STRUCTURAL FEATURES OF THE SPECIFICITY OF SERINE PROTEASES

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Specificity is one of the most important properties of enzymes, distinguishing them from nonbiological catalysts. Enzymes are capable of "distinguishing" compounds with different structures of groups remote from the reaction center and with different configurations at the asymmetric centers and even the conformers of the substrate. In the present paper, we shall consider the causes of the specificity of enzymes with respect to the structure of the substrates, using as example the serine proteases and the most studied representative of them – α-chymotrypsin.

The hypothesis according to which an enzyme on reacting with its substrate forms a sorption complex – a Michaelis complex – which then decomposes immediately or after a number of intermediate stages with the regeneration of the enzyme and the formation of the reaction products is generally accepted [1]. In the case of hydrolytic reaction catalyzed by chymotrypsin (E), the process for several substrates (S) is described by the three-stage scheme [2-7]

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2,$$
$$\downarrow P_1$$

where ES is the Michaelis complex; ES' is the acylated enzyme; and P_1 and P_2 are, respectively, the alcoholic (amine) and acyl reaction products.

This scheme includes, together with the ordinary Michaelis complex, at least one other intermediate compound – the acylated enzyme – the product of the transfer of the acyl moiety of the substrate to one of the catalytically active groups of the enzyme. The hydrolysis kinetic parameters that enter the Michaelis–Menten expression (1) are effective [2] and are connected with the parameters of the "elementary" stages by expressions (2) and (3):

$$V = \frac{k_{cat}[E]_0 \cdot [S]_0}{K_{M(app)} + [S]_0}; \quad (1)$$

$$k_{cat} = \frac{k_2 - k_3}{k_2 + k_3}, \quad (2)$$

$$K_{M(app)} = \frac{k_3}{k_2 + k_3} \cdot \frac{k_{-1} + k_2}{k_1}; \quad (3)$$

here

$$\frac{k_{-1} + k_2}{k_1} = K_M \text{ and } \frac{k_{-1}}{k_1} = K_S.$$

It would appear that the best measure of the specificity of an enzyme is represented by the constants of the individual stages. Nevertheless, the situation is complicated by the phenomenon of so-called nonproductive binding. Effective catalysis can take place only when the substrate group being split off is correctly oriented in relation to the catalytic groups of the enzyme. For an asymmetric substrate, however, 12 types of mutual orientation of the substituents with respect to the corresponding sections in the enzyme are possible. Of these, only four represent productive complexes [8, 9]. It has been shown [10, 11] that nonproductive binding affects not only the deacylation constant (k_3) but also the ratio of the catalytic constant to the Michaelis constant ($k_{cat}/K_{M(app)} = k_2/k_3$). It is just these magnitudes that may also be taken

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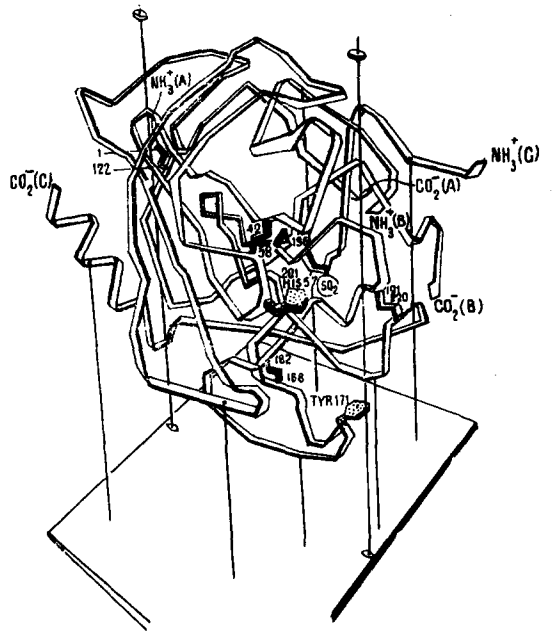


Fig. 1. Tertiary structure of α-chymotrypsin.

as a measure of the kinetic specificity of the enzyme. The second magnitude is in fact the rate constant of the bimolecular reaction between the substrate and the enzyme and reflects the specificity of the free enzyme for the substrate. In this respect it characterizes most accurately the capacity of the enzyme for binding and transforming the substrate.

The binding and transformation of the substrate take place in a limited region of the enzyme called the active center. There is no doubt that the groups of the enzyme "responsible" for these two sides of the catalytic process must be spatially adjacent. Consequently, when the catalytically active groups have been localized in the structure of the enzyme it is possible to state that the binding sections in the active center must be at a distance from these groups not greater than the distance from the bond of the substrate that is being cleaved to those groups of the substrate that are responsible for its sorption on the enzyme. In α-chymotrypsin, as the results of numerous investigations have shown, the catalytically active groups are the hydroxy group of serine residue 195 [12-17] and the imidazole residue of histidine 57 [18-21]. It is obvious that these groups must be adjacent in the tertiary structure of the protein (Fig. 1). In addition, the groups participating in the binding of the substrate must be localized close to these catalytic groups.

The problem of the determination of the mutual spatial position of the binding and catalytic sections of the active center may therefore be reduced to the problem of determining the conformation of the substrate in the Michaelis complex and in other enzyme–substrate complexes.

The following stage is the localization of the binding sections in the primary structure of the enzyme. This problem is undoubtedly more complex and at the present time is solved unambiguously only by x-ray structural analysis.

Among the methods of studying the connection between the structure and specificity of an enzyme, x-ray structural analysis, both because of its informativeness and, incidentally, because of its complexity, occupies a special place. However, this method, taken in isolation, is incapable of elucidating all the features of enzyme–substrate interaction in the dynamic aspect.

Historically, the first method – and by no means the last in importance – for studying the specificity of enzymes is the method of kinetic substrate and inhibitor analysis.

The natural substrates of chymotrypsin are peptides with aromatic amino-acid residues and, to a smaller extent, such amino acids as leucine, methionine, and asparagine [22]. Nevertheless, the overwhelming bulk of investigations has been performed with amides and esters of N-acylated amino acids – phenylalanine, tyrosine, and tryptophan [23]. The results of a comparison of the specificity of chymotrypsin for a number of acylated amino acids shows that substrate properties are possessed by compounds containing a voluminous hydrophobic substituent in the β position (Table 1).

The fact that the binding of an aromatic substituent in the active center of chymotrypsin has a hydrophobic nature follows from the existence of a clear correlation between the constants of the inhibition of chymotrypsin by substrate-like compounds and also by aromatic hydrocarbons and the distribution coefficients of these substances between water and a nonpolar phase [24].

A priori, it can be stated that a single interaction of the aromatic side chain of the substrate with the enzyme is not sufficient for the correct orientation of the group undergoing cleavage of an acylamine acid derivative, all the more since it is incapable of ensuring the stereospecificity of the enzymatic process. In actual fact, the replacement of an acetamido group in a substrate by the isosteric acetoxy group [25] sharply reduces the suitability of the substrate and the stereospecificity of the hydrolysis.

A careful thermodynamic analysis of the constants for the deacylation of acylchymotrypsins containing a lateral acylamino group and the corresponding compounds not containing this group permits the conclusion

TABLE 1. Some Synthetic Substrates of α-Chymotrypsin $R-CH_2CH(R')COX$

Compound	R	R'	X	k_{cat}, sec^{-1}	$K_{M(app)}$, mmole	$k_{cat}/K_{M(app)}$, $mole^{-1} \cdot sec^{-1}$
1	Indol-3-yl	$NHCOCH_3$	OCH_3	27,7	0,01	$2,77 \times 10^6$
2	C_6H_5-	$NHCOCH_3$	OCH_3	57,7	1,5	$3,8 \times 10^4$
3	C_6H_{11}-	$NHCOCH_3$	OCH_3	15,2	0,19	$8,0 \times 10^4$
4	$(CH_3)_2CH$-	$NHCOCH_3$	OCH_3	4,6	2,9	$,6 \times 10^3$
5	n-C_4H_9-	$NHCOCH_3$	OCH_3	13,4	1,64	$8,1 \times 10^3$
6	C_6H_5-	$OCOCH_3$	OCH_3	1,2	6,5	$1,8 \times 10^2$
7	n-HOC_6H_4-	$N(CH_3)COCH_3$	OCH_3	0,026	8,4	3,1
8	C_6H_5-	$NHCOCH_3$	NH_2	0,04	37,0	1,1

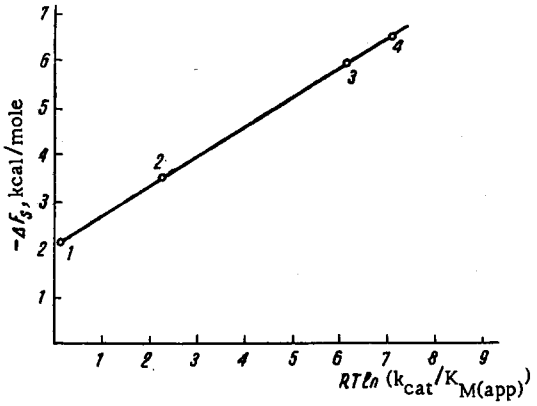


Fig. 2.

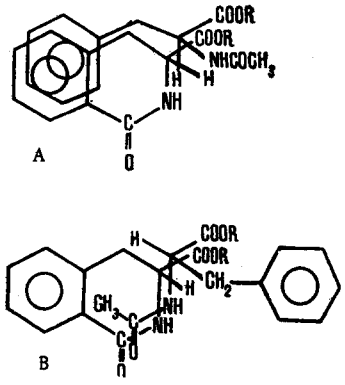


Fig. 3.

Fig. 2. Graph of the relationship between the free energy of hydrolysis and RT ln $(k_{cat}/K_{M(app)}$ for derivatives of acetylphenylalanine: 1) amide; 2) p-nitroanilide; 3) methyl ester; 4) ethyl ester.

Fig. 3. Possible ways for the mutual arrangement of the methyl ester of N-acetyl-L-phenylalanine and the methyl ester of D-3,4-dihydroisocarbostyryl-3-carboxylic acid in the active center of chymotrypsin.

that the acylamino group does actually make a fundamental contribution to specificity, this contribution having an entropy character [26]. The difference in the thermodynamic characteristics of the two groups of compounds amounts to 10 eu; i.e., the change in free energy is about 3 kcal/mole.

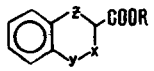
The introduction of voluminous substituents into the α or the β position of the substrate leads to a sharp fall in the rate of hydrolysis [27, 28]. This fact is usually interpreted as an indication of steric hindrance in the corresponding region of the active center of the enzyme. However, it must be observed that these and a number of other modifications of the substrate may change its conformational state and, thus, influence the capacity of the substrate for binding to the enzyme.

A modification of the group that is split off mainly affects the electrophilicity of the ester or amide carbonyl. A good correlation exists between the rate of hydrolysis of acylamino acid derivatives differing in the nature of the bond undergoing cleavage and the Hammett or Taft constants of the departing groups [23]. Moreover, differences in the rates of enzymatic hydrolysis of esters and amides of one and the same acylamino acid are due mainly to differences in the free energy of hydrolysis of these compounds, since a good correlation is observed between these magnitudes (Fig. 2) [29].

Consequently, in the active center of chymotrypsin, in addition to the catalytic center there are two binding regions – a hydrophobic section and an acylamino-group-binding section which, as may be assumed, is a group acting as a hydrogen-bond acceptor.

The catalytic and binding sections in chymotrypsin are separated spatially and are apparently independent. Thus, it has been shown that the selective modification of the catalytic section – namely, the methylation of the N^{ε} atom of the catalytically active histidine-57 residue – while completely depriving the enzyme of catalytic properties does not affect the capacity of the enzyme for binding the substrate at all [30, 31].

TABLE 2. Cyclic Substrates and Inhibitors of Chymotrypsin



Compound	z	x	y	R	Configuration (sign of rotation)	k_{cat},* sec^{-1}	$K_{M(app)}$,* mmole	$k_{cat}/K_{M(app)}$,* $mole^{-1} \cdot sec^{-1}$	K,† mmole
9	CH_2	NH	CO	CH_3	*D*	12,1	0,7	$0,13\times10^4$	—
10	CH_2	O	CO	CH_3	D	35,0	0,7	$5,0\times10^4$	—
11	CH_2	CO	NH	C_2H_5	(—)	0,08	1,92	$0,42\times10^2$	—
12	CH_2	$N-CH_3$	CO	CH_3	*DL*	—	—	—	7,1
13	CH_2	CH=	CH=	CH_3	*S*	—	—	$6,0\times10^2$	—
14	NH	CO	NH	C_2H_5	*DL*	—	—	—	1,0

* Conditions [44]: pH 7.2; 25° C; 4 vol. % of CH_3CN.

† Substrate: methyl ester of acetyl-L-phenylalanine.

The spatial separation of the catalytic and hydrophobic sections of the active center of chymotrypsin appears clearly in an investigation of the properties of so-called bifunctional inhibitors. These inhibitors include alkaneboronic acids [32-35]. The boric acid residue in these compounds forms a complex with the imidazole group of the histidine-57 residue and with the hydroxy group of the serine-195 residue, and the alkyl group is capable of interacting with the hydrophobic section [32]. It has been found that the constant of the inhibition of chymotrypsin by alkaneboronic acids depends strongly on the length of the alkyl chain, while the contribution of the aliphatic residue appears at values of n of from 3 to 6. From this it may be concluded that the hydrophobic section is at a distance of not less than two methylene groups from the catalytic section and has an extent of the order of four methylene groups. This agrees well with the dimensions of the substrates. Similar results have been obtained in a study of the rates of deacylation of acylchymotrypsins with alkyl chains of different lengths [36].

Nevertheless, these conclusions have only a relative value, since in no case is the conformation of a substrate or an inhibitor in the active center of the enzyme known. To investigate this question it is very useful to use substrates and inhibitors with a limited conformational mobility. This type of substrate was first proposed by Hein and Niemann [37] in 1960. It was established that 3-methoxycarbonyl-3,4-dihydroisocarbostyryl (9) – a cyclic analog of an ester of N-acetyl-L-phenylalanine – is hydrolyzed by chymotrypsin as effectively as the latter, but the enzyme is specific for the D form of this compound (Table 2).

Since these two substrates compete with one another, and also with indole, because of the active center of the enzyme [38], it may be assumed that the cyclic substrate is bound to the same section of the active center as acetylphenylalanine. In recent years, a large number of substrates of this type have been synthesized [27, 39-44] (see Table 2). It has been found that they are all hydrolyzed in the D form. It is characteristic that in contrast to linear substrates, the replacement of a lactam group by a lactone group (compound 10) does not affect the substrate properties of the cyclic analogs. However, compounds of the same type have been found which possess poor substrate properties or do not undergo enzymatic hydrolysis at all (compounds 12 and 14).

The similarity of the kinetic parameters of the enzymatic hydrolysis of D-methoxycarbonyldihydroisocarbostyryl and of that of the ester of N-acetyl-L-phenylalanine permits the assumption that the reaction conformation of the latter must be similar to a certain extent to the conformation of the cyclic compound.

However, the cyclic analog of acetylphenylalanine does not have a completely rigid conformation – there is a freedom of conformational transitions of the methoxycarbonyl group from the equatorial orientation into the axial. Consequently, before comparing the conformations of a cyclic and linear compound a choice between these two possibilities must be made. The axial conformation appeared more attractive since in this case a greater difference existed in the orientations of the L- and D-isocarbostyryls [40, 45]. There is also indirect information in favor of the equatorial conformation [46, 47]. This question could be answered by comparing the conformational state of a number of cyclic compounds in solution with their substrate properties. An investigation by the NMR method of a number of cyclic analogs of chymotrypsin substrates has shown [48] that in the majority of cases the equatorial conformation is preferred both in nonpolar solvents and in aqueous solutions. However, a compound with the equatorial conformation is not necessarily a good substrate. On the other hand, compounds with the axial conformation are not hydrolyzed by the enzyme. Consequently, the equatorial conformation is a necessary, but not sufficient, condition for high substrate properties of cyclic compounds of the type considered.

TABLE 3. State Constants of the Enzymatic Hydrolysis of the Methyl Ester of N-Acetyl-L-phenylalanine (1) and of D-3-Methoxycarbonyl-3,4-dihydroisocarbostyryl (9) [50].

Compound	$K_{M(app)}$, M·10⁴	k_{cat}*, sec⁻¹	K_S, M·10⁴	k_2, sec⁻¹	k_3, sec⁻¹	k_2/K_S, mole⁻¹·sec⁻¹
1	12,0	10,7	64	56	13	0,88×10⁴
9	5,6	1,6	14	4,1	2,6	0,28×10⁴

*Conditions: pH 6.0; 25° C; 0.1 N phosphate buffer.

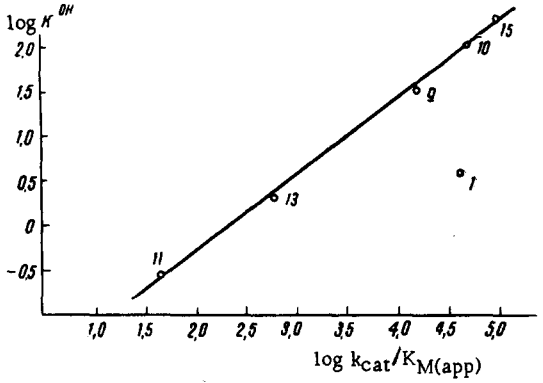


Fig. 4. Graph of the relationship between the constants of enzymatic and alkaline hydrolysis.

An independent confirmation of the equatorial conformation follows from the behavior of the nitrophenyl esters of cis- and trans-cinnamic acids. It has been shown that the cis-cinnamic ester, which is conformationally similar to the substrate in the axial conformation, reacts with the enzyme several orders of magnitude more slowly than the trans-cinnamic ester [49].

Thus, it might be justifiably assumed that the cyclic substrate of chymotrypsin is bound to the enzyme in the equatorial conformation. Since it is impossible completely to superpose the L and D enantiomers, two possibilities arise: either in the active center both substrates are bound by the aromatic groups to one and the same locus of the enzyme, in which case the amide and the lactam groups must be oriented differently, or both substrates orient their NH groups similarly, and then the positions of the aromatic groupings must be different.

Consequently, two basically possible mutual orientations of cyclic and linear substrates arise (Fig. 3). Even on the basis of information on the competing inhibition of the hydrolysis of a cyclic substrate by indole and also that given above on the dimensions of the sorption section of chymotrypsin it may be considered that the second scheme is unlikely. In order to exclude this possibility completely, it was necessary to show that the lactam group of isocarbostyryl does not take part in binding with the enzyme; i.e., it is oriented differently from the NH group of acetylphenylalanine. The behavior of methoxycarbonyldihydroisocoumarin [27] is a confirmation of this; however, it does not exhaust the problems, since other analogs modified in the lactam group and possessing low substrate properties exist (see Table 2).

If the cyclic substrate, unlike the linear substrate, does not take part in the formation of a H bond with the enzyme, differences must be expected in the specificity of the enzyme for these two substrates at different stages of the enzymatic process. At the acylation stage, when the cyclic substrate is bound "by a single point" – only by its aromatic group – the correct orientation of the group being split off must be hindered, and at this stage the enzyme will perform catalysis less effectively. However, in the case of the linear substrate binding by "two" points (aromatic group and NH group), catalysis should be substantially more effective. At the deacylation stage, these differences should be appreciably less, since here the cyclic substrate is fixed more rigidly by an additional covalent bond with the enzyme.

The results of the determination of the stage rate constants for the hydrolysis of linear and cyclic substrates have shown [50] that it is just this situation that is actually realized (Table 3). The acylation constants of the two substrates differ by a factor of 15 and the deacylation constants by a factor of approximately 5.

It may be concluded from this that methoxycarbonyldihydroisocarbostyryl does not react by its NH group with the hydrogen-bond acceptor in the enzyme and that the relative orientation of linear and cyclic substrates may be represented by formula A (see Fig. 3).

Why, then, do cyclic substrates with a modified lactam grouping that are slowly hydrolyzed by chymotrypsin exist? The answer to this question is clear for compounds possessing the axial conformation in solution, while for compounds with the equatorial conformation it follows from the results of a comparison of the rate constants of enzymatic and alkaline hydrolysis. For cyclic substrates of chymotrypsin, a good correlation has been observed between these two parameters (Fig. 4) [44]. Thus, the differences in the rates of enzymatic hydrolysis are due to electronic and steric factors of the substrates themselves and not to any difference in the nature of their binding to the enzyme. It is interesting to observe that the linear

TABLE 4. Constants of the Enzymatic Hydrolysis of the Methyl Ester of N-Acetylphenylalanine (1) and of 3-Benzylmorpholine-2,5-dione (15)

Compound	k_{cat},* sec^{-1}	$K_{M(app)}$, mM	$C = k_{cat}/K_{M(app)}$, $M^{-1} \cdot sec^{-1}$	C_{st}†	k^{OH^-}, $M^{-1} \cdot sec^{-1}$
1-*L*	19,3	3,45	$5,6 \times 10^3$	$5,6 \times 10^3$	3,3
1-*D*	Not hydrolyzed		—	—	—
15-*L*	124	1,25	$1,0 \times 10^5$	$1,9 \times 10^3$	179
15-*D*	Not hydrolyzed		—	—	—

* Conditions: pH 7.2; 25° C; 20 vol. % of dimethylformamide.
† The value of C has been corrected for the difference in the rate of hydrolysis under nonenzymatic conditions.

TABLE 5. Results of a Comparison of Conformations (degrees)

Parameter	Conformations		Most suitable conformation of N-acetyl-L-phenylalanine methylamide acc. to calc. [56]
	compound 1 according to kinetic substrate analysis (Fig. 6)	formyl-L-tryptophan, from x-ray structural analysis [55]	
$\psi(C_\alpha - C')$	130—140	180	120
$\varphi(C_\alpha - N)$	40	118	43
$\chi_1(C_\alpha - C_\beta)$	300	308	300
$\chi_2(C_\beta - C_\gamma)$	150 (—30)	170	85

substrate of chymotrypsin departs from this correlation. This becomes understandable if one considers that in solution the methyl ester of N-acetyl-L-phenylalanine possesses free internal rotation around the $C_\alpha - N$ and $C_\alpha - C_\beta$ bonds which does not exist in its cyclic analog. Because of this, the rate of its alkaline hydrolysis is low. Bonding with the enzyme limits free internal rotation and thereby the intrinsic reactivity of the substrate, i.e., the reactivity to a nucleophilic agent as strong as the hydroxyl ion, is increased. The increase in the intrinsic reactivity of the substrate on sorption by the enzyme may be one of the reasons for the high efficiency of enzymatic catalysis.

Before summarizing the results of an analysis of the conformation of the substrate in the active center of the enzyme, we must dwell on another question: on the conformation of its acylamino group. A modification of this group does not affect the capacity of the substrate for binding with the enzyme but strongly affects the catalytic constant. Starting from this Erlanger [52], and then Knowles [51], suggested that the enzyme binds the amide group not in the usual trans conformation but in the cis conformation. Then the fact that the free energy of binding is independent of the capacity of the substrate for being the donor of a hydrogen bond in the enzyme—substrate complex becomes understandable, since the difference in the free energies of the cis and trans conformers can compensate the energy of the hydrogen bond. Investigations of a new type of substrate of chymotrypsin with limited conformational mobility – namely 3-benzylmorpholine-2,5-diones (15) – have shown that chymotrypsin is capable of discriminating between the L and D forms of substrates containing fixed cis-acylamino groups (Table 4) [53]. The hypothesis of the cis conformation of the acylamino group of the substrate encounters a certain difficulty because of the very high barrier to the trans—cis transition in amides, which is about 20 kcal/mole [54]. Consequently, it may be stated that the enzyme is incapable of "selecting" cis isomers from a solution. In this case, the limiting stage would be trans—cis isomerization. However, it may be suggested that the enzyme binds the substrate in the trans form, but in the first Michaelis complex definite steric interactions arise which lower the barrier to the trans—cis transition, and isomerization to the cis form takes place actually within the complex. Thus, the question of the conformation of the acylamino group in the enzyme—substrate complex cannot yet be considered as solved, but it is obvious that the first stage consists in an interaction between the enzyme and the substrate with the trans conformation of the amide group.

The results of an investigation of the substrate properties of the morpholinediones (15) also enables us to gain an idea of the orientation of the carbonyl group undergoing cleavage in a typical substrate (type 1). Because of the nonplanar structure of the heterocycle in the morpholine diones (15), the $C_\alpha - C'$ dihedral angle in these compounds is about 140°. It is obvious that the corresponding angle ψ in the substrate will also be close to this figure. This agrees with the angle φ in indolylacryloylchymotrypsin, calculated on the basis of the results of x-ray analysis [65].

Summarizing what has been stated above, the conformation of the methyl ester of N-acetyl-L-phenylalanine may be represented in the active center of chymotrypsin as is shown in Fig. 5.

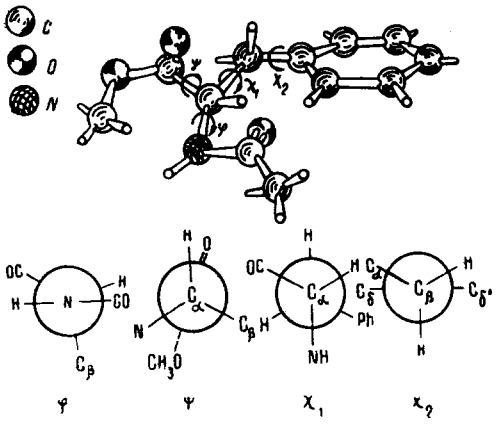


Fig. 5. Reaction conformation of the methyl ester of N-acetyl-L-phenylalanine.

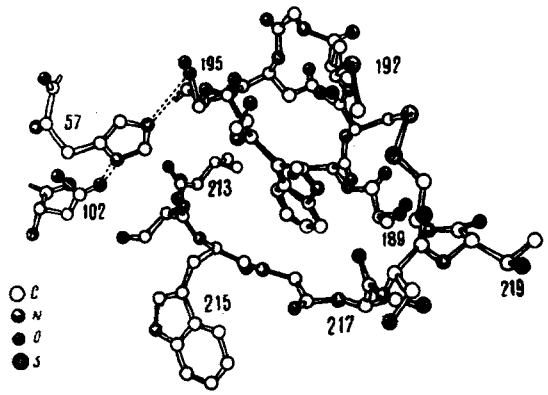


Fig. 6. Active center of α-chymotrypsin.

It is interesting to compare this conformation deduced as the result of a kinetic substrate analysis, with, in the first place, the most preferred conformations in solution and with, in the second place, the conformation of a substrate-like inhibitor – N-formyl-L-tryptophan – obtained by Steitz et al., by x-ray structural analysis (Table 5).

On comparing the conformational parameters of the ester of acetyl-L-phenylalanine in the active center of the enzyme and the parameters of an acetyl-L-phenylalanine derivative in solution, obtained both by theoretical conformational analysis [56] and by NMR spectroscopy [57], it can be seen that the enzyme binds the substrate in a conformation differing from the energetically most suitable conformation by the value of the angle χ_2 and by a small change in the angle ψ. The reaction conformation of the substrate differs in enthalpy from this most favorable conformation by ≈ 3 kcal/mole [56]. This value is substantially lower than the change in the enthalpy of the system in the formation of Michaelis complex (for the ethyl ester of N-acetyl-L-tryptophan, $\Delta H^\circ = -9.9$ kcal/mole [58]). It may be imagined that the binding of the substrate in the active center of chymotrypsin is accompanied by a rotation of the benzene ring by approximately 60° and by a rotation of the carbonyl group around the $C_\alpha - C'$ bond by 10-20°, and part of the energy of the enzyme–substrate interaction is consumed in this.

So far as concerns the conformation of N-formyl-L-tryptophan obtained on the basis of x-ray structural analysis, the differences here are somewhat more substantial. They relate to the orientation of the carboxy group which, because of the presence of a negative charge, cannot be arranged in the same way as ester or amide groups of the substrate, and to the orientation of $C_\alpha - N$ bond. The latter is apparently due to features of the crystal packing of the enzyme, which has a bimolecular cell in which the two molecules are in contact in a region close to the active center [59]. Consequently, the formylamino group of the inhibitor differs from the position which it occupies in the true enzyme–substrate complex.

As can be seen, the results of the x-ray structural analysis nevertheless largely agree with the results of the kinetic analysis, and the observed differences are explained rather by the deficiencies of the first method. A comparison of the two methods obviously gives the best approximation to reality.

X-ray structural analysis gives the most complete information on the structure and stereochemistry of the active center of the enzyme. For α-chymotrypsin this information has become available thanks to the work of Blow's group at Cambridge [59-62].

It has been found that near the catalytically active Ser-195 residue in chymotrypsin there is a region of reduced electron density, the so-called "tosyl hole," formed by the residues of the amino acids 189-195 and 213-220 of chain C of chymotrypsin. The catalytically active hydroxy group of serine-195 and imidazole group of histidine-57 are located, as it were, in this hole (Fig. 6). The hole is formed mainly by residues of nonpolar amino acids with small side chains, the side chains being, as a rule, located outside this hole. The dimensions of the hole correspond to the dimensions of the tryptophan side chain. Even the side chain of p-iodophenylalanine is not accommodated in this cavity and the corresponding substrate is not hydrolyzed by the enzyme.

The hydroxy group of Ser-195, the nitrogen atoms of the imidazole ring of His-57, and the carboxy group of the residue of Asp-102 located in the immediate vicinity of this ring form a single system of hydrogen bonds i.e., a charge "relay system" [63]. This system ensures a high nucleophilicity of the hydroxyl of Ser-195, in view of the fact that the Asp-102 residue is localized in a hydrophobic pocket and, apparently, possesses an anomalously high value of pK_a.

Apart from the catalytically active groups mentioned, the N-terminal amino group of the Ile-16 residue, the carboxy group of the Asp-194 residue, and the side chain of the Met-192 residue are important for the functioning of α-chymotrypsin. Chemical modification of these groups strongly affects the activity of the enzyme [64].

According to the results of x-ray structural analysis [60], in the region of neutral pH values the first two of the groups mentioned form an ion pair the presence of which is possibly responsible for the optimun pH of the catalytic activity of the enzyme. The side chain of the Met-192 residue in the free enzyme is screened in solution and does not interfere with the access of the substrate to the hydrophobic hole. Further, in the acylated enzyme its orientation changes in such a way that it screens the side chain of the substrate from contact with water [65]. It is interesting to note that in the region of the active center there is a special type of conformation of the peptide chain with a hydrogen bond between peptide bonds separated by two residues [61]. This type of hydrogen bond is possible only "with a hairpin bend," and for it to be realized the third amino acid must be glycine. Apparently, the presence in many serine proteases of glycine in a position adjacent to the catalytically active serine is connected with this. This conformation ensures the rigidity of the peptide chain and the accurate positioning of the functionally important residues in the active center. In the free enzyme, the hydrophobic hole is occupied by four molecules of water, which are displaced from this hole on the binding of substrates or inhibitors. The x-ray structural analysis of the complex of chymotrypsin with N-formyl-L-tryptophan shows that on the one side the aromatic group of this quasisubstrate interacts with the 190-191 and 191-192 peptide bonds and, on the other side, the indole ring interacts with the 215-216 bond. Although direct information on the acceptor group of the hydrogen bond with the substrate is not given by x-ray structural analysis, there are nevertheless weighty arguments in favor of the assumption that this group is the carbonyl group of the Ser-214 residue. This residue, by its OH group, is connected by a hydrogen bond with one of the oxygen atoms of the Asp-102 residue, in conquence of which the carbonyl group of Ser-214 is rigidly fixed with respect to the catalytic groups Ser-195 and His-57. Finally, the carboxy group of the pseudosubstrate interacts with the peptide NH groups of the Gly-193 and Ser-195 residues. Some arguments in favor of the hypothesis of the trans–cis isomerization of the enzyme–substrate complex have already been given. Yet another consideration in favor of this hypothesis follows from an analysis of a spatial model of the active center of the enzyme. It is known that the rate of cleavage of polypetides depends on the configuration of the residue connected with the bond undergoing cleavage [67, 68]. It has been shown, for example, that this secondary stereospecificity of the enzyme is very clearly expressed in the case of enantiomeric pairs of tripeptide substrates (for L-Val–L-Tyr–Gly-NH_2, $K_M = 7.82$ mM and $k_{cat} = 1.45$ sec^{-1}, while D-Val–L-Tyr–Gly-NH_2 is an inhibitor with $K_i = 26$ mM [69]). The fact that enantiomers containing a N-terminal amino-acid residue in the D form are inhibitors shows that the binding of this residue prevents the correct orientation of the bond to be cleaved. If we compare the mutual orientation of the D-Val–Tyr and L-Val–Tyr residues in the active center of the enzyme, then in the case of a trans amide bond between Val and Tyr no fundamental obstacles whatever to the cleavage of the D-Val residue are found. However, in the cis conformation the L residue is cleaved satisfactorily, and the D residue cannot be cleaved because of the steric hindrance created by the side chain of Met-192.

Let us dwell further on the question of the conformational changes of chymotrypsin taking place in the binding and transformation of a substrate. According to the results of a Fourier differential synthesis of chymotrypsin, its complex with a quasisubstrate – formyltryptophan – [55], and the acyl enzyme indolylacryloylchymotrypsin [65], the binding of the quasisubstrate does not change the conformation of the active center at all, and in the acylation of the enzyme considerable changes in the position of the individual groups in this section of the protein molecule are observed. Thus, the oxygen atom of the serine hydroxyl is displaced by about 2.5 Å, and the imidazole group is displaced by 0.3 Å in the direction of the solvent. A shift in the sulfur atom of the Met-192 residue by 1 Å and of the Cys-191–220 SS bridge by 0.35 Å is also observed. The methionine side chain is shifted, hiding, as it were, the side chain of the substrate from contact with water and forming a peculiar "lid" for the hydrophobic section [65].

Thus, at the stage of the formation of the Michaelis complex no fundamental conformational changes whatever in the enzyme are observed, and all the changes at the stage of the formation of the acyl enzyme are apparently due to a change in the covalent state of the substrate and of the serine hydroxyl of the enzyme. They are possibly a consequence of a disturbance of the charge "relay system" on acylation. This is supported by the fact that chymotrypsin selectively methylated at the N^{ε} atom of the histidine-57 residue exhibits, on x-ray structural analysis, practically the same conformational changes as indolylacryloylchymotrypsin [70]. The system of hydrogen bonds between the Asp-102, His-57, and Ser-195 residues pos-

sibly plays the role of a peculiar "trigger" for the conformational changes of chymotrypsin. It has been shown [71] that the enzyme methylated at the N^{ε} atom of His-57 is substantially less resistant to denaturation by urea than native chymotrypsin, the difference in the free energies of uncoiling of the proteinglobule of the native and the methylated enzyme amounting to about 4-5 kcal/mole, which is close to the energy of a hydrogen bond.

The fact that changes in the active center lead to the destabilization of the whole molecule of the enzyme is obviously connected with the similar general and local (in the region of the active center) conformational stabilities of chymotrypsin. This has been shown by a comparison of the rates of change of a number of parameters characterizing the state of the whole globule (ORD) on heat denaturation and on denaturation by urea with parameters characterizing the state of the active center (the sorption of proflavine, catalytic activity) [72, 73].

The conclusion of the conformational rigidity of the active center of chymotrypsin is in apparent contradiction to the known lability of the protein under very different influences. It can be seen from the diagram of state of chymotrypsin that even in the region of neutral pH values and ordinary temperatures this block exists in two thermodynamically different states, A_b and A_f [74]. Some conformational transitions in chymotrypsin can be connected with a change in complete definite sections of the molecule. Thus, for example, it has been shown [75] that chymotrypsin modified by nitration at the Tyr-146 and 171 residues possesses luminescent properties and in a study of the polarization of the luminescence as a function of the pH, two pH-dependent conformational transitions in the acid and neutral regions have been found, which may be connected with a change in the state of the C-terminal fragment of chain B of the protein, which contains the luminescent label.

However, under fixed conditions the enzyme apparently does not change its state on forming the enzyme–substrate complex. It is obvious that the "recognition" of the substrate by the enzyme will be the more effective the more rigid the conformation of the enzyme is. The considerable conformational changes taking place in the acylation of the enzyme by the substrate apparently have functional importance as well. The second step of the catalytic reaction – deacylation – requires the participation of a new nucleophile – water. From the results obtained by Henderson [65] from an x-ray structural analysis of indolylacryloyl-chymotrypsin it follows that it is just the conformational change in the position of the hydroxy group of serine and of the imidazole ring of histidine-57 that permits the water molecule to be oriented accurately with respect to the group of the acyl enzyme that is being split off.

Thus, summarizing the causes of the substrate specificity of chymotrypsin we may emphasize certain features of the structure of its active center.

1. In the active center there is a hole with dimensions sufficient for fixing the side chain of the residues of the aromatic amino acids of the substrate. This hole is hydrophobic in nature and is capable of retaining a lyophilic side chain.

2. There is a rigidly fixed carbonyl group of a Ser-214 residue, which is an acceptor of the hydrogen bond of an acylamino group of the substrate.

3. The substrate is bound to a protein with only a slight change of its optimum conformation in aqueous solution. It is not excluded, particularly for polypeptide substrates, that after the formation of the complex trans–cis isomerization of the acylamino residue of the substrate takes place through secondary interactions.

4. The binding of the residues of L-amino acids in the manner described above accurately orients the group undergoing cleavage with respect to the nucleophilic group of the enzyme – the hydroxy group of serine-195 – and the departing group is arranged in such a way that the proton from the imidazole N atom is capable of being transferred to this departing residue.

5. The binding of the substrate does not cause any conformational rearrangements in the enzyme whatever. The strain of the active center of the enzyme is electronic and not conformational. Such electronic strain, brought about by the charge "relay system," leads to a high nucleophilicity of the serine hydroxyl. The same system apparently stabilizes the conformation not only of the active center but of the whole protein globule.

6. The transfer of the acyl residue of the substrate to the enzyme is accompanied by conformational changes permitting a water molecule effectively to attack the carbonyl group of the acylated enzyme. In this process imidazole takes part as a common base.

It may be assumed that the conclusions presented for chymotrypsin are also applicable to other serine proteases very similar to it – in particular, trypsin and elastase.

The most important difference between these three enzymes consists in their different substrate specificities. As is well-known, trypsin is specific for basic amino acids and elastase for amino-acid residues having small side chains of the type of alanine [23]. It is obvious that these differences must have their structural explanations. In actual fact, it has been shown that in trypsin in place of the Ser-189 residue, which, as it were, closes the hydrophobic hole of chymotrypsin, there is an aspartic acid residue [75]. The interaction of the positively charged side chain of the substrate with the carboxy group of this residue ensures the required specificity. In elastase, in place of the same residue there is a valine residue, the side chain of which fills the sorption section and permits binding only with a small substrate of the type of alanine [76].

Thus, an accurate stereochemical correspondence of the substrate and the groups of the active centers of enzymes at each stage of the enzymatic process ensures the maximum degree of productive binding and a high efficiency of enzymatic catalysis.

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