand, as discussed in other reviews in this Special BBA issue. These enzymes are responsible for a multitude of biosynthetic processes as well as for the metabolism of xenobiotics. A fully functional P450 system consists of the heme enzyme cytochrome P450 and its redox partners, including iron-sulfur proteins and flavoproteins, with specific variations present in different biological species, from prokaryotes to eukaryotes, and from plants to animals. Our knowledge about the P450 field is quickly growing, with 3000 new research papers appearing in PubMed database every year.

The P450 enzymes exhibit broad diversity with respect to their biological occurrence and biochemical functions and properties, such as location in cells or tissues, solubility, types of redox partners, catalyzed substrates or chemical transformations. However, all of them share similar structural features and functional properties. All cytochromes P450 apparently originate from a single ancestor CYP51, [2] and thus exhibit a common protein fold. Furthermore, all P450s contain the same heme prosthetic group (Fe - protoporphyrin IX) with cysteine ligating heme iron, [3,4] and all of them utilize a similar catalytic cycle that activates the molecular oxygen or hydrogen peroxide. These similarities in structure and catalysis provide an opportunity for extrapolation of information obtained for some isozymes to studies of others, as well as for making certain predictions about functional properties of previously unknown P450 enzymes, just based on their basic physico-chemical characteristics. Such information can be also applied to directed design of P450 with modified properties employed in whole cell synthesis of fine chemicals, an approach that is quickly becoming one of the most promising directions in medical and biotechnology fields. [5,6,7,8]

All enzymes that belong to the cytochrome P450 family exhibit similar spectroscopic properties, allowing the identification of a P450 enzyme by its unique spectral signature; e.g., all carbonmonoxy adducts of ferrous CYPs exhibit split Soret absorption maximum near 450 nm. Furthermore, each step in P450's enzymatic cycle is characterized by specific changes in oxidation and spin states of heme iron, planarity of the porphyrin ring or disposition of the dioxygen moiety, providing its own specific spectral signature that can be

easily detected by the application of certain spectroscopic techniques. These spectroscopic markers can serve as a source of indispensable information about CYPs structure, dynamic and functional properties, which cannot be otherwise assessed. The optical absorption (UV-Vis) spectra and magnetic circular dichroism (MCD) are useful for the study of binding and dissociation of distal ligands, as well as redox changes in porphyrin ring and heme iron [9,10]. The resonance Raman (rR) and infrared (IR) spectra provide insight into heme vibrational structure, oxidation and spin state of iron ion, linkage with endo- and exogenous ligands, perturbations of porphyrin macrocycle, protonation and hydrogen bonding of dioxygen and its reduced forms [11,12,13,14]. Electron paramagnetic resonance (EPR) and electron nuclear double resonance (ENDOR) are capable of detecting the oxidation state and spin state of iron, and the protonation state of ferric peroxide intermediates. [15,16] The nuclear magnetic resonance (NMR) spectroscopy provides the most detailed information about substrate positioning in the active site, as well as about protein-protein interactions and related conformational as well as dynamic perturbations in the complex formed by P450 and redox partner proteins. [17]

In this article we provide a systematic summary of the most popular spectroscopic tools used in P450 studies which offer unique mechanistic insights into details of the biophysics and biochemistry of cytochromes P450. This review is not exhaustive, because of size limitations, and mostly reflects the authors' preferences and interests. Thus, special focus is on optical absorption, electron paramagnetic resonance and resonance Raman spectroscopies, and less attention is paid to other methods, which are briefly mentioned with references to original works and other reviews. [15,17,18,19,20]

1.1 Enzymatic Cycle

The cytochromes P450 are Nature's most versatile catalysts because of their extraordinary ability to perform highly regio- and stereo-selective oxidation reactions on an enormous number of complex, sometimes relatively inert, molecules such as alkanes, fatty acids, steroids and pharmaceuticals. The cytochromes P450 perform this impressive chemistry utilizing a sophisticated catalytic reaction mechanism (Scheme 1). [21] In brief, substrate binding to the six-coordinate (6c) low-spin (LS) ferric enzyme, called resting state, displaces the water cluster coordinated as the sixth ligand of the heme iron and changes the spin state to the five-coordinate (5c) high-spin (HS) state. The 5cHS ferric substrate bound form exhibits more positive reduction potential, rising by 80 – 130 mV, resulting in much easier reduction to a ferrous state. [21,22,23] Oxygen binding to the ferrous state leads to formation of the relatively stable ferric superoxo intermediate. The subsequent one-electron reduction of the oxy P450 complex forms a nucleophilic ferric peroxo intermediate (Fe-O-O⁻). The peroxo species is typically short-lived owing to fast addition of a proton to the terminal (distal) oxygen atom of the Fe-O-O fragment to produce the ferric hydroperoxo intermediate (Fe-O-O-H). Addition of a second proton to the terminal oxygen atom triggers heterolytic O-O bond cleavage and formation of highly reactive ferryl heme π -cation radical, Compound I, and a water molecule. The oxygen atom of Compound I is transferred to the substrate via a "hydrogen abstraction/oxygen rebound" mechanism ("Groves rebound" mechanism), [24,25,26] forming monooxygenated product and regenerating the ferric resting state.

For decades the major challenge of cytochrome P450 research was to understand the underlying reaction mechanism of each enzymatic step and to identify and characterize the powerful oxidizing intermediates of this catalytic cycle. Compound I species is now generally accepted as the most commonly encountered active oxidant, [27,28,29,30], however, it is recognized that in some cases the final oxidation reaction involves the ferric peroxo- and hydroperoxo-intermediates. For example, in the presence of a susceptible (electrophilic) substrate, the nucleophilic peroxo intermediate can react as a catalytic intermediate. [31,32] It is also noted that there are several possible unproductive side reactions, referred to as uncoupling, that result in lowered substrate turnover, including: the autoxidation of the oxy complex, the dissociation of peroxide or hydroperoxide anion, called a “peroxide shunt” and reduction of ferryl intermediate to water, called “oxidase uncoupling”. [33,34,35,36] These side reactions are often used to probe the functional properties of a given cytochromes or to generate, trap and study the unstable intermediates, e.g., artificial oxidants like *m*-chloroperbenzoic acid can be used as alternative oxidant.

Spectroscopic methods can provide multifaceted and detailed information about each of these enzymatic steps for various P450s (Scheme 2). As will be discussed in detail below, substrate binding usually results in a change of ligation state of the heme iron and consequently of its spin state, which can be monitored by UV-VIS absorption spectroscopy (blue shift of Soret band, termed Type I changes), EPR and rR spectroscopy. The same is true for inhibitor binding, if Type II changes are seen in UV-VIS spectra (red shift of Soret band), EPR (changes in the low-spin spectra) or rR spectroscopy (changes in spin state marker bands). Perturbations of the heme porphyrin ring and side chains can be detected by rR spectroscopy, while NMR spectroscopy can be used to monitor substrate positioning relative to the heme iron. Next, reduction of the heme iron from ferric to ferrous state also results in characteristic changes of UV-VIS spectra, EPR, and rR, as well as Mossbauer and EXAFS. Oxygen binding and associated electronic and vibrational changes are seen in UV-VIS and rR, while Mossbauer and EXAFS are also sensitive, although they require much more sample and cryogenic temperatures. Formation of transient peroxo- and hydroperoxo-ferric intermediates was not observed in steady-state experiments at ambient conditions and requires special cryotrapping methods, as briefly reviewed below. Recently these cryoradiolytically generated intermediates have been characterized using EPR, UV-Vis and rR spectroscopies. [37,38,39,40,41] Compound I is also very unstable and only recently has been trapped in several P450 enzymes using peroxide shunt pathway with artificial oxidant. [20,29] Sometimes a product complex can be identified by EPR and ENDOR following annealing of cryoreduced oxy complexes in several cytochromes P450. [42,43,44,45] This summary illustrates great value of spectroscopic methods for providing detailed information about all of the elementary steps in the P450 cycle, making this system a textbook case in chemistry and biochemistry of oxygen based catalytic oxidation and oxygenation. [1,46,47,48,49,50,51]

1.2 Ancillary techniques

Many spectroscopic methods have been applied to study the ferrous and ferric states of cytochromes P450 and, in some cases, the dioxygen adduct, because these forms are relatively stable at ambient conditions. However, the interrogation of the subsequent highly

oxidizing intermediates has been hampered by the rapid decay of these species for reactions conducted in solution. Fortunately, the obstacles to characterization of these key species have been overcome by application of the cryoradiolytic reduction, a technique initially pioneered by the Symons group [52,53,54] and by Davydov [55,56,57] and more recently refined and extended by Hoffman, Sligar and coworkers, [15,38,39,42,58] with the most recent advance being the application of rR detection by the Sligar and Kincaid groups. [41,59,60]

There are also intrinsic difficulties in studying mammalian, full-length, membrane-bound cytochromes P450, because of their tendency to aggregate in solution. [61,62,63] A common practice to avoid such uncontrolled aggregation is to use solubilizing agents, such as Emulgen 913, CHAPS or other detergents. [64,65,66] However, detergents can introduce complications in studying the structural effects of physiologically relevant substrates, by competing with the natural substrates for binding at the active site. The recent development of the Nanodisc technology provides a new and convenient system for solubilization of full length mammalian P450 molecules together with their redox partners in lipid bilayers, closely mimicking the native biological membranes. [34,61,67,68] These two innovative approaches which now facilitate the study of previously inaccessible short-lived intermediates of cytochromes P450 under conditions similar to their natural environment, are briefly described directly below.

1.2.1 Cryoreduction—The basic concept involved with heme protein cryoreduction is to freeze-trap dioxygen adducts of heme proteins within an aqueous buffer containing glycerol (G), as shown in Scheme 2. Upon subsequent irradiation with γ -rays at 77K or lower temperature, free-electrons and organic radicals are produced. [38,69] The electrons, being mobile, migrate to the Fe-O-O fragment, while other movements, including proton transport, are generally restricted at 77 K and a freeze-trapped Fe-O-O⁻ fragment is generated. [55,57] While in certain cases the active site structure may permit protonation even at 77 K, in many situations the heme-bound peroxo fragment is trapped and can be spectroscopically characterized. [42,43,58] The sample can be then carefully annealed to permit proton transfer, thereby generating a trapped hydroperoxo-heme fragment (Fe-O-O-H). Subsequent annealing to higher temperatures allows delivery of another proton to facilitate O-O bond cleavage and formation of Compound I or Compound II species, or sometimes loss of the bound hydroperoxo-fragment, depending upon the properties of the protein being studied. [15,42,43,44,45,58,70,71,72,73,74] The ⁶⁰Co nuclide is the most often used source of γ -rays needed for the efficient sample radiolysis (~ 4 Mrad), however, the access to high intensity ⁶⁰Co is sometimes difficult. The use of common [³²P] phosphate buffer solutions can offer a convenient, although slow, approach to generate these reduced states. [38,75] Cryoradiolytically generated intermediates can then be studied by EPR, ENDOR, Mössbauer, UV-Vis and resonance Raman spectroscopies, to structurally characterize these intermediates in the P450 enzymatic cycle. For more detailed description of cryoreduction and its application, the reader is referred to several earlier reviews. [15,38,76,77]

1.2.2 Nanodisc Technology—The nanodisc approach utilizes an encircling amphipathic membrane scaffold protein (MSP) which can solubilize a circular fragment of phospholipid

bilayer and incorporate membrane proteins in a native-like environment. [61,62,67,68,78,79,80] Monomeric membrane proteins and their functional complexes, including their reductase partners, can be incorporated in Nanodiscs, as shown in Figure 1. These stable and fully functional preparations are optimally suited for biochemical and biophysical studies, with precise control over protein oligomerization state, stoichiometry of complexes, as well as lipid composition. The properties of nanodiscs and various applications of Nanodisc technology for structural, analytical and functional studies of membrane proteins are described in several recent reviews. [68,79,81,82,83,84]

2. Substrate binding

As mentioned above, substrate binding initiates the enzymatic cycle, usually converting the ferric six-coordinate low spin (6cLS) resting state (substrate-free, SF) to a five-coordinate high-spin (5cHS) substrate-bound form (SB), the spin state change reflecting the elimination of a coordinated water molecule of the SF form by the incoming substrate. The efficiency of the conversion varies for different substrates; *e.g.*, if the water cluster is not completely expelled from the active site, the binding of substrate can result in a population containing both LS and HS states, reflecting coordination of a residual water molecule to the heme iron of some of the substrate-bound forms. Applications of various spectroscopic methods for detection and quantitative studies of substrate binding to cytochromes P450 have been recently reviewed. [85,86]

2.1 Electronic absorption spectroscopy

UV-VIS spectroscopy is the most popular method commonly used for quick detection of substrate binding to cytochromes P450, quantitative analysis of substrate binding isotherms, comparison of various substrates and for evaluation of extrinsic effects on cytochrome P450 affinity. [87,88] In addition, absorption spectra of carbonmonoxy-ferrous complex in cytochrome P450 routinely serve for concentration measurements in membrane suspensions and as a quality standard for evaluation of functional enzyme after isolation and purification. [89,90] Strong optical absorption of the heme provides signal at the Soret absorption band at 380 – 450 nm, which can be easily measured at sub-micromolar concentrations in a standard optical cell with 1 cm pathlength, or even at lower concentrations for measurements with high affinity substrate binding using 10 cm pathlength. [91] This signal indicates changes in ligation state or perturbation of the distal ligand to the heme iron. An incomplete list of possible ligands includes H₂O, CN⁻, imidazole, pyridine and multiple inhibitors containing nitrogen atom, capable of coordinating to the ferric heme iron, [92] or O₂, CO, and various nitrogen-containing ligands for Fe²⁺. [93] Magnetic circular dichroism (MCD) in UV-VIS range is also very sensitive to the perturbations caused by various substrates and can detect small variations of heme iron environment, which are not easily observed by other spectroscopic methods. [94]

High sensitivity and simple instrumentation, with no special requirements for experimental conditions, make UV-VIS spectroscopy a method of choice for thermodynamic and kinetic studies of cytochromes P450, as well as when employing cryogenic methods, [38,41,59,60,76,95] high pressure, [96,97,98,99] flash photolysis and photoacoustic methods

in nanosecond range, [100,101,102,103,104] etc. Optical absorption spectra can be used for monitoring binding mode and for comparing substrate analogs, [105,106,107] or binding of the same substrate to single point mutants. [7,23,108,109] In addition, spectral changes can be used for direct observation of allosteric effects in substrate binding. One such example is described in a recent work, [107] where a significant spin shift was observed upon addition of monomeric testosterone (TST) to CYP3A4 with a TST-dimer already bound in the active site. Another interesting application of such an approach is used in the study of “decoy” molecules in order to improve binding and catalytic properties of cytochromes P450 with respect to non-native substrates. This method is developed for improving hydroxylation of small hydrocarbons (such as propane, butane, or benzene) by CYP102A1 using perfluorinated carboxylic acids, which occupy most of the volume of substrate binding cavity and push the substrate towards catalytic site at the heme iron. [110,111] Such mechanism is directly confirmed by additional spin shift observed by UV-VIS spectroscopy in the presence of ‘decoy’ molecules. [105]

In the case of a single substrate binding event, experimentally measured spectral amplitude is proportional to the fraction of P450 molecules which bind substrate, because the total spectrum is a sum of spectra of substrate free and substrate bound enzyme. The latter represents a homogeneous population of P450 molecules with substrate bound close to the heme iron, resulting in destabilization and displacement of the axial ligand, in most cases water molecule. However, in the case of multiple binding events, the experimentally observed spectrum contains contributions of all possible binding intermediates, i.e. P450 molecules with one, two, or three bound substrates. Some of these binding events are spectrally silent, because of substrate binding to sites located remotely from the heme. Such cases of spectrally silent substrate binding are documented for CYP3A4, [102,112] CYP2B4, [113] and CYP2E1. [114,115] Variations of spectral titration curves calculated for three-site binding with identical signal from all three binding events, and for spectrally silent first binding, are shown in Figure 3. This figure also shows calculated populations of all three binding intermediates and illustrates the problem of simultaneous resolution of unknown binding constants and fractional spectral amplitudes, as described. [112,116,117]

Usually this problem is bypassed by either implying the same signal from all binding events, or by assuming that one of these binding events is spectrally silent. In the first case this equation simplifies to the multi-site binding isotherm, [117,118,119] or, alternatively, experimental results are analyzed using Hill equation, [116,117,120] which also implies the same signal from all binding events. However, in general, all unknown parameters cannot be resolved from one equation, and additional experiments are required to provide more information about a particular system. For instance, the equation for three binding sites contains at least six unknown parameters and cannot be resolved in the general case. [121] Simultaneous analysis of multiple sets of experiments is necessary to resolve all stoichiometric binding constants, as described in several recent papers and reviews. [112,116,117,122,123] Various aspects of analysis of spectral titration experimental data for Type I binding of multiple ligands have been discussed in multiple studies with CYP3A4 [112,121,122,124,125,126] and other works with CYP102, [127] CYP107, [128] CYP1A2 [129] and CYP2E1. [130] Binding stoichiometry can be addressed using Job’s titration, as

described by Halpert and coworkers, [131] or by competitive binding with inhibitors. [130,132]

It is noted that unlike Type I substrates, Type II substrates and inhibitors usually do not show cooperativity, although in some cases simple Michaelis-Menten behavior may be perturbed. [130] In addition, heterotropic cooperativity may be observed between Type II inhibitor and substrate, as described for CYP102A1 with imidazolyl-derivatives of fatty acids and substrate lauric acid. [133] Variations in spectral response for Type II binders are more pronounced than for Type I, because of different ligand strengths of ligands coordinating to the heme iron at 6th position. [9,134,135]

UV-VIS spectroscopy is also used to probe effects of mutations on substrate binding, as shown for CYP101A1 [136,137], CYP101D2 [138], CYP102 [139], CYP107 [140], CYP121 [140] CYP3A4, [109,141] CYP152L1. [142] Comparison of spectral titration results obtained with wild-type and mutant protein reveals variations in binding constant, as well as in degree of spin-shift at saturating amounts of substrate, the latter parameter often correlates with crucial functional properties, such as the rate of metabolism and coupling. [143]

It is worth mentioning that very sensitive detection of substrate binding and discrimination between Type I and Type II ligands is provided by localized surface plasmon resonance (LSPR) spectra, observed with cytochrome P450 immobilized on the surface of solubilized silver nanoparticles, as described for CYP101A1 [144] and CYP3A4 in Nanodiscs [145]. The binding of substrates or inhibitors to the P450 enzyme is monitored by the shift of the LSPR band of nanoparticles in the visible range, 450 – 700 nm. LSPR provides a higher sensitivity and requires less material due to the strong response of the localized plasmon mode upon changes of refractive index in the immediate vicinity of a nanoparticle (see reviews [146,147]). The response is even stronger if the plasmon band of the Ag nanoparticles overlaps with the optical absorption band of the target, in this case the heme group of CYP3A4. Because of this resonance coupling between spectral changes of the heme upon substrate binding and the plasmon band, the overall sensitivity is further increased. [148] For CYP3A4 on Ag nanoparticles this effect allowed observation of Type I or Type II ligand binding by detection of the 2 – 8 nm blue- or red-shifted plasmon band.

Electronic absorption spectroscopy is an obvious method of choice for studies of fast kinetics, due to the strong signal and fast acquisition times attainable (from picoseconds to seconds) in solution and at ambient conditions. Rates of substrate binding, and reduction kinetics can be successfully measured using UV-VIS spectroscopy [109,149,150,151] or fluorescence spectroscopy [86] employing rapid mixing of substrate free cytochrome P450 and substrate solution in stopped flow devices. Typical binding rates for most efficient soluble bacterial cytochromes P450 CYP101A1 [152,153] and CYP102A1 [154] were documented in the range $10^5 - 10^7 \text{ M}^{-1} \text{ s}^{-1}$, corresponding to observed rates of $10^2 - 10^4 \text{ s}^{-1}$ at typical substrate concentrations. Similar fast binding of TST to the human CYP3A4 in Nanodiscs, with various lipid compositions, was observed by Atkins and colleagues. [149] Stopped-flow studies of binding kinetics of several inhibitors to CYP3A4 with fluorescence detection permitted monitoring of multi-step kinetic processes with fast formation of

encounter complex, followed by slower penetration of ligand into the substrate binding pocket. [155] Applications of stopped-flow methods to kinetic measurements, specifically for cytochromes P450, were recently reviewed. [156]

Microsecond kinetics of spin-state relaxation after temperature-jump (T-jump) perturbation was also studied using absorption spectra in the Soret region. Temperature-dependent equilibrium between low-spin and high-spin state in CYP101A1 with various substrates was perturbed by fast discharge of capacitor through the sample solution and concomitant increase of temperature by ~3 degrees. [157] The rates of spin shift were measured at 283 – 286 K and varied from 75 to 2300 s⁻¹ correlating with the fraction of high-spin state for a given substrate. Similarly, using T-jump as a probe of spin-state relaxation monitored by optical absorption, the difference between wild-type CYP102A1 and F87G mutant was observed. [158] Faster relaxation in the mutant (2500 s⁻¹ at 298 K, compared to 800 s⁻¹ in wild-type protein) was attributed to the partially rate-limiting role of Phe87, and also to coupling of this conformational relaxation at the distal site of the heme to the proximal site and axial iron ligand.

The next step in P450 enzymatic cycle, i.e. reduction of ferric to ferrous state can also be monitored by UV-VIS spectroscopy, both in rapid kinetic mode, to measure rates of the first electron transfer from redox partner, and in equilibrium redox titration mode, in order to determine redox potentials. Reduction kinetics of CYP101A1 saturated with substrate camphor by reduced redox partner putidaredoxin was studied using stopped flow spectroscopy. [136,159,160,161] Rates in the range 40 – 60 s⁻¹ at 298 K were observed, indicating that the first electron transfer is not a rate-limiting step in CYP101A1 in the presence of substrate. For CYP102A1 the rate of first electron transfer is significantly faster, in the range 130 – 220 s⁻¹ with various substrates [154], also faster than turnover rates measured at 20 – 30 s⁻¹. Several human cytochromes (CYP1A2, CYP2C9, CYP2E1, CYP3A4) and rabbit CYP1A2 were also reduced rapidly in a reconstituted system with a 2-fold excess of CPR and cytochrome b5, although in most cases reduction was bi-exponential with 30% – 60% amplitude measured at the slower phase [162].

It is noted that spectroelectrochemical titration with dithionite, using UV-VIS monitoring, is a convenient method for measurements of redox potential of cytochromes P450, as described for CYP101A1 [163], CYP102 [164], CYP107H1 [165] CYP121 [140], CYP3A4 in Nanodiscs [166] and CYP51 [167], all of which employed immersed calomel, platinum, or gold electrode for direct potential measurement. Alternatively, instead of using an electrochemical system, similar titrations can be performed with redox sensitive dyes with known midpoint potentials, as described for CYP11A1 [168] and CYP101D1 [169]. The same spectropotentiometric method with optical absorption monitoring was utilized for measurements of redox potentials of cytochrome P450 reductase in Nanodiscs with various lipid compositions [170]. Incorporation of CPR in Nanodiscs with 100% POPC lipids resulted in considerable up shift of mid-point potentials of both flavins, while in 50% POPC-50% POPS Nanodiscs, this effect was less pronounced.

2.2 EPR spectroscopy

EPR spectra are sensitive to the nature of heme iron axial ligands and thus help to identify chemical nature of sixth ligand in cytochromes P450. In the absence of substrate it is usually water, resulting in low-spin signal from ferric heme iron with g -values 2.45, 2.26, 1.91, as reported for CYP101A1. [16] Substrate binding generates significant population of pentacoordinated high-spin iron with g -values 7.85, 3.97, 1.78. [16] Importantly, spin state equilibrium strongly depends on temperature, with low-spin state more favorable at low temperatures, i.e. quantitative comparison of this equilibrium measured by EPR at cryogenic temperatures with the one at ambient conditions is not straightforward. Useful information can be obtained by comparison of effects on EPR spectra caused by mutations, such as G248 mutants in CYP101A1. [171] Both G248T and G248V mutations completely eliminated low-spin signal in EPR spectra of camphor-saturated enzyme, indicating displacement of water molecule(s) presented near iron in the wild-type protein. Similar changes in EPR spectra of CYP2B4 caused by mutations in the active site and by various substrates have been documented by Coon and coworkers [172].

Sometimes EPR spectra for substrate-free cytochromes P450 indicate the presence of hydroxide as a sixth ligand, as reported for substrate free CYP152L1, where $g_z \sim 2.49$. [173] Upon addition of substrate fatty acids g_z shifted to 2.52, indicating direct coordination of carboxyl groups to the heme iron. Carboxyl coordination to iron was also reported in A264E mutant of CYP102A1, with $g_z=2.56$, as compared with $g_z=2.42$ in EPR spectra of substrate free wild-type CYP102A1. [174] Addition of substrate results in appearance of predominant population of pentacoordinated heme iron with high-spin EPR signal $g_z=8.18$. Ligation of nitrogen of azole group upon inhibitor binding results in low-spin EPR spectra with $g_z=2.58$, [174] and can be significantly perturbed by variations in inhibitor structures, as described for CYP51 [175]. Useful collections of EPR spectra of CYP101A1 with various substrates and inhibitors are tabulated in [16,176]. This can be compared with EPR spectra of CYP101A1 and several other P450 enzymes expressed in *E.coli* and measured in the whole cell preparations. [177]

A thorough spectroscopic study of several complexes of CYP2C9 and CYP125A1 with azole and pyridine inhibitors using UV-VIS and NIR absorption spectra together with MCD and EPR revealed fine details of binding and the role of axial water ligand. [178] Based on difference absorption spectra in the Soret region, all systems could be attributed to the Type II, although with obviously different spectral patterns. Analysis of EPR spectra and MCD revealed, that in some cases, the inhibitor does not displace water as the sixth ligand, and azole nitrogen is not coordinated to iron. Alternatively, it may be hydrogen bonded to the water molecule ligated to iron, which exhibits spectral changes similar to the Type II changes.

The same approach based on displacement of the 6th ligand by substrate is not straightforward for the reduced cytochromes P450, because ferrous heme is already pentacoordinated, and no spectral difference is observed between substrate-free and substrate-bound proteins. However, this difference may be introduced by adding another ligand, which can coordinate to the ferrous iron in the absence of substrate and thus can be monitored by optical spectroscopy. Displacement of this ligand by substrate binding at the

catalytic site is also detected spectroscopically. Such a method was described for measuring affinity of ferrous CYP102A1 mutants at F393 position to several fatty acids by monitoring UV-VIS spectral changes in the presence of 4-cyano pyridine. [179] As predicted by thermodynamic considerations, [88] binding of substrates is several-fold tighter when cytochrome P450 is reduced. In addition, linear correlation between experimentally observed energy of the metal-to-ligand charge transfer band at 628 – 647 nm and redox potential of the heme iron provides a good express method of predicting heme iron redox potential.

2.3 NMR for substrate binding and dynamics

NMR spectroscopy can be very useful in studying substrate binding to cytochromes P450 and for probing orientations and dynamics of substrate molecules inside catalytic active site. [180,181,182,183,184,185,186,187,188] While obtaining high-resolution structural information for the protein part requires isotope labeling and sequential assignment, NMR can monitor signals from other players. Paramagnetic relaxation of substrate protons provides crucial information of substrate positioning with respect to the heme iron, and can effectively serve as a convenient probe of substrate relocations caused by P450 reduction [180], temperature changes [182], or interactions with allosteric effectors [185]. Dawson and coauthors used ^{19}F NMR to probe dynamics of fluorinated camphor binding to reduced CYP101 (high-spin paramagnetic Fe^{2+}) and CO-bound ferrous CYP101A1 (low-spin paramagnetic Fe^{2+}). [187,188] Also, ^{13}C and ^{15}N labeled substrates can be employed, as described for complexes of selectively labeled acetaminophen analogs with CYP1A1 and CYP2B1 from rat liver [189]. Alternatively, signal from ^{13}C labeled methyl isocyanide can be used as a probe of binding of unlabeled substrates which can strongly perturbs NMR spectra of this ligand, as was shown for camphor binding to CYP101A1 and benzphetamine binding to CYP2B4 [190]. Notably, in both cases, multiple peaks were observed with substrate bound, indicating multiple conformational sub-states. Furthermore, high mobility of perdeuterated substrate adamantane in CYP101A1 active site was also detected by solid-state deuterium magic angle spinning NMR spectroscopy [181]. Other examples of NMR application to studies of substrate binding and for structural evaluation of substrate positioning and dynamics in P450 catalytic site have been described. [106,191,192]

It is important to note that the structural and dynamic changes in cytochromes P450 upon substrate binding have been also probed by NMR even without comprehensive sequential signal assignments. Incorporation of non-native amino-acids into CYP119 was used for detection of shifts between open and closed conformational sub-states upon binding substrates. [193] Three mutant proteins were created by replacing phenylalanines F144, F153 and F162 with ^{13}C - p -methoxyphenylalanine, and two-dimensional ^1H , ^{13}C -HSQC spectra were acquired for substrate free forms and in the presence of Type I (lauric acid) and Type II (imidazole and 4-phenylimidazole) ligands, both in oxidized and reduced CO-bound states. Positions of these Phe probes were selected at or near highly mobile F-G loop based on the known X-ray structures. In all cases, binding of substrate or inhibitor resulted in the appearance of new resonances, characteristic for the closed CYP119 form, with clearly observed residual resonances from the open form typical for substrate-free CYP119. In the later extension of this work, binding of the set of azole inhibitors to CYP119 was probed

using a similar method with ^{15}N -labeled phenylalanines introduced in a number of single-point mutants. [194]

Finally, when structural assignment is available, as was accomplished for CYP101A1 in several works by the Pochapsky group [195,196,197], NMR spectroscopy yields the most thorough picture of structural and dynamic changes associated with substrate binding. [198,199,200] By combining distance restraints obtained from residual dipole coupling with molecular dynamics simulations, resolution of the structural ensemble of CYP101A1 in solution was accomplished, with important differences being observed when compared with the X-ray structures. These NMR studies once again highlight flexible and dynamic architecture of P450 catalytic sites, which is true even for such an efficient isozyme as CYP101A1 with high substrate specificity.

2.4 Resonance Raman spectroscopy

Substrate binding to ferric cytochrome P450 gives very distinct and clear response in the rR spectra. Not only oxidation and spin state changes can be easily monitored but also changes in disposition of the heme peripheral groups, heme macrocycle deformations and key Fe-S bond between heme iron and protein thiolate residue. [11,13,201] Detection of out-of-plane deformations and changes in disposition of the propionic acid and potentially conjugated vinyl peripheral substituents, as well as monitoring strength of the Fe-S bond, are especially important because all of these factors are considered as effective structural determinants of heme reactivity.

2.2.1 Skeletal Modes and Heme Peripheral Groups—The modes in high frequency rR spectra of heme proteins are sensitive to oxidation and spin state changes or alterations of heme core size. [12,13,202] The nature of these structure-sensitive modes and the assignment of rR bands have been elucidated using isotopic labeling and normal mode calculations of model compounds and heme proteins. [203,204,205,206,207,208,209,210,211,212,213] For the substrate-free and camphor-bound forms of cytochrome P450cam (Figure 4), the oxidation state marker, ν_4 , appears at $1372\text{--}1373\text{ cm}^{-1}$, as expected for a ferric oxidation state (Table 1). The core size marker bands of the substrate-free form, the ν_3 , ν_2 and ν_{10} , which are sensitive to spin state and coordination number, appear at 1503 , 1584 and 1637 cm^{-1} , respectively, indicating a 6 coordinated low spin state form (Table 1). [13,201,214,215] Binding of camphor substrate causes down-shifts of these spin state marker modes to lower frequencies, with the ν_3 and ν_2 modes being now observed at 1488 and 1570 cm^{-1} , respectively, this behavior being indicative of a five coordinate, high spin complex (5cHS) arising in response to displacement of a water molecule upon binding of substrate (*vide supra*) (Figure 4, trace B). The unresolved wide envelope of overlapping bands appearing in the region of $1620\text{--}1635\text{ cm}^{-1}$ is attributable to the overlap of the ν_{10} spin state marker and the two vinyl $\nu(\text{C}=\text{C})$ stretching modes. [215]

The two vinyl $\nu(\text{C}=\text{C})$ stretching modes appearing at 1619 cm^{-1} and 1631 cm^{-1} in the SF form indicate the presence of at least two distinct vinyl conformers in the SF form and the rR studies of isotopically labeled cytochrome P450cam revealed that both vinyl stretching modes are unaffected by binding of camphor. [215] Early work with heme model

compounds by Bocian and coworkers, [216] as well as the more recent studies by Schelvis and coworkers on cytochrome P450 BM3, [217] suggest that the lower frequency $\nu(\text{C}=\text{C})$ stretching modes correspond to a nearly in-plane vinyl conformation, whereas higher frequency values are associated with more out-of-plane orientations, a suggestion consistent with the careful analysis by Smulevich and Marzocchi, [218] which defines the relationship between $\nu(\text{C}=\text{C})$ vinyl stretching modes and the orientation of vinyl groups with respect to the pyrrole ring.

In recent studies by Kincaid and coworkers, [219] the rR spectra of human CYP3A4 solubilized in detergent Emulgen 913 show no difference in SF and SB forms; i.e., the spectra of the SF enzyme, as well as in the presence of 600 μM testosterone (TST), showed that both samples have a mixture of spin states, containing approximately 40 % of HS (spectra A and B in Figure 5). [219] It was argued that the “substrate-free” sample shows components of HS state owing to the presence of the Emulgen 913 that might acts as a substrate, [64] and therefore compete with TST binding that is reflected in lack of further conversion to the HS state upon addition of excess of substrate molecules. On the contrary, the substrate-free sample of CYP3A4 incorporated into Nanodiscs shows rR spectral characteristic of predominantly 6cLS state, whereas addition of saturating amounts of TST molecules results in almost complete spin state conversion to the 5cHS state ($\sim 85\%$) (spectra C and D of Figure 5). The point to be made is that the Nanodisc sampling methodology, that had been previously shown to produce well-behaved functional properties of membrane bound enzymes, provides new systems with well-defined *spectral parameters* that are reliably responsive to substrate binding in a manner that is consistent with observed functional changes. The reliability of these rR studies was satisfyingly reinforced recently by crystallographic studies of CYP3A4 complex with bromocriptine, as discussed in a recent review, [220] where it is stated that substrate binding leads to out-of-plane distortions of the heme macrocycle and reorientation of vinyl groups, with the authors noting that these structural changes had been already deduced in an earlier rR study. [219] In more recent studies, structural and functional aspects of binding of multiple substrate molecules to CYP3A4 were studied by rR (and UV-Vis) spectroscopy using synthetic testosterone dimers of different configurations, *cis*-TST₂ and *trans*-TST₂. [107] It has been shown, for the first time that the binding of two steroid molecules, which can assume multiple configurations inside the substrate binding pocket of monomeric CYP3A4, can lead to active site structural changes that affect functional properties; e.g., the degree of spin shift and binding affinity strongly depend on the geometry of TST dimers. Furthermore, the interaction of TST monomer with CYP3A4 at the remote site of the enzyme significantly increases spin shift and binding affinity for the *trans*-TST₂, [107] supporting earlier observations of allosteric effect caused by binding of steroids at the allosteric site. [102,117,123]

It is worth noting that rR spectroscopy also provides a valid methodology for quantitative determination of the extent of spin state conversion upon substrate binding. The rR studies of cytochrome P450cam containing internal standards permitted the generation of reliable relative rR cross-sections of spin-state marker bands in substrate-free and substrate-bound forms of CYP101. [215] The intensities of the ν_3 modes (I_{LS}) and (I_{HS}) were compared to that of an internal standard of sodium sulfate (I_{IS}) to yield relative intensities for the two spin states; e.g., $Y_{\text{LS}} = I_{\text{LS}}/I_{\text{IS}}$. It was determined that the ($Y_{\text{HS}}/Y_{\text{LS}}$) ratio of the cross

sections for the ν_3 modes was 1.24. The application of this ratio was shown to permit a reliable calculation of relative populations of the two spin states from rR spectra of several other cytochromes P450, including drug metabolizing (CYP2B4, CYP3A4) and steroidogenic (CYP17) mammalian cytochromes P450, matching quite well with the values obtained from electronic absorption spectral studies of low concentration samples.

The most dominant mode in the low frequency region of the spectra of SF and SB samples is assigned to the ν_7 mode at around 675 cm^{-1} , while another intense in-plane skeletal mode, the ν_8 mode, is seen near 345 cm^{-1} (Figure 6). It has become increasingly apparent in recent years that certain heme modes in this region, such as bending motions of the vinyl and propionate substituents, as well as the out-of-plane modes (γ_i) activated by distortions of the heme macrocycle from planarity, are especially sensitive to binding of substrates, effectors and redox partners. [217,221,222,223,224] Importantly, convincing arguments have been presented for the significant impact of all of these structural features on heme protein functional properties, including heme reduction potential and affinities for endogenous and exogenous ligands. [225,226,227]

The so-called “vinyl bending” modes are usually observed in the $400\text{--}440\text{ cm}^{-1}$ region. Similar to the $\nu(\text{C}=\text{C})$ stretching counterparts in the high frequency region, the $\delta(\text{C}_\beta\text{--C}_\alpha\text{--C}_b)$ vinyl bending modes are generally considered to be sensitive to the orientation of the vinyl groups with respect to the planes of the adjacent pyrrole rings. The modes observed in the lower half of this frequency region are typically associated with an in-plane vinyl group orientation, while modes observed in the higher range are linked with vinyl groups experiencing out-of-plane distortions. [216,217,218,223,228] It is important to explain that while these modes have been designated as the “vinyl bending” modes, they are more accurately described as deformations of pyrrole rings I or II that contain significant vinyl bending contributions, as more fully discussed in recent work using several methyl deuterated protohemes. [229,230] Finally, in agreement with the behavior of the vinyl stretching modes discussed above, substrate binding generally has little or no effect on vinyl group dispositions, as judged by the lack of significant changes for the vinyl mode envelopes observed near 403 and 424 cm^{-1} in traces A and B of Figure 6.

The “propionate bending” modes, $\delta(\text{C}_\beta\text{--C}_c\text{--C}_d)$, typically occur in the region of $\sim 360\text{--}380\text{ cm}^{-1}$ with those features observed in the higher end of this range being ascribed to stronger H-bonding of the terminal carboxyl groups with active site residues. [217,221,228] Similar to the vinyl groups, this mode is also sensitive to the deuteration of heme methyl groups adjacent to III and IV pyrrole rings, indicating its proper assignment to the deformation of pyrrole rings with propionate bending contributions. [229,230] More importantly, it has been suggested that the propionate groups can also alter the heme iron redox potential. [225,227] The band assigned to the “propionate bending” mode in the spectrum of SF form of CYP101 is seen at 380 cm^{-1} . Upon binding of the substrate, a new mode appears near 367 cm^{-1} . [217,222] This mode was originally attributed to an out-of-plane γ_6 mode, [222,231] presumably activated by substrate binding in analogy with the γ_7 counterpart. However, Schelvis and coworkers [217] argued for assignment of this new, substrate-activated mode, to a second propionate mode, associated most reasonably with the propionate group at the 7-position. This issue was finally resolved by extended rR studies of cytochrome P450cam

reconstituted with deuterated hemes and employing systematic deconvolution procedures to provide convincing arguments that the 367 cm^{-1} feature contains contributions from *both* the γ_6 and propionate bending modes. [214] Finally, binding of substrate causes heme plane deformation that are reflected by activation out-of-plane modes (OOP), such as γ_7 , and OOP modes at around 500 cm^{-1} .

2.4.2 The Fe-S Bond—A direct measure of the strength of the Fe-S bond is of obvious importance and is most readily accomplished by resonance Raman (rR) spectroscopy using excitation within the $S \rightarrow \text{Fe}$ charge transfer transition envelope that occurs near 360 nm in the ferric HS form, far to the blue from the Soret transition. [11,13,201,232] The rR studies of isotopically labeled protein show that the 351 cm^{-1} feature seen in the spectrum of native P450_{cam} shifts by $+2.5\text{ cm}^{-1}$ upon ^{54}Fe substitution and -4.9 cm^{-1} upon ^{34}S isotope incorporation, confirming its assignment to the $\nu(\text{Fe-S})$ mode. [232] It is important to note that rR studies of substrate-free CYP101 samples show no evidence of the $\nu(\text{Fe-S})$ mode, the lack of enhancement being most reasonably attributed to the changes in the Fe-S charge-transfer transition associated with the low spin to high spin transition.(EXPLAIN?)

The $\nu(\text{Fe-S})$ mode is usually not sensitive to the structure of the distal pocket-bound substrate; e.g., the Fe-S stretch in P450_{cam} is not substantially different for samples containing different substrates (*i.e.*, camphor, adamantanone, norcamphor or TMCH (3,3,5,5-tetramethylcyclohexanone)). [233] Similar lack of substrate effects on the Fe-S linkage was observed for P450_{2B4}, [223] P450_{c21}, [234] CYP19A1, [235] and CYP17A1. [236,237] On the other hand, the changes in the proximal heme side, such as modulation of H-bonding network, can affect the strength of the Fe-S bond; *e.g.* H-bond donors are predicted to cause a lowering of the $\nu(\text{Fe-S})$ mode. [238] Early work led to unexpected results; *i.e.*, removal of existing H-bond donors led either to no effect on the observed $\nu(\text{Fe-S})$ frequency or to an unexpected *decrease*. [239] Conversely, introduction of a new H-bond donor residue to the proximal pocket of CYP102 (*i.e.*, F393H) failed to induce any observable shift of its $\nu(\text{Fe-S})$ mode. [217] The results obtained for the CYP101 mutations remain somewhat ambiguous, [240] while the absence of any changes on the $\nu(\text{Fe-S})$ mode for the F393H mutant of CYP102 mutant was reasonably attributed to the fact that the histidyl residue was not in a proper orientation to H-bond to the proximal cysteine thiolate fragment. [217] Though such apparently well-designed studies failed to document the expected impact of an H-bonding interaction, more recent rR spectroscopic studies of another cytochrome P450 and its mutant have provided convincing support for this effective intramolecular control mechanism. [241] The proximal pocket of CYP2B4 contains a F429 residue positioned near the Fe-S bond. In the F429H mutant the newly introduced histidine residue is apparently well-positioned to interact, leading to a tell-tale 6 cm^{-1} lowering of the $\nu(\text{Fe-S})$ stretching mode, as expected. A relatively strong feature appearing at 353 cm^{-1} in the BHT-bound WT enzyme (Figure 7) shifts down to 347 cm^{-1} upon introduction of the H-bond provided by the F429H mutation. The observed 6 cm^{-1} shift of $\nu(\text{Fe-S})$ to lower frequency for F429H is qualitatively consistent with expectations based on the reduction of effective negative charge on the thiolate, in agreement with recent computations. [238]

The bacterial cytochrome P450_{cam}, CYP101, is one of the most studied using rR spectroscopy. [11,201,214,215,232,242,243,244,245] Comprehensive early rR studies of

CYP101 expressed in *Escherichia coli* were performed by Wells et al. [201] CYP102, which served well as an early model for mammalian P450s, was another intensively studied P450. [217,231,246,247,248,249,250,251] The thermophilic cytochrome P450, CYP119 from *Sulfolobus solfataricus*, is stable up to ~85 °C and became an obvious subject of many investigations. [252,253,254] Resonance Raman studies of a few other bacterial P450 enzymes include peroxygenases CYP152B1 (P450_{SP α}) and CYP152A1 (P450_{BS β}), [255,256] several cytochromes P450 from *Mycobacterium tuberculosis*, [175,257,258,259,260] cytochrome P450BioI [261] involved in synthesis of biotin in *Bacillus subtilis* and a fungal CYP55 (P450_{nor}). [262]

The microsomal CYP2B4 from rabbit was one of the first mammalian P450 studied by rR. [223,263,264,265,266] The inactivation of CYP2B4 by the acetylenic inhibitor 4-(*tert*-butyl) phenylacetylene, was reported recently, showing, for the first time, the utility of rR technique to studying mechanism-based inactivation process. [267] There are several studies of CYP2C13, [268,269] CYP1A2, [270,271,272] CYP2A6, [271] CYP2C9, [271] CYP2E1, [273] CYP2D6 [271,274,275] and CYP3A4, [219,270,271] including the first application of rR technique to studying Nanodisc-incorporated assemblies (*vide supra*). [219] CYP11A1 (P450_{SCC}), the crucial enzyme that initiates the first step of steroid synthesis converting cholesterol to pregnenolone was the first steroidogenic cytochrome P450 investigated by rR spectroscopy. [263,276,277,278,279] The rR study of this enzyme by Tsubaki *et al.* showed that cholesterol binding to the SF form of P450_{SCC} causes substantial spin state change, similar to that observed for CYP101A1 [278]. Kitagawa and coworkers investigated substrate-induced structural changes in the heme pocket of human aromatase CYP19A1 that converts C19 androgens to estrogens. [280] These studies were followed by study of bovine cytochrome P450 steroid 21-hydroxylase (P450c21) that catalyzes hydroxylation at the C-21 carbons of progesterone (PROG) and 17-hydroxyprogesterone (17-OH PROG) to produce 11-deoxycorticosterone and 11-deoxycortisol, respectively. [234] In more recent studies of steroidogenic P450s, incorporated into Nanodiscs, Kincaid, Sligar and coworkers investigated the ferric and ferrous CO adducts of human CYP17A1. [236] This human cytochrome is essential in sex hormone biosynthesis and catalyzes the 17 α -hydroxylation of pregnenolone (PREG) to 17 α -hydroxypregnenolone (17-OH PREG) and progesterone (PROG) to 17 α -hydroxyprogesterone (17-OH PROG). The hydroxylated products are then used for production of corticoids or undergo a second CYP17A1-catalyzed transformation resulting in C₁₇–C₂₁ bond cleavage.

3. Dioxygen adducts

Binding of dioxygen to a ferrous cytochrome P450 forms the last relatively stable intermediate in the P450 enzymatic cycle (Scheme 1). This nominally ferrous dioxygen adduct is diamagnetic and EPR silent, but yields a Mössbauer quadrupole splitting for the iron in the oxy form that is characteristic of the ferric oxidation state. [281] A relatively low $\nu(\text{O-O})$ stretching mode seen near 1140 cm⁻¹ in the rR spectrum [282,283] is indicative of a ferric superoxide (Fe³⁺-O₂⁻) species. The Fe-O-O fragment is inherently bent, with the Fe-O-O angle being 130° – 140°, as observed by X-ray crystallography in CYP101A1, [284,285] similar to those observed in myoglobin (122°) [286], hemoglobin (124° and 126° in α - and β -subunits) [287], cytochrome c peroxidase (133°) [288], horseradish peroxidase

(126°) [289], guanylate cyclase H-NOX domain (114° – 134°) [290], FixL heme domain (119°) [291] and heme oxygenase (110°) [292]. Subsequently, the X-ray structures of oxy-ferrous complexes in mutant CYP101A1 [285], CYP107A1 [293] and CYP158A2 [294] were solved. Recently determined high resolution X-ray structure of oxy-complex of an iron picket fence porphyrin may serve as the best available model compound, with the angle Fe-O-O = 118.2° and the bond lengths Fe-O = 1.811 Å and O-O = 1.281 Å, also consistent with predominantly superoxide formulation. [295] However, this assignment is still debated, [296] in part because equilibrium between ferrous-oxy and ferric-superoxo forms can be easily shifted by hydrogen bonding to the dioxygen ligand [297] together with other factors [298], as recently reviewed. [299]

3.1. Electronic absorption spectroscopy

The UV-Vis spectra of the ferrous O₂ adducts of cytochrome P450 have been first reported for bacterial CYP101A1 by Peterson and co-workers in 1971 [300], followed by further studies of the same P450cam [301,302,303,304], CYP102, [305,306,307,308] CYP108, [305] CYP119, [252] CYP2B4 [309,310,311], CYP11A1 [312,313], CYP19A1 [314,315] and CYP3A4 [102,316]. The electronic absorption maxima of the oxygenated ferrous CYPs, called Soret band, is usually observed at 417–418 nm or in some cases at 424 – 427 nm, as in CYP3A4, [102,316] and in CYP2B4 [311] where position of absorption maximum can be significantly shifted in the presence of certain substrates. The Q-band has a maximum at 552–557 nm with a shoulder at ~580 nm. [75,304,315]

The UV-VIS spectra are commonly used to study kinetics of formation and decay of the oxy-complex in cytochromes P450 *via* autoxidation pathway with release of superoxide O₂^{•−} from ferric enzyme. [301,302,303,304,305,316] The stability of the oxy-complex in P450 enzymes is limited, with autoxidation rates ranging from ~200 s^{−1} for substrate-free CYP3A4 [316] to 0.004 s^{−1} for CYP101A1 saturated with camphor at room temperature. [153] Importantly, the rates of autoxidation of cytochromes P450 strongly depend on temperature, because of high activation energy barriers, from ~15 to ~23 kcal/mol. [316] This indicates relatively large scale conformational changes as a rate-limiting step, most likely related to the “open-close” concerted movement of the F and G helices. [317]

3.2. Mössbauer spectroscopy

The dioxygen adduct of CYP101A1 studied by Mössbauer spectroscopy and reported by Sharrock et al. [281] was found to be diamagnetic, but exhibited a combination of quadrupole splitting and isomer shift that are characteristic for the Fe³⁺, similar to those found in oxy hemoglobin [318] and HRP Compound III [319]. Temperature-dependent Mössbauer spectra of iron oxy-complex in the picket fence model compound show a significant conformational mobility of dioxygen ligand and possible vibronic contribution to reduced quadrupole splitting at higher temperatures. [295] Experimentally observed variations and temperature dependence of Mössbauer parameters in various oxyhemoglobins have been reviewed elsewhere. [320]

3.3 EXAFS/XANES spectroscopy

Dawson et al. structurally characterized oxygenated CYP101 using EXAFS spectroscopy [321]. The authors confirmed, by comparing EXAFS spectra of oxy-CYP101 to that of oxy-chloroperoxidase, that these two complexes had identical heme iron coordination spheres as had been proposed earlier [322] and that the discrepancies in their UV-vis absorption and MCD spectra were due to differences in the distal heme environment. The Fe-S bond distance of oxy-CYP101A1 was calculated to be 2.37 Å. The mechanistic implications of having a thiolate ligand in the P450 system have been the subject of many discussions, having been summarized previously [10]. Comparison of XANES spectra of oxy-forms of CYP101A1, HRP and Mb [323] showed that the geometry of iron ligation and the Fe-O-O bond angle are similar among these hemoproteins. Furthermore, the π -electron donation from the thiolate sulfur to the Fe-O-O moiety is substantiated in the XANES spectrum of CYP101A1. [296,323] This observation is clearly consistent with CYPs biological function where the weakening of the oxygen-oxygen bond favors its heterolytic scission and formation of the main ferryl-oxo catalytic intermediate.

3.4 Resonance Raman spectroscopy

The modes associated with the dioxygen adduct of cytochromes P450 are usually well enhanced in the rR spectra of these thiolate ligated proteins. The $\nu(\text{O-O})$ stretching mode is typically observed in 1125–1145 cm^{-1} range. The first rR spectrum of a dioxygen adduct was reported by Champion and co-workers, [282] with the $\nu(\text{O-O})$ mode being detected at 1140 cm^{-1} in CYP101A1, exhibiting the expected 66 cm^{-1} downshift upon $^{18}\text{O}_2$ substitution. [283] It has been shown that the $\nu(\text{O-O})$ stretching mode is sensitive to the steric crowding imposed by the bound substrate; e.g., the spectra of adamantanone-bound samples contain two $\nu(\text{O-O})$ modes. [283] Similarly, the broadening of the $\nu(\text{O-O})$ mode in the oxy D251N mutant of CYP101A1 was attributed to the existence of two or three structural conformers of the Fe-O-O group, [70] an observation later supported in a study by Kincaid and co-workers [59] who proposed that the difference in the frequencies of two major conformers is associated with changes in H-bonding interactions (*vide infra*). [324,325,326]

The $\nu(\text{Fe-O})$ modes are usually seen in the 517–541 cm^{-1} range and the first $\nu(\text{Fe-O})$ stretching mode was first identified by Hu *et al.* at 540 cm^{-1} for CYP101A1. [283] The 29 cm^{-1} shift of this mode upon $^{16}\text{O}/^{18}\text{O}$ exchange is larger than expected and was rationalized by invoking a coupling interaction with an adjacent heme mode [283] or, alternatively, explained in terms of an effective “three body oscillator” model. [327] The lowering of the $\nu(\text{Fe-O})$ frequency in the spectra of CYPs, as compared to the corresponding modes in the spectra of histidyl ligated globins, [12,202] reflects stronger electron donating properties of the CYPs proximal thiolate axial ligand. Finally, the $\delta(\text{Fe-O-O})$ bending mode was identified by Macdonald et al. at 401 cm^{-1} , shifting down by 19 cm^{-1} upon $^{18}\text{O}_2$ substitution; a three-body normal coordinate calculation was conducted to fit the experimental data, yielding a calculated angle of 125–130° for the Fe-O-O, [327] a value close the 130° – 142° extracted from X-ray crystallographic studies. [284,285]

The progress in development of *Escherichia coli* expression systems and optimization of purification methods of mammalian P450s [328,329] permitted characterization of these key Fe-O-O oxy fragments in these biologically and medically important systems. Kitagawa and co-workers reported the rR spectra of CYPc21 in the presence of its natural substrates, progesterone (PROG) and 17 α -OH progesterone (17-OH PROG). [234] CYPc21 is responsible for hydroxylation of PROG and 17-OH PROG at C-21 carbons, the essential steps in the biosynthesis of aldosterone and cortisol. The authors observed only one $\nu(\text{O-O})$ mode in the spectra of the PROG-bound sample while the 17-OH PROG sample exhibited two $\nu(\text{O-O})$ modes, an indication of the presence of one and two Fe-O-O conformers, respectively. The authors proposed that the lower frequency conformer has stronger H-bonding interactions formed between the substrate's hydroxyl group and the terminal oxygen atom of this Fe-O-O fragment. Also, rR spectroscopy was used to characterize the active site structures of dioxygen adducts of full length human CYP19A1, which is responsible for the conversion of androgens to estrogens. [235] The conversion of androstenedione (AD) to an aromatic C18 estrogen, estrone, involves two consecutive oxidations at the C19 methyl group and a third "lyase" step that culminates in cleavage of the C10-C19 bond of the C19-aldehyde and A ring aromatization. [330] It has been shown that the vibrational spectral patterns for both oxy adducts with the first (AD) or third (19-oxo AD) substrates [235] are consistent with H-bond donation to the terminal oxygen. (May be cut this statement in the light of CYP17A1 – OH-PROG situation, it is also H-bonded at distal oxygen, but goes through peroxo)

An especially effective demonstration of the potential of rR spectroscopy to reveal functionally significant H-bonding interactions with Fe-O-O fragments is given by the study of CYP17A1. [237] This cytochrome is responsible for standard hydroxylase chemistry, proceeding through the Compound I pathway, converting pregnenolone (PREG) and progesterone, to 17 α -OH pregnenolone (17-OH PREG) and 17 α -OH progesterone, respectively. [1,331,332] These hydroxylated products then participate in a second oxidative cycle in which the 17C-20C carbon-carbon bond is cleaved, in a "lyase" reaction, to form dehydroepiandrosterone (DHEA) and AD. [331,332] Interestingly, human CYP17 exhibits a higher product formation for 17-OH PREG over 17-OH PROG, prompting a long-standing debate as to the chemical mechanisms of this lyase activity.

The rR spectra of the dioxygen adducts of full length human CYP17 incorporated into Nanodiscs reveal a distinct difference in hydrogen bonding to the ferrous dioxygen intermediates. [237] In Figure 8 Panel A are shown rR spectra obtained for the $^{16}\text{O}_2$ adduct of PROG-bound CYP17. The $\nu(^{16}\text{O}-^{16}\text{O})$ mode is observed at 1140 cm^{-1} and the $\nu(\text{Fe-O})$ stretching mode appears at 536 cm^{-1} (difference traces in Panel A); neither of them exhibiting sensitivity to the $\text{H}_2\text{O}/\text{D}_2\text{O}$ exchange. The frequencies of these modes are quite similar to those observed when the Fe-O-O fragment is only weakly hydrogen bonded to P450 active-site residues. [14,59] The spectra of 17-OH PROG-bound CYP17 are shown in Panel B, where the $\nu(^{16}\text{O}-^{16}\text{O})$ appears at 1131 cm^{-1} ; i.e., 9 cm^{-1} lower than in the spectrum of the PROG bound sample. This behavior indicates that the Fe-O-O fragment is H-bonded, a suggestion that is supported by the telltale 2 cm^{-1} shift to higher frequency samples prepared in D_2O buffer (Panel B, lower right). [14,59,333,334] Significantly, the lowering of the $\nu(\text{O-O})$ mode is accompanied by a 6 cm^{-1} increase in the frequency of the

$\nu(\text{Fe-O})$ mode relative to its value in the PROG-bound enzyme. The changes in the frequency of the oxygen sensitive modes are most reasonable attributed to a hydrogen-bonding interaction of the Fe-O-O fragment with the newly introduced C17-OH(D) functionality of the hydroxylated substrate.

The corresponding spectra for CYP17 containing PREG and 17-OH PREG substrates are shown in Figure 8, panels C and D, respectively. The $\nu(\text{O-O})$ and $\nu(\text{Fe-O})$ modes of the PREG-bound enzyme are observed near 1140 and 535 cm^{-1} , virtually identical to these seen for PROG bound samples (panel A), indicating that the interactions of PROG and PREG with the Fe-O-O fragment are quite similar. However, as shown in Panel B, the $\nu(\text{O-O})$ mode of the 17-OH PREG sample, is shifted down by only 5 cm^{-1} compared with its value for the PREG-bound form and exhibits a barely detectable shift in deuterated solvents. Most importantly, the $\nu(\text{Fe-O})$ mode is observed at 526 cm^{-1} i.e., exhibiting a 9 cm^{-1} shift to *lower* frequency compared to the value observed for the PREG substrate, which lacks a H-bond donor. It is emphasized that quite dramatic differences are observed when comparing the samples bound with 17-OH PROG and 17-OH PREG. Though introduction of the 17-OH group causes downshifts of the $\nu(\text{O-O})$ modes for both hydroxylated substrates, the corresponding $\nu(\text{Fe-O})$ modes shift in *opposite* directions; i.e., the 17-OH PROG yields a 6 cm^{-1} upshift while the 17-OH PREG shows a 9 cm^{-1} downshift. It is noted that since rR spectra showed no evidence for changes in the trans Fe-S bond strength in all four samples, all changes in the Fe-O-O fragment arise mainly from distal side interactions.

It has been shown previously in several studies of the thiolate proteins and their model compounds that the modes associated with the Fe-O-O fragment are quite responsive to distal pocket H-bonding interactions. The H-bonding to the terminal oxygen atom O_T lowers the frequency of $\nu(\text{O-O})$ stretch by 7–12 cm^{-1} [59,234,235,237,335,336,337] and sometimes exhibits a tell-tale 2–3 cm^{-1} up-shift of the $\nu(\text{O-O})$ mode upon H/D exchange, as was elegantly shown by Naruta and coworkers in their studies of well-designed model compounds. [333,334] On the other hand, H-bonding to the proximal oxygen O_P does not affect the $\nu(\text{O-O})$ stretch substantially, but interestingly does reduce the frequency of the $\nu(\text{Fe-O}_2)$ mode by approximately 10 cm^{-1} . [237,335,336,338] These observations are consistent with the rR studies of globins and their mutants. [339,340,341,342] Furthermore, DFT calculations on histidine-ligated oxy complexes predict that, other factors being held constant, hydrogen-bond donation to the proximal oxygen atom (O_P) will weaken both bonds by withdrawal of electrons into the nonbonding sp^2 orbital on the O_P , thus causing both the $\nu(\text{Fe-O})$ and the $\nu(\text{O-O})$ modes to shift in concert to lower frequency (positive correlation). [14] In contrast, hydrogen-bond donation to the terminal oxygen atom, O_T , results in a straightforward increase in back-bonding; i.e., one expects a negative correlation, where the $\nu(\text{O-O})$ decreases while the $\nu(\text{Fe-O})$ increases.

In summary of the CYP17A1 data, [237] the positions of the $\nu(\text{Fe-O})$ and $\nu(\text{O-O})$ modes in spectra of 17-OH PROG bound CYP17 samples, relative to those with PROG-bound, indicate hydrogen bonding to the *terminal* oxygen (O_T) of the Fe- O_P - O_T fragment, whereas spectra obtained for the 17-OH PREG-bound sample implicate hydrogen bonding to the *proximal* oxygen (O_P). The authors pointed out that to the extent that these interactions persist in the subsequently reduced peroxo-intermediates, the latter interaction is expected to

inhibit O-O bond cleavage relative to the former, permitting nucleophilic attack of the peroxo intermediate on the 20-carbonyl, the crucial initial step triggering the lyase process. Thus, the observation of differential H-bonding interactions, satisfactorily explains lower susceptibility to lyase activity of the 17-OH PROG relative to that of the 17-OH PREG. Indeed, a recent review summarising CYP17A1 mechanism, makes the point that these rR data acquired for the CYP17A1 provide first experimental explanation for selectivity in the lyase step. [343]

4. Peroxo and hydroperoxo intermediates

There had long been a considerable lack of experimental information on the structural properties of the reduced dioxygen moiety due to its short lifetime and rapid decay in reactions performed in solution. The theoretical calculations predicted a significant lengthening of the O-O bond in the peroxo-ferric, as compared to the oxy-ferrous complex, and a further increase in O-O distance in the hydroperoxo-ferric intermediate (Scheme 1). [344,345,346,347,348] In heme enzymes the structure of the peroxo species is η^1 -Fe (end-on) and low spin. Though many early attempts to generate and stabilize the reduced oxy P450 failed, the application of cryoradiolytic reduction of oxy-ferrous precursor in frozen aqueous/organic solutions at 77 K, resulted in preparation of high yields of these trapped unstable species expediting their spectroscopic characterization. [15,37,38,41,42,57,59,60,75,76,77,349]

4.1. Electronic absorption spectroscopy

Electronic absorption spectra of hydroperoxo-ferric intermediate have been obtained by cryogenic radiolytic reduction of oxy-complex in CYP101A1 (with ^{32}P phosphate). [75] The authors showed that the absorbance maximum of the oxy-P450 spectrum seen at 417 nm decreased with concomitant increase in the absorbed dose of ^{32}P radioactivity and was accompanied by the appearance of a new peak at ~440 nm. These changes reflected the progress of one-electron reduction of oxy-P450 and the formation of reduced oxy-P450 complex, i.e., the hydroperoxo intermediate. The irreversible evolution of this hydroperoxo intermediate upon its careful annealing from 77 to 240 K was also monitored by UV-vis spectroscopy, revealing the formation of the low-spin ferric cytochrome P450 as an intermediate step before the product release that was previously determined by EPR spectroscopy. [42] Failure to trap the peroxo-intermediate is a result of the exceptionally efficient proton delivery system, even at 77 K, in the wild type CYP101A1 active site and is consistent with the EPR studies of CYP101A1 (*vide infra*). The electronic absorption spectrum of peroxo species has been obtained by cryoradiolytic reduction of oxyCYP101A1 D251N mutant that contains a perturbed proton delivery network in its active site, and exhibits the absorption maximum blue shifted by 2–3 nm with respect to the hydroperoxo intermediate (Figure 9). The results of these and subsequent investigations have been summarized previously [38,39,350] and show the UV-vis characterization of the peroxo and hydroperoxo states generated by low-temperature radiolysis of the ferrous-oxy complex in CYP101A1, [59,75,77] CYP119A1, [252] CYP3A4 [351] and chloroperoxidase [352] among other systems.

4.2. Electron paramagnetic resonance spectroscopy

The intermediates generated by one-electron reduction of diamagnetic oxy adducts of heme proteins are paramagnetic making electron paramagnetic resonance (EPR) spectroscopy especially valuable in characterizing the peroxo and hydroperoxo states of cytochromes P450. [15] The first EPR and ENDOR spectra of P450 peroxo intermediate states was reported by Davydov et al. using CYP101A1 and its mutants, Thr252Ala and Asp251Asn. [37,42,56,57,71] The oxygenated complexes were cryoreduced at 77 K employing ^{60}Co γ -irradiation. The primary reduced oxy-P450 species of wild type enzyme at 77 K was identified as a hydroperoxo-ferric heme complex with $g = [2.29, 2.166, 1.96]$ for CYP101A1, [42,353] where an efficient proton delivery system in the wild type CYP101A1 active site prevented accumulation of the peroxo-ferric intermediate. The additional EPR experiments contributed further to the understanding of the active site H-bond network and the role of the “acid-alcohol pair” (Asp251-Thr252 in CYP101). It was observed that Asp251Asn mutation perturbs the proton delivery mechanism and allows accumulation of the ferric peroxo intermediate in the cryoreduced samples at 77 K. (Figure 10) The following careful annealing studies of the Asp251Asn mutant lead to formation of the hydroperoxo intermediate and finally the ferric state and the product, similar to the WT protein. [37,42] On the other hand, while the Thr252Ala mutation allowed trapping of the hydroperoxo intermediate, it did not yield product whereas the wild-type and D251N mutants do, implying that this mutation perturbs the delivery of the second proton. Furthermore, it was concluded that the hydroperoxo state is a key intermediate at the branch point that leads either to product formation or to “uncoupling” in which O–O bond cleavage is stymied by H_2O_2 formation.

The characterizations of the peroxo- and hydroperoxo-ferric intermediates in CYP101A1 and its mutants [37,42] provided clear EPR signatures for the unprotonated peroxo-ferric ($g_1 < 2.27$) and protonated hydroperoxo-ferric ($g_1 > 2.27$) intermediates and prompted further experimental studies of other heme proteins, such as myoglobin, hemoglobin, horseradish peroxidase and heme oxygenase (see Table 3.2 in ref. [33]). It is noted that although the first immediate product of cryoradiolytic reduction of the oxy-complex is the peroxo anion, in some cases, such as with wild-type CYP101A1, protonation can occur even at 77 K, with the peroxo anion being trapped only with cryoreduction in liquid helium.. This temperature dependence of the proton transfer events provides a recipe for stepwise annealing of the trapped peroxo anion to follow the transformation of metastable intermediates along the reaction coordinate through to product formation. The catalytic competence of the cryoradiolytic reduction of CYP101A1 can be directly demonstrated by analysis of product formation, with the overall yield proportional to the irradiation dose [42].

The further EPR and ENDOR work on CYP101A1 demonstrated important variations in proton delivery to the coordinated dioxygen ligand induced by a set of different substrates [71]. Systematic annealing studies revealed that the presence of substrates increases the stability of the hydroperoxo-ferric complexes with observed half-lives of 20-fold, or longer, than for substrate-free CYP101A1. Changes in ^{14}N , ^1H hyperfine couplings in the ENDOR spectra indicated multiple conformational substates at the heme iron. It was shown that in some cases, annealing of these conformers and disappearance of the various hydroperoxo-

ferric intermediates occur with drastically different rates. In addition, the T252A mutant, which does not yield a product with normal substrates, produced the epoxide of (1*R*)-methylenyl camphor, tentatively attributed to direct catalysis by hydroperoxo-ferric intermediate, often termed Compound 0. [347] These results clearly illustrate important role of substrates in modulating the chemical properties of peroxo- and hydroperoxo-ferric intermediates and selection of specific "active oxygen" species for catalysis. [71]

EPR and ENDOR studies also provided detailed insight into mechanism of proton delivery and Compound I formation in CYP101A1 by careful comparison of substrate camphor with site-specifically deuterated 5,5-dideutero-camphor. [74] Hydroxylation of protiated substrate that followed cryoreduction and annealing to 190 K, gave ENDOR signal from two protons, one from product hydroxyl C5-OH_{exo} and another from C5-H_{endo}, with subsequent exchange of the former to deuterium in D₂O buffer and its disappearance at 230 K. When deuterated camphor was used as a substrate, no such signal was observed in 190 K annealed samples, but the signal from H_{exo} proton appeared at 230 K in H₂O buffer as a result of the same proton exchange. This example clearly illustrates the extraordinary selectivity of ENDOR spectroscopy which allows monitoring single protonation or proton exchange events in the act of P450 catalysis.

Direct involvement of amino acids in the immediate vicinity of the heme iron in the proton delivery mechanism was demonstrated by Makris et al. by mutating the G248 position in CYP101A1 [171]. The G248 mutants which retain the native acid-alcohol pair of D251 and T252, show significant perturbation of the proton delivery, even though they can still catalyze camphor hydroxylation in a reconstituted system. Functional studies suggest that protonation of the hydroperoxo-anion is also inhibited by mutations at the 248 position. The EPR spectrum of the cryoreduced oxy-complex shows that the first protonation is also impeded, since the immediate product of the cryoreduction at 77 K is almost completely the unprotonated peroxo-anion, in contrast to the wild-type and the T252A mutant, for which cryoreduction at 77 K produces the hydroperoxo state (*vide supra*) [37,42,43].

Employing the low-temperature oxygenation protocols developed for the preparation of unstable oxy-complexes in cytochromes P450 and NOS [76,77,308,352,354,355,356], cryoradiolytic reduction and characterization of the peroxo- and hydroperoxo-ferric intermediates have been carried out for the drug metabolizing mammalian cytochrome CYP2B4, [72] as well as for the steroid metabolizing P450s, such as CYP19A1 [315], CYP17A1, and CYP11A1 [73]. As with CYP101A1 and heme oxygenase [357], the immediate product of cryoradiolytic reduction in CYP2B4 with or without substrate was the protonated hydroperoxo-ferric complex characterized by $g_1 > 2.27$.

The peroxo- and hydroperoxo-ferric intermediates in the mammalian cholesterol side-chain cleaving cytochrome P450 (CYP11A1) had been recently documented [73] in the presence of the first substrate, cholesterol, (58) as well as the second and third substrates, 22(R)-hydroxycholesterol (22-CH) and 20 α ,22(R)-dihydroxycholesterol (20,22-CH). [44] The radiolytically reduced oxy-complex of CYP11A1 with cholesterol present showed that the main cryoreduced intermediate had an EPR signal with $g_1=2.34$, characteristic of a protonated hydroperoxo-ferric complex, with two minor signals with $g_1=2.214$ and $g_1=2.28$

indicating the presence of unprotonated peroxo-ferric intermediates. These latter intermediates converted to the hydroperoxo-ferric intermediate after annealing at 145 K. Annealing at 185 K and further to 220 K, resulted in decay of the hydroperoxo-ferric intermediate and formation of the 22-CH product. This step also featured a substantial solvent H/D isotope effect, consistent with the expected partially rate-limiting second proton-transfer step, which is necessary for formation of the catalytically active Compound I. This suggests that the C-C bond scission of a vicinal diol, as is the case in the generation of pregnenolone from cholesterol by CYP11A1, uses Compound I as the main catalytically active intermediate. The follow up studies of irradiated CYP11A1 bound to 22-CH or 20,22-CH substrates [44] showed that the primary cryoradiolysis species is superoxo-ferrous intermediate with minor fraction of the hydroperoxo specie in sample containing the second substrate, 22-CH. When the third substrate is present, the 20,22-CH, only hydroperoxo intermediates are detected. Annealing of the sample containing 22-CH to higher temperatures leads to conversion of the superoxo ferrous species to hydroperoxo at 145 K, followed by product formation at 170–180 K with a large solvent kinetic isotope effect. A similar conversion was observed for a sample with 20,22-CH substrate. Based on these elegant studies the authors concluded that Compound I is the active oxidant in each of the three sequential oxygenation reactions catalyzed by CYP11A1.

4.3 Resonance Raman spectroscopy

4.3.1. CYP101A1—The first efforts to couple the cryoradiolysis approach with rR spectroscopy of heme proteins were made using cryoreduced oxy myoglobin, [349] followed up by investigation of bacterial cytochrome P450cam, CYP101A1. [41,59] The rR data of irradiated CYP101A1 confirmed the fact that the initial species obtained after cryoradiolysis, at 77 K, is the hydroperoxo-form, not the peroxo-intermediate, in agreement with previous EPR studies (*vide supra*). [37,42] Following up on the study of wild-type CYP101A1, [41] studies were carried out for the D251N mutant of CYP101 with its hampered delivery of the first proton. [37,42,358,359] Indeed, the peroxo-ferric intermediate was observed as the initial species obtained after radiolytic reduction. [59] The rR spectra of irradiated oxy D251N samples show two isotopically sensitive spectral features, a $\nu(\text{Fe-O})$ at 553 cm^{-1} and a $\nu(\text{Fe-O})$ at 792 cm^{-1} , both exhibiting the expected isotopic shift upon $^{16}\text{O}_2/^{18}\text{O}_2$ exchange and lack of the $\text{H}_2\text{O}/\text{D}_2\text{O}$ sensitivity, an observation most consistent with the assignment of these features to the modes of the peroxo species. The same irradiated samples of D251N mutant were further annealed to 185 K and then cooled to 77 K for acquisition of the rR spectra. The difference patterns revealed two new frequencies, a lower frequency mode appearing at 564 cm^{-1} and a higher frequency one at 774 cm^{-1} . Both of these modes now shifted down by 2 and 4 cm^{-1} , respectively, when using the D_2O buffer. Those H/D shifts are in a good agreement with previously published rR data for known metallo-hydroperoxo species [40,41,349,360,361,362,363] and are consistent with the hydroperoxo formulation. It is noted that the comparison with the earlier studies on wild-type (WT) CYP101A1, [41] showed that the $\nu(\text{O-O})$ stretching mode in the WT is 25 cm^{-1} higher than that in the mutated protein. Thus, not only does the mutation of D251 residue retard proton delivery to the Fe-O-O-fragment, but the replacement of the acidic Asp251 acid side chain with the amide also leads to substantial active-site differences that significantly affect the disposition of the Fe-O-O fragment, as reflected in the large downshift of the $\nu(\text{O-O})$ vibrational mode.

Collectively, the data acquired for CYP101 D251N provide the first clear opportunity to directly compare experimental vibrational data for these superoxo/peroxo/hydroperoxo heme derivatives in similar environments. Upon reduction from oxy to peroxo species, there is a large shift of $\nu(\text{O-O})$ from $\sim 1135\text{ cm}^{-1}$, characteristic of bound superoxide, to 792 cm^{-1} , a frequency indicative of a bound peroxo-fragment. At the same time, the observed $\nu(\text{Fe-O})$ mode shifts to higher frequencies by $\sim 16\text{ cm}^{-1}$, from 537 to 553 cm^{-1} . Protonation to yield the hydroperoxo derivative causes the $\nu(\text{O-O})$ to decrease further by 18 cm^{-1} , whereas the $\nu(\text{Fe-O})$ simultaneously increases by 11 cm^{-1} , to 564 cm^{-1} . The changes in vibrational parameters in proceeding from the parent oxy through the peroxo and hydroperoxo intermediates are generally consistent with those previously calculated.

[344,345,346,348,364] An exception is the behavior of the $\nu(\text{Fe-O})$ modes in proceeding from oxy to peroxo, where strengthening of this bond is evident from the vibrational data, whereas a weakening (longer bond) is predicted from the calculations. [344,345,346] However, it is particularly satisfying to note that virtually all computational work correctly predicts the negative correlation, involving increased Fe-O and decreased O-O bond strengths upon formation of the hydroperoxo intermediate. The weaker O-O bond in hydroperoxo-ferric intermediate has been rationalized in terms of the higher electron density on the dioxygen fragment, filling the antibonding π^* orbital of hydroperoxide, as compared to unprotonated peroxide. This is consistent with the overall lower spin density on the distal and proximal oxygen atoms in hydroperoxo-than in peroxo-ferric complex, as suggested on the basis of the proton ENDOR measurements, [42] as well as with the similar predictions of DFT studies. [344,345,346,348,364]

4.3.2. CYP17A1—The cryoradiolytically reduced samples of mammalian full length CYP17A1 bound with PREG or with 17-OH PREG were interrogated by rR spectroscopy [60] to study the structures of the crucial intermediates involved in the CYP17A1 catalyzed $\text{C}_{17}\text{-C}_{20}$ bond cleavage reaction. The mechanism of this lyase reaction had been debated for over three decades. [331,332] One proposed mechanism, illustrated by the red arrows in Scheme 3, left, suggests that the conversion is initiated by attack of the nucleophilic Fe-O-O fragment of the peroxo-intermediate on the electrophilic C_{20} carbon of the substrate, generating an unstable peroxo-hemiacetal derivative that would then decay to yield DHEA and acetic acid via homolytic or heterolytic scission of the dioxygen bond. [60,330,331,332,365] Another mechanism (Scheme 10, right), invokes protonation of ferric-peroxo intermediate and formation of Compound I. [366,367] As shown in more detail below, the cryoradiolytically treated samples of oxygenated CYP17A1 with PREG and 17-OH PREG allowed to generate, trap and spectroscopically characterize several distinct intermediates including an unstable peroxo-hemiketal derivative (Scheme 3, left).

The sample containing PREG (Figure 11, a) exhibits two sets isolated $\nu(\text{O-O})$ and $\nu(\text{Fe-O})$ vibrational modes in the initial cryoreduced samples, signaling the presence of two intermediates. One species shows a $\nu(\text{O-O})$ mode at 802 cm^{-1} , with its corresponding $\nu(\text{Fe-O})$ at 554 cm^{-1} , these features don't shift for the samples prepared in D_2O buffer (not shown), consistent with a trapped ferric *peroxo*-intermediate of CYP17A1. The second species in this sample exhibits a $\nu(\text{O-O})$ mode at 775 cm^{-1} and a corresponding $\nu(\text{Fe-O})$ at 572 cm^{-1} ; the spectra of samples prepared with D_2O reveal that these modes shift

significantly, confirming the identification of this species as the hydroperoxo-derivative. These data indicate that, when PREG is bound to CYP17A1, a significant fraction of the peroxo-intermediate is converted to the hydroperoxo-ferric intermediate, even at 77 K. Thus, position of PREG in the active site favors efficient proton transfer required to facilitate the Compound I-mediated classical hydroxylation reaction producing 17-OH PREG.

In the case of the 17-OH PREG-bound sample (Figure 11, b) an additional H-bonding (R-OH) fragment is introduced to the immediate heme environment by the substrate. The initial product of cryoradiolysis at 77 K exhibits a $\nu(\text{O-O})$ mode at 796 cm^{-1} with the corresponding $\nu(\text{Fe-O})$ mode occurring at 546 cm^{-1} . The spectra obtained for the samples prepared with D_2O (not shown) reveal that these modes are insensitive to the H/D exchange; i.e., this species is a ferric peroxo-intermediate, though with a slightly different disposition than that seen for the PREG-bound sample. In fact, the lowered $\nu(\text{Fe-O})$ frequency of the peroxo-form of the 17-OH PREG sample (546 cm^{-1}), relative to that of the PREG-bound sample (554 cm^{-1}), suggests that an H-bonding interaction occurs between the hydroxyl group of the substrate and the proximal oxygen atom of the $\text{Fe-O}_\text{p}\text{-O}_\text{t}$ peroxo-fragment; i.e., the H-bonding seen in the dioxygen adduct *does* persist in the peroxo-species, as had been suggested in the earlier work on the dioxygen adducts. [237] This is an important new finding, because such a specifically directed H-bonding interaction to the O_p of the peroxo-fragment is expected to facilitate its involvement in the lyase phase of catalysis. [345,368] Therefore, this critical result, obtained by rR spectroscopy, suggests the 17-OH PREG-bound peroxo-intermediate is poised for attack upon the susceptible electrophilic C20 carbon of the bound substrate.

The following annealing studies showed that warming of the PREG-bound sample to 165 K (Figure 11, c) causes clean conversion of the peroxo-intermediate only to more of the hydroperoxo-intermediate, consistent with progress towards the hydroxylation pathway. In contrast, similar annealing of the 17-OH PREG-bound sample provides no evidence for any new $^{16}\text{O}/^{18}\text{O}$ sensitive bands (Figure 11, d). Lack of new bands can be the result of conversion to a species with a different absorption maximum, a process confirmed by separate UV-VIS experiments that revealed a new intermediate absorbing at 405 nm. Indeed, rR measurements using 406 nm excitation, yielded bands appearing at 791 cm^{-1} ($^{16}\text{O}_2$) and 749 cm^{-1} ($^{18}\text{O}_2$) (Figure 12) that are associated with a new “peroxo-like” intermediate; it is not a hydroperoxo-species, based on lack of H/D shift (Figure 12, bottom trace) and is attributable to the intermediate depicted in the center of enzymatic cycle depicted in Scheme 3 on the left. While an alternate pathway involving Compound I (Scheme 3, right), has been suggested, [367] it is ruled out by the published rR data. [60] The Compound I, which might arise from O-O bond cleavage of the Fe-O-O-H fragment, could form a similar Fe-O-O-C peroxo hemiketal intermediate, by attack on the $\text{C}_{17}\text{-}^{16}\text{OH}$ fragment of the substrate. [366,367] However, the experiment using $^{18}\text{O}_2$ substitution clearly eliminated such an alternative. Thus, employing $^{18}\text{O}_2$, the resultant $\nu(\text{O-O})$ appears at 749 cm^{-1} , consistent with the $^{18}\text{O}\text{-}^{18}\text{O}$ bond being intact (Figure 12), i.e., Compound I did not form prior to the hemiketal being generated. If Compound I does participate in this reaction, one would expect to observe a $\nu(\text{O-O})$ mode of the $^{16}\text{O}\text{-}^{18}\text{O}$ bond at around 770 cm^{-1} (Figure 12). Clearly, such a feature is not seen in the rR spectra, eliminating the possibility of the Compound I as intermediate in this reaction. Furthermore, this conclusion is in satisfying

agreement with the results of recent DFT computational work [369], which also concludes that a Compound I – mediated lyase reaction for this system is not feasible and strongly supports catalysis by the peroxo intermediate.

Collectively, these spectroscopic data provide the first experimental documentation for the long-proposed, but previously unsubstantiated, peroxo-hemiketal species depicted in the center of Scheme 3, left. In closing this section, it is important to emphasize that a significant advantage of the rR technique for monitoring the changes occurring upon irradiation and subsequent annealing procedures is that structures of virtually all of the key species, including precursors, intermediates and product states (even non-paramagnetic) can be effectively interrogated. The sequential appearance and gradual conversion of most important intermediates starting from oxy-ferrous complex can be monitored on the same sample by cryoradiolytic reduction followed by stepwise annealing, as described above.

6. Higher-valent intermediates

The main catalytically active intermediate in cytochrome P450s enzymatic cycle is the (P)Fe(IV)=O, ferryl-oxo complex possessing a π -cation radical localized on porphyrin ring. It was termed Compound I following nomenclature used for corresponding species in peroxidases. [370,371,372,373] During the C-H bond activation, the Compound I abstracts hydrogen atom from substrate yielding a transient Compound II species and substrate radical.

The Compound I intermediate in cytochromes P450 was first observed (albeit with low yield) using stopped-flow experiments by mixing *m*-chloroperobenzoic acid (*m*-CPBA) with P450cam [374,375] and with ferric CYP119. [376] Using electronic absorption spectra, a characteristic Soret band was observed near 370 nm, with another low intensity broad band at ~690 nm. Recently a thorough spectroscopic study of this intermediate was reported by Rittle and Green, [29] where the authors generated the Compound I using CYP119A1 from thermophile *Sulfolobus acidocaldarius* by reacting ferric enzyme with *m*-CPBA using stopped-flow technique and characterized this intermediate using optical absorption, EPR and Mossbauer spectroscopy. The maximum yield ~75% was obtained at 35 ms with the UV-Vis spectra exhibiting typical Soret band at 367 nm and broad band at 690 nm entirely consistent with the presence of porphyrin π -cation radical, i.e. Compound I in P450 enzyme with thiolate as an axial ligand. Subsequently, the same group documented spectral characterization of Compound I in CYP101A1 [20] and Compound II in CYP158A as well as in the L316Y mutant CYP119 [30]. The latter study also directly measured $pK_a = 11.9$ of Fe(IV)hydroxide in Compound II in P450 enzymes by monitoring deprotonation of this intermediate between pH 8 and 14 using UV-VIS and Mossbauer spectroscopy. A stopped flow instrument with double mixing was used in order to obtain spectra before protein unfolding at high pH. Both protonated and unprotonated forms of Compound II revealed split Soret bands with maxima at 371 nm and 437 nm at high pH, and 370 nm and 426 nm at low pH. The importance of highly basic Fe(IV)hydroxide in P450 enzymes is highlighted by thermodynamic analysis [30,377] of the hydrogen abstraction step by Compound I and critical role of thiolate proximal ligand.

Another experimental measurement of pK_a of Fe(IV)hydroxide, (Compound II in heme-thiolate peroxxygenase from *Agrocybe aegerita*, APO), using the same stopped-flow double mixing approach with UV-VIS detection was described by Groves and colleagues. [378] The presence of proximal thiolate determines high $pK_a = 10.0$, meaning that this intermediate is protonated under turnover conditions. The spectra of Compound I and Compound II are very similar to those in cytochromes P450, with maxima at 361 nm and 694 nm for the former, and the split Soret at 428 nm and 370 nm and Q bands at 535 and 567 nm for the latter.

Compound I was also observed by Makris and co-workers [379] as a transient intermediate at high yield using stopped flow UV-VIS spectroscopy in reaction of cytochrome P450 OleT (CYP152L1) with H_2O_2 in the presence of perdeuterated substrate eicosanoic acid. Signature spectra of Compound I with a Soret maximum at 370 nm and broad band at 690 nm were observed 15 ms after mixing, followed by biphasic decay with rates $80\ s^{-1}$ (90% amplitude) and $8\ s^{-1}$, independently of H_2O_2 concentration. Importantly, the same experiments with protiated substrate did not show any notable transient accumulation of Compound I, indicating much faster decay caused by high kinetic isotope effect for hydrogen atom abstraction by this intermediate from substrate.

Compound I has also been successfully characterized using EPR spectroscopy. The EPR spectra of Compound I for CYP119A1 [29] and CYP101A1 [20] are very similar but have different shapes as compared to that previously reported for chloroperoxidase (CPO), another thiolate ligated heme protein [380]. These spectra can be successfully fitted to the $S=1$ Fe(IV)-oxo unit coupled with $S=1/2$ porphyrin radical, resulting in significantly higher ratio of the exchange coupling (J) to zero-field splitting (D) for CYP119 ($J/D=1.30$) and for CYP101A1 ($J/D = 1.38$) than for CPO ($J/D=1.02$) [20,29]. The higher J value in both P450 enzymes was tentatively attributed to either a higher spin density on the thiolate sulfur atom or a shortened Fe-S bond. The Mössbauer parameters measured for the CYP119 and for CYP101A1 Compound I species are both similar to those of CPO [380], with the isomer shift $\delta=0.11$ mm/s (0.13 mm/s for CPO) and quadrupole splitting $E_Q=0.96$ or 0.90 mm/s [20] (0.90 mm/s for CPO). These parameters also correspond to the ferryl-oxo $S=1$ unit exchange coupled to the porphyrin radical ($S=1/2$).

Although the Compound I in cytochromes P450 has yet to be characterized using rR spectroscopy, the rR spectra of this species have been reported for horseradish peroxidase (HRP) and chloroperoxidase (CPO), the latter being a thiolate-ligated protein. The $\nu(Fe=O)$ stretching modes were shown to be in the range of $786\text{--}794\ cm^{-1}$ for HRP, depending on pH, [381,382,383] and at $\sim 790\ cm^{-1}$ for CPO. [381,383,384] Kitagawa and coworkers were able to observe an oxygen sensitive mode at $790\ cm^{-1}$ for CPO using mixed-flow apparatus that shifted to $756\ cm^{-1}$ when generated using $H_2\ ^{18}O_2$, the frequency and magnitude of isotopic shift suggesting assignment to a ferryl π cation radical, Compound I, species. [384,385] The $Fe=O$ mode was detected using the 363.8 nm excitation line, in resonance with the maximum of electronic absorption of Compound I at 367 nm. Hosten *et al.* performed extensive studies of various ferric derivatives, the ferrous form as well as the Compound I and Compound II species of CPO in the region of $600\text{--}1800\ cm^{-1}$. [383] The authors showed that the high frequency spectral pattern on Compound II (generated in reaction with peracetic acid in the presence of ascorbic acid) is relatively similar to that of

CPO in resting state, while the spectral pattern of Compound I (generated in reaction with peracetic acid) significantly differ from these two; *e.g.*, the intensity of the ν_4 mode of Compound I is strongly diminished and the ν_2 , ν_{11} and ν_{37} modes are strongly shifted to higher frequency. The $\nu(\text{Fe}=\text{O})$ modes of Compounds I was assigned by the Turner group to a band at 792 cm^{-1} . [383]

The early rR studies of another CPOs higher valent intermediate, Compound II, were unsuccessful in characterization of the $\text{Fe(IV)}=\text{O}$ mode. It is noted that the authors probed the $700\text{--}800\text{ cm}^{-1}$ spectral region where the ferryl stretching modes of Compound II had been observed for other heme proteins. [386,387,388,389] Hosten *et al.* [383] did initially assign the mode at 759 cm^{-1} to a $\nu(\text{Fe}=\text{O})$ stretching mode of Compound II; however, as the authors noted, this 759 cm^{-1} mode was overlapping with the ν_{15} heme mode and did not show expected ^{18}O sensitivity. The Compound II exhibits two Soret bands, one at 436 nm and the other at 373 nm ; [384] however, neither use of UV [383,390] or visible [384] excitation lines were successful in detecting $\nu(\text{Fe}=\text{O})$ mode in the middle frequency region of rR spectra of CPO. [384] These early rR studies of ferryl species are reviewed by Turner *et al.* [381] In newer studies, however, Green and coworkers successfully generated, trapped and characterized the Compound II intermediate of CPO using rR spectroscopy. [389] Based on previous X-ray absorption measurements [391] and calculations, [392] it was predicted that the CPO Compound II is actually an iron(IV)-hydroxide species, with the $\nu(\text{Fe}-\text{O})$ mode being expected near 560 cm^{-1} in the rR spectrum. Indeed, a feature was observed at 565 cm^{-1} in the spectrum of $\text{H}_2\text{ }^{16}\text{O}_2/\text{H}_2\text{O}$ sample of CPO Compound II, which shifted by 22 cm^{-1} and 13 cm^{-1} to lower frequency upon $^{16}\text{O}/^{18}\text{O}$ and D_2O exchange, respectively. The observed isotopic shifts are in good agreement with that predicted for a harmonic oscillator, strongly supporting the assignment of this newly characterized intermediate to a $\text{Fe(IV)}-\text{OH}$. [389]

7. Interactions of cytochromes P450 with redox partners

In addition to the intermediates of catalytic cycle described above, there is one more essential aspect of cytochrome P450 functional system, i.e., interaction of the heme enzyme with their redox partners, which are necessary for timely and efficient electron delivery during two reduction steps. These interactions are also efficiently studied by various spectroscopic methods, which can probe not only the structural and electronic perturbations of the heme and iron axial ligands, but also protein conformational changes and dynamics, as well as specific alterations of particular steps in catalytic cycle. Although comprehensive discussion of all aspects of mutual effects of redox partner proteins and cytochromes P450 is beyond the scope of our review, it is useful to mention several important studies which used spectroscopic methods to probe these interactions.

From the early years of research in P450 field, the idea of modulation of P450 functional properties by interactions with redox partners was widely discussed and commonly accepted [278,303,393,394]. The effect of putidaredoxin (Pdx) binding to CYP101A1 has been probed by EPR [16], NMR [395,396,397,398], rR [399] (*vide infra*), IR [400] and electronic absorption spectroscopy [233]. The formation of encounter complex is of substantial importance in electron transfer, because binding of putidaredoxin to CYP101A1 is relatively

weak with dissociation constants ~ 10 μM . [401] Small shift of spin state equilibrium towards high spin state was observed upon binding of two flavodoxins from *Bacillus subtilis* to P450 BioI, and dissociation constants of 1.9 and 5.0 μM for these redox partners were derived from titration curves monitored by electronic absorption spectroscopy. [402]

A modest increase of the high spin fraction (by 10 – 20%) is also observed by electronic absorption spectroscopy when CYP3A4 interacts with the reductase domain of CYP102A1. [403] Titration experiments reveal tight binding with dissociation constants $K_d = 0.14$ μM for CYP3A4 in liposomes and 0.1 μM for CYP3A4 in Nanodiscs. A similar experimental approach applied for soluble CYP3A4 and a reductase domain of CYP102A1, (termed BMR), resulted in dissociation constant 0.48 μM , [404] and 0.37 μM for CYP2B4 and BMR [405], all measured under similar conditions at pH 7.4 in 0.1 M HEPES buffer. A small but measurable increase of the high-spin fraction in CYP2B4 reconstituted in detergents or in lipid vesicles with CPR and with cytochrome b_5 was also described in earlier study. [406]

NMR spectroscopy is particularly useful for detecting structural and dynamic changes caused by protein-protein interactions, especially if site-specific signals can be identified using selective isotopic labeling and sequence assignments. [17,407] Conformational changes in CYP101A1 caused by interactions with isotopically labeled Pdx and with perdeuterated cytochrome b_5 have been studied by multidimensional NMR methods using Fe-CO complex as a stable diamagnetic model of oxy-ferrous P450 intermediate. [197] Based on previously assigned NH resonances, multiple amino acids were identified as sensitive to binding with Pdx. In addition to the primary binding site, which includes the first three turns of the C-helix (residues 107–118), several neighboring residues in A helix, A-beta1 loop, and in B' helix, as well as residues in other parts of CYP101A1, were also perturbed by Pdx binding. Interactions of Pdx with CYP101A1 have also been studied by paramagnetic NMR spectroscopy using lanthanide tags attached to cysteines at multiple positions. [408] Based on a total of 446 distance constraints, the solution ensemble of 10 structures was calculated, in a very good agreement with the X-ray structure of CYP101A1-Pdx complex, also determined in this study. Several new residues were found at the interface and their importance for CYP101A1-Pdx interactions was confirmed in kinetic measurements. [409] Another recent NMR study further explored the multiple configurations of encounter complexes of Pdx and CYP101A1 and located the preferred configurations at the most favorable spots on the map of electrostatic potential. [401]

Solution NMR of selectively ^{15}N enriched human CYP17A1 revealed multiple structural conformers, the population of which was dependent on the substrate present in solution (PROG or 17OH-PROG). [410,411] Titration with the soluble domain of unlabeled cytochrome b_5 induced a shift towards conformers typical for 17OH-PREG, indicating allosteric effect favoring substrate positioning for lyase catalysis. [411] Further work extended labeling of CYP17A1 to uniform (^{13}C , ^2H , ^{15}N) triple labeling for three-dimensional NMR and probed binding of ^{15}N labeled cytochrome b_5 , unlabeled cytochrome b_5 as well as FMN domain of CPR. Moreover, these studies included competitive binding experiments, because it has been shown that these reductases partly share a common binding site. [412] It was found that binding of FMN domain is tighter and induces more pronounced line broadening in CYP17A1 ^{15}N bands. More importantly, it was established that affinity of

FMN-CYP17A1 is stronger in the presence of the substrate 17OH-PROG, than with 17OH-PREG, which is consistent with stronger enhancement of lyase reaction by cytochrome b_5 with the latter substrate. [412] In addition, interaction between FMN domain and cytochrome b_5 was also detected, although with lower affinity.

The effect of the membrane incorporation on interaction between CYP2B4 and cytochrome b_5 was also studied by solution NMR using Nanodiscs assembled with amphipathic 22-mer peptides. [413] 2D ^1H - ^{15}N TROSY heteronuclear single-quantum coherence (HSQC) spectra of ^{15}N labeled cytochrome b_5 with and without CYP2B4 in Nanodiscs were compared in order to identify residues on cytochrome b_5 that participate in direct interactions with P450. In addition to several previously known residues, Leu75 was also identified as a part of cytochrome b_5 – CYP2B4 interface. This difference between Nanodisc results and those obtained earlier using bicelle incorporation [414] is attributed to possible variations in membrane incorporation modes in these two model systems, or to the presence of detergents in the bicelle samples.

Resonance Raman spectroscopy also can be used to detect conformational changes caused by interactions of cytochromes P450 with their redox partners, particularly when these interactions perturb heme modes or iron-axial ligand interactions. For example, much stronger perturbations of the heme modes were observed in the resonance Raman spectra of CYP101A1 in the complex with Pdx, than in CYP101D1 in the complex with Adx. [169] These differences indicated the presence of substantial effector role of Pdx in CYP101A1 activity, as was observed in comparison of functional properties and autoxidation rates of CYP101A1 and CYP101D1. However, Adx significantly increased the fraction of high spin in CYP101D1 in the presence of camphor, as monitored by UV-VIS spectra, [169] in contrast to the opposite tendency observed for CYP101A1 in the presence of Pdx. [233] In this study, the $\nu(\text{Fe-S})$ stretching mode exhibited a shift of $\sim 3\text{ cm}^{-1}$ to higher frequency when bound with Pdx, a shift that occurred in concert with an increase in low spin component, as reflected in the behavior of the high frequency spin markers; e.g., addition of oxidized Pdx to bacterial cytochrome CYP101 increases the low spin state population. [233] The increased LS population in CYP101-Pdx complex was explained by invoking distal pocket changes that allow water molecules to enter the heme pocket. A similar effect of redox partner on spin state was observed for CYP102; *i.e.*, Deng *et al.* compared the rR spectra of ferric heme domain and holoenzyme (heme domain fused with its reductase domain) showing that the presence of reductase domain results in larger amount of LS component in the palmitate-bound samples. [231] On the other hand, addition of adrenodoxin to mitochondrial ferric cytochrome P450_{SCC} (CYP11A1) resulted in complete conversion of a mixture of spin states to a pure high spin state. [278]

The rR technique was also applied to interrogate the changes in active site structure of another mammalian P450, CYP2B4, [223] in its interaction with cytochrome P450 reductase (CPR) and the Mn(III) analog of cytochrome b_5 (Mn Cyt b_5), the manganese derivative being employed to avoid complications involving interference by rR lines of the native (iron-containing) Cyt b_5 . [415] It was found that the binding of Mn Cyt b_5 increases the percentage of high spin component, whereas CPR does not substantially alter the spin state populations. [223] Interrogating the impact of redox partners on the Fe-S bond, addition of

Mn Cyt b_5 causes a slight (2 cm^{-1}) shift to lower frequency of the $\nu(\text{Fe-S})$ mode compared to the spectra of reductase-free samples, while addition of CPR does not change the position of the Fe-S stretch. [223] In the newest studies of Mn Cyt b_5 interactions with human CYP17A1 incorporated in Nanodiscs, the addition of redox partner resulted in small increase of LS component and in 3 cm^{-1} upshift of Fe-S, [416] suggesting possible contribution of Cyt b_5 as an allosteric effector in CYP17A1 catalysis.

In as much as the interaction with redox partners are generally associated with changes in the Fe-S bond, they might also affect the *trans*-axial Fe-X-Y bonds, such as Fe-C-O, Fe-O-O or Fe-N-O; the CO and NO adducts being useful probes of structural differences in the distal heme pocket. It has been shown that addition of Pdx to the ferrous CO adduct of camphor-bound CYP101A1 causes a small up shift of the $\nu(\text{Fe-CO})$ mode (2 cm^{-1}), [417,418] with the $\nu(\text{C-O})$ mode concomitantly shifting from 1940 cm^{-1} to 1932 cm^{-1} . [418] The authors suggested that Pdx binding induces structural and/or electrostatic perturbations on the heme proximal side of the CYP101 that, in turn, enhance an electron donation from the thiolate ligand to the d_z^2 orbital of the iron. [417,418] As a result, π back donation would account for the relatively small increase in $\nu(\text{Fe-CO})$ that was accompanied by large decrease in $\nu(\text{C-O})$ frequency. Interestingly, it was shown that binding of ferrous Cyt b_5 has no effect on the Fe-C-O fragment of CYP101 or its heme structure, [417] leading to the conclusion that there are significant differences in the interactions between CYP101A1 with Pdx and with Cyt b_5 . In mammalian systems, the study of binding of CPR and Mn Cyt b_5 by CYP2B4 showed that there is no effect on the position of the $\nu(\text{Fe-CO})$ mode. [223] Interestingly, the only molecular fragments of the active site that were significantly affected by binding these reductases were the vinyl groups, as reflected in changes of the vinyl bending modes, which appear as two equal intensity bands at 410 and 419 cm^{-1} ; e.g., in the complex with Mn Cyt b_5 the lower frequency feature was enhanced, while binding of CPR selectively enhanced the higher frequency vinyl bending mode. [223] These rR spectral data provide important evidence that CPR binding causes active site perturbations that generate more out-of-plane vinyl groups, while binding of Mn Cyt b_5 leads to a higher fraction of *in-plane* vinyl groups.

Finally, the rR spectroscopy also permits interrogation of the effects of a redox partner interactions with oxygenated forms of cytochromes P450. [70,399] Sjodin *et al.* [70] showed that the Fe-O-O fragment of the D251N mutant of CYP101A1 is significantly perturbed by the addition of Pdx; e.g., binding of reduced Pdx to the D251N mutant results in increased population of a lower frequency Fe-O-O conformer. The authors suggested that the structural perturbations of the Fe-O-O fragment are related to the effector function of Pdx and most reasonably involve changes in the electron-donating properties of the thiolate ligand. [70]

8. Summary

Several spectroscopic methods are capable of providing detailed characterization of each elementary step in the cytochrome P450 enzymatic cycle. The electronic absorption, EPR, NMR and rR spectroscopic techniques can provide information regarding electronic state, oxidation and spin states, substrate positioning in an active site. The rR can additionally monitor changes in heme planarity, disposition of peripheral groups and strength of key Fe-S

bond. Oxygen binding is easily monitored by UV-VIS and rR as well as Mossbauer and EXAFS, although the later require much more sample and cryogenic temperatures. While transient peroxo- and hydroperoxo-ferric intermediates are not observed in steady-state experiments at ambient conditions, they can be generated, trapped and characterized using cryoreduction and such generated intermediates have been characterized using EPR, electronic absorption and rR spectroscopies. It is worth mentioning that rR spectroscopy can provide unprecedented insight into the nature of functionally important H-bonding interaction with the Fe-O-O fragment of the oxy, peroxo- and hydroperoxo-intermediates. This is important because subtle changes in the directionality of these H-bonds can result in dramatic alterations of the CYPs chemistry. It is noted that the product complex can be identified by EPR and ENDOR following annealing of cryoreduced oxy complexes in several cytochromes P450. Compound I intermediate is very unstable and only recently was successfully trapped in several P450 enzymes using peroxide shunt and characterized using electronic absorption, EPR and Mossbauer spectroscopies. In addition, detailed spectroscopic data provide indispensable reference points for theoretical quantum chemical mechanistic calculations. It is expected that these spectroscopic techniques will continue to serve scientists well in their quest to unveil reaction mechanisms of various cytochromes P450, including multifunctional enzymes that are crucial in human physiology as well as these that hold great promise for biotechnological purposes.

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Figure 1.

Left - the Nanodisc-CYP assembly. The cytochrome P450 molecule is shown in green, with heme presented in red sticks. The phospholipid bilayer is shown in orange with oxygen atoms in red, while the scaffold protein encompassing the lipid bilayer is shown as a blue cartoon representation. Right - CYP3A4 and CPR reconstituted into Nanodisc.

Figure 2.
Electronic absorption spectra of human cytochrome P450 CYP3A4 in Nanodiscs without substrate (red line) and saturated with bromocryptine (blue line).

Figure 3.

Titration curve calculated for non-cooperative binding to the protein with three binding sites and the same spectroscopic signal measured for all three sites (red) or from only second and third binding events with the first binding spectrally silent (blue). Populations of protein with one, two or three sites occupied with ligand are shown in black and marked (1), (2) and (3) correspondingly.

Figure 4.

The high frequency rR spectra of cytochrome P450_{cam} in substrate-free (A) and substrate-bound (B) forms. Spectra were measured with 406.7 nm excitation line.

Figure 5.

The high frequency rR spectra of 60 μM ferric cytochrome CYP3A4 in detergent without substrate (A), and with 600 μM testosterone (B), as well as rR spectra of ferric cytochrome 3A4 in nanodiscs (100 μM) without substrate (C), with 1200 μM of testosterone (D).

Figure 6.

The low frequency rR spectra of cytochrome P450_{cam} in substrate-free (A) and substrate-bound (B) forms. Spectra were measured with 406.7 nm excitation line.

Figure 7. Low-frequency rR spectra of ferric CYP2B4: A) wild-type BHT-bound; B) F429H mutant BHT-bound. Spectra measured with 356 nm excitation line and normalized to the ν_7 mode at 676 cm^{-1} . Inserts show graphical representation of H-bond formation to CYP2B4 proximal cysteine upon Phe to His mutation.

Figure 8.

The rR spectra of $^{16}\text{O}_2$ adducts of ND:CYP17 in H_2O buffer with PROG (panel A), 17-OH PROG (panel B), PREG (panel C) and 17-OH PREG (panel D). The lower section of each panel shows $^{16}\text{O}_2$ - $^{18}\text{O}_2$ difference plots in H_2O buffers in low (left) and high (right) frequency regions.

Figure 9. Electronic absorption spectra of CYP101A1 in its oxy (1), peroxy (2) and hydroperoxy (3) forms. [59]

Figure 10.

EPR spectra of oxy-complex in CYP101A1, D251N mutant, cryoreduced in liquid nitrogen at 77K. Spectra were measured at 14 K after annealing for 1 minute at indicated temperatures, with g_1 -values shown for each spectrum. The first spectrum with $g_1 = 2.25$ is typical for unprotonated peroxo-ferric intermediate, which is protonated to hydroperoxo-ferric complex after annealing at 173 – 190 K with $g_1 = 2.29$. Annealing at higher temperatures results in product hydroxyl-camphor formation with transient coordination complex of product and heme iron ($g_1 = 2.62$ and $g_1 = 2.51$) and gradual relaxation to the

low-spin ferric CYP101A1 after warming up to the room temperature. Reproduced from reference [42] with permission from the American Chemical Society.

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Figure 11.

Resonance Raman spectral data for irradiated dioxygen adducts of CYP17A1. All spectra were measured with 442 nm excitation line at 77 K, total collection time of each spectrum was 6 hrs. The rR $^{16}\text{O}_2 - ^{18}\text{O}_2$ difference traces in H_2O of irradiated oxy CYP17A1 samples (before annealing) with PREG (a) and 17-OH PREG (b) and corresponding samples after annealing to 165 K (c) and (d).

Figure 12.

The $^{16}\text{O}_2 - ^{18}\text{O}_2$ difference traces of irradiated dioxygen adducts of CYP17A1 samples with 17-OH PREG annealed at 190 K measured with 406 nm excitation line at 77 K.

Scheme 1.
Cytochrome P450 catalytic cycle.

Scheme 2.

Schematic representation of heme protein cryoreduction; glycerol (G).

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Table 1
Cytochrome P450cam heme core marker bands for substrate free (SF) and substrate bound (SB) enzymes.

Form	Oxidation state	Coordination and spin state	ν_4	ν_3	ν_2	ν_{10}
P450						
P450 SF	FelII	6cLS	1373	1503	1584	1637
P450 SB	FelII	5cHS	1372	1488	1570	1623
P450 SF	FelII	5cHS	1345	1468	1563	1601
P450 SB	FelII	5cHS	1345	1466	1564	1601