chapter 6

THE BIOLOGICAL ACTIVITY OF PROTEINS IN RELATION TO STRUCTURE

We may almost define the life sciences as those concerned with the elucidation of the mechanisms by which molecules exert their specific actions on living cells. In the case of many simple inorganic ions and organic molecules it has been possible to arrive at an approximate understanding of their mechanism of action. We have some understanding, for example, of the physiological sequelae of raising or lowering the tonicity of body fluids by the administration or withdrawal of sodium chloride. Similarly, the aberrations in the synaptic transmission of nerve impulses following the administration of physostigmine may be partially explained by the action of this drug on the enzyme acetylcholinesterase. It is a tribute to cellular complexity that even such well-studied systems as these continue to be frontiers of research and speculation among those individuals who are fully conversant with them.

Protein chemists naturally feel that the most likely approach to the

understanding of cellular behavior lies in the study of structure and function of protein molecules. This is, perhaps, not an entirely unreasonable point of view. Except for those rare phenomena in biology which are purely physical, the "aliveness" of cells is basically the summation of enzymic catalysis and its regulation.

The field of protein chemistry has now reached a stage of relative sophistication allowing us to think of proteins as organic chemicals rather than as conglomerates of amino acids. In spite of their enormous complexity we now can measure the extent of such phenomena as "denaturation" in terms of rather well-defined changes in specific types of chemical bonds, some of which have been discussed in Chapter 5. Because of this happy situation, we may approach, in a rational way, the relation of specific aspects of covalent and noncovalent protein chemistry to biological activity. Proteins, as *molecules*, must be thought of as consisting of one or more polypeptide chains, cross-linked and coiled through a variety of chemical bonds of varying strengths. Modifications of any of these lead to an entity which is not identical to the original native molecule and which, in a purist sense, may be considered "denatured." From the standpoint of function, however, we may take a somewhat narrower view. The nativeness of an enzyme, *as estimated by its ability to catalyze a reaction*, need not involve the whole of its structure.

Studies of the effect of specific degradation on biologically active proteins are fairly recent. It was observed, however, more than twenty years ago, that various reactive groupings on proteins could be modified by substitution, or conversion to other forms, without loss of function. Perhaps the best-known example of such work is the series of investigations by Herriott and Northrop of the activity of pepsin during progressive acetylation.[1] Pepsin was treated with ketene which converted free amino groups and tyrosine hydroxyl groups to their acetyl derivatives. By this method Herriott was able to prepare a crystalline derivative which contained seven acetyl groups per mole of pepsin and had approximately 60 per cent of the enzyme activity of the original pepsin. He demonstrated that the ultraviolet absorption spectrum of this 60 per cent active material was modified to the extent to be expected for the masking of three tyrosine hydroxyl groups. Further, by cautious hydrolysis at pH 0.0 or at pH 10.0, three acetyl groups could be removed with a simultaneous increase in enzyme activity. These, and other, studies indicated that tyrosine residues were somehow involved in activity and also that acetylation of a number of the free amino groups in the protein *had no effect on function.*

Experiments of this sort have now become relatively commonplace, and there is no doubt that the structures of a large number of enzymes and hormones may be tampered with without inactivation. In spite of such information, the concept persisted, until very recently, that the structure of biologically active proteins was more or less "inviolable" and that the complete and integrated three-dimensional architecture of these proteins was required for function. This concept was nurtured by several theoretical considerations in which the protein molecule was pictured as a network of resonating pathways through which electrons might surge and redistribute during the process of catalysis. The observations of immunology added further support to this concept, since it was well known that relatively minor changes in, for example, the structure of a hapten could lead to a profound modification in the effectiveness of reaction with a specific antibody.

The idea of the "inviolability" of protein structure is now slowly being replaced with the idea of the "functional adequacy of less than the whole." Shortly after Sanger and his colleagues had completed their monumental studies on beef insulin, it was shown by Lens[2] that a known, degradative change in the hormone, namely, the removal of the *C*-terminal alanine residue of the *B* chain, did not lead to loss of biological activity. (The evolutionary implications of this observation were not particularly obvious at the time since the experiment was the first of its kind and had to be thought of as an isolated example. Now, however, as the result of many similar observations, we must concern ourselves (see Chapter 11) with the question of why the *C*-terminal alanine residue is preserved in insulin as a constant structural feature if it serves no function in the biological activity of the hormone.)

Insulin has been subjected to other, more extensive, studies of this sort. However, for the purpose of illustration of the extent to which proteins may be degraded without inactivation, we shall consider instead three other examples about which we have somewhat more information: the pituitary hormone, ACTH, the pancreatic enzyme, ribonuclease, and the plant enzyme, papain. In the following discussion of these examples, two quite different approaches to the structural basis of biological activity will be considered more or less simultaneously. One aim is to show that active polypeptides can be degraded without loss of function, and is concerned mainly with indicating the magnitude of *unessential* parts of structure. The other is directed deliberately toward the delineation of the *essential* parts, that is, the active centers.

Adrenocorticotropic Hormone

Complete sequences have been elucidated for adrenocorticotropic hormone (ACTH) isolated from three species, hog, beef, and sheep. The problem of species variation will be discussed elsewhere. For our present purposes we shall consider only the material isolated from hog pituitaries since the relation between structure and function appears to be the same for the hormones of all three species. Adrenocorticotropic hormone is one of a large number of polypeptides which can be demonstrated in extracts of the anterior pituitary gland. The hormone has been prepared in highly purified form and has been shown to cause the selective stimulation of the adrenal cortex with the release of adrenal steroid hormones. It also causes depletion of the ascorbic acid and cholesterol stores of the adrenal gland and is capable of stimulating the repair of histological changes owing to hypophysectomy.

The sequence of pig ACTH is shown in Figure 67. The polypeptide is composed of 39 amino acid residues and contains several intriguing groups of amino acids, notably the sequence of three hydroxyamino acids, Ser.Tyr.Ser, at the *N*-terminus and the highly basic grouping, Lys.Lys.Arg.Arg, in positions 15 to 18. The action of carboxypeptidase removes three residues from the *C*-terminal end of the chain without loss in hormonal activity. A more severe degradation results during limited pepsin digestion with the removal of the eleven *C*-terminal amino acids, still without change in activity. These experiments establish that, as a maximum, only the first 28 residues of ACTH are required for hormonal function. Paul Bell and his co-workers have recently reduced this estimate, reporting that by mild acid hydrolysis of the chain the last 15 residues may be removed without inactivation.[3]

It has also been possible to characterize the activity of ACTH in terms of specific aspects of structure which *are* essential. Thus it has been found that degradation of the *N*-terminal end of the chain with leucine aminopeptidase results in complete loss of activity after only one or two residues have been removed. Recent studies on the action of fibrinolysin by White and Gross[4] have further underlined the importance of the first half of the hormone. As is shown in Figure 67, this proteolytic enzyme cleaves the chain at two points, following residues 8 and 15, with complete inactivation.

These various degradative studies establish that at least 16, and perhaps as many as 24, of the amino acids of ACTH are essential ones. The ultimate elucidation of the exact structural requirements

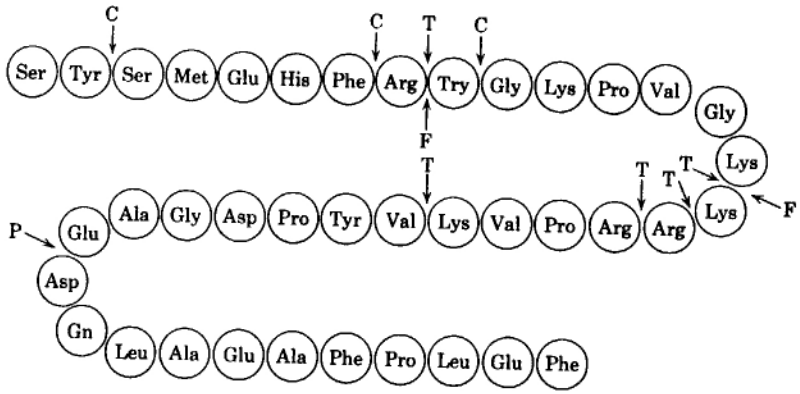

Figure 67. The structure of porcine α-corticotropin. Points of attack by chymotrypsin (C), trypsin (T), pepsin (P), and fibrinolysin (F) are indicated by the arrows.

for ACTH activity must probably await the step-by-step synthesis of the sequence, and such studies are in progress in Klaus Hofmann's laboratory,[5] where the unequivocal synthesis of a peptide corresponding to at least the first 14 amino acids in the polypeptide has been achieved. At last report, the synthetic material had not yet reached sufficient length to exhibit hormonal activity. In the meantime, Boissonnas and his colleagues[6] have reported the synthesis of a biologically active peptide consisting of the first 20 *N*-terminal amino acids of ACTH. Although the synthesis is not entirely unequivocal, the results give strong support to the conclusions drawn from the degradative work.

The studies on ACTH are of particular interest since they have to do with a substance which is assayed by *in vivo* methods. Investigations of the changes in activity during the degradation of enzymes must generally be controlled by *in vitro* tests. The possibility that functional adequacy in the test tube does not give a true picture of activity in an organized cellular environment adds an aspect of uncertainty to the interpretations. Discounting the rather slim possibility that degraded ACTH is reconverted to native ACTH during activity assays, we have, for this hormone, clear-cut evidence for the "functional adequacy of less than the whole."

Papain

Papain is a powerful proteolytic enzyme present in papaya latex which has been crystallized, both as the native protein and as the

mercuri-derivative, by Emil Smith and his colleagues.[7] The enzyme is of considerable interest as a model for the study of the relationships between structure and function because it appears to contain a great deal of structure which is "superfluous" for activity. The molecule contains six sulfhydryl groups, five of which are not reactive with the usual sulfhydryl reagents in the native protein but appear from within the inner regions of the structure only after suitable denaturation. The single reactive sulfhydryl is essential for activity, and complete inactivation occurs when two molecules of the protein react with a single molecule of mercuric ion to form the dimer mercaptide. No other sulfur is present beyond the six cysteine residues, and cross-linkage of the 180 amino acid residue chain through cystine bridges is therefore excluded.

The most exciting development in the studies on papain has been the observation by Hill and Smith[8] that approximately 80 residues may be removed from the *N*-terminal end of the chain by leucine aminopeptidase without loss in catalytic activity. The remaining *C*-terminal stretch of amino acids is relatively poor in basic amino acids but rich in tyrosine and valine. The noncritical nature of lysine side chains is indicated by the fact that the ε-amino groups may be converted to the guanido derivatives, by treatment with O-methylisourea, without activation.

Papain is reversibly inactivated by strong urea solutions and, to some extent, irreversibly by guanidinium ions. Under such conditions of denaturation the shape of the molecule is considerably altered as evidenced by changes in the optical rotatory, viscosity, and ultracentrifugal properties. Therefore, although only a portion of the papain chain is essential for catalysis and is apparently not dependent on covalent cross-linkages, there are obviously important secondary and tertiary structural features involved in the determination of its catalytically active center.

Ribonuclease

The present status of our knowledge of the sequence of ribonuclease has been reviewed in an earlier chapter together with a consideration of some of the physical properties of this enzyme, both in its native state and under denaturing conditions. With this background we can now examine some of the covalent and noncovalent aspects of its structure in connection with the problem of catalytic activity. As we have seen, the protein contains two rather longish "tails," one at the *N*-terminal end consisting of 25 residues and one at the

C-terminal end consisting of 14 (Figure 62, Chapter 5). Both of these parts of the chain have been subjected to controlled hydrolysis with proteolytic enzymes. It was first shown by F. M. Richards[9] that native ribonuclease, when digested with the bacterial enzyme subtilisin, was rapidly and specifically hydrolyzed at the Ala.Ser bond (residues 20–21) in the *N*-terminal "tail." The resulting derivative, in which the 20 residues of the "tail" portion were still attached to the body of the enzyme through some particularly strong noncovalent interaction, could be isolated on ion exchange columns and was shown to retain the full activity of the original protein. This active, degraded ribonuclease molecule now also contains, in addition to the *N*-terminal lysine residue characteristic of the native enzyme, a new *N*-terminal serine residue.

The *C*-terminal end of the chain can also be degraded without inactivation. It has been possible to remove the *C*-terminal valine residue by carboxypeptidase treatment, together with a fraction of the serine and alanine residues which immediately precede the valine. In such samples of degraded protein no evidence for inactivation has been observed.

The site of these noninactivating cleavages with proteolytic enzymes may be assigned to specific portions of the linear polypeptide chain. Some active products of more extensive digestion have also been described for which, unfortunately, the exact sites of cleavage cannot be pinpointed at present. Uziel, Stein, and Moore,[10] for example, have reported the isolation of active derivatives of ribonuclease which has been subjected to trypsin digestion under conditions of partial unfolding of the protein in dilute urea solutions. Similarly, Kalnitsky and Rogers[11] describe experiments using carboxypeptidase digestion in which the digesting enzyme apparently contained other proteases of unknown types which caused extensive degradation of the molecule without loss of much activity. As the analysis of these latter studies becomes more complete and it becomes possible to assign the changes that have occurred to definite parts of the polypeptide chain, we shall be able to make much more useful statements about the location and nature of the "active center" of this enzyme.

We may also derive useful information from experiments designed to determine the minimum changes necessary to cause *inactivation* of the enzyme. Two particular experiments are of special interest here. The first has to do with the inactivation of ribonuclease activity during photooxidation. Weil and Seibles[12] have shown that the enzyme, when exposed to light in the presence of methylene blue, undergoes slow loss of activity and that this loss parallels, at least during the

early stages of photooxidation, the disappearance of one (possibly two) of the four histidine residues that occur in this protein. Although the exact location of the histidine residue (or residues) involved has not been determined, it should be noted that two of the four are found in the *C*-terminal twenty amino acids of the chain.

A second sort of inactivating treatment which may ultimately help to elucidate the chemical basis of ribonuclease activity involves pepsin digestion under carefully controlled conditions. When the enzyme is exposed to pepsin at pH values in the neighborhood of 1.8, there occurs an extremely rapid hydrolysis of what appears to be a single, particularly labile, peptide bond.[13] The macromolecular portion of the reaction mixture may be isolated on ion exchange columns and is completely devoid of activity. The only other detectable fragment is a tetrapeptide, Asp.Ala.Ser.Val, the sequence of which indicates that it was derived from the *C*-terminal end of the chain by the cleavage of the peptide bond following the phenylalanine residue at position 120 in the polypeptide chain (see Figure 62). It has not been possible to detect the presence of cleavages elsewhere in the inactive macromolecular derivative, and both physical studies and *C*-terminal amino acid analysis support the conclusion that nothing else has happened beyond this unique hydrolysis.

Since the earlier studies on the carboxypeptidase treatment indicate the nonessentiality of the three *C*-terminally located amino acid residues, these results with pepsin suggest that the aspartic acid residue located at position 121 has some special function in the maintenance, or mechanics, of the catalytically active center. We shall discuss this point further in connection with observations on the spectral properties of ribonuclease and its various active and inactive derivatives.

Although the combined consideration of the degradative experiments indicates that some portions of the sequence of ribonuclease have greater importance in activity than others, we cannot conclude that catalysis by this enzyme requires only a simple linear sequence of amino acids. Both oxidized ribonuclease and ribonuclease that has been converted to a random, open chain by reduction of the disulfide bridges are completely inactive. It seems very likely that one or more portions of the sequence, perhaps quite widely separated in the sense of linear distance along the chain, are essential, and that their relation to one another in space is fixed by restrictions introduced by disulfide bridges and by other bridges of a noncovalent nature.

Some recent experiments on the relation between controlled, stepwise reduction of the disulfide bridges in relation to activity are of

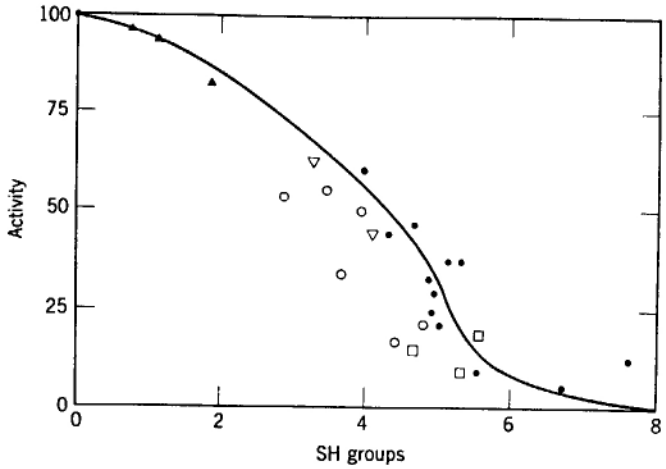

Figure 68. Activity of ribonuclease at various stages of reduction (expressed as percentage of the specific activity of native ribonuclease) as a function of the number of moles of sulfhydryl per mole of enzyme. Solid triangle, reduction in absence of urea; solid circle, reduction in 8 *M* urea; open square, reoxidation of fully reduced, inactive ribonuclease; open circle, reoxidation of samples containing more than six sulfhydryl groups per average molecule; open triangle, reoxidation of samples containing about four sulfhydryl groups per average molecule. From M. Sela, F. H. White, and C. B. Anfinsen, *Science*, **125**, 691 (1957).

interest in this regard.[13] The disulfide bonds may be reduced by thioglycollic acid and by various other reducing agents as discussed in Chapter 5. It has been found that the reduction of one of the four bridges in ribonuclease goes fairly rapidly in the absence of urea, but that in 8 *M* urea, in which the three-dimensional structure of the protein has been disoriented and "loosened," all four are easily cleaved. During reduction the activity falls off in a manner suggesting that one, and perhaps two, of the bridges may be dispensed with from the standpoint of enzymatic activity (Figure 68). Thus when an average of one SS link has been reduced, we can still demonstrate the presence of 80 to 90 per cent of the initial activity in spite of the absence of all but traces of native enzyme. Even at an average level of four SH groups per mole of protein (equivalent to the rupture of two SS bridges), considerably more than half the activity remains. It has recently been possible to separate some of the intermediates of early stages of reduction on ion exchange columns, and the identification of the bridges that are most susceptible to reductive cleavage is now under investigation. Preliminary results indicate that nearly

full activity is retained after the disulfide bridge between the first and sixth half-cystine residue is cleaved.

The reduction experiments, and the proteolytic studies discussed earlier, permit us to perform a certain amount of paper surgery on the enzyme and to construct a two-dimensional picture of the maximum structural requirement for activity (Figure 69). It seems likely that amputations of other parts of this structure may be possible without inactivation. We can already safely conclude, however, that considerable portions of the covalent structure of ribonuclease must either be unimportant evolutionary vestige or involve aspects of ribonuclease function and intracellular behavior about which we are still in complete ignorance.

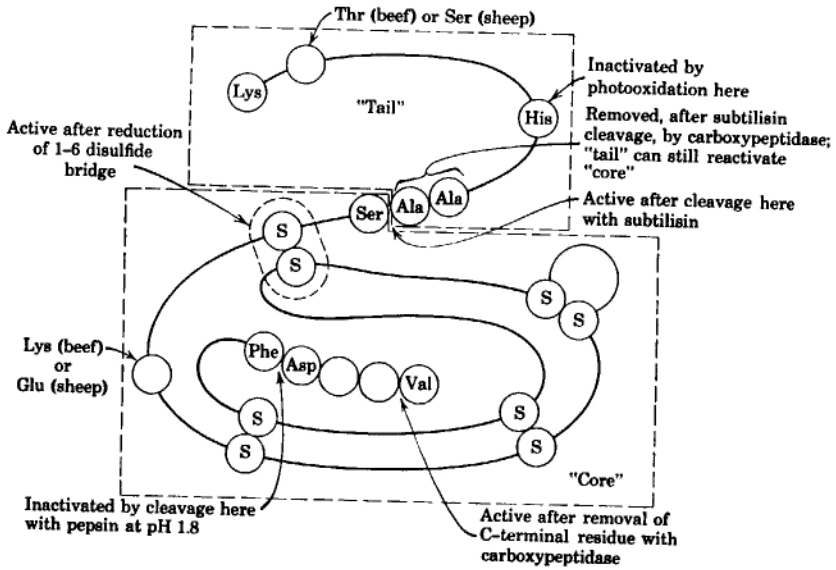

Figure 69. A schematic drawing of the ribonuclease molecule in two dimensions (see also Figure 62). Various experimental modifications of the native molecule are indicated, together with the consequences of such modifications in terms of enzyme activity. Also included are some results of studies on the comparative structures of bovine and ovine pancreatic ribonucleases (see Chapter 7). The data summarized in this figure make it tempting to suggest that much of or all the *N*-terminal "tail" of the molecule, together with only a portion of the "core," might eventually be shown to be sufficient for catalytic activity. The "active center" must obviously be a complicated three-dimensional structure but may not necessarily involve more than a fraction of the entire protein. This drawing is extremely speculative and is included here only to indicate some of the directions that research on the biological activity of this enzyme may take in the future.

Before leaving the subject of enzymatic structure in relation to function (and the reader must excuse my preoccupation with a specific enzyme about whose properties I happen to be able to write with less labor than certain others of equal illustrative value), let us consider a few aspects of noncovalent structure in terms of catalytic activity.[13] We have referred, in Chapter 5, to some of the physical characteristics of ribonuclease under conditions of reversible denaturation by urea. The rather large changes that occurred in the structure of the enzyme under such conditions (e.g., the intrinsic viscosity of ribonuclease in 8 *M* urea shifts to about 0.085 as compared with a value of 0.036 in dilute salt solution) made it of interest to examine whether or not activity was retained in 8 *M* urea solutions. It was quite surprising to find that hydrolysis of RNA was unimpaired and, indeed, even somewhat stimulated under these conditions of hydrogen bond disorientation. The conclusion was drawn that activity was independent of organized, three-dimensional structure. That this conclusion might be premature, however, was suggested when it was subsequently observed that low concentrations of phosphate ions or other polyvalent anions (e.g. arsenate) could reverse the unfolding effect of urea. The natural substrate for ribonuclease, RNA, is itself a polyanion and might also cause refolding of the enzyme under the conditions of assay. Various technical difficulties having to do with the spectral and optical properties of ribonucleic acid made the direct test of this hypothesis difficult. However, model substances such as polymetaphosphate and uridine-3′-phosphate (which is the product of ribonuclease attack on the synthetic substrate, uridylic-2′,3′-cyclic-phosphate) could also cause a refolding of the molecule as based on measurements of its spectrum, optical rotation, and intrinsic viscosity (Table 6). The refolding was not complete, as evidenced by the somewhat lower optical rotation of the enzyme in 8 *M* urea in the presence of these agents as compared with that of the native enzyme in water, and by an incomplete regeneration of viscosity behavior. However, *spectral* differences were completely abolished.

Although these experiments suggested that complete "nativeness" might not be essential, as indicated by the partial but significant irreversibility of the optical rotatory and viscosity properties of the enzyme to anions, it appeared that the portion of the three-dimensional structure of the enzyme which is responsible for the peculiar spectral properties of several of the six tyrosine residues (page 120, Chapter 5) must be in the proper configuration.

In an effort to support this hypothesis, the spectra of various ac-

TABLE 6

Some Physical Properties of Ribonuclease and Modified Ribonucleases

Ribonuclease	Unfolding Agent	Anion	$\Delta_{\epsilon.10^{-3}}$ [a]	$[\alpha]_D^{20}$	$\eta_{sp/c}$ [b]
Native	–	–	1120	−71.7°	0.036
Native	8 *M* urea	–	0	−103.7°	0.085
Native	8 *M* urea	0.4 *M* chloride	110	−98.8°	
Native	8 *M* urea	0.15 *M* phosphate	1120	−81.0°	0.050
Native	8 *M* urea	0.15 *M* arsenate	1120	−80.7°	
Native	8 *M* urea	0.15 *M* uridylate			0.052
Oxidized	–	–	0	−91.6°	0.116
Reduced and carboxymethylated	–	–	0		0.149
Pepsin inactivated	–	–	0	−83.8°	0.039
Subtilisin digested	–	–	1120		

The concentration of the proteins was 2.5–2.7 grams/liter for spectrophotometric readings, 25–30 grams/liter for polarimetric, and 14–15 grams/liter for viscosimetric measurements. All measurements were carried out at pH 6–7. Anions were added as their sodium salts.

The active derivative of ribonuclease produced by limited subtilisin digestion was purified according to Richards (see page 132).

[a] Change in extinction relative to the absorption of ribonuclease in 8 *M* urea in the absence of salts, at 285 mμ.

[b] Reduced viscosity, in (grams/100 ml)$^{-1}$. All viscosity measurements were carried out in 0.1 *M* KCl.

tive and inactive derivatives of ribonuclease were examined. It was found that all the derivatives of ribonuclease that possessed activity showed a normal "shifted" spectrum, whereas all those that were inactive possessed the spectral characteristics of tyrosine residues with unmodified resonance properties. We might tentatively conclude from these studies that the presence of a shifted, "native," spectrum leads to a "diagnosis" of catalytic activity and that factors which cause changes in the spectrum may be expected to inactivate.

To summarize our impressions at this point, it appears that at least one bond in the *N*-terminal and part of the *C*-terminal "tail" are dispensable, and that one, and perhaps two, of the SS bridges may be opened with impunity. Various findings suggest that a part of the

structure in the vicinity of the *C*-terminal "tail" has special importance, specifically the aspartic acid residue at position 121. Finally, there appears to be good correlation between the presence of a "shifted" spectrum, as occurs in the native enzyme, and activity. Since the evidence is now rather convincing that such shifted spectra may involve hydrogen bonding between carboxylate groups in the protein and hydroxyl groups on tyrosine residues, it may be suggested, as one alternative, that the free carboxylate group of the aspartic acid residue at position 121 is involved in such a linkage and functions as one of the determinants of the three-dimensional form of the active center of the enzyme.

The importance of considering the enzymatic activity of a protein in terms of three-dimensional structure is strongly emphasized by an especially intriguing aspect of ribonuclease chemistry, recently reported by F. M. Richards.[9] In a continuation of his studies on the enzymatically active derivative prepared by limited subtilisin digestion, he has investigated the nature of the noncovalent bond or bonds (see page 132) which hold the *N*-terminal "tail" peptide to the macromolecular portion of the protein. The attachment may be broken by treatment of the derivative with trichloroacetic acid, and the *N*-terminal peptide fragment may be separated by dialysis and purified by electrophoretic methods. Richards has shown that both the peptide and the macromolecular component are inactive, but that upon mixing the two activity is completely regenerated as shown in Figure 70.

Richards has also examined the spectral properties of the separate fragments and of the reconstituted, active mixture. The 20-amino acid peptide, which contains no tyrosine, of course shows no absorption at 280 mμ. The large piece, however, has the expected absorption in this region of the ultraviolet, but *shows none of the spectral shift* observed with the native protein or with the subtilisin-digested enzyme before separation of the two fragments. Its spectrum is extremely similar to that observed for native ribonuclease in urea or after treatment with inactivating reagents such as pepsin. Upon mixing the two components, the shifted tyrosine spectrum reappears almost completely, indicating that the peptide "tail" plays an important part in the determination of that part of the three-dimensional structure of the protein which is responsible for the anomalous tyrosine absorption.

We have, in Richards' experiments, an instance of biological function in a relatively small peptide which is strongly reminiscent of the action of some of the peptide hormones of the anterior and posterior

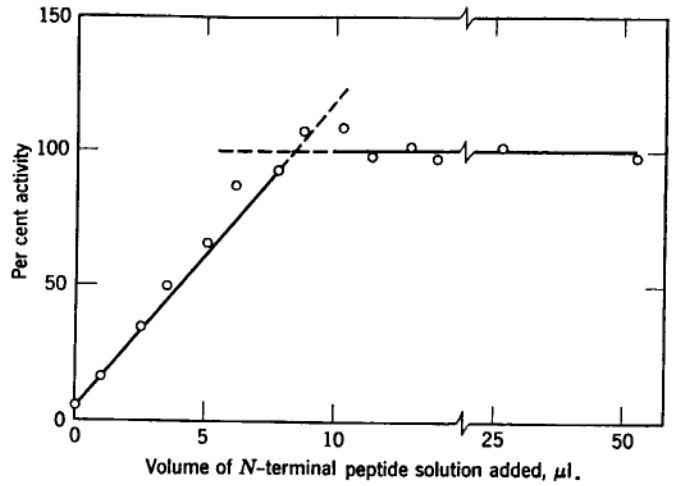

Figure 70. The regeneration of ribonuclease activity by the addition of the *N*-terminal peptide (which is split off from the native molecule by subtilisin) to the inactive macromolecular "core." As described in the text, the "core" was precipitated from the subtilisin digest with trichloroacetic acid. The *N*-terminal polypeptide, containing twenty amino acid residues, causes complete regeneration of activity when it has been added in amounts approximately equimolar with the concentration of the macromolecular component. From F. M. Richards, *Proc. Soc. Natl. Acad. Sci. U.S.*, **44**, 162 (1958).

parts of the pituitary gland. The complete regeneration of ribonuclease activity occurs at concentrations of the order of 10^{-6} *M*, a level within quite reasonable limits for hormonal action. On the basis of these studies alone, the *N*-terminal peptide fragment does not necessarily have to be an intrinsic part of the active center. It may conceivably function only as a determinant of proper folding in the rest of the molecule. Whatever its role, the forces that bind this small polypeptide to the body of ribonuclease must be multiple and highly specific.

In addition to being an essential partner to the catalytic "center" of ribonuclease, Richards' peptide fragment also appears to possess the ability to stabilize the rest of the molecule. The active subtilisin derivative is stable in aqueous solution, reflecting the considerable strength of the association between the two components of the complex. In urea, however, where these components dissociate, as they do in trichloroacetic acid, the macromolecular fraction is rapidly denatured and cannot be reactivated by the addition of the peptide, even following removal of the dissociating agent. The specific, cata-

lytically active configuration in the intact subtilisin derivative is thus dependent on the participation of certain bonds and amino acid residues contributed by the macromolecular body of the derivative, stabilized by the peptide "tail" fragment.

It is much too early to attempt to synthesize the various observations on the chemical basis of ribonuclease into a coherent whole. It is a tribute to the progress of modern protein chemistry, however, that we can even hope to do so in the near future. The pattern already emerging for ribonuclease, in which certain widely separated areas of the protein are strongly implicated as components of an active center, and other portions (at least one disulfide bond and an assortment of amino acid residues at various points in the chain) are not, is an object lesson in the necessity for reorienting our thinking about proteins from planar to solid geometry.

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