On the Mechanism of Action of Streptococcal Proteinase. III. The Effect of pH, Organic Solvents, and Deuterium Oxide on the Proteinase-Catalyzed Hydrolysis of *N*-Acylamino Acid Esters†

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ABSTRACT: The pH dependence of proteinase-catalyzed hydrolysis of *p*-nitrophenyl and phenyl *N*-Z-L-alaninate and of phenyl α-*N*-Z-L-lysinate has been determined. The pH–k_{cat} profile was sigmoidal, which is consistent with the supposition that for specific ester substrates k_{cat} is a measure of k_3, the deacylation rate constant, and indicates that deacylation is dependent on a basic group of pK = 4.1. The pH–k_{cat}/K_m profiles were also found to be dependent on a single ionizable basic group of pK = 4.44. The ionization of an acidic group with p$K \sim$ 8.5, usually attributed to the SH group of the sulfhydryl enzymes, was not observed in proteinase-catalyzed hydrolysis of the above esters. Determination of the pH–k_{cat}/K_m for the papain-catalyzed hydrolysis of phenyl *N*-Z-L-alaninate yielded a bell-shaped curve indicating that, for papain, acylation (as measured by the k_{cat}/K_m ratio) depends on two ionizable groups, a basic group of pK = 3.56 and an acidic group of pK = 8.07. The effect of deuterium oxide on the proteinase-catalyzed hydrolysis of several ester substrates was determined. A deuterium oxide isotope effect (k^{H_2O}/k^{D_2O}) of 2–3 is found and this is attributed to the involvement of a proton transfer in the rate-determining step (deacylation) of proteinase hydrolysis. A similar deuterium oxide isotope effect was noted for papain. The effect of acetonitrile and dimethyl sulfoxide on the kinetic constants, k_{cat} and K_m, was determined. Acetonitrile was found to decrease significantly the k_{cat} with only a small decrease in the K_m. Dimethyl sulfoxide had no effect on the k_{cat} but increased the K_m, as is typical of a competitive inhibitor. These results suggest that acetonitrile does not bind at the active site of proteinase as does the dimethyl sulfoxide but that it can compete with water in deacylation to cause a decrease in k_{cat} (k_3).

In the preceding paper, kinetic evidence was presented consistent with a three-step mechanism *via* an acyl-enzyme intermediate in the streptococcal proteinase-catalyzed hydrolysis of activated *N*-acylamino acid ester substrates (Kortt and Liu, 1973b).

In the present work we have determined the pH dependence of the proteinase-catalyzed hydrolysis of several ester substrates, *N*-Z-L-Ala-pNph,[1] *N*-Z-L-Ala-Ph, and α-*N*-Z-L-Lys-Ph, to further characterize the pH dependence of proteinase and to determine the pK values of the ionizable groups involved in its mechanism of action. In order to provide a direct comparison with papain, the pH dependence of papain-catalyzed hydrolysis of the substrate *N*-Z-L-Ala-Ph was determined in the present work.

The use of acetonitrile as a solvent for the ester substrates and as an additive in buffers to enhance the solubility of some esters, and more recently the use of a dimethyl sulfoxide buffer system in the preparation of proteinase by affinity chromatography (Kortt and Liu, 1973a), prompted the examination of the effect of these organic solvents on proteinase activity. The effect of D_2O on proteinase-catalyzed hydrolysis of some "activated" ester substrates was also determined and these results are reported.

Experimental Section

Materials. The *N*-benzyloxycarbonyl-L-amino acid esters used as substrates have been described earlier (Kortt and Liu, 1973b). Streptococcal proteinase and papain were prepared and activated as previously described (Kortt and Liu, 1972a). The normality of the enzyme solutions was determined by the rate assay with *N*-Z-Gly-pNph as a secondary standard, as described in the preceding article (Kortt and Liu, 1973b). Buffers were prepared from analytical grade reagents. Formate (pH 3.42–4.33), acetate (pH 4.55–5.53), and Tris (pH 7.4–8.65) buffers were used. The ionic strength of the buffers was 0.2. The kinetic constants appear to be independent of the nature and concentration of the buffers. pH measurements were made with a Radiometer pH meter 4, standardized as described by Bates (1964). The buffers containing organic solvents (up to 20% v/v) were prepared by mixing the appropriate volume of organic solvent with a suitable volume of the standard acetate buffer, pH 5.53, I = 0.2. Potassium chloride was added to maintain constant ionic strength. The addition of acetonitrile increased the pH of the buffers as follows: 5% v/v, ΔpH 0.15; 10% v/v, ΔpH 0.23; 15% v/v, ΔpH 0.31; 20% v/v, ΔpH 0.48. Dimethyl sulfoxide increased the pH of the buffers as follows: 5% v/v, ΔpH 0.02; 10% v/v, ΔpH 0.11; 15% v/v, ΔpH 0.24; 20% v/v, ΔpH 0.37. These increases are with respect to the acetate buffer, pH 5.53, I = 0.2, used in these experiments.

Stock solutions of esters were prepared in acetonitrile (Matheson Coleman and Bell, Spectro Grade) except for α-*N*-Z-L-lysine esters, which were made up in 10% v/v H_2O–acetonitrile. For the experiments with dimethyl sulfoxide the stock solutions were made up in dimethyl sulfoxide (Aldrich, Spectra Grade).

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[1] Abbreviations used are: pNph, *p*-nitrophenyl ester; oNph, *o*-nitrophenyl ester; Ph, phenyl ester; Z, benzyloxycarbonyl; Bz, benzoyl; Ac, acetyl; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

D_2O was obtained from Savannah River Plant, U. S. Atomic Energy Commission Aiken, S. C. The D_2O–phosphate buffer, $I = 0.1$, was prepared from disodium hydrogen phosphate and potassium dihydrogen phosphate salts oven dried at 100° for 2 hr and then dissolved in 99.8% D_2O. All other chemicals used were analytical grade reagents.

Methods. Rate measurements were made at 25.0 ± 0.1° with a Cary-15 recording spectrophotometer equipped with 0–0.1 and 0–1.0 absorbance slide-wires and a thermostated cell compartment. The wavelengths used and the general procedure for the rate measurements are described in the preceding paper (Kortt and Liu, 1973b). For the D_2O experiments, the volume of enzyme in aqueous solution was 25 μl for each rate measurement and the maximum amount of water added was 0.83%. The substrates were in acetonitrile except the α-*N*-Z-L-lysinates, which were in 10% v/v H_2O–acetonitrile, and the maximum amount of H_2O added was 0.32%. Proteinase appeared stable in the D_2O–phosphate buffer solution as indicated by enzyme assay carried out in aqueous solution. The pD was determined with a glass electrode, and a correction of 0.40 unit was made (Lumry *et al.*, 1951; Zerner and Bender, 1961).

The steady-state parameters, k_{cat} (= $V/[E]_0$) and K_m, were determined from initial velocity measurements at varying substrate concentrations from the least-mean-squares plot of $1/V$ *vs.* $1/S$. Calculation of the kinetic constants V_m and K_m was done with the CDC 6600 computer by the method of Hanson *et al.* (1967) as modified by K. Thompson of this department. Corrections for spontaneous hydrolysis of the substrate were applied when required. Michaelis–Menten kinetics were observed with all substrates studied. The ΔE values for each Lineweaver–Burk plot were determined either by following the reaction to completion at two or more substrate concentrations or by an independent measurement of the hydrolysis of the substrate under the same conditions. The values of k_{cat}/K_m were also determined under second-order conditions ($K_m \gg [S]_0$) from the apparent first-order rate constant (k_{obsd}) as previously described (Kortt and Liu, 1973b). The kinetic data obtained for the pH dependence of proteinase-catalyzed reactions were analyzed according to the procedures outlined for other proteolytic enzyme systems (Bender *et al.*, 1964; Whitaker and Bender, 1965) since kinetic evidence for an acyl-enzyme mechanism for proteinase has been documented (Kortt and Liu, 1973b). The dissociation constants and the theoretical values arising from them were calculated as previously described (Massey and Alberty, 1954; Laidler, 1955; Bender *et al.*, 1964). The stability at the higher pH values was demonstrated for proteinase by rate assay experiments, and has been reported for papain (Whitaker and Bender, 1965). The kinetic constants for proteinase in the presence of organic solvents and in the D_2O–phosphate buffer system were determined from Lineweaver–Burk plots as described above. The k_{cat} values for papain determined in the D_2O–phosphate buffer were measured under zero-order conditions (see Table V). The values of $[S]_0$ and $[E]_0$ are given in the tables.

1,3-Dibromoacetone was synthesized according to Weygand and Schmied-Kowarzik (1949) by treating acetone with Br_2 in the presence of acetic acid. The product, 1,3-dibromoacetone, was collected by distillation under reduced pressure (69–71° (5 mm)) and recrystallized repeatedly from hexane to give colorless needles; mp 27–28° (*Anal.* Calcd for $C_3H_4OBr_2$: C, 16.69; H, 1.87; Br, 74.03. Found: C, 16.58; H, 1.87; Br, 73.8); nmr spectrum measured with a Varian T-60 spectrophotometer in deuteriochloroform, (δ) 4.15 (s). No other peaks were observed.

TABLE I: Kinetic Constants for the Proteinase-Catalyzed Hydrolysis of *p*-Nitrophenyl *N*-Z-L-Alaninate.[a]

Buffer	pH	k_{cat} (sec^{-1})	K_m ($\times 10^4$ M)	k_{cat}/K_m (M^{-1} sec^{-1} $\times 10^{-5}$)
Formate	3.42	35.7 ± 3.96	2.67 ± 0.36	1.34
	3.71	44.8 ± 2.63	2.10 ± 0.15	2.13
	4.04	60.3 ± 4.5	1.88 ± 0.16	3.20
	4.33	73.4 ± 5.15	1.54 ± 0.14	4.77
Acetate	4.55	76.0 ± 6.2	1.43 ± 0.13	5.32
	4.74	93.7 ± 5.22	1.26 ± 0.11	7.44
	5.00	102.6 ± 7.28	1.14 ± 0.13	8.95
	5.53	110.2 ± 6.01	1.14 ± 0.22	9.67
Phosphate	5.94	127.4 ± 7.48	1.17 ± 0.10	10.89
	6.21	115.7 ± 8.77	1.05 ± 0.08	10.99
	6.73	128.2 ± 6.19	1.21 ± 0.09	10.58
	7.25	108.6 ± 2.97	1.02 ± 0.04	10.86
Tris	7.73	109.5 ± 9.5	1.00 ± 0.15	10.95

[a] Determined at 25.0 ± 0.1°, $I = 0.2$ buffer, 3.17% v/v acetonitrile, $[S]_0 = 0.277$–1.106×10^{-4} M, $[E]_0 = 0.498$–1.29×10^{-8} N.

Fully active streptococcal proteinase (0.05 μmol) prepared by a Sepharose–mercurial column (Sluyterman and Wijdenes, 1970; Kortt and Liu, 1973a) was treated with 1,3-dibromoacetone (0.05 μmol in 0.2 ml of acetone) in 0.05 M sodium acetate buffer, pH 5.5 (10 ml), essentially according to Husain and Lowe (1968a,b). Inhibition of the enzyme was completed in less than 10 min. The inhibited enzymes were dialyzed against several changes of water for 2 days and subjected to amino acid analysis before and after performic acid oxidation. The oxidation was performed by the procedure of Hirs (1956) at −10° for 6 hr. Portions of the inhibited proteinase and proteinase were further treated with equimolar amounts of iodoacetate at pH 5.5 for 30 min, dialyzed against water, and subjected to amino acid analysis (Table VI).

Results

pH Dependence of Proteinase-Catalyzed Hydrolysis. The kinetic constants for the proteinase-catalyzed hydrolysis of *p*-nitrophenyl *N*-Z-L-alaninate and phenyl α-*N*-Z-L-lysinate are listed in Tables I and II. The $k_{cat}/K_m(k_{obsd}/[E]_0)$ values for phenyl α-*N*-Z-L-lysinate determined from the apparent first-order rate constant ($K_m \gg [S]_0$) are also given in Table II. The effects of pH on k_{cat} and k_{cat}/K_m for these two substrates are shown in Figures 1 and 2. The experimental data showed the rate constant, k_{cat}, to be dependent on an ionizable group with a dissociation constant, K_1, in the acid region

$$k_{cat} = k_{cat}(lim)/(1 + ([H^+]/K_1)) \quad (1)$$

The reciprocal form of eq 1 gives a straight line, and a plot of $1/k_{cat}$ *vs.* $[H^+]$ allows determination of the values of $k_{cat}(lim)$ and K_1 (Massey and Alberty, 1954; Laidler, 1955; Bender *et al.*, 1964). The solid curves in Figures 1 and 2 represent the theoretical values calculated from the values of $k_{cat}(lim)$ and

TABLE II: Kinetic Constants for the Proteinase-Catalyzed Hydrolysis of Phenyl α-*N*-Z-L-Lysinate.[a]

Buffer	pH	k_{cat} (sec^{-1})	K_m ($\times 10^4$ M)	k_{cat}/K_m (M^{-1} sec^{-1} $\times 10^{-4}$)	k_{cat}/K_m (M^{-1} sec^{-1} $\times 10^{-4}$)
Formate	3.42	45.4 ± 1.76	15.2 ± 0.93	2.98	2.53
	3.71	60.8 ± 3.08	11.76 ± 0.83	5.17	4.73
	4.04	94.1 ± 2.67	12.38 ± 0.87	7.60	8.89
	4.33	114.5 ± 1.94	9.49 ± 0.32	12.06	13.32
Acetate	4.55	142.6 ± 4.23	8.33 ± 0.5	17.1	18.28
	4.74	150.5 ± 3.12	8.02 ± 0.35	18.76	18.84
	5.00	163.2 ± 8.20	7.01 ± 0.70	23.28	23.61
	5.39				28.52
	5.53	176.1 ± 10.80	6.67 ± 0.80	26.40	28.52
	5.94				29.50
Phosphate	6.21	182.4 ± 9.87	6.64 ± 0.78	27.46	31.82
	6.48				28.80
	6.73	178.0 ± 3.76	6.05 ± 0.36	29.42	30.25
	6.99	189.5 ± 2.59	6.37 ± 0.17	29.74	29.74
	7.25	173.7 ± 3.15	5.88 ± 0.42	29.54	30.08
Tris	7.73				31.16
	8.65				29.6

[a] Determined at 25.0 ± 0.1°, I = 0.2 buffer, 3.17% v/v acetonitrile, $[S]_0 = 0.293–1.17 \times 10^{-3}$ M, $[E]_0 = 0.641–1.797 \times 10^{-8}$ N. k_{cat}/K_m values determined under second-order conditions ($[S]_0 \ll K_m$) from the apparent first-order constant, k_{obsd} ($k_{obsd}/[E]_0 = k_{cat}/K_m$), $[S]_0 = 4.88 \times 10^{-1}$ M; $[E]_0 = 7.59 \times 10^{-6}$ N.

TABLE III: Effect of pH on the Proteinase- and Papain-Catalyzed Hydrolysis of Phenyl *N*-Z-L-Alaninate.[a]

		Proteinase	Papain
Buffer	pH	k_{cat}/K_m (M^{-1} sec^{-1} $\times 10^{-5}$)	k_{cat}/K_m (M^{-1} sec^{-1} $\times 10^{-6}$)
Formate	3.42	0.28	1.33
	3.71	0.51	3.10
	4.04	0.84	5.35
	4.33	1.16	7.34
Acetate	4.55	1.47	8.09
	4.74	1.81	8.90
	5.00	2.07	9.12
	5.39	2.32	9.73
	5.53	2.56	9.65
Phosphate	6.21	2.61	9.15
	6.48	2.57	9.64
	6.73	2.54	9.53
	6.99	2.62	9.65
Tris	7.40	2.74	8.74
	7.73	2.64	7.52
	8.00	2.92	5.69
	8.19	2.84	4.15
	8.65	2.88	2.20

[a] Determined at 25.0 ± 0.1°, I = 0.2 buffer, 3.17% v/v acetonitrile. The k_{cat}/K_m values were determined under second-order conditions ($[S]_0 \ll K_m$) from the apparent first-order constant, k_{obsd} ($k_{obsd}/[E]_0 = k_{cat}/K_m$). For proteinase, $[S]_0 = 2.86 \times 10^{-5}$ M; $[E]_0 = 7.43 \times 10^{-8}$ N. For papain, $[S]_0 = 1.43 \times 10^{-5}$ M; $[E]_0 = 7.43 \times 10^{-8}$ N.

K_1 determined from such plots of the experimental data. At the higher pH values, the k_{cat} for both substrates was found to be independent of pH. This result is consistent with the conclusion that for these substrates k_{cat} reflects k_3 (Kortt and Liu, 1973b) and indicates that the deacylation reaction is dependent on a single prototropic group with a pK of about 4.10.

The pH dependence of k_{cat}/K_m was unexpected (Figures 1 and 2). The k_{cat}/K_m ratio was dependent on a single ionizable group with a dissociation constant, K_1', in the acid region. The dependence on a second ionizable group, expected by analogy with papain, was not observed. The k_{cat}/K_m ratio was independent of pH, up to 7.73 for *N*-Z-L-Ala-pNph, and up to 8.65 for α-*N*-Z-L-Lys-Ph (Tables I and II). The k_{cat}/K_m(lim) and K_1' values were calculated from an equation analogous to eq 1, and the theoretical curves are shown in Figures 1 and 2. The experimental values showed a good fit to the theoretically calculated curves. The k_{cat}/K_m appears to be dependent on a single prototropic group with a pK of about 4.46 over the pH range studied. The same pH dependence was also found for proteinase-catalyzed hydrolysis of *N*-Z-Gly-pNph.

This unusual pH dependence of the k_{cat}/K_m ratio for the proteinase-catalyzed hydrolysis of *N*-acylamino acid esters was confirmed by comparison of the pH dependence of proteinase- and papain-catalyzed hydrolysis of *N*-Z-L-Ala-Ph over the pH range 3.42–8.65. The k_{cat}/K_m values determined for both enzymes under second-order conditions ($K_m \gg [S]_0$) are given in Table III. The k_{cat}/K_m(lim) and K_1' values for proteinase were calculated as described above. The k_{cat}/K_m(lim), K_1, and K_2 values for papain were calculated from an equation analogous to eq 1 which accounted for the second dissociation, with constant K_2, of an ionizable group in the alklaine region observed experimentally (Laidler, 1955). The

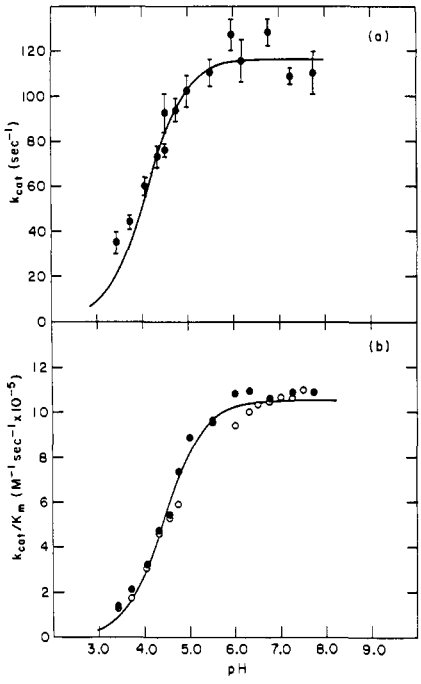

FIGURE 1: Effect of pH on k_{cat} and k_{cat}/K_m for the proteinase-catalyzed hydrolysis of *p*-nitrophenyl *N*-Z-L-alaninate; conditions as in Table I. The solid lines are theoretical ones calculated for $pK_1 = 4.11$ and $k_{cat}(lim) = 116.2$ sec^{-1} (curve a) and $pK_1 = 4.46$ and $k_{cat}/K_m(lim) = 10.5 \times 10^5$ M^{-1} sec^{-1} (curve b). The closed circles are experimental values from initial velocity measurements, and the open circles (curve b) are the k_{cat}/K_m values obtained from measurements of the apparent first-order rate constant, k_{obsd}, under second-order conditions ($[S]_0 << K_m$); the conditions were: $[S]_0 = 5.53 \times 10^{-6}$ M; $[E]_0 = 2.933 \times 10^{-8}$ N; in $I = 0.2$ buffer, 3.17% v/v acetonitrile at 25.0 ± 0.1°.

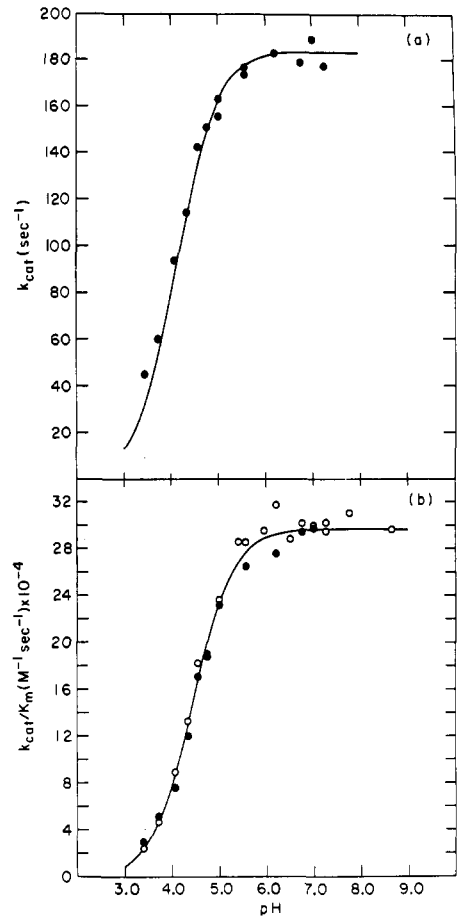

FIGURE 2: Effect of pH on k_{cat} and k_{cat}/K_m for the proteinase-catalyzed hydrolysis of phenyl α-*N*-Z-L-lysinate; conditions as in Table II. The solid lines are theoretical ones calculated for $pK_1 = 4.13$ and $k_{cat}(lim) = 192$ sec^{-1} (curve a) and $pK_1 = 4.46$ and $k_{cat}/K_m(lim) = 29.85 \times 10^4$ M^{-1} sec^{-1} (curve b). The closed circles are experimental values from initial velocity measurements, and the open circles (curve b) are the k_{cat}/K_m values obtained from measurements of the apparent first-order rate constant, k_{bosd}, under second-order conditions ($[S]_0 << K_m$).

experimental values and the theoretically calculated curves are shown in Figure 3 for both enzymes. For proteinase, the k_{cat}/K_m ratio is dependent on a single ionizable group with $pK = 4.4$. For papain, the k_{cat}/K_m ratio is dependent on two ionizable groups with $pK_1 = 3.56$ and $pK_2 = 8.07$, which is consistent with previous reports (Bender and Brubacher, 1966; Williams and Whitaker, 1967). These results clearly demonstrate that the pH dependence of the k_{cat}/K_m ratio for proteinase differs from that for papain. The K_m for proteinase-catalyzed hydrolysis of *N*-Z-L-Ala-pNph and α-*N*-Z-L-Lys-Ph was found to be independent of pH above pH 5.0 (Tables I and II).

The Effect of Organic Solvent. The effect of acetonitrile and dimethyl sulfoxide on the kinetics of proteinase-catalyzed hydrolysis of *p*-nitrophenyl and phenyl *N*-Z-L-alaninate is shown in Table IV. The data show that the effect of acetonitrile differs from that of dimethyl sulfoxide.

Acetonitrile, at the highest concentration tested, decreases the k_{cat} value by a factor of 6.6–7.7 and the K_m value by 61%. The inhibition observed may be described as a mixed type (Dixon and Webb, 1964). Dimethyl sulfoxide causes competitive inhibition; the k_{cat} values are virtually independent of the Me_2SO concentration, and the K_m values increase to a maximum of about fourfold at the highest concentration used. The effects of both solvents appear to be independent of the type of leaving group present in the ester substrate (Table IV). The effect of acetonitrile is primarily on k_{cat}, whereas that of dimethyl sulfoxide is on K_m. Both have a similar if not identical effect on the k_{cat}/K_m ratio for both substrates (Table IV). These solvents exhibit the same effects with other ester substrates such as *N*-Z-Gly-pNph, α-*N*-Z-L-Lys-pNph and -Ph, and α-*N*-Z-L-Glu-Ph. The stability of proteinase at the highest concentration of organic solvent used was determined by rate measurements with *N*-Z-Gly-pNph, and proteinase was found to be stable for at least 3 hr at 25° under these conditions.

Effect of D_2O. The kinetic constants, k_{cat} and K_m, for the proteinase-catalyzed hydrolysis of three *N*-Z-L-alaninates, two α-*N*-Z-L-lysinates, and *N*-Z-Gly-pNph were determined in D_2O– and H_2O–phosphate buffers (Table V). The effect of D_2O on the k_{cat} of all the *N*-Z-L-amino acid esters used was essentially the same. The k_{cat} was found to be 2.09- to 2.68-fold higher in H_2O at pH 5.95 than in D_2O at pD 6.39. In this pH region, k_{cat} is essentially independent of pH (Tables I and II). This large D_2O effect on k_{cat} may be attributed to the effect on k_3, the rate-limiting step of ester hydrolysis. Kinetic data for proteinase-catalyzed hydrolysis of "activated" *N*-acylamino acid esters are consistent with the conclusion that k_{cat} is a measure of k_3 (Kortt and Liu, 1973b). The effect of D_2O on the k_{cat} values for the papain-catalyzed hydrolysis of these esters (Table V) showed that the k_{cat} in H_2O was 2.24- to 2.74-fold higher than in D_2O. These results are consistent

TABLE IV: Effect of Acetonitrile and Dimethyl Sulfoxide on the Proteinase-Catalyzed Hydrolysis of *p*-Nitrophenyl and Phenyl *N*-Z-L-Alaninates.[a]

	N-Z-L-Ala-*p*-Nph[b]			*N*-Z-L-Ala-Ph[c]		
	k_{cat} (sec^{-1})	K_m (×10^4 M)	k_{cat}/K_m (M^{-1} sec^{-1} × 10^{-6})	k_{cat} (sec^{-1})	K_m (×10^4 M)	k_{cat}/K_m (M^{-1} sec^{-1} × 10^{-5})
			% (v/v) Acetonitrile			
3.13	115.9 ± 11.7	0.97 ± 0.18	1.19	83.2 ± 13.8	3.25 ± 0.74	2.56
8.00	67.9 ± 1.94	0.76 ± 0.05	0.89	44.4 ± 6.8	2.31 ± 0.55	1.92
12.8	41.1 ± 0.8	0.65 ± 0.03	0.63	28.6 ± 5.0	2.38 ± 0.60	1.20
17.6	29.4 ± 0.93	0.65 ± 0.05	0.45	20.32 ± 1.44	2.33 ± 0.25	0.87
21.4	17.6 ± 4.32	0.59 ± 0.04	0.30	10.81 ± 6.43	1.98 ± 0.19	0.55
			% (v/v) Dimethyl Sulfoxide			
3.13	132.9 ± 11.5	0.94 ± 0.18	1.41	118.1 ± 4.25	4.49 ± 0.21	2.63
8.00	132.9 ± 11.5	1.54 ± 0.22	0.86	105.5 ± 28.3	6.37 ± 0.21	1.66
12.80	131.4 ± 13.9	2.18 ± 0.33	0.60	107 ± 29.5	10.0 ± 1.00	1.07
17.6	142.9 ± 20.1	3.26 ± 0.59	0.44	110 ± 30.0	15.4 ± 1.54	0.72
21.4	139.4 ± 14.1	4.19 ± 0.52	0.33	110 ± 32.0	23.5 ± 2.35	0.47

[a] Kinetic constants determined in acetate buffers, $I = 0.2$, containing acetonitrile or dimethyl sulfoxide (3.13–21.4% v/v), pH 5.5; at 25.0 ± 0.1°. [b] $[S]_0 = 0.476\text{–}1.19 \times 10^{-4}$ M; $[E]_0 = 1.09\text{–}2.10 \times 10^{-8}$ N. [c] $[S]_0 = 0.651\text{–}1.63 \times 10^{-4}$ M; $[E]_0 = 2.84\text{–}5.72 \times 10^{-7}$ N.

with previous reports on the effect of D_2O on papain hydrolysis (Whitaker and Bender, 1965; Brubacher and Bender, 1966).

For proteinase, the K_m was found to be lower in D_2O than in H_2O for all the substrates examined, by 4.67- to 7.56-fold (Table V). Lower K_m values in D_2O for papain have been reported for α-*N*-benzoyl-L-arginine ethyl ester and amide, by a factor of 2.4 and 2.9, respectively (Whitaker and Bender, 1965). These results are consistent with the reported effect of D_2O on α-chymotrypsin-catalyzed hydrolysis (Bender and Hamilton, 1962).

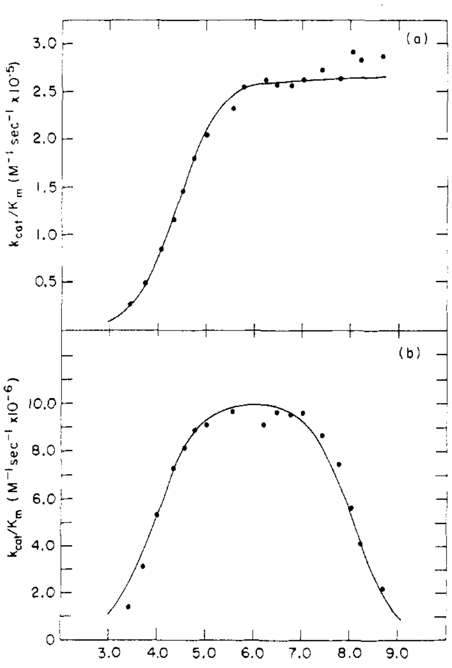

FIGURE 3: Effect of pH on k_{cat}/K_m for (a) the proteinase- and (b) the papain-catalyzed hydrolyses of phenyl *N*-Z-L-alaninate; conditions as in Table III. The solid lines are theoretical ones, calculated for $pK_1 = 4.4$ and $k_{cat}/K_m(\text{lim}) = 2.63 \times 10^5$ M^{-1} sec^{-1} for proteinase, and for $pK_1 = 3.56$, $pK_2 = 8.07$, and $k_{cat}/K_m(\text{lim}) = 10.0 \times 10^6$ M^{-1} sec^{-1} for papain. The k_{cat}/K_m values were determined from the apparent first-order rate constant, k_{obsd} measured under second-order conditions ($[S]_0 << K_m$).

Reaction of Reduced Streptococcal Proteinase with 1,3-Dibromoacetone. Reaction of streptococcal proteinase with 1 molar equiv of 1,3-dibromoacetone resulted in rapid and complete loss of enzymic activity. The results of the stoichiometric inhibition of proteinase by 1,3-dibromoacetone at pH 5.5 showed that complete inhibition was achieved at a ratio of [dibromoacetone]/[SH groups] = 1.08. The amino acid analyses of proteinase and 1,3-dibromoacetone-inhibited proteinase are compared in Table VI. No loss of histidine residue in the inhibited enzyme is evident. Positive identification of 1- or 3-carboxymethylhistidine in the performic acid oxidized sample of the inhibited proteinase revealed the absence of these histidine derivatives. The absence of *S*-carboxymethylcysteine in the 1,3-dibromoacetone-inhibited proteinases that have been subsequently treated with iodoacetate (Table VI) strongly suggests that the inhibition of proteinase is due to the reaction of the active-site cysteine with 1,3-dibromoacetone. Thus, the bifunctional reagent, 1,3-dibromoacetone, appears to have reacted only with the active cysteine in streptococcal proteinase and does not bridge to any histidine.

Discussion

The minimal kinetic scheme for proteinase-catalyzed hydrolysis of ester substrates probably requires a three-step mechanism (Kortt and Liu, 1973b)

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} \underset{P_1}{\underset{+}{ES'}} \xrightarrow{k_3} E + P_2 \qquad (2)$$

TABLE V: Proteinase-Catalyzed Hydrolyses of *N*-Benzyloxycarbonyl-L-amino Acid Esters in Water and Deuterium Oxide at 25°.[a,b]

Ester	H_2O			D_2O			Papain	
	k_{cat} (sec^{-1})	K_m ($\times 10^4$ M)	k_{cat}/K_m (M^{-1} sec^{-1} $\times 10^{-5}$)	k_{cat} (sec^{-1})	K_m ($\times 10^4$ M)	k_{cat}/K_m (M^{-1} sec^{-1} $\times 10^{-5}$)	$k_{cat}(H_2O)$ / $k_{cat}(D_2O)$	$k_{cat}(H_2O)$ / $k_{cat}(D_2O)$
N-Z-L-Ala-pNph[c]	126.3 ± 7.5	1.08 ± 0.1	1.17 (1.13)[d]	60.4 ± 4.69	0.173 ± 0.039	0.35	2.09	2.59[e]
N-Z-L-Ala-oNph[f]	34.6 ± 8.3	6.45 ± 1.76	0.53 (0.55)[g]	12.9 ± 0.84	0.98 ± 0.11	1.31 (1.30)	2.68	
N-Z-L-Ala-Ph[h]	76.4 ± 13.7	3.83 ± 0.87	2.00 (2.48)[i]	33.8 ± 2.15	0.68 ± 0.09	5.02 (4.92)	2.26	
Z-Gly-pNph[j]	7.31 ± 1.2	1.54 ± 0.3	0.475	3.16 ± 0.10	0.33 ± 0.02	0.958	2.31	2.74[k]
α-*N*-Z-L-Lys-pNph[l]	215.3 ± 67.9	4.81 ± 2.0	4.48	83.17 ± 2.49	0.95 ± 0.06	8.75	2.60	2.43[m]
α-*N*-Z-L-Lys-Ph[n]	178 ± 3.99	6.69 ± 0.80	2.66	67.5 ± 8.08	0.884 ± 0.07	7.64	2.64	2.24[o]

[a] Kinetic constants determined in phosphate–H_2O buffer, $I = 0.1$, pH 5.95, and in phosphate–D_2O buffer, $I = 0.1$, pH 6.39 (corrected value; pD = pH + 0.4), 3.17% v/v acetonitrile at 25.0 ± 0.1°. The D_2O reaction was carried out in 99.2% D_2O. [b] k_{cat} values for papain-catalyzed hydrolysis determined under zero-order conditions ($[S]_0 \gg K_m$). [c] $[S]_0 = 0.348$–1.158×10^{-4} M; $[E]_0 = 1.80 \times 10^{-8}$ N. [d] Values in parentheses were determined from the apparent first-order constant, k_{obsd} measured under second-order conditions ($[S]_0 \ll K_m$); $[S]_0 = 1.93 \times 10^{-5}$ M; $[E]_0 = 1.80 \times 10^{-8}$ N. [e] $[S]_0 = 1.81 \times 10^{-4}$ M; $[E]_0 = 2.77 \times 10^{-8}$ N. [f] $[S]_0 = 0.356$–119×10^{-9} M; $[E]_0 = 1.13 \times 10^{-7}$ N. [g] $[S]_0 = 0.356 \times 10^{-4}$ M; $[E]_0 = 1.13 \times 10^{-7}$ N. [h] $[S]_0 = 0.46$–1.53×10^{-4} M; $[E]_0 = 1.06 \times 10^{-7}$ N. [i] $[S]_0 = 0.46 \times 10^{-4}$ M; $[E]_0 = 1.06 \times 10^{-7}$ N. [j] $[S]_0 = 0.229$–0.762×10^{-4} M; $[E]_0 = 1.13 \times 10^{-7}$ N. [k] $[S]_0 = 0.762 \times 10^{-4}$ M; $[E]_0 = 0.259 \times 10^{-7}$ N. [l] $[S]_0 = 0.564$–1.82×10^{-4} M; $[E]_0 = 1.14 \times 10^{-8}$ N. [m] $[S]_0 = 9.26 \times 10^{-4}$ M; $[E]_0 = 1.83 \times 10^{-4}$ M; $[E]_0 = 1.795 \times 10^{-8}$ N. [o] $[S]_0 = 1.83 \times 10^{-4}$ M; $[E]_0 = 2.77 \times 10^{-8}$ N.

TABLE VI: Basic Amino Acids and Cysteine in the Hydrolysates of Streptococcal Proteinase and 1,3-Dibromoacetone-Inhibited Streptococcal Proteinase.

Amino Acid[a]	Streptococcal Proteinase	1,3-Dibromoacetone Inhibited Streptococcal Proteinase	
		After 10 min	After 60 min
Lysine	17.00	17.00	17.00
Histidine	7.79	7.74	7.70
Arginine	8.18	8.22	8.06
Cysteine[b]	0.95	0.06	0.04

[a] Determined by ion exchange chromatography (Spackman *et al.*, 1958). The values reported are averages of duplicate analyses. The results are expressed as the calculated number of residues per molecule based on lysine equals 17. Proteins were hydrolyzed without performic acid oxidation with 4.0 N methanesulfonic acid at 115° for 22 hr (Liu and Chang, 1971). [b] Determined as *S*-carboxymethylcysteine. Streptococcal proteinase and 1,3-dibromoacetone-inhibited streptococcal proteinase were treated with an equimolar amount of iodoacetate as described in the text.

where ES is the Michaelis–Menten complex, K_s is its equilibrium constant, ES′ is the acyl-enzyme intermediate involving the SH group of the essential cysteine residue, and P_1 and P_2 are the alcohol and acid portions of an ester substrate. The constants in this equation are related to those of the usual Michaelis–Menten equation

$$E + S \overset{K_M}{\rightleftharpoons} ES \xrightarrow{k_{cat}} E + P_1 + P_2 \tag{3}$$

by the relations

$$k_{cat} = k_2k_3/(k_2 + k_3) \tag{4}$$

$$K_m = k_3/(k_2 + k_3)K_s \tag{5}$$

The influence of pH on the Michaelis–Menten parameters (Massey and Alberty, 1954; Laidler, 1955; Dixon and Webb, 1964) and on the kinetic constants of reactions involving an acyl-enzyme intermediate (Bender *et al.*, 1964; Whitaker and Bender, 1965; Kaplan and Laidler, 1967; Kasserra and Laidler, 1969) has been described in detail. A number of general kinetic schemes involving two ionizable groups have been outlined for several acyl-enzyme systems (Bender *et al.*, 1964; Whitaker and Bender, 1965; Kasserra and Laidler, 1969), and by an approximate steady-state treatment the following general expressions are obtained for the effect of pH on the kinetic parameters k_{cat} and k_{cat}/K_m

$$k_{cat} = \frac{k_{cat}(lim)}{1 + ([H^+]/K_1) + (K_2/[H^+])} \tag{6}$$

$$\frac{k_{cat}}{K_m} = \frac{k_2}{K_s} = \frac{k_{cat}/K_m(lim)}{1 + ([H^+]/K_1) + (K_2/[H^+])} \tag{7}$$

The pH dependence of proteinase-catalyzed hydrolysis was analyzed in terms of these general equations.

In Figures 1 and 2 are shown the pH dependences for the proteinase-catalyzed hydrolysis of *N*-Z-L-Ala-pNph and α-*N*-Z-L-Lys-Ph, respectively. For both substrates, the k_{cat} values were found to be dependent on the ionization of a single prototropic group of pK = 4.11. For the specific ester substrates used, it is reasonable to assume that $k_{cat} = k_3$, the deacylation rate constant (Kortt and Liu, 1973b). and the sigmoidal pH–rate profile found is consistent with such an assumption. This result is in agreement with the work of Liu *et al.* (1969), who found a sigmoidal pH–rate profile with a pK of 4.5 for the substrate α-*N*-Z-L-Lys-*p*-Nph, but is in disagreement with the work of Gerwin *et al.* (1966), who inferred a pK_1 of 6.4 for a catalytically important group in the free enzyme from the values of K_m and V_{max} for the proteinase-catalyzed hydrolysis of *N*-Z-Phe-Tyr.

The pH dependences of k_{cat}/K_m for *N*-Z-L-Ala-pNph, α-*N*-Z-L-Lys-Ph, and *N*-Z-Ala-Ph are shown in Figures 1–3. The k_{cat}/K_m ratio depends on a single ionizable group with pK = 4.44. In the pH range studied (3.4–8.7), a second prototropic group with p$K \approx 8.5$, characteristic of the SH group, was not found for the three substrates. In the absence of any perturbing effects, such as the ionization of a prototropic group on the substrate, the effect of pH on the experimentally determined ratio $k_{cat}/K_m = (k_2/K_s)$ should reveal the ionizable groups on the enzyme which are essential in the reaction of the enzyme–substrate complex and the acylation reaction (Peller and Alberty, 1959; Kasserra and Laidler, 1969). There is ample evidence that the SH group of the single cysteine residue in streptococcal proteinase is essential for its activity (Liu and Elliott, 1971; Kortt and Liu, 1973a,b). The existence in the proteinase of a sulfhydryl group with a pK of around 8.4 was suggested by the work of Gerwin (1967), who studied the pH dependence of the inhibition of streptococcal proteinase with chloroacetic acid and chloroacetamide. Using *N*-Z-Phe-Tyr as a substrate, Gerwin *et al.* (1966) noted in a plot of pK_m *vs.* pH an inflection at pH 8.4. This inflection, which indicates the existence of a group with a pK of around 8.4, has also been suggested to reflect the ionization of this SH group in the proteinase. In view of the above findings, it was expected that the ionization of this SH group would be revealed in the pH dependence of k_{cat}/K_m for the ester substrates studied. The unexpected failure to observe the bell-shaped profile in the porteinase-catalyzed hydrolysis of the substrates *N*-Z-Ala-pNph, α-*N*-Z-Lys-Ph, and *N*-Z-Ala-Ph prompted us to examine the behavior of papain toward the substrate *N*-Z-Ala-Ph under identical experimental conditions. As can be seen in Figure 3, a bell-shaped profile was obtained for papain, and the ratio k_{cat}/K_m was dependent on two prototropic groups with a pK_1 of 3.56 and a pK_2 of 8.07. These pK values agree with the data of Brubacher and Bender (1966) and clearly demonstrate a difference in the pH dependence for proteinase- and papain-catalyzed hydrolysis of ester substrates in the alkaline pH region.

The apparent discrepancy between the results of Gwerin *et al.* (1966) and the present studies on the pK of a catalytic entity in the streptococcal proteinase-catalyzed hydrolysis of peptide and ester substrates illustrates the uncertainty involved in the classical methods for assigning kinetic pK values for the kinetically important groups in an enzyme and emphasizes the necessity of having independent chemical evidence for assigning a particular functional group at the active site of an enzyme. It should be noted that the peptide substrate used by Gerwin *et al.* (1966) is anionic at pH >4 whereas the ester substrates used in the present studies are cationic or neutral. The pK value derived from the studies on the dependence of k_{cat}/K_m on pH reflects the ionization of the enzyme and should be identical for all substrates provided there is no pH-dependent interaction of the charged substrate with noncatalytic groups on the enzyme, which would affect this value. Such an effect might be present in the pH ranges studied since the anionic substrate *N*-Z-Phe-Tyr and the neutral substrate *N*-Z-Ala-pNph exhibited different pH dependences of k_{cat}/K_m for streptococcal proteinase. It is possible that the second catalytic group involved in the acylation reaction of streptococcal proteinase has a pK value higher than 9 and therefore the influence of its dissociation on the enzymic activity would be manifested only at higher pH values. The use of ester and amide substrates stable at pH >9 will be required to determine the ionization of the prototropic group of pK >9 in proteinase.

The dependence of acylation and deacylation steps on a group with pK = 4.1–4.5 has been demonstrated for a number of ester substrates, both charged and uncharged, for streptococcal proteinase (Figures 1–3; Tables I–III). It cannot be determined from these data, however, whether this group acts as a general base catalyst rather than as a nucleophilic catalyst in the deacylation. Indication that the basic group acts as a general base catalyst comes from the finding of a large D_2O solvent isotope effect ($k_{cat}^{H_2O}/k_{cat}^{D_2O}$ = 2.1–2.7) for a number of ester substrates (Table V), for which $k_{cat} \approx k_3$ (Kortt and Liu 1973b). Similarly large D_2O solvent isotope effects on the values of k_3 have been reported for α-chymotrypsin (Bender and Hamilton, 1962) and for papain (Whitaker and Bender, 1965; Brubacher and Bender, 1966). The data shown in Table V also reveal that the values of K_m were significantly lower in D_2O than in H_2O ($K_m(H_2O)/K_m(D_2O)$ = 4.7–7.6). This decrease in the K_m value is most likely not due to a large decrease of K_s in D_2O, since the solvent properties of D_2O would be expected to cause but a small decrease in K_s values (Krishenbaum, 1951; Bender and Hamilton, 1962). The complex nature of K_m ($= k_3/(k_2 + k_3)K_s$) with deacylation (k_3) the rate-limiting step for ester hydrolysis makes it difficult to draw any conclusion regarding the effect of D_2O solvent isotope on the acylation step. However, the results obtained in the present study are in general agreement with the suggestion that in the proteinase-catalyzed hydrolysis of ester substrates there is a rate-limiting proton transfer in the deacylation as well as in the acylation step.

The two aprotic solvents, acetonitrile and dimethyl sulfoxide (Me_2SO), exhibited different effects on the kinetic constants of the proteinase-catalyzed hydrolysis of ester substrates (Table IV). Acetonitrile caused a large decrease in k_{cat} and only a small decrease in K_m. Since $k_{cat} \approx k_3$ for these substrates, the effect of acetonitrile is to lower the rate of deacylation by competing with water for the acyl-enzyme in the deacylation step, as has been suggested for methanol (Kortt and Liu, 1973b). However, the data shown in Table IV and Figure 4 reveal that, unlike methanol, acetonitrile does not influence the binding of the substrate to the enzyme. No significant effect on k_{cat} was observed upon addition of Me_2SO to the reaction mixture. The data in Table IV reveal rather than the solvent Me_2SO behaved as a typical competitive inhibitor to the ester substrate for proteinase. These results could be explained by a scheme in which the methyl groups of Me_2SO bind in the area of substrate binding in the free enzyme (Kortt and Liu, 1973b) and thus Me_2SO behaves as a competitive inhibitor, whereas CH_3CN, being a smaller molecule, can enter the active site of the acyl-enzyme during the deacylation step and thus influences the rate of deacylation. Me_2SO is too bulky to enter the active site of the acyl-enzyme in the deacylation step and thus has no effect on k_{cat} ($= k_3$). It is obvious, in the light of the

above discussion, that in the acyl-enzyme intermediate of the proteinase, there is little space for solvent molecules larger than a water molecule. The effect of acetonitrile on proteinase reactions is markedly different from its effect on papain, for which rate accelerations have been observed (Hinkle and Kirsch, 1970).

The identity of the group with $pK = 4.1$–4.5 seen in both acylation and deacylation reactions of streptococcal proteinase is as yet unresolved. Chemical modification of a histidine residue in the proteinase with α-*N*-bromoacetylarginine methyl ester resulted in complete loss of enzymic activity (Liu, 1967). This result was taken to indicate that a histidine residue is present in the active-site region of proteinase.

On the basis of these observations it is tempting to assign the $pK = 4.1$–4.5 to an imidazole group with an unusually low pK, as has been proposed in the case of papain (Lowe, 1970). Support for such a conclusion comes from the fact that the acid limb of the pH–rate profiles for proteinase closely resembles those found for papain, which suggests that both enzymes may utilize the same catalytic group and share a basically common mechanism. However, in the absence of more direct evidence such as X-ray diffraction data, the possibility cannot be ruled out that this pK value may reflect the ionization of a carboxyl group which either acts as a catalytic entity or maintains an active enzyme conformation. Even in the case of papain, in which chemical modification studies (Husain and Lowe, 1968a) and X-ray diffraction studies (Drenth *et al.*, 1970) have presumably shown the presence of a histidine and a cysteine in close proximity in the active site, correlation of these groups with the kinetic data for pH dependence of hydrolysis reactions and alkylation reactions with α-halo acids is far from conclusive. It is noteworthy that the bifunctional alkylating reagent, 1,3-dibromoacetone, which reacts first with the active cysteine in papain, ficin, and bromelain (Husain and Lowe, 1968a,b) and then reacts intramolecularly with an active histidine residue, reacts only with the cysteine in streptococcal proteinase (this report) and does not bridge the histidine.

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