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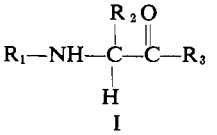
On the Specificity of Papain*

John de Jersey†

ABSTRACT: Good substrates for papain such as *p*-nitrophenyl hippurate contain an α-acylamino group. To investigate the interaction between this group and the active site of the enzyme, a number of *p*-nitrophenyl esters in which the group has been modified or removed have been examined as substrates. A quantitative estimate of the importance of the acylamino group in the acylation reaction has thus been obtained. The oxazolin-5-one and *p*-nitrophenyl ester of Bz-DL-Ala have been used to show that the acylamino group is the major factor in establishing the stereospecificity of both acylation and deacylation reactions. The effects of indole, benzene, and benzyl alcohol on the acylation of papain by 4,4-dimethyl-2-phenyloxazolin-5-one and on the corresponding deacylation reaction have been determined.

Papain (EC 3.4.4.10), one of several proteolytic enzymes present in papaya latex (Kunimitsu and Yasunobu, 1967), has been reported to have a broad specificity (Smith and Kimmel, 1960; Hill, 1965). However, the main emphasis has been on the determination of the specificity of the enzyme for the side chain of the L-α-amino acid contributing the carboxyl group to the bond being hydrolyzed (R_2 in I). The nature of this side chain is a major specificity determinant



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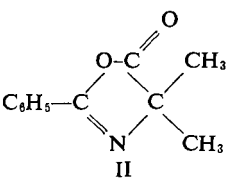
in catalysis by trypsin and α-chymotrypsin. By contrast, kinetic constants (k_{cat} and K_m) for papain-catalyzed hydrolyses are relatively insensitive to variation in R_2. For example, k_{cat} for the best substrate known, α-*N*-Z-L-LyspNp[1] (46 sec^{-1}) (Bender and Brubacher, 1966) is only a factor of 8 greater than k_{cat} for *N*-Z-GlypNp (5.9 sec^{-1}). Because of this, an attempt has been made to determine the importance of some of the other possible interactions between the enzyme and the substrate. In particular, the importance of the α-acylamino group (R_1—NH—) has been investigated by testing as substrates for papain a series of compounds in which the acylamino group has been progressively removed or modified.

Several experiments have indicated that the active site of papain may contain a hydrophobic region which interacts with the leaving group of the substrate. For example, Brubacher and Bender (1966) found that L-tryptophanamide was about 100 times as efficient as glycinamide in catalyzing the deacylation of *trans*-cinnamoyl-papain. D-Tryptophanamide was only about half as efficient as glycinamide. On this basis, it was proposed that hydrophobic compounds might affect the rates of the acylation and deacylation reactions of papain in much the same way as indole increases the rate of deacylation of acetyl-chymotrypsin (Foster, 1961). 4,4-Dimethyl-2-phenyloxazolin-5-one (II) was chosen as a substrate to test the effect of indole, benzene, and benzyl alcohol on these reactions. This oxazolinone has been considered previously as a substrate for papain (de Jersey and



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[1] Abbreviations used are: Z, benzyloxycarbonyl; Bz, benzoyl; But, butyryl; pNp, *p*-nitrophenyl ester; Et, ethyl ester; Sar, sarcosine.

TABLE I: Kinetic Constants for the Papain-Catalyzed Hydrolyses of *p*-Nitrophenyl Esters at 25°.[a]

Acyl Group	k_{cat}/K_m (M^{-1} sec^{-1})	k_{OH^-} (M^{-1} sec^{-1})
$C_6H_5CONHCH_2CO$[b]	$(3.55 \pm 0.19) \times 10^5$	7,040[c]
$C_6H_5CON(CH_3)CH_2CO$[d]	6.24 ± 0.21	80[e]
$^+H_3NCH_2CO$[f]	5.30 ± 0.62	44,300[g]
CH_3CO[h]	6.12 ± 0.21	12.6[i]

[a] Determined in 0.05 M phosphate–EDTA–dithiothreitol buffer (pH 6.09)–3.2% v/v acetonitrile, except for the hydrolysis of the glycine ester which was observed in 0.05 M formate buffer (pH 3.30), containing 2.5×10^{-4} M dithiothreitol and 3.2% v v dimethylformamide. [b] $[S]_0 = (1.25\text{–}10.4) \times 10^{-5}$ M; $[E]_0 = 2.08 \times 10^{-8}$ M. [c] de Jersey *et al.* (1969). [d] $[S]_0 = (3.85\text{–}15.4) \times 10^{-5}$ M; $[E]_0 = 1.28 \times 10^{-5}$ M. [e] de Jersey (1969). [f] $[S]_0 = (4.2\text{–}21) \times 10^{-5}$ M; $[E]_0 = 3.26 \times 10^{-5}$ M. [g] Kortt (1969). [h] $[S]_0 = (0.80\text{–}8.03) \times 10^{-4}$ M; $[E]_0 = 1.28 \times 10^{-5}$ M. [i] Unpublished result of K. A. Connors.

Zerner, 1969a). The acylation reaction may be observed directly and yields a stable acyl-enzyme. 4,4-Dimethyl-2-phenyloxazolin-5-one has a further advantage in that it lacks a normal leaving group. Hence, interaction of a hydrophobic compound with a site on the enzyme designed to interact with the leaving group of a substrate should not be sterically hindered by the presence of the oxazolinone at the active site.

Experimental Section

Materials. The preparation and properties of *p*-nitrophenyl acetate, *p*-nitrophenyl hippurate, *N*-Bz-DL-AlapNp, *N*-Z-GlypNp, 2-phenyloxazolin-5-one, DL-4-methyl-2-phenyloxazolin-5-one, and 4,4-dimethyl-2-phenyloxazolin-5-one have been described previously (de Jersey *et al.*, 1969). Glycine *p*-nitrophenyl ester was prepared from *N*-Z-GlypNp by treatment with HBr in glacial acetic acid. *N*-Z-β-AlapNp was prepared from *N*-Z-β-Ala (mp 103°) and *p*-nitrophenol by the action of dicyclohexylcarbodiimide in ethyl acetate. Recrystallization from chloroform–hexane gave colorless crystals, mp 94°. *Anal.* Calcd for $C_{17}H_{16}N_2O_6$: C, 59.29; H, 4.68; N, 8.14. Found: C, 58.97; H, 4.93; N, 8.04. Complete hydrolysis of the ester in NaOH gave 97% of the calculated amount of *p*-nitrophenol.

N-Z-γ-NH_2ButpNp (mp 82–84°) was a gift from Dr. B. Zerner. α-*N*-Bz-L-ArgEt was obtained from Mann Research Laboratories, dithiothreitol and L-Cys-HCl from Sigma Chemical Co., iodoacetic acid from British Drug Houses, and β-mercaptoethanol from Fluka AG. Indole (British Drug Houses) was recrystallized several times from petroleum ether (bp 30–60°). Sephadex G-25 was obtained from Pharmacia. Buffers were prepared using analytical grade reagents. pH measurements were made using an Horiba M-5 pH meter, and are accurate to ±0.02 pH unit. Acetonitrile (Eastman Organic Chemicals Spectrograde) and benzene (British Drug Houses Analar) were used without further purification. Benzyl alcohol was redistilled before use. Papain (twice crystallized) was obtained from Worthington Biochemical Corp. as a concentrated suspension (~28 mg/ml).

Methods. Kinetic experiments were performed using a Cary 14 PM-50 recording spectrophotometer equipped with 0–0.1- and 0–1.0-absorbance slide-wires and a thermostatted cell compartment. Kinetic measurements were made at 25 ± 0.2°. Typically, 3 ml of buffer solution was equilibrated and the reaction was started by the addition of an aliquot of a solution of the substrate (usually in acetonitrile) on a flat-ended stirring rod. Hydrolysis of the *p*-nitrophenyl esters was followed at 347.5 mμ, the isobestic point of *p*-nitrophenol and *p*-nitrophenolate ion. In each set of experiments, Δε was checked using a standard solution of *p*-nitrophenol. The hydrolysis of 2-phenyloxazolin-5-one was followed by the decrease in absorbance at 260 mμ (Δε −3630). The reaction of DL-4-methyl-2-phenyloxazolin-5-one with papain was also followed at 260 mμ. Kinetic data obtained under steady-state conditions were evaluated using the HYPER program of Cleland (1967).

Solutions of papain were prepared by dilution of the papain suspension into 0.05 M phosphate buffer (pH 6.09), containing 10^{-3} M EDTA and either 2.5×10^{-4} M cysteine or 2.5×10^{-4} M dithiothreitol (hereinafter referred to as phosphate–EDTA–cysteine and phosphate–EDTA–dithiothreitol buffers). The same final activity was reached in each case. Papain solutions were routinely assayed using either α-*N*-Bz-L-ArgEt or *N*-Z-GlypNp as substrate, in 0.05 M phosphate buffer (pH 6.09), containing 10^{-3} M EDTA (hereinafter referred to as phosphate–EDTA buffer). In the α-*N*-Bz-L-ArgEt assay, the increase in absorbance at 253 mμ was measured using the 0–0.1-absorbance scale, with an initial substrate concentration of 5×10^{-4} M (K_m = 0.014 M, Whitaker and Bender, 1965). In the *N*-Z-GlypNp assay, the release of *p*-nitrophenol was measured at 347.5 mμ, again on the 0–0.1-absorbance scale, with an initial substrate concentration of 7×10^{-5} M ($K_m = 7 \times 10^{-6}$ M). In this assay, a small correction for spontaneous hydrolysis must be made. The *N*-Z-GlypNp assay was used except in the experiments involving reactivation of stable acyl-enzymes. In these experiments, it was desirable to include a high concentration of cysteine (5 mM) to ensure that all of the free enzyme was in the active form during the assay, and hence, it was necessary to use the α-*N*-Bz-L-ArgEt assay.

Bender *et al.* (1966) correlated an assay of papain using α-*N*-Bz-L-ArgEt as substrate with the titration of papain solutions at low pH using *N*-Z-L-TyrpNp. In the present work, an α-*N*-Bz-L-ArgEt assay under very similar conditions (0.05 M acetate buffer (pH 5.20), 10^{-4} M in EDTA, and 2.5×10^{-4} M in dithiothreitol; $[S]_0 = 5 \times 10^{-4}$ M) was correlated with the *N*-Z-GlypNp assay. Hence, the actual enzyme concentration used in each set of kinetic runs could be calculated. The Worthington twice-crystallized papain was found to be 52% active enzyme, based on a molecular weight of 23,000 (Drenth *et al.*, 1968) and an $\epsilon_{280\ m\mu}$ of 56,600 (Glazer and Smith, 1961).

[2] Microanalysis performed by the Australian Microanalytical Service, Melbourne, Victoria.

TABLE II: Kinetic Constants for the Papain-Catalyzed Hydrolyses of p-Nitrophenyl Esters of General Formula $ZNH(CH_2)_n$-C(O)OR at 25°.[a]

Substrate	n	k_{cat} (sec^{-1})	k_{cat}/K_m (M^{-1} sec^{-1})	k_{OH^-} (M^{-1} sec^{-1})
N-Z-GlypNp[b]	1	5.94 ± 0.33[c]	(7.85 ± 0.89) × 10^5	156[d]
Z-β-AlapNp[e]	2	0.0482 ± 0.0016	(6.88 ± 0.23) × 10^2	20
Z-γ-NH_2ButpNp[f]	3		(5.93 ± 0.26) × 10	10[g]

[a] Kinetic constants determined in 0.05 M phosphate–EDTA–dithiothreitol buffer (pH 6.09). [b] $[S]_0$ = (3.6–40.6) × 10^{-6} M; $[E]_0$ = 8.1 × 10^{-9} M; 1.6% v/v acetonitrile; K_m = (7.57 ± 1.23) × 10^{-6} M. [c] Williams and Whitaker (1967) reported a k_{cat} of 5.2 sec^{-1}. [d] de Jersey *et al.* (1969). [e] $[S]_0$ = (1.22–12.1) × 10^{-5} M; $[E]_0$ = 4.25 × 10^{-6} M; 3.2% v/v acetonitrile; K_m = (7.04 ± 0.46) × 10^{-5} M. [f] $[S]_0$ = (1.32–6.62) × 10^{-5} M; $[E]_0$ = 1.28 × 10^{-5} M; 3.2% v/v acetonitrile. [g] Kortt (1969).

TABLE III: Comparison of the Reactions of Papain with *p*-Nitrophenyl Acetate and *N*-Z-GlypNp at 25°.[a]

Enzyme	*p*-Nitrophenyl Acetate		*N*-Z-GlypNp	
	Act. (× 10^{-8} M/sec)	% Control Act.	Act. (× 10^{-8} M/sec)	% Control Act.
Enzyme not activated[b]	0.17	2.5	0.18	1.8
Enzyme inactivated by iodoacetate[c]	0.16	2.3	0.07	0.7
Enzyme assayed at pH 7.10[d]	7.02	104	10.8	106
Control enzyme[e]	6.75	100	10.2	100

[a] Assays were performed in 0.05 M phosphate–EDTA buffer (pH 6.09) or in 0.05 M phosphate buffer (pH 7.10). In the *p*-nitrophenyl acetate assays, $[S]_0$ = 8.0 × 10^{-4} M, $[E]_0$ = 1.9 × 10^{-5} M. In the *N*-Z-GlypNp assays, $[S]_0$ = 5.9 × 10^{-5} M, $[E]_0$ = 2.3 × 10^{-8} M. [b] Enzyme made up in 0.05 M phosphate–EDTA buffer, pH 6.09. [c] Enzyme made up in 0.05 M phosphate–EDTA–dithiothreitol buffer (pH 6.09) then incubated for 30 min in the presence of 0.01 M iodoacetate before assay. [d] Enzyme made up in 0.05 M phosphate buffer (pH 7.10), containing 2.5 × 10^{-4} M dithiothreitol. [e] Enzyme made up in 0.05 M phosphate–EDTA–dithiothreitol buffer (pH 6.09).

Results

Substrates Lacking an α-Acylamino Group. *p*-Nitrophenyl hippurate is a good substrate for papain. Considering *p*-nitrophenyl hippurate as a reference compound, the α-acylamino group has been modified in three ways: N methylation, giving *N*-BzSarpNp; removal of the benzoyl group, giving GlypNp; and removal of the benzoylamino group, giving *p*-nitrophenyl acetate. These three compounds were compared as substrates for papain, and in each case, the initial velocity was directly proportional to the substrate concentration, indicating that, under the conditions used, $K_m \gg [S]_0$. Hence, the slope of the plot of $v/[E]_0$ *vs.* $[S]_0$ is equal to k_{cat}/K_m, according to the Michaelis equation. Experimental details and results are given in Table I.

In a second approach, *N*-Z-GlypNp was used as a reference compound. Corresponding compounds with the Z-amino group in the β and γ positions were tested as substrates for papain, and the results are given in Table II. The reaction of the *N*-Z-β-AlapNp with papain was also studied in 0.05 M formate buffer (pH 3.7), containing 10^{-3} M EDTA and 2.5 × 10^{-4} M dithiothreitol ($[S_0]$ = 1.3 × 10^{-4} M and $[E]_0$ = 10^{-5} M). Under these conditions, a burst of *p*-nitrophenol was observed indicating that $k_{+2} > k_{+3}$, *i.e.*, that $k_{cat} \approx k_{+3}$.

Because of the low values of k_{cat}/K_m obtained for *p*-nitrophenyl acetate and some of the other substrates, the reaction of papain with *p*-nitrophenyl acetate was investigated further. Three experiments were carried out to compare the characteristics of the reactions of papain with *p*-nitrophenyl acetate and *N*-Z-GlypNp. Experimental details and results are listed in Table III.

Comparison of p-Nitrophenyl Esters and Oxazolin-5-ones as Substrates for Papain. *p*-Nitrophenyl hippurate and the corresponding oxazolinone, 2-phenyloxazolin-5-one, have been considered as substrates for papain. Both of these compounds may be regarded as activated esters of hippuric acid, and therefore, the acyl-enzyme intermediate formed in each case would be hippuryl-papain. The reactions of *p*-nitrophenyl hippurate and 2-phenyloxazolin-5-one with β-mercaptoethanol were also studied to provide a comparison of their reactivities toward a thiolate ion. The measured rate constants are collected in Table IV. Included in Table IV are the rate constants previously reported for the reaction of *p*-nitrophenyl hippurate and 2-phenyloxazolin-5-one with imidazole (de Jersey *et al.*, 1969).

The reactions of Bz-DL-AlapNp and the corresponding racemic oxazolinone, DL-4-methyl-2-phenyloxazolin-5-one, were investigated with the aim of comparing the stereo-

TABLE IV: Kinetic Constants for Some Reactions of *p*-Nitrophenyl Hippurate and 2-Phenyloxazolin-5-one at 25°.

Constant	Substrate		Ratio Ester/Oxazolinone
	p-Nitrophenyl Hippurate	2-Phenyloxazolin-5-one	
k_{cat}[a,b]	2.59 ± 0.03	3.32 ± 1.04	0.78 ± 0.25
K_m[a,c]	7.29 ± 0.45	1060 ± 420	
k_{cat}/K_m[a,d]	(3.55 ± 0.19) × 10^5	(3.13 ± 0.27) × 10^3 3.7 × 10^3[e]	113 ± 16
k_{RS^-}[d,f]	275	4420	0.06
k_{Im}[d,g]	1.56	~30	0.05

[a] Determined in 0.05 M phosphate–EDTA- dithiothreitol buffer (pH 6.09)–1.5% v/v acetonitrile. For *p*-nitrophenyl hippurate, $[E]_0 = 2.08 \times 10^{-8}$ M, $[S]_0 = (1.25\text{–}10.4) \times 10^{-5}$ M. For 2-phenyloxazolin-5-one, $[E]_0 = 2.28 \times 10^{-6}$ M, $[S]_0 = (0.78\text{–}3.72) \times 10^{-4}$ M. [b] In sec^{-1}. [c] μM. [d] M^{-1} sec^{-1}. [e] Determined under second-order conditions ($[S]_0 \ll K_m$); $[S]_0 = 7.8 \times 10^{-5}$ M, $[E]_0 = 2.28 \times 10^{-6}$ M. The apparent first-order constant k_{obsd} was measured; $k_{obsd}/[E]_0 = k_{cat}/K_m$. [f] $RS^- = \beta$-mercaptoethanol anion. k_{obsd} was determined in 0.05 M phosphate buffer (pH 6.09) containing a range of β-mercaptoethanol concentrations from 0 to 17.4 mM. k_{RS^-} was calculated assuming that reaction is due solely to the anion, and assuming a pK_a' of 9.51 (Kirsch and Igelström, 1966). k_{RS^-} for Z-GlypNp determined under the same conditions, was found to be 283 M^{-1} sec^{-1}, in fair agreement with the previously reported value of 325 M^{-1} sec^{-1} (Kirsch and Igelström, 1966). [g] de Jersey *et al.* (1969).

specificities of the reactions. When an aliquot of DL-4-methyl-2-phenyloxazolin-5-one was added to a solution of papain in 0.05 M phosphate–EDTA–cysteine buffer (pH 6.09) and the decrease in absorbance at 260 mμ was followed, a burst of 0.15 absorbance unit was observed ($[S]_0 = 2.7 \times 10^{-4}$ M and $[E]_0 = 5.7 \times 10^{-6}$ M), after which no further enzymatic hydrolysis of the oxazolinone occurred. An aliquot of the reaction mixture, assayed against *N*-Z-GlypNp, had ≤1% of the original activity. This behavior is readily understood in terms of

$$E + \text{L-oxazolinone} \rightarrow \text{Bz-L-Ala-E} \rightarrow E + \text{Bz-L-Ala}$$
$$E + \text{D-oxazolinone} \rightarrow \text{Bz-D-Ala-E (stable)}$$

From the size of the burst and $[E]_0$, and from $\Delta\epsilon_{260\ m\mu}$ (−4700),

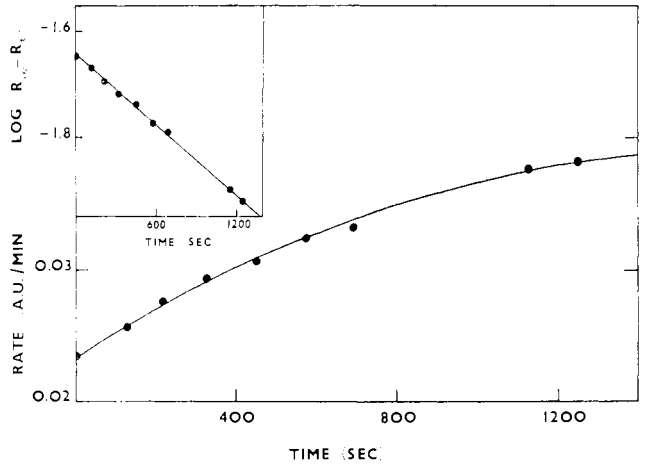

FIGURE 1: The deacylation of isolated Bz-D-Ala-papain, followed by assaying aliquots of the reaction mixture against α-*N*-Bz-L-ArgEt. Zero time is the time at which the first aliquot was assayed, the activity at zero time being due to partial deacylation during the isolation procedure. $R_\infty = 0.044$ absorbance unit/min.

the stereospecificity ratio for the acylation reaction may be calculated as

$$\left(\frac{k_{cat}}{K_m}\right)^L \Big/ \left(\frac{k_{cat}}{K_m}\right)^D \approx 6$$

A more detailed discussion of a similar system, the reaction of α-chymotrypsin with DL-4-(*p*-hydroxybenzyl)-2-phenyl-oxazolin-5-one, has been given previously (de Jersey and Zerner, 1969a).

The reaction of Bz-DL-AlapNp with papain was investigated under steady-state conditions ($[E]_0 = 2.26 \times 10^{-8}$ M; [L isomer]$_0 = (0.73\text{–}7.3) \times 10^{-5}$ M; 1.5% v/v acetonitrile; 0.05 M phosphate–EDTA–dithiothreitol buffer, pH 6.09). Under these conditions, only the L isomer was hydrolyzed. For Bz-L-AlapNp, $k_{cat} = 10.7 \pm 0.2$ sec^{-1}, $K_m = 5.8 \pm 0.4$ μM, $k_{cat}/K_m = (1.85 \pm 0.10) \times 10^6$ M^{-1} sec^{-1}. Using much higher concentrations of papain (up to 3.7×10^{-5} M), attempts were made to detect enzymatic hydrolysis of the D isomer. However, even at such high concentrations, the rate of hydrolysis in the presence of enzyme was not significantly higher than the rate of spontaneous hydrolysis. In this way, an upper limit of 10 M^{-1} sec^{-1} could be established for k_{cat}/K_m in the papain-catalyzed hydrolysis of Bz-D-AlapNp.

The absence of stereospecificity in the acylation of papain by DL-4-methyl-2-phenyloxazolin-5-one permitted the isolation of Bz-D-Ala-papain. An aliquot (100 μl) of DL-4-methyl-2-phenyloxazolin-5-one in acetonitrile was added to 5 ml of papain in 0.05 M phosphate–EDTA–cysteine buffer, pH 6.09 ($[S]_0 = 3.7 \times 10^{-4}$ M and $[E]_0 = 3.4 \times 10^{-5}$ M). After 4 min, assay with α-*N*-Bz-L-ArgEt revealed that ≤1% of the original activity remained. A 3-ml aliquot of the reaction mixture was passed through a column of Sephadex G-25 (1.6 × 21 cm), equilibrated with phosphate–EDTA buffer. The inactive enzyme was eluted in the void volume (~20 ml), and was effectively separated from excess substrate and product. The fraction containing the enzyme (~5 ml) was equilibrated at 25°, and 200-μl aliquots were assayed against

TABLE V: Effect of Modifiers on Kinetic Constants for Papain-Catalyzed Hydrolyses of 4,4-Dimethyl-2-phenyloxazolin-5-one and *N*-Z-GlypNp at 25°.

Substrate	Modifier	Acylation[a] $10^2 k_{obsd}$ (sec^{-1})	Deacylation[b] $10^4 k_{obsd}$ (sec^{-1})
4,4-Dimethyl-2-phenyl-oxazolin-5-one	None	1.01, 1.01	2.13, 2.17
	4 mM Indole[c]	1.16	2.19
	6 mM Benzene[c]	1.10	2.16
	50 mM Benzyl alcohol	1.23, 1.25	
	100 mM Benzyl alcohol	1.46, 1.49	3.4[d]
		k_{cat}/K_m (M^{-1} sec^{-1})	k_{cat} (sec^{-1})
N-Z-GlypNp[e]	None[f]	$(7.85 \pm 0.89) \times 10^5$	5.94 ± 0.33
	100 mM Benzyl alcohol[g]	$(11.58 \pm 0.63) \times 10^5$	21.3 ± 0.8

[a] In 0.05 M phosphate–EDTA–dithiothreitol buffer (pH 6.09). $[S]_0 = 9.34 \times 10^{-5}$ M; $[E]_0 = 5.4 \times 10^{-7}$ M; 200-μl aliquots assayed against *N*-Z-GlypNp; k_{obsd} shown to be proportional to $[S]_0$. [b] In 0.05 M phosphate–EDTA buffer (pH 6.09); 200-μl aliquots assayed against α-*N*-Bz-L-ArgEt in 0.05 M phosphate–EDTA buffer (pH 6.09) containing 5 mM cysteine. [c] Reaction mixtures also contained 2% v/v acetonitrile. [d] 96 mM benzyl alcohol. [e] Kinetic constants determined in 0.05 M phosphate–EDTA–dithiothreitol buffer (pH 6.09). [f] $K_m = 7.57 \times 10^{-6}$ M. [g] $K_m = (18.4 \pm 1.6) \times 10^{-6}$ M.

α-*N*-Bz-L-ArgEt in 0.05 M phosphate–EDTA buffer containing 5 mM cysteine (pH 6.09). The rate of deacylation of Bz-D-Ala-papain could thus be measured. Figure 1 shows a plot of rate (in the α-*N*-Bz-L-ArgEt assay) *vs.* time, and the corresponding first-order plot. k_{obsd} for the deacylation of Bz-D-Ala-papain under these conditions was found to be $(1.00 \pm 0.02) \times 10^{-3}$ sec^{-1}.

Effect of Hydrophobic Compounds on Papain-Catalyzed Reactions. The reaction of 4,4-dimethyl-2-phenyloxazolin-5-one (II) with papain provides a convenient system for the direct observation of the effect of modifiers on both acylation and deacylation reactions. The acylation of papain by 4,4-dimethyl-2-phenyloxazolin-5-one may be observed directly (at 250 mμ), or monitored by taking aliquots and assaying against *N*-Z-GlypNp (de Jersey and Zerner, 1969a). The acylation reaction was followed by the *N*-Z-GlypNp assay method in the presence and absence of indole, benzene, and benzyl alcohol (Table V). The effect of 0.1 M benzyl alcohol on k_{cat} and K_m for the papain-catalyzed hydrolysis of *N*-Z-GlypNp was also determined (Table V). Indole (4 mM) and benzene (6 mM) had no significant effect on k_{cat} and K_m for this reaction.

Papain reacts with 4,4-dimethyl-2-phenyloxazolin-5-one to give a stable acyl-enzyme, *N*-benzoylaminoisobutyryl-papain. This acyl-enzyme was prepared by reaction of papain ($\sim 3.4 \times 10^{-5}$ M) with the oxazolinone (3.5×10^{-4} M) in 0.05 M phosphate–EDTA–cysteine buffer (pH 6.09). After 5 min, the residual activity was $\leq 3\%$ of the original activity. The acyl-enzyme was isolated and its reactivation was studied in the same way as has been described in detail for Bz-D-Ala-papain. Deacylation rate constants were determined in the presence and absence of modifiers, and are included in Table V.

Discussion

In recent years, good evidence has been accumulated that papain-catalyzed hydrolyses proceed *via* acyl-enzyme intermediates. The scheme of eq 1 accounts for most, if not all, of the experimental observations. ES is the Michaelis complex,

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} ES' \xrightarrow[H_2O]{k_{+3}} E + P_2 \qquad (1)$$
$$\qquad\qquad\qquad\qquad\quad + \\ \qquad\qquad\qquad\qquad\quad P_1$$

ES′ the acyl-enzyme, P_1 the leaving group of the substrate, and P_2 the carboxylic acid. The kinetic constants, k_{cat} and K_m, are related to the constants of eq 1 as follows (Gutfreund and Sturtevant, 1956):

$$k_{cat} = k_{+2}k_{+3}/(k_{+2} + k_{+3})$$

$$K_m = \frac{k_{3+}(k_{-1} + k_{+2})}{(k_{+2} + k_{+3})k_{+1}}$$

$$k_{cat}/K_m = k_{+1}k_{+2}/(k_{-1} + k_{+2})$$

In the present studies, activated derivatives have been used to permit determination of deacylation rate constants. Lowe and Williams (1965) obtained presumptive evidence that deacylation is rate limiting in the hydrolysis of *p*-nitrophenyl hippurate. Hubbard and Kirsch (1968) observed the acylation of papain by *N*-Z-GlypNp by a stopped-flow method, obtaining direct evidence that for this substrate, k_{cat} is a measure of k_{+3}. Regardless of which step is rate limiting, the ratio k_{cat}/K_m is a measure of specificity in the acylation reaction.

Substrates Lacking the α-Acylamino Group. Before the present study, the only reported substrates for papain which lacked an α-acyl-L-amino substituent were *N-trans*-cinnamoylimidazole (Brubacher and Bender, 1966) and 4,4-dimethyl-2-phenyloxazolin-5-one (de Jersey and Zerner, 1969a). The deacylation rate constant for *trans*-cinnamoyl-papain is reported to be 3.7×10^{-3} sec^{-1}, compared with 2.6 sec^{-1} for hippuryl-papain. For most of the substrates examined

TABLE VI: Positive and Negative Effects of α-Methyl Substitution on Deacylation Rate Constants at 25°.[a]

Acyl-enzyme	α-Methyl Groups L	α-Methyl Groups D	k_{+3} (sec^{-1})
Hippuryl-papain	−	−	2.6
Bz-L-Ala-papain	+	−	10.7
Bz-D-Ala-papain	−	+	1.00×10^{-3}
$BzNH_2Ibu$-papain	+	+	0.22×10^{-3}

[a] Experimental details have been listed in the Results section.

here, it was not possible to estimate k_{cat} and K_m. This indicates that K_m was much higher than the highest substrate concentration used. In the papain-catalyzed hydrolyses of activated derivatives of α-acylamino acids, K_m is determined largely by the k_{+3}/k_{+2} ratio. Therefore, it can be argued that the loss of the acylamino group affects k_{+2} more than k_{+3}.

The estimates of k_{cat}/K_m given in Tables I and II give a quantitative estimate of the importance of the acylamino group in the acylation reaction. N Methylation of *p*-nitrophenyl hippurate results in a 5×10^4-fold decrease in k_{cat}/K_m. Similar decreases in k_{cat}/K_m occur when the benzoyl group and the benzoylamino group are removed. Displacement of the acylamino group to the β position resulted in a somewhat smaller decrease in k_{cat}/K_m (~10^3). For this substrate, *N*-Z-β-AlapNp k_{cat} ($=k_{+3}$) could also be measured. The insertion of one methylene group causes an 100-fold decrease in k_{+3}. The insertion of a second methylene group (as in *N*-Z-γ-NH_2ButpNp) results in a further tenfold decrease in k_{cat}/K_m.

k_{cat}/K_m may also be regarded as the second-order rate constant for the reaction of enzyme and substrate. Hence, values of k_{cat}/K_m may be compared directly with values of k_{OH^-} and k_{RS^-}. Because of the low values of k_{cat}/K_m for several of the substrates, the question must be asked as to whether substrates such as *p*-nitrophenyl acetate react with papain in the same way as good substrates. *p*-Nitrophenyl acetate is a good acylating agent, and could conceivably react with several groups in the protein which may not be involved in the normal catalytic reaction. For example, Piszkiewicz and Bruice (1968) have shown that lysozyme catalyzes the hydrolyses of several nitrophenyl esters including *p*-nitrophenyl acetate, and that this catalysis is due to a histidine residue far removed from the active site. Two results (Table III) indicate that the reactive sulfhydryl group of papain is necessary for reaction with *p*-nitrophenyl acetate: the reaction does not occur if the enzyme has not been activated, and is abolished by incubation of the enzyme with iodoacetate. The observation (Table III) that k_{cat}/K_m is the same at pH 7.10 as it is at pH 6.09 indicates that, as in the hydrolysis of good substrates, ESH rather than ES^- is probably the active species. Therefore, the evidence suggests that the papain-catalyzed hydrolyses of *p*-nitrophenyl acetate and *N*-Z-GlypNp occur by similar mechanisms.

The presence of an α-acylamino group in a substrate is necessary for a rapid rate of papain-catalyzed hydrolysis. It is interesting, therefore, to note the difference in the pH dependence of k_{cat} for the papain-catalyzed hydrolyses of the neutral substrates, *N-trans*-cinnamoylimidazole and *N*-Z-GlypNp. k_{+3} for the deacylation of *trans*-cinnamoyl-papain depends on a basic group of $pK_a' = 4.69$ (Brubacher and Bender, 1966), whereas k_{-3} for the deacylation of *N*-Z-Gly-papain depends on a group of $pK_a' = 3.85$ (Williams and Whitaker, 1967). This difference could be explained by the formation of a hydrogen bond between the NH group of a good substrate and a carboxylate ion at the active site. Such a hydrogen bond would lower the pK_a' of the carboxyl group. The X-ray crystallographic studies of Drenth *et al* (1968) have shown the proximity of Asp-105 and Asp-160 to the reactive cysteine residue (Cys-25). It should be noted that k_{+3} for the deacylation of α-*N*-Z-L-Lys-papain depends on a group of $pK_a' = 3.33$ (Bender and Brubacher, 1966). However, this value may be complicated by the presence at the active site of the charged ε-amino group of the lysine.

The nature of the acyl portion of the acylamino group seems to have a considerable effect on the kinetic constants for papain-catalyzed hydrolyses. k_{cat} for *N*-Z-Gly-pNp is about twice k_{cat} for *p*-nitrophenyl hippurate. Both of these compounds have hydrophobic acyl groups. Kortt (1969) has shown that k_{cat} for *N*-AcGlypNp is less than k_{cat} for *p*-nitrophenyl hippurate by a factor of ~5, while K_m is greater by a factor of ~30. Hence, in this case, a hydrophobic acyl group has a quite small effect on the deacylation reaction but quite a large effect on the acylation reaction. In this regard, Schecter and Berger (1968) have shown that an important interaction occurs between a hydrophobic group in this region of the substrate and the active site. Peptides of the form -Phe-X-X- were found to be cleaved specifically and rapidly at the X–X bond.

Oxazolin-5-ones as Substrates for Papain. Oxazolin-5-ones have proved to be useful activated derivatives of *N*-acylamino acids in the study of proteolytic enzymes (de Jersey and Zerner, 1969a,b). They differ from other activated derivatives of *N*-acylamino acids in that they do not contain an acylamino group, nor do they possess a normal leaving group. However, they give the same acylaminoacyl-enzyme intermediates as other derivatives. Comparing 2-phenyl-oxazolin-5-one and *p*-nitrophenyl hippurate as substrates for papain (Table IV), the k_{cat} values are the same within experimental error. This indicates that the breakdown of hippuryl-papain is rate-limiting in both cases. However, k_{cat}/K_m for the ester is 100 times k_{cat}/K_m for the oxazolinone, indicating that acylation of papain by the ester (as measured by the second-order rate constant k_{+2}/K_s) occurs much faster than acylation by the oxazolinone. In contrast, 2-phenyloxazolin-5-one is about 20 times as reactive as *p*-nitrophenyl hippurate in the nonenzymatic acylation of imidazole and β-mercaptoethanol (Table IV). The decreased rate of acylation of papain by 2-phenyloxazolinone could be due to the absence of the C(O)NH group, or the leaving group, or both. In view of the results obtained with other substrates lacking the α-acylamino group, it seems likely that the absence of the acylamino group is the major factor.

The importance of the acylamino group in determining the stereospecificity of papain-catalyzed hydrolyses was shown by comparing the reactions of papain with the racemic oxazolinone and *p*-nitrophenyl ester of *N*-benzoylalanine. For the oxazolinone, the stereospecificity ratio in the acyla-

tion reaction is ~6. For the *p*-nitrophenyl ester, the stereospecificity ratio is $\geq 2 \times 10^5$.

The use of DL-4-methyl-2-phenyloxazolin-5-one as a substrate for papain has allowed the first quantitative estimate of stereospecificity in the deacylation reaction to be made. Using this racemic oxazolinone, Bz-D-Ala-papain may be isolated and its deacylation followed (Figure 1). k_{+3} for this reaction (1.00×10^{-3} sec^{-1}) may be compared with k_{+3} for the deacylation of Bz-L-Ala-papain (10.7 sec^{-1}). Thus, the stereospecificity ratio in the deacylation reaction is $\sim 10^4$.

We are now in a position to postulate that the major interaction responsible for this factor of $\sim 10^4$ is between the acylamino group of the substrate and the enzyme. The effects (positive and negative) of the interaction of the α-methyl group of substrates derived from L-alanine with the enzyme can be seen in the data of Table VI. The presence of the L-α-methyl group produces a fourfold *increase* in k_{+3} in the absence of the D-α-methyl group. In the presence of the D-α-methyl group, the L-α-methyl group causes a fourfold *decrease* in k_{+3}. Clearly, the interaction between the acylamino group and the enzyme in Bz-D-Ala-papain (and not the interaction between the α-methyl group and the enzyme) is responsible for the major part of the stereospecificity ratio. This interaction maintains the D-α-methyl group in a position which results in a much slower reaction (presumably for steric reasons).

Effect of Indole, Benzene, and Benzyl Alcohol on Papain-Catalyzed Reactions. A number of examples are now known in which the interaction of a compound with part of the active site of an enzyme causes an increased rate of reaction of the enzyme with partial substrates (see, for example, Inagami and York, 1968). The effect of hydrophobic compounds on the papain-catalyzed hydrolyses of 4,4-dimethyl-2-phenyloxazolin-5-one and *N*-Z-GlypNp has been determined (Table V). In both cases, it was possible to determine the effect on both acylation and deacylation reactions. In the deacylation reaction, indole and benzene (in the accessible concentration range) failed to have any measurable effect. Enhancement of deacylation by benzyl alcohol is due, in all probability, to reaction with the acyl-enzyme, giving the benzyl ester. Brubacher and Bender (1966) showed that benzyl alcohol accelerates the deacylation of *trans*-cinnamoyl-papain.

Considering the acylation reaction, indole, benzene, and benzyl alcohol all produced small increases in the rate of reaction of papain with 4,4-dimethyl-2-phenyloxazolin-5-one. Benzyl alcohol produces a similar increase in k_{cat}/K_m for the papain-catalyzed hydrolysis of *N*-Z-GlypNp. k_{obsd} for the acylation of papain by 4,4-dimethyl-2-phenyloxazolin-5-one increased in a linear fashion for concentrations of benzyl alcohol from 0 to 100 mM, indicating that the maximum effect on the reaction has not been observed. The results do suggest that the effect of hydrophobic modifiers is more marked in the acylation reaction than in the deacylation reaction. Whitaker (1969) has recently determined the effect of a number of compounds on the papain-catalyzed hydrolysis of α-*N*-Bz-L-ArgEt. In several cases, k_{cat} is enhanced by the presence of the compound, but as in the present work, the effects are small.

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