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Enzyme Substrate and Inhibitor Interactions

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Enzyme substrate and inhibitor interactions

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[Plate 1]

An enzyme is designed to bind most tightly to a substrate when it is in the transition state of the reaction which the enzyme catalyses. The consequent reduction of the activation energy of the reaction constitutes the catalytic mechanism. The energetic contributions of different features of the interaction can only be crudely assessed, but they are dominated by entropically driven effects. The binding site of trypsin orients the substrate so that the reacting groups are correctly placed for reaction to occur. Apart from two side chains which take part in chemical steps of the reaction, the enzyme behaves almost as a rigid body. The full binding interactions are only developed when the substrate is in an intermediate stage of the reaction.

The tightly bound complexes of trypsin with protein trypsin inhibitors have proved amenable to structural analysis. Enzyme inhibitor interactions, which account for almost 80 kJ mol^{-1} of interaction energy, are known fairly accurately. The similarity of the two known trypsin inhibitor structures, close to the primary binding site, indicates a high specificity, even for this simple interaction. In cases where no large conformational changes occur the specificity of an enzyme should be predictable from accurate knowledge of its tertiary structure.

The notion that enzyme catalysis is a means of accelerating a reaction by reducing its activation energy has been current for a long time. Haldane (1930) showed how this implies that the binding site of an enzyme must fit to a form of the substrate intermediate between its original state and that of the reaction product. Pauling (1946) stated more directly that an enzyme must be designed to bind to the transition state of the reaction that it catalyses. These ideas were implicit in the proposals for the mechanism of lysozyme presented at a Discussion Meeting of this Society in 1966 (Blake *et al.* 1967).

Strain mechanisms, which consider distortion of the substrate towards its transition state by the enzyme, are most easily envisaged in terms of geometric strain, where the geometry of the substrate is altered (Eyring, Lumry & Spikes 1954; Jencks 1966). Geometric strain is only one of the mechanisms which can bring the configuration of the substrate to one which is closer to the transition state, and from which it is more likely to pass through the transition state. Another very important type of mechanism involves bond polarization, induced by the surrounding charge distribution, and especially by the possibility of proton transfer (Blow & Steitz 1970). Such a polarization occurs in the active site of lysozyme when the differing pK_a of two carboxyl groups allow them to be in different states of protonation (Parsons & Raftery 1970); it occurs in the 'entatic' environment of a metal ion bound to an enzyme like carboxypeptidase (Vallee & Williams 1968); and at the active site of the trypsin family of enzymes, where the charge relay system encourages proton transfer (Blow, Birktoft & Hartley 1969). In all of these cases, the unusual environments of the polar groups involved, evidently stabilized by the overall folding of the enzyme, favour a charge distribution on the substrate which will occur in the transition state of the reaction.

Proximity and entropic analysis

Intramolecular reactions are often very much more rapid than analogous reactions which occur between different molecules. This effect of 'proximity' has often been treated as essentially one of mass action, in which an 'effective concentration' of a reacting group in an intermolecular reaction is obtained by comparing a unimolecular rate constant with an analogous bimolecular rate constant. Page & Jencks (1971) have shown how such rate accelerations can be considered in terms of additional order which must be imposed on the system to allow it to reach the transition state. If two molecules are to react, they must first come together in the right way, with a loss of freedom in their relative translational and rotational motions. If, on the other hand, a substrate is already immobilized on the surface of an enzyme, there is much less further order to be imposed before a reaction step can take place. It may only need a simple rotation about a single bond, for example, and may be many powers of 10 more likely than the uncatalysed reaction.

The great importance of this approach is that it brings the proximity factors and the strain factors into the same conceptual framework. In order to reach the transition state, both the enthalpy and the entropy of the system must alter. Together these factors account for the free energy of activation of the reaction. An enzyme catalysed reaction will be accelerated by a reduction of this free energy of activation, whether the enthalpic or the entropic component. Jencks & Page (1972) show that in enzyme reactions the entropic factor is overwhelmingly important.

The main loss of mobility of a substrate occurs when it binds to the enzyme, forming a Michaelis complex, before any chemical change has taken place. A substrate binding site is folded so that there is a release of free energy when the substrate binds to it. This means that it must be in a state of relatively high free energy in the absence of substrate. The folding of the enzyme molecule results in a structure which can release energy when the substrate is bound.

The binding site in the trypsin family of enzymes

At the active site of chymotrypsin is a hydrogen-bonded system composed of the side chains of aspartic acid 102, histidine 57 and serine 195. The aspartic acid side chain is buried, polarizing the histidine side chain at the enzyme surface, and inducing some negative charge on the histidine nitrogen adjacent to Ser 195. This 'charge relay' system would facilitate the transfer of a proton from Ser 195 to His 57 (Blow *et al.* 1969).

Although the importance of the charge relay system is emphasized by its independent occurrence in the enzyme subtilisin (Alden, Wright & Kraut 1970), various lines of reasoning indicate that the major contribution to the catalytic activity of trypsin and chymotrypsin comes from the precise orientation of the scissile bond achieved by the enzyme's binding site (Fersht & Sperling 1973) The primary binding site, a pocket which can accommodate a lysine or arginine side chain in trypsin, or a flat, hydrophobic side chain in chymotrypsin, defines the positions of the C^{β} and C^{α} atoms of the side chain fairly precisely (Steitz, Henderson & Blow 1969; Stroud, Kay & Dickerson 1974). Hydrogen bonds from the NH of this amino acid residue (to CO 214 on the enzyme) and to its CO (from NH 193 and NH 195) fix the scissile bond precisely (figure 1).

The mechanism of these enzymes involves two acyl transfer reactions, in which the 'leaving group' of the scissile bond is attacked first by the oxygen of the enzyme's active serine, and

secondly by a water molecule (Bender & Kézdy 1964; Fastrez & Fersht 1973). In each of these steps, the trigonally coordinated carbon of the scissile bond is attacked by an oxygen ligand which approaches almost perpendicularly to the plane of the bond, passing through a tetrahedral form (Henderson 1970; Henderson, Wright, Hess & Blow 1971). The transition state of each step of the reaction is thought to be close to the tetrahedral conformation. In each step a proton needs to be stored temporarily, and the charge relay system makes His 57 favourable for this purpose. We may say that the charge relay system stabilizes the charge distribution at the transition state.

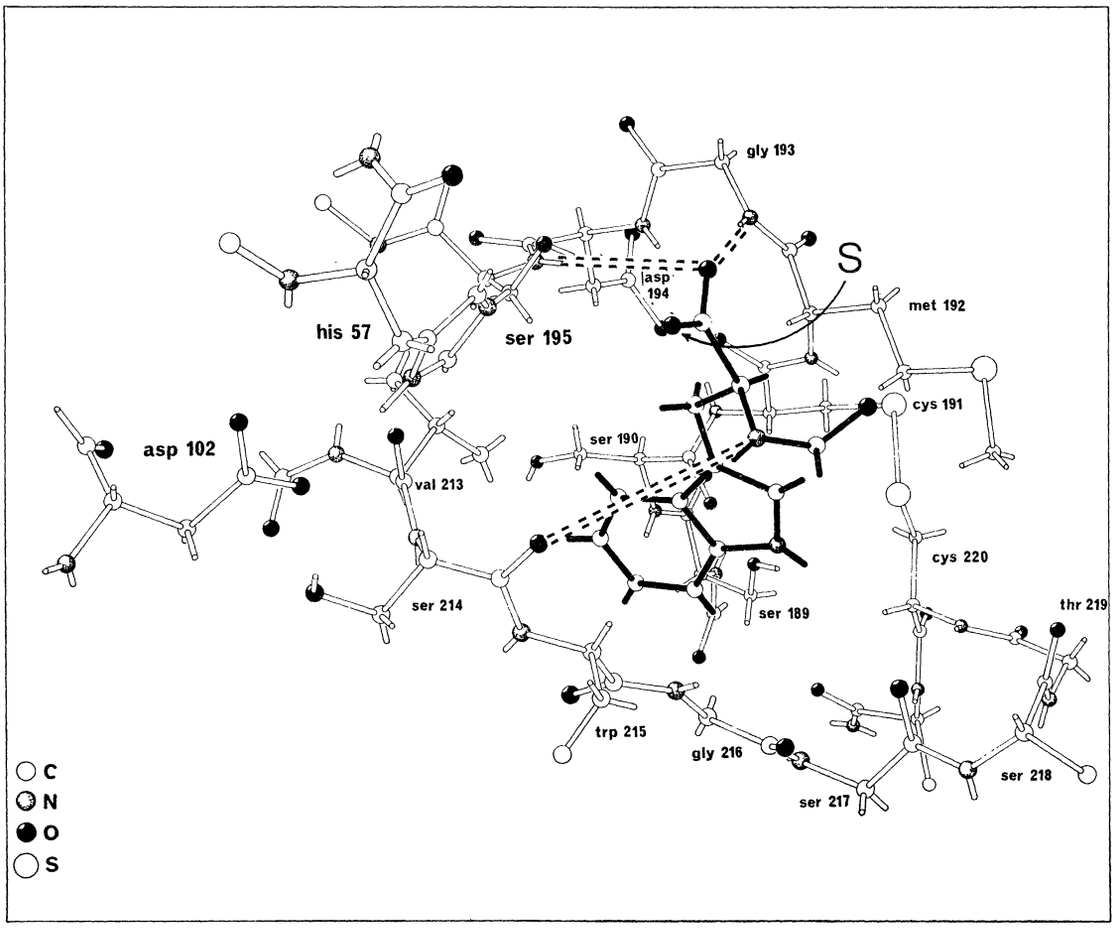

FIGURE 1. Binding of formyl-L-tryptophan to chymotrypsin as determined crystallographically (Steitz *et al.* 1969). The stabilizing hydrogen bonds are indicated. In a peptide substrate, the scissile peptide bond would replace the oxygen atom marked S.

The atoms involved in the chemical steps of the reaction are arranged so the reaction can proceed with a minimum of atomic movements (figure 2). The entropy of activation of the various reaction steps is thus very low.

A further contribution to catalysis of the first step of the reaction (the acylation step) for peptide substrates comes from the interactions between enzyme and the part of the substrate which is displaced in this step (the leaving group). This is the rate-limiting step for the natural amide substrates, but not for ester substrates. Fersht, Blow & Fastrez (1973) showed that in the reverse reaction, deacylation by amines, amino acid amides act far more effectively in

deacylation than water or even hydroxylamine. As there was no kinetic evidence for favourable binding of the amino acid amide, this effect again has to be interpreted as transition state stabilization. We were able to interpret it from accurate model building studies, based on the binding of the pancreatic trypsin inhibitor (Blow *et al.* 1972), as follows.

There is a hydrophobic binding site for an amino acid side chain of the leaving group, against the two sulphurs of a cystine bridge (Cys 42–58), and on top of the indole ring of His 57. This can only accept L amino acids. The hydrophobic contacts are mainly with C^{α}

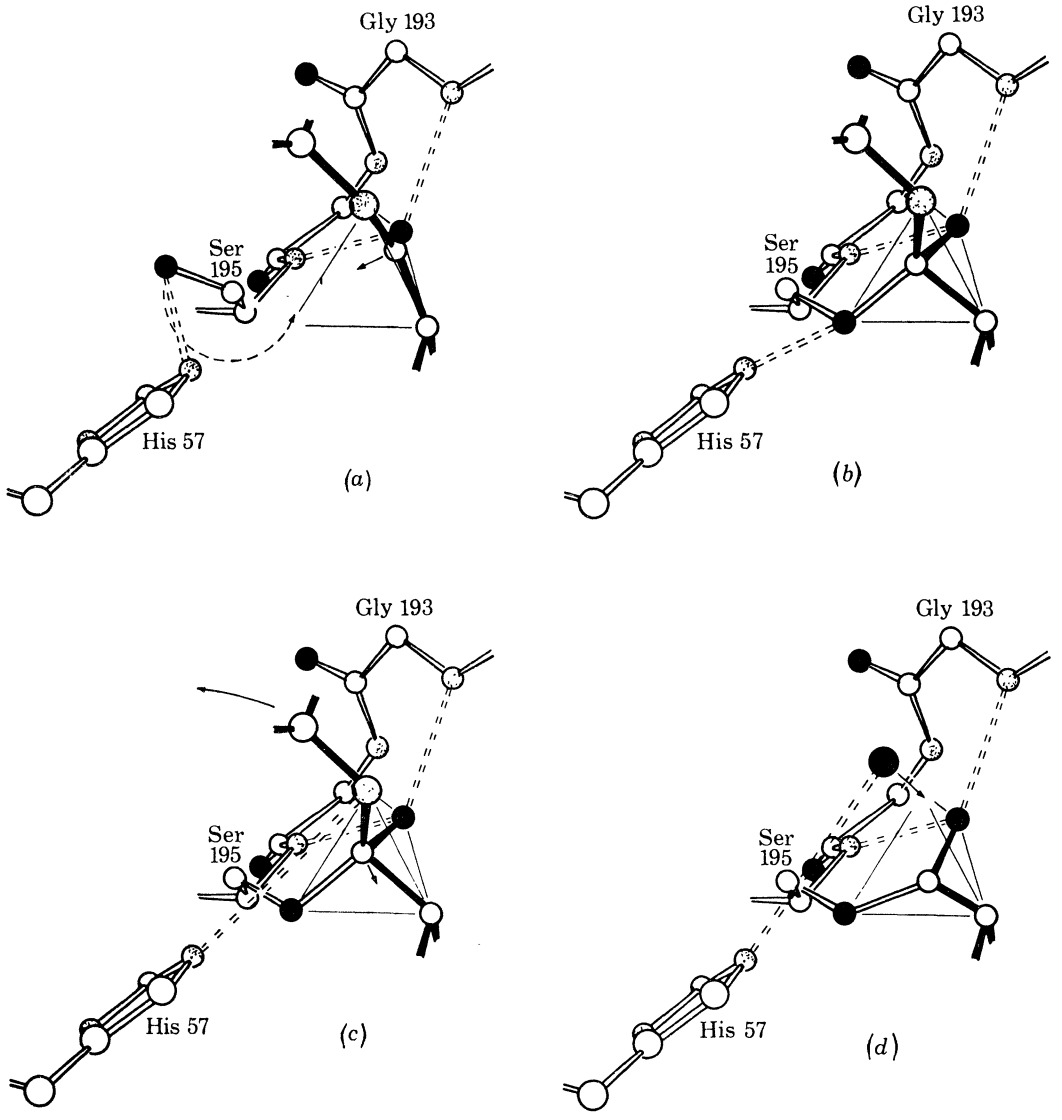

FIGURE 2. Steps in the formation of the acyl enzyme, illustrating the geometrical relationship between the attacking groups. Bonds between atoms in the peptide substrate are indicated in black. (*a*) Rotation of serine oxygen to attack the carbonyl carbon of the substrate, which moves into a tetrahedral conformation. This must be accompanied by some displacement of His 57 to avoid steric hindrance. (*b*) The tetrahedral intermediate, as observed in the inhibitor complexes. (*c*) The hydrogen bond from the displaced His 57 is transferred from the serine oxygen to the peptide nitrogen of the substrate. The leaving group is then expelled. (*d*) The tetrahedral carbon then moves into the plane defining the ester group of the acyl enzyme. The leaving group is replaced by a water molecule, which can move in to attack the ester group, initiating the deacylation process.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

and C^{β}, so virtually any L amino acid can be accommodated. However, to place a peptide with one side chain in the primary binding site, and the next making these contacts, results in overcrowding at the active site, as indicated in figure 3. These bad contacts would be relieved by the movement of O^{γ} (Ser 195) which is required for formation of the tetrahedral intermediate. On going past the intermediate, when the peptide bond is broken the two fragments must move apart, which again destroys the optimal contacts. Proper binding can thus only be achieved when the complex is close to the tetrahedral form. It is interesting that the overcrowding is only a matter of a few hundredths of a nanometre, indicating that the enzyme must be rather exactly rigid for there to be any effect. It also means that we might not have trusted the accuracy of our coordinates without independent confirmation from the structures of trypsin inhibitor complexes.

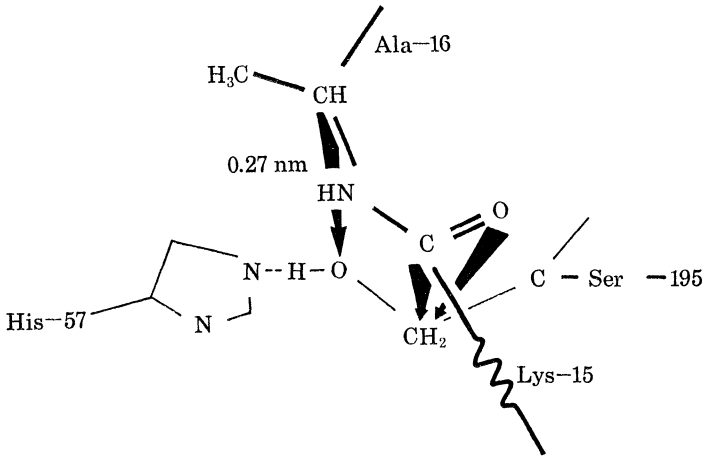

FIGURE 3. Overcrowding found in a model of the complex of pancreatic trypsin inhibitor with chymotrypsin proposed by Blow *et al.* (1972). The broad black arrows represent contact distances of 0.28 nm or less, which indicate that the serine oxygen must move towards its position in the tetrahedral intermediate before the full intermolecular contacts can be developed. (Fersht *et al.* 1973.)

ACCURACY OF ORIENTATION

A variety of 'locked' substrates of chymotrypsin exist, which can assume conformations consistent with the conformation of simple substrates observed crystallographically (Blow 1971; Kenyon & Fee 1973). Such substrates give information relevant to estimating the entropy of the transition state, and to answer the questions 'How accurately must the atoms of the scissile bond be oriented in the active site?' or 'How accurately must the locking groups be defined?'

Consider the series of three locked substrates shown in figure 4 (Hayashi & Lawson 1969). N.m.r. measurements show that the two possible ways of puckering the five-membered ring are in equilibrium at room temperature (Rodgers 1974). Productive binding would require the conformations illustrated. A crystallographic structure determination shows that the carbonyl carbon atoms of the compounds with an asymmetric 5-membered ring lie 26 pm from the median plane through the linked 6-membered ring (Rodgers 1974). In this series of compounds the movement of the scissile bond by 26 pm in going from **1** to **2** has only a small effect on the activity, but the further movement of 26 pm from **2** to **3** has a very large effect (table 1). While this is only a single case, it does suggest the order of magnitude of accuracy required for orientation.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

The allowable movement seems to be the same order of size as the size of a bonding orbital. This provides further evidence of the rigidity of the active site, since any flexibility in the enzyme conformation would increase the range of allowable movement.

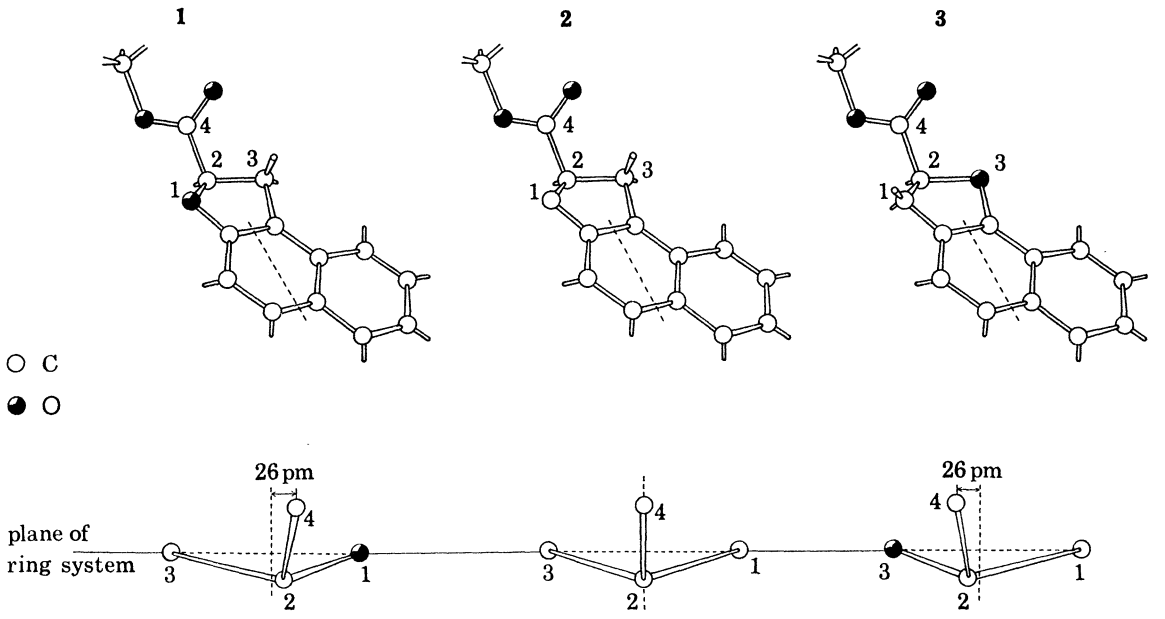

FIGURE 4. Three locked substrates of chymotrypsin: **1** D-methyl 1,2-dihydronaptho[2,1-*b*]furan-2-carboxylate (Hayashi & Lawson 1969); **2** (−)-methyl 4:5-benzindan-2-carboxylate (Pattabiraman & Lawson 1972); **3** L-methyl 2,3-dihydronaptho[1,2-*b*]furan-2-carboxylate (Hayashi & Lawson 1969). The lower diagrams show the ester group viewed in the direction of the dotted lines of the upper diagrams, and the vertical dashed lines indicate the median plane through the adjacent 6-membered ring.

TABLE 1. KINETIC CONSTANTS OF LOCKED SUBSTRATES

in 50% dimethyl sulphoxide, pH 7.0 (Pattabiraman & Lawson 1972)

substrate (figure 4)	**1**	**2**	**3**
Michaelis constant K_M/(mmol l^{-1})	1.46	1.94	14.9
activity corrected for intrinsic rate of hydrolysis $((k_{cat}/K_M)/k_{OH})$/(mol^{-1} s^{-1})	80	48	0.54
specificity ratio D:L $(k_{cat}/K_M)_D/(k_{cat}/K_M)_L$	38000	209†	4

† Specificity ratio (−):(+).

TRYPSIN INHIBITOR COMPLEXES

We now know the crystal structures of the complexes of two different protein trypsin inhibitors with trypsin (Rühlmann *et al.* 1973; Sweet *et al.* 1974). These structures show a number of common features:

(1) There is a relatively small area of contact, amounting to only 7 nm² (J. Janin & C. H. Chothia, manuscript in preparation).

(2) The conformation of the polypeptide chain at the binding site is identical (within experimental error), despite total dissimilarity of the remainder of the two inhibitors.

(3) The active site peptide is bound like a substrate, and all the interactions and hydrogen bonds expected for polypeptide substrates are made by the inhibitors.

(4) In each case the complex is in the form of a tetrahedral adduct. These inhibitors act as 'super-substrates', which bind so tightly to the active site that they never get away for long. The exclusion of water from the active site hinders the hydrolytic reaction, though in the case of the soybean trypsin inhibitor it proceeds slowly.

These structures directly confirm the stabilization of the transition state of a reaction by the enzyme. The latest results (Huber *et al.* 1974) indicate an abnormal bond length between the serine oxygen of the enzyme and the carbonyl carbon of the inhibitor in the tetrahedral adduct, defining the stablized form even more closely.

Biological recognition

A single molecule in a cell of typical size gives a concentration of the order of 10^{-9} M. The binding constants of up to 10^{-14} M achieved by the trypsin inhibitors (corresponding to a stabilization energy up to 77 kJ mol^{-1}) (Vincent & Lazdunski 1972) are thus ample to keep a single molecule in a cell almost permanently bound to a binding site or receptor. It is hard to see how a greater binding efficiency than this could be biologically necessary, and it is likely that the binding site for the trypsin inhibitors is as extensive as any binding site would need to be.

The role of hydrogen bonding in the molecular recognition process may be largely a negative one. If the interactions between two molecules were to cause a potential hydrogen-bonding group to become buried without a satisfactory partner, this would be energetically unfavourable. When an intermolecular interaction causes two hydrogen-bonding groups to combine, the new hydrogen bond replaces hydrogen bonds formed to water molecules. Not enough is known about the free energy change of such a transition, but it is probably small, unless several hydrogen bonds are made cooperatively.

The primary driving force for the observed interactions is mainly the exclusion of water from interacting hydrophobic surfaces (recently estimated at 10 kJ nm^{-2}) (Chothia 1974). Ultimately this energy derives from the protein folding process which stabilizes the original hydrophobic surfaces.

Conclusion

The results were not obtained in the logical order in which they have been presented. In our work on the trypsin family of enzymes our interpretations were very cautious, and almost every step of the argument has been tested experimentally. These firmly based conclusions may help us to tackle future problems in a more radical style.

A most important problem is the correct delineation of the binding site, which requires the development of accurate contacts (with the exclusion of water) by a correctly shaped substrate. The shapes are hard to visualize from skeletal models, and space-filling models are difficult to build with great accuracy. For a correct visualization of the substrate we need something like a mould of an accurate space-filling model. Such a mould would have sharp protrusions, and there would be sections joined on by narrow channels, while a space-filling model of a molecule could not have a sharper curvature than that of a hydrogen atom, whose radius is about 0.1 nm. Moreover a 'neck' joining two regions of density must, for a protein, be at least as wide as a CH_2 group or a peptide group.

We have developed a computer program to map a surface which will give the best fit to an

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

active site, following these rules. By marking coloured areas on the surface, corresponding to unsatisfied hydrogen-bonding groups on the enzyme, we have produced a rather accurate statement of the requirements of the binding site of trypsin and other enzymes (figure 5, plate 1). We hope that such methods may be useful in defining specificity in proteins where less information may be available in advance.

We thank A. R. Fersht and R. Henderson for helpful comments.

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Discussion

L. E. Sutton, F.R.S. (*Inorganic Chemistry Laboratory, South Parks Road, Oxford OX1 3QR*)

I should like to ask if one can say in general terms what is the effect of the complicated processes which Dr Blow describes?

If we consider the extremely simple case of two molecules A and B reacting reversibly and rapidly together to form initially a complex AB which then undergoes a slower and more drastic change to form a new substance or substances, it is useful to represent the course of events by plotting the free energy of the system as a function of some reaction coordinate, as in

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

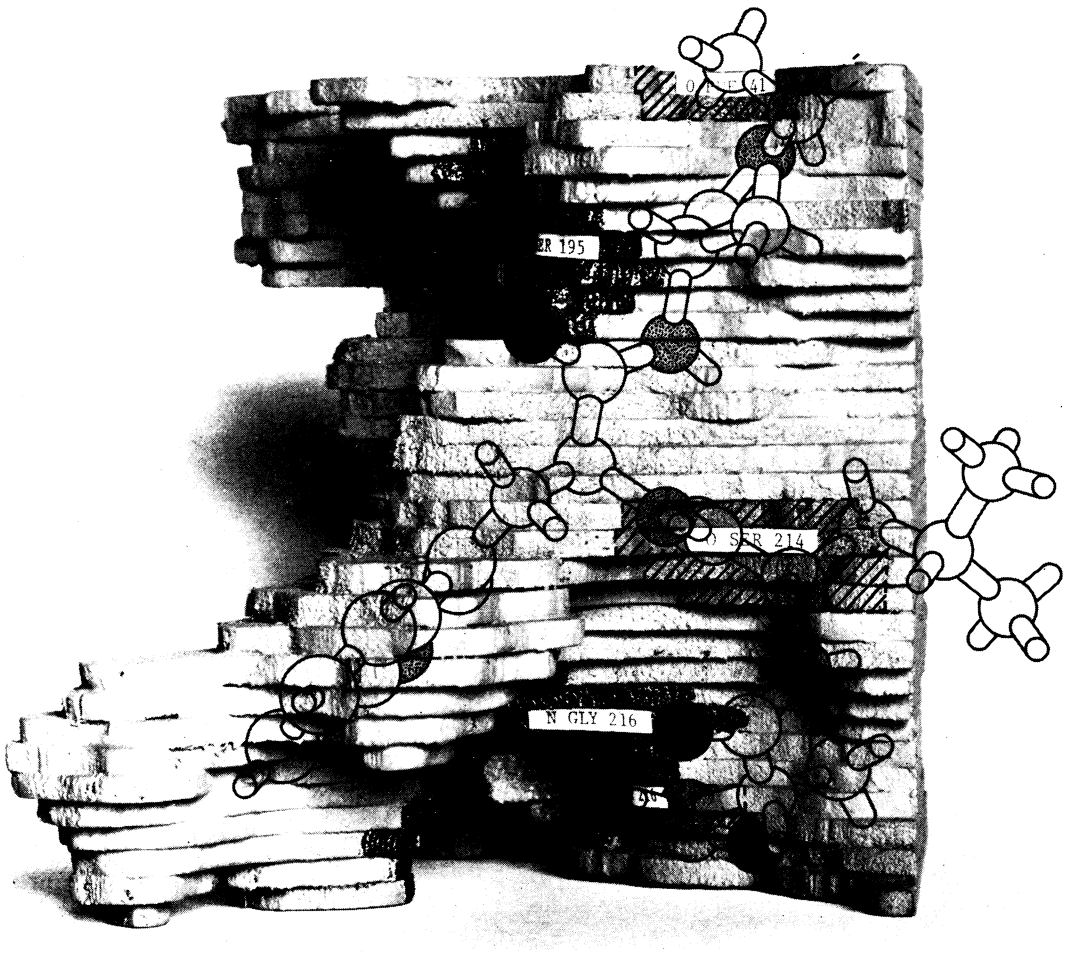

FIGURE 5. A model representing the surface conformation of chymotrypsin around the active site. The model defines the volume accessible to substrate atoms, as described in the text. The painted regions refer to unsatisfied hydrogen bond donors and acceptors on the enzyme surface: the darker regions are donors and the cross-hatched lighter regions acceptors. The line drawing shows how Ala-Leu-Trp-Ala could be accommodated.

(*Facing p.* 94)

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

figure 6. This 'reaction coordinate' is a single variable which, we suppose, can express the changes in the relative positions of the atoms as the reaction proceeds. Starting from the level G_0 the free energy will fall to $G_{1(a)}$ if the complex is a strong one, or rise to $G_{1(b)}$ if it is a weak one. Then it will rise higher to $G^{\ddagger}$, the free energy of the transition state of the major reaction, and finally fall to G_2, that of the product or products.

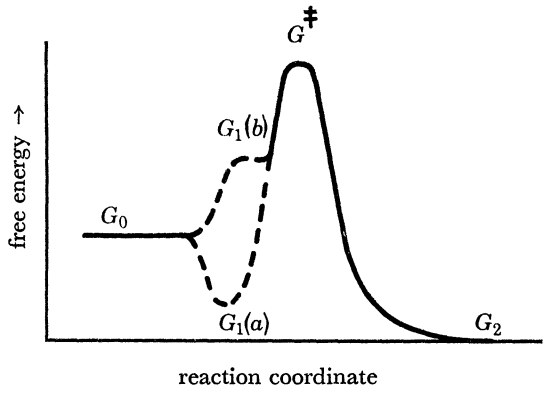

FIGURE 6

By simple algebra it can be shown that, provided the complexing does not alter $G^{\ddagger}$, the overall rate of change from G_0 to G_2 is:

$$\text{rate} = Ca(\text{A})a(\text{B}) \exp[-(G^{\ddagger} - G_0)/RT],$$

where $a(\text{A})$ and $a(\text{B})$ are the activities of A and of B when complexing has occurred and C is a constant. This is a well-known result.

The free energy of the complex, G_1, does not appear in the exponential index. Complexing can only make $a(\text{A})$ and $a(\text{B})$ less than their values before complexing; so it can only reduce the overall rate. It may, indeed, reduce almost to zero the one which is initially the smaller, and so stop the reaction. An increase of forward rate must, therefore, imply a reduction of $G^{\ddagger}$. But can complexing reduce $G^{\ddagger}$? If the sequence is as shown in figure 6 the answer must be that it cannot; for the free energy is a single-valued, unique function of the reaction coordinate. What has gone before does not affect the value of $G^{\ddagger}$ at the value of the coordinate describing the transition state. The only possible way in which a change of $G^{\ddagger}$ may occur is that complexing opens up a new pathway for the reaction, i.e. a new reaction coordinate sequence, which means that figure 1 no longer describes the sequence. A new figure with a new reaction coordinate would be needed to do this: the initial supposition that the reaction coordinate is a unique, one-dimensional variable would be wrong.

This ultra simple model does not correspond well to that of enzyme catalysis which more resembles heterogeneous catalysis, where the reaction takes place in the presence of a third substance X on which A and B are held and from which the product has to be released. Almost fifty years ago Hinshelwood showed† that effective enthalpy (not free energy) of such a reaction is that of the homogeneous reaction reduced by the heat of desorption of the reactants but increased by that of the products. For gas reactions on solids, when estimates could be made of the reduction of activation enthalpy by the action of a catalyst they were found to be

† C. N. Hinshelwood 1926 *The kinetics of chemical change in gaseous systems*, 1st edn, Chap. 7. Oxford.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

too great to be accounted for by the heat of desorption of the reactants; so he concluded that the effect of the heterogeneous catalyst in these examples was to change the reaction mechanism. I believe that this conclusion still stands.

From these analogies, I suspect that what the enzyme does is also to open up a new pathway for the reaction. Does Dr Blow agree?

Dr W. J. Albery and Professor J. R. Knowles consider that the course of an enzyme catalysed reaction probably involves passage over at least two free energy maxima; and they suggest that this gives so much scope for adjustment of the path that a highly efficient and specific catalyst may result from such changes during or by an evolutionary process.†

D. M. Blow, F.R.S.

Certainly I agree that if an enzyme participates in a reaction, we ought to say that the reaction pathway is changed. As Dr Sutton suggests, in many cases a new intermediate is involved.

The reaction scheme he describes may be written:

$$\begin{array}{ccccc} A+B+X & \rightleftharpoons & (A+B)^{\ddagger}+X & \rightleftharpoons & C+D+X \\ \updownarrow & & & & \updownarrow \\ (A+B).X & \rightleftharpoons & (A+B)^{\ddagger}.X & \rightleftharpoons & (C+D).X \end{array}$$

The upper pathway is an uncatalysed reaction, while in the lower pathway reactants and products are adsorbed onto the catalyst. I suppose we could say the mechanism is similar if the two transition states $(A+B)^{\ddagger}$ and $(A+B)^{\ddagger}.X$ have similar characteristics, in particular if the enthalpy of activation of the uncatalysed reaction is the same as the enthalpy of activation of the adsorbed reactants in the catalysed reaction. The relative rates depend on the free energy difference of the two transition complexes. Much of the free energy of adsorption accounts for the loss of entropy of the reactants which are immobilized on the catalyst. For this reason the activation energy of the reaction may be reduced by far more than the enthalpy of adsorption. This is discussed lucidly by Jencks & Page (1972 *Proc. 8th FEBS Meeting* **29**, 45–58).

K. Dalziel (*Biochemistry Department, Oxford*)

In discussions of rate enhancement and the greater affinity of enzymes for the transition state of the substrate, I wonder whether a possible advantage of 'unimolecular catalysis' in a complex with a large protein molecule has been overlooked. I understand that unimolecular reactions involving large molecules are often much faster than would be expected from absolute reaction rate theory, and that this is often correlated with the complexity of the molecule, because there are many vibrational degrees of freedom to contribute to the activation energy. Perhaps one of the features of enzyme protein structure is that thermal energy can be 'funnelled' to the active centre for substrate activation. This may be related to the protection of many enzymes by their substrates or competitive inhibitors against thermal denaturation. It seems odd that a small ligand can protect a large protein molecule against denaturation unless the process begins at the active centre most readily.

L. J. Dunne (*Physics Department, Guy's Hospital Medical School, London Bridge, London SE1 9RT*)

With regard to the point raised by Dr Dalziel concerning the mode of action of enzymes it is pertinent to recall that Professor Frohlich (*Nature, Lond.* **228**, p. 1093, 1970) pointed out that

† See *Chemistry in Britain* 1974 **10**, p. 261 (no. 7).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

many of the remarkable properties of enzymes may be understood if we consider the collective electrical excitations of the entire molecule and not just the chemistry involved at the active site.

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Referring to Dr Dalziel's comments: Rather than an enzyme having all the degrees of freedom just implied, it seems instead to be a highly constrained structure. The results just presented by Dr Blow illustrate this rather convincingly. Therefore it seems unlikely that an enzyme's action is to collect, channel, and 'upgrade' thermal energy to drive reactions. Instead an enzyme seems to be a relatively rigid device which acts by binding reactants and imposing a favourable geometric arrangement of reactants and catalytically participating groups.

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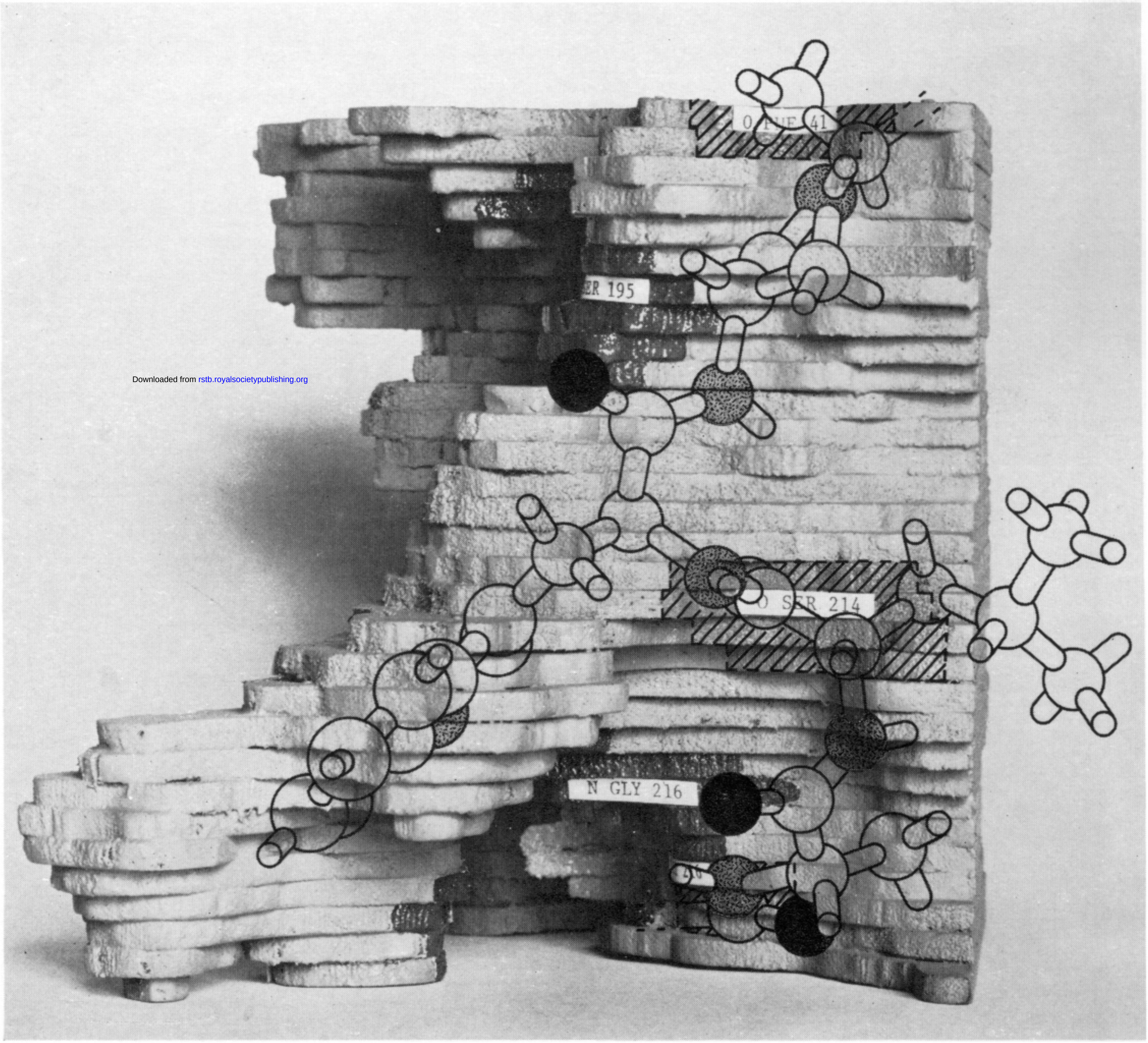

IGURE 5. A model representing the surface conformation of chymotrypsin around the active site. The model defines the volume accessible to substrate atoms, as described in the text. The painted regions refer to unsatisfied hydrogen bond donors and acceptors on the enzyme surface: the darker regions are donors and the cross-hatched lighter regions acceptors. The line drawing shows how Ala-Leu-Trp-Ala could be accommodated.

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