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Stereochemical Aspects of Peptide Hydrolysis Catalyzed by Serine Proteases of the Chymotrypsin Type

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The steric course of peptide hydrolysis catalyzed by serine proteases has been studied on the basis of the available, extensive structural data and taking into account the stereoelectronic theory of Deslongchamps (*Heterocycles*, 7, 1271 (1977)). These studies allowed elucidation of the structure of intermediates, in particular of the tetrahedral intermediate, and of the main structural events taking place during catalysis. They reveal a difficulty inherent in the generally accepted mechanism of peptide hydrolysis: protonation of the leaving nitrogen in the configuration arising from nucleophilic attack of Ser-195 on the carbonyl carbon cannot take place internally from His-57. Two alternative mechanisms are discussed which are compatible with all implications of the stereoelectronic theory. The main features of the more probable mechanism are: (i) a conformational change allowing the imidazole ring of His-57 to occupy two distinct positions; in one position a proton is abstracted from O^{γ} of Ser-195, and in the other this proton is donated to the leaving nitrogen; (ii) a configurational change (inversion) of the pyramidal leaving nitrogen reorienting the lone-pair orbital developed during nucleophilic attack; in one orientation C–O bond breaking, and in the other C–N bond breaking, is allowed. This inversion process confers on the nitrogen the property of a switch controlling the breakdown of the tetrahedral intermediate.

NOMENCLATURE AND DEFINITIONS

For describing a generic substrate general formulas of the type

$$E_x\text{–}L_{xn}\text{–}\cdots\text{–}L_{x2}\text{–}L_{x1}\text{–}\overset{\downarrow}{}L_{y1}\text{–}L_{y2}\text{–}\cdots\text{–}L_{ym}\text{–}E_y$$

are used in which the position of the amino acid residues is defined with reference to the susceptible bond (↓). Accordingly, the symbols L_{x1}, L_{x2}, etc., are used whenever the position, but not the nature, of a particular residue needs to be specified. They are also used in the text and in the figures to indicate which residue single atoms of the substrate belong to; e.g., $O(L_{x1})$ signifies the carbonyl oxygen of residue L_{x1}.

TR–B	trypsin–benzamidine complex
TR–PTI	trypsin–pancreatic trypsin inhibitor complex
Tos-CHT	tosylchymotrypsin

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INTRODUCTION

The mechanism of the reactions catalyzed by chymotrypsin and related pancreatic serine proteases is known to involve, after formation of an enzyme–substrate complex, a first acyl-transfer step in which the acyl moiety of an ester or amide substrate is transferred to the hydroxyl oxygen of the active serine of the enzyme (acylation) and a second acyl-transfer step in which the acyl moiety is transferred to the oxygen of a water molecule (deacylation) (*1, 2*). This mechanism is summarized in the following scheme:

$$E + A \rightleftharpoons EA \rightleftharpoons E' + P \rightleftharpoons E + Q,$$

where EA is the enzyme–substrate complex, E′ the acyl enzyme, P the alcohol or amine product, and Q the carboxylic acid product. The acyl-transfer steps are assumed to pass through tetrahedral intermediates (*3–5*) which result, for acylation, from attack of the hydroxyl of Ser-195 on the carbonyl of the substrate; and for deacylation, from attack of water on the carbonyl of the acyl enzyme.

In order to obtain more detailed information about the mechanism and in particular, about the steric course of the reaction, precise information on the structure of intermediates such as enzyme–substrate complex, tetrahedral intermediates, and acyl enzyme is required. These intermediates are transient species which normally do not appear in sufficiently large proportion to allow direct structure determination by the current physicochemical techniques. In contrast, direct structural information is available for several enzyme derivatives or complexes with substrate analogs which are related to these intermediates. In cases in which the relationship between intermediate and these stable enzyme species is clearly discernible in terms of the structural differences between substrate and substrate analog, the structure of a particular intermediate can be deduced from the structure of one or more related stable enzyme species. For this purpose the trypsin–benzamidine and trypsin–pancreatic trypsin inhibitor complexes and tosylchymotrypsin seem to be quite appropriate examples. Information on these structures has become available in great detail during the past 10 years from high resolution X-ray diffraction studies. The significance of this information in relation to the mechanism of action of these enzymes has recently been reviewed by the authors of the crystallographic studies (*9, 10*).

Model building represents a suitable aid for carrying out the modifications needed to transform the structure of stable enzyme species into that of the intermediates. The necessary manipulations of the models are performed along lines dictated by the framework of the structural relationships between substrate and substrate analog and should be supported as much as possible by theoretical considerations. It is essential that these theoretical considerations rely on well-established principles worked out in the field of organic chemistry.

In this paper we would like to follow up this method of indirectly elucidating the structure of the intermediates and the structural events taking place during reaction. The structure of the tetrahedral intermediate is of special interest since it constitutes the key intermediate for understanding the mechanism of acylation. An important contribution to the model building of this species is expected from

taking into account the stereoelectronic effect (*6*) discovered by Deslongchamps. Experimental work by Deslongchamps and co-workers on the nonenzymatic hydrolysis of model compounds such as cyclic *ortho*-esters (*7*) and imidate salts (*8*) has shown that selective breakdown of the tetrahedral intermediates is controlled by the orientation of the lone-pair orbitals of the hetero atoms bound to the central carbon. This principle, which is believed to be of general importance for the study of acyl transfer steps, has hitherto not been used in the investigation of enzymatic reactions.

MODEL BUILDING

The structure of the stable enzyme species elucidated, trypsin–benzamidine complex (TR–B) (*11*), trypsin–pancreatic trypsin inhibitor complex (TR–PTI) (*12, 13*), and tosylchymotrypsin (Tos-CHT) (*14*) which are related to enzyme–substrate complex, tetrahedral intermediate, and acyl enzyme, respectively, were chosen as a structural basis for the mechanistic considerations described in this paper. Kendrew-type skeletal models for the active site portion of the three stable enzyme species were constructed according to the atomic coordinates published in the literature (*14*) or provided by the authors of the X-ray studies.[1] The original coordinates of TR–B and TR–PTI were previously transformed into new sets of coordinates having as a common coordinate system the one defined for Tos-CHT. Models of two intermediates, an enzyme–substrate complex and a tetrahedral intermediate were constructed by appropriate modification of the models of the stable enzyme species. For building these models a new method was used which allows simple and rapid fixation of the model pieces according to coordinates (method to be published elsewhere).

Models of the Stable Enzyme Species

Comparison of the TR–B, TR–PTI, and Tos-CHT models indicates that no gross conformational changes of the main chain sections carrying the catalytic residues take place during catalysis. With regard to the side chains of the catalytic residues it is important to note that in the structures considered the torsion angles χ_1 about the $C^\alpha - C^\beta$ bond of Ser-195 are all near $-80°$. The corresponding "down position" (*14*) of O^γ (Ser-195) is therefore assumed to be maintained all over the catalytic process.[2] The models show, however, a relevant difference concerning the position of the imidazole ring of His-57. There seem to be two distinct positions: one, occurring in the TR–PTI complex, is characterized by a torsion angle χ_1 of $+62°$ (in-position); the other is observed in TR–B and Tos-CHT with χ_1 $+92°$ and $+97°$, respectively (out-position). From the structural point of view the

[1] We thank Dr. R. Huber and Dr. W. Bode for providing us the coordinates of the TR–PTI and TR–B complexes.

[2] This is in disagreement with a previous stereochemical study (*15*) published in 1974 when the structural information on the trypsin complexes was not yet available.

most important difference of the two positions of the imidazole ring concerns its ability to form hydrogen bonds with O^{γ} of Ser-195 and the leaving nitrogen $N(L_{y1})$ of the substrate. For reasons of simplicity, in these considerations the position of the nitrogen atom $N(L_{y1})$ of the substrate is assumed to be the same as that of N(Ala-16 I) in the TR–PTI complex. In the other two structures this atom is located at a position which coincides closely with that observed in the TR–PTI complex taking the main chain sections carrying the catalytic residues as a reference.

The in-position allows the formation of a good hydrogen bond between $N^{\epsilon 2}$ of His-57 and O^{γ} of Ser-195 (2.7 Å, linear) but the distance between $N^{\epsilon 2}$ and $N(L_{y1})$ is too long (4.2 Å) for a hydrogen bond to be formed. Conversely, the out-position is suitable for $N^{\epsilon 2}$ to form a hydrogen bond with $N(L_{y1})$ (2.9 Å, linear) but not with O^{γ} of Ser-195: in Tos-CHT the distance between $N^{\epsilon 2}$ and O^{γ} is 3.9 Å; in TR–B this distance is 3.2 Å but the orientation of donor and acceptor is such that a hydrogen bond would have to be strongly bent. With regard to the absence of a good hydrogen bond between O^{γ} (Ser-195) and $N^{\epsilon 2}$(His-57) in TR–B it is worth noting that this feature is also shared by other native proteases of the chymotrypsin family (*16*) and by the evolutionary related proteases of *Streptomyces griseus* (*17*).

On the basis of these model considerations it seems very likely that the in- and out-positions are related to two distinct functional roles of His-57 and that interconversion of the two positions takes place during catalysis. It could therefore be expected that interconversion could occur by simple rotation about the C^{α}–C^{β} bond of His-57 from 92° to 62°. Model inspection, however, indicates that this rotation alone would disrupt the important hydrogen bond between $N^{\delta 1}$ of His-57 and $O^{\delta 2}$ of Asp-102. Indeed, generation of the in-position in the TR–B model by decreasing χ_1 by 30° reduces the distance between $N^{\delta 1}$ (His-57) and $O^{\delta 2}$ (Asp-102) from 2.9 to 1.8 Å, whereas analogous generation of the out-position in the TR–PTI model increases this distance from 2.7 to 3.7 Å. Consequently it must be assumed that the carboxyl group of Asp-102 is able to follow the movement of the imidazole ring during the interconversion of the two positions. This carboxyl group, however, is firmly held in its position by a network of hydrogen bonds involving the backbone NH groups of Ala-56 and His-57 and the side-chain hydroxyl group of Ser-214. Thus interconversion of the two imidazole positions cannot take place without concomitant movement of the backbone part carrying the groups involved in this network of hydrogen bonds. These assumed structural changes of the backbone could involve a relatively large section of the structure and thereby be split up into several small changes which are not easily detectable by means of model comparison.

Model of the Enzyme–Substrate Complex

In the TR–PTI complex the inhibitor residues Lys-15 I and Ala-16 I correspond to the residues adjacent to the susceptible bond, L_{x1} and L_{y1}, in a peptide substrate. As a consequence of incipient nucleophilic attack to the lysine carbonyl carbon by O^{γ} (Ser-195) the distance between these atoms was found to be 2.5 Å

and the angle θ^4, which expresses the extent of pyramidalization of the carbonyl carbon, to be of 34° (*13*).

To produce a model of the enzyme–substrate complex a dipeptide substrate containing the reactive residues of PTI, Ac-Lys–Ala-NH_2, was fitted into the active site of the enzyme in a TR–PTI model from which the PTI part had been removed. Orientation and conformation of this dipeptide substrate was first made to coincide with those of the corresponding dipeptide section of PTI in the TR–PTI structure. Since the susceptible peptide group in this truncated TR–PTI complex is deformed out of planarity but is planar in the enzyme–substrate complex, the following adjustments involving these residues were made. The distance between O^γ of Ser-195 and the carbonyl carbon of the substrate, was increased from 2.5 to 3.0 Å, the normal Van der Waals contact distance between these atoms. Part of this increment was achieved by allowing the carbonyl carbon to revert to planarity and part by slightly displacing the substrate peptide chain away from the enzyme surface. This movement, however, is not shared to an equal extent by all substrate atoms; it is large for the central carbon and adjacent atoms but is allowed to decrease with distance on account of peptide chain flexibility. The most important contact distances between substrate and enzyme atoms are summarized in Table 1. As can be seen in this table the only major increase in distance arises between S^γ of Cys-42 and C^β of residue L_{y1}, but this is mainly a consequence of the pyramidal to planar conversion of the geometry of N (L_{y1}).

Model of the Tetrahedral Intermediate Formed in Acylation

For building this model the truncated TR–PTI complex described in the preceding section was modified as follows. The central carbon (carbonyl carbon of L_{x1}) and the leaving nitrogen (nitrogen of L_{y1}) were allowed to assume fully tetrahedral geometry. This was achieved by displacing the carbonyl carbon toward the serine oxygen and the nitrogen away from it. Evidence showing that carbonyl carbon pyramidalization is accompanied by nitrogen pyramidalization

TABLE 1

RELEVANT INTERATOMIC DISTANCES[a]

	$N(L_{x1})$	$O(L_{x1})$	$N(L_{y1})$	$C^\alpha(L_{y1})$	$C^\beta(L_{y1})$	$N(L_{y2})$
O(214)	*3.4/3.3/2.6*					
N(193)		*2.5/2.9/3.0*				
N(195)		*3.1/3.1/2.6*				
C^β(195)		3.0/3.1/2.4	3.8/3.7/3.0	4.0/3.9/3.2		
O(41)						*2.9/3.0/2.6*
S^γ(42)					5.8/3.7/3.3	
$C^\alpha(L_{y1})$		2.8/2.7/2.5				

[a] The first, second, and third numbers refer, respectively, to the distances (Å) measured in the model of the enzyme-substrate complex, calculated from the coordinates of the TR–PTI complexes and measured in the model of the tetrahedral intermediate formed in acylation. Numbers in italic express values for hydrogen bonding distances.

and that the apices of the pyramides develop, as assumed, in opposite sense has been recently provided by crystal structure analysis of naphthalene derivatives in which incipient nucleophilic attack of a dimethylamido group by an oxygen nucleophile is determined by the close proximity of these substituents (*18*).

The distance between O^{γ}(Ser-195) and the central carbon was reduced from 2.5 to 1.4 Å, the normal value for an ester bond. Rotation of O^{γ}(Ser-195) about the C^{α}–C^{β} bond by approximately 10° toward the central carbon contributed little to decrease this distance, so that a moderate translation of the substrate peptide chain toward the enzyme surface was required. This translation can be carried out with confidence since it is guided by two important hydrogen bonds, the one between CO(Phe-41) and NH(L_{y2}) and the one between CO(Ser-214) and NH(L_{x1}). In comparison with TR–PTI the length of these hydrogen bonds decreases considerably from, respectively, 3 and 3.3 Å to about 2.6 Å. In a parallel manner, the carbonyl oxygen is slightly shifted within the oxyanion hole (*19*) from a position closer to NH(Gly-193) to one closer to NH(Ser-195) (see Table 1). It is interesting to note that the resulting direction of approach of O^{γ}(Ser-195) to the carbonyl carbon of the substrate is consistent with the Bürgi–Dunitz theory of nucleophilic attack on carbonyl (*20*).

Final adjustment, carried out in order to optimize the contact distances between enzyme and substrate groups in the crowded region around the new C–O bond, mainly concerned the torsion angles about bonds linking the central carbon to its substituents. These torsion angles are 60° for the ideal fully staggered arrangement shown in Fig. 1. In the actual model they were allowed to deviate somewhat from this value so that the strain arising from the 1,3-*syn*-periplanar arrangement of the carbon atoms C^{β} (Ser-195) and C^{α} (L_{y1}) is considerably relieved, as their distance increases from 2.5 to 3 Å. Consequently the conformations about the O^{γ} (Ser-195)–C(L_{x1}) bond and the C(L_{x1})–N(L_{y1}) bond become intermediate between eclipsed and staggered as it is shown by the Newman projections of Fig. 2. In contrast, the distances of O(L_{x1}) from C^{β} (Ser-195) and C^{α} (L_{y1}) remain considerably smaller than the Van der Waals contact distances (see Table 1) when these

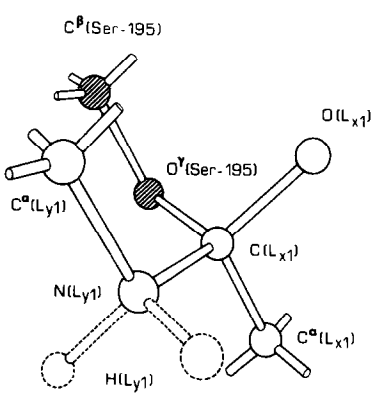


FIG. 1. Schematic representation of the spacial arrangement of the reacting atoms in the tetrahedral intermediate. The shaded circles indicate atoms belonging to the enzyme, the open circles to the substrate. The dotted lines indicate the two possible positions of H(L_{y1}).

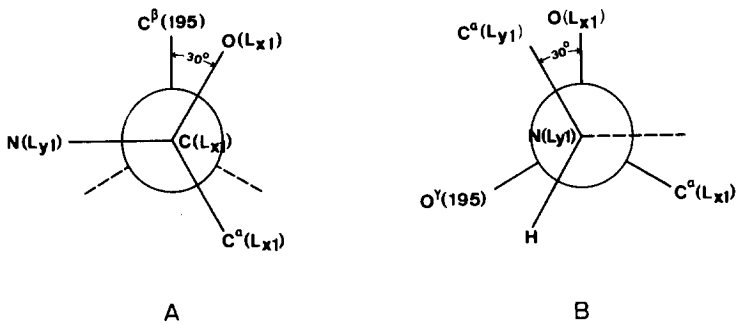


FIG. 2. Newman projections showing the conformations about the O^{γ}(Ser-195)–$C(L_{x1})$ bond (A), and the $C(L_{x1})$–$N(L_{y1})$ bond (B). The dotted lines represent lone-pair orbitals.

rotations are carried out and must be regarded as a strain factor intrinsic to formation of the tetrahedral intermediate from the enzyme–substrate complex. Pictures of the relevant part of this model are given in Figs. 3 and 4.

STEREOELECTRONIC CONTROL AND ITS IMPLICATIONS FOR THE STERIC COURSE OF THE REACTION

As revealed by Fig. 4 orientation and conformation of the substrate part of the tetrahedral intermediate are determined by the enzyme–substrate interactions.

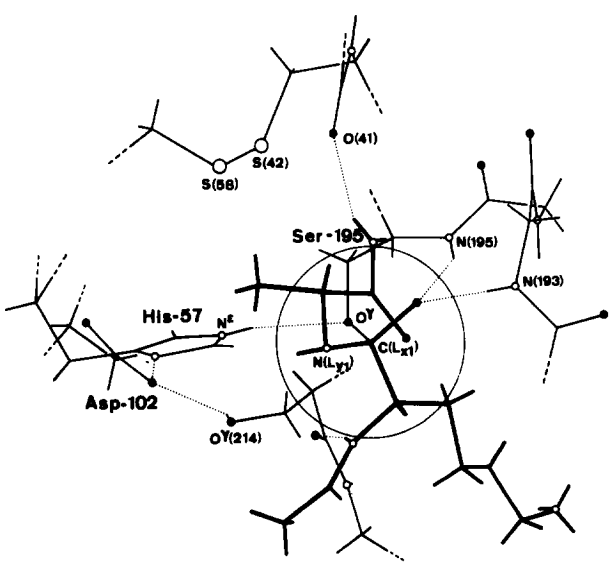


FIG. 3. Designation of the relevant residues in the model of the tetrahedral intermediate formed in acylation. Thin lines represent the enzyme part, heavy lines the substrate Ac-Lys–Ala-NH_2. •, Oxygen; o, nitrogen; O, sulfur. The circle helps to locate the reacting atoms as represented schematically in Fig. 1.

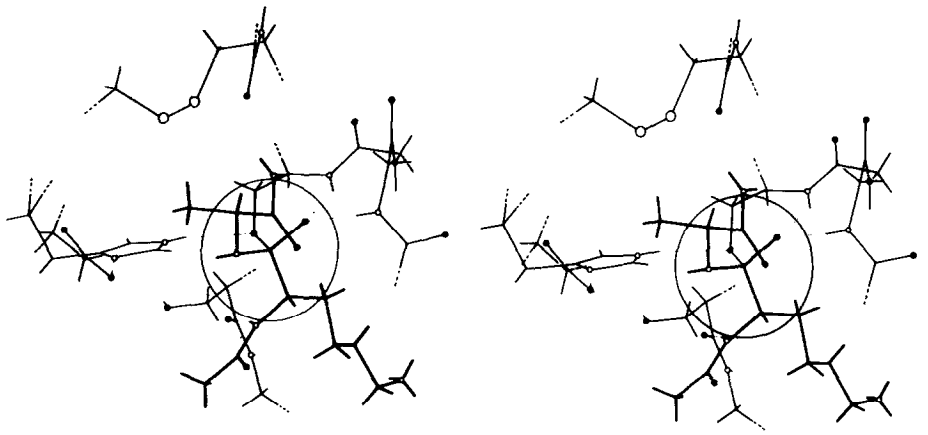


FIG. 4. Stereopicture of the tetrahedral intermediate formed in acylation. Notations as in Fig. 3.

This steric arrangement also determines the orientation of the lone-pair orbitals at O^{γ}(Ser-195). What cannot be obtained from model building is the stereochemistry of $N(L_{y1})$. This information can only be deduced by applying the stereoelectronic theory recently proposed by Deslongchamps and co-workers on the basis of experimental results (*6, 7*) and supported by theoretical calculations carried out by Lehn *et al.* (*21, 22*). According to this theory, specific cleavage of the tetrahedral intermediate is controlled by the orientation of the lone-pair orbitals of the hetero atoms, in that the cleavage of a C–N or a C–O bond occurs only when each of the two other hetero atoms has a lone-pair orbital *anti*-periplanar to the cleaved bond. From the principle of microscopic reversibility it follows that the lone-pair orbitals developing on the hetero atoms upon nucleophilic attack on an amide or an ester group must be *anti*-periplanar to the new C–N or C–O bond. Application on this stereoelectronic theory to nucleophilic attack of Ser-195 on the susceptible bond of a peptide substrate leads to the arrangement shown in Fig. 5A. From this figure it can be seen that the hydrogen atom of the leaving nitrogen points toward the imidazole ring of His-57 and that the lone-pair orbital points toward the solvent. As was pointed out in a previous communication (*23*) this observation provides the explanation for the unreactivity of peptide substrates

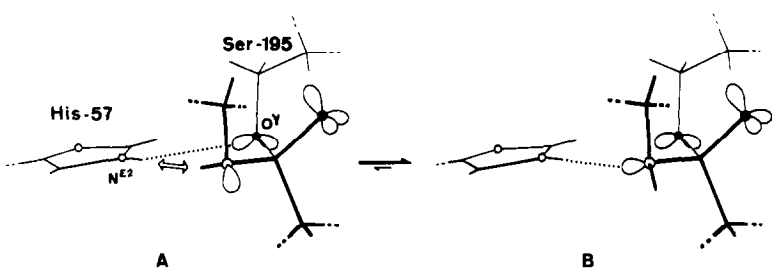


FIG. 5. Stereoisomeric forms of the tetrahedral intermediate differing in the configuration of the leaving nitrogen. In (A) and (B) the leaving nitrogen presents the (*R*) and (*S*) configuration, respectively. The dipole–dipole repulsion between the NH of His-57 and the NH of the leaving group in (A) is indicated by an arrow.

carrying alkyl substituents on the leaving nitrogen. The distance between the leaving nitrogen and the imidazole ring in the tetrahedral intermediate is only 3.5 Å, and is just sufficient to accommodate a hydrogen atom. For peptides carrying substituents on the leaving nitrogen steric hindrance prevents formation of the tetrahedral intermediate in the required configuration.

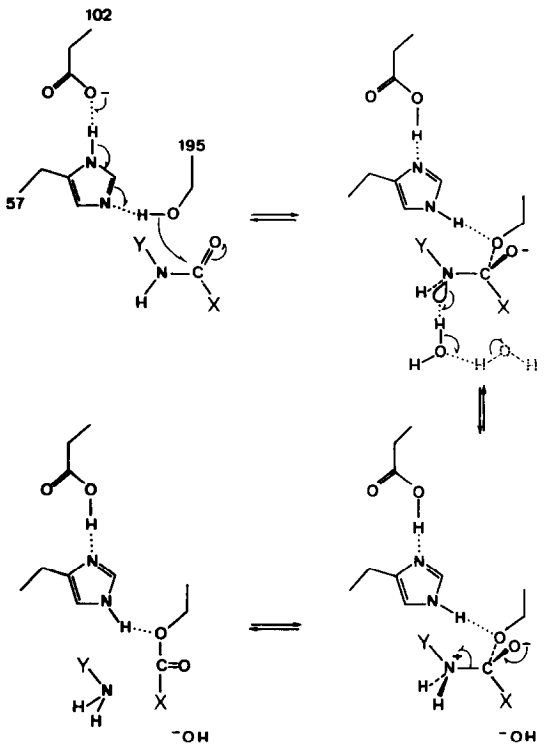
In the subsequent breakdown of the tetrahedral intermediate the susceptible carbon–nitrogen bond is split and the amine product released. For cleavage of this bond to occur the stereoelectronic theory requires that each of the two oxygen atoms geminal to the leaving nitrogen has a lone-pair orbital *anti*-periplanar to this C–N bond. From Fig. 5A it can be seen that the conformation about the O^{γ}(Ser-195)–C(L_{x1}) bond turns out to be such that this stereoelectronic requirement is actually met. Since the other oxygen atom, O(L_{x1}), has three lone-pair orbitals, one of which will occupy the required position, the conformation about the C(L_{x1})–O(L_{x1}) bond is not critical.

Whereas the orbitals on the oxygen atoms are suitably oriented for specific cleavage of the C–N bond, the orientation of the lone-pair orbital on the leaving nitrogen N(L_{y1}) deserves further consideration. Since this atom cannot depart in the anionic form, its protonation has to occur prior to or at least simultaneously with cleavage of the C–N bond. It has been suggested (*12*) that the proton is donated to the leaving nitrogen by $N^{\epsilon 2}$ (His-57) which had accepted a proton from O^{γ} (Ser-195) in the preceding step. Inspection of the structure of the tetrahedral intermediate (Fig. 5A) reveals that the configuration of the leaving nitrogen is not suitable for this proton transfer to occur since the nitrogen lone-pair orbital which should accept the proton points toward the solvent and not toward His-57. This finding is not consistent with an earlier proposed mechanism (*24*) in which one-step proton transfer from O^{γ}(Ser-195) to the leaving nitrogen via $N^{\epsilon 2}$(His-57) acting as a stationary proton relay is postulated: such one-step proton transfer would require the lone-pair orbital of the leaving nitrogen to be on the proton pathway. For the same reason a recently proposed, SN_2-like mechanism of acylation with amide substrates (*25*) does not seem to be likely. Two alternative mechanisms for the breakdown step, differing with respect to the mode of protonation of the leaving nitrogen, can therefore be proposed.

Mechanism 1

The proton is donated by a water molecule of the solvent, toward which the lone-pair orbital of the leaving nitrogen is directed (Scheme 1). Thus protonation would not be carried out by His-57, as hitherto generally assumed, but would occur externally with respect to the enzyme.

In this case it is conceivable that the proton transferred from O^{γ}(Ser-195) to $N^{\epsilon 2}$(His-57) remains bound to the latter (but still forming a hydrogen bond with O^{γ}) after that the tetrahedral intermediate has collapsed to acyl enzyme. From the principle of microscopic reversibility this mode of reaction implies that His-57 is not involved as a general-base catalyst in the reverse direction, i.e., attack of the acyl enzyme by the amine NHR′ and, by analogy, attack of any other nucleophile, including water. Consequently in the deacylation step, which involves addition of



SCHEME 1

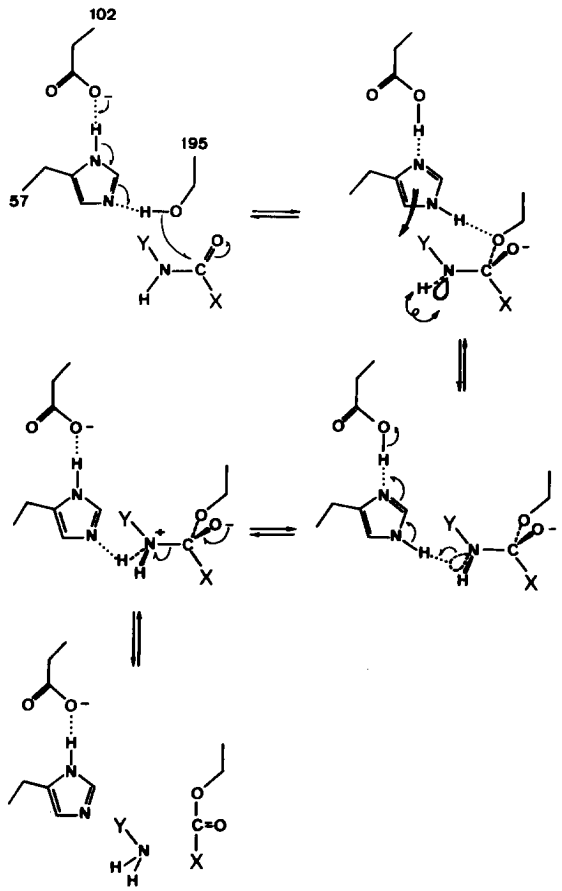
water to the carbonyl of the acyl enzyme, a proton of the attacking water molecule has to be released into the solvent.

Mechanism 2

Protonation occurs internally from His-57 (Scheme 2). This proton transfer can only occur when the lone-pair orbital of the leaving nitrogen is oriented toward His-57. Since in the tetrahedral intermediate, as it is first generated, this is not the case, an additional step, involving inversion of the leaving nitrogen, must be postulated. Nitrogen inversion, by changing the configuration of this atom from (*R*) to (*S*) leads to a new tetrahedral intermediate in which the positions of N–H bond and lone-pair orbital are interchanged (Fig. 5B). Mechanism 2 differs essentially from Mechanism 1 in that no solvent participation is required to protonate the leaving nitrogen: the same proton which is first abstracted from O^{γ}(Ser-195) is subsequently donated to the leaving nitrogen. The second of these proton transfers triggers the breakdown of the tetrahedral intermediate and the formation of the acyl enzyme. Apart from the now recognized necessity for N-inversion, the internal protonation mode corresponds to the current views on the mechanism of acylation.

Comparison of the Two Mechanisms

Mechanism 1 is well compatible with the overall rate of C–N cleavage for



SCHEME 2

specific peptide substrates; this rate, which at 25°C is never found to exceed 50 s^{-1} (*26, 27*), is far lower than the rate of proton transfer from water as can be predicted from the nitrogen p*K*, according to Eigen's classical investigations (*28*). Assuming a p*K* of 8–9 for the leaving nitrogen in the tetrahedral intermediate (*29*) the pseudo-first-order rate constant of proton transfer from water to this nitrogen should in fact be in the range 10^4–10^5 s^{-1} (*30*). Thus, on account of the high p*K* value that the leaving nitrogen assumes in the tetrahedral intermediate, the protonated $N^{\epsilon 2}$ of His-57 acting as general acid catalyst would not be necessary to assist expulsion of the leaving amine. According to this mode of protonation the catalytic role of His-57 would solely consist in facilitating the formation of the tetrahedral intermediate by efficient proton abstraction from O^{γ}(Ser-195).

On the basis of the extensive body of kinetic data presently available Satterthwait and Jencks proposed six alternative mechanistic schemes (*31*). With respect to the mode of involvement of His-57 our Mechanism 1 is best compared with Satterthwait's Scheme 2. This scheme postulates rate-limiting attack of Ser-195 on the susceptible carbonyl of a peptide substrate assisted by concerted

general base catalysis by His-57. Mechanism 1 is compatible with this scheme since it assigns the same catalytic role to His-57. The similarity is stressed by the fact that in Mechanism 1 this catalytic role is exerted exclusively during the rate-determining step of Scheme 2, i.e., nucleophilic attack by Ser-195.

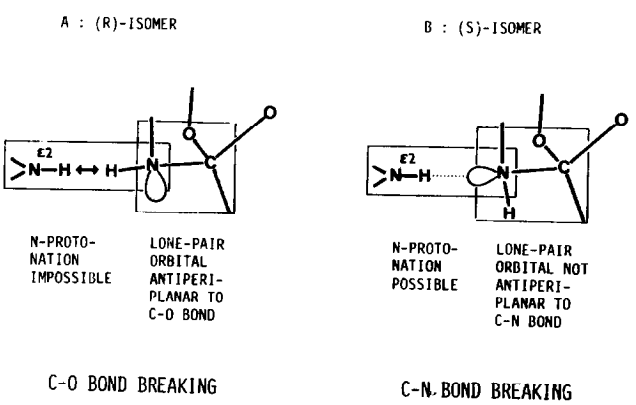
An important point of concern for discriminating between the two proposed mechanisms is related to the positions of the imidazole ring as revealed by the stable enzyme species. For Mechanism 1 it is only the in-position which is functionally relevant since the hydrogen bond between O^{γ}(Ser-195) and $N^{\epsilon 2}$ (His-57) allows rapid proton transfer between these atoms to occur. It could therefore be expected that the imidazole ring would remain in this position during the whole catalytic process. However, the in-position is found neither in TR–B, an analog of the enzyme substrate complex, nor in Tos-CHT, an analog of the acyl enzyme. In both species the out-position is realized, though this position would not be functionally relevant according to Mechanism 1.

In this context it is worth noting that the position of the imidazole ring found in TR–B is not consistent with the hypothesis that the function of the charge relay mainly consists in conferring enhanced intrinsic nucleophilicity to Ser-195, since effective transfer of negative charge on O^{γ}(Ser-195) would require the associated proton transfers to occur along preformed hydrogen bridges. As pointed out by Kraut (*32*), this hypothesis is questionable not only on account of the unfavorable position of His-57 in the TR–B complex—this position might improve upon binding of a specific substrate—but also on account of the p*K* values of the Asp-102–His-57 diad, 6.8, and of serine, approximately 14. With this large p*K* difference no effective proton transfer is expected to occur in the thermodynamically unfavorable direction: proton transfer can only occur in concert with nucleophilic attack. Thus the role of the His–Asp diad appears more likely to consist in preventing formation of positive charge on O^{γ}(Ser-195) by providing a good acceptor for the hydroxyl proton. The expression "proton-relay system" proposed by Kraut (*32*) seems therefore more appropriate than "charge-relay system."

Further complications are met with Mechanism 1 when the deacylation step is considered. If the imidazole ring remains protonated after breakdown of the tetrahedral intermediates it will not be able to act as general base during water attack on the carbonyl of the acyl enzyme. This implies that the hydroxyl group should be more reactive than water in nucleophilic attack and, in general, that an unprotonated nucleophile should be more reactive than a protonated one. However, it has been shown (*33*), that the pH dependence of the rate of nucleophilic attack of monoisonitrosoacetone on acetylchymotrypsin presents a bell-shaped profile dependent on p*K* 7, due to ionization of His-57, and p*K* 8.3 which coincides with the p*K* of the nucleophile. This result was interpreted to mean that the nucleophile enters the reaction only in its protonated form. This argument, together with the fact that only one of the two imidazole positions becomes functionally significant, makes Mechanism 1 not very probable.

The most relevant feature of Mechanism 2 consists in the participation of His-57 in two successive transfers of the same proton: in acylation the proton is abstracted from O^{γ}(Ser-195) and donated to the leaving nitrogen and in deacyla-

tion the proton is abstracted from the entering nucleophile and donated to O^{γ}(Ser-195). This complex function of His-57 has two important consequences which deserve to be discussed in detail. The first one is the necessity for the leaving nitrogen to undergo inversion in order to accept the proton from His-57. As shown in Fig. 5 this additional step leads to a new tetrahedral intermediate in which the configuration of the leaving nitrogen is reversed from (*R*) to (*S*). Inversion of pyramidal nitrogen atoms carrying only hydrogen or carbon substituents is normally a very rapid process. For example the thermal inversion frequency of ammonia at 300 K is 2×10^8 s^{-1} (*34*). In the case of the tetrahedral intermediate inversion could even be faster since steric hinderance due to 1,3-*syn*-periplanar type of contact between C^{β}(Ser-195) and $C^{\alpha}(L_{yl})$ is decreased in the transition state of the inversion process, as a result of the pyramidal-to-trigonal change of the nitrogen. The equilibrium between the two tetrahedral intermediates of Fig. 5 is likely to be largely on the side of the (*S*) isomer since the (*R*) isomer is destabilized by the unfavorable dipole–dipole interaction between the N–H group of His-57 and the N–H group of the leaving nitrogen. In the (*S*) isomer this unfavorable interaction is replaced by a favorable hydrogen bond interaction between the N–H group of His-57 and the lone-pair orbital of the leaving nitrogen. It is interesting to note that due to this particular equilibrium position the tetrahedral intermediate is soon locked into a configuration where reversion to the enzyme–substrate complex is no more possible, since the bond between O^{γ}(Ser-195) and the carbonyl carbon of the substrate no longer has an *anti*-periplanar lone-pair orbital on the nitrogen. This N-inversion step implies that the leaving nitrogen acts as a switch controlling which of the two critical bonds of the tetrahedral intermediate, the C–O bond or the C–N bond, is broken. In the switch position corresponding to the (*R*) configuration of the nitrogen (Scheme 3, A) C–O bond breaking is allowed by the stereoelectronic principle, whereas C–N bond breaking is prevented by lack of nitrogen protonation. In the other switch position corresponding to the (*S*) configuration of the nitrogen (Scheme 3, B) C–O bond breaking is forbidden by the stereoelectronic principle but the possibility of nitrogen protonation allows the C–N bond to be broken. Since the tetrahedral intermediate is more stable in the (*S*) than in the (*R*) configuration its breakdown



SCHEME 3

in the forward direction is favored over that in the backward direction. Thus the functional significance of such a switch lies in a protein-controlled suppression of the reverse reaction.

The second consequence of the complex function of His-57 is that its imidazole ring must be able to swing between a position allowing formation of a hydrogen bond between $N^{\epsilon 2}$ (His-57) and O^{γ}(Ser-195), and one allowing formation of a hydrogen bond between $N^{\epsilon 2}$ and the leaving nitrogen. This imidazole mobility is consistent with X-ray diffraction data of the stable enzyme species showing that the two extreme positions of this movement, the in-position and the out-position, can really be occupied. As mentioned above interconversion between these two positions, besides rotation of the imidazole ring, requires adjustment of the hydrogen bond network in which His-57 and Asp-102 are involved. It is tempting to speculate that this conformational change is linked with the inversion of the leaving nitrogen: the imidazole ring cannot approach to the leaving nitrogen as long as the latter is in the (*R*) configuration, because of the dipole–dipole repulsion between the two NH groups (Fig. 5A); only after inversion this approach becomes possible. The requirement for a conformational change involving His-57 fits well into the first of the six mechanistic schemes enumerated by Satterthwait and Jencks (*31*). According to this scheme a rate-limiting conformational change of the enzyme during acylation had to be assumed in order to rationalize kinetic data. It seems quite reasonable to identify the conformational change assumed in this scheme with that proposed in our Mechanism 2. The description given above serves therefore to define in structural terms the conformational change first postulated by Satterthwait and Jencks on a phenomenological basis.

In conclusion we believe that Mechanism 2 is more probable than Mechanism 1 since the role of His-57 in the former is more consistent with the structural data. This opinion still needs to be confirmed on a kinetical basis.

ANALYSIS OF THE STRUCTURAL EVENTS

The structural and theoretical considerations concerned with the formation and further reaction of the tetrahedral intermediate have led to the proposition of a minimum set of four structural events which must be assumed to take place during reaction. In view of providing a sound basis for the understanding of the molecular dynamics of a full reaction cycle, it remains to be shown, how the individual structural events are integrated into the catalytic process. Plausible sequence and direction of occurrence of these events are indicated in the circular diagram of Fig. 6. This diagram shows the four structural events on the four levels represented by concentric rings: association and dissociation of ligands (level 1), imidazole shift (level 2), bond-making and bond-breaking steps (level 3), and configurational change of $N(L_{y1})$ (level 4). The enzyme species which are interconverted by these structural events are indicated on the edges of the sectors a–g and g′–a′ in the circular diagram and their chemical nature is shown in Scheme 2. In the free enzyme the imidazole ring occupies the out-position, where it is probably hydrogen bonded to a solvent water molecule (*12*). This interaction

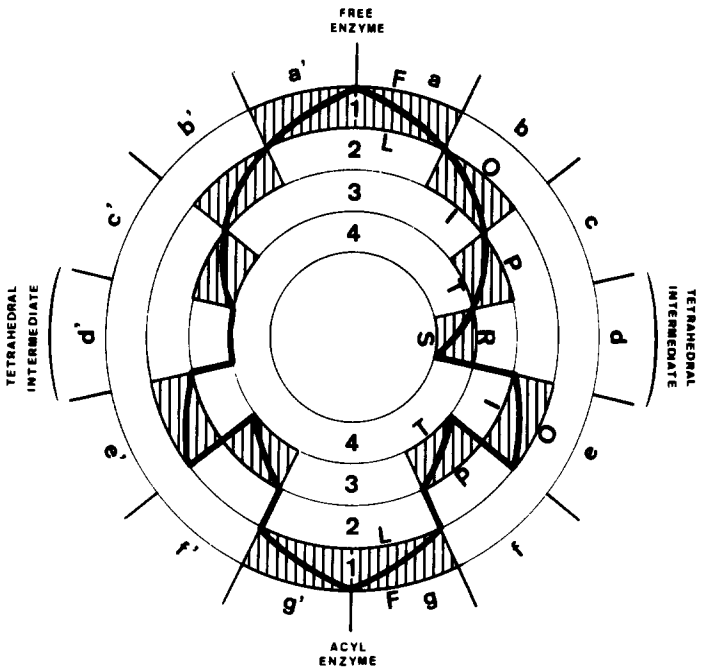


FIG. 6. Structural events occurring during catalysis. The four relevant structural events are represented on the four levels of the circular diagram. (1) Association and dissociation of ligands. Enzyme state: F, free; L, liganded. (2) Imidazole shift. Imidazole positions: I, in; O, out. (3) Bond making and breaking. Geometry of $C(L_{x1})$ and $N(L_{y1})$: P, planar; T, tetrahedral. (4) Configurational change of $N(L_{y1})$. Configurations: (*R*) and (*S*). The solid line indicates the sequence of events: when the line crosses a level the corresponding event takes place. The crossing direction shows the direction of occurrence of the events.

of the imidazole ring is suggested by the fact that in the TR–B complex[3] a solvent molecule, 702-OH, is observed in a position compatible with the existence of such hydrogen bond (*11*). When a specific substrate is bound to the active site (sector a, F → L) water is displaced. Shielding of the imidazole ring from solvent stabilizes its in-position and leads to the out–in imidazole shift (sector b, 0 → I). After this conformational change has taken place the imidazole ring is in a position in which the hydrogen bond between $N^{\epsilon 2}$(His-57) and O^{γ}(Ser-195) can be formed and hence nucleophilic attack of the latter on the carbonyl carbon of the substrate can be assisted by the proton transfers from O^{γ} to $N^{\epsilon 2}$ and from $N^{\delta 1}$(His-57) to $O^{\delta 2}$(Asp-102). During this step the geometry of the carbonyl carbon $C(L_{x1})$ and that of the leaving nitrogen $N(L_{y1})$ change from planar to tetrahedral (sector c, P → T). According to the stereoelectronic theory the tetrahedral intermediate is generated in the (*R*) configuration. In this steric arrangement the NH group of imidazole points toward the NH of the leaving group thus creating a dipole–dipole repulsion

[3] Since the benzamidine inhibitor occupies only the binding pocket for the side chain of L_{x1}, TR–B can be considered, for this purpose, a model for the free enzyme.

which prevents the imidazole ring from approaching $N(L_{y1})$; furthermore, the lone-pair orbital of the latter is not in the position where it can accept the proton from imidazole. Nitrogen inversion (sector d, R → S) relaxes this repulsion so that the imidazole ring can move back to the out-position (sector e, I → O) in which its $N^{\epsilon 2}$ forms a hydrogen bond with the now properly oriented lone-pair orbital of $N(L_{y1})$. With the imidazole ring in the out-position breaking of the $C(L_{x1})$–$N(L_{y1})$ bond can be assisted by proton transfer from $N^{\epsilon 2}$(His-57) to $N(L_{y1})$ and from $O^{\delta 2}$(Asp-102) to $N^{\delta 1}$(His-57). During this step the geometry of the central carbon $C(L_{x1})$ reverts from tetrahedral to planar (sector f, T → P) thus producing the acyl enzyme. The amine product P carrying the leaving nitrogen is subsequently released into the solvent (sector g, F → L). The acyl enzyme may undergo attack by a new nitrogen nucleophile as in the case of a transpeptidation reaction. This process is the exact reverse of acylation and is therefore described by the right side of the circular diagram in the sequence g → a. Under physiological conditions, however, the prosecution of the reaction normally involves acyl enzyme hydrolysis (deacylation). It is reasonable to assume that in analogy to attack by a nitrogen nucleophile the structural events occurring during deacylation are the same as in acylation but take place with opposite sequence and direction, as shown in the circular diagram (sectors g′–a′). A break in the symmetry of this diagram is apparent at level 4, since no inversion occurs when the reacting atom of the entering nucleophile is an oxygen atom.

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