Published in final edited form as:

Biochim Biophys Acta. 2011 January ; 1814(1): 223–229. doi:10.1016/j.bbapap.2010.05.017.

Cytochromes P450 in Nanodiscs

Iliia G. Denisov and **Stephen G. Sligar**

Department of Biochemistry, School of Molecular and Cellular Biology, The University of Illinois, 505 South Goodwin Avenue Urbana, IL 61801 USA

Abstract

Nanodiscs have proven to be a versatile tool for the study all types of membrane proteins, including receptors, transporters, enzymes and viral antigens. The self-assembled Nanodisc system provides a robust and common means for rendering these targets soluble in aqueous media while providing a native like bilayer environment that maintains functional activity. This system has thus provided a means for studying the extensive collection of membrane bound cytochromes P450 with the same biochemical and biophysical tools that have been previously limited to use with the soluble P450s. These include a plethora of spectroscopic, kinetic and surface based methods. Significant improvements in homogeneity and stability of these preparations open new possibilities for detailed analysis of equilibrium and steady-state kinetic characteristics of catalytic mechanisms of human cytochromes P450 involved in xenobiotic metabolism and in steroid biosynthesis. The experimental methods developed for physico-chemical and functional studies of membrane cytochromes P450 incorporated in Nanodiscs allow for more detailed understanding of the scientific questions along the lines pioneered by Professor Klaus Ruckpaul and his array of colleagues and collaborators.

Keywords

Cytochrome P450; Monooxygenase; Membrane protein; Nanodisc; Reaction Intermediates

1. Introduction

This review is dedicated to Professor Dr. Klaus Ruckpaul, whose works on cytochromes P450 constitute an indispensable part of the fundamental knowledge base of this field. His work, reaching a pinnacle through the “Berlin Buch Group” in the 1970s and 1980s, is briefly described in an autobiographical historical review [1]. Results obtained through the efforts of his group provide a classic paradigm forming studies during “Biophysical and Biochemical Period” [2] which mapped the landscape and paths for future generations investigating this class of enzymes that play a central role in human health and disease [3]. These include application of spectroscopic methods for substrate binding and spin-shift thermodynamics and kinetics [4–6], studies of interaction with redox partners for hepatic and mitochondrial cytochromes P450 [7–8], cryogenic spectroscopy of binding intermediates and non-equilibrium states obtained by cryoradiolytic reduction and cryogenic photolysis [9–10], magneto-optical methods [11], novel spin labeled and fluorescent dye labeled components

Contact Information: Stephen G. Sligar, School of Molecular and Cellular Biology, The University of Illinois, 505 South Goodwin Avenue, Urbana, IL 61801 USA, s-sligar@uiuc.edu, Telephone: 217 244-7395.

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[12], and other methods of modern physical chemistry which have often led in developing new tools for biochemistry of membrane proteins.

The current work in our laboratory continues and extends the application of novel biophysical methods for mechanistic studies of P450 catalysis and biomolecular recognition in soluble and membrane bound monooxygenase systems. During the last decade we have developed a new and versatile tool for the field of membrane biophysics and biochemistry, the soluble nanoscale lipid bilayers termed Nanodiscs. Through a simple self-assembly process, membrane proteins can be incorporated into a nanometer scale lipid bilayer that places the target protein in its native-like membrane environment yet renders the complex soluble via an encircling amphipathic helical “belt”. To date (March, 2010) numerous membrane associated cytochromes P450 have been successfully incorporated in Nanodiscs, including in chronological order: CYP2B4 [13], CYP6B1 [14], CYP3A4 [15-25], CYP73A5 [26], CYP19 [27-28], CYP86A8 [29]. Incorporation of the broad spectrum of membrane proteins into Nanodiscs has been recently reviewed by Duan et al. [29], Boldog et al. [30], Borch et al. [31], Nath et al. [32], Ritchie et al. [33] and Bayburt et al. [34]. The general approach to functional reconstitution of membrane proteins into Nanodiscs from cholate solubilized mixture of components was originally developed using cytochrome P450 reductase (CPR) [35-36].

Incorporation of cytochromes P450 into Nanodiscs results in stable soluble, homogeneous and monomeric preparations in native-like environment of lipid bilayer. As a brief example of this approach, Nanodisc preparations showed that it is possible to achieve full conversion of the spin-state in Type I substrate binding of CYP3A4 with testosterone and bromocriptine [15,20-22,37], preparation and stabilization at low temperature of oxy-complex in CYP3A4 and CYP19 [27], measurement of the activation parameters of autoxidation in these enzymes with and without substrates [21,28], immobilization of CYP3A4 on silver nanoparticles for ultrasensitive plasmon resonance monitoring of Type I and Type II binding of substrates and inhibitors [38], measurement of midpoint potential of CYP3A4 and CPR in bilayers and evaluation of effects of substrate binding and presence of anionic lipids [37,39]. In a beautiful application of single molecule biophysical techniques, Nanodisc incorporated CYP3A4 were used to monitor substrate binding dynamics [25]. In addition, Nanodiscs can be formed with different compositions of synthetic lipids, as well as with lipids from native membranes [14] to explore the role of lipids in ligand binding, catalytic mechanism, and macromolecular recognition.

2. Novel methods for P450 investigations enabled by Nanodisc incorporation

Historically, the first functional incorporation of CPR and CYP2B4 into prototype of Nanodiscs, i.e. reconstituted high density lipoproteins (HDL) have been described in [13,35-36] and used for the atomic force microscopy (AFM) characterization on the surface. CPR was assembled in HDL using DPPC and full length human apolipoprotein A (ApoA) at 37° C by dialysis of cholate solubilized reconstitution mixture of all components [35]. After dialysis at 37° C, CPR incorporated in HDL were purified from heterogeneous mixture of HDL particles having multiple sizes by size exclusion chromatography on Sephadex 200 HR10/30 column at room temperature. Successful incorporation of CPR in HDL was confirmed by the release of soluble CPR by tryptic hydrolysis of the membrane binding N-terminal domain and SDS electrophoresis. HDL containing CPR have been absorbed on the flat surface of freshly cleaved mica in a fluid cell and characterized by atomic force microscopy (AFM) in the tapping mode. Lipoprotein particles with CPR have been detected as objects protruding ~3.5 nm higher above surface as compared with flat HDL particles with no CPR. Subsequent analysis of force-distance measurements at each pixel image gave

better statistics and, by allowing extrapolation to zero force, generated precise measurement of the height of CPR above bilayer as 5.6 nm [36]. Similar treatment by trypsin caused loss of these particles from the image as well as loss of activity of CPR activity as measured by cytochrome c reduction. Subsequent incorporation of the full length CYP 2B4 purified from rabbit liver into DPPC – ApoA lipoprotein particles was accomplished using the same method of cholate dialysis [13]. AFM measurements on a mica surface provided the first single-molecule evaluation of the topology of membrane incorporation of the mammalian hepatic cytochrome P450, envisioned as the insertion of the N-terminal tip of the triangular prism of the protein into the lipid bilayer, as shown schematically in the Figure 6, ref [13].

In addition to the approach based on self-assembly of the purified membrane proteins, lipids and the membrane scaffold protein MSP [15,20,30,34,40-43], incorporation of the cytochromes P450 into Nanodiscs can be accomplished by direct solubilization of the membrane fraction of heterologous expression system in the mixture of detergent and membrane scaffold protein MSP with subsequent dialysis of detergent [14,26]. CYP6B1 [14], CYP73A5 and housefly reductase have been expressed separately in Sf9 cells and transfected by baculovirus expression vectors [14,26]. Microsomal membranes solubilized with cholate are mixed with MSP, dialyzed to remove detergent, and the Nanodisc incorporated proteins are separated by affinity chromatography on a Ni-chelated column using a His₆-tag on the MSP construct [14]. Subsequent size-exclusion chromatography allows for separation of fractions containing cytochrome P450 [14] or complex of cytochrome P450 and reductase [26].

In a more recent review [29] the general strategy of using Nanodiscs for the high-throughput approach to the analysis and screening of heterologously expressed cytochromes P450 and reductases has been outlined. Here the application of Nanodiscs provides a breakthrough due to the possibility of using different affinity tags to separate multiple target proteins from non-labeled membrane proteins in one pass, yielding a much higher purity and stability of cytochrome P450 incorporated in Nanodiscs and significantly better signal/noise ratio than is usually obtained in liposomal or microsomal samples.

The opportunity to incorporate additional functionalities provided by Nanodiscs and the encircling MSP has been used in several studies where the goal is to obtain stable and functional preparations of membrane cytochromes P450 and other membrane proteins inserted into Nanodiscs while immobilized on surfaces [25,38,44]. For example, affinity tags engineered on the scaffold protein or on the lipid molecules in Nanodiscs provide for specific tight binding on the metal or other activated surface eliminate the need of modification of cytochrome P450 for direct immobilization on solid substrate, which potentially can perturb the structure and binding properties of the heme enzyme. In addition, incorporation of monomeric cytochromes P450 in Nanodiscs greatly improves their stability against inactivation and formation of P420.

In the first single-molecule fluorescence study of the human cytochrome P450 [25], the CYP3A4 in Nanodiscs have been attached to the avidin treated glass slides by using 5% fraction of biotinylated lipids in disc preparation. Binding and dissociation of the model substrate fluorescent dye Nile Red was monitored in a single-molecule mode and compared to the equilibrium titration measurements in solution. Single molecule detection allowed for separation of the dissociation events from the Nanodisc lipid bilayer (fast, $\sim 30 \text{ s}^{-1}$) and from the active site of the heme enzyme (slow, $\sim 1.5 \text{ s}^{-1}$). Allosteric effect of alpha-naphthoflavone (ANF) on the monomeric CYP3A4 in Nanodisc was revealed through the changes in dissociation rate of Nile Red in the presence of effector.

Another current method for label-free ultra-sensitive detection and screening of small molecule binding to the membrane cytochromes P450 based on localized surface plasmon resonance (LSPR) was also used. Nanodiscs for immobilization and stabilization of the enzyme on the surface of the gold or silver nanoparticles [38]. The surface of silver nanoparticles on a glass slide was modified by a monolayer of 11-mercaptoundecanoic acid (11-MUA), and Nanodiscs with incorporated human hepatic cytochrome CYP3A4 were covalently attached by EDC coupling between the terminal carboxylic groups of 11-MUA and protein amino groups. Detection of Type I or Type II binding was accomplished by monitoring the blue- or red-shift of the LSPR band of the nanoparticles. A much higher sensitivity of this method, as compared to standard SPR, for quantitating the binding of low-molecular weight substrates and inhibitors of mammalian cytochromes P450 incorporated in the native-like lipid bilayer is realized. This is due to an electronic coupling of the heme transition moment with the resonant wavelength of the plasmon resonance. Approximately 100 Nanodiscs with cytochrome P450 can be adsorbed to one nanoparticle and measurement of LSPR in a single particle mode is possible before and after substrate binding extending assays to the attomolar detection level of small molecule binding. LSPR response can be optimized by the choice of nanoparticles with the maximal overlap with the absorption spectrum of the protein. As a result, such methods can be considered as prototype for monitoring of drug interactions with membrane bound cytochromes P450 on the nanoscale and for design of new high-throughput screening methods of chemical libraries.

Incorporation of membrane cytochromes P450 into Nanodiscs opens a straightforward path to creation of micro-arrays for simultaneous screening of multiple isozymes and/or multiple drugs using functional groups on lipids or scaffold protein for selective binding with high affinity on nanoparticles (LSPR) or surfaces and for creating of microarrays [45]. Detection of binding events can be accomplished either by monitoring an LSPR optical signal, as described above, or by direct observation of binding or release of fluorescent substrate or substrate analog [16,24-25].

Our recent works describe additional advantages provided by homogeneous and monodisperse preparations of CYP3A4 in Nanodiscs for various modern spectroscopic methods. One example is the first solid-state NMR study of CYP3A4 in POPC Nanodiscs [46] which demonstrated that high resolution spectra can be obtained for these large metalloenzyme oxygenases. In these procedures, the P450 enzyme retains full functional activity after resuspension in buffer. In a subsequent solid state NMR experiment (Kijac et al., *in press*) protein-lipid interactions were documented by monitoring magnetization transfer. As a final example, resonance Raman spectra of CYP3A4 in Nanodiscs were obtained which allowed determining the packing of different substrates such as testosterone, bromocriptine and erythromycin into the active site and the subsequent protein conformational changes as monitored by the heme and Fe-CO vibrational modes (Mak et al., *in preparation*).

3. Equilibrium and steady-state kinetic properties of monomeric CYP3A4 in Nanodiscs

Stable homogeneous preparations of CYP3A4 and other cytochromes P450 incorporated into the lipid bilayer provide a vitally important basis for quantitative mechanistic studies of equilibrium and steady-state biophysical and biochemical properties of these heme oxygenases. A detailed understanding of essential functional properties of monomeric cytochromes P450 in the native membrane environment is necessary to distinguish between the characteristics of monomeric enzyme and the role of homo- and hetero-oligomers which can be formed *in vivo*. Nanodiscs provide an ideal tool for such studies because they make possible the isolation of stable and homogeneous populations of fully functional monomeric

cytochrome P450 embedded into a lipid bilayer with a controlled composition. This approach eliminates ambiguities caused by the presence of high concentrations of detergents, or by coexistence of multiple forms of the enzyme with different properties caused by oligomerization [47].

In the first study of homotropic cooperativity of substrate binding by human cytochrome P450 in Nanodiscs [15] we have shown that CYP3A4, when purified and incorporated into POPC bilayer in a monomeric form, can bind up to three testosterone (TS) molecules eliciting an almost complete shift in the ferric spin state equilibrium (~92%) with a high apparent cooperativity (Hill coefficient >2). Analysis of these binding data with the Adair equation for three binding events revealed a very low spectral signal from the first binding event. As we discussed in our original publication [15], this could be a result of either very high binding cooperativity, or substantially different spectral response for sequential binding events, with the first occupancy being spectrally silent. The latter possibility was suggested to be the case, although the answer could not be proved based solely on the results of spectral titration. This led to the use of Nanodiscs to co-incorporate reductase with the P450 to allow deconvolution of the activities of each substrate bound state yielding an understanding of the functional activity of xenobiotic metabolizing cytochromes P450 and the role of homotropic and heterotropic cooperativity in their function [20,48-49].

Thus we developed the method of co-incorporation of the functional complex of CYP3A4 and CPR into Nanodiscs in 1:1 ratio [20,50]. The mechanism of functional homotropic cooperativity of TS metabolism by CYP3A4 was resolved using simultaneous analysis of the results of several different experiments (spectral titration, steady-state kinetics of NADPH consumption, and kinetics of the product formation, all as a function of TS concentration) performed under identical conditions [20]. Such an approach allows applying the same set of binding constants to all experiments to resolve the equilibrium populations of binding intermediates, i.e. the fraction of the enzyme with one, two, or three molecules of substrate bound. At any given substrate concentration, the overall response of the system is determined by fractional contributions from all these intermediates. Results of the global analysis revealed that the high homotropic cooperativity of TS hydroxylation by monomeric CYP3A4 is due to essentially “all-or-none” dependence of the rate of product formation on the number of substrate molecules bound [20,49]. While no product was formed by CYP3A4 with only one TS molecule bound, the binding of the second TS molecule switched the CPR-CYP3A4-substrate complex to a catalytically active state. Importantly, the actual microscopic dissociation constants for all three binding events are very similar, and thus there is no binding cooperativity present in this system despite the highly cooperative Type I spectral titration curves. The optical signature is simply due to a very small spectral response caused by the first binding event, as compared to the almost 100% spin shift caused by the second substrate association [20]. These results are consistent with many similar observations of the presence of the spectrally silent and/or non-productive binding in several other cytochromes P450 which are capable of binding more than one molecule of substrate. This approach, which builds on the work of Professor Ruckpaul, has been recently reviewed [48]. It is thus clear that the mechanisms of functional cooperativity may be common for all xenobiotic metabolizing cytochromes P450, most of which are known for their extremely broad range of substrates efficiently metabolized, as there is no requirement for the presence of highly selective binding and specific interaction between binding sites.

This same global analysis approach can be applied to the case of heterotropic cooperativity, when different substrates can bind simultaneously to one cytochrome P450. The association of an additional substrate or effector can trigger the same response as binding of a second molecule of the same substrate and thus result in heterotropic, or allosteric, positive effect

on the substrate metabolism. This could be erroneously attributed to an interaction free energy between these two compounds. However, like in the case of homotropic functional cooperativity of CYP3A4 with TS, where a lack of cooperative interactions on the level of binding is compatible with a heterotropic functional cooperativity, a specially designed set of experiments is needed to resolve the mechanistic basis of this interaction. Working on the general problem of drug-drug interactions mediated by human cytochromes P450, we developed a new method for the detection of heterotropic cooperativity in CYP3A4, termed a “mixed titration approach” [23]. This method is based on the comparison of the titration with a mixture of two substrates at a constant concentration ratio with the results of titrations with the pure substrates. For example to resolve the effect of ANF, which activates TS hydroxylation by CYP3A4 with elimination of cooperativity of this reaction, we compared the results of mixed titration experiments with different TS:ANF ratios with the results of similar titrations with pure substrates. In all cases, the mixed titration data can be described by the simple additive effect of two substrates without any synergistic cooperative interaction between them. Thus, the allosteric effect of ANF on TS binding is simply due to the presence of an additional substrate in the system and does not demand that there be any specific interactions between the two entities.

A detailed study of binding of several substrates to empty Nanodiscs and to CYP3A4 incorporated in Nanodiscs have addressed an important question as to the distribution of small hydrophobic molecules between the membrane lipid bilayer and active center of cytochrome P450 [24]. Because of the well known composition and stoichiometry, Nanodisc system provides an ideal tool for simultaneous measurement of partition coefficient for a given drug between bilayer and aqueous phase and of determination of the actual affinity of the P450 embedded into this bilayer. As described in the original publication [24] and in a recent Ph.D. theses [51], different substrates can bind preferentially to CYP3A4 or partition into the membrane, and the overall distribution depends on the volume of the lipid phase and concentration of the heme enzyme.

Other benefits accrue when using Nanodiscs to investigate the cytochrome P450s. Working in the Ruckpaul laboratory in 1980s for a two week lecture visit, it was noticed that one investigated human P450s by conducting experiments in ice buckets. Why, when the enzyme normally lives at 37° C? Most eukaryotic cytochromes P450 are unstable in detergent solubilized mixtures and loose activity within 1 – 2 days. However, CYP3A4 incorporated in POPC Nanodiscs is stabilized against temperature inactivation as well as P420 formation induced by high pressure [17]. In addition, characteristics of Type I titration curves of monomeric CYP3A4 in Nanodiscs with testosterone do not change at high pressure, contrary to the behavior of this enzyme in oligomeric state in solution.

An important result has been obtained by applying the method of spectropotentiometric titration for the measurement of the midpoint potential E' of the CYP3A4 in Nanodiscs in the substrate free and substrate bound states. Previous attempts to measure the effect of the substrate binding on redox properties of the membrane bound cytochromes P450 have been inconclusive and reported either the presence of up-shift of the midpoint Fe^{3+}/Fe^{2+} potential, or the lack of any real effect, and yet for CYP3A4 a dramatic acceleration of the rate of reduction by the presence of some substrates including TS is well established. The problem can be traced to aggregates of P450, and their inability to interact productively with an electrode surface or mediator. We measured an upshift of the midpoint potential by +80 mV in the presence of the Type I substrates as TS and bromocriptine (BC) when CYP3A4 is self-assembled into Nanodiscs [37] and a lack of such change in redox potential in the presence of erythromycin. This placed the membrane bound P450 on the same footing as the soluble CYP101 in the correlation of the presence or absence of a ferric spin shift with redox potential. Recall that binding of TS and BC induce an almost complete spin-shift in

CYP3A4 embedded in Nanodiscs, while significantly lower effects (~50 – 60%) have been reported in earlier works [52-53]. Thus the origin of differing conclusions on the coupling of spin state and redox free energies seem due to the non-native state of the monooxygenase, with a common mechanism being present in both soluble and membrane bound P450s.

An additional opportunity provided by Nanodiscs is the ability to systematically control the lipid composition in the immediate vicinity of incorporated membrane protein. This is of crucial importance in documenting the effect of the membrane bilayer on the various functional properties of membrane bound proteins and enzymes. For example, changes in the redox potential of CPR induced by the lipid bilayer have been measured using spectropotentiometric titration in solution [39]. In pure POPC Nanodiscs, the midpoint potentials of both FMN and FAD undergo a shift to more positive values as compared to the soluble CPR. However, in the presence of the negatively charged POPS, the redox potentials of both flavins become more negative, thus favoring the electron transfer to the cytochrome P450. These changes are much more pronounced for the semiquinone/hydroquinone couple than for oxidized/semiquinone couple [39].

4. Stopped-flow kinetic studies of monomeric cytochromes P450 in Nanodiscs

Evaluating the critically important features of catalytic mechanism of membrane bound cytochromes P450 requires understanding the rate-limiting steps and branching points under steady-state conditions. However, the catalytic cycle of cytochromes P450 includes more than seven distinct intermediate states with different stability and multiple reaction pathways [54].

This complexity makes straightforward analysis difficult. One natural way to obtain such information is to study separately the properties of catalytically important intermediates. Equilibrium and kinetic studies of substrate binding and determining electron transfer kinetics are one such need. In addition, documenting the kinetics of oxygen binding and the stability of oxy-complex is also critical to determine the mechanisms of autoxidation and non-productive decomposition of the oxy-complex as represented in an uncoupling of pyridine nucleotide utilization. This is especially true for the membrane-bound cytochromes P450, where stability of oxy-complex is usually lower than in the well-studied soluble CYP101. Execution of these measurements, typically involving stopped-flow or temperature jump methods is problematic in the case of membrane proteins. Historically they have been investigated either in an aggregate state or incorporated into liposomes, which are not stable in these biophysical approaches. Again, the cassette approach of Nanodisc self-assembly has been enabling. Monomeric, homogeneous and stable preparations of CYP3A4 and CYP19 in Nanodiscs allowed for isolation and characterization of these intermediates [21-22,28].

Using stopped-flow kinetic spectroscopy, we documented formation and decay of oxy-complex in CYP3A4 incorporated in POPC Nanodiscs with no substrate present, and at saturating concentrations of TS and BC [21]. Similarly to CYP101, substrate binding significantly extends the life-time of oxygenated intermediate, and this stabilizing effect depends on the substrate, being more pronounced for BS than TS. Despite the relatively faster autoxidation of CYP3A4 compared to other cytochromes P450 [21], the apparent activation barriers for this reaction as estimated from Arrhenius plots are very similar in all cases. Subsequent study [22] revealed that significant stabilization of the oxy-complex, as well as a substantial increase of the amplitude of CO geminate rebinding, is caused by conformational changes coupled to the binding of a single TS molecule. This means that the first binding event, which does not cause detectable spin shift [20] and apparently happens at

the remote site far from the heme iron, plays an important functional allosteric role serving as a modulator of the first uncoupling channel of CYP3A4.

A stopped-flow study of autoxidation of CYP19 saturated with the substrate androstenedione in Nanodisc revealed a similar fast autoxidation rate (0.7 s^{-1} at 37°C) and high activation energy (18 kcal/mol) [28]. These findings provided a guideline to the preparation of oxy-complexes of these and other human cytochromes P450 for cryoradiolytic experiments, as reviewed in the next section.

The kinetics of CYP3A4 reduction by dithionite was studied using stopped flow [18] to evaluate the role of oligomerization of the enzyme and conformational heterogeneity with respect to the observed allosteric properties of cytochromes P450. Monomeric CYP3A4 in Nanodiscs was reduced in a single exponential reaction, both in the presence and in the absence of substrate bromocriptine. Analogous effects have been reported recently for reduction of CYP3A4 by the flavin domain of cytochrome P450 CYP102 [19]. As with dithionite, the reduction of monomeric CYP3A4 in Nanodiscs was almost complete, contrary to only 50% reduction of oligomeric CYP3A4 in liposomes observed with a low lipid:protein ratio (2:1:0.6 phosphatidylcholine, phosphatidylethanolamine and phosphatidic acid). Direct comparison of results obtained with low (76 lipids per CYP3A4) and high (910 lipids per CYP3A4) ratios in liposomes revealed the definitive role of oligomerization of CYP3A4 in incomplete reduction by flavoprotein reductase. These works demonstrate the importance of studying the equilibrium and kinetic properties of monomeric cytochromes P450 and to separate effects caused by aggregation and / or oligomerization.

5. Cryogenic radiolytic reduction and the spectroscopy of unstable intermediates in membrane cytochromes P450

The incorporation of human cytochromes P450 into Nanodiscs provided an opportunity for the isolation and entrapment of unstable catalytic intermediates in P450 catalysis using the same cryogenic methods developed recently for mechanistic studies of oxygen activation in the soluble P450 heme enzymes [55-63]. The overall stability of heme oxy-complexes increases dramatically at low temperatures due to the high activation barriers for this reaction (15 – 25 kcal/mol [21,28,64-65]). For example, the half-life of the oxy-complex in CYP3A4 in the presence of testosterone increases from ~10 ms at 37°C to hundreds of seconds at -30°C , making possible the oxygenation of anaerobically reduced heme enzyme by manual mixing. The advantage of working at subzero temperatures is especially important for the mammalian cytochromes P450, which usually are autoxidized very quickly at ambient conditions. However, the work in solution at $< -30^\circ \text{C}$ requires the use of aqueous cryosolvents. The most popular solvents used in cryoenzymology are glycerol and ethylene glycol, which are preferable due to their inability to dissolve lipids and to disrupt Nanodiscs, as opposed to such alternatives as methanol, ethanol or dimethyl sulfoxide. In separate control experiments we confirmed that CYP3A4 or CYP19 Nanodiscs remain intact after the addition of 75% glycerol, and the enzymes still show full activity and lack of P420 formation. The usage of high glycerol or ethylene glycol concentration thus allows the formation of a transparent glassy frozen sample suitable for optical absorption spectroscopy at cryogenic temperatures [59].

The oxy-complex in CYP19 incorporated in Nanodiscs was prepared by mixing of reduced enzyme with an excess of oxygenated cold 90% glycerol/phosphate buffer solution at -30°C and cooled to liquid nitrogen temperatures as described [27]. Absorption spectra of these samples measured at cryogenic temperatures are very similar to those resolved from stopped-flow kinetic experiments [28]. Samples for EPR spectroscopy were prepared similarly by mixing directly in quartz tubes and frozen in liquid nitrogen. Radiolytic

reduction was accomplished by γ -irradiation from ^{60}Co source at liquid nitrogen temperature as described [59-60]. As a result of one-electron reduction of the oxy-complex in CYP19, the unprotonated peroxo-ferric complex was formed with characteristic g-values $g_1 = 2.254$ and $g_2 = 2.163$ (g_3 could not be determined since it overlaps with radicals generated in frozen glycerol matrix). This initial peroxo-anion complex was stable at temperatures up to 180 K, and then gradually converted to the low-spin ferric complex with $g_1 = 2.39$ at 190 – 220 K. Stabilization of unprotonated peroxo-ferric complex in CYP19 indicates potentially important difference in the proton delivery mechanism between this enzyme and other heme enzymes such as CYP101 and CYP2B4 [56-57]. As the active intermediate in the aromatization reaction catalyzed by CYP19 is suggested to be the peroxo-anion complex, the active site of aromatase may be optimized to maintain this complex in an unprotonated state. Further investigation of these intermediates in CYP19 using ENDOR and Q-band EPR is in progress through collaboration with Professor Brian Hoffman and Dr. Roman Davydov at Northwestern University.

Using the same methods, we have also prepared and measured the UV-VIS spectra of the oxy- and peroxo- complexes in CYP3A4 self-assembled into POPC Nanodiscs. The spectra of oxy-complex of CYP3A4 saturated with TS and BC and measured in 65% glycerol at $T = 100$ K are shown in Figure 2. Similar to the spectra measured in stopped-flow experiments [21], the position of the main Soret band is slightly different for the CYP3A4-TS and CYP3A4-BC complexes. The different shape of Soret and Q-bands is better resolved at low temperatures and may indicate the presence of multiple conformers of oxygen molecule coordinated to iron atom, similarly to the conformational heterogeneity of the oxy-complex of CYP101 documented by resonance Raman spectroscopy [66-67].

After cryoradiolytic reduction, a (hydro)peroxo-ferric complex is formed with the estimated yield $\sim 50\%$. The optical spectrum of this intermediate is shown in Figure 3 together with the spectrum of the same sample before irradiation. The shape and positions of split Soret bands at 439 nm and at ~ 367 nm are similar to the spectrum of the same intermediate in CYP101 [59].

6. Conclusions and Summary

We have attempted in this brief review to link the pioneering investigations of Professor Ruckpaul on the cytochromes P450 with future efforts at unraveling the deep secrets of the membrane bound form of these important monooxygenases. Early mechanistic work in the 1970-1990 time period utilized the soluble microbial P450s as a model of the important membrane bound forms which compose the vast majority of the $\sim 11,000$ P450 genes so far identified. When these membrane bound P450s are removed from their native environment, aggregation and inactivation often occur and the sophisticated biochemical and biophysical techniques used with their soluble brothers were often not applicable. What was needed is a means for converting these aggregating and often inactive forms into a soluble monodisperse entity that would allow mechanistic details to be revealed. The self-assembled Nanodisc system has proven to open this door to advanced quantitative analysis. Highly purified homogeneous fully functional and stable preparations of membrane bound P450s have now been used by many laboratories in their quest to understand the workings of one of the most prevalent enzymes in Nature's basket of biotransformations.

Acknowledgments

The works of many members of the Sligar laboratory cited in this review is gratefully acknowledged. Our research on cytochromes P450 has been supported for nearly thirty years by two grants from the National Institute of Health GM31756 and GM33775.

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Abbreviations

AFM	Atomic force microscopy
ANF	alpha-naphthoflavone
BC	bromocriptine
CPR	Cytochrome P450 reductase
DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
HDL	High-density lipoprotein
LSPR	localized surface plasmon resonance
MSP	Membrane scaffold protein
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
SPR	surface plasmon resonance
TS	testosterone

Figure 1.

A molecular model of a 1:1 functional complex of CYP3A4 and CPR incorporated in a Nanodisc. The coordinates of the POPC bilayer in a liquid crystalline state are based on molecular dynamics simulations available at: <http://www.lrzmuemchen.de/~heller/membrane/membrane.html> [68]. The membrane scaffold protein (MSP1E3) [69] (blue) is modeled as an ideal α -helix. CYP3A4 (red), from the Protein Data Bank file 1TQN.pdb [70] and CPR (yellow) from Protein Data Bank file 1AMO.pdb [71] are pictured in a close contact in their docked conformation for illustrative purposes.

Figure 2.
The absorption spectra of the oxy-ferrous complex of CYP3A4 in Nanodiscs both with testosterone (red) and bromocriptine (blue) bound at the active site. Potassium phosphate buffer /65% glycerol, T=100 K.

Figure 3. The absorption spectra of the oxy-ferrous (red) and hydroperoxo-ferric (blue) complexes of CYP3A4 in Nanodiscs with bromocriptine bound.

Scheme 1.
Catalytic cycle of cytochrome P450. Distinct intermediates are numbered 1 through 7.
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