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The past and present of P450cam structural biology

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A brief history

What follows is a brief accounting of the who and how surrounding the solution of the P450cam crystal structure. Given that Gunny's memory of those days is far better than mine, I at first was reluctant to try to relate events. Fortunately, my old notebooks are still in good shape and records uncharacteristically neat and dated. It started in the summer of 1978, when I first met Gunny at the Scripps Institute of Oceanography in La Jolla, California, very near the UCSD campus. He was attending a symposium honoring Martin Kamen in celebration of Martin's 65th birthday [1]. At the time Martin was in the Chemistry Department at UCSD, where I was working in Joe Kraut's lab one floor up from the Kamen lab. I was quite close to the Kamen group owing to common interests in heme proteins and was honored when Joe allowed me to present a paper at the Kamen symposium.

I had been working on my first structure, cytochrome *c* peoxidase (CCP), and by 1978 the light was at the end of the tunnel and it was clear that the structure would actually be solved. I went looking for something new and exciting and P450 looked to be something that might be important. I didn't know much about P450 in those days. I had an inkling that these were important enzymes but I had no idea how important nor did I fully appreciate how critical Gunny's P450cam was to the field. I knew Gunny would be there so I introduced myself and suggested that I work on P450cam. How truly naive. I had no idea of the history behind P450cam, that Dick Dickerson had made a try but was not very interested owing to his shift into DNA structures, and that Gunny was deadly serious about getting the P450cam structure solved. My first shock was when Gunny pulled out a notebook filled with crystallization information and pictures of crystals. This prompted me to suspect Gunny was hunting for a crystallographer and that the Kraut lab was on the list. As with most collaborations I expected things to go slowly, that Gunny would return to Urbana, and that we would all think about this for a while before anything actually happened. However, Gunny was in a hurry and it was either the next day or shortly thereafter that Gunny, Joe, and I got together to talk this over and it was decided that I should visit Urbana and get to know the P450cam world.

Gunny no doubt remembers the specific dates, but I recollect that it was days later that I visited Urbana and first met Gerry Wagner, Gunny's right-hand man. Gerry had been setting up many crystallization trials and had some promising results from what was then touted as the magic crystallization agent, polyetheylene glycol. I took a look at some of these setups and immediately caught a glimpse of a tetragonal form. This looked promising and we decided to try to improve on these. I went back to UCSD and thence came the steady supply of purified P450cam from Gunny's lab made by Gerry and his assistant, Mary Perez. The PEG crystallization worked out and by November of 1978, only a few months after getting started, I had crystals that could be placed in the X-ray beam. These turned out to be in a tetragonal space group with one 255-Å unit cell edge. This would not be much of a limitation today, but back then this could cause some major data collection problems. Gerry and I decided to keep trying for another crystal form and if we did not hit on something within a few months, we'd find a way to work with the large unit cell edge. I kept an optimistic front on this problem but I knew that to tackle the large unit cell edge would require a major retooling of hardware. In other words, I was worried that we might get stuck with this far less than optimal crystal form.

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We had talked over various methods of protein crystallization and perhaps because the free-interface diffusion method had been worked out by Ray Salemme in the Kraut lab [2], Gerry decided to try this method. Gerry was far less reluctant to consume large amounts of protein than I was, and he set up crystallization trials using mM concentrations of P450cam at 50 μl per shot. Gerry obtained the first crystals of the orthorhombic form that were used to solve the structure using the free interface diffusion method in small test tubes which required about 2 mg of protein per tube. The first crystals arrived from Urbana in May 1979 and the first X-ray photos were taken on May 29, 1979, and completed the next day. That was a big day for all of us. It was immediately clear that these crystals diffracted well (Fig. 1), were in everyone's favorite space group ($P2_12_12_1$), and had manageable unit cell edges. Repeating the crystallization was a bit tricky and required the careful control of temperature and ammonium sulfate concentration. Setting up several tubes with such large amounts of protein was going to strain the protein supply, although this was probably not a correct assumption on my part. At the time I did not know that Gunny had a P450cam factory going in Urbana so protein supply should not have been a concern. Nevertheless, to save protein and anticipating the day that I would have to purify P450cam myself, I switched to a capillary method so only 10 μl of protein need be used rather than 50 μl. This did not hurt the quality of the crystals and actually made it easier to recover crystals. It took a minimum of 63 different crystallization setups consisting of several capillaries each to get the structure. I say "minimum" since this is the last entry in my notebook.

The search for heavy atom derivatives started on May 31, 1979, 2 days after the first X-ray photos were taken. The last notebook entry for heavy atoms was March 16, 1982, at heavy atom trial 110. Heavy atom screening was carried out using precession photos like the one shown in Fig. 1, which took about 2 days per heavy atom so close to a year was spent just on heavy atom screening. For those that looked promising, full data sets were obtained using a single crystal diffractometer. Approximately 2 weeks were required to collect and work up a full low-resolution data set and several months for a high-resolution set since the data from several crystals had to be merged together. Hence, over a year was required to collect the data finally used for solving the structure. The first electron density map was calculated in 1981, but it took until the summer of 1984 before the structure was solved and refined.

There were several problems with interpreting the first electron density maps. The sequence of P450cam had not yet been completed when the crystal structure work started, although I was being fed information prior to publication. The thiolate–heme interaction was fairly clear in the map but the question was which Cys residue was coordinated to the heme. From the limited information available at the time, it looked like Cys357 or Cys334 was the ligand. I incorrectly eliminated Cys357 as the ligand based on the following information. Any Cys that could react with thiol-selective agents could not be the ligand. The Cys357 tryptic peptide could be chemically modified to give a distinctly colored

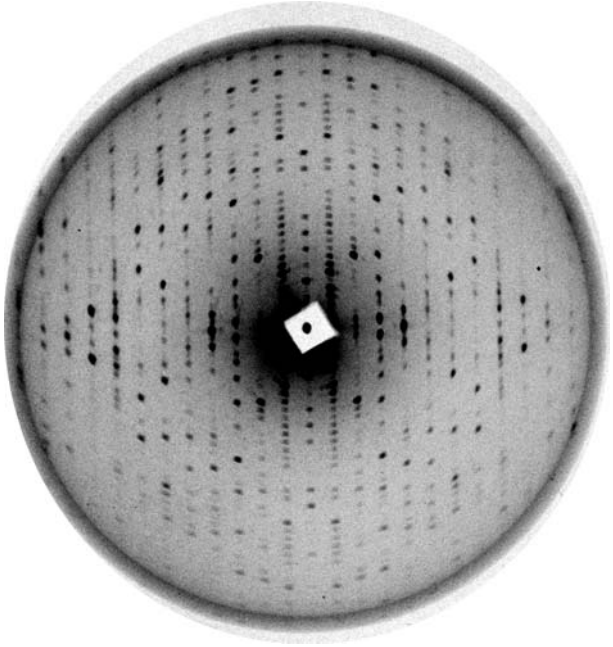

Fig. 1. The first X-ray precession photo taken of the orthorombic $P2_12_12_1$ crystal form used to solve the P450cam structure. This required about a 24-h of exposure. The edge of the photo is approximately 3 Å.

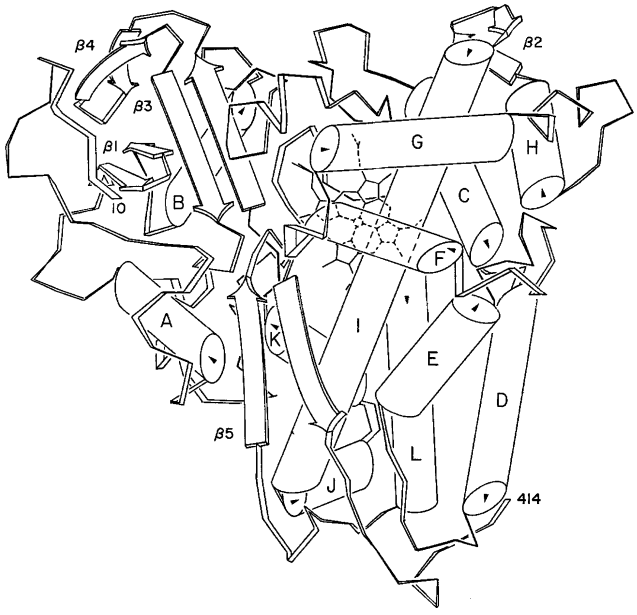

Fig. 2. The first cartoon drawing of P450cam (taken from Ref. [6]). Note that the B′ helix is missing.

peptide indicating that Cys357 rapidly reacted with the reagent and hence could not be the ligand. The problem was that the Cys357 tryptic peptide also contains His at position 361, which was misidentified as Ser in the initial sequence [3]. It is very likely that one of the 4 His residues in this peptide had reacted with the reagent, giving a colored peptide leading to the incorrect conclusion that Cys357 had been modified and hence could not be the ligand. Given this information together with the reasonable sequence homology around Cys334, I assumed Cys334 was the ligand and fit the entire electron density map based on this assumption. Unfortunately, I did not know about similar work in Jud Coon's lab on P450LM2, published in 1985, where the correct ligand was identified using chemical modification methods [4]. In addition to the ligand problems, Trp55–Thr56 were missing from the original sequence, which caused a somewhat less serious problems. While helix I and several other helices were correctly fit, it was not possible to proceed with all the inconsistencies between the sequence and electron density map, especially in the most critical region of the structure. In fact, the initial tracing of the polypeptide backbone had the polypeptide going through electron density that belonged to a strong ion

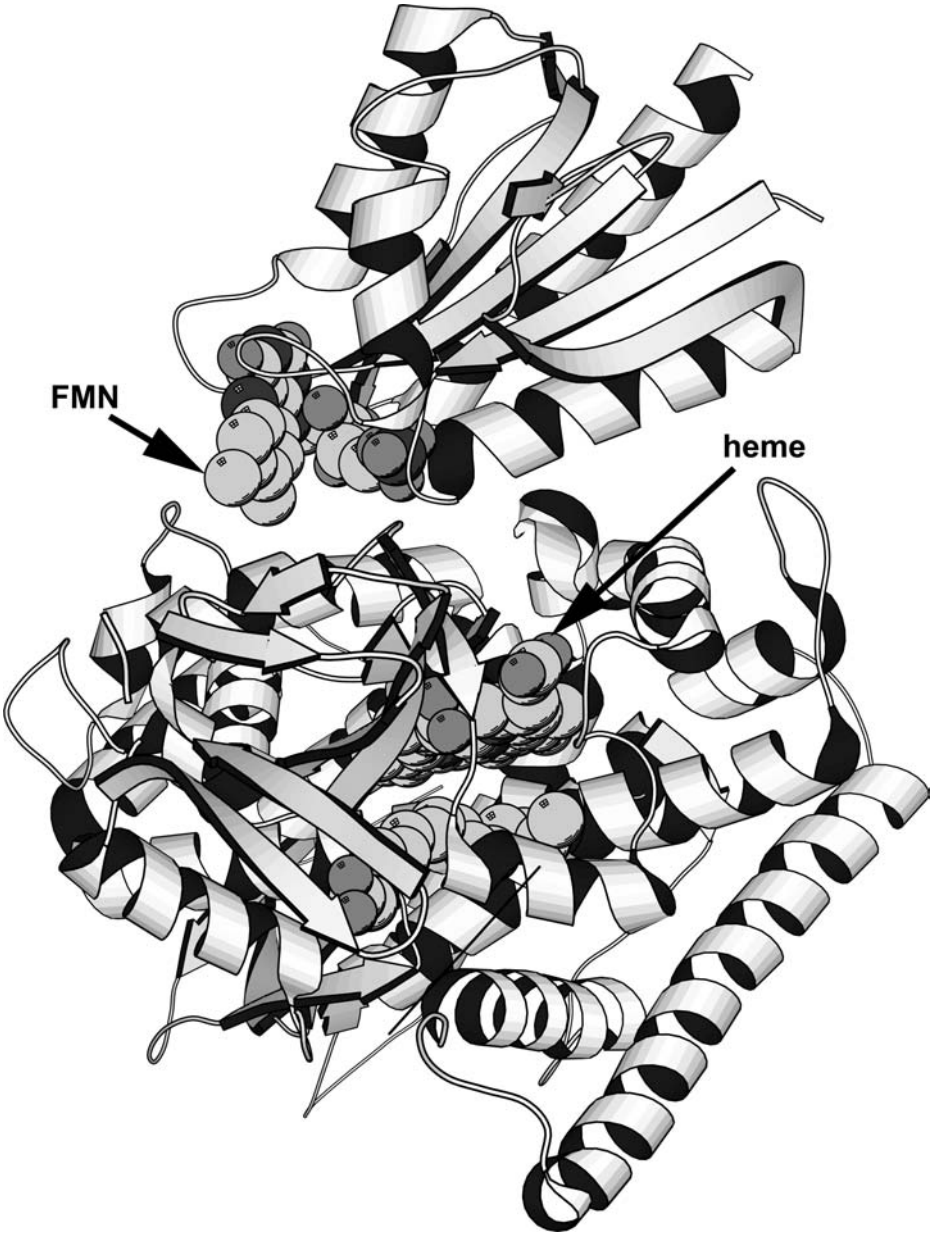

Fig. 3. Crystal structure of the complex formed between the heme and FMN domains of P450BM-3 [10]. The FMN domain docks on the proximal surface of the heme domain which provides the closest approach of the heme to the molecular surface. The FMN and heme are about 18 Å apart, although the FMN directly contacts a section of the heme domain polypeptide that might serve as an electron transfer conduit to the heme.

pair. This incorrect tracing was a consequence of the incorrect identification of the Cys ligand.

During this critical period I moved to a new job at Genex Corp. (located in Gaithersburg, MD) in October 1983, which disrupted the P450cam work for a time. Work resumed at the beginning of 1984. The structure determination was rescued by the DNA sequence which Steve Sligar provided to me in 1984 prior to publication [5]. Things fell into place rapidly at this point and during the summer of 1984 I lived in front of the graphics system correcting the model, while Barry Finzel, a former student in the Kraut lab who also moved to Genex, provided invaluable assistance with refinement. At the Brighton Microsomes and Drug Oxidation Meeting in 1984, Gunny shared his time with me and that was the first public presentation of the P450cam structure. While we were writing up the first P450cam structure paper [6], Andy Howard and I collected the 1.6-Å data set on the new Xentronics area detector that Genex had purchased. The structure refined with these data is the one most widely used today [7].

The structure shown in Fig. 2 is the hand-drawn version created by Gerry and me, mostly Gerry who spent much time on this figure. It is surprising that over the years people are not more curious as to why the B′ helix is not the C helix. The fact is that in the initial publication, it was not clear that the B′ helix was a helix. This region contains the K^+ binding site and Tyr96, which forms a specific H-bonding interaction with camphor. At 2.6 Å the K^+ site was not identified and appeared as a large lobe of density that blended into the protein. The C-terminal end of the helix adopted an odd conformation in order to form the K^+ site and was quite difficult to properly fit. At 2.6 Å this was all very messy

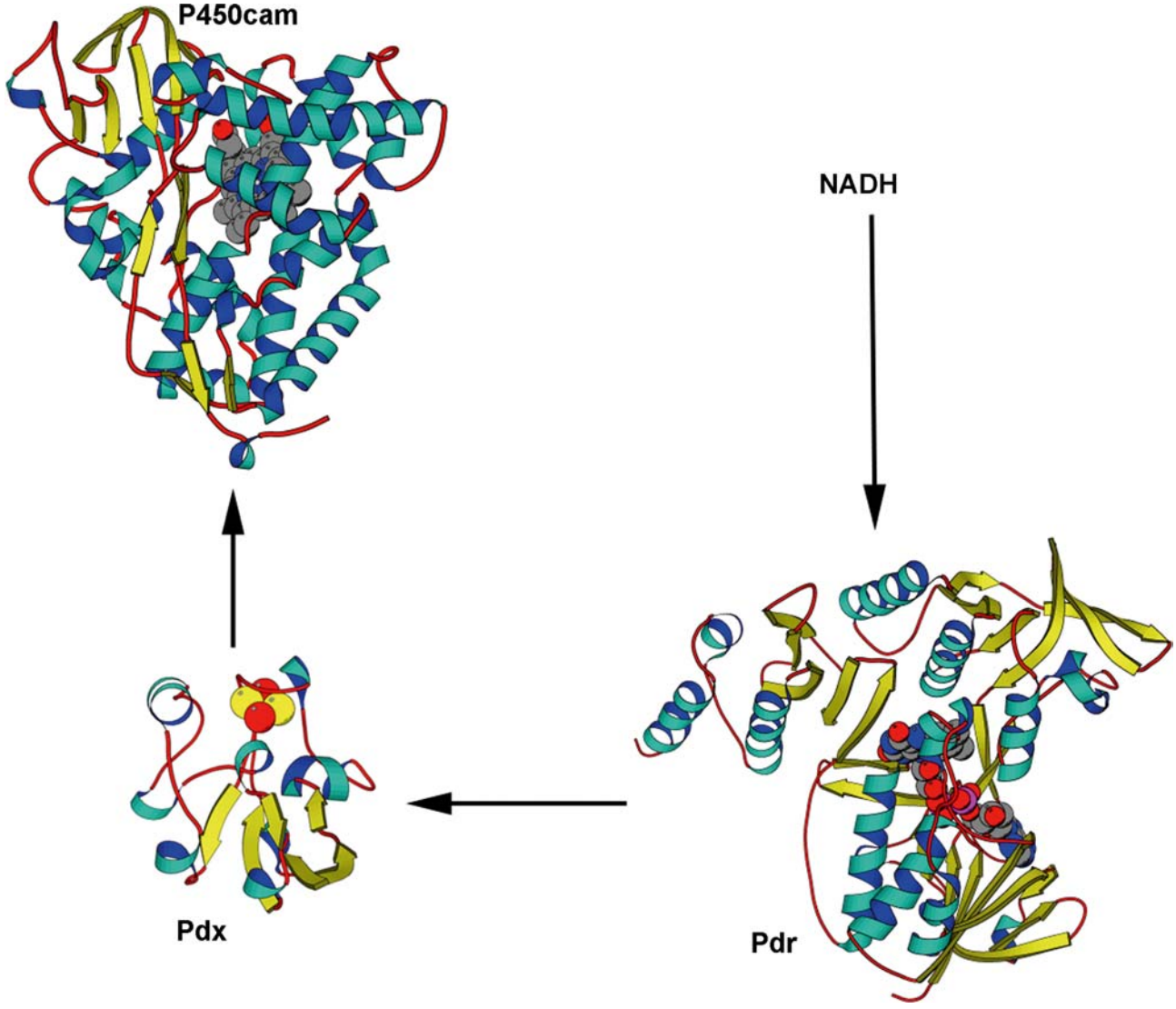

Fig. 4. The structure of all three components of the P450cam monooxygenase system. The Pdx and Pdr structures were solved by Irina Sevrioukova and Huiying Li in the author's lab and will be published soon.

so I did not assign this as a helix. When the higher resolution structure was completed, these details were worked out, but we were faced with the decision to rename all the secondary structure or call the new helix B′. It seemed less confusing to leave the assignments as is and call the new helix B′. This is only worth mentioning since the B′ helix has turned out to be critical in substrate recognition and binding.

These were clearly some of the most exciting times in my early career. Gunny stuck with me through the many ups and downs in solving the P450cam structure and his support was essential in seeing me through some tough times, especially with the move to Genex, which placed enormous stress on my growing family. Gunny introduced me to big-time science and taught me the value of collaborations. Until P450cam, I had pretty much worked in isolation with a fairly myopic view, but Gunny showed me by example that this approach is too limiting. Equally important was my introduction by Gunny to the broader P450 community, which is populated with some exceptional scientists and people. This has led to some long-standing collaborations and friendships. It also became evident that Gunny casts a giant shadow. Once I got to Genex, I found that Gunny was a consultant for Genex. Should I have been surprised? Probably not given his preeminence in the world of microbiology. His shadow stretched to UC Irvine as well. UCI began the courtship with me and my wife, Andrea Tenner, in 1990. Finally the details were worked out and we moved to Irvine in January 1992. Little did I know at the time that the chair of my department, Krishna Tewari, had been hired by Gunny to run the New Delhi wing of the International Center for Genetic Engineering and Biotechnology.

Current efforts

P450 structural biology moved fairly slowly after the P450cam structure. The next breakthrough was the P450BM3 heme domain structure in Bill Peterson's lab in 1992 [8]. As more P450s are cloned and expressed, the data base has and will steadily grow and we can expect at least a few new structures per year for the foreseeable future. It now seems clear that the field is well on the way to understanding how the basic P450 fold adapts to the requirements of substrate specificity. A more challenging problem is to understand how the nonselective drug-metabolizing P450s adapt to substrates of different sizes and shapes. There are indications that the F/G loop and regions nearby may shape themselves around the substrate [9] but it remains to be seen if microsomal P450s operate in a similar way.

Also lacking is detailed structural information on the various redox complexes in P450 systems, but this now is changing. To date, there is one structure of a P450 redox complex, which is the P450BM3 heme domain complexed with the FMN domain [10] (Fig. 3) and the structure of the adrenodoxin–adrenoxdoxin reductase structure also is known [11]. At long last, too, the components of the P450cam system, putidaredoxin (Pdx) and pudiaredoxin reductase (Pdr), are known, both of which were solved by Irina Sevrioukova and Huiying Li (unpublished) (Fig. 4). Unfortunately, these structures do not reveal why P450cam is so selective for Pdx as the electron donor and what critical role the C-terminal Trp plays in electron transfer [12]. Clearly, direct structural information on the complexes is required. A major advance has been made in this area. Using NMR methods, Pochapsky's lab has made some important discoveries on long-range structural effects when P450cam forms a specific complex with Pdx [13]. The structural pieces now are in place to finally "underderstand" the entire P450cam puzzle, a goal that is achievable in the very near future. So stay tuned, Gunny. What you started is still bearing fruit.

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