Structure and Activity of ENZYMES

Edited by

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FEDERATION OF EUROPEAN BIOCHEMICAL SOCIETIES
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PREFACE

The Federation of European Biochemical Societies came into being on 1 January 1964. It was planned at a meeting of delegates of 17 European Biochemical Societies, held in Oxford in July 1963. All the Societies subsequently agreed to join the Federation and the number has grown to 19, representing Austria, Belgium, Bulgaria, Czechoslovakia, Denmark, Finland, France, Germany, Great Britain, Hungary, Italy, The Netherlands, Norway, Poland, Spain, Sweden, and Switzerland.

Membership of the Federation is conferred automatically on members of each constituent Society. The Federation exists to promote closer co-operation between biochemists in Europe, among other things by holding annual meetings. The symposium printed here took place on 24 March 1964, during the first Federation Meeting on 23–25 March 1964 in London. The Biochemical Society of Great Britain, which organized the first meeting for the Federation, is grateful to Dr. F. Sanger and Professor T. W. Goodwin who arranged the symposium, to Drs. B. S. Hartley and J. I. Harris for editing the manuscripts, to the Royal Society and the Wellcome Trust for financial assistance to the speakers and, foremost, to the speakers themselves.

The Federation has a brief, informal list of Statutes. It does not propose to have a permanent secretariat. The administration changes as each Society in turn holds a Federation Meeting. In its short existence the Federation has already promoted much closer relations between European biochemists and practical benefits have emerged. For example, over 500 biochemists took part in Federation air charters from London and Paris to the Sixth International Congress of Biochemistry in New York in July 1964. The Federation has planned summer schools for training in advanced research methods and "Gordon-style" research conferences are envisaged for the future. This symposium is the first in a series which will emanate from the annual meetings.

As the first officers of the Federation, appointed by The Biochemical Society, we have been delighted by the success of the first year's activities and grateful for the support of all the adhering Societies. The fact that European scientists in other disciplines are now planning co-operative ventures modelled on that of the biochemists is a welcome compliment.

F. C. Happold *Chairman*
W. J. Whelan *Secretary*
S. P. Datta *Treasurer*

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Morning Session

Chairman : P. Desnuelle

Introduction by F. Sanger†

The chemical processes of living matter are almost entirely dependent on the catalytic activity of enzymes, so that the problems of what enzymes are and how they work are two of the most fundamental in biochemistry. It is clear that this activity must depend on the exact chemical structure of the molecule and particularly on the nature of that part of the enzyme that comes into contact with the substrate and is generally referred to as the "active site". In the past, many studies have used indirect approaches to deduce something of the nature of active sites, but these have been greatly hampered by a lack of the knowledge of the chemical structure of the enzymes involved. During recent years there has been considerable progress in studies of protein structure, both in the determination of amino acid sequences by chemical methods and in the elucidation of the configuration by the X-ray method, so that we are rapidly approaching a situation where it will be possible to give the exact chemical structure of an enzyme and probably to explain the details of its catalytic mechanism. This is the subject of the present symposium. The problem will be dealt with mainly from the point of view of the protein chemist; however, there are many other aspects of enzymology that must be considered in any attempt to understand enzymes.

Because of the great amount of work that is going on in this subject, it is only possible to deal with a few special topics. We have chosen to deal with three proteins about which much is known from the chemical point of view, and to have a special section on the new methods for specifically labelling active sites. The inclusion of haemoglobin in a symposium on enzymes seems justified since much is known about its active site, and a consideration of its structure and activity is likely to teach us much about enzymes.

† Read in his absence by P. Desnuelle

RIBONUCLEASE

Structure and Activity of Ribonuclease

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Since the first report of its purification by Kunitz (1940), ribonuclease isolated from bovine pancreas has been the subject of a rapidly increasing number of studies in the field of protein chemistry. The great stability and low molecular weight have been important factors in the frequent choice of the enzyme. As a macromolecule, ribonuclease now occupies the position of a primary standard for the calibration of many physical and chemical techniques. However, its mechanism of action as an enzyme is still unknown. Some aspects of the chemistry of this protein are discussed in this paper.

The initial studies of Anfinsen and the work of Hirs, Stein and Moore and their colleagues have provided the entire covalent structure of this molecule (see Figure 1). Knowledge of the sequence has encouraged the detailed study of the chemical modification of a number of functional groups. In the case of limited reaction, the modified residues can usually be definitively located in any part of the sequence. A number of such residues whose covalent structure has been modified by the addition or substitution of one or more atoms are shown in the circles in Figure 1.

CHEMICAL MODIFICATION OF RIBONUCLEASE-A

Hirs (1962) has made a detailed study of the reaction between 1-fluoro-2,4-dinitrobenzene (FDNB) and RNase-A. The principal products of the initial stages are mono-DNP derivatives of the α-amino group of residue 1 and the ε-amino group of residue 41. The former derivative has partial enzymic activity, the latter is inactive. The reactivity of the group at position 41 is almost two orders of magnitude greater than that of a similar amino group in a simple peptide. A number of competitive inhibitors of the enzyme prevent this specific reaction. Further substitution occurs at residue 7 but prior modification of 41 appears to be mandatory. This work provides an excellent example of the delicate use of a relatively non-specific reagent under conditions where the specificity is supplied by the protein. The ability to draw firm conclusions then depends entirely on careful characterization of the reaction products. In the present instance it is clear that lysyl residue 41 is at, or close to, the active site of the enzyme.

The interaction of the enzyme with haloacetates is another example of the same approach. Barnard and Stein (1959) have shown that a major product of the reaction involving bromoacetate is an enzymically inactive derivative

containing a carboxymethyl group on the histidyl residue in position 119. In an extensive series of experiments using iodoacetic acid, Gundlach, Stein and Moore (1959) and Crestfield, Stein and Moore (1963) have shown that alkylation of histidine 119 occurs on nitrogen-1 of the imidazole ring. A minor component of the reaction mixture contains a nitrogen-3 substituted histidyl residue at position 12. This derivative shows only a trace of residual activity. Surprisingly, the substitutions at these two positions are mutually exclusive, and the whole effect is seen only with iodoacetate ion and not with iodoacetamide. The peculiar reactivity of these two residues is related to the conformation of the catalytically active protein and is destroyed by denaturation (Stark *et al.*, 1961). The reactions are also slowed down or stopped in the presence of divalent anions such as sulphate or phosphate. However, the enzyme is catalytically active even in very high concentrations of sulphate ion (Doscher and Richards, 1963). The relation of iodoacetate reaction to enzymic activity is considered below in the discussion by Mathias. A more detailed description of Crestfield's experiments will be given by Moore (1964) at the anniversary meeting of the Société de Chimie Biologique.

Denatured ribonuclease reacts readily with iodoacetate (or iodoacetamide) to form derivatives involving the methionyl residues. Alkylation takes place at the sulphur atom to yield a sulphonium salt. This reaction occurs very slowly or not at all with the native enzyme (Neumann *et al.*, 1962; Crestfield *et al.*, 1963). The four methionyl side chains are masked, or unavailable, in the absence of denaturing conditions. They appear to play an important role in maintaining the conformation of the protein. Sulphonium salt formation in certain of these residues causes marked structural change and loss of catalytic activity. There is no evidence that any of the methionines serve as contact residues at the active site. They are considered below in relation to RNase-S.

The tyrosines represent another class of residues that appears to have an important structural role. The peculiar ionization and spectral properties of three of the six groups were discovered by Shugar (1952). Subsequent studies (Tanford *et al.*, 1955; Bigelow, 1961) have shown the interrelation between the native structure and the anomalous properties and how both are affected by changes in the solvent environment. From this work it is clear that three tyrosine residues are distinguishable from each other and from simple phenols in aqueous solution. These three anomalous residues are clearly not so accessible to the solvent as are the other groups of this class.

More recently, Cha and Scheraga (1963) have checked the chemical reactivity of the phenolic groups with the iodination reaction. Both mono- and di-iodotyrosines can be formed depending on the extent of the reaction. In the absence of denaturing conditions it was found that the tyrosine residues in positions 25 and 97 do not react with iodine. These are presumably two of the three residues whose spectral properties are anomalous. The third residue with an abnormal phenolic ionization is close enough to the aqueous environment to react normally with iodine. The iodination of the ring occurs ortho to the phenolic hydroxyl group. It does seem odd that a large attacking group such as I_3^- can get so close to a hydroxyl function which is having difficulty

dissociating a proton. If the effect on ionization is electrostatic rather than steric, then the required local negative potential would surely repel the iodinating reagent.

MODIFICATION OF RIBONUCLEASE-S

Ribonuclease-S is itself a derivative of the native enzyme produced by limited proteolysis (Figure 1). The only known change in covalent structure is the loss of the peptide bond between residues 20 and 21. The ability to separate and recombine the two parts (designated S-protein, and S-peptide respec-

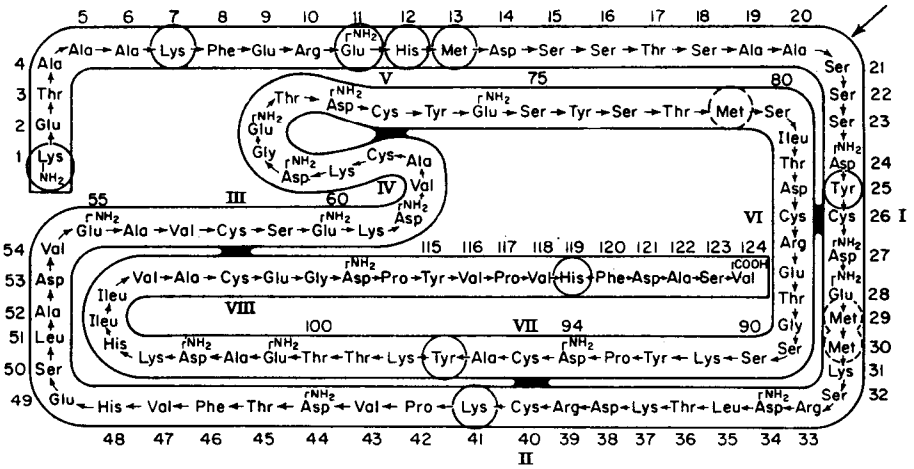

FIGURE 1. Diagram of the covalent structure of bovine pancreatic ribonuclease-A (Smyth, Stein and Moore, 1963). The heavy circles enclose some of the residues that have been specifically altered by chemical modification. The nature of the modifications and the effects produced are discussed in the text. The arrow at the upper right indicates the bond cleaved by proteolysis to yield ribonuclease-S (Richards and Vithayathil, 1959). The smaller component of the latter substance, residues 1–20, is designated S-peptide. The other component, residues 21–124, is referred to as S-protein.

tively) of this molecule has proved useful. Two factors of general interest are: (1) the very strong binding between the two parts at neutral pH and ambient temperature; and (2) the reappearance of enzymic activity which accompanies this interaction. The two effects may not be directly connected.

Richards and Vithayathil (1960) have summarized the results of a series of chemical modifications of the functional groups on the smaller component, S-peptide. Substantial changes in the net charge of the peptide, through acetylation or esterification, have little effect on either the binding or the activity of the complex. However, the methionine residue in position 13 makes an important contribution to the interaction. Conversion to a sulphonium salt by reaction with iodoacetate lowers the peptide association constant by a factor of at least a thousand. However, the catalytic activity of the complex is not markedly affected. There is tentative evidence that the

amide group in position 11 is required for catalytic activity but has no influence on the binding (Vithayathil and Richards, 1961a). Crestfield *et al.* (1963) have inferred that histidine 12 is either at or very close to the active

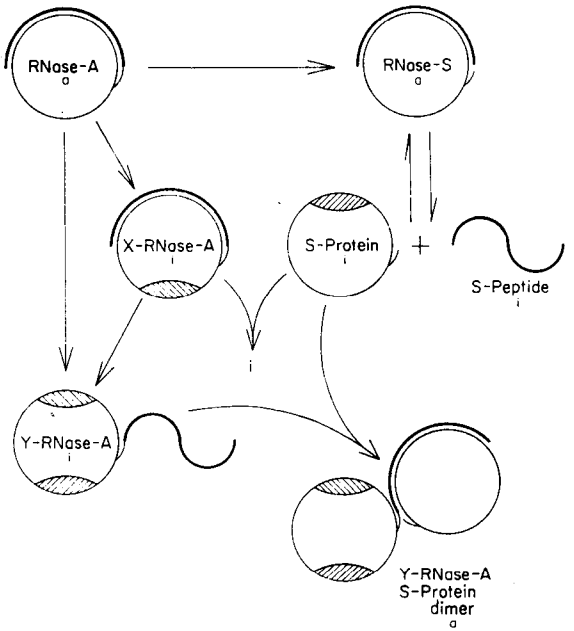

FIGURE 2. Schematic diagram of the ribonuclease system. The irreversible conversion of RNase-A to RNase-S is shown at the top, followed on the right by the reversible dissociation of RNase-S into S-peptide and S-protein. Altered forms of RNase-A are shown on the left which either do not, or do, result in a loosening of the amino-terminal portion of the molecule. In the former case no interaction occurs with added S-protein. In the latter a dimer may be formed which regenerates catalytic activity. The various species are indicated as either enzymically active or inactive with the small letters a and i. The shaded areas are the regions of presumed structural alteration.

centre. It is interesting that an adjacent residue (methionine 13) can be so drastically modified without marked influence on the enzymic activity.

A schematic diagram of the ribonuclease system is shown in Figure 2. The reaction of the protein and peptide components shown on the right has just been discussed. The protein component can also be used to investigate the state of association of the amino-terminal portion of the native enzyme (Richards and Vithayathil, 1960). For example, treatment with strong alkali rapidly causes complete loss of activity in RNase-A and considerable disruption of the native conformation. If this denatured material is mixed with S-protein, full enzymic activity is restored, the "tail" of the opened molecule complexing with the undenatured S-protein. Other drastic modifications such as complete acetylation of amino groups or esterification of carboxyl groups give similar results. More subtle inactivation, however, may produce a

different effect. The fully guanidinated material described by Klee and Richards (1957), although itself inactive, will not regenerate activity in the presence of S-protein. Denaturation of the guanidinated preparation permits recovery of activity. A similar observation was made with the carboxymethyl histidine-119 derivative of Crestfield (Vithayathil and Richards, 1961b). In the latter two cases the absence of enzymic activity in the derivatives is not associated with any conformational change sufficiently drastic to free the amino-terminal portion of the molecule. This is evidence to confirm that the altered residues are a part of, or very close to, the active site of the enzyme.

In addition to chemical modification of the functional groups, attempts have been made to define, by degradation and by synthesis, that portion of S-peptide which is essential for binding and activity. Following the initial observations (Richards, 1958) on activity loss after tryptic or chymotryptic digestion, Allende and Richards (1962) made a more detailed study of the reaction with trypsin. Of the two susceptible bonds, 7–8 and 10–11, the latter is split much more rapidly and the two decapeptides can be obtained in fair yield. These peptides, either separately or combined, showed no ability to regenerate activity when mixed with S-protein. No interaction of any sort was detected at the usual assay concentration ($\sim 10^{-6}$ M). (Apparently some proteolysis of S-protein by trypsin can occur without abolishing the interaction with the peptide; however, the picture here is complicated.) A minor component in some ribonuclease preparations lacks the amino-terminal lysine and will give a 19 residue S-peptide otherwise identical with the normal one (Gordillo *et al.*, 1962). Two stages of the Edman degradation were applied to a diguanidinated derivative of S-peptide. The product showed substantial enzymic activity with little change in binding to S-protein (M. Doscher, unpublished results). Attempts to get smaller identifiable peptides by degradation from the amino terminal end with leucine amino peptidase have so far proved to be unsuccessful.

Recently Potts, Young and Anfinsen (1963) have studied the degradation of S-peptide with carboxypeptidase-A. The removal of the five carboxyl terminal amino acids yielded a fifteen residue peptide which still showed full activity when mixed with S-protein at a 1:1 molar ratio and assayed with RNA. Whether or not there is any change at all in binding constant cannot be stated with certainty since the differential binding of RNA biases the estimate of the protein–peptide equilibrium constant.

Figure 3 summarizes these degradative studies. The maximum number of required residues cannot be greater than those in positions 3 through 15.

Work on the synthesis of S-peptide has been reported recently by Hofmann *et al.* (1963), and by Rocchi, Marchiori and Scoffone (1963) and Marchiori, Rocchi and Scoffone (1963). The complete 20 residue unit has not yet been assembled but interaction has been observed between S-protein and some of the smaller pieces of S-peptide. Some of this work is summarized in Figure 3. It is seen that a peptide containing residues 1 through 13 is capable of regenerating activity with the protein component although the binding constant is considerably reduced. Comparing this result with that of Potts

et al. (1963), it appears that residues 14 and 15 play an important role in the binding but are not directly connected with the activity of the complex.

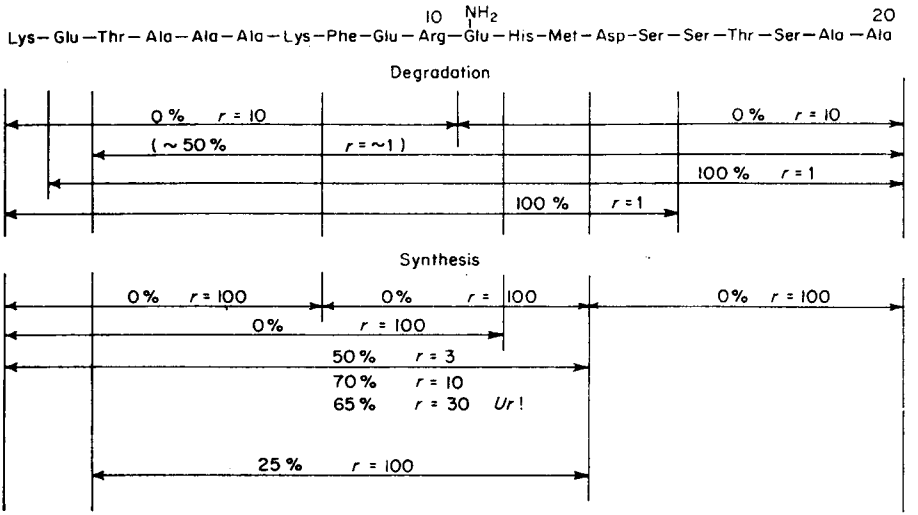

FIGURE 3. Summary of data on the fragments of S-peptide obtained either by degradation or synthesis. The individual peptides were assayed with a fixed quantity of S-protein. The numbers indicate activity observed as a percent of that shown by an equivalent quantity of RNase-S. The symbol *r* gives the molar ratio of peptide fragment to S-protein used in the assay mixture. The substrate was ribonucleic acid unless otherwise indicated. (Ur! = cyclic uridylic acid.) Estimates of the peptide–protein binding constants can be obtained from data on activity as a function of *r*. In general, structural changes in the peptide may be expected to alter the binding constant or the catalytic activity of the complex or both. Literature references to the various studies are given in the text.

A 13 residue peptide has also been produced in another degradative study employing cyanogen bromide cleavage of the native enzyme (Parks, Baranick and Wold, 1963). The carboxyl-terminal residue in this case is homoserine lactone rather than methionine. High activities are obtained for the complex with S-protein but the binding constant appears to be lower than with the methionine-containing tridecapeptide. This observation is further evidence of the importance of methionine 13 in providing an important part of the binding energy for the peptide–protein interaction.

REACTION IN MICELLES

The low reactivities of methionyl and certain tyrosyl residues in ribonuclease are examples of the masking of functional groups, a phenomenon found in many proteins. A commonly offered explanation is that such groups are buried in "hydrophobic regions in the interior of the macromolecule." The

implication of the statement appears to be that the kinetic parameters are altered by the hydrocarbon environment, by steric effects or by both. Micelles provide small hydrocarbon volumes which have dimensions comparable to those of macromolecules and are dispersed in aqueous solution. In principle, one could alter the kinetics of a bimolecular reaction simply by separation of the reactants, one being excluded from the micelle and the other strongly absorbed. The most clear-cut examples of this effect come from systems where one of the reactants has a formal charge of the same sign as that of the micelle. Thus, complex formation between divalent copper ions and a neutral porphyrin is strongly inhibited in the presence of a cationic detergent (Lowe and Phillips, 1961); the reaction of 1-fluorodinitrobenzene (FDNB) and glycylglycine is restricted to the aqueous phase in the presence of sodium dodecyl sulphate since the amine anion is excluded from the micelle (Herries *et al.*, 1964). For these two examples, the converse effect, rate enhancement, is observed if micelles of opposite charge are used. Less easily explained is the lack of kinetic change for systems where the two reactants are uncharged even though the partition coefficients are very different. The free base form of glycineamide is polar enough to be excluded from the micelle interior while FDNB partitions strongly in favour of the micelle. From a reaction in pure aqueous solution to one in a detergent concentration such that 75% of the total FDNB is absorbed to the micelles, there is a change in reaction rate of less than 5% (Herries *et al.*, 1964). If one assumes that the reaction occurs on the surface of the micelle, then some fortuitous cancellation of effects must be invoked to explain the lack of detectable effect on the reaction kinetics. Since charge separation is involved, the rate in the non-polar interior region would almost certainly be much lower. The available evidence on this subject is insufficient to provide a clear picture of all the factors involved.

So far these studies have reinforced the general observations made on the modification of proteins. A particular amino acid residue may exhibit strong interaction with other parts of the protein and contribute to the stability of the native structure; a functional group located on this residue may or may not have altered chemical reactivity depending entirely on the nature of the local environment and of the reagent used.

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DISCUSSION

A. P. MATHIAS: Dr. Richards has found that ribonuclease is active in the crystalline state. Has it been possible to observe any conformational change when the substrate diffuses into the crystal?

F. M. RICHARDS: Yes, we have studied the activity of ribonuclease-S in the crystalline state. The major part of the work by M. Doscher on this problem has appeared in the *Journal of Biological Chemistry* in July 1963. The general qualitative conclusion is that the molecules in the crystal lattice interior to the surface are, in fact, catalytically active. One can arrange a crystal on the X-ray camera in such a position that it is completely immersed in a liquid which can be flowed over the crystal while the diffraction pattern is being observed. When substrate (cytidine 2′,3′-phosphate) is added to the liquid, the intensities of some of the X-ray reflections are changed with little or no changes in unit cell dimensions. The intensity changes were reversed when the substrate was washed out of the crystal. The significance of these intensity changes will probably remain unclear until the complete three dimensional structure of the enzyme is known. Speculation on the conformation changes during catalytic activity is unwarranted on the basis of these observations alone.

In addition to RNase-S we have looked at crystalline carboxypeptidase-A and rabbit muscle aldolase. The first appears to be catalytically active and the second not in the crystals with which we are working. The lack of activity in the aldolase crystals is attributed to the great sensitivity of the reaction to ionic strength rather than to any effect of the crystal lattice. Mr. Florante Quiocho is attempting to work out the kinetic parameters of crystalline carboxypeptidase-A for comparison with the values in solution. Water or dilute salt solutions which can be used with carboxypeptidase are much more convenient solvents than the very concentrated ammonium sulphate solution required to keep the ribonuclease crystals from dissolving.