Minireview

JUD COON: 35 YEARS OF P450 RESEARCH, A SYNOPSIS OF P450 HISTORY

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ABSTRACT:

The year 2004 marks the 50th anniversary of the discovery of cytochrome P450. Minor J. (Jud) Coon has been a leader in this field for the last 35 years. This review summarizes his contributions to P450 research by discussing six of his most significant publications; not surprisingly, these papers serve as landmarks for the major directions followed in P450 research.

1. Role of Hemoprotein P-450 in Fatty Acid ω-Hydroxylation in a Soluble Enzyme System from Liver Microsomes


As noted in his recent “Reflections” article (Coon, 2002), Minor J. (Jud) Coon (Fig. 1) did not set out in 1966 to purify cytochrome P450. His interest was in fatty acid oxidation, and earlier studies by Bill Peterson and others in his laboratory (Peterson et al., 1966; Peterson and Coon, 1969) (Table 1). Although others had tried earlier to solubilize and purify catalytically active cytochrome P450, the keys to success were the recognition that reconstitution of activity might require the combination of multiple fractions from liver, following the precedent set by Peterson in his studies on the Pseudomonas system, and the inclusion of glycerol in the solubilization buffers to prevent enzyme denaturation. Indeed, there was widespread concern that these membrane-bound enzymes could not function outside of their native membrane environment. Also critical to success was the use of detergent-solubilized P450 reductase, rather than the lipase-released cytochrome P450-δ reductase commonly studied at that time (Williams and Kamin, 1962); lipase preparations were contaminated with proteases that removed the N-terminal membrane-binding segment of the reductase and prevented it from interacting with cytochrome P450. The procedure for separation and reconstitution of the cytochrome P450 system described by Lu and Coon became the standard approach for subsequent studies on mammalian P450 around the world, and by 1975 close to 20 papers were published on the purification of cytochrome P450. The Coon laboratory turned its attention to the role their newly found P450 played in drug metabolism, and to determine whether isozymes of P450 were present in liver.
2. Purified Liver Microsomal Cytochrome P-450: Separation and Characterization of Multiple Forms


The important question of how many forms of P450 exist in liver was hotly contested in the early 1970s. It had been widely recognized that administration of various inducers of P450, typified by phenobarbital and 3-methylcholanthrene, altered drug metabolism profiles and increased or altered the carbon monoxide absorption peak attributed to P450, but it was unclear whether this was due to changes in the protein or to changes in the membrane environment. Although this indirect evidence for multiple forms was provided as early as 1966 by the Sato and Mannering laboratories (Imai and Sato, 1966; Sladek and Mannering, 1966), the difficulty of purifying P450 from microsomes made proof difficult.

In two papers authored by David Haugen, the Coon laboratory applied expertise in protein purification to obtain evidence that at least four isoforms of P450 were present in rabbit liver (Haugen et al., 1975) (Fig. 2), and subsequently purified and characterized the principal phenobarbital-inducible and β-naphthoflavone-inducible forms (Haugen and Coon, 1976). This latter paper established beyond doubt the presence of at least two P450 isoforms in liver (CYP2B4 and CYP1A2), as evidenced by different molecular masses, carboxy-terminal sequences, amino acid compositions, spectral properties, and catalytic activities. Several other groups, one of which included Anthony Lu, also reported the purification of either the phenobarbital- or methylcholanthrene-inducible form at this time (Imai and Sato, 1974; Kawalek et al., 1975). It should be noted that, despite its accomplishments, the Coon laboratory was not infallible: it was eventually made proof difficult.

Although a number of additional forms of liver P450 were purified by the Coon group in subsequent years, the difficulty in separating highly similar P450 proteins by column chromatography limited the success of this approach. Nonetheless, these studies established the approach for P450 characterization studies for the next decade, until the advent of cDNA cloning and heterologous expression.

3. Purified Liver Microsomal NADPH-Cytochrome P-450 Reductase: Spectral Characterization of Oxidation States


Not overlooked by the Coon group in their studies on the P450 system was the requisite electron-transfer partner for cytochrome P450, NADPH-cytochrome P450 reductase. This was the first in a series of papers from the Coon laboratory on the catalytic mechanism of cytochrome P450 reductase. Although the flavoprotein had been first characterized in whole liver as a cytochrome c reductase (Horecker, 1950), its predominant location on the endoplasmic reticulum (Williams and Kamin, 1962) indicated that cytochrome c was not a natural substrate; the role of the reductase in the P450 system was established by Lu and Coon in their 1968 paper. Although some of the properties of the reductase had been established earlier with a protease-solubilized form of the protein (Iyamag and Mason, 1973), this form of the protein was not competent in electron transfer to P450, and so Janice Vermilion set out in her dissertation work to characterize the detergent-solubilized reductase. These studies took advantage of the earlier purification studies of Dignam and Strobel (1975) (Henry Strobel was a former postdoc with Coon) and, later, the affinity purification procedure developed in Bettie Sue Masters’ laboratory (Yasukochi and Masters, 1976).

The Coon laboratory was also able to take advantage of the extensive expertise in flavoprotein enzymology at the University of Michigan provided by Vince Massey, Charles Williams, and David Ballou. Their combined expertise in protein purification and flavin enzymology allowed this group to establish the electron acceptor (FAD) and donor (FMN) flavins in the reductase (Vermilion et al., 1981), and the redox states of these flavins during catalysis (Fig. 3). They were also able to resolve the controversy about the air-stable state of the reductase semiquinone, which, because it has two flavin groups, could contain either one or two added electrons; they clearly showed that

### TABLE 1
Reconstitution of fatty acid hydroxylation (adapted from Lu and Coon, 1968)

<table>
<thead>
<tr>
<th>Components</th>
<th>Activity (Percentage Complete)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>100</td>
</tr>
<tr>
<td>−P450</td>
<td>17</td>
</tr>
<tr>
<td>−Lipid</td>
<td>9</td>
</tr>
<tr>
<td>−P450 reductase</td>
<td>10</td>
</tr>
<tr>
<td>−NADPH</td>
<td>0</td>
</tr>
<tr>
<td>−O₂</td>
<td>10</td>
</tr>
</tbody>
</table>

Fig. 2. Polyacrylamide gel electrophoresis of cytochrome P450 fractions prepared from rabbit liver.

Microsomes from phenobarbital-treated (PB) and normal (N) animals, and four fractions enriched in P450 (A–D) were resolved on a 7.5% polyacrylamide gel in the presence of 0.1% SDS and stained with Coomassie blue. The four proposed isoforms of P450 were designated 1, 2, 4, and 7, based on their mobility. Forms 2 (fraction A) and 4 (fraction D) correspond to CYP2B4 and CYP1A2, respectively. SDS, sodium dodecyl sulfate. Reprinted with permission from Haugen DA, van der Hoeven TA, and Coon MJ (1975) J Biol Chem 250:3567–3570.
Coon MJ (1981) P450. Reprinted with permission from Vermilion JL, Ballou DP, Massey V, and FMN hydroquinone (2 electron-reduced flavin) serving as the electron donor to important roles in toxicology (Coon and Koop, 1987) and alcohol-al., 1989; Peng and Coon, 2000). This P450 also has proven to have studies on its role in ethanol metabolism (Morgan et al., 1982; Koop and Coon, 1978), later shown to reside in the FMN group (Vermilion et al., 1981).

Although this series of papers has served as a benchmark for kinetic studies on this and related flavoproteins, including nitric oxide-synthase, many aspects of the redox-cycling of these unusual flavoproteins remain controversial, and prominent groups continue to dissect and explore their complicated redox kinetics (Murataliev and Feyereisen, 1999; Gutiérrez et al., 2003).

4. Purification and Characterization of a Unique Isozyme of Cytochrome P-450 from Liver Microsomes of Ethanol-Treated Rabbits


By the late 1970s, the multiplicity of P450 isoforms was well established, and the key questions were how many isoforms existed and how they differed in physical and catalytic properties. The Coon laboratory attacked these problems with vigor. Of particular interest was the ethanol-inducible isocorr, CYP2E1 (earlier designated LM3a in rabbits). The proposed role of this P450 in ethanol metabolism was a controversial subject that could best be resolved by purifying the enzyme and characterizing its activity in a reconstituted system. Dennis Koop and Eddie Morgan were new postdocs in the Coon laboratory, and together they initiated a series of studies on CYP2E1 that eventually led to over 40 publications on this P450, including studies on its role in ethanol metabolism (Morgan et al., 1982; Koop and Coon, 1985), the cloning of its cDNA and gene (Khani et al., 1987, 1988), and studies on the regulation of its expression (Porter et al., 1989; Peng and Coon, 2000). This P450 also has proven to have important roles in toxicology (Coon and Koop, 1987) and alcohol-induced liver pathology (Cederbaum et al., 2001). The paper by Koop et al. was the first to describe the purification and properties of CYP2E1, and brought to five the number of P450s purified and characterized in the Coon laboratory.

The approach taken by Koop et al. to purify CYP2E1, standard column chromatography with a variety of exchange resins and carefully selected elution conditions, represented the zenith of P450 purification studies, and was gloriously illustrated in Fig. 1 of that paper with five immaculately purified P450 isoforms (reproduced here in Fig. 4). But this study also heralded the end of the era of traditional protein biochemical approaches to P450 purification. These traditional fractionation techniques lacked the power to separate readily the many highly similar isoforms of P450 being identified, and the ability to compare proteins purified in different laboratories, even from the same species, was becoming increasingly difficult. Investigators could not be sure that their P450s corresponded to those purified by similar techniques in another laboratory, and so the number of P450 isoforms multiplied with every laboratory that joined the effort. Clearly a new approach to P450 identification was needed. Although not widely recognized by the P450 protein biochemistry community, that approach had arrived in the guise of molecular biology just a year earlier: Yoshiaki Fuji-Kuriyama’s group had cloned the first cDNA to a P450, CYP2B1 (Fuji-Kuriyama et al., 1981). When the sequence of that P450 was published a year later (Fuji-Kuriyama et al., 1982) it became obvious to all that a new paradigm was in place and would greatly change the way P450 was studied. The Coon group wasted little time in adopting this new approach to P450 biochemistry.
The most widely used method for the characterization of cloned P450s, and well over a hundred papers have been published using this technique. The paper by Larson et al. added a second surprising finding to the P450 story, in that the N-terminal segment, which serves as a signal for membrane insertion in mammalian cells, proved unnecessary for membrane incorporation in bacteria or catalytic activity of CYP2E1. This result suggested that additional membrane-binding segments were present in P450, and subsequent studies in Eric Johnson’s laboratory showed that an internal segment of the enzyme, close to the substrate access channel, conveyed this additional contribution to membrane association (Cosme and Johnson, 2000). Deletion and modification of these segments led to a soluble, catalytically active enzyme and allowed the first crystallization and structural determination of a mammalian P450 (Williams et al., 2000). Thus, the study of Larson et al. not only led to the most successful method for P450 expression and characterization, but also opened the door for determination of the three-dimensional structure of the cytochrome, a much sought-after goal in P450 biochemistry.

6. Peroxo-Iron and Oxenoid-Iron Species as Alternative Oxygenating Agents in Cytochrome P450-Catalyzed Reactions: Switching by Threonine-302 to Alanine Mutagenesis of Cytochrome P450 2B4


If there is one overriding challenge in P450 biochemistry, it is to understand the mechanism by which molecular oxygen is activated and inserted into generally unreactive compounds by this versatile enzyme. This challenge has occupied the Coon laboratory for most of the last 35 years. The generally accepted mechanism for oxygen activation involves the generation of an iron-oxene species \((\text{Fe}^{2+}/\text{H}_{11005}\text{O})\) derived from the heterolytic cleavage of \(\text{O}_2\). Studies by Ron White, Steve Sligar, and Jud Coon (White et al., 1980) indicated that an active oxygen species might also be generated by the homolytic cleavage of peroxide, and raised the possibility that other oxygenating species derived from iron-peroxy complexes might also be relevant to catalysis. In the paper above by Vaz et al., mutation of threonine 302, a highly conserved residue thought to be involved in proton delivery to the P450 heme-\(\text{O}_2\) complex (Raag et al., 1991), was expected to impair generation of the iron-oxenoid species, leaving the peroxy heme as the principal oxidizing species. Indeed, oxene-dependent reactions were decreased and peroxy-dependent reactions were enhanced 10-fold. This paper and a subsequent report (Vaz et al., 1998) that demonstrated that hydroperoxo-iron can serve as an alternative electrophilic oxidant thus provided convincing evidence that P450 can utilize multiple active oxygen species for catalysis.

Close to 50 publications from the Coon laboratory have addressed the catalytic mechanism of cytochrome P450. The well-known catalytic cycle (reproduced here in Fig. 5) first appeared in a paper in 1973 (Coon et al., 1973),\(^2\) and was updated in a review by White and Coon (1980) that is a classic for its clarity and insight. Although many other researchers have also made significant contributions to our understanding of P450 catalysis (such as Fred Guengerich, a former postdoc with Coon, and Steve Sligar), perhaps no other biochemist has devoted as much time and effort to resolving this most challenging problem in P450 biochemistry. And these studies continue: a recent review (Newcomb et al., 2003) summarizes the complexity of reactions involving peroxo-iron, hydroperoxo-iron, and iron-oxene as oxygenating species, as shown in Fig. 6. Quoting a recent review, “The occurrence of multiple oxidizing species may contribute to the remarkable versatility of the P450 family” (Coon et al., 1998). Jud Coon is clearly not ready to give up the effort to understand the impressive versatility of cytochrome P450.

\(^2\) Ron Estabrook published a similar version of the catalytic cycle in the same issue of this journal (Estabrook et al., 1973) and an earlier, less elaborate version in 1971 (Estabrook et al., 1971).
Epilogue. Not surprisingly, many important developments in the mammalian P450 field have not been covered here, and much of our understanding of P450 biochemistry has been built on studies of prokaryotic P450s, most notably the work that came out of the laboratory of Irwin Gunsalus on P450cam (CYP101) (e.g., Poulos et al., 1985). Also notable in their absence are studies on the expression and regulation of this superfamily of enzymes, from early studies on induction to more recent studies on the nuclear receptors involved in their expression. Although studies in the Coon laboratory touched on these topics, they were not a major focus of his group and, thus, are not discussed here. This in no way diminishes their importance to our understanding of P450 biology.  

Jud Coon will close his laboratory at the end of 2003, after 60 years in scientific research. Well over 90 scientists have passed through his laboratory, and many have gone on to make significant contributions to P450 science in their own right. The National Library of Medicine currently lists Jud as an author in 187 publications on P450. When he published his first paper on P450 in 1968, there were less than 40 papers on this topic; by 2003 there have been over 32,000 publications, with over 1000 appearing in the first six months of that year. Not surprisingly, many important developments in the P450 field have not been covered here, and much of our understanding of P450 biochemistry has been built on studies of prokaryotic P450s, most notably the work that came out of the laboratory of Irwin Gunsalus on P450cam (CYP101) (e.g., Poulos et al., 1985). Also notable in their absence are studies on the expression and regulation of this superfamily of enzymes, from early studies on induction to more recent studies on the nuclear receptors involved in their expression. Although studies in the Coon laboratory touched on these topics, they were not a major focus of his group and, thus, are not discussed here. This in no way diminishes their importance to our understanding of P450 biology.  

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References


Several reviews on the early history of P450 have recently appeared; in addition to those cited here (Lu, 1998; Coon, 2002; Klingenberg, 2003), personal accounts by Gil Manning (Manning, 2001) and Allan Conney (Conney, 2003), and an early account by David Cooper (Cooper, 1973) are also of interest.

Todd D. Porter received a B.S. in Biochemistry from the University of Illinois in 1976 and his Ph.D. in Pharmacology from the same institution in 1981. His graduate work was with Martin Schulman on the induction of drug-metabolizing enzymes by ethanol feeding. He carried out post-doctoral research with Charles Kasper at the University of Wisconsin, working on cytochrome P450 reductase, and was an Assistant Professor under Jud Coon at Michigan from 1986 to 1991, where they pioneered the use of bacteria for the expression of cytochrome P450. He has continued his research in this area, publishing a number of articles on the use of bacteria for biotechnological and enzymological investigations of cytochrome P450 and cytochrome P450 reductase.

Dr. Porter is an Associate Professor in the College of Pharmacy at the University of Kentucky and also serves as the Director of Graduate Studies for the Graduate Center for Toxicology under Dr. Mary Vore. He is on the Editorial Board of the Journal of Biochemical and Molecular Toxicology.