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EPR on the $P450_{cam}$ system in the late 1960s with Gunny and his gang: insights concerning the structure of Fe-S proteins and the spin states of P450

Helmut Beinert*

Institute for Enzyme Research and Department of Biochemistry, College of Agricultural and Life sciences, University of Wisconsin–Madison, 1710 University Avenue, Madison, WI 53726-4087, USA

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In the 1940s, when I had the privilege to be a graduate student and then postdoc at the Kaiser-Wilhelm-Institute in Heidelberg, we had a beautiful library, with a large window-front facing up the river toward the old city of Heidelberg and the castle, which lies on the down-slope of the Odenwald mountains toward the Rhine valley. Unfortunately, at that time the library was of little use, as we were cut off from almost all of the foreign literature, where the most exciting results in biochemistry were appearing. When, after the war, the first issues were trickling in, I eagerly went after the *Annual Reviews of Biochemistry* and devoured what was being reported. As there had been a gap of several years compared to the volumes we had in the library, I quickly realized that now a new generation was at work around the world. Old familiar names that had been frequently cited, such as Warburg, Theorell, von Euler, Meyerhof, Krebs, Barker, and Elvehjem, were now thinned out by new and to me at least so far unknown ones, among which the easiest to remember for me were those of Umbreit, Snell, and Gunsalus. I also noticed that while previously nutrition or biochemistry of humans and laboratory animals had been the principal objects of study, now the most exciting work originated from studies on microbes; it seemed that microbiologists had taken the role of telling biochemists what to look for and what to go after. It was the time when all sorts of factors that were necessary for bacterial growth were described. I remember the chase after the "citrovorum," the *Lactobacillus casei*, the *Lactobacillus bulgaricus*, or the pyruvate oxidation factor. In the identification of the pyruvate factor, Gunny played a major role after his previous successes with pyridoxal phosphate had become common knowledge. The pyruvate oxidation factor turned out to be lipoic acid. This is how I first ran into Gunny. Then, at the annual FASEB-ASBC meetings in Atlantic City, I first heard and saw him in action. I also noticed that, when it mattered, he was standing his ground.

After I had moved somewhat up the academic ladder at the University of Wisconsin, I was invited to serve a term on the Biochemistry Study Section of he NIH, then under the rule of the legendary Ed Larsson, who was succeeded soon after that by Sanford Birnbaum. There I entered an illustrious group of biochemists, three of whom later even received the Nobel Prize, namely, Fritz Lipmann, Ed Fischer, and Bob Holley; and sure enough, this guy Gunny was also there. The terms of Dan Koshland, Myron Bender, Merton Utter, and Frank Westheimer also partly overlapped with mine. At that time specialization of study sections had not gone as far as today, as is apparent from the mix of specialties represented by that group.

It was the time when Gunny, then at Urbana, laid the groundwork for his subsequent endeavors on the P450 system of *Pseudomonas putida*. It had become clear that the proximal reductant of the P450 heme was a ferredoxin type Fe-S protein, named putidaredoxin. He liked my work, in which we had replaced the iron in a ferredoxin from azotobacter with a stable isotope with nuclear spin; this allowed us to definitely show from the observed nuclear hyperfine structure (HFS) that the electron added to the Fe-S cluster on reduction was sensing two iron nuclei. He actually took me as guest to a National Academy of Sciences meeting to present this

* Corresponding author. Fax: +608-265-2904.
E-mail address: hbeinert@facstaff.wisc.edu.

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work. We were, of course, discussing whether a similar experiment could be done with the labile sulfide. There were two obstacles to this at the time, which made it questionable whether one could expect a decisive answer: first, the only sulfur isotope with nuclear spin available was ^{33}S, and this was only about 30% enriched; and second, ^{33}S has a nuclear spin of 3/2, which would lead to a 4-line HFS pattern for a single sulfur and to 7 lines for a pair of sulfurs.

I was interested in doing these experiments with putidaredoxin, because it was better characterized and more readily available than the azotobacter protein we had in Madison. Gunny said that he might be able to "kid the guys at Oak Ridge out of some ^{33}S of higher enrichment." We agreed that if ~50% enrichment were to become available, Gunny would come up to Madison with some putida protein to do the experiment with us, because we had the best equipped EPR setup for biochemical work in the area. Well, Gunny, of course, succeeded in getting the isotope and we could at least show that sulfide interacts with the electron, added on reduction, but the number of sulfides involved could not be established because the hyperfine lines were not sufficiently separated [1].

In the meantime Gunny's postdoc John Tsibris had been trying, with some success, to incorporate selenide instead of sulfide into putidaredoxin. This would have three great advantages for our planned experiment: Se has several stable isotopes with and without nuclear spin. The stable isotope ^{80}Se has no nuclear spin and could be used for comparison, and ^{77}Se has the desired spin of 1/2 and was available in ~90% enrichment. It was then with the Se-labeled proteins that we were successful in obtaining the unambiguous answer that both sulfides sense the electron added on reduction [2]. In the seleno-Fe-S proteins the cluster environment is sufficiently distorted, leading to a considerable shift of g values, so that the EPR spectrum looks quite different from that of the original Fe-S proteins; however the 1:2:1 line pattern at low field is unambiguous (Fig. 1, left). We were curious to take a look at the mammalian counterpart of putidaredoxin, namely, adrenodoxin, which Nan Orme-Johnson had prepared from pig adrenals and labeled with ^{77}Se and ^{80}Se. Here, the Se and S spectra, though shifted, were more similar (Fig. 1, right) and represented an excellent example of the use of electron–nuclear interactions in EPR to obtain information on the electronic structure of metal complexes.

Today this is, of course, trivial and common knowledge; however, in the 1960s and early 1970s this may have been assumed, postulated, or even been likely; but it had to be unambiguously demonstrated by a relevant experiment, and that is what we accomplished in our collaboration.

For the various sessions with putidaredoxin and then $P450_{cam}$ Gunny always came up from Urbana with quite an entourage of international composition. I think it was a good idea of his to let the students and/or postdocs experience themselves what one can do with methods other than the usual microbiological and biochemical ones, to get information on the products of their labor. Among the students and postdocs that were in the group, many such as Randy Tsai, Chang An Yu, John Lipscomb, and Stephen Sligar are now particularly well known.

Following the work on the Fe-S protein of the $P450_{cam}$ system of *P. putida* we concentrated on the heme component, namely, P450 itself. Optical absorption spectra had been determined and from them, together with what was known from other heme proteins,

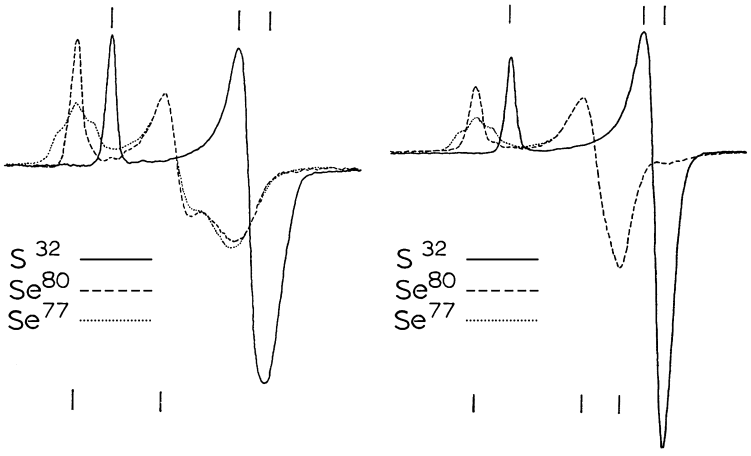

Fig. 1. (Left): Superposition of EPR spectra of putidaredoxin in natural form (^{32}S) and with the labile sulfur substituted by Se isotopes. The ordinate is the first derivative of the absorption; the abscissa, the magnetic field strength. Conditions of spectroscopy: 27 mW, temperature, 82 K at 9221.7 MHz. The upper field markers, from left to right, are at 326.2, 339.1, and 342.3 mT; the lower markers at 322.7 and 331.5 mT. (Right): Superposition of EPR spectra of adrenodoxin in natural form and with Se substitution of the labile sulfur. The conditions of EPR were 9 mW and 82 K at 9213.4 MHz. The upper field markers are at 325.9, 339.4, and 347.6 mT; the lower markers at 322.2, 333.2, and 337.0 mT.

it appeared that there was a change in spin state of P450 when substrate was bound. The bacterial system lent itself well to further investigation of this observation, as it has a defined substrate, namely, camphor, and the components of the system were available in quantity and high purity. The EPR spectra unequivocally showed that a change from low to high spin did take place on addition of substrate and we investigated the kinetics of the changes by EPR with our rapid freeze-quench technique. With the help of Bill Blumberg and Jack Peisach of New York on the theoretical and interpretative side, a complete quantitative evaluation was made. It was also found that $P450_{cam}$, in the presence of camphor shows the most rhombic high-spin state of any heme investigated by that time, with an E/D of 0.087, which represents 29% rhombicity [3].

Altogether we published five papers from all this work [1–5], which had both of our names and those of between five and seven other collaborators on them. From my laboratory, Bill and Nan Orme-Johnson and Ray Hanson were variously represented.

Actually, the University of Illinois at Urbana, compared to other universities in those days, had a particularly strong interest in physical and theoretical approaches in chemistry and biology, and Gunny certainly was one who appreciated their significance and fostered and supported this direction of work. There was also strong support for this tendency from a group working in the area of photosynthesis; the name Govindje comes to my mind as one of the representatives in this field at the time. Among the younger generation there were no lesser people than Martin Karplus and Rudolph Marcus. The well-known Swiss physicist Hans Frauenfelder had settled at Urbana and had brought Mössbauer spectroscopy to the University, which was taken over later by Peter Debrunner. In 1969 there was a meeting at an estate of the University out in the countryside, Allerton House at Monticello, dedicated to the use of Mössbauer spectroscopy of biological systems and other physical and theoretical approaches. I remember that Cyrus Levinthal gave us an introduction to what is now called the "Levinthal paradox" in protein folding. It was at the occasion of this meeting that I first met Eckard Münck, a young Mössbauer spectroscopist, who had just arrived from Germany as a postdoc in Hans Frauenfelder's group. I had no idea at the time that this newcomer to the US and also to biological problems would one day become one of the most productive and respected Mössbauer spectroscopists in the biological field. The Mössbauer spectra of reduced putidaredoxin that Debrunner and Münck recorded in 1972 clearly showed that the added electron is not shared by the iron ions but is localized at one iron which becomes ferrous. For the EPR nonspecialist it should be said here that the electron spin nevertheless senses the two nuclear spins in its neighborhood.

While much or all of the above is now common knowledge, it is fun for me to look back at those days of our collaboration, when the basis for work with the then novel techniques was established, and it was by far not as costly as today's novel techniques.

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