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Chymotrypsin: Tertiary Structure and Enzymic Activity

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In addition to the electron-density map of α-chymotrypsin at high resolution (Sigler, Blow, Matthews & Henderson, 1968) electron-density maps have been prepared at lower resolution for chymotrypsinogen (Kraut, Sieker, High & Freer, 1962), and for π-, δ- and γ-chymotrypsins (Kraut, Wright, Kellerman & Freer, 1967; Matthews, Cohen, Silverton, Braxton & Davies, 1968). The process leading from the zymogen to the various forms of the enzyme involves no major refolding, and the activation step appears to depend on the insertion of the α-amino group of Ile-16 into an interior position in the molecule where it can interact with the β-carboxylate group of Asp-194. It is proposed that when Asp-194 is drawn into the interior, the active-centre residues, including His-57 and Ser-195, are brought into the correct stereochemical relationship.

The crystals have been shown to exhibit specific chymotryptic activity, though at reduced rates, despite the fact that the crystals are only stable up to about pH6·3. In attempts to discover the mode of binding of the substrate, the binding positions of *N*-formyl-*p*-iodophenylalanine and of *N*-formylphenylalanine have been studied in three dimensions. A detailed study has been made of the differences of structure between the native enzyme and the tosylated enzyme.

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Mechanisms of Enzyme-Catalysed Hydrolysis Reactions: Present Status and Outstanding Problems

By H. Gutfreund. (*Department of Biochemistry, University of Bristol*)

The exciting developments in the three-dimensional structure analysis of enzyme molecules have confronted the enzyme mechanic with reality. In the case of some enzymes, the combination of suitable substrates with techniques that permit the resolution and characterization of individual enzyme–substrate compounds, the approach of modern chemical kinetics has proved itself dominant in the elucidation of mechanisms and in the design of meaningful crystallographic and chemical experiments. In other cases, such as lysozyme (Blake *et al.* 1967) and carboxypeptidase (Reeke *et al.* 1967), studies of the reactions in solutions gave only limited results before the concrete information obtained from crystallographic studies. In these latter systems, the structural data are the ones that show the way to the design of meaningful chemical and kinetic experiments.

Here I am mainly concerned with enzyme systems that have been fruitfully studied by the methods of chemical kinetics. The hydrolytic enzymes that fall into this group are those that have either a specially reactive serine hydroxyl group or a cysteine thiol group at the active site. These two large groups of enzymes probably have a number of important common features. One must be aware of the dangers of generalizations, but one should not forget the special importance of phenomena that are found to occur widely. I shall discuss only three enzymes of 'serine active site' group in any detail and we shall try to learn our lessons from these: chymotrypsin, trypsin, and alkaline phosphatase from *Escherichia coli*.

One important lesson that can be learned from studies of these three enzymes is that the use of over-simplified methods can provide a great deal of invaluable information, but becomes dangerously misleading if not rapidly abandoned at the right time. I am referring to the use of pseudo-substrates and the extensive interpretation of steady-state kinetics. The first of these is probably the major problem of the future. We must obtain information about the interaction of the natural substrates with hydrolytic enzymes. The specific conformation and reactivity of the enzyme–substrate complex is surely dependent on the specific substrate used. Yet nobody will deny that we have learned a lot about hydrolytic enzymes from studies with pseudo-substrates, and even with reagents that bear no structural relation to the substrate. We are, however, at the point of making dangerous extrapolations from these studies. This major problem of substrate specificity in the widest sense will benefit most from complementary studies between crystallography and chemical kinetics.

In the area of reaction mechanisms for the three enzymes mentioned, we have been faced with the problem that one of the principal arguments involved in the characterization of acyl and phosphoryl enzyme intermediates by steady-state

kinetics is wrong, is still widely used and leads to quantitative results that are misleading. This opens up the second major problem to be solved in detail, which is closely related to the first one: the response of the enzyme to interaction with their specific substrates. The problem can be most clearly stated by a discussion of the hydrolysis catalysed by *E. coli* phosphatase. Phosphate esters with widely different leaving groups are hydrolysed at identical rates. Phosphoryl enzyme can be shown to occur as a reaction intermediate, and the obvious explanation for the identical reaction rates was the hypothesis that the hydrolysis of the intermediate was rate-determining in the case of all the substrates. Rapid-flow techniques have enabled us (Trentham & Gutfreund, 1968; Fernley & Walker, 1966; Aldridge, Barman & Gutfreund, 1964) to show that at optimum pH it is, in fact, the phosphorylation of the enzyme that is rate-determining and identical with a range of substrates. This indicates that there is some step between the Michaelis complex and the phosphoryl enzyme common to the reaction of different substrates, which is independent of the leaving group. The response of the enzyme to form the reactive intermediate could provide some explanation for this.

With trypsin and chymotrypsin, a series of experiments with chromophoric substrates led us to the conclusion that these enzymes too formed another significant intermediate between the Michaelis complex and formation of the acyl enzyme (Barman & Gutfreund, 1966; Bernhard, 1968). These findings will be elaborated in relation to the exploration of the reactivity of the active site. The fact that during amide and peptide hydrolyses, none of the enzyme intermediates, except the initial Michaelis complex, occur at a significant concentration has to be borne in mind. Only carbonyl derivatives of specific amino acids with good leaving groups have so far permitted the detection of other intermediates.

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COMMUNICATIONS

The Protection of Peptides from Biological Sources for Use in the Chemical Synthesis of Proteins

By T. A. Backer and R. E. Offord. (*Laboratory of Molecular Biophysics, Department of Zoology, University of Oxford*)

A common approach to the chemical synthesis of polypeptide chains involves starting with amino acids having protected side-chain functional groups, and producing protected oligopeptides by stepwise condensation. These larger fragments are subsequently coupled to produce the final sequence.

Since many such projects involve the production of sequences close to those found in nature, we have investigated the protection of tryptic peptides of proteins for use in place of synthetic ones. The peptides were purified from digests of hen's-egg lysozyme, initially in batches of tens of milligrammes. Since most normal methods of protection were developed for free amino acids, it was not always clear whether they would cause peptide-bond cleavage or other side reactions in peptides, or even if they would work at all. The benzylation method of Cipera & Nicholls (1955), for example, cleaved labile peptides. However, benzylation with phenyldiazomethane (Sarin & Fasman, 1964) in ethanol–water (5:1, v/v) at pH4·5 (cf. diazomethane: Chibnall & Rees, 1958) gave seemingly quantitative conversion of the peptides studied into products with properties consistent with the benzylation their carboxyl function.

If protection is possible, it is necessary to free the terminal carboxyl and amino groups (to allow coupling to proceed) without unblocking the side-chain groups (which must remain protected throughout). This can be done if protection is applied to the whole protein before tryptic digestion, and it is this method that is being developed for side-chain amino protection (to be published). Although preliminary experiments show that this may be done for carboxyl groups also, it has been found easier to protect them at the tryptic-peptide stage. The terminal carboxyl only may be removed very rapidly by trypsin (in one case roughly 50%/min. at 37° with 1% of enzyme by weight) which is an esterase (Schwert, Neurath, Kaufman & Snoke, 1949; Kloss & Schröder, 1964) bound to cleave groups in such a position. It was essential to find a