THE NATURE OF GENERAL BASE-GENERAL ACID CATALYSIS IN SERINE PROTEASES

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Abstract.—The high reactivity of the serine residue at the active site of serine proteases is often attributed to the formation of a hydrogen bond between this serine and a histidine residue. In the case of the serine protease subtilisin, the catalytic serine residue can be specifically replaced by a cysteine residue and this modified enzyme is called thiol-subtilisin. By studying the D_2O effect on acyl-enzyme formation with subtilisin and thiol-subtilisin, we present evidence that thiol-subtilisin but not subtilisin may contain a hydrogen bond. Based on the comparison of the catalytic activities of subtilisin and thiol-subtilisin, a rigid active site model for the serine proteases is proposed in which the histidine residue operates in a fixed steric position both as a general base and as a general acid, and this, rather than the formation of a hydrogen bond, accounts for the high nucleophilicity of the serine residue.

Since the unusual reactivity of a serine residue and the catalytic function of a histidine residue in chymotrypsin, trypsin, elastase, subtilisin, and other serine proteases became known, the high nucleophilicity of this particular serine side chain has drawn special attention. A number of hypotheses have been put forward suggesting that a hydrogen bond is formed between the nitrogen atom of the imidazole ring and the hydroxyl group of the serine residue.[1-3] Recent studies on the tertiary structure of chymotrypsin[3] and subtilisin[4] support the idea of the formation of a hydrogen bond. On the other hand, kinetic investigations have indicated that thiol-bacterial proteinase Novo, but not bacterial proteinase Novo, may contain a hydrogen bond at the active site.[5] Here we present evidence in favor of the latter suggestion and try to reconcile the apparent contradiction between kinetic and X-ray diffraction studies.

In analogy of a general approach in physical organic chemistry, in which the variation of the structure of reactants is utilized, we have studied the kinetics of thiol-bacterial proteinase Novo and thiol-subtilisin (Carlsberg) in which enzymes the reactive serine residues were replaced by cysteine side chains. The hydrolyses by both the serine proteases and their thiol-derivatives proceed via the mechanism of equation (1).

$$E + S \overset{k_1}{\rightleftharpoons} ES \overset{k_2}{\rightarrow} ES' \overset{k_3}{\rightarrow} E + P_2 + P_1 \qquad (1)$$

where the symbols have their usual meaning.[6]

When *p*-nitrophenyl acetate is used as substrate, the rate-limiting step is deacylation; that is, the hydrolysis of the intermediate acetyl-enzyme and acetyl-thiol-enzyme, respectively. Thus the over-all rate constant of this reaction, k_{cat} is equal to k_3. From the pH dependence of k_{cat}, one can determine the pK

of that catalytic group which operates in the *acyl-enzyme*. On the other hand, the pH dependence of k_{cat}/K_m is characteristic of the catalytic group of the *free enzyme*.[7] By the measurement of the kinetic parameters it was shown that bacterial proteinase Novo and the Carlsberg subtilisin, as well as their thiol-derivatives possess in their acyl-enzyme form an ionizable group with a pK of about 7. This group, which appears to be a histidine residue, is also demonstrated in the free serine enzymes but not in their free thiol-derivatives.[5, 8, 9] Thus in the free Carlsberg thiol-subtilisin, the pK value is shifted to 6.15 and in thiol-bacterial proteinase Novo it is somewhat lower than 5.5. As the most reasonable explanation for this observation it has been suggested[5, 9] that a hydrogen bond is formed between the thiol and the imidazole groups of the free thiol-enzymes. This bond should stabilize the imidazole group against protonation, and the apparent pK of the histidine residue should consequently be lowered. In thiol-subtilisin (Carlsberg) this hydrogen bond appears to be weaker (pK = 6.15) than in thiol-bacterial proteinase Novo (pK < 5.5). The absence of a shift in pK in the serine enzymes indicates that in the serine enzymes no hydrogen bond is formed.

Independent evidence on the formation of a hydrogen bond in serine or thiol-enzymes should be obtained from a study of the effect of D_2O on the kinetic constants of the hydrolysis of *p*-nitrophenyl acetate. It is known that the velocity of a reaction with rate-limiting proton transfer decreases to about one-third in D_2O. The formation of a hydrogen bond can be considered as a partial proton transfer. Accordingly, if a hydrogen bond were formed between the catalytic groups, the formation of the acetyl-enzyme during the hydrolysis of *p*-nitrophenyl acetate would not be affected markedly in D_2O. It is important to use substrates with good leaving groups for such investigations. In this case in particular, the acyl-enzyme formation requires only general base catalysis, that is, the transfer of one proton from the serine to the histidine residue.[9] When simple ester or amide substrates are used, general acid catalysis,[6] that is, another transfer of the same proton from the imidazolium to the leaving group, may interfere with the D_2O effect.

Table 1 shows the effect of D_2O on the pH independent rate constants of the hydrolysis of *p*-nitrophenyl acetate catalyzed by the Carlsberg subtilisin and its thiol-derivative k_{cat} refers to deacetylation and k_{cat}/K_m, a complex second-order rate constant involving binding, refers to acetyl-enzyme formation. It is seen from Table 1 that the rate of deacetylation is approximately three times lower with both enzyme species in D_2O than in water, in accordance with the idea of the occurrence of a rate-controlling proton transfer in this catalytic step. In

TABLE 1. *Effect of D_2O on the kinetic constants (limit) of p-nitrophenyl acetate hydrolysis by subtilisin and thiol-subtilisin (Carlsberg).**

	Subtilisin	Thiol-subtilisin
$k_{cat}^{H_2O}/k_{cat}^{D_2O}$	3.2	3.4
$\frac{(k_{cat}/K_m)^{H_2O}}{(k_{cat}/K_m)^{D_2O}}$	2.5	1.0

* 25.0°, 3.3% (v/v) acetonitrile-water.

the data relevant to the formation of the acetyl-enzyme it is seen that there is a rate-determining proton transfer only in the serine enzyme. This indicates that the imidazole group abstracts the proton from the hydroxyl group only in a rate-controlling transition state but not in the free enzyme. The lack of a D_2O effect with the thiol-enzyme supports the suggestion that there is a hydrogen bridge in the free thiol-enzyme.

There is an important point in the above reasoning which we should like to make clear. Namely, the question might be raised whether a thiol group is indeed capable of forming a hydrogen bond since it is known that hydrogen bond formation is very poor in H_2S. However, the thiol group is a poor proton acceptor but an excellent proton donor, and so it can easily lend its proton to a nitrogen atom with a free electron pair as we suggest to be the case in thiol-subtilisin.

By postulating the formation of a hydrogen bond between the serine and the histidine residues, the outstanding reactivity of the hydroxyl group of the serine enzymes could simply be explained.[1] This hypothesis was further developed recently by Wang.[2] He emphasized the importance of a rigidly and accurately held hydrogen bond which would facilitate the proton transfer—by analogy of proton transfer in ice crystals—in the catalysis by the serine proteases. He further suggested that in thiol-subtilisin the hydrogen bond has been disrupted and that this is the reason for the inefficiency of the thiol-enzyme in catalyzing the hydrolysis of simple ester, amide, or peptide substrates. This idea is just the opposite of what we have suggested on the basis of the data in Table 1. We suggest that the reactivity of the hydroxyl group of serine enzymes can be explained without postulating a preformed hydrogen bond. If there is only a van der Waals contact between the nitrogen atom of the imidazole ring and the hydrogen atom of the hydroxyl group, a favorable collision between the carbonyl carbon atom of the substrate and the oxygen atom of the hydroxyl group may initiate the abstraction of the proton by the nitrogen atom which will, in turn (as proposed by Wang), offer the same proton to the leaving moiety of the substrate. Such a general base-general acid catalysis requires an elaborate spatial arrangement of the reactive groups and, as can be seen in Figure 1, this is not fulfilled in the thiol-enzyme because the greater atomic radius and the different bond angle of the sulfur atom relative to the oxygen atom result in a distortion of the active site. Accordingly, it is this distortion of the active site which would account for the drastic decrease in the catalytic rate of thiol-subtilisin.

In the mechanism illustrated in Figure 1, the role played by the histidine residue consists of accepting the proton from the hydroxyl group of the serine side chain and then lending it to the leaving moiety of the substrate. It is seen from Figure 1 that in the successive two-proton transfers the sp^2 orbital of the unshared electron pair of the nitrogen atom must operate in two different directions in space. There are two main types of mechanisms whereby this can be achieved. (1) One may assume that the serine and the histidine residues are in approximately the same steric position during general base-general acid catalysis, in which case the proton cannot be transferred from the oxygen to the

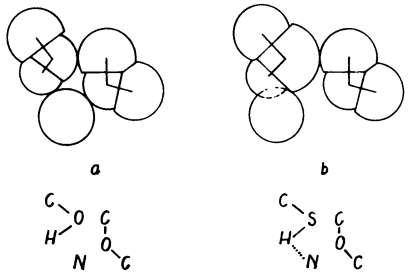

FIG. 1.—A possible conformation of the active site of subtilisin ($a\epsilon$) and thiol-subtilisin (b) in the ground state of the reaction with a specific substrate.

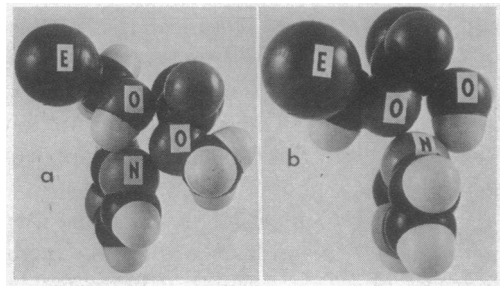

FIG. 2.—(a). Model of the serine and the histidine residues of a serine protease reacting with the methyl ester of a specific substrate. E stands for the rest of the enzyme. (b) Model of the acyl-enzyme.

nitrogen atom and then to the leaving group in the most favorable way (where the donor, the hydrogen, and the acceptor atoms lie on a straight line). (2) Alternatively, the imidazole ring may move between the general base-general acid catalytic steps, thereby rendering it possible for the proton to move in the first transfer along the straight line between the oxygen and nitrogen atoms and in the second transfer along the straight line between the same nitrogen atom and the hetero atom of the leaving group.

From the two alternative mechanisms the first one, depicted by the rigid active-site model, is supported by the following considerations:

(1) The fastest chemical reaction is the proton transfer, particularly if it occurs between atoms with appropriate bond angles as in ice crystals. The rate constant of such a reaction is several orders of magnitude higher than that of the fastest enzymatic reaction. It is not reasonable to assume that acyl-enzyme formation or deacylation proceed via a mechanism consisting of two very rapid proton transfers with an interposed slow change in the position of the histidine residue. This would not be consistent with the experimentally found D_2O effect which shows that proton transfer is a rate-limiting step. Moreover, the nitrogen atom would lose its proton before it could lend it to the leaving group if the positional change of the imidazole ring is slower than deprotonation.

(2) It is reasonable to postulate that the attacking oxygen atom of the serine residue approaches the substrate in a line perpendicular to the plane of the sp^2 hybrid orbitals of the carbonyl carbon atom. Namely, this geometry leads to maximum overlap of the bonding orbital of the nucleophile and the π electron cloud of the carbonyl carbon atom. In this case, as seen in Figure 1, the hydrogen atom of the serine residue and the oxygen atom of the leaving moiety of the substrate can get in close proximity which allows the nitrogen atom of the imidazole ring to interact in a fixed position with both the donor and the acceptor atoms. Of course, a double interaction of the nitrogen atom in the ground state of the reaction is not an essential requirement. It is equally possible that the proton acceptor atom of the leaving group only contacts the imidazolium ring in the course of covalent bond formation between the serine oxygen and the carbonyl carbon atoms. The assumption of such a geometry of

the reacting atoms appears to be one of the most important clues leading to the solution of the catalytic mechanism of serine proteases. The electron cloud which penetrates into the space between the hydrogen donor and acceptor atoms may help the proton to proceed rapidly in a double transfer even though the bond angles are not optimal for a single proton transfer. This proposal is strongly supported by previous observations that thiol-subtilisins do not catalyze the hydrolysis of specific substrates.[8, 10, 11] Figure 1 accounts for this observation by showing that the distance between the donor and the acceptor atoms must be larger in the thiol-enzyme if the carbonyl carbon atom approaches the nucleophile from the same direction as in the serine enzyme, which would be the case with specific substrates. It is important to note that the direction of the approach to the sulfur atom by the carbonyl carbon atom of a specific substrate is not favorable if it occurs according to the proposed model. Namely, the orbitals of the sulfur atom are practically not hybridized (bond angle = 92°), whereas the bond angle of the oxygen atom is approximately tetrahedral (105°). Therefore, the most favorable bond-making between the sulfur atom and the carbonyl carbon atom of the substrate would occur in an orientation approximately perpendicular to the plane of the S—C and S—H bonds.

(3) Specific substrates of the subtilisins are not hydrolyzed at a measurable rate by the thiol-enzymes, whereas *p*-nitrophenyl acetate, a nonspecific substrate is hydrolyzed at a reasonable rate. This indicates that the steric position of the imidazole nitrogen atom is relatively stable during catalysis. If we consider the deacetylation step of *p*-nitrophenyl acetate hydrolysis, it is obvious (cf. ref. 12) that the acetyl group of the acetyl-thiol-enzyme should be able to move freely to approach the nitrogen atom, in contrast to the acyl moiety of a specific substrate which is more rigidly attached to the binding site. In fact, the rate of hydrolysis of the acetyl-thiol-subtilisins is higher by more than an order of magnitude than that of the cinnamoyl-thiol-enzymes.[5, 9]

It has recently been published that the k_{cat} values of thiol-subtilisin for the hydrolysis of *p*-nitrophenyl esters of specific amino acid derivatives are higher than the k_{cat} of the hydrolysis of *p*-nitrophenyl acetate.[13] This is not consistent with the catalytic role of the histidine residue developed above since deacylation is the rate-limiting step in these reactions and it has been argued above that with a "distorted" thiol-enzyme the rate of deacetylation ought to be higher than the rate of deacylation with a specific substrate. We have pointed out elsewhere[5, 8, 14] that a reliable k_{cat} cannot be obtained for nitrophenyl esters of specific carboxylic acids in the hydrolysis by thiol-subtilisins, because the thiol-enzyme preparations always exhibit some additional activity and this interfering reaction does not proceed through the acyl-thiol-enzyme intermediate. Nevertheless, a rough correction can be made for this extra activity, and one can estimate in this way that the rate of hydrolysis of *p*-nitrophenyl *N*-benzyloxycarbonylglycinate catalyzed by thiol-subtilisin cannot be higher than the rate for the *p*-nitrophenyl acetate reaction.

(4) The principle of symmetry in acylation and deacylation,[6] which is a very important feature of the catalytic mechanism with alkyl ester substrates, is satisfied by the rigid model in Figure 2. The models of acylation and deacylation

shown in Figure 2 demonstrate that not only the catalytic groups of the enzyme but also the carbonyl carbon atom of the substrate can be held in a fixed position, disregarding the slight difference which is due to the fact that the van der Waals radii of the oxygen and the carbon atoms in the enzyme-substrate complex are substituted by the covalent radii in the acyl-enzyme. The rearrangement of the chemical bonds in the course of formation of the acyl-enzyme does not imply a gross change in the position of the carbonyl carbon atom. However, the plane of its sp^2 orbitals should be reoriented in the course of the reaction when the substrate is fixed both by the ester bond of the acyl-enzyme and by the binding of its aromatic ring to the enzyme surface. This assumption is supported by the ingenious work of Charney and Bernhard[15] which indicates that a β-arylacryloyl serine enzyme has an *s-cis* configuration in contrast to the small molecule model compounds which are predominantly in the *s-trans* configuration. *s-cis–s-trans* Isomerization with regard to the acryloyl carbon-carbon single bond can only take place if the plane of the sp^2 orbitals of the carbonyl carbon atom changes its position relative to the α,β double bond.

(5) A fixed position of the imidazole ring is indicated by recent X-ray studies of chymotrypsin, subtilisin, and elastase which show that N,[1] the nitrogen atom of the histidine residue, which does not participate directly in the proton transfer, may lend its hydrogen atom to the carboxyl group of an aspartate residue.[3, 4] If the hydrogen bond formed in this way plays an important role in the catalysis, which is likely the case, then both nitrogen atoms of the histidine residue must operate simultaneously, and this is most easily achieved with a fixed imidazole ring. It is important to note that the possibility of a significant dislocation of the histidine residue has only been demonstrated by a difference electron density map of free and arylsulfonyl-enzyme.[4, 16]

X-ray diffraction studies have revealed the existence of a hydrogen bond between the serine and the histidine residue at the active site.[3] This result was interpreted as strong support for the idea that a mechanistically important hydrogen bond is formed.[3] However, the diffraction measurements were performed with chymotrypsin crystallized at pH 4.2, where the histidine residue is in a protonated, catalytically inactive form. Therefore, from these measurements it is difficult to draw any conclusion regarding the mechanism. However, it is possible to obtain important information about the nature of this hydrogen bridge. The data show that the hydrogen bond is of the N—H···O type.[3] The suggestion that a hydrogen bond is formed between a protonated imidazole and a hydroxyl group is plausible on chemical grounds, but it is not likely that a normal hydrogen bond would form between a hydroxyl and an unprotonated imidazole nitrogen atom, with the hydroxyl acting as donor, since the basicity of the alcoholate ion is far greater than that of the imidazole group. In fact, the X-ray studies of subtilisin[4] indicate a distance of 3.5 Å between the oxygen and the nitrogen atoms in question. This is certainly larger than that expected for a normal hydrogen bond and indicates that either the bond is a very weak hydrogen bridge or that the contact is of the van der Waals type. On the other hand, when the oxygen is replaced by a sulfur atom in subtilisin, the distance between sulfur and nitrogen atoms will be more suitable for hydrogen bond formation.

(The atomic radius of sulfur is greater by 0.4 Å than that of the oxygen atom.) Moreover, the difference between the basicity of the sulfur and the nitrogen atoms is greatly reduced compared to the difference between oxygen and nitrogen atoms, and this provides a chemical basis for the formation of hydrogen bond in thiol-subtilisin.

The recent discovery of the hydrogen bond between the carboxyl group of a buried aspartic acid residue and the N^1 nitrogen atom of the catalytically important histidine side chain enabled Blow *et al.*[3] to modify a weak point in Wang's hypothesis of a pre-transition state mechanism,[2] which suggests that the reactive serine residue is in a dissociated form at a pH as low as 8. The proposed "charge relay system" would allow the transfer of the negative charge from the acid group to the serine residue which can thus act as a powerful nucleophile in the acylation step of peptide or acyl-anilide substrates. However, one cannot expect that in the hydrogen bonding system of carboxylate-imidazole-hydroxyl groups the proton is bound more strongly to the carboxylate ion than to the serine oxygen atom since the alcoholate ion is a stronger base by many orders of magnitude. Moreover, based on the conclusion that there cannot be a normal hydrogen bond between the serine and histidine residues, one should postulate that simultaneously no hydrogen bond between the carboxylate ion and the N^1 atom of the imidazole ring is formed either, as it is shown in Figure 3, since it would raise the pK of the imidazole group above the observed normal value. It seems to be more likely that the main function of the hydrogen bonding system is to stabilize the imidazole ion against a possible release of its proton between the steps of general base and general acid catalyses.

The experimental data[17, 18] which led Wang to propose the pretransition state protonation in the acylation step of anilides can also be explained by assuming that a rate-limiting general acid catalysis, that is, a proton transfer from the imidazolium ion to the nitrogen atom of the leaving group, takes place in the transition state.

In conclusion, the obtained pH-rate profiles of acylation together with the data of kinetic isotope effect on acylation indicate that there cannot be a normal

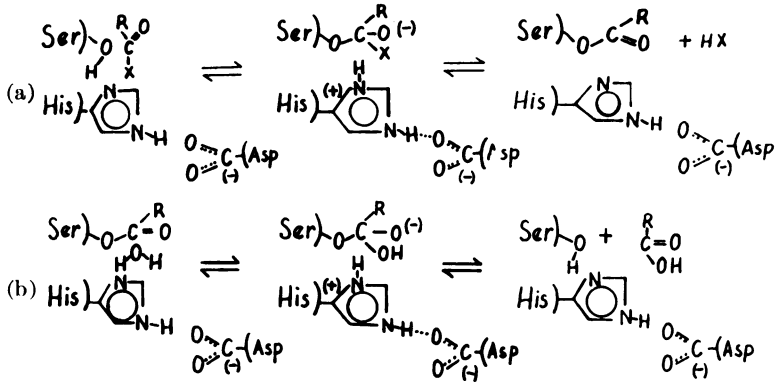

FIG. 3.—A possible mechanism of the formation (*a*), and the hydrolysis (*b*) of the acyl-enzyme in the catalysis by a serine protease. *X* represents the leaving group of the substrate.

hydrogen bond between the serine and the histidine residues at the active site of serine proteases. A van der Waals contact or a very weak hydrogen bond may only exist between the two groups in question. The proton acceptor atom of the leaving group of the substrate is in a close proximity to the hydroxyl group of the serine residue and this allows the nitrogen atom of the imidazole ring, which is fixed in space, to interact simultaneously with both the proton donor and acceptor atoms. A favorable collision between the carbonyl carbon atom of the substrate and the oxygen atom of the serine residue triggers the catalytic reaction. Bond making, that is, the formation of a probably tetrahedral intermediate, and proton transfer to the imidazole ring proceed in a concerted action. This step involving general base catalysis is followed by a general acid-catalyzed step in which a proton is transferred from the imidazolium ion to the leaving group, which takes place in a concerted action with bond breaking. Between the two general catalyses the imidazolium ion is stabilized by the aspartate-imidazole hydrogen bond system. We suggest that the rate of acylation is determined by the rate of formation of the (tetrahedral) intermediate with alkyl ester substrates[9] and by the rate of decomposition of the intermediate with peptides and some anilides. With some further substrates which contain a good leaving group the formation of acyl-enzyme requires only general base catalysis.[2, 9] Deacylation with any substrate is simply the reverse of acyl-enzyme formation with alkyl ester substrates, when water substitutes for the serine residue and the ester group of the acyl-enzyme for the substrate.[6] This proposal for the mechanism of action of serine proteases is based on the work of Bender and Kézdy[6] and incorporates many of the features of the hypotheses of Wang[2] and Blow *et al.*[3]

[1] Cunningham, L. W., in *Comprehensive Biochemistry*, ed. M. Florkin and E. K. Stotz (Amsterdam, London, New York: Elsevier Publishing Company, 1965), vol. 16, p. 85.
[2] Wang, J. H., *Science*, **161**, 328 (1968).
[3] Blow, D. M., J. J. Birktoft, and B. S. Hartley, *Nature*, **221**, 337 (1969).
[4] Wright, Ch. Sch., R. A. Alden, and J. Kraut, *Nature*, **221**, 235 (1969).
[5] Polgár, L., and M. L. Bender, *Biochemistry*, **6**, 610 (1967).
[6] Bender, M. L., and F. J. Kézdy, *Ann. Rev. Biochem.*, **34**, 49 (1965).
[7] Peller, L., and R. A. Alberty, *J. Am. Chem. Soc.*, **81**, 5807 (1959).
[8] Polgár, L., *Acta Biochim. Biophys. Acad. Sci. Hung.*, **3**, 397 (1968).
[9] Polgár, L., and M. L. Bender, in preparation.
[10] Polgár, L., and M. L. Bender, *J. Am. Chem. Soc.*, **88**, 3153 (1966).
[11] Neet, K. E., and D. E. Koshland, Jr., these PROCEEDINGS, **56**, 1606 (1966).
[12] Bender, M. L., F. J. Kézdy, and C. R. Gunter, *J. Am. Chem. Soc.*, **86**, 3714 (1964).
[13] Neet, K. E., A. Nanci, and D. E. Koshland, Jr., *J. Biol. Chem.*, **243**, 6392 (1968).
[14] Polgár, L., and M. L. Bender, *Biochemistry*, **8**, 136 (1969).
[15] Charney, E., and S. A. Bernhard, *J. Am. Chem. Soc.*, **89**, 2726 (1967).
[16] Sigler, P. B., D. M. Blow, B. W. Matthews, and R. Henderson, *J. Mol. Biol.*, **35**, 143 (1968).
[17] Inagami, T., S. S. York, and A. Patchornik, *J. Am. Chem. Soc.*, **87**, 126 (1965).
[18] Parker, L., and J. N. Wang, *J. Biol. Chem.*, **243**, 3729 (1968).