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Biochemical and Biophysical Research Communications 312 (2003) 103–107
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My passion for extreme conditions to solve the cytochrome P450 and NO synthase reaction mechanisms

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Received 2 October 2003

Abstract

Cytochrome P450, and especially its reaction mechanism, has always been a major research interest of Professor I.C. Gunsalus. The reaction cycle of this enzyme is complex, containing many elementary steps and intermediates, and constitutes a challenge for the scientific community. During his repeated stays in our laboratories in Paris and in Montpellier, he contributed decisively to our study of the P450 reaction mechanism under extreme conditions, i.e., at subzero temperatures and at high pressure. From this initial impulse, we continued the work with different forms of cytochrome P450 and later on with nitric oxide synthase. This paper gives an overview of the insights into these enzymes gained by the use of extreme conditions. These exciting achievements were initiated by numerous discussions with Professor Gunsalus and also reflect a long-lasting collaboration and friendship.

Keywords: Cytochrome P450; Nitric oxide synthase; Cryoenzymology; High pressure; Enzyme mechanism

I came to know Professor Gunsalus ("Gunny" to everybody) as a young postgraduate student in 1976 in Pierre Douzou's laboratory at the Institut de Biologie Physico-Chimique in Paris. Gunny visited us several times in Paris, and later on also in Montpellier. I think he appreciated very much being in France. He even spent a sabbatical year in our Paris laboratory, where he appeared to be happy at his hand-made desk with a big wine container in one corner. He was here together with Dorothy, his wife, who took singing lessons at the nearby *Schola Cantorum*. They both enjoyed life in the *Quartier Latin*. Being a newcomer in the P450 field, I was quite impressed to meet one of the most distinguished researchers of the field. Yet, to my surprise—and as I understood later, this is a characteristic of all great men—he behaved astonishingly simply, being very kind, and it has always been my pleasure to have discussions with him. Undoubtedly, his ability to fascinate people with his huge scientific knowledge, his love for unconventional approaches combined with a very sharp eye for details, but also his immense human qualities, marked me for the rest of my career.

Cytochrome P450cam

At that time, the group of Pierre Douzou, comprising Pascale Debey, Gaston Hui Bon Hoa, Franck Travers, and Claude Balny, had developed a cryoenzymologic approach to studying elementary steps of enzyme mechanisms [1]. The interest in working at subzero temperatures (in the presence of organic antifreeze co-solvents) is evident: at low temperature, enzyme reactions are slowed down, according to the Arrhenius law. This permits one—in principle—to resolve complex enzyme mechanisms into their elementary steps, and to trap intermediate states that are elusive at ambient temperature [2]. Gunny was very interested in this new approach, and—as a member of the U.S. National Academy of Sciences—communicated one of the early papers applying this method to PNAS [3]. At that time this approach was brand new. It was in fact a simple method, with the potential to answer mechanistic problems of many enzymes. Yet, to be fully applicable,

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0006-291X/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2003.10.083

many technical but also theoretical problems had to be solved. Notably, thermodynamic parameters, such as dielectric constant, viscosity and pH had to be controlled in the presence of organic antifreeze solvents and at subzero temperatures. Care had to be taken to maintain the integrity of the enzymes under these conditions, and the spectroscopic instruments had to be adapted. But in the second half of the 1970s, these difficulties were overcome. Therefore, Gunny came to Paris exactly at the right moment, to apply this new method to an enzyme system which was destined to become one of the most studied in the following decades: cytochrome P450. He was intrigued by the possibility of applying this method to study "his" enzyme: cytochrome P450cam from *Pseudomonas putida* [4].

My project was to study the mechanism of the spin transition of P450cam at low temperatures. In its ferric form, this heme-thiolate protein undergoes a transition from low spin to high spin upon substrate (camphor) binding. Furthermore, its spin state is in thermal equilibrium, and it depends on several parameters such as pH, ionic strength, etc. The spin state is also important for the function of the enzyme, as it governs the redox state of P450 by facilitating its reduction as the final component of an electron transfer chain [5]. The spin state transition is difficult to study under normal conditions, since, at equilibrium, the enzyme is nearly 100% low spin or high spin, and the transition between the two states occurs very rapidly. However, at low temperatures (−20 to −40 °C), it became possible to study this transition with ease. Equilibrium spectral data and kinetics showed a close link between the spin state and P450 protein conformation which could be modulated by the physical chemical properties of the solvent [6–8]. The results of this study were beyond expectation: since we could "dissect" the reaction mechanism into individual steps, it became possible to gain information about how local structural elements could modulate them. I remember I was quite enthusiastic about these findings, as they brought us closer to my dream of a structural explanation of the enzyme mechanism. However, things are never that easy, I learned again from Gunny. Once, when I showed him a first manuscript of these results, he told me "never forget a basic principle in science—an article is like a jewel; it has always to be crystal clear..."—well, I think, I understood what he meant. That was his delicate way to say that he disliked the paper in its initial form.

Cytochrome P450scc

Later on, in our second laboratory (in Montpellier, headed by Claude Balny), together with Christian Larroque, we applied a similar approach to other forms of cytochrome P450, such as the cholesterol side-chain-cleaving P450scc. This is an important enzyme in adrenal cortex mitochondria, where it is responsible for the biosynthesis of pregnenolone, the precursor of steroid hormones. We wanted finally to apply the potential of the subzero temperature method to the most interesting aspect of the cytochrome P450 reaction mechanism: the activation of oxygen. Indeed, after binding of substrate, reduction by a first electron, and oxygen binding, the resulting ferrous oxygen complex of P450 is reduced by a second electron. This is followed by an activation of oxygen leading ultimately to substrate hydroxylation and liberation of one molecule of water. The precise nature of intermediates involved in this reaction were, however, not known. A combination of the subzero temperature method with experiments using cholesterol peroxide as both an oxygen and an electron donor enabled us to detect a reactive intermediate species with epr characteristics of an iron IV π-cation radical [9,10]. The reaction cycle of cytochrome P450 therefore appeared to show an intermediate state similar to compound I of peroxidases. This was certainly an important breakthrough in decoding the P450 reaction

Fig. 1. Prof. I.C. Gunsalus as a chairman at the 12th International Conference on Cytochrome P450, September 2001, at La Grande Motte, France.

mechanism. Nevertheless, despite numerous further studies, P450 still retains part of its mystery: its precise reaction mechanism is not yet fully established.

It is noteworthy to recall here that Gunny followed all these mechanistic investigations with great interest. It was therefore not astonishing to find him participating in most of the cytochrome P450 conferences. Thus, in the 12th International Conference on Cytochrome P450, which I organized in 2001, he acted as chairman of a special session devoted to young P450 researchers [11,12]. The photo in Fig. 1 shows him during this conference.

Nitric oxide synthase

Gradually, my activity shifted from cytochrome P450 to nitric oxide synthase (NOS). Of course, here again we used the low-temperature method, in a way similar to what we had started with Gunny. NOS catalyzes the conversion of L-arginine to N^G-hydroxy-L-arginine (NOHLA) by the cycle shown in Fig. 2 and then the conversion of the latter compound to NO and citrulline by a second similar cycle. As it is also a heme-thiolate protein, a reaction mechanism similar to that of cytochrome P450 was expected [13]. Nevertheless, the actual mechanism must be more complex: to be active, NOS requires the presence of two additional cofactors: Ca/calmodulin and tetrahydrobiopterin (BH4). It is especially the latter whose function has long been a subject of debate. Our low-temperature approach was simple:

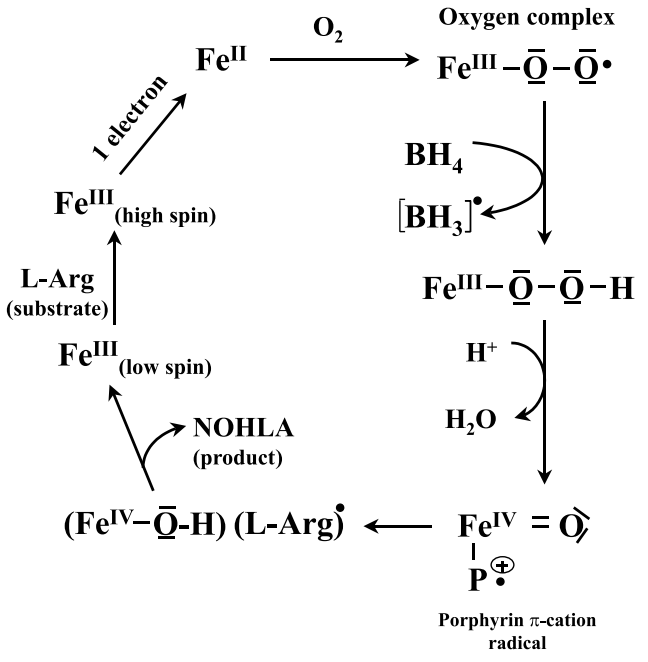

Fig. 2. The reaction mechanism of nitric-oxide synthase deduced from subzero temperature studies. The scheme shows the first reaction cycle, leading to the formation of N^G-hydroxy-L-arginine (NOHLA).

under single turnover conditions, we mixed reduced NOS at −30 °C with molecular oxygen and then recorded the spectral evolution of the system. In another experiment, the reaction was quenched immediately after oxygen addition, and the reaction products were analyzed by HPLC. The experiments were carried out with the oxygenase domain of NOS, i.e., in the absence of the reductase domain. However, the reaction mixture contained BH4. To our surprise, we observed an intermediate spectrum which had some similarities to the one observed with P450scc. Furthermore, we could demonstrate that the presence of BH4 as an electron donor was required (and was sufficient) for NOS activity. This was the first time that a redox role of BH4 had been observed [14,15]. Of course, we looked immediately afterward at the nature of oxidized BH4. Here again, the subzero temperature method was used: immediately after oxygen addition, the reaction mixture was frozen and then analyzed by epr. Our data demonstrated the transient formation of a protonated BH3 radical [16,17]. Independently, Hurshman et al. [18] came to a similar conclusion. Subsequently, we could show that BH4 was an electron donor in both the first and the second reaction cycle of NOS biosynthesis. Interestingly, the power of the subzero temperature approach to studying the NOS reaction mechanism is now also used by other laboratories [19].

High-pressure method

However, subzero temperatures were not the only extreme condition in which Gunny had an interest. During his sabbatical year in Paris, in the 1970s, and later on also in Montpellier, Gaston Hui Bon Hoa and Claude Balny started to elaborate an alternative approach: enzymology under high pressure [20]. Pressure affects both chemical equilibria and kinetics in a different way from temperature. Pressure affects specifically electrostatic and hydrophobic interactions. These weak chemical interactions often play an important role in enzyme-catalyzed reactions. In contrast to low temperature, which has always a decelerating effect, high pressure can increase or decrease reaction rates depending on the type of chemical interactions [21]. Thus, pressure may be used as an interesting tool to modulate individual steps of complex enzyme reactions, in order to gain a better structural understanding of the process [22]. For this purpose, Claude Balny had designed a stopped-flow instrument capable of functioning under various pressures up to 200 MPa. We used this facility to study further the mechanism of ligand binding of reduced NOS. As shown in Fig. 3, CO binding to reduced NOS is rapid in the absence of substrate, but slow in the presence of substrate, whatever the nature of the substrate and the cofactor. However, with increasing

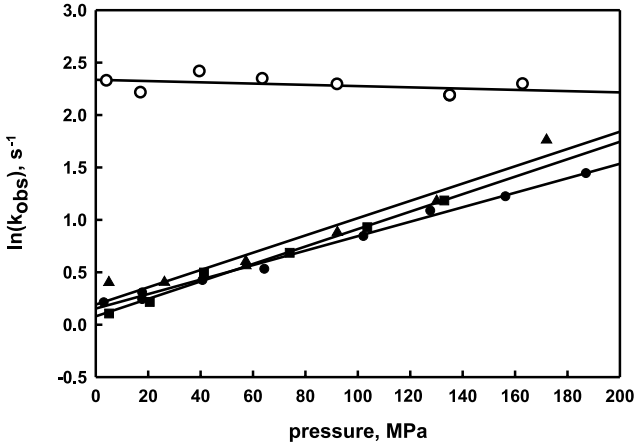

Fig. 3. CO binding to neuronal nitric-oxide synthase, measured by high-pressure stopped flow. In the absence of substrate (○), the binding is rapid and does not depend on pressure. In the presence of 200 μM L-arginine (●), 200 μM L-arginine and 25 μM BH4 (■), and 500 μM NOHLA (▲) the binding is slow, but is accelerated by high pressure.

pressure, the CO binding rate in the presence of substrate increases up to a rate comparable to that in the absence of substrate [23]. In NOS, substrate appears thus to obstruct the entrance of CO into the active site. One may argue that substrate is expelled from the active site at high pressure by liberating the entrance of CO. Currently, we are using the same high-pressure approach with CO replaced by oxygen. Furthermore, the high-pressure method is going to be used more and more to unravel the structure–function relationships in cytochrome P450 [24,25].

Conclusion

The examples shown above deliver an important message: if one wants to resolve complex enzyme mechanisms, an elegant approach is to study the enzyme under extreme conditions, such as subzero temperatures or high pressure. These conditions will disturb the normal process so that individual steps can be observed. However, it was an adventure to use these extreme conditions. Especially at the beginning, it meant stepping into a field which had not been marched on by others. Gunny and his friend Pierre Douzou, who met often in St. Guillem le Desert, a medieval village near Montpellier, have certainly been the pioneers in this adventure. Gunny supported these approaches by his continuous encouragement, and also by introducing us to the mechanistic world of cytochrome P450.

Acknowledgment

I thank my colleague Claude Balny for many helpful discussions in preparing the manuscript.

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