Antonini, E., Wyman, J., Moretti, R., and Rossi-Fanelli, A. (1963), *Biochim. Biophys. Acta 71*, 124.
Brown, W. D., and Mebine, L. B. (1969), *J. Biol. Chem. 244*, 6696.
Bucher, D., Richards, E. G., and Brown, W. D. (1970), *Anal. Biochem. 36, 368.*
Cann, J. R. (1969), *Biochemistry 8*, 4036.
Dickerson, R. E. (1964), *Proteins 2*, 603.
Gottlieb, A. J., Robinson, E. A., and Itano, H. A. (1967), *Arch. Biochem. Biophys. 118*, 693.
Gutter, F. J., Sober, H. A., and Peterson, E. A. (1956), *Arch. Biochem. Biophys. 62*, 427.
Holden, H. F. (1936), *Austr. J. Exp. Biol. Med. Sci. 14*, 291.
Kendrew, J. C. (1962), *Brookhaven Symp. Biol. 15*, 216.
Kiehs, K., Hansch, C., and Moore, L. (1966), *Biochemistry 5*, 2602.
Kurihara, K., and Shibata, K. (1960), *Arch. Biochem. Biophys. 88*, 298.
Lamm, O., and Polson, A. (1936), *Biochem. J. 30*, 528.
Lemberg, R., and Legge, J. W. (1949), Hematin Compounds and Bile Pigments, New York, N. Y., Interscience Publishers, p 176.
Merrett, T. (1966), *Biochim. Biophys. Acta 124*, 389.
Mommaerts, W. F. H. M., and Aldrich, B. B. (1958), *Biochim. Biophys. Acta 28*, 627.
Moore, B., and Roaf, H. E. (1906), *Proc. Roy. Soc., Ser. B 77*, 86.
Morris, C. J. O. R. (1964), *J. Chromatogr. Sci. 16*, 167.
Pauling, L., and Coryell, C. D. (1936), *Proc. Nat. Acad. Sci. U. S. 22*, 159.
Perutz, M. F., Bolton, W., Diamond, R., Muirhead, H., and Watson, H. C. (1964), *Nature (London) 203*, 687.
Richards, E. G., Teller, D. C., and Schachman, H. K. (1968), *Biochemistry 7*, 1054.
Rossi-Fanelli, A., Antonini, E., and Caputo, A. (1964), *Advan. Protein Chem. 19*, 105.
Samejima, T., and Yang, J. T. (1964), *J. Mol. Biol. 8*, 863.
Schoenborn, B. P. (1965), *Nature (London) 208*, 760.
Schoenborn, B. P. (1967), *Nature (London) 214*, 1120.
Schoenborn, B. P., Watson, H. C., and Kendrew, J. C. (1965), *Nature (London) 207*, 128.
Taylor, J. F., Pugh, D. P., and van Osdol, B. F. (1966), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 25*, 928.
Theorell, H., and Ehrenberg, A. (1951), *Acta Chem. Scand. 5*, 823.
Whitaker, J. R. (1963), *Anal. Chem. 35*, 1950.
Wishnia, A., and Pinder, T. W. (1966), Abstracts, Biophysical Society Meeting, Boston, Mass.

Specificity and Mechanism of Clostripain Catalysis*

Perry W. Cole,† Kazuo Murakami,‡ and Tadashi Inagami§

ABSTRACT: The mechanistic basis for the unique specificity of bacterial protease clostripain for arginyl residues in preference to lysyl substrates was studied by the steady-state kinetic method using synthetic substrates and inhibitors. The binding affinity of alkylguanidine, as determined by K_i was two to three orders of magnitude higher than alkylamines. Only a small difference was observed in k_{cat} for an identically substituted pair of arginine and lysine substrates. Therefore, it was proposed that the specific action on arginine substances was due to a specifically elevated affinity for the alkylguanidine structure of its side chain. Contrary to trypsin, no catalysis was observed with a neutral, nonspecific glycine substrate, nor was the inductive activation of catalysis by alkylguanidines or amines demonstrable. Again differing from trypsin, esters of acylhomoarginine and -norarginine were not hydrolyzed. On the other hand, *p*-nitrophenyl *p*-guanidinobenzoate, an inhibitor of trypsin, was hydrolyzed by clostripain. The amide and ester of identically substituted arginines were found to have identical k_{cat} values indicating the deacylation of the acyl-enzyme intermediate is the rate-limiting step. The pH profiles of k_{cat} and k_{cat}/K_m revealed the presence of catalytically functional groups with pK's of 6.7 and 8.2. The energy of activation of the clostripain catalysis was found to be considerably lower than the values for trypsin and papain.

Clostripain (EC 3.4.4.20) isolated from the culture filtrate of *Clostridium histolyticum* is a sulfhydryl protease (Kocholaty and Krejci, 1948). Unlike other sulfhydryl enzymes from plants and animals this enzyme has a very narrow substrate specificity. Ogle and Tytell (1953) have shown that, of all synthetic substrates studied, only esters and amides of arginine and lysine are hydrolyzed. Amides and peptides of neutral amino acids were not attacked. Using chromatographically purified enzyme, Labouesse and Gros (1960) were able to show that clostripain has a rigid substrate specificity analogous to that of trypsin (EC 3.4.4.4). However, in contrast to trypsin, clostripain hydrolyzed arginyl substrates much more readily than identically substituted lysyl substrates. Using a number of peptides and proteins of known sequence, Mitchell and Harrington (1968) have demonstrated that clostripain can be used for the selective cleavage of the arginyl peptide bond without significant lysyl peptide-bond hydrolysis. Mitchell (1968) has also reported that the

* From the Department of Biochemistry, School of Medicine, Vanderbilt University, Nashville, Tennessee 37203. *Received May 24, 1971.* This work has been supported by National Science Foundation Research Grant GB 27583, National Institutes of Health Grant HE 14192, and General Research Support SO1-FR-5424, and constitutes a part of the dissertation submitted by P. W. C. to the Graduate School of Vanderbilt University in partial fulfillment of the requirement of the M. S. degree.

† Recipient of a National Defense Education Act Fellowship. Present address: Department of Biochemistry and Molecular Biology, Louisiana State University School of Medicine, Shreveport, La.

‡ Recipient of a Tennessee Heart Association Postdoctoral Fellowship.

§ To whom to address correspondence.

Arg-Pro peptide linkage, which is hydrolyzed only very slowly by trypsin (Ando *et al.*, 1959; Ishii *et al.*, 1967), is readily hydrolyzed by clostripain.

Elucidation of the mechanism underlying such a uniquely specific action is of great value in understanding the problem of biological specificity. Such a study will be particularly fruitful when carried out in comparison with trypsin which does not differentiate between arginine and lysine. With these objectives in mind a detailed kinetic investigation of clostripain has been undertaken.

Hydrolysis of esters and amides of various analogs of arginine and lysine was studied in a quantitative manner to determine the structural requirement for substrates. The specific action of an enzyme can arise through the selective binding of a substrate to the enzyme as well as by the induction of the active conformation of the enzyme resulting from the specific binding of the substrate. Such a mechanism of interaction between enzyme and substrate can be analyzed by using specific inhibitors possessing part of the substrate structures. The studies on the competitive inhibition of clostripain by guanidine and amine derivatives, which had been known to inhibit trypsin (Inagami, 1964; Mares-Guia and Shaw, 1965; Inagami and York, 1968), revealed a remarkably strong affinity for the guanidine compounds in preference to the amine derivatives suggesting that the unique specificity for arginine substrates is primarily due to the high affinity of clostripain for the guanidino group of the specific substrates.

Materials

Clostripain was prepared from a Worthington crude collagenase preparation lot CLS-95, which contained a high clostripain activity, by the procedure of Mitchell and Harrington (1968). The preparation obtained did not contain collagenase activity as determined by the procedure of Wunsch and Heidrich (1963) using the chromogenic substrate 4-phenylazobenzyloxycarbonyl-L-prolyl-L-leucylglycyl-L-prolyl-D-arginine hydrochloride. The final enzyme preparation showed a specific activity of approximately 50 μM min^{-1} mg of enzyme^{-1} which was reproducible for several preparations. It was stored in solution at $-20°$ without any appreciable loss of activity.

Substrates. Bz-Arg-OEt,[1] Bz-Arg-NH_2, and 4-phenylazobenzyloxycarbonyl-L-prolyl-L-leucylglycyl-L-prolyl-D-arginine hydrochloride were obtained from Mann Research Laboratories, Inc., New York, N. Y.; Bz-Lys-OMe, Bz-Lys-NH_2, Ts-Arg-OMe, and Ts-Arg-NH_2 were products of Cyclo Chemical Corp., Los Angeles, Calif.; NPGB was prepared by the procedure of Chase and Shaw (1969); Ac-Gly-OEt was synthesized according to the procedure of Inagami and Mitsuda (1964); Bz-Arg-NA was synthesized by Nishi *et al.* (1970) and kindly made available for this study.

Inhibitors. Benzamidine was purchased from Aldrich Chemical Co., Milwaukee, Wis.; Ts-Har-OMe and Ts-Nar-OMe were kindly provided by Dr. D. T. Elmore. Methyl-, ethyl-, 1-propyl-, and 1-*n*-butylamine were obtained from Eastman Organic Chemicals, Rochester, N. Y. Their hydrochloride salts were recrystallized and stored in a desiccator. The concentration of the alkylamine solutions was measured by the method of Habeeb (1966) using TNBS. Methyl-, ethyl-, 1-propyl-, and 1-*n*-butylguanidine, synthesized previously in the sulfate form (Inagami and York, 1968), were converted to their hydrochloride salts by passing through a column of Amberlite IR-120 resin in the chloride form.

DTT was purchased from Nutritional Biochemicals, Cleveland, Ohio; Tris was the Ultra Pure grade of Mann Research Laboratories; MES was from Calbiochem., Los Angeles, Calif.; TNBS was obtained from Eastman Organic Chemicals.

Methods

Activation of clostripain by SH compounds was necessary for full enzyme activity. In all rate determinations, the enzyme was preincubated in 10 mM DTT for 16 hr at 10° before use. The activation was slow. The maximum enzyme activity level was attained in approximately 5 hr. It remained essentially at the plateau level thereafter for 72 hr. However, when frozen in the presence of DTT, the enzyme lost its activity completely. Clostripain was also activated by KCN under similar conditions, but was only 80% as active as the preparation activated with DTT.

Determination of the initial rate of hydrolysis of various synthetic substrates was carried out either by the spectrophotometric method (Schwert and Takenaka, 1955), using a Cary 15 recording spectrophotometer or by the potentiometric method (Schwert *et al.*, 1948), using a Radiometer pH-Stat recorder assembly TTT1c-SBR2c. In the spectrophotometric method the initial rate of catalysis was determined with a reaction mixture containing 0.1 M KCl, 3 mM DTT, 40 mM buffer of an appropriate pH, substrate, and clostripain in a cuvet of 1-, 2-, or 10-mm optical path length. The reference cuvet contained all of the components except for the enzyme. In the titrimetric method 3.2–64 μg of the enzyme was used in a 2-ml reaction mixture containing 0.1 M KCl, 3 mM DTT, and substrate. The temperature of the reaction mixture was maintained by a circulating water bath connected to the jacket of the cuvet holder or of the titrator vessel. The protein concentration was determined according to Lowry *et al.* (1951) and a molecular weight of 50,000 (Mitchell and Harrington, 1968) was used to compute the first-order rate constant of the catalysis, k_{cat}.

The $K_{m,app}$ and V_{max} values were obtained from the plot of the initial rate v_0 *vs.* v_0/[S], according to Eadie (1942). The slope of the linear plot, which was identical with $-K_m$ was calculated using the linear regression program 219 of Olivetti Underwood calculator Model Programa 101. The competitive inhibition constant was obtained as described earlier (Inagami, 1964).

Results

The apparent Michaelis constant $K_{m,app}$ and V_{max} were determined for the clostripain-catalyzed hydrolysis of various substrates. The concentration of substrates was varied over a 50-fold range. Results obtained showed the typical Michaelis–Menten kinetics without evidence of substrate inhibition or substrate activation. Plots of initial rate v_0 *vs.* v_0/[S] for Bz-

[1] Abbreviations used are: Ac-Gly-OEt, acetylglycine ethyl ester; Bz-Arg-NA, N^{α}-benzoyl-L-arginine *p*-nitroanilide; Bz-Arg-NH_2, N^{α}-benzoyl-L-argininamide; Bz-Arg-OEt, N^{α}-benzoyl-L-arginine ethyl ester; Bz-Lys-OMe, N^{α}-benzoyl-L-lysine methyl ester; Bz-Lys-NH_2, N^{α}-benzoyl-L-lysinamide; DMF, *N,N*-dimethylformamide; DTT, dithiothreitol; MES, 2-(*N*-morpholino)ethanesulfonic acid; NPGB, *p*-nitrophenyl *p'*-guanidinobenzoate; Ts-Arg-NH_2, N^{α}-*p*-tosyl-L-argininamide; Ts-Arg-OMe, N^{α}-*p*-tosyl-L-arginine methyl ester; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Ts-Har-OMe, N^{α}-*p*-tosyl-L-homoarginine methyl ester; Ts-Nar-OMe, N^{α}-*p*-tosyl-L-norarginine methyl ester; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

TABLE I: Clostripain Catalysis.

Substrate	$K_{m,app}$ (mM)	k_{cat} (sec^{-1})	$k_{cat}/K_{m,app}$ (mM sec^{-1})
Bz-Arg-OEt[a]	0.25	91	364
Bz-Arg-OEt[b]	0.24	92	382
Bz-Arg-NH_2[a]	1.3	87	67
Bz-Arg-NA[c]	0.8	86	108
Bz-Lys-OMe[b,e]	3.0	22	7.3
Bz-Lys-NH_2[d,e]	>10		0.08
Ts-Arg-OMe[a]	0.022	9.8	440
Ts-Arg-NH_2[a]	0.25	10	40
Ac-Gly-OEt[b]		0	
Ts-Nar-OMe[a]		0	
Ts-Har-OMe[a]		0	
NPGB[a]	0.26	31	120

Comparison of Kinetic Constants with Trypsin and Papain

	Trypsin				Papain			
	$K_{m,app}$ (mM)	k_{cat} (sec^{-1})	$k_{cat}/K_{m,app}$ (mM sec^{-1})	Ref	$K_{m,app}$ (mM)	k_{cat} (sec^{-1})	$k_{cat}/K_{m,app}$ (mM sec^{-1})	Ref
Bz-Arg-OEt	0.0043	14.0	3400	*f*	14.5	15.7	1.1	*n*
Bz-Arg-NH_2	2.5	2.8	1.1	*g*	32.3	8.5	0.26	*n*
Bz-Lys-OMe	0.017	17	1000	*h*				
Ts-Arg-OMe	0.013	60	4600	*i*				
Ts-Arg-NH_2	7.4	5.2	0.7	*g*				
Ac-Gly-OEt	800	0.028	3.5×10^{-5}	*j*				
Ts-Nar-OMe	0.69	38	57	*k*				
Ts-Har-OMe	0.29	4	14	*l*				
NPGB	Inhibition			*m*				

[a] Determined spectrophotometrically in a 1-cm cuvet at 25° and pH 7.8 with a 1-ml assay mixture containing 40 mM Tris, 100 mM KCl, 3 mM DTT, and 0.06–0.30 μM enzyme. Absorbance change at 253 and 247 nm was observed with N^{α}-benzoylated substrates and N^{α}-tosylated substrates, respectively. The following difference molar extinction coefficients between the substrates and their hydrolyzed products were used: Bz-Arg-OEt, 1150 cm^{-1} M^{-1} (Trautschold and Werle, 1961); Bz-Arg-NH_2, 750; Ts-Arg-NH_2, 595 (Wang and Carpenter, 1968); Ts-Arg-OMe, 740 (Hummel, 1959); and Bz-Lys-OEt, 1160 (determined in this study). [b] Determined potentiometrically at 25° and pH 7.8 with a 2-ml assay mixture containing 100 mM KCl, 3 mM DTT, and 0.06–1 μM enzyme using 0.20 N NaOH as titrant. [c] Determined spectrophotometrically under the same condition as in footnote *a*. Absorbance changes at 410 nm were observed, and a difference molar extinction coefficient of 8800 (Erlanger *et al.*, 1961). [d] Determined spectrophotometrically with a 1-mm cuvet under the same condition as in footnote *a*. A difference molar extinction coefficient of 750 (Wang and Carpenter, 1968) was used. [e] Determined in 2% dimethylformamide. The stock solutions of these substrates were made in 20% dimethylformamide. [f] Inagami and Sturtevant (1960). [g] Wang and Carpenter (1968). [h] Elmore *et al.* (1967). [i] Curragh and Elmore (1964). [j] Inagami and Mitsuda (1964). [k] Baird *et al.* (1965). [l] Baines *et al.* (1964). [m] Chase and Shaw, 1969). [n] Whitaker and Bender (1965).

Arg-OEt, Bz-Arg-NH_2, Ts-Arg-OMe, and Ts-Arg-NH_2 are shown in Figure 1. Kinetic parameters thus obtained for various substrates hydrolyzed by clostripain are tabulated in Table I. The catalytic rate constant k_{cat} of the ethyl ester of N^{α}-benzoyl-L-arginine (91 sec^{-1}) is practically identical with k_{cat} of its amide (87 sec^{-1}). Its *p*-nitroanilide, Bz-Arg-NA, showed a conspicuous substrate inhibition. However, extrapolation of the uninhibited portion of the Eadie plot allowed the estimation of its k_{cat} as 86 sec^{-1}, which was practically identical with its ester and amide. Similarly k_{cat} for the ester (9.8 sec^{-1}) of N^{α}-tosyl-L-arginine is also practically identical with its corresponding amide (10 sec^{-1}). These results indicate that the deacylation of the acyl-enzyme intermediates, which are common to the ester and the corresponding amide, is the rate-limiting step.

The labile ester NPGB had been found to inhibit trypsin by forming a stable *p*-guanidinobenzoyl-enzyme (Chase and Shaw, 1969). Unlike trypsin, clostripain hydrolyzed this ester at a k_{cat} of 31 sec^{-1} comparable to normal substrates. Since DTT used as an activator of clostripain was found to catalyze the hydrolysis of NPGB, the enzyme activity determination was corrected for this nonenzymatic hydrolysis by running a control experiment at each NPGB concentration.

Ac-Gly-OEt has no amino acid side chain which will be involved in a specific interaction with the enzyme. It was not possible to demonstrate the hydrolysis of this compound by clostripain. Experiments carried out with a large amount of the enzyme (1 μM as compared to 0.06 μM used for arginine substrates) at an unusually elevated concentration of substrate of 0.75 M and at an elevated temperature of 35° failed to

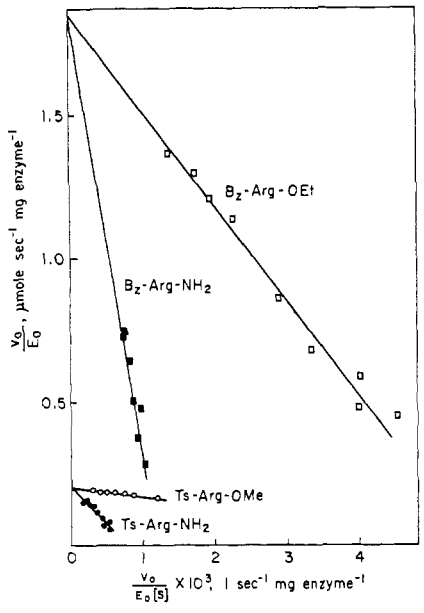

FIGURE 1: Eadie–Hofstee plots of clostripain-catalyzed hydrolyses of various arginine substrates. Initial rate v_0 was determined at 25° and pH 7.8 spectrophotometrically with a 1-ml reaction mixture contining 0.1 M KCl–0.04 M Tris–3 mM DTT. Clostripain was added to 0.06–0.16 μM. Concentration of all four substrates was changed from 0.1 to 0.75 mM.

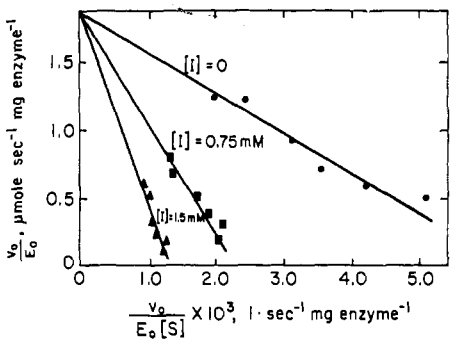

FIGURE 2: Competitive inhibition of clostripain by benzamidine. Assay conditions are identical with those given in Figure 2. The concentration of the substrate Bz-Arg-OEt was changed from 0.1 to 0.75 mM.

detect any hydrolysis beyond the level of a nonenzymatic rate. Under these conditions the reaction with a k_{cat} of 0.05 sec^{-1} should have been easily detected. In trypsin catalysis, the rate of the Ac-Gly-OEt hydrolysis was enhanced by the addition of an alkylamine (Inagami and Murachi, 1964) or an alkylguanidine (Inagami and York, 1968). These compounds did not induce the hydrolysis of Ac-Gly-OEt by clostripain.

The $K_{m,app}$ value of 3.0 mM for the lysine substrate Bz-Lys-OMe is approximately one order of magnitude higher than the $K_{m,app}$ of 0.25 mM for the similarly substituted arginine substrate Bz-Arg-OEt. On the other hand, k_{cat} for the lysine substrate (22 sec^{-1}) was only four times lower than that of Bz-Arg-OEt (91 sec^{-1}). The compounded effect of the unfavorable $K_{m,app}$ and k_{cat} for the lysine substrate is reflected in its specificity constant $k_{cat}/K_{m,app}$ (Zerner *et al.*, 1964a,b), which is 50 times as low as the corresponding value for Bz-Arg-OEt. Unfortunately $K_{m,app}$ for Bz-Lys-NH_2 could not be obtained due to the insufficient solubility of this substrate. The $k_{cat}/K_{m,app}$ was estimated from the second-order rate constant.

Ester substrates derived from homologs of arginine such as homoarginine and norarginine, which contain side chains longer and shorter than arginine by one methylene unit, respectively, were examined. Neither Ts-Har-OMe nor Ts-Nar-OMe was found to be hydrolyzed by clostripain under the conditions in which a catalysis with a k_{cat} of 0.05 sec^{-1} could be easily detected. As will be discussed later, the former was found to be a potent competitive inhibitor. The homoarginine and norarginine esters are hydrolyzed by trypsin (Baird *et al.*, 1965; Baines *et al.*, 1964). Clostripain seems to have a higher degree of specificity than trypsin with respect to the length of the basic amino acid side chain.

Competitive inhibition of clostripain by various analogs of substrates were studied by comparing their K_i values. While maintaining the inhibitor at a fixed concentration, a series of initial rate determinations at different concentrations of Bz-Arg-OEt used as substrate produced v_0 *vs.* $v_0/[S]$ plots compatible with the competitive inhibition as shown by an example in Figure 2. The K_i values, computed from the slope of the plots, are listed in Table II.

It is evident from the data in Table II that the alkylguanidines are stronger inhibitors than their respective counterparts in the amine series by several orders of magnitude. Elongation of the hydrocarbon chain in the alkylguanidines was accompanied by an increase in inhibitory effect, from the K_i of 0.13 mM for methylguanidine to 0.019 mM for 1-*n*-butylguanidine. With the alkylamines there was no such systematic effect due to the size of the hydrocarbon moiety. All of their K_i values fell in the range between 10 and 30 mM.

Ts-Har-OMe was found to inhibit clostripain very strongly with a K_i of 0.026 mM. On the other hand, Ts-Nar-OMe, when tested up to 25 mM, caused no change in the rate of the

TABLE II: Comparison of the Competitive Inhibition of Clostripain and Trypsin by Amines and Guanidines.[g]

Inhibitor	K_i (mM)	
	Clostripain	Trypsin
Ts-Nar-OMe	>25.0[a]	Substrate, K_m = 0.69[c]
Ts-Har-OMe	0.026[a]	Substrate, K_m = 0.29[d]
Benzamidine	0.36[a]	0.18[e]
Alkylguanidines		
Methylguanidine	0.13[a]	11.0[f]
Ethylguanidine	0.054[a]	2.0[f]
Propylguanidine	0.051[a]	0.69[f]
Butylguanidine	0.019[a]	1.7[f]
Alkylamines		
Methylamine	28.0[b]	260.0[f]
Ethylamine	27.0[b]	62.0[f]
Propylamine	8.5[b]	8.7[f]
Butylamine	27.0[b]	1.7[f]

[a] pH 7.8. [b] pH 6.9. [c] Baird *et al.* (1964). [d] Baines *et al.* (1965). [e] Mares-Guia and Shaw (1965). [f] Inagami and York (1968). [g] Initial rates of catalysis were determined spectrophotometrically in a 1-cm cuvet at 25° with a 1-ml assay mixture containing 40 mM Tris, 100 mM KCl, 3 mM DTT, 0.06 μM enzyme, an inhibitor at a fixed concentration, and varying concentrations of substrate Bz-Arg-OEt.

TABLE III: Effect of pH on Kinetic Constants for Clostripain-Catalyzed Hydrolysis of Bz-Arg-OEt.[a]

pH	k_{cat} (sec^{-1})	K_m (M)	Buffer
5.50	83	4.8	Sodium acetate
6.00	79	0.94	Mes
6.47	81	0.30	Mes
6.88	94	0.39	Tris
7.37	90	0.41	Tris
7.89	80	0.45	Tris
8.50	47	0.42	Tris
8.95	15.7	0.35	Sodium borate
9.39	6.6	0.31	Sodium borate

[a] Determined spectrophotometrically in a 1-cm cuvet at 25° with 1-ml assay mixtures containing 50 mM buffers, 0.1 M KCl, 3 mM DTT, 0.1–0.75 mM Bz-Arg-OEt as substrate, and 0.06–0.12 μM enzyme.

hydrolysis of Bz-Arg-OEt. Benzamidine inhibited clostripain with a K_i of 0.36 mM. This was weaker than its inhibition of trypsin, for which the K_i value was 0.018 mM (Mares-Guia and Shaw, 1965).

pH Profile. When the enzyme activity was investigated as a function of pH at a fixed concentration of 0.5 mM of the substrate Bz-Arg-OEt, a sharp optimum was observed near pH 7.0 in agreement with the finding of Ogle and Tytell (1953) and Weil and Kocholaty (1937). Such information on the optimum pH was useful for the purification of the enzyme and some preliminary studies.

In contrast to the results obtained at a fixed concentration of substrate, k_{cat} determined by the Eadie plots over a range of pH produced an entirely different pH profile (Table III). Plot of log k_{cat} for the hydrolysis of Bz-Arg-OEt against pH according to Dixon (1953) gave a profile consisting of a horizontal line over a wide neutral pH range and a sloping straight line in the alkaline pH region (Figure 3a). This straight line intersected with the horizontal line at pH 8.2 and

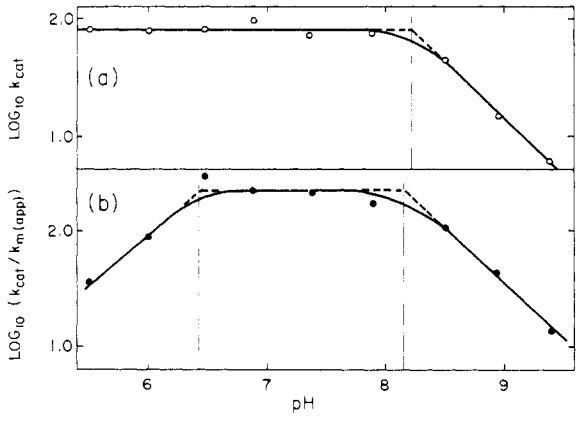

FIGURE 3: (a) Effect of pH on k_{cat} for the clostripain-catalyzed hydrolysis of Bz-Arg-OEt. (b) The pH profile of $k_{cat}/K_{m,app}$. The enzyme activity was determined spectrophotometrically with a 1-ml mixture containing 0.1 M KCl, 0.05 M buffer, 3 mM DTT, and 0.062 M enzyme maintained at 25°. Buffers used are sodium acetate at pH 5.50; MES at pH 6.00 and 6.47; Tris; Tris at pH 6.88, 7.37, 7.89, and 8.50; sodium borate at pH 8.95 and 9.39.

TABLE IV: Effect of Temperature on the Kinetic Parameters of Clostripain Catalysis.[a]

	$K_{m,app}$ (mM)			k_{cat} (sec^{-1})		
Substrate	35°	25°	15°	35°	25°	15°
Bz-Arg-OEt	0.32	0.25	0.24	113	91	67
Bz-Arg-NH_2	1.3	1.3	1.3	113	87	62
Bz-Lys-OEt	2.9	3.0	2.9	32	22	13

[a] Conditions are the same as given in Table Ia.

had a slope of −1, indicating that ionization of a single group with a pK_a of 8.2 is intimately related to the clostripain catalysis. Below pH 5.0 enzymatic activity could not be reproducibly determined presumably due to the denaturation of the enzyme.

The temperature-dependent change in $K_{m,app}$ and k_{cat} was studied at 15, 25, and 35° with three different substrates Bz-Arg-OEt, Bz-Arg-NH_2, and Bz-Lys-OMe. As shown in Table IV the $K_{m,app}$ values of all three substrates were not appreciably affected by temperature. The energy of activation, E_a, was obtained from the slope of the plot of log k_{cat} *vs.* $1/T$ as shown in Table V. Little difference was observed between the E_a values of the lysine ester and the arginine ester, or between the arginine ester substrate and the corresponding amide substrate.

Discussion

One of the most interesting features of clostripain catalysis is its highly restricted substrate specificity. Among the various synthetic substrates tested by Ogle and Tytell (1953), Gros and Labouesse (1960), and Mitchell and Harrington (1968), only those containing arginine and lysine were hydrolyzed by this enzyme. Furthermore, between the two basic amino acids, arginine seems to be strongly preferred. When peptides were used as substrate, it was possible to limit the action of clostripain to arginyl peptide bonds without cleaving lysyl bonds (Mitchell and Harrington, 1968; Shih and Hash, 1971).

It is remarkable that neither Ts-Har-OMe, which has a side chain longer than Ts-Arg-OMe by only one methylene unit, nor Ts-Nar-OMe, which is shorter by one methylene

TABLE V: Comparison of Energy of Activation of Catalysis by Clostripain, Trypsin, Papain, and Bromelain.

Enzyme	E_a (kcal $mole^{-1}$)		
	Bz-Arg-OEt	Bz-Arg-NH_2	Bz-Lys-OMe
Clostripain[a]	7.3	6.0	6.4
Trypsin[b]	11.2	14.9	
Papain[c]	12.8		
Bromelain[d]	7.3	12.8	

[a] Present study. [b] Schwert *et al.* (1948); Schwert and Eisenberg (1949). [c] Hammond and Gutfreund (1959). [d] Inagami and Murachi (1963).

unit, is hydrolyzed by clostripain. As shown in Table I trypsin hydrolyzes these homologs of Bz-Arg-OMe at appreciable rates. Furthermore, the nonspecific substrate Ac-Gly-OEt is not attacked by clostripain, whereas trypsin hydrolyzes this substrate. These results clearly demonstrate that the substrate specificity of clostripain is considerably more restricted than that of trypsin.

The selective attack of arginine in preference to lysine is a feature unique to clostripain not shared by trypsin and many other trypsin-like proteases. However, the ester substrate of N^{α}-benzoyl-L-lysine is hydrolyzed at an appreciable rate. Its amide is also hydrolyzed at a diminished yet finite rate (Table Ia). The k_{cat} value of Bz-Lys-OMe is only four times lower than Bz-Arg-OEt. On the other hand, the $K_{m,app}$ value for the same lysine substrate is ten times higher than the arginine substrate. Thus the difference in these two parameters, when considered separately, does not seem to be large enough to account for the remarkable preference for arginine. However, the specificity constant $k_{cat}/K_{m,app}$ (Zerner *et al.*, 1964a,b), in which the differences in the k_{cat} and $K_{m,app}$ are compounded, gives a 50-fold difference between these two ester substrates. A somewhat larger difference was observed between the amide substrates of N^{α}-benzoyl-L-lysine and N^{α}-benzoyl-L-arginine. On the other hand, trypsin has k_{cat} values of similar magnitude for both Bz-Arg-OEt and Bz-Lys-OMe. The $K_{m,app}$ for the former substrate, however, is three times lower than for the latter. Consequently, there is only a threefold difference in $k_{cat}/K_{m,app}$ (Table Ib).

The preference of clostripain for arginine suggested stronger interaction with the guanidine group of arginine than with the amino group of lysine. The lower $K_{m,app}$ value for the former was considered as a support to such a hypothesis. However, since $K_{m,app}$ is usually not a true dissociation constant of an enzyme–substrate complex, its value cannot be used directly for the comparison of the affinity between the enzyme and various substrates. In contrast to $K_{m,app}$, the inhibition constant, K_i, is the true dissociation constant for an enzyme–inhibitor complex, hence it can be used directly as the index of the affinity of the enzyme to analogs of a substrate acting as an inhibitor. The results summarized in Table II demonstrate the remarkable affinity of clostripain for alkylguanidines. For example, 1-*n*-butylguanidine is bound 1400 times more strongly than 1-*n*-butylamine. Although alkylamines are weaker inhibitors compared with alkylguanidines, their K_i values for clostripain are within the same range of K_i values for trypsin. On the other hand, K_i values for alkylguanidines for the clostripain catalysis are one to two orders of magnitude lower than for trypsin. Clostripain seems to be able to recognize the guanidines and distinguish them from the amines. There seems to be little doubt that such specific affinity for the guanidine group is one of the major factors in shaping the remarkable specificity of this enzyme for arginine. One additional aspect in the comparison of alkylamines to alkylguanidines is that in the guanidine series the size of the alkyl group has a profound effect on the K_i value whereas in the amine series such effect is not apparent. It looks as if the aliphatic chain of a proper dimension significantly enhances the binding affinity of the amino acid side-chain group when the cationic moiety is affixed to the specificity site in a correct manner.

An inductive activation of the catalytic activity by alkylguanidines and alkylamines was postulated as an important mechanism for the specific action of trypsin (Inagami and Murachi, 1964; Inagami and York, 1968). By analogy a similar, strong activation of clostripain by alkylguanidine and a weak activation by alkylamines had been expected. The absence of the detectable activation may be interpreted that such inductive mechanism is not the major factor in determining the specificity of the enzyme. However, it is equally likely that the technique successfully applied to trypsin is not adequate for detecting a similar effect in the clostripain catalysis.

That Ts-Har-OMe is a potent inhibitor rather than a substrate is somewhat analogous to the case of thrombin (Curragh and Elmore, 1964) and is in contrast to trypsin, which hydrolyzes this substrate at a diminished yet finite rate. Ts-Nar-OMe has a very weak affinity for clostripain and is not hydrolyzed by this enzyme, whereas it is a good substrate of trypsin. Benzamidine is a very strong competitive inhibitor for trypsin, but its affinity for clostripain is weaker. These results suggest that the specificity determining site of clostripain would be considerably different from that of trypsin. Elucidation of the structure of the specific binding site of clostripain is awaited. NPGB inhibits trypsin by forming a stable acyl-enzyme intermediate (Chase and Shaw, 1969). Contrary to trypsin, clostripain was found to hydrolyze NPGB continuously. This may be partly due to difference in the reactivity of the intermediate hydroxyl ester and thiol ester formed by the guanidinobenzoylation of trypsin and clostripain respectively, but it is also possible to attribute the difference in the reaction to NPGB to certain difference in the specificity sites of these enzymes.

In the clostripain catalysis the catalytic rate constants k_{cat} for the ester (91 sec^{-1}), amide (87 sec^{-1}), and *p*-nitroanilide (86 sec^{-1}) derivatives of benzoylarginine are essentially identical. Likewise the k_{cat} values for the methyl ester (9.8 sec^{-1}) and amide (10 sec^{-1}) of tosylarginine are also practically identical (Table Ia, Figure 1). Since k_{cat} is identical for the ester, anilide, and amide of identically substituted amino acids, the rate-limiting step for hydrolysis must involve an intermediate common to the amide and the ester. It is most likely that such an intermediate is an acyl-enzyme and that the sequence of the catalysis involves at least three distinct steps similar to those proposed for many other proteases (Gutfreund and Sturtevant, 1956; Hartley and Kilby, 1952; Bender and Kezdy, 1964; Whitaker and Bender, 1965). Other SH-proteases such as papain (Whitaker and Bender, 1965) and bromelain (EC3.4.4.24) (Inagami and Murachi, 1963) hydrolyze the amide substrate Bz-Arg-NH_2 at a considerably reduced rate compared with Bz-Arg-OEt. The present finding that the rate of hydrolysis of Bz-Arg-NH_2 by clostripain is as high as 90 sec^{-1} and is equal to the hydrolysis of Bz-Arg-OEt indicates that this enzyme has an uniquely efficient catalytic mechanism for the hydrolysis of an amide linkage.

The pH profile of k_{cat} of clostripain was found to be essentially constant from pH 6.5 to 8.0. Plot of log k_{cat} *vs.* pH (Figure 3a) according to the method of Dixon (1953) gave two straight lines with their intersection at pH 8.2, indicating that an ionizing group with a pK of 8.2 in the acyl-enzyme intermediate participates in catalysis. The slope of -1 of the straight line on the alkaline side indicates that ionization of only this group is responsible for the profile in the alkaline region. Although clostripain is known to contain an essential SH group at its active site, there are no convincing mechanistic explanation and experimental data which justify the assignment of this pK value to the ionization of the SH group. The possible participation of an imidazole or amino group cannot be excluded. Papain has a bell-shaped pH profile of k_{cat} with pK_1 of 4.04 and pK_2 of 9.10 (Whitaker and Bender, 1965). The presence in clostripain of another ionizing group with a

pK of approximately 4 cannot be excluded. However, investigation of this pH region was not possible due to instability of the enzyme. The pH profile of $k_{cat}/K_{m,app}$, which shows the ionization state of the active site of the free enzyme (Bender *et al.*, 1964), is bell shaped indicating the involvement of acidic and basic groups. The plot of log $(k_{cat}/K_{m,app})$ in Figure 3b indicates the acidic ionizing group has a pK_1 of 6.4 and the latter has a pK_2 of 8.2. Although further work is needed for the identification of these functional groups, the basic group of pK_2 = 8.2 may be the same one which controls k_{cat}. Other SH-proteases such as papain (Smith and Parker, 1958; Whitaker and Bender, 1965) and ficin (Hammond and Gutfreund, 1959) are controlled by a group with a pK_2 of 8.0–8.5. It is likely that a common functional group is responsible for this mechanism. The pK_1 of 6.4 is much higher than pK_1's found in other SH-proteases which range from 4.0 to 4.3 (Smith and Parker, 1958; Whitaker and Bender, 1965; Hammond and Gutfreund, 1959; Inagami and Murachi, 1963). It may be an imidazole group which can be inferred to be in the catalytic site by analogy with papain (Husain and Lowe, 1968). However, participation of a carboxyl group ionizing with such a high pK value can not be excluded.

Very little difference was observed in the activation energy for Bz-Arg-OEt, Bz-Arg-NH_2, and Bz-Lys-OMe (Table V), suggesting that the hydrolysis of these substrates involves a similar intermediate and transition state. It is interesting to note that the activation energy of the clostripain catalysis is only half of that for the papain or trypsin catalysis. The clostripain value is closer to that for the bromelain-catalyzed ester hydrolysis. These results combined with previously discussed data may be interpreted that the clostripain catalysis may involve an intermediate similar to that in the papain catalysis, but the catalytic mechanism for the hydrolysis of this intermediate may be somewhat different.

It is also interesting to note that the $K_{m,app}$ values of Bz-Arg-OEt, Bz-Arg-NH_2, and Bz-Lys-OMe remained practically unchanged between 15 and 35°. This may be explained by assuming equal changes in the numerator and the denominator in $K_{m,app}$. This constant for the hydrolysis of Bz-Arg-OEt by papain was also reported to be insensitive to temperature change, while k_{cat} increased with temperature (Smith and Parker, 1958) in a similar manner as with clostripain.

Acknowledgment

The authors are greatly indebted to Dr. William M. Mitchell for stimulating discussion and for enzyme preparations made available to this study in its initial phase. Generous gifts of tosyl-L-homoarginine methyl ester and tosyl-L-norarginine methyl ester from Dr. D. T. Elmore and benzoyl-L-arginine *p*-nitroanilide from Drs. J. Noguchi, S. Tokura and N. Nishi are gratefully acknowledged.

References

Ando, T., Ishii, S., and Yamasaki, M. (1959), *Biochim. Biophys. Acta 34*, 600.

Arrhenius, S. (1889), *Z. Phys. Chem. 4*, 226.

Baines, N. J., Baird, J. B., and Elmore, T. T. (1964), *Biochem. J. 90*, 470.

Baird, J. B., Curragh, E. F., and Elmore, D. T. (1965), *Biochem. J. 96*, 733.

Bender, M. L., Clement, G. E., Kezdy, F. J., and Heck, H. d'H. (1964), *J. Amer. Chem. Soc. 86*, 3680.

Bender, M. L., and Kezdy, F. (1964), *J. Amer. Chem. Soc. 86*, 3704.

Chase, T., and Shaw, E. (1969), *Biochemistry 8*, 2212.

Curragh, E. F., and Elmore, D. T. (1964), *Biochem. J. 93*, 163.

Dixon, M. (1953), *Biochem. J. 55*, 161.

Eadie, G. S. (1942), *J. Biol. Chem. 146*, 85.

Elmore, D. T., Roberts, D. V., and Smith, J. J. (1967), *Biochem. J. 102*, 728.

Erlanger, B. F., Kokowski, N., and Cohen, W. (1961), *Arch. Biochem. Biophys. 95*, 271.

Gros, P., and Labouesse, B. (1960), *Bull. Soc. Chim. Biol. 42*, 559.

Gutfreund, H., and Sturtevant, J. M. (1956), *Proc. Nat. Acad. Sci. U. S. 42*, 719.

Habeeb, A. F. S. A. (1966), *Anal. Biochem. 14*, 328.

Hammond, B. R., and Gutfreund, H. (1959), *Biochem. J. 72*, 349.

Hartley, B. S., and Kilby, B. A. (1952), *Biochem. J. 50*, 672.

Hummel, B. C. W. (1959), *Can. J. Biochem. Physiol. 37*, 1393.

Husain, S. S., and Lowe, G. (1968), *Biochem. J. 108*, 855.

Inagami, T. (1964), *J. Biol. Chem. 239*, 787.

Inagami, T., and Mitsuda, H. (1964), *J. Biol. Chem. 239*, 1388.

Inagami, T., and Murachi, T. (1963), *Biochemistry 2*, 1439.

Inagami, T., and Murachi, T. (1964), *J. Biol. Chem. 239*, 1395.

Inagami, T., and Sturtevant, J. M. (1960), *J. Biol. Chem. 235*, 1019.

Inagami, T., and York, S. (1968), *Biochemistry 7*, 4045.

Ishii, S., Yamasaki, M., and Ando, T. (1967), *J. Biochem. (Tokyo) 61*, 687.

Kocholaty, W., and Krejci, L. E. (1948), *Arch. Biochem. 18*, 1.

Labouesse, B., and Gros, P. (1960), *Bull. Soc. Chim. Biol. 42*, 543.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. H. (1951), *J. Biol. Chem. 193*, 265.

Mares-Guia, M., and Shaw, E. (1965), *J. Biol. Chem. 240*, 1579.

Mitchell, W. M. (1968), *Science 162*, 374.

Mitchell, W. M., and Harrington, W. F. (1968), *J. Biol. Chem. 243*, 4683.

Nishi, N., Tokura, S., and Noguchi, J. (1970), *Bull. Chem. Soc. Jap. 43*, 2900.

Ogle, J. D., and Tytell, A. A. (1953), *Arch. Biochem. Biophys. 42*, 327.

Schwert, G. W., and Eisenberg, M. A. (1949), *J. Biol. Chem. 179*, 665.

Schwert, G. W., Neurath, H., Kaufman, S., and Snoke, J. E. (1948), *J. Biol. Chem. 172*, 221.

Schwert, G. W., and Takenaka, Y. (1955), *Biochim. Biophys. Acta 16*, 570.

Shih, J. W., and Hash, J. H. (1971), *J. Biol. Chem. 246*, 994.

Smith, E. L., and Parker, M. J. (1958), *J. Biol. Chem. 233*, 1387.

Trautschold, I., and Werle, E. (1961), *Hoppe-Seyler's Z. Physiol. Chem. 325*, 48.

Wang, S. S., and Carpenter, F. H. (1968), *J. Biol. Chem. 243*, 3702.

Weil, L., and Kocholaty, W. (1937), *Biochem. J. 31*, 1255.

Whitaker, J. R., and Bender, M. L. (1965), *J. Amer. Chem. Soc. 87*, 2728.

Wunsch, E., and Heidrich, H. G. (1963), *Hoppe-Seyler's Z. Physiol. Chem. 333*, 149.

Zerner, B., Bond, R. P. M., and Bender, M. L. (1964a), *J. Amer. Chem. Soc. 86*, 3674.

Zerner, B., Bond, R. P. M., and Bender, M. L. (1964b), *J. Amer. Chem. Soc. 86*, 3669.