RECOLLECTIONS

The joys and vicissitudes of protein science

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When invited to look back over a previous life in protein chemistry, I was a little uncertain as to how to proceed but thought that some of my experiences as an author and the inevitable interactions with editors in some of my past research would be appropriate. I believe that scientific discoveries really fall into three categories: first, the serendipity category ("chance to the prepared mind" as Pasteur stated it), of which Roentgen's finding of X-rays may be the classic example; second, the strategic campaign, somewhat like the deliberate assault on a mountain peak, of which Fred Sangers's sequencing of insulin might be an example; and third, the solving of the puzzling anomaly, of which the development of the quantum mechanical atom to replace the Bohr atom because atomic spectral details could not be explained by the Bohr atom is an example. My initial interest in enzyme mechanism was influenced importantly by the classic papers of Hans Neurath and Robert Alberty on enzyme kinetics and focused my early work on enzyme mechanism, enzyme kinetics, and protein reagents. I thought a brief account of my experiences in protein chemistry and where they fitted into the categories listed would provide some illumination of the scientific process.

I ran into the third category, the "puzzling anomaly" category, in my work on the induced-fit theory. At the time I was a beginning investigator at the Brookhaven National Laboratory and was asked to give a speech at a scientific meeting. As I was preparing the talk about our

work on ATP hydrolysis in muscle function, it occurred to me that there was an anomaly in the fact that hexokinase was not an ATPase. If one believed in the classic template, or key-lock, theory of Emil Fischer, one could explain, for example, why glycerol was not a good substrate for hexokinase, because glycerol was so different in structure from substrate glucose that it would have a much lower binding affinity to the enzyme surface. There was, however, no mechanism based on the key-lock theory to explain why water was excluded as a substrate. Water is small, and at 55 M concentration water should be at the active site in effective concentrations. The hydroxyl group of water is just as reactive as the hydroxyl of glucose, so on the template model it should react almost as well as the substrate glucose when present at saturation. Once I began thinking along the lines that the key-lock theories of Emil Fischer needed change, there seemed to be many other phenomena that really could not be explained by a key-lock enzyme. For example, "noncompetitive inhibition" was a kinetic term to explain the phenomenon of inhibitors that did not interfere with substrate binding but did prevent enzyme action. That was a useful phrase to describe the kinetics, but it provided no good mechanism from known protein chemistry as to how a distant site in a template-type enzyme could inactivate the enzyme without affecting substrate binding.

As most protein chemists are now aware, I postulated the induced-fit theory, which stated that the substrate induced a new active conformation of the protein. In other words, the ligand-protein interaction is more like a hand in a glove than like the fit of two pieces in a jigsaw puzzle. It required the substrate or an activator of an enzyme to produce a new shape in order to activate catalysis. That led us to many predictions, of which the central one was that enzymes like hexokinase would undergo conformational changes on binding to substrates. Another was that a molecule, which itself did not react, could help a "deficient substrate" by inducing the proper protein shape for activity, e.g., by adding xylose to hexokinase to make it an ATPase, or ethylamine to make the glycine ethylester a substrate for trypsin. Both were found to be true. Many

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other phenomena could be explained by the idea of a flexible enzyme. We were able to explain anomalies in the specificity of beta amylase and phosphoglucosmutase, for example. The acceptance of this new idea was not simple. The established scientific journals and many scientists at that time thought the induced-fit theory was crazy. One journal rejection letter said in effect, "The Fischer-template theory is now well-established, and a suggestion that it is not adequate to explain enzyme action is ridiculous at this stage of knowledge."

The arguments from specificity and kinetic experiments, which we developed with a number of enzymes such as beta amylase, 5' nucleotidase, hexokinase, etc., were convincing to us, but we knew we had to do direct protein experiments to convince the doubters. The difference between the "flexible" enzymes of induced-fit and the "rigid" enzyme of the template hypothesis is that the average positions of the protein atoms change on binding substrate in induced-fit, whereas in a "template" enzyme they don't. In both cases there are rapid, reversible, small fluctuations and vibrations of the protein atoms around average localized positions. Since X-ray and NMR had not been developed at that time, we needed a direct protein test that would give results for as expected an induced-fit enzyme but that would not be expected for a template-type. We chose the reactivity of amino acids to a protein reagent before and after binding a substrate as a test. "Template" or "rigid" enzymes would have one or two amino acids "buried," or less accessible to a protein reagent, after substrate binding but would not expose any previously buried amino acids (the classical "protection" experiment), whereas "induced-fit" or flexible enzymes might cause exposure of new amino acids by the induced conformational change. Of course, we couldn't predict which amino acids in any protein would be useful for the test, because X-ray structures were not available, so we used a variety of protein reagents to see if any amino acids became more exposed as a consequence of binding substrate (induced-fit prediction) instead of more buried (template-type prediction). We chose the enzyme phosphoglucosmutase because it, like hexokinase, showed no hydrolytic activity, whereas template-type theory would predict it should. The experiment worked: an SH group becomes more reactive to iodoacetamide after binding substrate (induced-fit prediction) instead of more buried (template-type prediction). We chose the enzyme phosphoglucosmutase because it, like hexokinase, showed no hydrolytic activity, whereas template-type theory would predict it should. The experiment worked: an SH group becomes more reactive to iodoacetamide after binding substrate than before such binding. The figure we used to explain the experiment is shown in Figure 1.

Still, resistance to the idea of induced-fit continued. I remember leaving one meeting in Gatlinburg, where I had received a particularly stony reaction to the ideas of a flexible enzyme. As we left the hall, Ronald Breslow turned to me and said, "Well, Dan, at least if you ever prove it to be correct, you can say, 'I remember when nobody believed me,'" which was not very encouraging at the time. I was depressed then to read a philosophical article by Polanyi, who said you never win a scientific argument by logic but only by outliving your opponents. I published

Fig. 1. Schematic illustration of flexibility in the action of phosphoglucosmutase. The upper part of the figure represents the enzyme molecule in the absence of substrate. The lower part of the figure represents the change in conformation leading to exposure of -SH burying of X, Y, Z, and W. (Reprint of Figure 10, Yankeelov, J.A., Jr. & Koshland, D.E., Jr., 1965, *J. Biol. Chem.* 240, 1593-1602. Reproduced with permission of The American Society for Biochemistry & Molecular Biology.)

the first papers on the subject in the *Proceedings of the National Academy of Sciences*, sponsored by Donald Van Slyke, because other journals thought it too crazy to publish. My pictures of the flexible enzyme in subsequent publications and in the *Journal of Biological Chemistry* and *Science* showed larger than necessary movements in order to clarify the active from the inactive conformation (Fig. 2), but as an organic chemist I thought an Å or so would be big enough. I remember Melvin Calvin saying to me after one speech, "Dan, you've got a good idea, but its going to be hell proving it, because small changes, which are difficult to demonstrate, would be enough to give the effect." Therefore, I greeted with enthusiasm the arrival of crystallography of proteins and the small changes that were shown by David Phillips in lysozyme

Fig. 2. Illustration of energetic relationships in a monomer undergoing conformational changes induced by substrate. **A:** Extreme pathways are K_sK_i (initial protein conformation change followed by binding of substrate to the new conformation) and $K_i'K_s'$ (initial binding of substrates followed by protein conformation change). $K_s''K_i''$ is an intermediate type pathway involving simultaneous conformation change and binding followed (K_i'') by further isomerization to final conformation. **B:** Illustrative example of changes shown in Figure 1A in which H, +, and - represent hydrophobic and electrostatic attractions between protein chain, substrate molecule, and solution ions. It should be noted that the new bond formation involves only a single hydrophobic bond. Formation of products will be followed by dissociation of product and refolding of protein to initial conformation by an unfolding process similar to the folding. (Reprint of Figure 4, Koshland, D.E., Jr. & Neet, K.E., 1968, *Annu. Rev. Biochem.* 37, 359-410. Reproduced with permission of Annual Reviews, Inc.)

and Fred Richards in ribonuclease, but many considered these changes too small to be significant.

Because the induced-fit concept explained a wide variety of phenomena, we became more assured that it was actually true and started to think of what would be the consequences of a ligand bound to one subunit in a multi-subunit protein. We came to the sequential model, which was developed together with Drs. George Nemethy and David Filmer. We had considerable difficulty publishing, but it finally appeared in *Biochemistry* in 1966. In the interim, Monod and his collaborators, Wyman and Chan-

geux, published their theory of cooperativity (*J. Mol. Biol.*, 1965), and it was intriguing to compare the differences of the two points of view. We considered the problem from the point of view of the binding of ligand inducing a conformational change in one subunit and how this could change the interactions with the neighboring subunit. Those assumptions provided the framework for the "sequential" (KNF) model. The Monod, Wyman, Changeux ("concerted" or MWC) model proceeded from an entirely different framework, assuming that a multi-subunit protein had to preserve symmetry when conformational "changes" occurred, and therefore the changes in individual subunits occurred in a concerted manner.

An advantage of the MWC theory was that it was far simpler mathematically, since there were only two total states of the protein and it predicted only positive cooperativity, which was all that was known at the time. Hence, it became the theory that most people initially believed and supported, and of course Monod was already a very famous scientist and I was much less well known.

A major distinction between the two was that the KNF theory predicted negative cooperativity, whereas such an effect could not exist in the MWC theory. So we set out to look for negative cooperativity. After some false starts, Abby Conway found negative cooperativity in glyceraldehyde-3-phosphate dehydrogenase. A little later Alex Levitzki, Cedric Long, and I showed "half-of-the-sites reactivity," which we concluded would be an extreme case of negative cooperativity (two initially identical sites were altered when the first ligand binds to cause a change at the second site so the second ligand didn't bind at all). These experiments fit into "the category of a strategic campaign" in which we did many experiments on cooperativity and protein structure to explore and substantiate our original "flexible" enzyme ideas both at the active site and in the distant allosteric site. These predictions of specificity and cooperativity added credibility and led many to consider the induced-fit theory more hospitably.

The elegant crystallographic experiments of Steitz and Lipscomb, which showed a very large conformational change in carboxypeptidase, then started a major movement for acceptance of the induced-fit and sequential model theories and also considerable confusion in all the terminology. By then the term "allostery" was used as an indication of a sigmoid curve, a site outside the active site and by some even as a synonym for conformational change. So some suggested that the whole excitement of that era was evidence for "allosteria" on the part of the scientists involved. Also, many biochemists who knew little organic chemistry came to me saying "conformational change" is so difficult to prove that it wasn't really a theory. What they had confused was that it was, at that time, difficult to devise experiments to show conformational changes in proteins, and they forgot that new techniques arise when there is a need. Anyone who reads the history of science knows that new theories generate the need

for new technologies, and new techniques generate new theories.

One of my most fond reminiscences was my first speech after our finding evidence for negative cooperativity, which I gave at the University of Wisconsin. During that speech I showed slides indicating how positive and negative and no cooperativity would be indicated by Scatchard, Hill, or double-reciprocal plots. When I finished, Phil Cohen was extraordinarily excited and grabbed me almost as I descended from the podium and whisked me to his office. There he introduced me to a graduate student who had apparently performed experiments that gave her a concave downward double-reciprocal plot, and she and Phil wouldn't publish it because she believed the results were impossible. Phil was delighted that she had an explanation, and she was delighted that she could finish her degree promptly. Subsequently many negatively cooperative enzymes were published, and they now outnumber positively cooperative enzymes.

One of the early believers was Carl Cori, who gave me a friendly introduction at a Federation Symposium lecture. That buoyed my spirits, because I had just overheard a conversation in the corridor in which one scientist said to another that the induced-fit theory "showed Koshland had become senile."

Today, it is known that essentially every protein undergoes large or small conformational changes on binding ligand. Large changes are certainly important, but I would still maintain our original hypothesis that the definition of a significant change should be based on function, i.e., enough ligand-induced change to make a kinetically effective catalyst. Fortunately, however, the first crystallographic evidence produced in the paper of Steitz and Lipscomb was a very large change in the amino acid side chains of carboxypeptidase, and the climate for accepting conformational changes in enzymes became much more favorable after that important paper. In recent studies with isocitrate dehydrogenase, we have found small but significant conformational changes that affect the reactivity of the enzyme, and we believe these will be found increasingly in other enzymes, just as negative cooperativity is found increasingly in enzymes and other proteins.

The difficulty of publishing revolutionary ideas didn't apply only to my own work. I recall that I received the original paper of Schoellman and Shaw on affinity labeling of chymotrypsin with TPCK as a reviewer for *Biochemical and Biophysical Research Communications*. *BBRC* was a good journal, but certainly not the first rank that I would have expected for such an important paper. I found that it had been rejected by some major journals of the time.

The history of finding more conventional papers easily accepted by journals and more revolutionary ideas harder to publish has led me as an editor to be friendly to the less conventional concept. There is rarely "a con-

spiracy" against a new idea by "the establishment" as some think. But scientists learn lots of facts and construct theories that become well established over time. It is easy, therefore, for peer reviewers to decide something startlingly new is undoubtedly wrong. So I have a rule of thumb that heavy criticism of a submitted paper frequently means you have hit on a truly new phenomenon, while immediate acceptance probably indicates it's competent but not that novel. Neither is quite fair, and most editors, like myself, find that most criticism is accurate, but editors must be ever aware that the true frontier is rarely pretty, or polished, or easily comprehended. Peer review must detect the sloppy, the incredible, and the overstated, and we must always keep our eye out for a new truth that is deemed at first unworthy but is really the new horizon.

It is a great deal of pleasure to look back on an idea that was viewed with skepticism originally and see it now widely proved and accepted in textbooks. I never felt personal hostility, and, in fact, had many extensive and delightful arguments with Jacques Monod, who once presented me with evidence from an art book for his advocacy of "symmetry as a universal standard of esthetics." I argued that "breaking symmetry is a universal standard of behavior."

The development of the cooperativity models from the initial induced-fit idea both theoretically and experimentally was an example of the second category of scientific discovery, the deliberate campaign. In the "deliberate campaign" the scientist is allowed to have a point of view in establishing a hypothesis, but he or she must always be alert to his prejudices. One of the best approaches in that category is to try to divine the critical experiment for your own theory. Each time you fail to prove it false, you are inevitably delighted, and the campaign moves one step further to fruition. If the theory is wrong, it has to be abandoned, but the sooner you find out, the better.

The final category, serendipity, occurred in our work on "the tryptophan reagent," 4-nitro, 2-hydroxybenzylchloride. I had developed the concept of "reporter groups" and wished to design a new alkylating agent that was colored and could be attached to proteins. Dr. Karkhanis, a postdoctoral fellow, and I applied it to chymotrypsin and got a seemingly well stuck yellow color that could not be removed by dialysis. It also showed the reporter group characteristics I wanted (a change in color on binding substrate to the enzyme). Unfortunately, the amino acid analysis showed that no amino acid in the enzyme had been modified. We expected a histidine or a methionine to be alkylated, but none was modified. We realized no journal would accept our paper unless we could show protein modification. Because no amino acid modification was detected, we had to admit extraordinarily tight noncovalent binding was a possible explanation. And then suddenly the answer became clear. The one amino acid that the amino acid analyzer machine of Stein and Moore

didn't measure was tryptophan. So tryptophan was a possibility that wouldn't show up in an amino acid analysis. That turned out to be true, and we had by mistake developed a "tryptophan reagent" at a time when specific protein reagents were very much needed. We and others used the reagent for this purpose; however, I remember well that it was not the result of a logical, ordered analysis, but of pure and very confusing luck.

Scientists are puzzle-solvers. When all else is said and done (and as the saying goes, more is usually said than done), we are rewarded for long hours of work at low pay for that dazzling, ecstatic moment when a puzzle is solved. The strategic campaign is usually all fun, despite the hard work, because one is on the trail of a beloved hypothesis. The solution of the important anomaly or the luck of serendipity are usually preceded by anguish, frustration, and heartache. But that moment of joy, when the

solution appears like a candle in the darkness, is worth all the travail. And reminiscing, even in the short intervals between experiments and grant writing—a luxury for which I am deeply appreciative to *Protein Science*—allows a scientist to go back over history, forgetting the anguish and happily recalling the joy that can come from scientific research.

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