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On the Reactivity of the Thiol Group of Thiolsubtilisin

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1. The reaction of thiolsubtilisin with iodoacetamide shows that in the alkaline pH-range the —SH group of the enzyme reacts like a simple thiol compound such as mercaptoacetate. On the other hand, around neutral pH the enzyme displays enhanced reactivity compared with that of mercaptoacetate. On the basis of measurements in ^2H_2O it is concluded that a mercaptide-imidazolium ion-pair is formed in thiolsubtilisin and this accounts for the enhanced reactivity around neutral pH.

2. Iodoacetamide reacts with the —SH group of the enzyme, as compared to mercaptoacetate, at a relatively higher rate than bromo-, chloro-, and fluoroacetamide. This might be due to the less polar environment around the —SH group on the surface of the enzyme than in water, as indicated by alkylation of mercaptoacetate with iodo- and chloroacetamide in the presence of dioxane.

3. By alkylating thiolsubtilisin with enantiomeric reactants, a slight conformational change at the active site, not measurable with ordinary physical methods, could be established above pH $\approx$ 9, whereas the overall protein structure is stable up to pH $\approx$ 11. D-2-Bromo-*n*-butyramide reacts with thiolsubtilisin about 60 times as fast as D-2-bromopropionamide. This indicates that hydrophobic interaction between the enzyme and a methyl group of the substrate can enhance the reaction rate by more than one order of magnitude. Model building of the active site suggests that Ala-152 and Thr-220 of thiolsubtilisin may form van der Waals interactions with D-2-bromo-*n*-butyramide if the alkylating agent is properly positioned for reaction. No such interaction is possible between D-2-bromopropionamide and thiolsubtilisin.

Thiolsubtilisin, an —SH analog of the serine protease subtilisin, can be produced by the replacement of the reactive serine residue at the active site by a cysteine residue [1]. Thiolsubtilisin can be regarded as a model of thiol proteases, such as papain, inasmuch as both thiolsubtilisin [2] and papain [3] have an imidazole ring in the immediate vicinity of the thiol group, as clearly indicated by X-ray diffraction studies. However, thiolsubtilisin is not a protease, it can only catalyze the hydrolysis of active esters [4].

In a recent paper we have pointed out that the catalytic action of papain depends on the formation of a mercaptide-imidazolium ion-pair at the active site [5]. On the basis of acylation of thiolsubtilisin, we have also suggested that the reactivity of the thiol group is highly affected by an interaction with the neighboring imidazole group [4, 6]. On the other hand, Neet *et al.* [7] have concluded from the alkylation of thiolsubtilisin by iodoacetamide that the reactivity of the thiol group is similar to that of a simple thiol compound.

The detailed study of alkylation of Carlsberg thiolsubtilisin is presented in this paper. Unlike acylation, alkylation is a simple nucleophilic displacement reaction, not complicated by the formation of an intermediate, therefore the study of alkylation, rather than of acylation, offers more conclusive evidence for the chemical behavior of the thiol group. Carlsberg thiolsubtilisin was preferred to the Novo enzyme because of its higher stability. We have found that around neutral pH values there is indeed an interaction between the thiol and imidazole groups, which significantly enhances the reactivity of thiolsubtilisin as compared with a simple thiol compound.

Abbreviation. Nbs_2, 5,5′-dithio-bis(2-nitrobenzoic acid) (formerly abbreviated DTNB).

Enzyme. Subtilisin (EC 3.4.4.16).

Table 1. *Yields and physical parameters of 2-bromocarboxylic acids*
Yields were measured after distillation. α_D^t values were measured on the neat product in a 1-dm polarimeter tube

Starting material	Product	Yield	Boiling point		α_D^t	
		%	°C		degree	
D-Alanine	D-2-Bromopropionic acid	71	76— 78	(at 4 mm Hg)	+46.05	(at 20 °C)
L-Alanine	L-2-Bromopropionic acid	68	72— 73	(at 2 mm Hg)	−46.20	(at 25 °C)
D-Butyrine	D-2-Bromo-*n*-butyric acid	79	75— 76	(at 3 mm Hg)	+49.45	(at 24 °C)
L-Butyrine	L-2-Bromo-*n*-butyric acid	83	79— 80	(at 4 mm Hg)	−49.40	(at 24 °C)
D-Norvaline	D-2-Bromo-*n*-valeric acid	82	111—112	(at 5 mm Hg)	+49.30	(at 24 °C)
L-Norvaline	L-2-Bromo-*n*-valeric acid	78	109—111	(at 5 mm Hg)	−49.60	(at 25 °C)

MATERIALS AND METHODS

Glutathione, 5,5'-dithio-bis(2-nitrobenzoic acid) (Nbs_2), phenylmethanesulfonyl fluoride and dioxane were commercial preparations.

Mercaptoacetic acid was redistilled twice at reduced pressure under nitrogen (b.p. 104—106 °C, 11 mm Hg).

Thiolacetic acid was redistilled twice (b.p. 87—89 °C). It was neutralized with sodium methoxide in methanol. (The pH of an aliquot diluted with water was about 5.5.) Methanol was removed *in vacuo* and the resulting powdery sodium thiolacetate was dried over phosphorus pentoxide *in vacuo* for 48 h and kept in a sealed tube in the refrigerator.

Dioxane was purified by refluxing first with hydrochloric acid then with sodium according to Fieser's method (a) [8] and was distilled from sodium before use.

Iodoacetamide and chloroacetamide were recrystallized from carbon tetrachloride and from water, respectively.

Bromoacetamide was obtained from bromoacetyl chloride by the method of Pomerantz and Connor [9].

For the preparation of fluoroacetamide the method of Bacon *et al.* [10] was employed with a slight modification. A mixture of fluoroacetic acid (20.7 g), absolute ethanol (15 g) and sulfuric acid (3.15 g) was refluxed for 6 h. After cooling the reaction mixture was neutralized and treated with about a two-fold excess of concentrated aqueous ammonia solution and shaken vigorously for 15 min. The crystals were collected at 0 °C, washed with water and dried over phosphorus pentoxide. The crude product was recrystallized from chloroform (m.p. 107 °C).

Enantiomers of 2-bromocarboxylic acid amides were prepared according to Scheme 1.

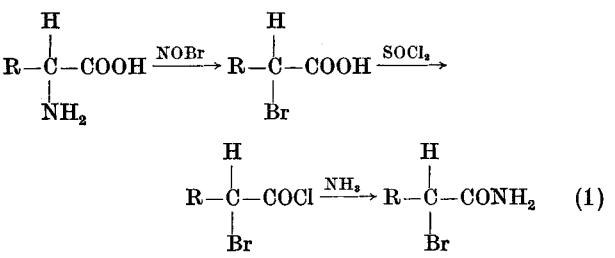


Table 2. *Boiling points of 2-bromocarboxylic acid chlorides at different pressures*

Compound	Boiling point	
	°C	
D-2-Bromopropionyl chloride	31—32	(at 16 mm Hg)
	35—36	(at 22 mm Hg)
L-2-Bromopropionyl chloride	31—32	(at 16 mm Hg)
	27—28	(at 12 mm Hg)
	42—43	(at 32 mm Hg)
D-2-Bromo-*n*-butyryl chloride	42—44	(at 17 mm Hg)
	38—39	(at 9 mm Hg)
L-2-Bromo-*n*-butyryl chloride	43—44	(at 18 mm Hg)
	38—39	(at 9 mm Hg)
D-2-Bromo-*n*-valeryl chloride	74—75	(at 20 mm Hg)
L-2-Bromo-*n*-valeryl chloride	72—74	(at 20 mm Hg)

Amino acids (D and L) of high optical purity (minimum 99.5%) were obtained by the resolution of *N*-chloroacetyl-DL-amino acids with acylase I [11].

D- and L-2-bromocarboxylic acids were obtained from the corresponding amino acids by the action of NOBr [12]. This method does not affect the configuration of the asymmetric carbon atom. Yields and physical parameters are compiled in Table 1.

D- and L-2-bromocarboxylic acid chlorides were prepared from the corresponding acids with thionyl chloride as described by Fu *et al.* [13] for D- and L-chloropropionic acid chlorides. The boiling points are shown in Table 2.

D- and L-2-bromocarboxylic acid amides were synthesized according to Skinner and Elmslie's procedure [14] modified as follows. Instead of acid bromides, acid chlorides, which are easier to prepare, were used. Reaction time and temperature were reduced. The preparation was carried out as follows: 100 ml sodium-dried petroleum ether (b.p. 40—70 °C) was saturated with dry ammonia at 0 °C, then 0.05 mol 2-bromocarboxylic acid chloride were added dropwise to the solution with intensive stirring and cooling. Introduction of ammonia and stirring were continued for a further 30 min while the internal temperature was kept 0 °C. The precipitat-

Table 3. *Yields and physical parameters of 2-bromocarboxylic acid amides*
Melting points are uncorrected. $[\alpha]_D^t$ was measured with $c = 2\%$ in ethanol using a 1-dm polarimeter tube

Compound	Yield	Melting point	$[\alpha]_D^t$		Calcd		Found	
					N	Br	N	Br
	%	°C	degree		%			
D-2-Bromopropionamide	57	129–131	+25.7	(at 25 °C)	9.25	52.6	9.32	52.6
L-2-Bromopropionamide	60	127–130	−25.2	(at 23 °C)	9.25	52.6	9.20	52.6
D-2-Bromo-*n*-butyramide	75	125–127	+34.8	(at 22 °C)	8.44	48.1	8.31	48.3
L-2-Bromo-*n*-butyramide	72	126–128	−34.5	(at 25 °C)	8.44	48.1	8.45	47.7
D-2-Bromo-*n*-valeramide	73	101–103	+50.8	(at 24 °C)	7.78	44.4	7.57	44.0
L-2-Bromo-*n*-valeramide	70	103–104	−49.9	(at 24 °C)	7.78	44.4	7.88	44.3

ed 2-bromocarboxylic acid amide and ammonium chloride were collected from the cold reaction mixture and washed with small quantities of petroleum ether and water to remove ammonium chloride. The product was dried *in vacuo* and recrystallized from dry benzene. Yields and physical constants of the 2-bromocarboxylic acid amides are presented in Table 3.

Subtilisin type Carlsberg and type Novo were purchased from Novo Industri A/S.

Carlsberg thiolsubtilisin was prepared as described previously [15] and was treated with phenylmethanesulfonyl fluoride in order to inhibit the parent serine enzyme present as a small contamination [16]. Novo thiolsubtilisin was prepared by the method previously described [4]. After gel filtration, the final step of the preparation, thiolsubtilisins were obtained in 0.005 M phosphate buffer pH 7.0 containing 0.04 M potassium chloride and 1 mM EDTA. The concentration of the protein was about 0.3 mM.

The concentration of the thiol groups was determined by titration with Nbs_2 [17].

The concentration of iodoacetamide was measured spectrophotometrically at 275 nm by using the molar absorption coefficient $\varepsilon = 372\ M^{-1} \cdot cm^{-1}$ [18]. In the case of the other halocarboxylic acid amides stock solutions were prepared on the weight basis.

Alkylations were followed at 25 °C under second or pseudo first-order conditions by taking aliquots from the reaction mixture at appropriate times and by measuring the concentration of the —SH group with Nbs_2. The reaction mixture contained 0.03–0.08 mM thiolsubtilisin or 0.2–0.8 mM mercaptoacetate, 1 M potassium chloride, 3 mM EDTA in 0.05 M phosphate, carbonate or glycine buffer.

In the presence of 37.5% dioxane, KCl was omitted from the reaction mixture because of the decreased solubility.

pH or p^2H was measured immediately after the reaction. The accuracy of pH-measurements was within 0.1 unit. $p^2H = pH + 0.4$ [19].

The errors in the k (limit) values calculated from the pH-dependence of alkylations are about ± 5%.

RESULTS AND DISCUSSION

Alkylation of Carlsberg Thiolsubtilisin with Iodoacetamide

Fig. 1 shows the pH-dependence of the reaction of thiolsubtilisin with iodoacetamide. At high pH values the experimental points fit the theoretical curve calculated for the dissociation of an ionizing group with $pK_a = 10.12$. With decreasing pH the experimental points deviate gradually from the theoretical curve and represent much higher reaction rates than expected on the basis of the dissociation of an ordinary thiol group. As a comparison the pH-rate profile of the reaction of mercaptoacetate with iodoacetamide is also shown in Fig. 1. It is seen that the experimental points for the model compound conform to the theoretical curve (solid line) in the pH-range studied. This indicates that the mercaptide ion is the reactive form of the thiol group even 3 pH units below its pK_a. Table 4 shows the pK_a and the pH-independent maximal rate constant, k(limit), values.

The high stability of thiolsubtilisin at alkaline pH allowed us to extend the measurements slightly above pH 11. It was shown by Markland [20] that the spectrophotometric titration of the tyrosine residues of Carlsberg subtilisin at 295 nm is completely reversible even after 3 to 4 h of incubation at pH values up to 10.8 and at ionic strength $I = 0.17$. We found thiolsubtilisin similarly stable as measured by the change in the absorption at 243 nm. At this wavelength the difference between the alkaline and the neutral spectra of the protein is significantly greater than at 295 nm; furthermore, it reflects not only the dissociation of the phenol groups, but also the dissociation of the thiol groups [21]. By means of this method we have not observed any appreciable changes in the absorption of thiolsubtilisin at pH values up to

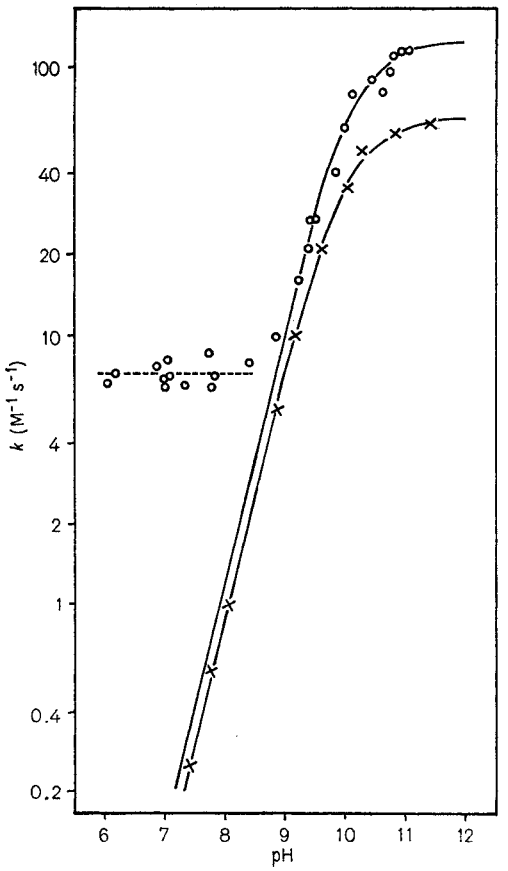


Fig. 1. *pH-dependence of the reaction of iodoacetamide with thiolsubtilisin (○) and mercaptoacetate (×).* Iodoacetamide was used in concentrations of 0.04—0.6 mM and 0.2 to 0.8 mM with thiolsubtilisin and mercaptoacetate, respectively. The solid lines are theoretical curves the parameters of which are shown in Table 4

11.2—11.3 during 30-min incubation whereas denaturation could easily be detected above pH 11.3. These measurements were performed in the presence of 1 M KCl, similarly to the alkylation of thiolsubtilisin, since KCl was found to increase the stability of the protein. The absence of denaturation around pH 11 is also supported by the fact that alkylation is not affected by preincubation of thiolsubtilisin at this pH for 15 min. Alkylations were followed for periods not longer than 15 min.

From the study of alkylation of thiolsubtilisin Neet *et al.* [7] came to the conclusion that the enzyme has a normal —SH group with a $pK_a = 8.5$. This is at variance with our finding shown in Fig. 1. It is also noteworthy that in the alkaline pH-range the values of the second-order rate constants calculated from their data are lower that those found in this work and do not conform to a normal dissociation curve. Hence it is scarcely justifiable to assign a $pK_a = 8.5$ to the —SH group. In some experiments

Table 4. *Kinetic parameters of the reaction of iodoacetamide with thiolsubtilisin and model compounds*
Measurements were made in the presence of 1 M KCl, at 25 °C. The best fit to the experimental points was found with curves calculated for the pK_a values indicated

Thiol compound	pK_a	k(limit)
		$M^{-1}s^{-1}$
Thiolsubtilisin	10.12	122
Mercaptoacetate	9.85	63.3
Digested thiolsubtilisin[a]	9.40	35
Glutathione	8.90	30

[a] Thiolsubtilisin was digested with pepsin (100:1) at pH 2.0 for 20 h at 25 °C.

we also measured significantly lower rate constants when thiolsubtilisin was partially decomposed during a longer reaction time possible due to digestion by the regenerated serine enzyme. It should be noted that even if the serine enzyme is treated with phenylmethanesulfonyl fluoride, protease activity cannot be completely excluded because of the slow hydrolysis of the phenylmethanesulfonyl derivative.

In the light of the three-dimensional structure of the active site of thiolsubtilisin [2], the most probable explanation for the pH-dependence of the alkylation of thiolsubtilisin (Fig. 1) is that the reaction of thiol group is promoted by the neighboring imidazole ring. If this is the case, after an appropriate structural change of the protein a normal dissociation curve should be obtained for the alkylation of thiolsubtilisin. In fact, with thiolsubtilisin previously digested by pepsin at pH 2, the pH-rate profile does not deviate from a normal dissociation curve. It is seen in Table 4, that after digestion pK_a and k(limit) become lower and, thus, k(limit) is more similar to that of a simple peptide —SH group, such as glutathione.

As to the promotion of the alkylation of thiolsubtilisin by the imidazole group of His-64 around neutral pH (Fig. 1), general base catalysis should be considered. This can be tested by measuring the effect of ^2H_2O on the reaction. Namely, general base-catalyzed reactions generally proceed about three times as fast in water as in ^2H_2O, although exceptions have been reported (*cf.* [22]). According to our measurements, the ratio of the second-order rate constants of alkylation in water and in ^2H_2O is 1.03 ± 0.10 rather than 3. This suggests that breaking of the S—H bond is not the rate-determining step of alkylation of thiolsubtilisin, *i.e.* the proton is transferred to the imidazole group before the transition state of the simple nucleophilic substitution. Therefore, *per definitionem* one cannot speak of general base catalysis.

Table 5. *Kinetic parameters of the reaction of haloacetamides with thiolsubtilisin and mercaptoacetate*
Measurements were made in the presence of 1 M KCl at 25 °C. The best fit to the experimental points was found with curves calculated for the pK_a values indicated

Alkylating agent	Thiolsubtilisin			Mercaptoacetate		A/C	B/C
	k (pH ≈ 6—8.5) (A)	k(limit) (B)	pK_a	k(limit) (C)	pK_a		
	$M^{-1}s^{-1}$			$M^{-1}s^{-1}$			
Iodoacetamide	7.17	122	10.12	63.3	9.85	0.11	1.93
Bromoacetamide	2.58	45.0	10.15	39.2	9.87	0.066	1.15
Chloroacetamide	0.0213	0.250	10.10	0.466	9.90	0.046	0.54
Fluoroacetamide	0.000533			0.0133[a]	9.87[b]	0.040	

[a] Calculated from rate constants measured in the pH-range of 8.9—10.2, by assuming a pK_a of 9.87 since fluoroacetamide is hydrolyzed at higher pH values and this parallel reaction interferes with alkylation.
[b] Average of the former values.

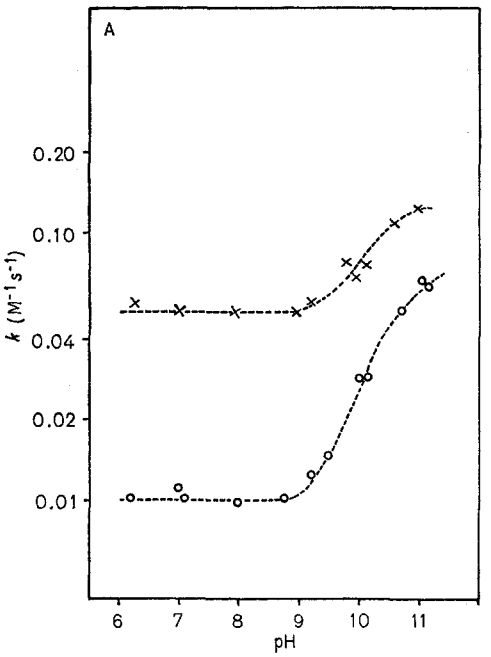
With proteins it is often argued that conformational changes may interfere with kinetic isotope effect, although, for example, in the catalysis by chymotrypsin the occurrence of a large or irreversible change in conformation has been ruled out [23, 24]. If conformational changes in ^2H_2O take place at all, they should be similar in thiolsubtilisin and in the parent serine enzyme, since they have the same tertiary structure [2]. Unfortunately, as a control reaction, alkylation of the "reactive" serine residue by iodoacetamide cannot be measured. However, both subtilisin and thiolsubtilisin can be acylated by *p*-nitrophenyl acetate and we have previously found that there is a significant kinetic isotope effect in the acylation of subtilisin but not in the acylation of thiolsubtilisin [6]. This suggests that in these proteins structural changes may not seriously interfere with the kinetic isotope effects.

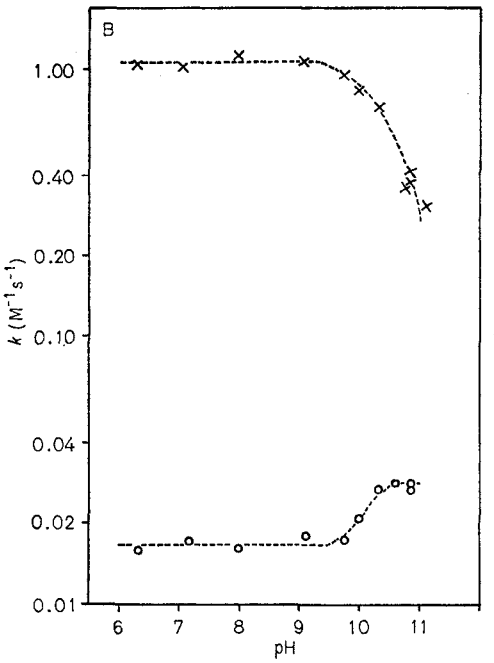
The complete lack of ^2H_2O effect makes also unlikely the formation of a hydrogen bond by partial donation of the proton from the thiol to the imidazole group. The formation of such a hydrogen bond could offer an explanation for the increased reactivity of the —SH group, but some ^2H_2O effect (between 1 and 3) would also be expected in this case. Accordingly, we assume that the proton of the thiol group is transferred to the nitrogen atom of the imidazole ring before the formation of the transition state. This implies that the reactive nucleophile of thiolsubtilisin is a mercaptide-imidazolium ion-pair and this accounts for the enhanced reactivity of the —SH group in the neutral pH-range. The mercaptide ion of the ion-pair may even be hydrogen-bonded to the imidazolium ion; however, in this case the imidazolium ion is the proton donor. With increasing pH the ion-pair decomposes. The free mercaptide ion formed at highly alkaline pH is not influenced by the positively charged imidazolium ion and reacts at a higher rate.

Effect of the Microenvironment of the —SH Group on Reactivity

Alkylation of Thiolsubtilisin by Haloacetamides. The polarity of the immediate environment of the —SH group may influence the reaction rate. This can be tested by using different haloacetamides. The polarizability of the haloatoms decreases in the order I > Br > Cl > F. Therefore, the reactivity of iodoacetamide may be affected to a greater extent by a charged group [25] or a less polar environment at the protein surface, than that of the other haloacetamides.

Table 5 shows that the pK_a values calculated from pH-dependence curves of all four haloacetamides are the same within experimental error with both thiolsubtilisin and mercaptoacetate. The rate constants, of course, decrease from iodo- to fluoroacetamide. Is it further seen from Table 5 that iodoacetamide reacts with the —SH group of the enzyme, as compared to mercaptoacetate, at a relatively higher rate than the other haloacetamides both around neutral and at highly alkaline pH values. This may be attributed to a weak electrophilic assistance or a less polar environment around the —SH group at the surface of the enzyme than in water. In the light of the three-dimensional structure of thiolsubtilisin [2], the only positive charge near the thiolate ion is the imidazolium ion, which only exists around neutral pH, whereas the relatively high reaction rate of iodoacetamide with thiolsubtilisin is preserved at highly alkaline pH values. In addition, even if the imidazolium ion exists, its steric position is unfavorable for the electrophilic assistance of the reaction. Table 6 shows that in the presence of 37.5% dioxane the alkylation rate of mercaptoacetate increases to a greater extent with iodoacetamide than with chloroacetamide. Thus, it is more probable that the less polar environment at the enzyme surface increases the rate of the reaction of the —SH group with iodoacetamide.





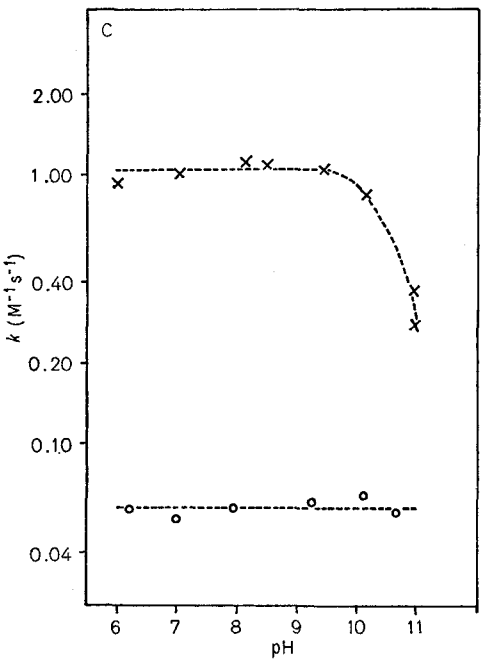


Fig. 2. *pH-dependence of the reaction of thiolsubtilisin with* D (×) *and* L (○) *enantiomers of (A) 2-bromopropionamide, (B) 2-bromo-*n*-butyramide and (C) 2-bromo-*n*-valeramide.* Reaction mixtures contained 7—28 mM D- or 13—130 mM L-2-bromopropionamide, 1—8 mM D- or 8—45 mM L-2-bromo-*n*-butyramide, 1—4 mM D- or 17—24 mM L-2-bromo-*n*-valeramide, respectively. The rate constants related to the pH-independent ranges are shown in Table 7 (first column)

Table 6. *Effect of dioxane on the rate of alkylation of mercaptoacetate by iodo- and chloroacetamide*
These measurements were performed in the absence of 1 M KCl at 25 °C. $k(\text{limit})_D$ was measured in the presence of 37.5% (v/v) dioxane

Alkylating agent	k(limit) (A)	$k(\text{limit})_D$ (B)	B/A
	$M^{-1}s^{-1}$		
Iodoacetamide	63.3	165	2.61
Chloroacetamide	0.420	0.683	1.63

Stereospecific Alkylations of Thiolsubtilisin. The possible structural alterations of the active site of thiolsubtilisin with the variation of pH, which may be related to the stability of the ion-pair, might be revealed by comparing the pH-dependences of reactions with D- and L-enantiomers of alkylating agents, as was carried out with papain [5]. Furthermore, one can study the contribution of hydrophobic interactions between the enzyme and the reactants to the reaction rate by using alkylating agents of different chain lengths.

Fig. 2 demonstrates the pH-dependences of the second-order rate constants for the reactions of thiolsubtilisin with the enantiomers of 2-bromopropionamide, 2-bromo-*n*-butyramide and 2-bromo-*n*-valeramide. It is seen that the D-antipodes react faster than the corresponding L-antipodes. The rate of alkylation is independent of pH between pH 6 and

Table 7. *Rate constants of alkylation of thiolsubtilisin and mercaptoacetate with 2-bromocarboxylic acid amides*
Measurements were made in the presence of 1 M KCl at 25 °C

Alkylating agent		Thiol-subtilisin	Mercapto-acetate	A/B
Name	Stereo-isomer	k (pH ≈ 6—9) (A)	k(limit) (B)	
		$M^{-1}s^{-1}$		
Bromoacetamide		2.58	39.2	0.066
2-Bromopropion-amide	D	0.0500	0.333	0.15
	L	0.0103		0.031
2-Bromo-*n*-butyr-amide	D	1.05	0.105	10.0
	L	0.0167		0.16
2-Bromo-*n*-valeramide	D	1.05	0.0950	11.1
	L	0.0583		0.61

9 in accord with our above suggestion, *i.e.* that around neutral pH an imidazolium-mercaptide ion-pair is the nucleophile. With increasing pH the ion-pair gives rise to imidazole base, proton and mercaptide ion, and the rate constants appear to approach a value characteristic of an ordinary —SH group.

Table 7 shows the pH-independent rate constants of alkylation of thiolsubtilisin between pH 6 and 9, as well as the k(limit) values for alkylation of mercaptoacetate. It is seen that the reactivity of the alkylating agents towards the model compound decreases with increasing chain length. (The enantiomers naturally react with identical rates.) Studying the reactions of thiolsubtilisin one can see (last column in Table 7) that in all cases the D-antipodes are the better alkylating agents. It is also shown that the relative rate (enzyme/model compound) of bromopropionamide does not appreciably differ from that of bromoacetamide. However, the "addition" of only one methylene group to D-bromopropionamide results in a more than 60-fold increase in the relative reaction rate, which may be due to a hydrophobic interaction between the enzyme and the reagent. "Lengthening" of the chain of 2-bromo-*n*-butyramide with a methylene group does not alter the reactivity of the D-enantiomer significantly but increases the relative rate of the L-enantiomer by a factor of about 4.

Study of the Stereochemistry of Alkylation of Thiolsubtilisin by Model Building. In the light of the three-dimensional structure of subtilisin [26], it is possible to study the stereochemistry of the alkylation of thiolsubtilisin. The structures of subtilisin and thiolsubtilisin are identical within experimental error, except that the sulfur is substituted for the oxygen atom [2]. Unfortunately, the X-ray diffraction pattern of Novo subtilisin has only been determined. However, since the amino acid residues in the neighborhood of Ser-221 are identical in the Novo and the Carlsberg enzyme [26], the steric structure of their active site region should be very similar. This assumption is supported by our finding, not demonstrated here, that the difference in the rates of alkylation of thiolsubtilisin type Novo with D- and L-2-bromo-*n*-butyramides is similar to that found with the Carlsberg enzyme.

Accordingly, on the basis of the atomic coordinates of Novo subtilisin [27] we have built the Kendrew model of the active-site region of thiolsubtilisin, a part of which is seen in Fig. 3 A and B. When in the model O_G of Ser-221 was replaced by the sulfur atom, the distance between S_G of Cys-221 and N_{E2} of His-64 became shorter than expected for a van der Waals or N—H . . . S hydrogen bond distance (0.35 nm). Therefore, we moved S_G of Cys-221 by rotating it around the C_A—C_B bond to a distance of 0.35 nm.

Inspection of the model shows that S_G of Cys-221 is situated in a fairly apolar medium, as we have suggested above. It is positioned at almost a van der Waals distance between the methyl groups of Thr-220 and Met-222.

Alkylation of a thiol group is a simple nucleophilic substitution. We have anticipated that D-2-bromo-*n*-butyramide approaches S_G of Cys-221 in a low-energy, expanded conformation, the bromine atom pointing backwards to the solvent. When the mercaptide ion of the ion-pair reacts with C_A of the bromobutyramide, C_G can be in a van der Waals contact with both C_B of Ala-152 and C_{G2} of Thr-220 (Fig. 3 A). In addition to this hydrophobic interaction, a hydrogen bond can also be formed between the carbonyl oxygen atom of D-2-bromo-*n*-butyramide and the amide group of the side chain of Asn-155. This hydrogen bond may be rather strong although it is accessible to water. Namely, the amide-amide N—H . . . O=C bond is stronger than amide-water bonds [28]. Furthermore, the formation of hydrophobic interaction between C_G of butyramide and the methyl groups of Ala-152 and Thr-220 facilitates the formation of the hydrogen bond by fixing the alkylating agent in the proper position. It may be noted that the methyl groups of Ala-152 and Thr-220, which are at van der Waals distance from each other, are parts of the hydrophobic binding site of subtilisin [29].

By means of the Kendrew model of the active site, it can also be shown that C_B of bromopropionamide cannot interact with the methyl groups of Ala-152 and Thr-220 during the reaction with the thiol group of Cys-221. Furthermore, "lengthening" of the chain of D-2-bromo-*n*-butyramide with a methylene group (D-2-bromo-*n*-valeramide) does not offer any new interactions with the protein.

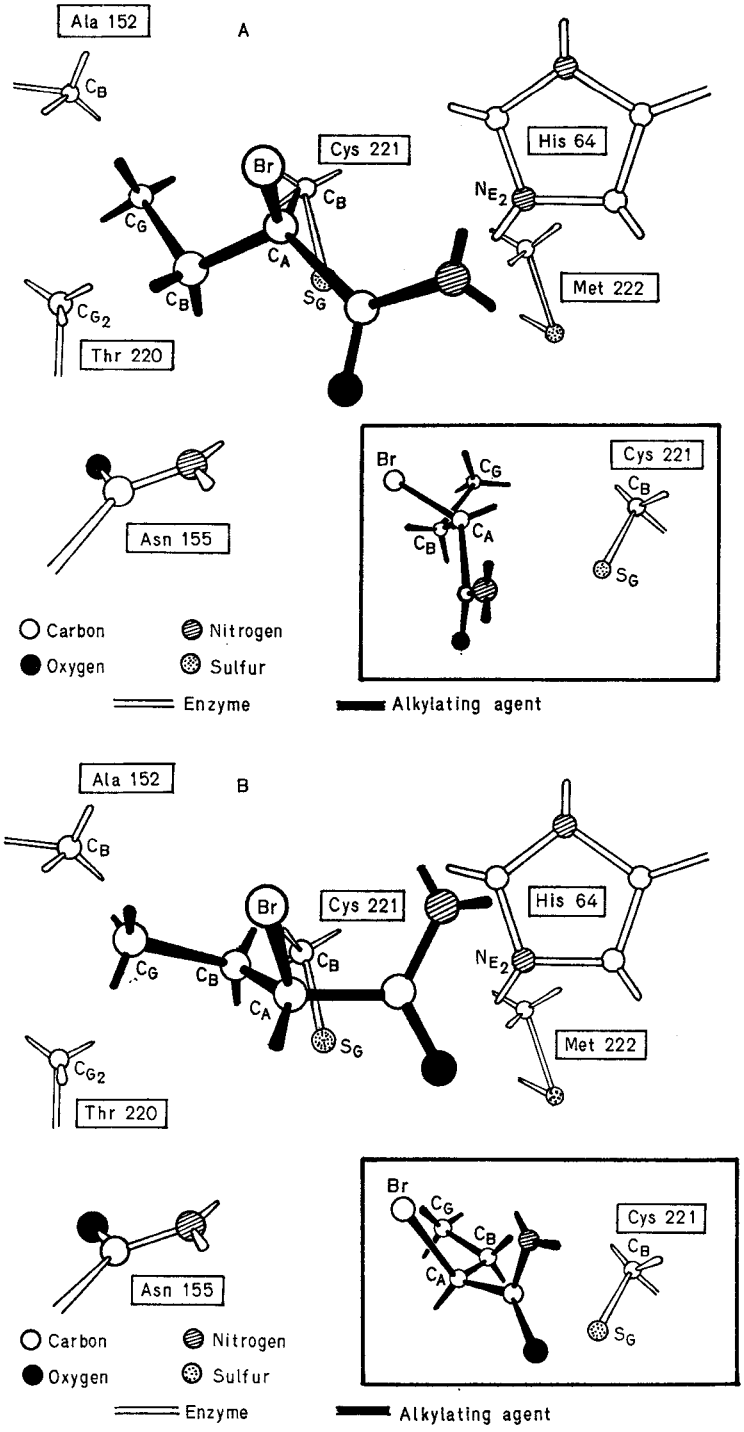


Fig. 3. *Probable atomic arrangement of the reaction of thiol-subtilisin with the two optical antipodes of 2-bromo-*n*-butyramide (A): D-antipode, (B): L-antipode.* In the frame the model is viewed from the right-hand side

The poor reactivity of L-2-bromo-*n*-butyramide can be explained in terms of the greater freedom of motion of the L-antipode in the transition state as compared with that of the D-antipode. A significant loss of entropy of activation in the reaction with the D-antipode occurs if the molecule is fixed in a proper orientation by the hydrophobic and the hydrogen bonds as shown in Fig. 3A. This is not possible,

however, with the L-antipode, since if proper orientation is achieved through the hydrophobic interaction, the hydrogen bond between the amide groups cannot be formed as shown in Fig. 3B. Moreover, as also seen in Fig. 3B, the attack on C_A by the sulfur atom is somewhat hindered by the amide group of the alkylating agent.

The considerable difference in the reactivities of D- and L-2-bromo-*n*-butyramides towards thiolsubtilisin, as interpreted above in terms of the three-dimensional structure of the active site, only exists between pH 6 and 9. It is seen in Fig. 2B that with increasing pH the ratio of the rate constants for the reaction of D- and L-enantiomers decreases significantly. The same is valid for 2-bromopropionamide and 2-bromo-*n*-valeramide (Fig. 2A and C), although the effects are not so conspicuous in these cases. The change in the ratios with pH indicates that some structural alterations occur in the immediate environment of the sulfur atom. This is conceivable since at high pH the mercaptide-imidazolium ion-pair decomposes, its proton dissociates into the solvent, and the polarity around the mercaptide ion becomes more negative. It is probable that after decomposition of the ion-pair above pH $\approx$ 9, the van der Waals interactions between C_G of D-2-bromo-*n*-butyramide and the methyl groups of Ala-152 and Thr-220 are weakened. It should be emphasized that such a conformational change in the active site may be a very subtle one which does not affect the overall conformation of the protein and therefore cannot be detected by ordinary physical methods, like spectrophotometric titration.

It was shown earlier that the rates of alkylation of creatine-phosphokinase [30] and yeast alcohol dehydrogenase [31–33] with iodoacetamide are independent of pH in a wide range. It follows from Fig. 2 that the rate constants measured around neutral pH may equal k(limit) with a particular reactant, such as L-2-bromo-*n*-valeramide, which gives the impression of a pH-independent reaction. In this case the ionizable group, on which the reaction actually depends, cannot be observed. Therefore, it is more meaningful to measure pH-rate profiles with several different reactants.

Finally, a comparison between the reactivity of thiolsubtilisin and papain shows that a mercaptide-imidazolium ion-pair is the nucleophile at the active site of both thiol enzymes. It is noteworthy that the rate constants of the reaction of the ion-pair with iodoacetamide are similar: 7.17 $M^{-1}s^{-1}$ for thiolsubtilisin and 12.7 $M^{-1}s^{-1}$ for papain [5]. However, the ion-pairs exist in considerably different pH-ranges: pH $\approx$ 6–8.5 for thiolsubtilisin and pH $\approx$ 5–6 for papain.

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