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Science with Gunny

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Abstract

Professor I.C. Gunsalus has not only performed outstanding research for more than 60 years but also is largely responsible for a much closer interaction between physicists and biologists. In this brief paper, I sketch the story of how this interaction came about. Published by Elsevier Inc.

The start

The story begins with the Mössbauer effect [1,2]. During a visit to Munich I was introduced by Heinz Maier-Leibnitz to one of his students, Rudolf Mössbauer, who had just made a major discovery [1]. I was impressed by this discovery—namely, the emission of very narrow gamma ray lines—and wrote a letter back to my group at the University of Illinois. Fortunately, Harry Lipkin, who was guiding my group while he was on leave of absence from the Weizmann Institute, immediately recognized the importance of this effect and started work with my group. We realized quickly that the best nuclide was not ^{191}I, used by Mössbauer, but ^{57}Fe. Thus started the Mössbauer work at the University of Illinois [3]. In 1962, the group was strengthened by the arrival of Peter Debrunner from Switzerland. For a few years, we used the Mössbauer effect to study nuclear and condensed matter problems. In condensed matter physics, the effect is very useful because it permits the observation of the internal electromagnetic field at the position of the nucleus. With Harry Drickamer, we measured, for instance, the internal field in iron as a function of pressure [4].

The research into condensed matter physics could have continued for a long time, but one day a new student, Roger Cooke, came into my office. He asked if I would mind if he used the Mössbauer effect to study a biological molecule. I did not know what biological molecules really were, but said yes anyway. And so began the interaction with biochemists. Peter Debrunner first established the contact with Professor Gunsalus, whom I knew only vaguely through our Viennese friends, who described him as a nearly mythical and fear-inducing person. I was also roped into the collaboration. After a short time it was obvious that we physicists did not know the biochemistry, and the biochemists did not really understand the physics. The situation was expressed by our invaluable radiochemist, Giovanni DePasquali (Peps): "I know little physics, but I know ten times more physics than you know chemistry." To remedy the situation, we started a weekly luncheon at the Urbana-Lincoln Hotel. Gunny's guidance in these discussions was invaluable, and we learned not to talk past each other. This observation is still valid today: A truly interdisciplinary collaboration requires extensive interactions and discussions. Otherwise, it is too easy for the physicists to work on unimportant problems with bad samples, and for the biochemist to misunderstand the capabilities of the instruments and the knowledge of the physicists. Gunny taught us to treat the biochemical side of the experiments with the same care we used on our own, the physics side. In any case, the interaction increased and resulted in a number of publications, for instance [5].

The 1969 Mössbauer conference

The University of Illinois has a beautiful estate, the Allerton Park. I organized the first Mössbauer conference

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at the Allerton House on June 5 and 6, 1960, before the Mössbauer effect became widely known [6]. Inspired by the memory of the successful first meeting, we organized a conference titled Mössbauer Spectroscopy in Biological Systems, on March 17 and 18, 1969. Gunny, a member of the small organizing committee, was crucial in attracting leading biologists. Speakers were not asked to provide manuscripts; graduate students and postdocs took notes and these notes were conserved [7]. These proceedings are actually the only place where Levinthal's original remarks are published. Here is a short excerpt: "How to fold gracefully. ... Let us ask ourselves how proteins fold to give such a unique structure. By going to a state of lowest energy? Most people would say yes and indeed, this is a very logical assumption. On the other hand, let us consider the possibility that this is not so. ..." The conference showed clearly that by 1969 the Mössbauer effect had made major contributions to biochemistry. Indeed the use of this technique has continued and is still an important tool today, for instance, in the hands of Eckard Münck, a former Illinois postdoc [8]. At the end of the conference I asked: "Everything I heard deals with the static picture of proteins. Proteins must move. What do we know about motions?" I was told that this problem was too difficult. This remark leads to the next part of the story in which Gunny plays again a crucial role.

Learning about dynamics

I started thinking how one could approach dynamics. As a nuclear physicist, radiation appeared to be a good tool. Could one start a reaction by using a light flash? Absence of knowledge can sometimes be useful. I did not know that stopped flow and flash photolysis had already been invented. We therefore found our own approach, with new help: Eli Greenbaum had joined the group as a postdoc and Bob Austin as a student. The first publishable study involved the photoreduction of $NADP^+$ [9]. There was, however, also a bigger target, cytochrome P450, a very important protein. It appeared ideally suited to dynamic studies. In contains a heme group with an iron atom in its center and different states, such as ligand-bound and ligand-free, can be distinguished spectroscopically. We had built a primitive flash photolysis system and proceeded to look at P450. It became clear quickly that P450 was so complicated that progress would be very slow or nonexistent. I therefore asked Gunny to find a protein that was simple and fully understood. Gunny thought for a week and came back with myoglobin. Myoglobin was considered a simple dioxygen storage protein. Binding was considered a one-step process, exponential in time, with no conformational changes involved, and with no Bohr effect. So we started using Mb, hoping to get back to P450 in a month or so. This was 30 years ago. We are still trying to understand Mb [10].

The myoglobin story

The Mössbauer effect had taught us that proteins should (and could) be studied at temperatures as low as a few kelvins. We therefore put carbonmonoxy myoglobin, MbCO, into our cryostat. Vince Marshall, one of Gunny's postdocs, used a flashlight and noticed that the color of the sample changed! Even the light from the flashlight had photodissociated the MbCO! Using a xenon flash tube, we started to look at the photodissociation and rebinding more carefully over the range from 40 to 300 K. Two things soon became clear. The first was that a xenon flash tube was not good enough for the experiments. Bob Austin took a week off from the data taking and built a 1-μs dye laser. The second insight was that even Mb was not simple. Both myoglobin and P450 showed a confusing richness of rebinding processes, extending over the entire temperature range [11]. The dream of understanding Mb fully in a short time was shattered. One observation was particularly challenging. Below about 170 K, rebinding unambiguously occurred from the inside of Mb, and this geminate process was nonexponential in time between about 50 and 170 K. Assuming that the binding process was controlled by a barrier of height H, the observed data over the entire temperature range could be explained by assuming that H was not uniqie, but was given by a distribution $g(H)$. Here $g(H)dH$ is the probability of finding a barrier with height between H and $H + dH$. Why does such a distribution occur? Long discussions, involving not only Gunny, but also a Russian friend, Vitalii Goldanskii, suggested a solution [12]. I quote from the paper: "A given primary structure results in a group of closely related tertiary structures that differ in activation energy. Myoglobin, then, would not just have one well-defined structure, but a number of closely similar ones,...". Thus we had here an answer to Cy Levinthal's question quoted earlier: The concept of a unique ground state for a protein is wrong.

A question remained: What were the other processes that were observed above about 200 K? This question actually has even now not been fully answered, but some of the major features became clearer after long discussions with Gunny and the other members of the group. The data showed four different processes. The simplest way to account for such a behavior is to assume a linear sequence with five states,

$$A \leftarrow B \leftrightarrow C \leftrightarrow D \leftrightarrow S,$$

where A is the state with the CO bound to the heme iron, B the state with CO close to the heme, and S the state with the CO in the solvent. With such a scheme, the data

at all temperatures could be fit. The nature of states *C* and *D*, however, was not clear and our original guesses were wrong. Only recent time-resolved X-ray diffraction experiments are yielding answers. The discussions with Gunny also led to another result. The nonexponential time dependence of the low-temperature binding can be caused in two different ways. (1) All Mb molecules can be identical, but CO binding to each is nonexponential in time. (2) Each Mb molecule is frozen into a different conformation with a different barrier height *H*. To distinguish between the two cases, we invented the multiflash technique, a primitive form of a single-molecule experiment. The answer was clear—case (2) applied and proteins are inhomogeneous. Writing a paper to describe all results took a long time. We started in December of 1973 and submitted a paper to *Biochemistry* 8 months later. In October 1974 Hans Neurath sent a reply with two very long and very critical reviews. The reviewers were incredibly careful and many (most?) of their points were well taken. We rewrote and rewrote, and the reviewers answered. The 12th version was submitted in August 1975. One reviewer, Allen P. Minton, finally got tired and asked that his name be given to us. Thus we could speed up the exchange and could settle the unclear questions by phone. The paper was accepted and finally published [13]. It is clear that without the dedicated reviewers, in particular, Allen P. Minton, the paper would have been far less clear and readable.

I have described one particular effort, the (partial) elucidation of the binding of CO to Mb, in detail because this was a prime example of the close collaboration with Gunny. It was not the only case. Peter Debrunner continued to interact strongly with Gunny and, together, they produced a number of important publications. Steve Sligar had joined the group and became a close collaborator of Gunny. Their work resulted in major advances in the biochemistry and biophysics of cytochrome P450. After Gunny left the University of Illinois, the intensity of the collaboration decreased, but not the friendship and the personal interaction. The close collaboration of Gunny with the physics community at the University of Illinois has resulted not only in major progress in getting biochemistry and physics closer together, but also in continuing human contacts. Gunny's dedication to excellence has had lasting effects.

References

[1] R.L. Mössbauer, Kernresonanzfluoreszenz von Gammastrahlung in ^{191}Ir, Z. Physik 151 (1958) 124–143.
[2] H. Frauenfelder, The Mössbauer Effect, Benjamin, New York, 1962.
[3] G. DePasquali, H. Frauenfelder, S. Margulies, R.N. Peacock, Nuclear resonance absorption and nuclear Zeeman effect in ^{57}Fe, Phys. Rev. Lett. 4 (1960) 71–73.
[4] D.N. Pipkorn, C.K. Edge, P. Debrunner, G. DePasquali, H.G. Drickamer, H. Frauenfelder, The Mössbauer effect in iron under very high pressure, Phys. Rev. 135 (1964) A1604–A1612.
[5] R. Cooke, J.C.M. Tsibris, P. Debrunner, R. Tsai, I.C. Gunsalus, H. Frauenfelder, Mössbauer studies on putidaredoxin, Proc. Natl. Acad. Sci. USA 59 (1968) 1045–1052.
[6] D.S. Lieberman, E. Lüscher, The Mössbauer effect—a conference report, Phys. Today 13 (1960) 20–23.
[7] P. Debrunner, J.C.M. Tsibris, E. Münck, Mössbauer spectroscopy in biological systems. University of Illinois Bulletin 67, Number 41, 1969.
[8] E. Cadieux, V. Vrajmasu, C. Achim, J. Powlowski, E. Münck, Biochemical, Mössbauer, and EPR studies of the Diion cluster of phenol hydroxylase from *Pseudomonas* sp. strain CF 600, Biochemistry 41 (2002) 10680–10691.
[9] E. Greenbaum, R.H. Austin, H. Frauenfelder, I.C. Gunsalus, Photoreduction of $NADP^+$ sensitized by synthetic pigment systems, Proc. Natl. Acad. Sci. USA 69 (1972) 1273–1276.
[10] P.W. Fenimore, H. Frauenfelder, B.H. McMahon, F.G. Parak, Slaving: solvent fluctuations dominate protein dynamics and functions, Proc. Natl. Acad. Sci. USA 99 (2002) 16047–16051.
[11] R.H. Austin, K. Beeson, L. Eisenstein, H. Frauenfelder, I.C. Gunsalus, V.P. Marshall, Dynamics of carbon monoxide binding by heme proteins, Science 181 (1973) 541–543.
[12] R.H. Austin, K.W. Beeson, L. Eisenstein, H. Frauenfelder, I.C. Gunsalus, Activation energy spectrum of a biomolecules: photodissociation of carbonmonoxy myoglobin at low temperatures, Phys. Rev. Lett. 32 (1974) 403–405.
[13] R.H. Austin, K.W. Beeson, L. Eisenstein, H. Frauenfelder, I.C. Gunsalus, Dynamics of ligand binding to myoglobin, Biochemistry 14 (1975) 5355–5373.