The 1972 Nobel Prize for Chemistry

The 1972 Nobel Prize for Chemistry has been awarded jointly to Christian B. Anfinsen at the National Institute of Arthritis and Metabolic Disease for "his work on ribonuclease, especially concerning the connection between the amino acid sequence and the biologically active conformation" and to Stanford Moore and William H. Stein at the Rockefeller University for their contribution to the understanding of the chemical structure and catalytic activity of the active center of the ribonuclease molecule. For more than two decades, the major part of the effort of the two laboratories has been devoted to studies of this single product of the bovine pancreas, the enzyme ribonuclease. Although this enzyme is of interest because of its role in the hydrolysis of ribonucleic acid, its importance undoubtedly lies both in the general laboratory procedures and in the theories of protein structure and function which its study has engendered. The work has spanned and in a major way contributed to the most exciting era in biochemistry so far.

The present story really begins with the isolation and crystallization of ribonuclease by M. Kunitz in 1941 at the Rockefeller Institute. Ribonuclease turned out to be a very small enzyme with a molecular weight in the neighborhood of 14,000. It was also extremely stable in that it appeared to withstand exposure to strongly acid solutions and to temperatures of 100°C. The seeds of some of Anfinsen's later conclusions were actually contained in these early observations, but their significance was not recognized at that time. The enzyme was subsequently prepared in very pure form in large quantities (more than a kilogram), in the late 1940's by Armour, Inc., of Chicago, one of the results of their work during World War II, when they were engaged on the fractionation of the components of blood plasma, a project that had been instituted and supervised at the laboratory of E. J. Cohn at the Harvard Medical School. The enzyme was offered at very low cost by Armour to protein chemists throughout the world and led to very rapid advances in the field as a whole and to the study of ribonuclease in particular. It was during this period that the three Nobel laureates began the work for which they have just been honored.

William H. Stein received his B.S. degree from Harvard in 1933 and his Ph.D. from Columbia in 1938. During this same period, Stanford Moore received his B.A. degree from Vanderbilt University and his Ph.D from the University of Wisconsin. Stein and Moore both arrived in 1939 at Rockefeller Institute. By the end of World War II, they were working closely together and since that time they have worked at Rockefeller and published together to the point where their names have become inseparable in the minds of most biochemists. Anfinsen received his undergraduate degree at Swarthmore College in 1937, and his Ph.D. from Harvard in 1943. He stayed on at Harvard for about 7 years, the last 3 years as an assistant professor, and, in 1950, he moved to the National Heart Institute in Bethesda.

In the mid-1940's, Fred Sanger reported his determination of the sequence of the two chains of insulin. This brilliant work (for which Sanger received the Nobel award in 1958) finally confirmed the peptide hypothesis of protein structure, and at one stroke changed the biochemical fraternity's entire attitude toward the kinds of chemical experiments that were possible in macromolecular systems. The work also served as a beautiful example of the extensive application of the paper chromatographic techniques set in motion only a few years earlier by Martin and Synge (received the Nobel award in 1952). This mid-1940's period also saw the start of a division among biochemists which persists to some extent to this day: the paper chromatographers on one side and the column chromatographers on the other. It was at that time and in the latter context that Stein and Moore began their extensive and detailed investigations of amino acids, peptides, and proteins.

Trained as chemists, Stein and Moore, by preference, wanted to actually see significant amounts of material at the end of a separation procedure: amounts which could, in principle, be weighed and identified by any of a variety of procedures. Column chromatographic methods appeared most suitable. They undertook the complete separation and quantitative analysis of amino acid mixtures. After successful, but often frustrating experiments, with starch as a supporting medium, they moved on to ion exchange resins and developed in detail the systems now used routinely. Of necessity, they invented the fraction collector, and, when even this very useful device became onerous for multiple analyses, they developed the completely automatic amino acid analyzer in collaboration with their colleague, D. H. Spackman. Routine analyses are now done by this method all over the world. The easily attainable accuracy of better than ± 3 percent by itself made the technique an enormously important contribution to biochemistry.

With the analytical technique in hand, Stein and Moore, with their colleague C. H. W. Hirs, began work on the sequence of ribonuclease with a careful investigation of the purity of the enzyme, again employing ion exchange chromatographic procedures. After the isolation of what has come to be recognized as one of the best examples of a pure protein, the actual sequence work started. This involved the careful dissection of the molecule into peptide fragments with the separation and quantitation of these units at each stage. With a helpful hint from their friend and earlier colleague, J. S. Fruton, they developed the now standard column procedures for peptide fractionation. The result was the first determination of the sequence of an enzyme ever to be reported.

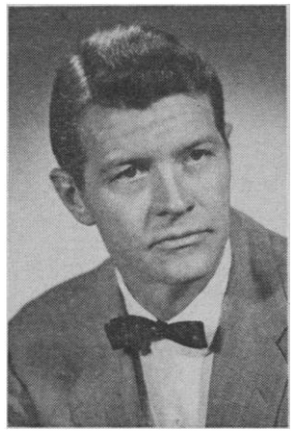
During and subsequent to the work on the sequence analysis, efforts on the chemical modification of the functional groups of this protein were proceeding. The enzyme is inactivated by iodoacetic acid. This was initially considered very strange, since it was clear that the enzyme itself contained no free sulfhydryl groups and the iodoacetic ion is normally considered a sulfhydryl reagent. E. A. Barnard and W. D. Stein (no relation) in England had indicated that the probable site of reaction of this reagent was histidine residue number 119. Detailed studies by Stein and Moore and their colleagues showed that, in fact, two histidine residues were modified, 119 and 12, and that the reaction of either of these groups induced complete loss of enzymatic activity, and that the reactions were mutually exclusive. The tentative conclusion that these two residues, although almost at opposite ends of the single peptide chain, must, in fact, be very close in three dimensions in the native enzyme, has been completely confirmed in the structures derived by others from single-crystal x-ray diffraction procedures. The later ex-

periments of Hirs, implicating lysine 41, Crestfield's elegant studies of the ribonuclease dimer, the observed effects of substrate and inhibitors all form part of the extensive chemical definition of the active site of this enzyme by Stein and Moore and their colleagues.

Anfinsen's work on ribonuclease has been equally important but of an entirely different character and style. Early on Anfinsen latched on to a large bottle of the Armour ribonuclease sample. By inclination, at least in those days, he was a paper man rather than a column man. His initial investigations centered on the size and shape of the enzyme and on establishing the single chain structure by end-group analysis, applying Sanger's technique. He determined the sequence of several residues at the amino terminal end of the chain. By that time, however, it was clear that the juggernaut at Rockefeller Institute was in full gear and that the sequence would be forthcoming in an inevitable and definitive manner. Anfinsen prepared a derivative inactivated by pepsin and showed that it lacked only a tetrapeptide at the carboxyl terminal end. This derivative has played an important role in recent studies at Rockefeller.

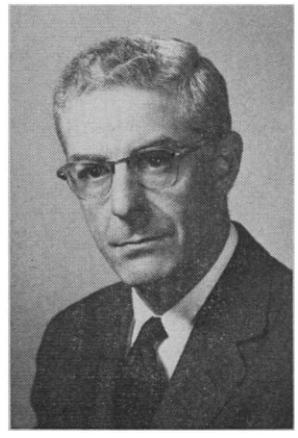
During a leave of absence, Anfinsen arrived with his precious bottle of ribonuclease at the Carlsberg Laboratory in Copenhagen, then under the leadership of K. U. Linderstrøm-Lang. This single event had a dramatic effect on the professional lives of a number of biochemists including A. Hvidt and S. O. Nielsen, who were, or shortly afterward became, associates of the Danish group; and, as visitors, William Harrington, John Schellman, J. I. Harris, and F. M. Richards. The importance of ribonuclease as a test substance in protein chemistry increased during this period which saw the beginnings of the extensive application of optical rotation to protein structural problems, the detailed investigation of denaturation phenomena, the effect of solvent composition, particularly substrate-related ligands, and the use of limited proteolysis as a probe, producing, in this case, the derivative ribonuclease-S.

On his return from Denmark, Anfinsen began to concentrate his efforts on the disulfide bonds of the enzyme. Whereas the Rockefeller group, for analytical purposes, had cleaved these covalent bridges by means of irreversible oxidation with performic acid,



Christian B. Anfinsen

Stanford Moore



William H. Stein

Anfinsen was cleaving them by reduction to the free (or protected) sulfhydryl groups. It soon became clear that reduction could not be accomplished in the native enzyme at reasonable temperatures at neutral *p*H. Conditions that led to the unfolding of the molecule were mandatory before reduction could be completed. (The unfolding process had been under investigation in Copenhagen.)

Anfinsen noted that enzymatic activity was completely lost when the four disulfide bonds were fully reduced, so that there were eight sulfhydryl groups. The crucial observation was that the totally reduced protein, freed of denaturant, began to recover enzymatic activity merely on standing in a beaker on the bench. Although this observation is recorded in notebooks of workers in other laboratories at about the same time, its significance was missed. In characteristic fashion, Anfinsen saw essentially the total picture at once, even though the data were not all in. The enzyme was capable of refolding itself into the native structure, re-forming by air oxidation the disulfide bridges with essentially complete regain of catalytic activity. An immense amount of work rapidly went into an investigation of this process. The implications were quite clear that the total "information" needed for the self-assembly of a three-dimensional structure of a native protein was in fact present in the sequence of the linear polypeptide chain. From today's vantage point, the intense arguments which occurred at that time had to be lived through to be imagined. There were the incredibly difficult question of residual structure in the presumed random coil, the question of whether the native structure was indeed formed, the question of how useful catalytic activity was in defining such a structure, and the like. The coup de grace for the skeptics, of course, was made only a few years ago when the total synthesis of this particular enzyme was accomplished by Gutte and Merrifield at Rockefeller University and by Denkewalter and Hirschman and their colleagues at Merck.

No possible "residual" structure could be imagined under those circumstances, and recovery of substantial amounts of enzymatic activity with the concurrent appearance of all the normal physical and chromatographic properties indicate that differences in this refolded material from the native structure, if any, must be very small indeed.

The idea that the information content in the linear sequence will, by itself, result in the rapid formation of a unique biologically active structure forms part of the central dogma of modern biochemistry and molecular biology. However, just as the translation of the genetic code into a linear peptide sequence is still today infinitely complex and intriguing, the mechanism by which such a chain folds up to give the native three-dimensional structure is equally poorly understood.

The ribonuclease saga forms the basis for continuing studies on active sites, on the mechanism of catalysis, on the problem of protein structure, and on chain folding that are going forward full speed in these two laboratories and in the laboratories of the many biochemists, who over the years have been associated with Anfinsen or with Stein and Moore.

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