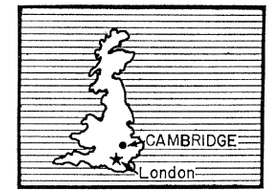
Laboratory of Molecular Biology, Cambridge, II



Cambridge, England. The relationship between the structure and function of enzymes looms large in both physical and chemical studies at the Laboratory of Molecular Biology here.

While pressing forward his x-ray diffraction studies of the three-dimensional structure of the blood-protein hemoglobin, laboratory chairman Max Perutz is also studying the structural change which occurs when hemoglobin gives up oxygen. Under the leadership of deputy chairman John C. Kendrew, also an x-ray crystallographer, determination of both an amino acid sequence and a three-dimensional structure for myoglobin, a protein resembling one of the four subunits of hemoglobin, are nearing completion. Frederick Sanger and his protein chemist colleagues are working on the complete amino acid sequences of large proteins and mapping the active regions of others.

A significant recent achievement, by B. S. Hartley of Sanger's group, was determination of the complete amino acid sequence of the digestive enzyme chymotrypsin. This enzyme has a sequence of roughly 240 amino acid residues, grouped into three chains. This sequence is almost five times as long as that of the 51-amino-acid protein insulin, worked out over a decade ago by Sanger, and about twice that of the 124-amino-acid enzyme ribonuclease, established in 1960 by C. H. Werner Hirs, Daniel H. Spackman, Derek G. Smyth, William H. Stein, and Stanford Moore, of the Rockefeller Institute in New York.

Hartley announced the sequence of chymotrypsinogen A, derived from beef pancreas, at the first meeting of the Federation of European Biochemical Societies, held in London from 23 to 25 March. At the same meeting, an almost complete sequence for the same enzyme independently determined, was presented by B. Keil and F. Sorm of the Institute of Organic Chemistry and Biochemistry of the Czechoslovak Academy of Sciences in Prague.

Both Keil and Hartley addressed an all-day symposium on enzyme structure and function that was the core of the conference, and in which scientists from the Laboratory of Molecular Biology figured prominently. The symposium was concerned with the structure and activity of chymotrypsin, ribonuclease, and hemoglobin, and with the specific labeling of active sites in such enzymes as the esterases, carbonic anhydrase, and dehydrogenases with SH groups implicated in their activity.

Hartley has worked for 6 years (two of them in the laboratory of Hans Neurath of the University of Washington) on bovine chymotrypsinogen A, which is converted into the active enzyme by the removal of four amino acid residues. He has used an intricate combination of chemical methods. Enzymes such as chymotrypsin itself, trypsin, pepsin, subtilisin, elastase, and papain have digested the material by cleaving it at different points into fragments of differing length. The "overlapping" of the fragments allows greater certainty about the sequence. Despite all the digestions, however, a "core" within the C-chain of the molecule still has not been separated cleanly.

Carboxypeptidase A was used to determine the amino acid at the carboxyl end of peptide fragments. To find the amino acid at the amino terminal, several chemicals were tried: fluorodinitrobenzene (FDNB), introduced by Sanger in 1945; phenyl isothiocyanate, reported by P. Edman in 1950; and *l*-dimethyl-amino-naphthalene-5-sulfonyl chloride. This last chemical, which Hartley and his co-worker W. R. Gray call "dansyl" chloride, is bound to peptides, single amino acids, or whole proteins before they go through various separation processes. While the first two chemicals distinguish between chemical groups bound to them by their color, "dansyl" chloride does so by its fluorescence. Under a strong ultraviolet lamp, as small a quantity of the "conjugates" of protein material and the identifying agent as 10^{-5} micromole will exhibit a yellow fluorescence. This quantity of marker chemical is at least 100 times smaller than the amount required for easy identification of conjugates with FDNB, Hartley and Gray have reported [*Biochem. J.* **89**, 60P (1963); *ibid.*, p. 379].

Acidic material was separated from basic material in automatic ion-exchange chromatographic columns similar to those used in the Rockefeller Institute work on ribonuclease. A high-voltage electrophoresis method separates the material further at various *p*H's. It was developed in 1951 by H. Michl of Vienna and was used in the last stages of Sanger's work.

To locate the five disulfide bridges within the molecule, Hartley and J. R. Brown, a NATO and U.S. Public Health Service postdoctoral fellow at the laboratory, developed a "diagonal" technique to isolate peptides containing the cysteic acids which contribute the sulfur to the bridges. With this technique they were able to examine a peptide containing the molecule's active serine—195 in the proposed sequence —and they found near it a methionine residue which is apparently alkylated by 1,2-epoxy-phenoxypropane. Thus the methionine-191 seems implicated in the enzyme's activity, because 1,2-epoxy-phenoxypropane halts 95 percent of chymotrypsin's activity.

Activity and Structure of Enzymes

Brown and Hartley reported this work at the London meeting. Also reported was work implicating histidine-57 in the activity of chymotrypsin, which was done by Hartley and L. B. Smillie of the University of Alberta, another visiting researcher. In the work on this histidine—brought close to the active site by a disulfide bridge

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—they used a chloromethylketone derivative of N-tosyl-*l*-phenylalanine called TPCK, very similar in structure to the epoxide used in the methionine study. Despite the similarity of the reagents, they bound with very different parts of the molecule. "The exploitation of the differences and similarities of these two reagents offers an approach to the elucidation of the configuration of the active center," Smillie and Hartley said in their report. They also found similarities in amino acid sequences around the histidines joined by a disulfide bridge in chymotrypsin, chymotrypsin B, and trypsin.

This kind of similarity between related large enzymes has also shown up in the work of J. Ieuan Harris, also of Sanger's group, on such SH enzymes as glyceraldehyde 3-phosphate dehydrogenase. Harris worked first in collaboration with Jane H. Park of Vanderbilt University's department of physiology and later with Richard N. Perham [*Nature* **197**, 154 (1963); *J. Mol. Biol.* **7**, 316 (1963)]. Labeling of active sites in glyceraldehyde 3-phosphate and yeast alcohol dehydrogenases indicates that each is made up of four similar subunits with a molecular weight around 35,000. Harris's work is part of a group of studies showing that dehydrogenases have similar sequences around their active regions, even though the enzymes may be found in cells as different as those of yeast and rabbit muscle. Such work on mapping of active sites provides a way of extending knowledge of enzymes which are so large that they are not suitable for crystal studies or complete amino acid determinations. Some of the enzymes Harris has been studying are five times as large as chymotrypsin.

In the work on the activity and structure of enzymes there has been much debate about what kind of structural changes take place when the enzyme forms a "complex" with the substrate [D. E. Koshland, Jr., *Science* **142**, 1533 (1963); C. Niemann, *ibid.* **143**, 1287 (1964)]. For the past 2 years Perutz has been actively interested in structural differences between reduced and oxygenated forms of hemoglobin. At the London meeting he was able to report that it had been possible to crystallize a reduced form of horse hemoglobin for direct comparison with horse oxyhemoglobin. The comparison showed that, in the reduced form, two of the molecule's four subunits, the beta chains, have moved apart, the distance between their two heme groups being thus increased by 7 angstroms as compared with that in the oxygenated form.

In his Nobel prize lecture in December 1962, Perutz had explained his interest in this work as follows.

"Hemoglobin acts as a carrier of oxygen from the lungs to the tissues and of carbon dioxide back to the lungs. The four iron atoms are in the ferrous state, and each is capable of combining reversibly with one molecule of oxygen without itself becoming oxidized in the process. The four iron atoms interact in a physiologically advantageous way, so that the combination of any one of them with oxygen increases the rate of combination of its partners. A similar reaction takes place when the oxygen is given up. Carbon dioxide is not carried by the iron atoms directly, but its uptake by the red cells or the serum is facilitated by the disappearance of an acid group from each quarter molecule of hemoglobin when it has given up its oxygen. Conversely the presence of acid in the tissues speeds up the liberation of the oxygen by hemoglobin.

"The hemoglobin molecule may, therefore, be regarded as an enzyme with two functions and several active sites, which interact in a complex and sophisticated manner. The explanation of this behavior is one of the main aims of our research."

Perutz seeks to conduct studies related to the function of the hemoglobin molecule, while he and his colleagues push the resolution of the three-dimensional model of hemoglobin, based on x-ray diffraction patterns, beyond the presently achieved 5.5 angstroms.

In earlier work, Perutz did not have crystals of reduced horse hemoglobin and so he was forced to use reduced human hemoglobin for comparison. He found a similar increase in distance between the heme groups of about 7 angstroms in the reduced human form, but this may have been a species difference.

Nonetheless, Perutz and Hilary Muirhead commented [*Nature* **199**, 633 (1963)] that "the structural change which accompanies the reaction of hemoglobin with oxygen suggests that there may be other enzymes which undergo a major change of structure on combination with their substrate." This idea gained interest, they said, from a proposal by Jacques Monod, François Jacob, and Jean-Pierre Changeux, of the Institut Pasteur in Paris, that there is a class of reactions, called "allosteric," in which the final product of a biosynthetic pathway slows its own manufacture by acting on a special inhibition site on an enzyme, usually the first in the pathway. This idea [*J. Mol. Biol.* **6**, 306 (1963)] calls for two special active regions in an enzyme, one for binding with the substrate and the other for binding with the inhibitor. Perutz and Miss Muirhead said, "the interaction between two sites resembles that between the oxygen-combining sites in hemoglobin."

Conclusion of Myoglobin Studies

Under Kendrew's leadership the studies of myoglobin are reaching a conclusion after two decades of effort. The resolution of the three-dimensional model of the myoglobin molecule is being carried to 1.4 angstroms. Instead of the 10,000 reflections and electron density calculations for 100,000 points in the molecule that were carried out for the 2-angstrom model of myoglobin achieved in 1960, the new work involves 25,000 reflections and 500,000 electron density values. At a resolution of 1.4 angstroms most of the 1260 nonhydrogen atoms of myoglobin can be distinguished from each other. The positions of nearly 1200 have been determined.

Working with H. C. Watson of the laboratory and L. Stryer of the Stanford University Medical School, Kendrew has been able to observe in detail how the few light atoms of an azide ion attach themselves to a modification of myoglobin called metmyoglobin, in which a water molecule occupies the position near the iron atom normally occupied by oxygen. This observation of azide ion attachment is made easily because the phases of x-rays used to describe the normal protein are so well known that new determinations of phases—a laborious process—can be avoided. The azide derivatives have been found to be isomorphous with native metmyoglobin. The "difference Fourier" method clearly points out new peaks of electron density associated with the new atoms.

This success [*J. Mol. Biol.* **8**, 96 (1964)] leads directly toward detailed studies of the action of myoglobin when the structure is finished at the atomic level. "It is anticipated," Kendrew says, "that the method we have

employed will be feasible for studying the interactions of substrates, co-enzymes and inhibitors with enzymes, once the structure of the native protein has been determined to atomic resolution."

Meanwhile, Kendrew's group is not neglecting the contribution protein chemistry techniques can make to elucidating the structure of myoglobin. Allen Edmundson, formerly a graduate student under Hirs at the Rockefeller Institute, is working on the last stages of an amino acid sequence for the molecule, which is a single chain of 153 residues. As Kendrew reported in his Nobel prize lecture in Stockholm in 1962, Edmundson's characterizations of tryptic and chymotryptic peptides have agreed almost entirely with characterizations of amino acid side chains which it was possible to make even at 2-angstrom resolution in the x-ray studies.

Studies of Muscle Filaments

Enzyme activity is also an aspect of Hugh Huxley's electron microscope study of muscle filaments. Over more than a decade Huxley has shown that muscles contract by the action of two types of fibers sliding past each other, one type being made of the protein actin and the other of the protein myosin. Recently Huxley has sought to show in detail how the two types of protein might act on each other to cause movement. He had to adopt some technique other than that of looking at thin sections of muscle, which he had used to show the difference between the two types of filament in rabbit psoas muscle. An obvious choice was the negative staining technique, first discovered by Huxley and then developed independently by Sidney Brenner and R. W. Horne. In this technique a sample is first put into a solution of some dense salt, then dried. The technique worked well for such material as viruses, only a few hundred angstroms in diameter. But muscle fibrils, which contain large numbers of filaments, are usually at least 1 micron in diameter.

Normal treatment in a centrifuge failed to break down the fibrils, presumably because of the strong cross-links between the actin and myosin filaments that Huxley had seen in his thin-section micrographs. To allow these cross-links to be broken and the filaments to be extracted from their "very robust lattice," Huxley had to find a suitable "relaxing medium," one which would inhibit the normal activity of enzymes which break down adenosine triphosphate (ATP).

The filaments were stained in uranyl acetate, which gives better contrast and less destruction of filaments than sodium phosphotungstate. Treated in this way, the separated filaments strongly resembled the structures found in the older thin-section micrographs. Furthermore, some of the dissociated filaments were allowed to recombine, and those which did recombine showed a polarization similar to that seen in the earlier micrographs.

Huxley's studies indicate that the actin filaments are about 65 angstroms in diameter and the myosin filaments 110 angstroms. Each actin filament interacts with three myosin filaments. Projections about 40 angstroms in diameter reach out from the myosin filaments to touch the actin, separated from the myosin by about 170 angstroms. The projections probably contain the enzymic site of the myosin [*J. Mol. Biol.* **7**, 281 (1963)].

Despite progress of this sort and prospects of more, students of enzyme structure and function, both at the Laboratory of Molecular Biology and at the London meeting of biochemists, express some discouragement. Both protein chemists and crystallographers cite the imperfections and limits of their methods for elucidating structure and activity.

Alpha Chymotrypsin

David M. Blow of the laboratory, reporting at the London meeting, a study of alpha chymotrypsin at a resolution of 5.8 angstroms, called for even finer resolution and reported that chymotrypsin has few or no alpha-helix or rodlike structures—structures which have rendered studies of myoglobin easier. Similar problems were reported by J. Kraut of the University of California, La Jolla, who is studying the zymogen precursor of chymotrypsin at a higher resolution, 4 angstroms.

The London sessions and conversations in Cambridge emphasize how physical and chemical methods reinforce each other in these studies of structure and activity. The physical researchers contribute three-dimensional maps of electron density computed from the x-ray diffraction patterns. The protein chemists contribute amino acid sequences either for whole enzymes or for their active sites; data on the speed of reactions; and studies of specific inhibitor chemicals which illustrate the role of specific amino acid residues in the enzyme's normal action on substrates.

Behind two decades of work lie decades of technical development, including development of the method of attaching heavy atoms to protein crystals to permit determination of the phase of x-rays. Much of this work was done at the Laboratory of Molecular Biology by Perutz, Kendrew, Howard M. Dintzis, and Gerhard Bodo. Other technical developments include methods of purifying enzymes, countless uses of radioactive "labeled" reagents, and the discovery of chemicals which permit protein chemists to use tiny amounts of hard-to-purify enzymes in analytic work. Sanger, Hartley, and Gray have figured prominently in developing these "micromethods."

Amino Acid Sequence

Although the sequences of amino acids are one-dimensional, researchers like Sanger emphasize that there is an important link between these sequences and the three-dimensional arrangements of proteins. In Stockholm, Kendrew expressed the idea this way: "The geneticists now believe—though the point is not yet rigorously proved—that the hereditary material determines only the amino acid sequence of a protein, not its three-dimensional structure. That is to say, the polypeptide chain, once synthesized, should be capable of folding itself up without being provided with additional information; this capacity has, in fact, recently been demonstrated by Anfinsen *in vitro* for one protein, namely ribonuclease. If the postulate is true it follows that . . . in the very long run, it should only be necessary to determine the amino acid sequence of a protein and its three-dimensional structure could then be predicted; in my view this day will not come soon, but when it does come the x-ray crystallographers can go out of business, perhaps with a certain sense of relief, and it will also be possible to discuss the structures of many important proteins which cannot be crystallized and therefore lie outside the crystallographer's purview."

—VICTOR K. MCELHENY