On the Mechanism of Action of Streptococcal Proteinase. II. Comparison of the Kinetics of Proteinase- and Papain-Catalyzed Hydrolysis of *N*-Acylamino Acid Esters†

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ABSTRACT: Kinetic evidence consistent with the formation of an acyl-enzyme intermediate in the proteinase-catalyzed hydrolyses of *N*-Z-L-alaninates and α-*N*-Z-L-lysinates has been obtained. The effect of adding a simple nucleophile, such as methanol, was consistent with the partitioning of a common acyl-enzyme intermediate between water and the alcohol. However, for the hydrolysis of *o*-nitrophenyl *N*-Z-L-alaninate and for the effect of methanol, the kinetics were more complex. The k_{cat} for *o*-nitrophenyl *N*-Z-L-alaninate was about 3.4-fold lower than the k_{cat} values for *p*- or *m*-nitrophenyl *N*-Z-L-alaninates. The rate of increase of k_{cat} with methanol concentration was dependent upon the nature of the leaving group of the *N*-Z-L-alanine ester. The k_{cat}/K_m ratio was also dependent upon the nucleophile concentration. These results are interpreted in terms of the binding of the leaving group and the methanol at the active site of proteinase, and indicate the existence of a kinetically important ternary enzyme–substrate–leaving group (nucleophile) complex. Proteinase showed specificity toward an unbranched carbon chain or an aromatic residue as the amino acid side-chain group of *N*-acylamino acid ester substrates; substitutions in the β-carbon position resulted in a marked decrease in activity. The presence of a charged group in the side-chain group, positive or negative, had no significant effect on the rate of proteinase hydrolysis. The most striking feature is the specificity of proteinase toward the *N*-acyl group of the substrate. The *N*-acyl group, benzyloxycarbonyl, was found to yield optimal activity with ester substrates and even small changes in this structure resulted in a dramatic decrease in activity. Evidence is presented indicating that the binding of a hydrophobic group in the *N*-acyl moiety, in a precise manner and at a precise distance with respect to the ester bond, is essential for optimal enzymatic activity and suggesting that the binding site for the *N*-acyl group in proteinase may be a relatively small inflexible hydrophobic cleft. The kinetic properties of proteinase are compared with those of papain and ficin.

The enzyme-catalyzed hydrolyses of *N*-acylamino acid esters and amides by sulfhydryl proteinases (such as papain and ficin) can be described minimally by the kinetic scheme (eq 1) where ES is an enzyme–substrate complex, K_s is its

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2 \qquad (1)$$
$$\qquad\qquad\qquad\quad + \qquad\qquad\quad P_1$$

equilibrium constant, ES′ is the acyl-enzyme, and P_1 and P^2 are the alcohol and acid portions of an ester substrate, respectively. The three-step kinetic scheme predicts a number of kinetic consequences (Zerner and Bender, 1964) which can be tested experimentally. In terms of eq 1, several studies of papain- and ficin-catalyzed hydrolyses of *N*-acylamino acid esters have provided kinetic evidence for an acyl-enzyme intermediate and a kinetic scheme with at least three steps (Lowe and Williams, 1965b; Kirsch and Igelström, 1966; Bender and Brubacher, 1966; Bender and Kezdy, 1965; Kortt, 1969). The observation of "titration" kinetics with papain, using a number of labile ester substrates (Bender *et al.*, 1966; de-Jersey *et al.*, 1969), and the partitioning of the deacylation step by the addition of nucleophiles (Brubacher and Bender, 1966, 1967; Fink and Bender, 1969) provided further support for a three-step kinetic mechanism. Furthermore, the spectrophotometric observation and isolation of the acyl-enzyme of papain (Lowe and Williams, 1965a; Brubacher and Bender, 1966; Hinkle and Kirsch, 1970) and of ficin (Lowe and Williams, 1965a) has provided direct evidence for the acyl-enzyme intermediate.

The Michaelis–Menten parameters for eq 1 can be derived by using the steady-state treatment and are given in eq 2 and 3.

$$K_m = [k_3/(k_2 + k_3)]K_s \qquad (2)$$

$$k_{cat} = k_2k_3/(k_2 + k_3) \qquad (3)$$

$$k_{cat}/K_m = k_2k_3/(k_2 + k_3)/k_3K_s/(k_2 + k_3) = k_2/K_s \qquad (4)$$

Streptococcal proteinase catalyzes the hydrolysis of small peptide and protein substrates (Elliott, 1950; Mycek *et al.*, 1952; Gerwin *et al.*, 1966) and of N-substituted amino acid ester and amide substrates (Liu *et al.*, 1969).

In this report, we have investigated the kinetics of streptococcal proteinase-catalyzed hydrolyses of a series of *N*-acylamino acid esters to obtain further information on: (1) the mechanism of action of proteinase and to provide some evidence for the validity of the proposed three-step kinetic scheme (eq 1) for proteinase; (2) the effect of an added nucleophile such as methanol on proteinase-catalyzed hydrolyses of some ester substrates; and (3) the specificity of proteinase (a) toward the amino acid side-chain group of ester substrates and (b) toward the *N*-acyl group of the *N*-acylamino acid ester substrates. Comparisons of the kinetic properties and specificity of proteinase were made with those of papain.

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TABLE I: Kinetic Constants for the Proteinase-, Papain-, and Ficin-Catalyzed Hydrolyses of *N*-Benzyloxycarbonyl-L-alanine Esters.[a]

N-Z-L-Ala Ester	Concn ($\times 10^4$ M)	k_{cat} (sec^{-1})	K_m ($\times 10^4$ M)	k_{cat}/K_m (M^{-1} sec^{-1} $\times 10^{-5}$)	k_{OH^-} (M^{-1} sec^{-1})
		Streptococcal Proteinase			
p-Nitrophenyl[b]	0.277–1.11	118 ± 2.40	1.32 ± 0.04	9.00 (8.86)[c]	79.6
m-Nitrophenyl[d]	0.265–1.06	119 ± 9.60	1.35 ± 0.09	8.65 (8.39)[e]	55.8
o-Nitrophenyl[f]	0.266–1.07	35.5 ± 8.75	6.58 ± 1.18	0.53 (0.55)[g]	48.0
Phenyl[h]	0.269–1.15	82.5 ± 5.10	3.64 ± 0.30	2.35 (2.28)[i]	8.9
		Papain			
p-Nitrophenyl[j]	0.096–0.38	27.3 ± 0.34 26.9[k]	0.065 ± 0.003	42.0	
o-Nitrophenyl[l]	0.294–1.18	26.0 ± 3.90	5.33 ± 0.940	0.49	
Phenyl[m]	0.383–1.53	26.04 ± 0.31	0.47 ± 0.016	5.43	
		Ficin			
p-Nitrophenyl[n]		31.0	0.059	52.5	

[a] Kinetic constants determined in acetate buffer, pH 5.5, $I = 0.2$; 3.17% v/v acetonitrile; at 25.0 ± 0.1°. [b] $[E]_0 = 0.67 \times 10^{-8}$ N. [c] Values in parentheses determined under second-order conditions ($[S]_0 \ll K_m$); $[E]_0 = 2.16 \times 10^{-8}$ N; $[S]_0 = 9.66 \times 10^{-6}$ M. The apparent first-order constant k_{obsd} was measured; $k_{obsd}/[E]_0 = k_{cat}/K_m$. [d] $[E]_0 = 1.43 \times 10^{-8}$ N. [e] As for footnote *c*, $[S]_0 = 2.67 \times 10^{-5}$ M, $[E]_0 = 2.3 \times 10^{-8}$ N. [f] $[E]_0 = 2.81 \times 10^{-8}$ N. [g] As for footnote *c*, $[S]_0 = 3.25 \times 10^{-5}$ M; $[E]_0 = 4.52 \times 10^{-8}$ N. [h] 5.28×10^{-8} N. [i] As for footnote *c*, $[S]_0 = 3.28 \times 10^{-5}$ M; $[E]_0 = 4.52 \times 10^{-8}$ N. [j] $[E]_0 = 1.82 \times 10^{-8}$ N. [k] k_{cat} determined under zero-order conditions ($[S]_0 \gg K_m$), $[S]_0 = 1.16 \times 10^{-4}$ M; $[E]_0 = 8.94 \times 10^{-8}$ N. [l] $[E]_0 = 0.87 \times 10^{-7}$ N. [m] $[E]_0 = 4.48 \times 10^{-8}$ N. [n] Determined in 0.1 M phosphate buffer, pH 6.0, 3.17% v/v acetonitrile (Kortt, 1969).

Experimental Section

Materials. The Ph, pNph, mNph, and oNph[1] esters of Z-L-alanine and the pNph esters of Z-glycine, *p*-NO_2-Z-glycine, cinnamoylglycine, acetylglycine, and benzoylglycine were synthesized by the dicyclohexylcarbodiimide method of Bodanszky and du Vigneaud (1962). The esters were purified by recrystallization from chloroform–hexane. α-*N*-Z-L-Lys-pNph, α-*N*-Z-L-Lys-Ph, and α-*N*-Z-Glu-Ph were available from a previous study (Liu *et al.*, 1969). The other α-*N*-Z-L-amino acid pNph esters used (see Tables V and VI) were purchased from Cyclo Chemical Co. and were recrystallized from chloroform–hexane. The melting points were in good agreement with previously published values. The purity of all esters used was 99 ± 1.0% based on the amount of nitrophenol or phenol released (measured spectrophotometrically) on complete hydrolysis of a known weight of ester in 0.1 N NaOH, compared with the theoretical amount of nitrophenol or phenol from the same weight of pure ester.

Benzoylglycine (Eastman) and acetylglycine (Cyclo) were used without further purification. *p*-Nitrobenzyloxycarbonylglycine was prepared from *p*-nitrobenzyloxycarbonyl chloride (Nutritional Biochemical Corp.) and glycine, mp 123° (Greenstein and Winitz, 1961) and 122–124°, respectively. *N*-Cinnamoylglycine was synthesized as follows. To 15 mmol of glycine methyl ester hydrochloride in 50 ml of cold chloroform was added 15 mmol of triethylamine. Cinnamoyl chloride (Eastman), 15 mmol in 10 ml of chloroform, was added in three lots with equal portions of triethylamine (15 mmol) over a period of 30 min. The reaction mixture was washed successively with 3 × 50 ml portions of H_2O, 0.5 N HCl, H_2O, saturated $NaHCO_3$, and H_2O in a countercurrent fashion and dried over $MgSO_4$. The chloroform was removed on a rotary evaporator to yield an oily residue. Crystalline *N*-cinnamoylglycine methyl ester was obtained from methanol–water. *N*-Cin-

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

TABLE II: Kinetic Constants for the Proteinase-, Papain-, and Ficin-Catalyzed Hydrolyses of α-*N*-Benzyloxycarbonyl-L-lysine Esters.[a]

α-*N*-Z-L-Lys Ester	k_{cat} (sec^{-1})	K_m ($\times 10^4$ M)	k_{cat}/K_m (M^{-1} sec^{-1} $\times 10^{-5}$)
	Streptococcal Proteinase		
p-Nitrophenyl[b]	199 ± 27.0	3.83 ± 0.66	5.2
Phenyl[c]	187 ± 3.53	8.92 ± 0.29	2.1
	Papain		
p-Nitrophenyl[d]	37.3 ± 1.8	0.026 ± 0.008	143.5
e	37.7	0.0316	120.0
Phenyl[f]	38.4 ± 1.62	0.155 ± 0.004	33.4
	Ficin		
p-Nitrophenyl[g]	35.0 ± 1.2	0.088	39.8

[a] Kinetic constants determined in acetate buffer, pH 5.5, $I = 0.2$, 3.17% v/v acetonitrile, at 25.0 ± 0.1°. [b] $[S]_0 = 0.42$–1.67×10^{-4} M; $[E]_0 = 0.81 \times 10^{-8}$ N. [c] $[S]_0 = 2.3$–9.18×10^{-4} M; $[E]_0 = 1.53 \times 10^{-8}$ N. [d] $[S]_0 = 1.11$–4.43×10^{-5} M; $[E]_0 = 0.993 \times 10^{-8}$ N. [e] Kinetic constants reported by Fink and Bender (1969); determined in phosphate buffer, pH 7.0, $I = 0.5$, 10% v/v acetonitrile. [f] $[S]_0 = 0.57$–2.3×10^{-4} M; $[E]_0 = 2.0 \times 10^{-8}$ N. [g] Determined in 0.1 M phosphate buffer, pH 6.0, 3.17% v/v acetonitrile (Kortt, 1969).

TABLE III: Effect of Methanol on the Proteinase-Catalyzed Hydrolyses of *N*-Benzyloxycarbonyl-L-alanine Esters.[a]

		p-Nitrophenyl Ester[b]			
[Methanol] (M)	0	0.5	1.0	1.5	2.0
k_{cat} (sec^{-1})	36.4 ± 0.8	44.2 ± 1.43	50.1 ± 2.7	58.1 ± 3.5	63.9 ± 5.1
K_m ($\times 10^4$ M)	0.94 ± 0.05	1.09 ± 0.07	0.94 ± 0.12	1.10 ± 0.14	1.03 ± 0.16
k_{cat}/K_m (M^{-1} sec^{-1} $\times 10^{-5}$)	3.85	3.93	5.31	5.28	6.21
		o-Nitrophenyl Ester[c]			
[Methanol] (M)	0	0.5	1.0	1.5	2.0
k_{cat} (sec^{-1})	10.8 ± 1.12	12.7 ± 0.71	15.6 ± 0.91	17.4 ± 3.1	20.3 ± 3.0
K_m ($\times 10^4$ M)	4.38 ± 0.63	4.60 ± 0.35	5.05 ± 0.38	4.82 ± 1.15	4.96 ± 0.96
k_{cat}/K_m (M^{-1} sec^{-1} $\times 10^{-5}$)	2.45	2.77	3.10	3.62	4.10
d	2.14	2.59	3.02	3.58	3.96
		Phenyl Ester[e]			
[Methanol] (M)	0	0.5	1.0	1.5	2.0
k_{cat} (sec^{-1})	27.4 ± 1.19	38.6 ± 2.8	50.4 ± 3.19	64.2 ± 5.44	75.8 ± 8.7
K_m ($\times 10^4$ M)	3.14 ± 0.23	4.08 ± 0.48	4.97 ± 0.47	5.47 ± 0.66	6.02 ± 0.99
k_{cat}/K_m (M^{-1} sec^{-1} $\times 10^{-5}$)	8.74	9.46	10.1	11.73	12.57
f	9.02	10.1	12.5		

[a] Kinetic constants determined in phosphate buffer, $I = 0.2$, pH 6.13, containing acetonitrile + methanol to 10% v/v; at 25.0 ± 1°. [b] $[S]_0 = 0.75$–1.87×10^{-4} M; $[E]_0 = 1.39 \times 10^{-8}$ N. [c] $[S]_0 = 0.86$–2.15×10^{-4} N; $[E]_0 = 1.04 \times 10^{-7}$ N. [d] Determined under second-order conditions ($[S]_0 \ll K_m$); $[S]_0 = 6.38 \times 10^{-5}$ M; $[E]_0 = 2.06 \times 10^{-7}$ N. [e] $[S]_0 = 1.33$–3.33×10^{-4} M; $[E]_0 = 1.10 \times 10^{-7}$ N. [f] As for footnote *d*; $[S]_0 = 8.31 \times 10^{-5}$ M; $[E]_0 = 2.18 \times 10^{-7}$ N.

TABLE IV: Effect of Methanol on the Proteinase-Catalyzed Hydrolyses of *p*-Nitrophenyl α-*N*-Z-L-Lysinate and *N*-Z-Glycinate.[a]

		α-*N*-Z-L-Lys-*p*-Nph[b]			
[Methanol] (M)	0	0.5	1.0	1.5	2.0
k_{cat} (sec^{-1})	41.5 ± 2.8	46.75 ± 2.5	53.7 ± 1.8	62.8 ± 7.8	70.5 ± 3.6
K_m ($\times 10^4$ M)	1.97 ± 0.23	2.06 ± 0.24	2.06 ± 0.12	2.17 ± 0.43	2.29 ± 0.17
k_{cat}/K_m (M^{-1} sec^{-1} $\times 10^{-5}$)	2.08	2.27	2.60	2.89	3.08
		N-Z-Gly-*p*-Nph[c]			
[Methanol] (M)	0	0.5	1.0	1.5	2.0
k_{cat} (sec^{-1})	2.40 ± 0.09	3.06 ± 0.13	4.18 ± 0.16	5.16 ± 0.36	6.43 ± 0.50
K_m ($\times 10^4$ M)	0.89 ± 0.06	1.05 ± 0.07	1.42 ± 0.14	1.52 ± 0.14	1.91 ± 0.19
k_{cat}/K_m (M^{-1} sec^{-1} $\times 10^{-5}$)	2.71	2.92	3.16	3.40	3.37

[a] Kinetic constants determined in phosphate buffer, $I = 0.2$, pH 6.13, containing acetonitrile + methanol to 10% v/v, at 25.0 ± 1°. [b] $[S]_0 = 0.56$–1.88×10^{-4} M; $[E]_0 = 0.63 \times 10^{-8}$ N. [c] $[S]_0 = 2.27$–7.56×10^{-5} M; $[E]_0 = 2.05 \times 10^{-7}$ N.

namoylglycine methyl ester, mp 80–82° (*Anal.* Calcd for $C_{12}H_{13}NO_3$: C, 65.74; H, 5.98; N, 6.39. Found: C, 65.30; H, 6.13; N, 6.16), was saponified to yield *N*-cinnamoylglycine, mp 194–196° (*Anal.* Calcd for $C_{11}H_{11}NO_3$: C, 64.38; H, 5.40; N, 6.83. Found: C, 64.39; H, 5.69; N, 6.61).

Buffers were prepared using analytical grade reagents. Buffers containing organic solvent were prepared by mixing the appropriate volume of solvent with a suitable volume of buffer. Potassium chloride was added to maintain constant ionic strength. Buffers containing methanol were prepared with the methanol plus acetonitrile concentrations = 10% v/v, *i.e.*, 50 M water. pH measurements were made using a Radiometer pH Meter 4, standardized according to Bates (1964), and are accurate to 0.01 pH unit. Concentrations of 10% v/v acetonitrile (and CH_3CN plus CH_3OH) resulted in an increase of 0.23 pH unit in the measured pH of the buffer. Concentrations of 20% v/v acetonitrile resulted in an increase of 0.46 pH unit.

Stock solutions of all esters were prepared in acetonitrile except α-*N*-Z-L-Lys-pNph and α-*N*-Z-L-Lys-Ph which were made up in 10% v/v H_2O–acetonitrile. Acetonitrile (Matheson Coleman and Bell) and methanol (reagent grade from Mallinckrodt) were used without further purification. All other materials were analytical reagent grade.

Streptococcal proteinase (herein referred to as proteinase) was obtained as the zymogen from Dr. Stuart Elliott of the Rockefeller University. The enzyme was prepared from the zymogen either by tryptic digestion and reduction or by autodigestion (Liu and Elliott, 1965). For kinetic studies, the enzyme was passed through sulfoethyl Sephadex C-25 (Liu *et al.*, 1963) and stored as the tetrathionate-inhibited enzyme. Papain (twice crystallized) was obtained from Worthington

TABLE V: Effect of Methanol on the Papain-Catalyzed Hydrolyses of Some *N*-Benzyloxycarbonyl-L-amino Acid Esters.[a]

[Methanol] (M)		0	0.5	1.0	1.5	2.0
α-*N*-Z-L-Lys-pNph[b]	k_{cat} (sec^{-1})	37.6	41.7	43.8	46.7	48.6
	c	37.7	39.9	40.7	40.6	43.5
α-*N*-Z-L-Lys-Ph[d]	k_{cat} (sec^{-1})	38.4	42.5	43.5	48.1	49.6
N-Z-L-Ala-pNph[e]	k_{cat} (sec^{-1})	23.4	26.6	27.6	29.0	32.4
N-Z-L-Ala-*o*-Nph[f]	k_{cat}/K_m (M^{-1} sec^{-1} × 10^{-5})	4.91	4.78	4.93	4.96	
N-Z-L-Ala-Ph[g]	k_{cat}/K_m (M^{-1} sec^{-1} × 10^{-5})	0.285	0.279	0.288	0.280	0.265

[a] Kinetic constants determined in phosphate buffer, I = 0.2, pH 6.13, containing acetonitrile + methanol to 10% v/v; at 25.0 ± 0.1°. k_{cat} values determined under zero-order conditions ($[S]_0 \gg K_m$) and k_{cat}/K_m values determined under second-order conditions ($[S]_0 \ll K_m$). [b] $[S]_0 = 1.88 \times 10^{-4}$; $[E]_0 = 4.53 \times 10^{-8}$ N. [c] Values for k_{cat} reported by Fink and Bender (1969). [d] $[S]_0$ 2.3×10^{-4} M; $[E]_0 = 7.37 \times 10^{-8}$ N. [e] $[S]_0 = 1.87 \times 10^{-4}$ M; $[E]_0 = 4.53 \times 10^{-8}$ N. [f] $[S]_0 = 6.38 \times 10^{-5}$ M; $[E]_0 = 1.34 \times 10^{-7}$ N. [g] $[S]_0 = 8.31 \times 10^{-5}$ M; $[E]_0 = 4.22 \times 10^{-8}$ N.

TABLE VI: Kinetic Constants for the Proteinase-Catalyzed Hydrolyses of Some *N*-Benzyloxycarbonyl-L-amino Acid Esters.[a]

Ester	Concn (×10^4 M)	k_{cat} (sec^{-1})	K_m (×10^4 M)	k_{cat}/K_m (M^{-1} sec^{-1} × 10^{-5})	$\frac{k_{cat}/K_m}{k_{cat\ Gly}/K_{m\ Gly}}$
	(i) In Acetate Buffer, I = 0.2, pH 5.5, 3.17% v/v CH_3CN				
N-Z-Gly-pNph[b]	0.189–0.758	9.4 ± 0.91	2.36 ± 0.28	0.398	1.00
N-Z-L-Ala-pNph[c]	0.348–1.160	118 ± 2.4	1.35 ± 0.09	8.74	21.96
α-*N*-Z-L-Lys-pNph[d]	0.559–1.863	199 ± 27.0	3.83 ± 0.66	5.19	13.04
N-Z-L-Ala-Ph[e]	0.269–1.15	82.5 ± 5.1	3.64 ± 0.3	2.26	
α-*N*-Z-L-Lys-Ph[f]	2.29–9.18	187 ± 3.5	8.92 ± 0.29	2.10	
N-Z-L-Glu-Ph[g]	4.75–11.73	57.5 ± 2.3	7.02 ± 0.61	0.82	
	(ii) In Acetate Buffer, I = 0.2, pH 5.99, 21.4% v/v CH_3CN				
N-Z-Gly-pNph[h]	0.295–1.104	1.9 ± 0.23	2.59 ± 0.43	0.73	1.00
N-Z-L-Ala-pNph[i]	0.52–2.08	14.9 ± 2.1	0.87 ± 0.03	17.12	23.60
N-Z-L-Val-pNph[j]	0.297–1.19	0.78 ± 0.01	0.31 ± 0.02	2.52	3.45
N-Z-L-Ile-pNph[k]	0.274–1.09	0.34 ± 0.01	0.24 ± 0.03	1.42	1.93
N-Z-L-Leu-pNph[l]	0.273–1.09	2.25 ± 0.17	0.84 ± 0.12	2.68	3.67
N-Z-L-Asn-pNph[m]	0.34–1.36	9.25 ± 1.57	2.19 ± 0.53	4.22	5.78
N-Z-L-Lys-pNph[n]	0.56–1.86	29.4 ± 8.9	2.83 ± 0.13	10.43	14.30
N-Z-L-Phe-pNph[o]	0.17–0.676	19.4 ± 8.4	4.03 ± 2.00	4.81	6.60

[a] Kinetic constants determined under conditions noted at 25.0 ± 0.1°. Enzyme concentrations were (×10^{-8} N). [b] 12.1. [c] 1.62. [d] 1.06. [e] 5.28. [f] 1.53. [g] 2.44. [h] 4.83. [i] 2.38. [j] 10.9. [k] 42.7. [l] 7.9. [m] 7.15. [n] 3.08. [o] 3.32.

Biochemical Corp. as a concentrated suspension (~24 mg/ml, lot no. Pap 9DA).

Methods. Rate measurements were made at 25.0 ± 0.1° with a Cary-15 recording spectrophotometer equipped with a 0–0.1 and 0–1.0 absorbance slide-wire and a thermostated cell compartment. Wavelengths used were: phenyl esters, 270 nm (Δε 1500); *p*-nitrophenyl esters, 317 nm or 340 nm below pH 7.0, 400 nm above pH 7.0; *o*-nitrophenyl esters, 352 nm (<pH 7.0) and 417 nm (>pH 7.0); *m*-nitrophenyl esters, 330 nm (<pH 7.0) and 393 nm (>pH 7.0). Reactions were generally performed as follows. The substrate (100, 75, 50, 40, 30, or 25 μl) in acetonitrile plus an aliquot of acetonitrile to make a final volume of 100 μl in acetonitrile was added to 3.0 ml of buffer solution in a 1-cm cell; spontaneous hydrolysis, if any, was observed for several seconds, and then a suitable volume (usually 50 μl) of proteinase was added (see Tables I–VII for $[S]_0$ and $[E]_0$). The enzyme was added on a glass stirring rod to achieve rapid mixing and recording was commenced 4–5 sec after the addition of the enzyme.

The steady-state parameters k_{cat} ($= V/E_0$) and K_m were determined from initial velocity measurements at the varying substrate concentrations from the least-mean-squares plots of $1/v$ *vs.* $1/S$. Calculations were carried out using the CDC 6600 computer according to the method of Hanson *et al.* (1967) as modified by K. Thompson of this department. When required, correction for spontaneous hydrolysis of the substrate was applied. No nonrandom deviation from Michaelis–Menten kinetics were observed. For each reciprocal plot, the reaction at two or more substrate concentrations was followed to completion to determine the Δε value. For a number of esters the condition $[S]_0 > K_m$ was not attainable due to limiting solubilities in water and high K_m values. Under conditions of

TABLE VII: Kinetic Constants for the Proteinase- and Papain-Catalyzed Hydrolyses of *p*-Nitrophenyl *N*-Acylglycinates and *N*-Acyl-L-alaninates.[a]

	Proteinase			Papain		
N-Acyl Group	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} $\times 10^{-5}$)
			N-Acyl-Gly-pNph			
Acetyl		Not hydrolyzed		1.16[b]	4.35	0.027
Benzoyl[c]	0.21 ± 0.024	4.22 ± 0.55	0.005	6.23[b]	0.134	4.65
Benzyloxycarbonyl[d]	9.4 ± 0.91	2.36 ± 0.28	0.398	6.81 ± 0.11[e]	0.11 ± 0.012	6.19
p-Nitrobenzyloxycarbonyl[f]	1.15 ± 0.12	0.63 ± 0.09	0.18	8.31 ± 0.12[g]	0.12 ± 0.005	6.81
tert-Butyloxycarbonyl[h]	0.147 ± 0.011	4.38 ± 0.39	0.0034	10.96 ± 0.31[i]	0.85 ± 0.008	1.30
Cinnamoyl[j]	1.52 ± 0.45	3.34 ± 1.07	0.046	6.65 ± 0.08[k]	0.019 ± 0.002	35
Benzyloxycarbonylglycine[l]	~0.06	~13.3		17.54 ± 1.79[m]	3.19 ± 0.47	5.50
			N-Acyl-L-Ala-pNph			
Benzyloxycarbonyl[n]	118 ± 2.4	1.32 ± 0.04	9.0	27.3 ± 0.08[o]	0.065 ± 0.003	42
tert-Butyloxycarbonyl[p]	4.31 ± 0.61	6.1 ± 0.98	0.07	26.1 ± 0.42[q]	0.25 ± 0.015	10.5

[a] Kinetic constants determined in acetate buffer, pH 5.5, $I = 0.2$; 3.17% v/v acetonitrile; at 25.0 ± 0.1°. [b] In 0.1 M phosphate buffer, pH 6.0, 3.17% v/v acetonitrile (Kortt, 1969). [c] $[S]_0 = 0.38$–0.95×10^{-4} M; $[E]_0 = 9.05 \times 10^{-7}$ N. [d] $[S]_0 = 0.19$–0.758×10^{-4} M; $[E]_0 = 1.21 \times 10^{-7}$ N. [e] $[S]_0 = 0.23$–0.756×10^{-4} M; $[E]_0 = 7.86 \times 10^{-8}$ N; deJersey (1970) reported a k_{cat} of 5.94 sec^{-1}. [f] $[S]_0 = 0.133$–0.33×10^{-4} M; $[E]_0 = 9.05 \times 10^{-7}$ N. [g] $[S]_0 = 0.133$–0.33×10^{-4} M; $[E]_0 = 7.86 \times 10^{-8}$ N. [h] $[S]_0 = 0.42$–1.06×10^{-4} M; $[E]_0 = 9.05 \times 10^{-7}$ N. [i] $[S]_0 = 0.32$–1.06×10^{-4} M; $[E]_0 = 7.86 \times 10^{-8}$ N. [j] $[S]_0 = 0.136$–0.34×10^{-4} M; $[E]_0 = 9.05 \times 10^{-7}$ N. [k] $[S]_0 = 0.1$–0.338×10^{-4} M; $[E]_0 = 8.48 \times 10^{-8}$ N. [l] Determined in acetate buffer, pH 5.99, $I = 0.2$, 21.4% v/v acetonitrile. $[S]_0 = 0.57$–1.95×10^{-4} M; $[E]_0 = 1.56 \times 10^{-6}$ N. [m] In acetate buffer, pH 5.99, $I = 0.2$, 21.4% v/v acetonitrile, $[S]_0 = 0.61$–2.05×10^{-4} M; $[E]_0 = 0.97 \times 10^{-7}$ N. [n,o] As for Table II. [p] $[S]_0 = 0.36$–1.19×10^{-4} M; $[E]_0 = 3.07 \times 10^{-7}$ N. [q] $[S]_0 = 0.30$–1.21×10^{-4} M; $[E]_0 = 2.93 \times 10^{-8}$ N.

$K_m \gg [S]_0$, good first-order kinetics were observed and values of k_{cat}/K_m ($k_{obsd}/[E]_p$) were calculated from the apparent first-order rate constants (k_{obsd}).

Tetrathionate-inhibited proteinase (Liu, 1967) in 0.04 M NaCl–0.001 M EDTA was activated (and diluted if required) in 0.1 M phosphate buffer, pH 7.0, containing 10^{-3} M dithioerythreitol and 10^{-3} M EDTA. The dithioerythreitol was removed by dialysis against oxygen-free 0.04 M NaCl–0.001 M EDTA. The same enzyme activity was observed before and after dialysis. The reduced activator-free proteinase was stored under N_2. Proteinase solutions were routinely assayed using *N*-Z-Gly-pNph as substrate in acetate buffer, pH 5.5, $I = 0.2$. The release of *p*-nitrophenol was measured at 317 mμ on the 0–0.1 absorbance scale, with an initial substrate concentration of 7.58×10^{-5} M. The normality of proteinase solutions (*i.e.*, the concentration of reduced SH groups) was determined by titration with DTNB as described by Ellman (1959). The DTNB titration has been shown to give an accurate measure of the concentration of reduced –SH groups of proteinase solutions and the –SH group concentration is proportional to the enzyme activity (Kortt and Liu, 1973a). Correlation of the assay of proteinase using N-Z-Gly-pNph as substrate and the titration of sulfhydryl group concentration with DTNB affords a method for calculating the actual enzyme concentration (the normality) used in each set of kinetic runs (assuming that the single essential cysteine sulfhydryl group of proteinase participates in catalysis). Proteinase solutions were found to be between 30 and 56% active enzyme, depending on the batch preparation, based on a molecular weight of 32,000 and on ϵ_{280} nm of 52,448 (Liu *et al.*, 1963).

Solutions of papain were prepared by dilution of the papain suspension as described for proteinase, followed by dialysis against 0.04 M NaCl–0.001 M EDTA under N_2 to remove the activator. Papain solutions were routinely assayed using *N*-Z-Gly-pNph as substrate (7.58×10^{-5} M) using the same conditions as for proteinase. The concentration of reduced –SH groups of papain solutions was determined by titration with DTNB, and as for proteinase, correlation of the rate assay and the DTNB titration of the sulfhydryl groups enabled the calculation of the actual enzyme concentration used in each set of kinetic runs. The value of k_{cat} for α-*N*-Z-L-Lys-pNph was 37.3 ± 1.8 sec^{-1} with papain concentration determined by DTNB titration. Fink and Bender (1969) report a k_{cat} of 37.7 sec^{-1}. These authors determined papain concentration from the rate assay of α-*N*-Bz-L-Arg-Et which has been correlated to the normality of papain determined by titration with *N*-Z-L-Tyr-pNph at low pH (Bender *et al.*, 1966). The Worthington twice crystallized papain was found to be 51.8% active enzyme based on a molecular weight of 23,000 (Drenth *et al.*, 1968) and an ϵ_{280} nm of 56,600 (Glazer and Smith, 1961). Thus the titration of the sulfhydryl group, with DTNB, of the sulfhydryl proteinase provides a rapid and convenient method for the estimation of the enzyme normality. The alkaline rate constants for the series of *N*-Z-L-alanine esters were determined in 0.001 N NaOH. The alkaline rate constant for *p*-nitrophenyl acetate was determined at the same time under the same conditions and the observed rate constants were then corrected by using a value of 12.6 M^{-1} sec^{-1} for the alkaline rate constant of *p*-nitrophenyl acetate (K. A. Conners, 1961, quoted by deJersey *et al.*, 1969).

Results

Hydrolysis of N-Z-L-Ala and α-N-Z-L-Lys Esters. To furnish kinetic evidence for an acyl-enzyme intermediate and the scheme shown in eq 1, proteinase-catalyzed rates of hydrolyses of a series of nitrophenyl *N*-Z-L-alaninates (Table II) and α-*N*-Z-L-lysinates (Table II) were measured to determine the effect of the reactivity of the leaving group on k_{cat} and K_m. To compare proteinase with other sulfhydryl proteinases, the rates of papain-catalyzed hydrolyses of the same series of esters were determined and the data, together with available data for ficin, are included in Tables I and II. For *N*-Z-L-Ala-pNph and *N*-Z-L-Ala-mNph the maximum substrate concentrations were $\simeq K_m$, but for *N*-Z-L-Ala-oNph and *N*-Z-L-Ala-Ph the maximum substrate concentrations were 6.15- and 3.17-fold lower than K_m, respectively. These conditions lead to a large uncertainty in the measurement of initial velocities from the experimentally obtained rate curves as little linearity is observed, even using the 0–0.1 absorbance slide-wire of the spectrophotometer. To check on the accuracy of k_{cat} and K_m determined by initial velocity measurements, k_{cat}/K_m ($= k_{obsd}[E]_0$) was determined from the apparent first-order constant, k_{obsd}, determined under second-order conditions ($K_m \gg [S]_0$). The k_{cat}/K_m values determined by both methods were in excellent agreement (Table I).

The finding that the k_{cat} for proteinase-catalyzed hydrolysis of *N*-Z-L-Ala-*o*-Nph was significantly lower than the k_{cat} values observed for the other *N*-Z-L-alaninate esters in this series, and that the k_{cat} did not reflect the reactivity of this ester (as measured by the alkaline rate constant, k_{OH^-}) prompted the investigation into the effect of adding a nucleophile, in this case methanol, on proteinase-catalyzed hydrolyses.

Effect of Methanol. The effect of methanol on the kinetic constants of proteinase-catalyzed hydrolysis of the series of nitrophenyl Z-L-alaninates (Table III), and of *p*-nitrophenyl α-*N*-Z-L-lysinate and *p*-nitrophenyl Z-glycinate (Table IV) was determined. The effect of methanol on the k_{cat} of the papain-catalyzed hydrolyses of *N*-Z-L-Ala-pNph, α-*N*-Z-L-Lys-pNph, and α-*N*-Z-Lys-Ph ($[S]_0 \gg K_m$) and the k_{cat}/K_m of papain-catalyzed hydrolysis of Z-L-Ala-oNph and Z-L-Ala-Ph ($K_m \gg [S]_0$) was determined (Table V) to provide a comparison for proteinase. High concentrations of organic solvents affect the solvent properties and thus may lead to secondary solvent effects (Clement and Bender, 1964). Studies on the effect of an inert organic solvent, such as acetonitrile, have shown that proteinase-catalyzed hydrolyses are sensitive to even low concentrations of this organic solvent. For acetonitrile, a mixed type of inhibition was observed; k_{cat} was significantly lowered with only a small effect on the K_m (Kortt and Liu, 1973b). To minimize secondary solvent effects, the concentration of acetonitrile plus methanol was kept constant to a concentration of 10% v/v, as the dielectric constant of 10% v/v acetonitrile is similar to that of 2.0 M methanol, the highest concentration of methanol used. The addition of methanol increased k_{cat} for the proteinase-catalyzed rates of all the esters studied; the K_m values increased for *N*-Z-L-Ala-oNph, *N*-Z-L-Ala-Ph, and Z-Gly-pNph with only a small increase in K_m for *N*-Z-L-Ala-pNph and α-*N*-Z-L-Lys-pNph. In all cases, the ratio k_{cat}/K_m was observed to increase. The effect of methanol for the papain-catalyzed rates of these esters was in agreement with the work of Fink and Bender (1969).

Specificity toward the Amino Acid Side-Chain Group. The effect of the side-chain group of the substrate on k_{cat} and K_m of proteinase-catalyzed hydrolyses was examined. Amino acid esters with the benzyloxycarbonyl group as the *N*-acyl group and the *p*-nitrophenyl leaving group were used. As the *p*-nitrophenyl ester of α-*N*-Z-L-glutamic acid was not available, the phenyl ester was used and compared with the phenyl esters of *N*-Z-L-alanine and α-*N*-Z-L-lysine. The kinetic constants for the proteinase-catalyzed rates of hydrolyses of the esters examined are given in Table VI. The low solubility of a number of esters in water made it necessary to use 20% v/v acetonitrile buffers. As the acetonitrile has a marked effect on the k_{cat} and K_m values of proteinase-catalyzed rates of hydrolyses (Kortt and Liu, 1973b), only results obtained under the same experimental conditions were compared.

The kinetic constants for the papain-catalyzed hydrolyses of *p*-nitrophenyl *N*-Z-L-phenylalaninate and phenyl α-*N*-Z-L-glutamate were also determined. For *N*-Z-L-Phe-pNph, $k_{cat} = 4.05 \pm 0.49$ sec^{-1} and $K_m = 0.086 \pm 0.006 \times 10^{-4}$ M, and the conditions were acetate buffer, pH 5.99, $I = 0.2$, 21.4% v/v acetonitrile, $[S]_0$ range 0.201–0.67 $\times 10^{-4}$ M, $[E]_0 = 6.195 \times 10^{-8}$ N; for α-*N*-Z-L-Glu-Ph, $k_{cat} = 25.7 \pm 3.3$ sec^{-1} and $K_m = 1.19 \pm 0.09 \times 10^{-4}$ M, and the conditions were acetate buffer, pH 5.50, $I = 0.2$, 3.17% v/v acetonitrile, $[S]_0$ range $2.96–11.83 \times 10^{-4}$ M $[E]_0 = 6.195 \times 10^{-8}$ N.

Specificity toward the N-Acyl Group. The effect of the structure of the *N*-acyl group of the substrate on the proteinase-catalyzed rates of hydrolyses was determined using a series of *p*-nitrophenyl *N*-acylglycinates (Table VII). The kinetic constants for papain-catalyzed hydrolyses of this series of esters were also determined and are presented in Table VII.

Discussion

Acyl-Enzyme Intermediate. To obtain evidence for an acyl-enzyme intermediate in streptococcal proteinase-catalyzed hydrolyses, the rates of hydrolyses of a series of activated *N*-Z-L-alanine and α-*N*-Z-L-lysine esters were determined. The kinetic consequences of the scheme shown in eq 1 predict two limiting cases: (1) if $k_2 \gg k_3$, *i.e.*, rate-limiting deacylation, then $k_{cat} = k_3$ and $K_m = k_3/k_2K_s$; and (2) if $k_2 \ll k_3$, *i.e.*, rate-limiting acylation, then $k_{cat} = k_2$ and $K_m = K_s$. Thus, for a series of esters with the same *N*-acylamino acid moiety but different leaving groups, if deacylation is rate limiting, k_{cat} ($= k_3$) describes the breakdown of a common acylenzyme intermediate and it should be constant. The independence of k_{cat} on the leaving group provides evidence for a common intermediate and the proposed scheme of eq 1. If acylation is rate limiting, k_{cat} ($= k_2$) describes the formation of the acyl-enzyme intermediate and reflects the reactivities of the esters in the series. The finding that the k_{cat} values for the pNph, mNph, and Ph of *N*-Z-L-Ala (Table I), and for the pNph and Ph of α-*N*-Z-L-Lys (Table II) are nearly independent of the leaving group is consistent with a common rate-determining step not involving the alcohol leaving group of the substrate. However, the k_{cat} value for *N*-Z-L-Ala-oNph is significantly lower than that for the other *N*-Z-L-Ala esters. This result suggests that the *o*-nitrophenyl leaving group may bind at the active site of the enzyme in such a way as to influence the rate-determining step.

In the case of papain (Table II), the k_{cat} values for the rates of hydrolysis of the *N*-Z-L-Ala esters are constant. This finding is consistent with a number of previous studies (Lowe and Williams, 1965b; Kirsch and Igelström, 1966; Bender and Brubacher, 1966) indicating that for the papain-catalyzed hydrolyses of activated *N*-acylamino acid esters deacylation is rate limiting.

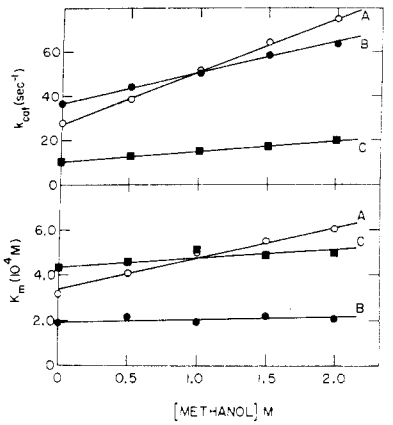

FIGURE 1: The effect of methanol on k_{cat} and K_m for the proteinase-catalyzed hydrolyses of *N*-Z-L-Ala esters: A, Ph, (○); B, pNph (●); C, oNph (■). The K_m values of B are one-half of that shown. Experimental conditions as for Table IV.

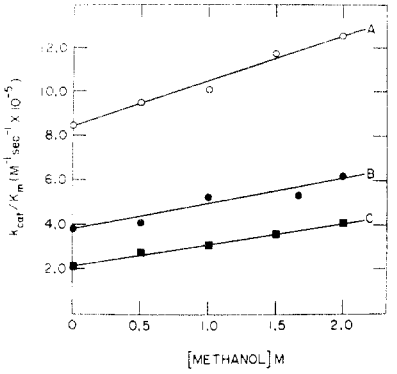

FIGURE 2: The effect of methanol on k_{cat}/K_m for the proteinase-catalyzed hydrolyses of *N*-Z-L-Ala esters: A, Ph (○); B, p-Nph (●); C, oNph (□). Experimental conditions as for Table IV.

To provide further evidence for the validity of the three-step scheme of eq 1 for the proteinase-catalyzed hydrolyses of ester substrates, the effect of an added nucleophile was investigated. The effect of an added nucleophile, such as methanol, on the acyl-enzyme mechanism (eq 1) will be to partition the breakdown of the acyl-enzyme intermediate, and this is shown in terms of a minimal scheme in eq 5, where ES′ is the acyl-

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \begin{cases} \xrightarrow{k_3[W]} E + P_2 \\ + \xrightarrow[P_1]{k_4[N]} E + P_3 \end{cases} \quad (5)$$

enzyme, k_3[W] and k_4[N] are the first-order rate constants for the breakdown of ES′ in the presence of water and nucleophile, respectively, and P_2 and P_3 are the products resulting from the attack on the acyl-enzyme by water and nucleophiles, respectively. This scheme predicts that if deacylation is rate limiting, k_{cat} (= k_3) and K_m (= k_3/k_2K_s) increase with increasing [N], while the k_{cat}/K_m (= k_2/K_s) ratio remains constant; if acylation is rate limiting, the addition of the nucleophile will not affect the kinetic constants provided that there are no secondary effects such as binding of the nucleophile to the active site of the enzyme.

The effect of methanol on the proteinase-catalyzed hydrolyses of a number of *N*-Z-L-amino acid esters was determined under turnover conditions. The addition of methanol causes an increase in both k_{cat} and K_m of all the esters examined as predicted by eq 5 (Tables III and IV; Figure 1). By using a fixed concentration of organic solvent, the concentration of water was kept constant to 50 M, thus eliminating effects caused by varying concentrations of water, and minimizing secondary effects which may be caused by the solvent. As the changes in the dielectric constant of the medium were kept to a minimum, the observed effect of methanol cannot be caused by changes in the dielectric constant. These data together with the results discussed (Tables I and II) support the acyl-enzyme mechanism of eq 1 and 5, and are not consistent with a simple one-step scheme which would require K_m to be independent of [N]. It is reasonable to assume that for the proteinase-catalyzed hydrolyses of *p*-nitrophenyl esters of *N*-Z-L-Ala, α-*N*-Z-L-Lys and Z-Gly deacylation is rate limiting (*i.e.*, $k_2 \gg k_3$) (see Tables I and II) in the absence of methanol. As the concentration of added methanol is increased, the rate of deacylation will increase (provided the nucleophile participates in the deacylation) until it exceeds that of acylation, when the rate will become independent of [N], since acylation will now be rate limiting. The results for the addition of methanol up to 2.0 M show an increase in the values of k_{cat} to about twofold over that of k_3[W] without becoming independent of the methanol concentration which indicates that $k_2 \gg k_3$, and provides confirmatory evidence that deacylation is indeed rate limiting for these substrates.

While the addition of methanol increases the k_{cat} and K_m values for the three *N*-Z-L-Ala esters (Table III), the rate of increase of k_{cat} with [methanol] for each ester is not the same as shown by the different slopes obtained for their k_{cat} *vs.* [methanol] plots (Figure 1). If the added methanol acts solely as a nucleophile in the rate-determining deacylation of a common intermediate, the slopes of the lines in Figure 1 should be independent of the leaving group. Such a relationship was demonstrated for papain-catalyzed hydrolyses of α-*N*-Z-L-Lys-pNph and Ph (Figure 3) under the present experimental conditions. The observation that the rate of increase of k_{cat} with increasing methanol concentration is dependent on the leaving group of the substrate for the proteinase-catalyzed hydrolyses of the *N*-Z-L-Ala esters studied suggests that the leaving group binds at the active site of the enzyme, and implies that the added nucleophile must compete with and displace the leaving group from the active site in order to participate in the deacylation. The observation that k_{cat}/K_m (= k_2/K_s) was dependent upon the concentration of methanol (Figure 2) cannot be explained solely by the participation of the nucleophile in deacylation and the minimal scheme of eq 5, but requires the interaction of the nucleophile with enzyme or the enzyme–substrate complex prior to acyl-enzyme formation. This in turn suggests the existence of a ternary enzyme–substrate–nucleophile complex, and provides evidence that a simple molecule such as methanol can bind at the active site of proteinase. The effect of methanol on the k_{cat}/K_m ratio implies that the bound nucleophile (methanol) causes a change in the acylation reaction and/or the dissociation constant of the enzyme–substrate complex. These results indicate that the leaving groups of *N*-Z-L-amino acid esters and simple nucleophiles, such as methanol, bind at the active site of proteinase, probably at the same site, and this binding has a profound effect on the deacylation and acylation, and also on the binding constant, K_s. Extension of the present work to a study of the effect of leaving groups and added

nucleophiles of a wider diversity of structure should provide further information as to the structural requirements of this binding site, and help to elucidate the mechanism of action of proteinase-catalyzed hydrolyses of ester substrates.

The effect of methanol on papain-catalyzed hydrolyses was determined for several esters (Table V) in order to have a direct comparison with proteinase. For papain, the addition of methanol results in an increase in k_{cat} while the k_{cat}/K_m ratio remains constant as predicted by eq 5. The effect of methanol on papain-catalyzed hydrolysis of α-*N*-Z-Lys-pNph was in fair agreement with the results of Fink and Bender (1969). However, the slope of the k_{cat} *vs.* [methanol] plot for α-*N*-Z-L-Lys-pNph was greater than the slope obtained from the data of Fink and Bender (1969) (Figure 3), with no decrease observed in k_{cat} values at the higher concentrations of methanol. The slopes of k_{cat} *vs.* [methanol] plots for the papain-catalyzed hydrolyses of α-*N*-Z-L-Lys-pNph and -Ph, and *N*-Z-L-Ala-pNph are constant (Figure 3). This result is consistent with methanol acting solely as a nucleophile in the deacylation reaction of papain. Thus, in contrast to proteinase, for the papain-catalyzed hydrolyses of the substrates examined, the binding of the leaving group of *N*-Z-L-amino acid esters and simple nucleophiles, such as methanol, at the active site of papain is not significant enough to influence the deacylation or the acylation reaction and/or the binding constant, K_s.

Specificity of Proteinase. The kinetic parameters of proteinase-catalyzed hydrolysis of substrates of the type R_1-$NHCHR_2C(O)R_3$, where R_1 is the amino protecting group, R_2 is the side chain of the amino acid, and R_3 is the alcohol leaving group, are given in Tables I, II, VI, and VII. The specificity of proteinase toward *N*-acylamino acid esters was examined with respect to R_1, R_2, and R_3 to provide information on the structural requirements of the active-site region of this enzyme.

The Effect of R_3. The effect of variation of R_3 for a series of *N*-Z-L-Ala esters is shown in Table I. The k_{cat} values are quite similar, when R_3 = *p*-Nph, *m*-Nph, and Ph, consistent with a rate-limiting deacylation of a common intermediate (*vide supra*). In this case, the lower k_{cat} value, for R_3 = oNph, is not a reflection of the lower reactivity of this ester, as measured by the alkaline rate constant, k_{OH^-}, since the k_{OH^-} values for both the mNph and oNph are similar and both values are significantly higher than the k_{OH^-} value for the Ph. A probable explanation is that for substrates with a sterically hindered group, such as the *o*-nitrophenyl group, the breakdown of an acyl-enzyme leaving group tetrahedral intermediate is rate limiting rather than the deacylation of the acyl-enzyme.

It is instructive to compare the kinetic constants presented in Tables I and II for the three sulfhydryl proteinases. Firstly, the k_{cat} values (presumably k_3 for the *p*-nitrophenyl esters) are about fivefold higher for proteinase than for papain and ficin which have very similar kinetic constants. Thus, proteinase is a much more efficient esterase in the hydrolyses of activated *N*-Z-L-amino acid esters than papain and ficin. Secondly, the K_m values for proteinase are about 25-fold higher than the corresponding values for papain and ficin. The k_{cat}/K_m ratio for proteinase is lower than for papain and ficin. This indicates that while proteinase shows faster deacylation rates ($k_{cat} = k_3$), the acylation rates and/or binding or both, as measured by k_{cat}/K_m ($= k_2/K_s$), are less efficient for proteinase than for the other two sulfhydryl proteinases. Furthermore, it is surprising to find that as the turnover rate increases in going from *N*-Z-L-Ala-pNph to α-*N*-Z-L-Lys-pNph, the K_m increases (Tables I and II). The reverse is generally observed with other proteinases, *i.e.*, the better the substrate the higher the k_{cat} and the lower the K_m. The high K_m values for proteinase suggest that the mode of binding of the substrate at the active site is different from that of papain and that the nonproductive modes of binding may contribute to the observed K_m values. The similarity between the kinetic constants for papain and ficin implies that these enzymes hydrolyze ester substrates *via* very similar mechanistic pathways and probably have very similar active-site topographies. On the other hand, proteinase shows distinctly different kinetic parameters and hence is expected to have a somewhat different active-site topography as compared to the plant proteinases.

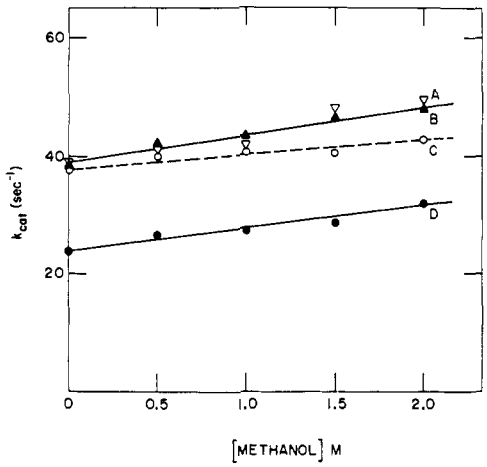

FIGURE 3: The effect of methanol on k_{cat} for the papain-catalyzed hydrolyses of: A, α-*N*-Z-L-Lys-Ph (▽); B, α-*N*-Z-L-Lys-pNph (▲); C, values of k_{cat} for α-*N*-Z-L-Lys-pNph reported by Fink and Bender (1969) (○); D, *N*-Z-L-Ala-pNph (●). Experimental conditions are given in Table VI.

The Effect of R_2. In Table VI, the relative reactivity (k_{cat}/K_m) of each ester substrate has been expressed with reference to Z-Gly-pNph as 1.0. Referring to this substrate as a basic structure, in which R_2 = –H, it can be seen that in terms of turnover rate ($k_{cat} = k_3$) the best substrate for proteinase is one in which R_2 has the side chain of lysine. The k_{cat} value is about 200 sec^{-1} which is nearly 20-fold higher than that of Z-Gly-pNph. The high reactivity of this lysine ester with proteinase does not appear to be solely due to the positive charge of the ε-amino group of the lysine side chain, since the esters of *N*-Z-L-Ala, *N*-Z-L-Asn, and *N*-Z-L-Phe are also excellent substrates with k_{cat} values about five- to tenfold higher than that of *N*-Z-Gly-pNph. Furthermore, the negatively charged ester *N*-Z-L-Glu-Ph is also a good substrate for the proteinase (Table VI). The notable resistance of substrates in which the R_2 is branched at the β-carbon (valyl, isoleucyl) suggests that steric hindrance is operative, and perhaps one of the catalytic groups of proteinase is prevented from attacking the carbonyl group of the ester bond.

For both papain and streptococcal proteinase, the k_{cat} values for *N*-Z-Gly-pNph are similar (Table VII), suggesting that, in the absence of a side-chain group, the deacylation reaction mechanism might be similar for the substrate. However, the k_{cat}/K_m ratio is 15.6-fold higher for papain than for proteinase. This implies that the acylation step and/or binding of this substrate are much more facile in papain than in proteinase. Introduction of a side-chain group into the substrate, *e.g.*, R_2 = $-CH_3$ or $-(CH_2)_4NH_3^+$, enhanced the turnover rate for both enzymes. The increase in k_{cat} values for proteinase in going from R_2 = –H to R_2 = $-CH_3$ is 12.5-fold, and

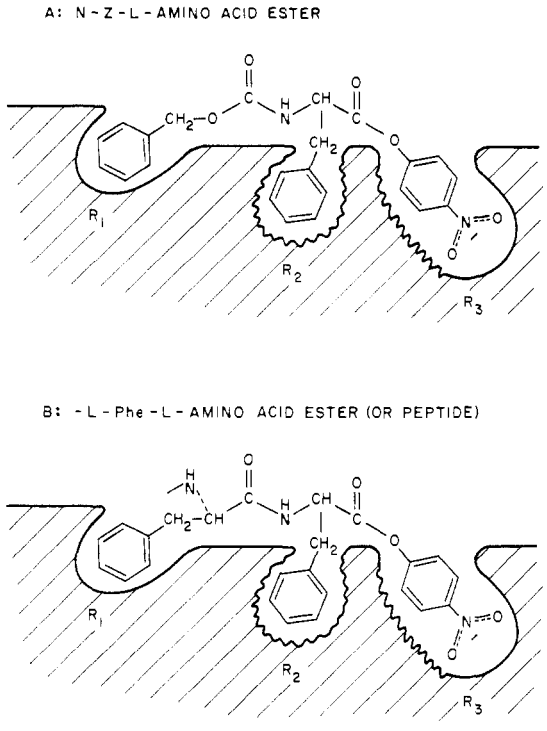

FIGURE 4: Schematic representation of the binding of an *N*-Z-L-amino acid *p*Nph substrate and a peptide type substrate, with a phenylalanine residue as the *N*-acyl group, -L-Phe-L-amino acid pNph at the active site of proteinase. In both cases, the R_2 position is shown with a $-CH_2C_6H_5$ side chain of a phenylalanine residue.

in going from $R_2 = -H$ to $R_2 -(CH_2)_4-NH_2$ is 21.2-fold. The increases in k_{cat} values for papain, for the same changes in R_2, are 4.0- and 5.48-fold, respectively. Thus, the enhancement of the turnover rate is three- to fourfold greater for proteinase than for papain with the same changes in the structure of R_2. Papain, like proteinase, shows little sensitivity to the presence of a charged group in the side-chain moiety. The k_{cat} values for papain-catalyzed hydrolysis of α-*N*-Z-Lys-Ph, *N*-Z-Glu-Ph, and *N*-Z-Ala-Ph were similar.

However, for the substrate *N*-Z-L-Phe-pNph, with $R_2 = -CH_2C_6H_5$, the rate was enhanced for proteinase but not for papain. The k_{cat} for proteinase is 19.4 sec^{-1} and for papain it is 4.05 sec^{-1} determined in acetate buffer, 21.4% v/v, CH_3CN, pH 5.99. At this concentration of acetonitrile, the k_{cat} for the proteinase-catalyzed hydrolyses of esters is diminished by about a factor of 7.5 (Kortt and Liu, 1973b), while the k_{cat} for papain-catalyzed hydrolyses is enhanced. A positive effect of inert organic solvents on the papain hydrolyses has been reported (Hinkle and Kirsch, 1970) and confirmed in the present study (see below). When corrections for the effect of acetonitrile are made, the k_{cat} for the proteinase is ~36-fold higher than the k_{cat} for papain for the substrate with $R_2 = -CH_2C_6H_5$. Thus the placement of an aromatic and planar substituent at the β carbon to the carbonyl functional group has a favorable effect on the rate of hydrolyses by proteinase but not papain. This result gives further support to the view that the nature of the R_2 group in the *N*-Z-L-amino acid ester substrates has a greater influence on proteinase than on papain in the deacylation reaction of activated ester substrates.

The Effect of R_1. The work of Gerwin *et al.* (1966) showed that the nature of the amino-terminal blocking group had a decisive effect upon the rate of hydrolysis of depeptide substrates. These authors found that when such a group was lacking altogether, or when it was small, hydrolysis was impaired. The exacting specificity of strepcococcal proteinase with respect to the *N*-acyl group, R_1, is provided by our present study.

Examination of the data in Table VII shows that for the proteinase-catalyzed hydrolysis of a series of *N*-acyl-Gly-pNph, variation of the structure of the *N*-acyl group, R_1, leads to a dramatic change in the reactivity of proteinase toward the substrate. Even a small change in the structure of R_1, such as the introduction of a para nitro group in the *N*-benzyloxycarbonyl function has a marked effect on k_{cat} and K_m. The presence of a hydrophobic group, in the *N*-acyl moiety, at a precise distance from the carbonyl carbon of the ester bond undergoing hydrolysis is decisive in determining the catalytic efficiency in the cleavage of the ester bond. With $R_1 = C(=O)CH_3$, as in *N*-Ac-Gly-pNph, no hydrolysis was observed, and within the solubility limit of the ester, $[S]_0 = 2.52 \times 10^{-4}$ M, it did not act as an inhibitor in the proteinase-catalyzed hydrolysis of *N*-Z-Gly-pNph. The benzoyl group is hydrophobic in character but the distance of the aromatic ring from the carbonyl group of the ester bond is about 2.6 Å shorter than that of the benzyloxycarbonyl group, and the relative reactivity, k_{cat}/K_m, for *N*-Bz-Gly-pNph is only 1/80th of *N*-Z-Gly-pNph. The cinnamoyl group has a phenyl ring about the same distance from the carbonyl group of the ester bond as that in the benzyloxycarbonyl group, but lacks the rotational freedom of the phenyl ring in the benzyloxycarbonyl group. For *N*-cinnamoyl-Gly-pNph the k_{cat} value is about sixfold lower and the K_m value is 1.27-fold higher than for *N*-Z-Gly-pNph. Addition of the para nitro group in the phenyl ring of Z decreases the k_{cat} eightfold and the K_m 3.75-fold. Replacement of the Z group with a bulkier *tert*-butyloxycarbonyl group resulted in a decrease of 68-fold in k_{cat} and an increase of about twofold in K_m. When the Z group is displaced about 3 Å further away from the carbonyl group of the ester bond, as in Z-Gly-pNph, a marked decrease in k_{cat} (~22-fold) and an increase in K_m (~5.6-fold) are observed. (The kinetic constants observed in Table VII for this substrate were corrected for the effect of organic solvent as described earlier.) It is clear from the above discussion that there is a decided preference for an unsubstituted phenyl group in the R_1 moiety of the substrate, and that this phenyl group must be situated at a distance of 7.0 ± 0.2 Å from the carbonyl group of the ester bond undergoing hydrolysis for maximum catalysis to occur. Rotational ability of this phenyl group is clearly an added advantage. Furthermore, the results show that the location of the hydrophobic group in the *N*-acyl moiety with respect to the carbonyl group of the ester bond is important in both the deacylation and acylation reactions in proteinase hydrolyses.

Studies of Gerwin *et al.* (1966) showed that in the proteinase-catalyzed hydrolysis of peptide substrates a Phe residue and/or a Z-*N*-acyl group in dipeptides was required in the N-terminal position for rapid cleavage to occur. The Z group closely resembles the structure of the side chain of Phe and could readily bind in the same manner as illustrated in Figure 4. The schematic representation of the structures and the positioning of the R_1, R_2, and R_3 groups of the *N*-acyl-amino acid *p*-nitrophenyl esters shown in Figure 4 were deduced from space filling models constructed by using CPK atomic models (Corey–Pauling–Koltun). The models clearly show that the phenyl group of an *N*-acylphenylalanine in the substrate, -L-Phe-L-Phe-pNph, has the same configuration and location with respect to the carbonyl group of the ester bond as the phenyl group of the *N*-acyl Z group of the substrate *N*-

Z-L-Phe-pNph as shown in Figure 4. These results support the conclusion that the specificity of proteinase is directed primarily to a hydrophobic moiety in the *N*-acyl group, such as the phenyl group of the benzyloxycarbonyl group or the phenyl group of an *N*-acylphenylalanine residue.

The data in Table VII show that for the papain-catalyzed hydrolyses of *N*-acyl-Gly-pNph's changes in R_1 from B_Z to Z, or *p*-nitro-Z or *tert*-butyloxycarbonyl or cinnamoyl or Z-Gly have only a small effect on k_{cat}. The higher k_{cat} value observed for Z-Gly-Gly-pNph can be attributed to the presence of organic solvent as the kinetic constants for this ester were determined in acetate buffer 21.4% v/v, CH_3CN, pH 5.99. For Z-Gly-pNph, the kinetic constants under these conditions were $k_{cat} = 10.5\ sec^{-1}$ and $K_m = 0.29 \times 10^{-4}$ M. A positive effect of an inert organic solvent for papain hydrolyses has been reported by Hinkle and Kirsch (1970). The effect on K_m shows that a hydrophobic *N*-acyl group has a large effect on the acylation reaction as is demonstrated by the ~30-fold greater K_m for Ac-Gly-pNph (Table VII). The K_m for cinnamoyl-Gly-pNph is ~20-fold lower than the K_m for Z-Gly-pNph indicating that the planar rigid structure of the cinnamoyl group is favored in the acylation reaction.

Thus, changes in the structure of R_1 appear to affect both the acylation and deacylation reactions of proteinase but only primarily the acylation reaction of papain. These results provide evidence that the binding site for the *N*-acyl group in proteinase is a relatively inflexible small hydrophobic cleft rather than a large, hydrophobic cleft region as found for papain.

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