Meeting Report

STRUCTURAL ASPECTS OF ENZYMATIC ACTIVITY

A REPORT OF THE BRITISH BIOPHYSICAL SOCIETY MEETING HELD IN LONDON ON 17 AND 18 DECEMBER 1968

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1. Introduction

The British Biophysical Society's timely meeting on "Structural Aspects of Enzymatic Activity" demonstrated the exciting complementation between recent X-ray crystallographic results and chemical experiments which has provided some remarkable advances towards the understanding of enzymatic activity. The structures which were discussed in detail included ribonuclease, chymotrypsin, elastase, carboxypeptidase, papain and lysozyme. With perhaps the exception of papain, the three-dimensional structures of these enzymes and their inhibitor-complexes have revealed the stereochemical interactions which provide the substrate specificity and the precise location and orientation of the groups in the active site. This information concerning the very special environment of the amino-acids at the active site has provided, in turn, a rationalisation for the properties of those groups involved in catalysis and an explanation for the crucial steps in the enzymatic action. Indeed because the enzyme action takes place at a special site whose structure and that of the enzyme-substrate complex can be probed, statically by X-ray crystallography and kinetically by relaxation spectrometry, it seems that enzyme mechanisms are becoming rather more easy to understand than those reactions catalysed in solution in the model systems of physical organic chemistry. However, as Professor C.A.Vernon (University College, London) pointed out in his stimulating lecture, the elucidation of any catalytic mechanism must include the determination of the structures of the intermediates of the mechanistic pathway and an understanding of the factors which affect the rates at which these intermediates convert. Although several of the enzymes mentioned above are rapidly approaching this stage, lysozyme remains the enzyme, at the present time, for which a catalytic mechanism has been elaborated in greatest structural detail.

2. Lysozyme and α-lactalbumin

On the basis of the crystallographic evidence and chemical data available three years ago, it was suggested that the hydrolysis of the $\beta(1-4)$ glycosidic linkage of the polysaccharide substrate of lysozyme proceeds through general acid catalysis by the acid residue GLU 35, resulting in the formation of a carbonium ion intermediate which is stabilised by another acid residue, ASP 52, which is in the carboxylate state. The distortion of a sugar residue of the substrate to a conformation which resembles the transition state of the catalysed reaction forms an important contribution to the rate enhancement. At the meeting Professor D.C.Phillips (Oxford) surveyed the crystallographic hypothesis in the light of recent chemical experiments. The specificity of the enzyme, which involves six saccharide binding sites with the distortion of the fourth sugar ring, has been confirmed by a number of binding experiments in solution and the involvement of the acid residues, GLU 35 and ASP 52, have been indicated in some elegant chemical modification studies. With regard to the

mechanism, Professor Phillips stressed the difficulties involved in designing definitive experiments. However, the recent experiments of Raftery which have involved the replacement of the hydrogen at the C_1 position with deuterium and those of Rupley in which the transglycosylation reaction has been studied in the presence of alcohols and their corresponding sulphur compounds, strongly indicate that the mechanism probably proceeds through a carbonium ion rather than a covalent intermediate. The *N*-acetyl side chain of the substrate does not appear to be essentially involved in the catalytic mechanism, although its participation has not been conclusively eliminated. The complex interaction between lysozyme and the cell wall substrate and the dependence of this interaction on ionic strength and pH have been considerably clarified by the recent experiments of Drs. R.C.Davies and B.M.Wilson (St. Mary's Hospital Medical School), which were reported at the meeting, and which again show no conflict with the proposed mechanism. In summary, it appears that these recent elegant chemical experiments support and confirm the crystallographic hypothesis. It is interesting to note that the role of distortion in the mechanism of action of lysozyme may be pertinent to the action of other glycosidases, such as *N*-acetyl-glucosaminidase and β-glucuronidase for which it is known that lactones, that are related to the substrate and which possess a similar structure to the postulated carbonium ion intermediate, are powerful inhibitors.

Dr. A.C.T.North (Oxford) discussed the relevance of the structure of lysozyme to α-lactalbumin. Homologies in sequence had enabled the construction of a hypothetical but highly plausible molecular model on the basis of the lysozyme structure, and this model was now undergoing crystallographic investigation. α-Lactalbumin is involved, together with a second protein termed the A-protein in the synthesis of lactose and initially, the functional basis for similarities in sequence and proposed structure between α-lactalbumin and lysozyme appeared temptingly obvious since one protein was involved in a reaction leading to the synthesis of a β(1–4) glycoside linkage and the other in the hydrolysis of such a linkage. However, the recent work of Brew, Vanaman and Hill has shown that the role of α-lactalbumin in lactose synthesis is somewhat less direct than was at first thought. The protein appears to act as a substrate-modifier which changes the specificity of the A-protein in such a way as to inhibit the synthesis of galactosyl-*N*-acetyl-glucosamine and to favour the synthesis of galactosyl-glucose.

3. Ribonuclease

The two papers on the enzymatic mechanism of ribonuclease demonstrated a very satisfactory agreement between the chemistry and the crystallography. Professor B.R.Rabin (University College London) summarised the relevant chemical evidence, most of which had been obtained some years in advance of the crystallography. The hydrolysis of RNA is known to proceed in two chemically separable steps, the first of which involved the formation of a 2′,3′-cyclic phosphate derivative of a pyrimidine nucleoside. This is hydrolysed in the second step to yield a pyrimidine nucleoside 3′-phosphate. Alkylation and kinetic studies demonstrated that two histidine residues (12 and 119) are part of the active centre and that they are involved functionally, one as an acid and the other as a base. In the first step it was suggested that the base residue abstracted a proton from the 2′-hydroxyl and the acid residue protonated the leaving oxygen of the P–O bond undergoing cleavage. Preliminary crystallographic results obtained nearly two years ago had confirmed the proximity of histidines 12 and 119 at the active site but the feasibility of a concerted acid-base type of mechanism was not immediately apparent. The recent work of Professor F.M.Richards and Dr. H.W.Wyckoff's group at Yale, which was described at the meeting by Miss N.Allewell, has clarified the situation considerably. The crystallographic data have been extended from 3.5 Å to 2 Å resolution, which has resulted in a more precise location of the amino-acid side chains, and studies on the structures of a number of inhibitor and substrate-analogue complexes have revealed the interactions involved in the inhibitor binding. The electron density map of the complex with cytidine 3′-monophosphate, for example, shows the binding of the pyrimidine ring to be stabilised by hydrophobic interactions and three hydrogen bonds which are rather similar to those involved in the structure of DNA. The 2′-hydroxyl of the ribose ring is hydrogen bonded to HIS 12. These features explain the specificity of ribonuclease

towards pyridimine nucleosides and various substituted pyrimidines. The electron density for the phosphate group is, surprisingly, not very definite and this is thought to arise from the displacement caused by the phosphate of HIS 119 which moves outwards towards an aspartic acid residue, ASP 121. The flexibility of HIS 119, observed also in the binding of arsenate and at high pH, has been the key to the understanding of the crystallographic results. With a substrate bound in this position the action may well proceed through concerted acid-base catalysis involving histidines 12 and 119, as predicted.

It was also reported that some additional binding force may be obtained from interaction between LYS 41 and the phosphate group. The separation of these groups is rather large for hydrogen bonding, and it appears that the electrostatic interaction is unlikely to interfere with the formation of the pentacoordinated phosphate intermediate with the attacking and departing groups in the apical positions of a trigonal bipyramid. The formation of such an intermediate would be very favourable in the light of recent work by Westheimer and his colleagues since the groups in the apical positions would both be protonated and thus strongly electronegative and the cyclic phosphate ring would span one apical and one basal position. The next step for the crystallographers is to determine the position of the second nucleotide binding site, which has proved difficult so far, possibly due to the lack of specificity of the enzyme at this site. We eagerly await their new results which should help to clarify the tentative proposals for the mechanism and may confirm yet another chemical result which had indicated that a proton donating group, possibly LYS 7, may interact with a purine ring of the nucleoside providing the 5′-oxygen of the bond undergoing cleavage.

4. The serine proteinases

The active site of chymotrypsin and a number of other proteinases, such as elastase, trypsin, thrombin and subtilisin, have been known for a long time to contain a particularly reactive serine residue, SER 195 in chymotrypsin. From the most recent work of Dr. D.M.Blow's Cambridge team on α-chymotrypsin, the reactive nature of this particular amino acid can now be explained in terms of tertiary structure. Mr. R.Henderson described how an improved electron density map has allowed a more definite assignment of hydrogen bonds. In particular, the side chain of the active site histidine (HIS 57) is hydrogen bonded both to SER 195 and a buried acid group, ASP 102. The hydrogen bonding scheme allows the transfer of the negative charge from the acid group to the serine which can thus act as a powerful nucleophile in the acylation step of the enzymatic mechanism. It was interesting to hear from Mr. Henderson that the conformation adopted by the polypeptide chain in the region of the active serine can only tolerate a glycine residue at certain positions. This perhaps explains the conservation of the sequence GLY-ASP-SER-GLY in serine proteinases since the glycines as well as the aspartic acid, which forms a charge-charge interaction with an α-amino group, may be important in maintaining the serine in the correct conformation. Although several mechanistic pathways have been proposed for the action of chymotrypsin, the crystallographers prefer not to commit themselves until they have obtained results for a longer oligopeptide inhibitor molecule than the formyl-tryptophan-complex which has so far been studied.

A highlight of the conference was the description by Mr. D.M.Shotton of the remarkably swift and beautiful determination of the structure of tosyl-elastase at 3.5 Å resolution carried out at Cambridge and Bristol by Dr. H.C.Watson and himself. The general conformation and orientation of the side chains are very similar to chymotrypsin and this was shown to be a consequence of the high degree of homology (78%) between those residues which occupy internal sites. This is considerably higher than the overall homology between elastase and chymotrypsin A, chymotrypsin B and trypsin which is only 37%. The same conservation of interior sites has already been shown to be an important factor in the stabilisation of the structure of myoglobins and haemoglobins. The different substrate specificities of elastase and chymotrypsin was shown to arise from small changes of the amino acids in the vicinity of the substrate side chain binding site. In particular, the substitution of a glycine in chymotrypsin with a valine in elastase blocks the entrance to the pocket in which the tosyl group binds in chymotrypsin. The different mode of binding the tosyl group required in elastase apparently

causes a displacement of the active site histidine and disruption of the hydrogen bonding scheme. Hence, although it is probable that the same charge relay system for the activation of the serine operates for elastase, this cannot be stated explicitly until the structure of native untosylated elastase is determined. Those who were present at the Royal Society Meeting the previous week, heard there from Professor J.Kraut (University of California) that bacterial subtilisin BPN', although differing considerably in structure from chymotrypsin, nevertheless contains a similar charge relay system for the activation of the serine. This represents a remarkable example of convergent evolution which has arrived at the same mechanism within different molecular frameworks.

5. Carboxypeptidase

Of the many beautiful structural features of carboxypeptidase which have been revealed by the X-ray crystallographic results of Professor W.N.Lipscomb's group at Harvard, perhaps the most exciting are the large conformational changes in the catalytic groups observed on binding substrate, which provide the clearest example so far of Koshland's induced fit theory of enzyme mechanism. These results were described at the meeting by Dr. T.A.Steitz. The substrate molecule glycyl-tyrosine is shown to be bound with its aromatic group in a pocket on the enzyme and its carboxylate group forming a salt bridge with the guanidinium group of ARG 145. In order to make this link the arginine moves about 2 Å. The shift causes the disruption of the hydrogen bonding scheme in this region and appears to trigger off the 12 Å motion of the hydroxyl group of TYR 248 which swings down to hydrogen bond with the substrate. The importance of the carboxyl group in this reorientation process is demonstrated by the finding that the binding of lysyl-L-tyrosine-amide does not give rise to these conformational changes. The carbonyl oxygen of the dipeptide substrate is not clearly seen, due to the displacement of a water molecule, but its position has been inferred from the location of the rest of the substrate and shows it to be co-ordinated to the zinc atom. The zinc thus serves a two-fold function. Firstly, it orientates the substrate and secondly it acts as a Lewis acid polarising the carbonyl bond of the substrate. It would also, of course, tend to stabilise the formation of a tetrahedral intermediate in the reaction. The mode of binding of glycyl-tyrosine in the crystal appears to be non-productive owing to a hydrogen bond between the α-amino group of the substrate and an acid residue, GLU 270. Model building studies have indicated that in the productive binding of a longer oligopeptide this hydrogen bond is no longer made. Once again, in spite of the very detailed features shown by the crystallography, it is difficult to distinguish between several possibilities for the mechanism. Those favoured by the crystallographers include general acid-nucleophilic attack involving the hydroxyl of TYR 248 as an acid and GLU 270 as a nucleophile with the intermediate formation of a mixed anhydride. A second possibility is that tyrosine 248 acts as an acid and glutamate 270 as a base, promoting the reactivity of water as an attacking nucleophile. It is of interest that an almost identical mechanism was suggested for other metal peptidases in 1958 and there exists the exciting possibility that these groups of enzymes may act by a common mechanism. This second possibility would seem more reasonable in view of the fact that carboxypeptidase does not catalyse transpeptidation reactions. Moreover a mechanism involving the formation of an anhydride with GLU 270 would require tyrosine 248 to act as a general base in the anhydride hydrolysis and this seems unlikely in view of the high pK of this group. The possible function of water or a hydroxyl ion co-ordinated to the zinc was also discussed.

6. Papain

Crystallographic and chemical studies on papain appear to be proceeding simultaneously. Dr. J.N. Jansonius described the results of the structure determination at 2.8 Å resolution by the Groningen group which has confirmed the chemical result of the proximity of a histidinyl and a cysteinyl residue at the active site. However, any further interpretation of the mechanism from crystallographic data awaits inhibitor and substrate binding studies which are now underway. Dr. L.A.AE.Sluyterman (Eindhoven) discussed the recent chemical experiments which elegantly elucidate the rate limiting steps for some substrates in

the reaction, which proceeds through an acyl-thiol intermediate, and also reported that the enzyme was fully active in the crystal. Possible interpretations of the pH rate profile of papain hydrolysis in terms of particular groups involved in the catalysis caused much discussion and those non-biochemists in the audience were grateful for Dr. H.Gutfreund's remarks on the limitations of this type of study. He stressed the need to examine the crystallographic structure and the special surroundings of the particular groups in order to interpret the kinetics. It was also pointed out that where more than one ionising group is involved in the catalysis the kinetically determined ionisation constants cannot be unambiguously assigned to individual ionising entities but are properties of the ionising system as a whole. The marked effect of the environment of the protein molecule on the behaviour of certain groups was emphasised again in a paper on carbonic anhydrase. Dr. R.J.P.Williams (Oxford) reported that, as yet, no model systems had been devised which gave the same ligand field spectra as cobalt (II) carbonic anhydrase in the alkali region.

7. Oligomeric enzymes

The meeting ended appropriately with a session on oligomeric enzymes – a subject which forms the next target for attack by X-ray crystallography. Already crystallographic work at 5 Å resolution on lactate dehydrogenase (A.J.Wonacott, Purdue) has revealed a shift in the subunits of the molecule of about 5 Å on association with co-enzyme while rapid reaction techniques (H.Gutfreund, Bristol) are yielding information concerning the kinetic framework for the consecutive steps in the reaction. Haemoglobin has, of course, for a long time remained our classic example of an oligomeric protein. Dr. M.F.Perutz gave a fascinating report of his investigation of the stereochemical consequences resulting from deletions or amino acid replacements of many different mutant haemoglobins. Examination of the molecular model, derived from X-ray analysis at 2.8 Å, showed that the molecule is insensitive to replacements at most amino acids on its surface but very sensitive to even quite small alterations of internal non-polar contacts, especially those near the haems. Replacements at the contacts between α and β subunits result in severe alteration of respiratory function. This report and those from the meeting in general indicate that, in the not too distant future, not only will the catalytic mechanisms of the enzymes involved in the different reactions of living organisms be understood, but also the consequences of inborn errors of metabolism.

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