Active Site Mapping of the Serine Proteases Human Leukocyte Elastase, Cathepsin G, Porcine Pancreatic Elastase, Rat Mast Cell Proteases I and II, Bovine Chymotrypsin A_α, and *Staphylococcus aureus* Protease V-8 Using Tripeptide Thiobenzyl Ester Substrates†

J. Wade Harper, Robert R. Cook, C. Justine Roberts,‡ Beth J. McLaughlin, and James C. Powers*

ABSTRACT: The primary subsite specificities of human leukocyte elastase, cathepsin G, porcine pancreatic elastase, rat mast cell proteases I and II, bovine chymotrypsin A_α, and the protease from strain V-8 of *Staphylococcus aureus* have been mapped with a series of tripeptide thiobenzyl ester substrates of the general formula Boc-Ala-Ala-AA-SBzl, where AA represents one of 13 amino acids. In addition, the effects of a P_2 Pro and P_4 methoxysuccinyl and succinyl groups were investigated. In an attempt to introduce specificity and/or reactivity into the substrate Boc-Ala-Ala-Leu-SBzl(X), the 4-chloro-, 4-nitro-, and 4-methoxythiobenzyl ester derivatives were studied. Enzymatic hydrolyses of the substrates were measured in the presence of 4,4′-dithiobis(pyridine) or 5,5′-dithiobis(2-nitrobenzoic acid), which provided a highly sensitive assay method for free thiol. The thio esters were excellent substrates for the enzymes tested, and in many cases, the best substrates reported here have k_{cat}/K_M values higher than those reported previously. The best substrate for human leukocyte elastase was Boc-Ala-Pro-Nva-SBzl(Cl), which has a k_{cat}/K_M of 130×10^6 M^{-1} s^{-1}. A very reactive rat mast cell protease substrate, Boc-Ala-Ala-Leu-SBzl(NO_2), was also found. The *S. aureus* V-8 protease was the most specific enzyme tested since it hydrolyzed only Boc-Ala-Ala-Glu-SBzl. Substituents on the thiobenzyl ester moiety of Boc-Ala-Ala-Leu-SBzl resulted in decreased K_M values with human leukocyte elastase and rat mast cell protease I when compared to the unsubstituted derivative. The 4-nitro group caused a 7-fold increase in reactivity (k_{cat}/K_M) toward rat mast cell protease I, and the 4-chloro derivative was 3.3-fold more reactive than the unsubstituted derivative toward human leukocyte elastase. This series could prove useful in mapping the active site of newly isolated serine proteases and has the advantage of being highly sensitive, thereby requiring smaller quantities of enzyme than other methods.

Serine proteases are a class of endopeptidases with very broad specificities toward synthetic substrates. These enzymes can be grouped into three very general catagories on the basis of their primary subsite specificities: chymotrypsin-like, trypsin-like, and elastase-like. Serine proteases are involved in a number of important cellular processes including blood coagulation, complement activation, phagocytosis, and turnover of damaged cell tissue. For example, human leukocyte (HL)[1] elastase and cathepsin G, which are located in the granule fraction of human leukocytes, are thought to be responsible for the elastin degradation that occurs in emphysema as well as the processing of damaged lung tissue (Powers, 1983). The two chymotrypsin-like enzymes, rat mast cell proteases I and II, have been isolated from typical and atypical mast cells, respectively, and have been implicated in processes such as chemotaxis and degradation of connective tissue (Woodbury & Neurath, 1980).

Synthetic peptide substrates are a valuable tool for determining enzyme specificity as well as detecting enzymes during isolation. The most convenient peptide substrates for serine proteases include peptide 4-nitroanilides, peptide thio esters, and peptide 7-amino-4-methylcoumarins. Enzymatic hydrolysis of thio esters is often much faster than with the corresponding nitroanilide or aminomethylcoumarin substrate. In addition, thiol release is easily detected by inclusion of 4,4′-dithiobis(pyridine) in the assay mixture. Therefore, peptide thio esters have distinct advantages over amide substrates such as 4-nitroanilides when limited amounts of enzyme are avaliable.

A number of studies with thio ester substrates have been reported. Amino acid and peptide thio esters have been used as substrates for chymotrypsin-like enzymes (Hirohara et al., 1974; Farmer & Hageman, 1975; Harper et al., 1982), trypsin-like enzymes (Green & Shaw, 1979), human tryptases (Tanaka et al., 1983), human leukocyte and porcine pancreatic elastases (Castillo et al., 1979), complement proteases (McRae et al., 1981a), proteases involved in blood coagulation (McRae et al., 1981b; Cho et al., 1984), and leucine aminopeptidase (Metrione, 1972). These studies indicated that peptide thio ester substrates are generally much more sensitive than the corresponding amide substrates.

In this paper, we report the hydrolysis of a series of 13 tripeptide thiobenzyl esters of the general formula Boc-Ala-Ala-AA-SBzl (where AA is one of 13 amino acids) by human leukocyte elastase, cathepsin G, porcine pancreatic elastase, rat mast cell proteases I and II, bovine chymotrypsin A_α, and the protease from strain V-8 of *Staphylococcus aureus* (Drapeau, 1976). In addition, the effects of P_2[2] proline and P_4 methoxysuccinyl and succinyl, as well as substituents on the thiobenzyl moiety, were investigated. This is the first

† From the School of Chemistry, Georgia Institute of Technology, Atlanta, Georgia 30332. *Received November 17, 1983.* This research was supported by the Council for Tobacco Research and by a grant to the Georgia Institute of Technology (HL 29307) from the National Institutes of Health.

‡ Coca-Cola Summer Undergraduate Research Student for 1983.

[1] Abbreviations: HL, human leukocyte; PP, porcine pancreatic; RMCP I, rat mast cell protease I; RMCP II, rat mast cell protease II; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Boc, *tert*-butyloxycarbonyl; NA, 4-nitroanilide; $NHC_6H_4CH_3$, 4-methylanilide; SBzl, $-SCH_2C_6H_5$; SBzl(Cl), $-SCH_2C_6H_4$-4-Cl; SBzl(NO_2), $-SCH_2C_6H_4$-4-NO_2; SBzl(OCH_3), $-SCH_2C_6H_4$-4-OCH_3; Nva, norvaline; Nle, norleucine; Met(O), methionine sulfoxide; MeO-Suc, methoxysuccinyl; Suc, succinyl; TLC, thin-layer chromatography.

[2] The nomenclature for the individual amino acid residues (P_3, P_2, and P_1) of a substrate and for the subsites (S_3, S_2, and S_1) of an enzyme is that of Schechter & Berger (1967).

0006-2960/84/0423-2995$01.50/0

Table I: Physical Properties of Tripeptide Thiobenzyl Ester Substrates

substrate	mp (°C)	R_f[a]	C, H, N anal. (calcd; found)
Boc-Ala-Ala-Ala-SBzl[b]	166–168	0.57	56.47, 7.22, 9.41; 56.48, 7.20, 9.38
Boc-Ala-Ala-Val-SBzl	102–103	0.53	59.38, 7.58, 9.02; 59.46, 7.63, 9.01
Boc-Ala-Ala-Nva-SBzl	140–141	0.61	59.38, 7.58, 9.02; 59.58, 7.62, 9.14
MeO-Suc-Ala-Ala-Nva-SBzl	160–162	0.66[c]	57.59, 6.99, 8.76; 57.47, 7.00, 8.73
Suc-Ala-Ala-Nva-SBzl	181–184	0.60[c]	56.75, 6.71, 9.03; 56.62, 6.74, 8.99
Boc-Ala-Pro-Nva-SBzl(Cl)	68–70	0.47[d]	57.06, 6.91, 7.99; 56.94, 6.95, 7.94
Boc-Ala-Ala-Leu-SBzl	123–124	0.64	60.10, 7.78, 8.76; 60.38, 7.84, 8.83
Boc-Ala-Ala-Leu-SBzl(NO_2)	153–154	0.55	54.95, 6.92, 10.68; 54.68, 6.96, 10.56
Boc-Ala-Ala-Leu-SBzl(Cl)	152–153	0.55	56.07, 7.06, 8.17; 55.88, 7.06, 8.11
Boc-Ala-Ala-Leu-SBzl(OCH_3)	143–144	0.55	58.91, 7.71, 8.25; 59.02, 7.76, 8.21
Boc-Ala-Ala-Nle-SBzl	143–145	0.62	60.10, 7.78, 8.76; 59.96, 7.81, 8.73
Boc-Ala-Ala-Ile-SBzl	60–64	0.72	60.10, 7.78, 8.76; 60.04, 7.79, 8.75
Boc-Ala-Ala-Phe-SBzl	113–115	0.66	63.14, 6.86, 8.18; 63.25, 6.92, 8.15
Boc-Ala-Ala-Met-SBzl[b]	65–67	0.50	55.51, 7.09, 8.44; 55.65, 7.14, 8.39
Boc-Ala-Ala-Met(O)-SBzl	160–161	0.41	52.85, 6.94, 8.04; 52.67, 6.86, 7.96
Boc-Ala-Ala-Ser-SBzl[e]	127–129	0.28	53.48, 7.05, 8.91; 53.43, 7.06, 8.89
Boc-Ala-Ala-Asn-SBzl	149–152	0.32	54.98, 6.71, 11.66; 54.71, 6.75, 11.59
Boc-Ala-Ala-Glu-SBzl[b]	100–103	0.33	54.74, 6.79, 8.33; 54.71, 6.63, 8.28
Boc-Ala-Ala-Pro-SBzl	85–90	0.50	59.59, 7.17, 9.06; 59.50, 7.22, 9.05

[a] Solvent system was $CHCl_3$/MeOH (9:1) unless otherwise specified. [b] Contains 0.5 water of crystallization. [c] Solvent system was $CHCl_3$/MeOH (5:1). [d] Solvent was diethyl ether. [e] Contains 1 water of crystallization.

report of primary subsite mapping of chymotrypsin- and elastase-like proteases with peptide thiobenzyl ester substrates. This series contains some of the most reactive substrates yet reported for these enzymes.

Materials and Methods

Human leukocyte elastase (Baugh & Travis, 1976) and cathepsin G (Travis et al., 1978) were generous gifts from Dr. James Travis and his research group at the University of Georgia and were shown to be homogeneous by SDS gel electrophoresis. RMCPs I and II were kindly provided by Dr. Richard Woodbury and Dr. Hans Neurath of the University of Washington and were prepared as previously described (Woodbury et al., 1981). Both rat mast cell proteases showed a single band on SDS gel electrophoresis and a single N-terminal amino acid sequence. Crystalline chymotrypsin A_α, PP elastase, and protease V-8 were purchased from Sigma Chemical Co., St. Louis, MO, and were of the highest purity avaliable. The 4,4'-dithiobis(pyridine) (Aldrithiol-4), 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent), and Hepes were purchased from Aldrich Chemical Co., Milwaukee, WI.

The active site concentration of each enzyme was determined by rate assays with synthetic substrates whose kinetic constants were based on titrated enzyme, except protease V-8, which was based on dry weight with an M_r of 27 000 (Drapeau, 1978). In general, hydrolysis rates at two or three substrate concentrations were measured under the appropriate conditions, and the measured velocities along with previously determined kinetic constants were used to calculate enzyme concentrations. The concentration of HL and PP elastase was determined with either MeO-Suc-Ala-Ala-Pro-Val-NA or the corresponding thiobenzyl ester (Castillo et al., 1979). The concentration of HL cathepsin G and chymotrypsin A_α was determined with Suc-Val-Pro-Phe-NA (K_M = 1.4 mM and k_{cat} = 9.6 s^{-1} and K_M = 0.079 mM and k_{cat} = 20 s^{-1}, respectively; 0.1 M Hepes, pH 7.5, 0.5 M NaCl, 10% v/v Me_2SO; unpublished results from this laboratory). The concentrations of both rat mast cell proteases were determined with Suc-Phe-Pro-Phe-NA in 50 mM phosphate buffer, pH 8.0, and 10% v/v Me_2SO (Yoshida et al., 1980).

Synthesis. All Boc amino acids as well as 1-hydroxytriazole were obtained from Chemical Dynamics Corp., South Plainfield, NJ. Carbonyldiimidazole, dicyclohexylcarbodiimide, (4-methoxybenzyl)mercaptan, (4-chlorobenzyl)mercaptan, and benzylmercaptan were purchased from Aldrich Chemical Co., Milwaukee, WI. The (4-nitrobenzyl)mercaptan was a product of Parish Chemical Co., Ovem, UT. The synthesis of CF_3CO-Lys-Ala-$NHC_6H_4CH_3$ has been reported (Renuad et al., 1983). Succinylations and methoxysuccinylations were carried out as previously described (Nakajima et al., 1979; Harper et al., 1981). Boc amino acid thiobenzyl esters, except for Boc-Ser-SBzl, were prepared by the carbonyldiimidazole method as described below for Boc-norvaline benzyl thio ester. Peptide couplings were generally carried out with dicyclohexylcarbodiimide as the coupling reagent. Some representative syntheses are given as are any syntheses of compounds not prepared by these general methods. The physical properties for each tripeptide thiobenzyl ester are summarized in Table I. Elemental analyses were performed by Atlantic Microlabs, Atlanta, GA. The assigned structures are consistent with their NMR spectra.

tert-Butyloxycarbonylnorvaline Benzyl Thio Ester. *tert*-Butyloxycarbonylnorvaline (10 mmol) was dissolved in dry tetrahydrofuran (10 mL), carbonyldiimidazole (1.62 g, 10 mmol) added, and the reaction stirred at 0 °C for 0.75 h. Benzylmercaptan (1.16 mL, 10 mmol) was added and the reaction mixture allowed to warm to room temperature overnight. After the solvent was removed under vacuum, ethyl acetate (20 mL) was added and the product washed with 10% citric acid, 4% $NaHCO_3$, and saturated aqueous NaCl. The ethyl acetate solution was then dried over $MgSO_4$ and filtered and the solvent removed. The solid residue was collected from hexane or petroleum ether (80% yield). The product gave one spot by TLC ($CHCl_3$/CH_3OH 5:1 and 9:1) and was used for the subsequent reactions without further purification.

tert-Butyloxycarbonylserine Benzyl Thio Ester. *tert*-Butyloxycarbonylserine (10 mmol), 1-hydroxybenzotriazole (0.135 g, 1.0 mmol), and benzylmercaptan (1.16 mL, 10 mmol) were dissolved in 20 mL of dimethylformamide and cooled to 0 °C. Dicyclohexylcarbodiimide (2.17 g, 10.5 mmol) was added and the mixture stirred at 0 °C for 12 h. The dicyclohexylurea precipitate was removed by filtration and the product washed as described for the carbonyldiimidazole procedure.

Norvaline Benzyl Thio Ester Hydrochloride. *tert*-Butyloxycarbonylnorvaline benzyl thio ester (3 mmol) was treated with 10 equiv of 2.2 N HCl in dioxane and allowed to stir for

0.75 h at 25 °C. The solvent was removed and ether added to solidify the product. The resulting hydrochloride salt was dried for 3 h under vacuum in the presence of KOH and P_2O_5. The product was used without further purification.

tert-Butyloxycarbonylalanylalanylnorvaline Benzyl Thio Ester. Norvaline benzyl thio ester hydrochloride (3 mmol), *tert*-butyloxycarbonylalanylalanine (0.781 g, 3.0 mmol), 1-hydroxybenzotriazole (0.608 g, 4.5 mmol), and triethylamine (0.42 mL, 3.0 mmol) were dissolved in 10 mL of dimethylformamide, and the solution was cooled to −10 °C in an ice/water/salt bath. Dicyclohexylcarbodiimide (0.678 g, 3.3 mmol) was added and the reaction stirred for 2 h at −10 °C. After the mixture stirred overnight at 25 °C, the dicyclohexylurea was filtered off, the solvent removed under reduced pressure, and the residue washed as described above. Recrystallization was accomplished with $CHCl_3$/petroleum ether with cooling.

tert-Butyloxycarbonylalanylalanylglutamic Acid α-Benzyl Thio Ester. Glutamic acid γ-*tert*-butyl α-benzyl thio ester hydrochloride (0.91 g, 2.6 mmol) and *tert*-butyloxycarbonylalanine hydroxysuccinimide ester (0.76 g, 2.6 mmol) were dissolved in 8 mL of acetonitrile. *N*-Methylmorpholine (0.58 mL, 5.28 mmol) was added and the reaction stirred overnight at 0 °C. Typical workup gave 0.87 g (68% yield) of *tert*-butyloxycarbonylalanylglutamic acid γ-*tert*-butyl α-benzyl thio ester, R_f = 0.70 ($CHCl_3$/CH_3OH, 5:1). The blocked compound was treated with 10 equiv of 50% trifluoroacetic acid in methylene chloride for 0.5 h at 0 °C. The reaction was allowed to warm to room temperature and stirred for 5 h. The solvent was removed and the residue dried overnight to yield alanylglutamic acid- α-benzyl thio ester trifluoroacetate as a foam (77%), R_f = 0.80 (BuOH/HOAc/H_2O 5:2:1). The deblocked derivative was treated with *tert*-butyloxycarbonylalanine hydroxysuccinimide ester as described above to yield 0.2 g (33%) final product.

tert-Butyloxycarbonylalanylalanylmethionine (Sulfoxide) Benzyl Thio Ester. *tert*-Butyloxycarbonylalanylalanylmethionine benzyl thio ester (0.37 g, 0.75 mmol) was dissolved in 6 mL of methanol and 30% H_2O_2 (0.030 mL, 1.05 mmol) added. After the reaction was allowed to stir for 12 h, 0.060 mL of H_2O_2 was added, and after 8 h, another 0.070 mL was added. After a total of 65 h, the reaction was 90% complete by TLC. The solvent was removed and the product purified via flash column chromatography on silica gel with methylene chloride as the eluant. The isolated yield was 50%.

Kinetic Measurements. The enzymatic hydrolysis of the thio ester substrates was measured with either 4,4′-dithiobis(pyridine) (Grassetti & Murray, 1967) or 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman, 1959). Assays were conducted at 25 °C in 0.1 M Hepes buffer, pH 7.5, containing 0.5 M NaCl and 10% v/v Me_2SO. Stock solutions of substrate were prepared in Me_2SO and stored at 0 °C. Aliquots of substrate stock solutions were diluted with buffer immediately before use. To a cuvette containing 2.2 mL of buffered substrate solution was added 0.025 mL of a 15 mM solution of 4,4′-dithiobis(pyridine) [or 5,5′-dithiobis(2-nitrobenzoic acid)] in Me_2SO. The spectrophotometer was zeroed and the initial absorbance increase measured upon the addition of a 10–50-μL aliquot of enzyme solution (0.2–20.0 nM final concentration). Absorbance increases were monitored at 324 (ϵ = 19 800 M^{-1} cm^{-1}) and 412 nm (ϵ = 13 600 M^{-1} cm^{-1}) for 4,4′-dithiobis-(pyridine) and 5,5′-dithiobis(2-nitrobenzoic acid), respectively. Background hydrolysis was negligible in most cases but, when significant, was corrected for by including substrate and thiol reagent in the reference cuvette. Between five and nine substrate concentrations were examined. Hydrolysis rates of Boc-Ala-Pro-Nva-SBzl(Cl) by HL elastase were measured in the presence of 0.15 mM CF_3CO-Lys-Ala-$NHC_6H_4CH_3$ and the kinetic constants determined as discussed under Results. The initial slopes were converted into molar rates and the kinetic constants obtained from Hanes plots (Hanes, 1932). Most correlation coefficients were 0.99 or greater and none were less than 0.98. In cases where the slope of the Hanes plot was near zero, the slope of a Lineweaver–Burk plot was used to estimate k_{cat}/K_M. This occurred when substrate solubility was apparently much less than K_M. A Beckman 35 spectrophotometer was used throughout the investigation.

Results

The kinetic constants for hydrolysis of several tripeptide thiobenzyl ester substrates by HL and PP elastases are given in Table II. Substrate solubility made it possible to determine only k_{cat}/K_M values for PP elastase catalyzed hydrolysis of Boc-Ala-Ala-Leu–SBzl(X) (X = Cl, OCH_3) since the K_M values are apparently much higher than the solubility limit (0.15 mM with 10% Me_2SO) and the Lineweaver–Burk plots went through the origin. The kinetic parameters for hydrolysis of several thiobenzyl ester substrates by HL cathepsin G, RMCPs I and II, and chymotrypsin A_α are given in Table III. As expected, the best substrates contained aromatic or bulky amino acids in P_1. The kinetic constants for the hydrolysis of Boc-Ala-Ala-Asn-SBzl by RMCP I could not be obtained due to severe curvature of the Hanes plot. While protease V-8 from *S. aureus* hydrolyzed Boc-Ala-Ala-Glu-SBzl (k_{cat}/K_M = 410 000 M^{-1} s^{-1}), tripeptide thiobenzyl esters containing uncharged polar amino acids (Asn, Ser) and alkyl amino acids (Leu, Nva, Ala) in P_1 were not hydrolyzed under the conditions employed (hydrolysis rates less than 5×10^{-12} M s^{-1} at [E] = 0.27 nM).

Thio ester hydrolysis rates were measured continuously by reaction of the released thiol with 4,4′-dithiobis(pyridine) or 5,5′-dithiobis(2-nitrobenzoic acid) present in the reaction mixture. The products of these reactions, 4-thiopyridone and the 3-carboxy-4-nitrothiophenoxide anion, have extremely high extinction coefficients (ϵ_{324} = 19 800 M^{-1} cm^{-1} and ϵ_{412} = 13 600 M^{-1} cm^{-1}, respectively), which allow hydrolysis rates with substrate concentrations as low as 1 μM to be measured with good reproducibility.

As shown in Table II, Boc-Ala-Pro-Nva-SBzl(Cl) binds very tightly to HL elastase (K_M = 0.08 μM). Preliminary investigation of this substrate with HL elastase indicated that the maximal velocity was maintained at substrate concentrations as low as 1–2 μM. Since measurement of hydrolysis rates of substrate concentrations lower than 1 μM is difficult due to small absorbance increases, the experimental procedures were modified in order to obtain a value for K_M. In the presence of a competitive inhibitor, the Michaelis-Menten equation takes the form $v = k_{cat}[E][S]/([S] + K_{M,app})$ where $K_{M,app} = K_M(1 + [I]/K_I)$. Therefore, inclusion of a suitably high concentration of a good competive inhibitor would result in a measureable $K_{M,app}$, which would allow K_M to be estimated. We chose to use CF_3CO-Lys-Ala-$NHC_6H_4CH_3$ (Renaud et al., 1983) as the competitive inhibitor, which has a K_I of 10 μM at pH 7.5, 10% Me_2SO. Inclusion of 0.15 mM inhibitor would raise K_M by a factor of 16. Under these conditions, we found $K_{M,app}$ to be 1.2 μM (K_M = 0.08 μM). This value is only an estimate at best due to the inherent inaccuracies of measuring hydrolysis rates at very low substrate concentrations.

The spontaneous hydrolysis of several peptide thiobenzyl esters was investigated at pH 7.5 (10% v/v Me_2SO). Boc-Ala-Ala-Nva-SBzl, Boc-Ala-Ala-Glu-SBzl, and Boc-Ala-

Table II: Kinetic Constants for the Hydrolysis of Tripeptide Thio Ester Substrates by Human Leukocyte Elastase and Porcine Pancreatic Elastase[a]

substrate P_4 P_3 P_2 P_1	kinetic constants[b]	enzyme: HL elastase	enzyme: PP elastase
Boc-Ala-Ala-Ala-SBzl	K_M	87	100
	k_{cat}	63	73
	k_{cat}/K_M	0.73	0.74
Boc-Ala-Ala-Val-SBzl	K_M	3.0	95
	k_{cat}	9.0	63
	k_{cat}/K_M	3.0	0.66
Boc-Ala-Ala-Nva-SBzl	K_M	2.2	84
	k_{cat}	20	570
	k_{cat}/K_M	9.2	6.8
MeO-Suc-Ala-Ala-Nva-SBzl	K_M	7.7	64
	k_{cat}	25	310
	k_{cat}/K_M	3.3	4.9
Suc-Ala-Ala-Nva-SBzl	K_M	590	42
	k_{cat}	110	150
	k_{cat}/K_M	0.18	3.7
Boc-Ala-Pro-Nva-SBzl(Cl)	K_M	0.08	100
	k_{cat}	10	230
	k_{cat}/K_M	130	2.2
Boc-Ala-Ala-Leu-SBzl	K_M	16	180
	k_{cat}	34	810
	k_{cat}/K_M	2.2	4.4
Boc-Ala-Ala-Leu-SBzl(NO_2)	K_M	12	210
	k_{cat}	47	680
	k_{cat}/K_M	4.0	3.2
Boc-Ala-Ala-Leu-SBzl(Cl)	K_M	5.2	
	k_{cat}	38	
	k_{cat}/K_M	7.4	3.3[c]
Boc-Ala-Ala-Leu-SBzl(OCH_3)	K_M	11	
	k_{cat}	34	
	k_{cat}/K_M	3.0	2.9[c]
Boc-Ala-Ala-Nle-SBzl	K_M	11	70
	k_{cat}	20	210
	k_{cat}/K_M	1.8	3.0
Boc-Ala-Ala-Ile-SBzl	K_M	1.4	7.6
	k_{cat}	5.4	7.5
	k_{cat}/K_M	3.9	0.98
Boc-Ala-Ala-Phe-SBzl	K_M	NR[d]	NR
	k_{cat}		
	k_{cat}/K_M		
Boc-Ala-Ala-Met-SBzl	K_M	96	42
	k_{cat}	82	71
	k_{cat}/K_M	0.86	1.7
Boc-Ala-Ala-Met(O)-SBzl	K_M	159	190
	k_{cat}	0.64	27
	k_{cat}/K_M	0.004	0.15
Boc-Ala-Ala-Ser-SBzl	K_M	290	250
	k_{cat}	66	110
	k_{cat}/K_M	0.23	0.43
Boc-Ala-Ala-Asn-SBzl	K_M	NR	NR
	k_{cat}		
	k_{cat}/K_M		
Boc-Ala-Ala-Glu-SBzl	K_M	NR	NR
	k_{cat}		
	k_{cat}/K_M		
Boc-Ala-Ala-Pro-SBzl	K_M	NR	NR
	k_{cat}		
	k_{cat}/K_M		

[a] Hepes buffer (0.1 M), pH 7.5, 0.5 M NaCl, and 10% v/v Me_2SO at 25 °C. Enzyme concentrations: 0.4–5.7 nM HL elastase; 0.3–6.8 nM PP elastase. [b] The units of k_{cat}, K_M, and k_{cat}/K_M are s^{-1}, μM, and 10^{-6} M^{-1} s^{-1}, respectively. [c] Only the ratio k_{cat}/K_m could be calculated due to substrate solubility. [d] NR, no reaction (hydrolysis rates were less than 5×10^{-12} M s^{-1} at substrate concentrations from 100 to 200 μM).

Ala-Leu-SBzl(X) (X = OCH_3, H) showed no release of thiol over a 40-min period at concentrations approaching their solubility limit (0.15 mM; less than 1% hydrolysis could have been detected under the conditions used). Boc-Ala-Ala-Leu-SBzl(NO_2) and Boc-Ala-Ala-Ser-SBzl decomposed slowly ($t_{1/2}$ = 160 min) while Boc-Ala-Ala-Asn-SBzl decomposed more rapidly ($t_{1/2}$ = 35 min).

Discussion

While several amino acid and peptide thio ester substrates have been reported for chymotrypsin-like and elastase-like proteases, most of these structures are analogous to previously known substrates with 4-nitroanilide or 4-nitrophenol leaving groups. Since no systematic investigation of the effects of the P_1 amino acid in peptide thio ester substrates on hydrolysis by chymotrypsin-like and elastase-like enzymes has been reported, we decided to synthesize a series of tripeptide thiobenzyl esters of the general sequence Boc-Ala-Ala-AA-SBzl, where AA represents one of 13 amino acids. As this study developed, we decided to investigate P_2 by replacing Ala with Pro and P_4 by replacing Boc with methoxysuccinyl and succinyl in the substrate Boc-Ala-Ala-Nva-SBzl(X) (X = H, Cl). In an attempt to increase selectivity and/or reactivity of Boc-Ala-Ala-Leu-SBzl(X), we replaced the P_1' thiobenzyl moiety with the 4-nitro-, 4-chloro-, and 4-methoxythiobenzyl groups.

Thio ester substrates have several advantages over amide substrates such as peptide 4-nitroanilides. The K_M values for thio esters are generally lower than those for amide substrates, which allows smaller quantities of substrate to be used in routine assays. In addition, the high reactivity of thio esters coupled with the high extinction coefficient of the thiol reagent allows smaller quantities of enzyme to be used than with amide substrates. For example, as little as 2 pM HL elastase can be assayed with MeO-Suc-Ala-Ala-Pro-Val-SBzl (Castillo et al., 1979) and as little as 1 pM RMCP I can be detected with Suc-Ala-Ala-Pro-Phe-SBzl (Harper et al., 1981). Kinetic measurements can be made in both the visible and ultraviolet wavelength regions, which may be beneficial when highly chromophoric species are present in the assay mixture.

Human Leukocyte Elastase. Human leukocyte elastase has received a great deal of attention lately, primarily because of its important role in the destruction of lung elastin, which occurs in emphysema (Powers, 1983). This enzyme is normally involved in turnover of dead lung tissue, but its leakage from leukocytes can result in severe elastin damage.

Several studies with peptide 4-nitroanilide substrates and peptide chloromethyl ketone inhibitors indicate that this enzyme prefers substrates with relatively small alkyl side chains such as Val in P_1 (Marossy et al., 1980; Nakajima et al., 1979; Powers et al., 1977; Zimmerman & Ashe, 1977; McRae et al., 1980). This was also found to be the case with the thio ester substrates investigated here (Table II). The best substrate for HL elastase, Boc-Ala-Pro-Nva-SBzl(Cl), has a k_{cat}/K_M of 130×10^6 M^{-1} s^{-1}, which is 110-fold higher than the most reactive substrate previously reported, MeO-Suc-Ala-Ala-Pro-Val-SBzl (Castillo et al., 1979). Substitution of Ala for the P_2 Pro results in a 14-fold decrease in k_{cat}/K_M primarily because of the increase in K_M from 0.08 to 2.2 μM. Substrates with Ala, Val, Leu, Nle, Ile, and Met in P_1 were intermediate in reactivity. The finding that Val in P_1 is better than Ala is in agreement with previous work (McRae et al., 1980; Marossy et al., 1980; Powers et al., 1977; Zimmerman & Ashe, 1977). The observation that oxidation of the methionine of Boc-Ala-Ala-Met-SBzl to methionine sulfoxide results in a 180-fold reduction in k_{cat}/K_M is in agreement with earlier studies where it was found that oxidation of the methionine side chain of MeO-Suc-Ala-Ala-Pro-Met-NA resulted in a 300-fold reduction in k_{cat}/K_M (McRae et al., 1980).

Table III: Kinetic Constants for the Hydrolysis of Tripeptide Thio Ester Substrates by Chymotrypsin-like Enzymes[a]

substrate P_4 P_3 P_2 P_1	kinetic constants[b]	enzyme: HL cathepsin G	chymotrypsin A_α	RMCP I	RMCP II
Boc-Ala-Ala-Ala-SBzl	K_M	NR[c]	120	NR	NR
	k_{cat}		0.40		
	k_{cat}/K_M		0.0035		
Boc-Ala-Ala-Val-SBzl	K_M	NR	NR	NR	NR
	k_{cat}				
	k_{cat}/K_M				
Boc-Ala-Ala-Nva-SBzl	K_M	33	7.6	6.1	13
	k_{cat}	6.9	12	71	9.6
	k_{cat}/K_M	0.21	1.6	12	0.74
MeO-Suc-Ala-Ala-Nva-SBzl	K_M	53	5.2	9.0	14
	k_{cat}	6.7	19	140	9.7
	k_{cat}/K_M	0.13	3.7	15	0.71
Suc-Ala-Ala-Nva-SBzl	K_M	32	6.7	9.4	11
	k_{cat}	8.3	16	119	11
	k_{cat}/K_M	0.26	2.4	12.7	0.99
Boc-Ala-Pro-Nva-SBzl(Cl)	K_M	20	4.7	4.1	2.7
	k_{cat}	5.4	15	98	12
	k_{cat}/K_M	0.26	3.2	24	4.3
Boc-Ala-Ala-Leu-SBzl	K_M	22	4.1	4.0	31
	k_{cat}	4.9	20	30	6.7
	k_{cat}/K_M	0.22	5.0	8.0	0.22
Boc-Ala-Ala-Leu-SBzl(NO_2)	K_M	12	4.1	0.68	9.4
	k_{cat}	3.1	19	38	5.1
	k_{cat}/K_M	0.25	4.6	56	0.54
Boc-Ala-Ala-Leu-SBzl(Cl)	K_M	21	1.8	1.4	4.6
	k_{cat}	3.6	13	38	4.0
	k_{cat}/K_M	0.17	7.4	29	0.87
Boc-Ala-Ala-Leu-SBzl(OCH_3)	K_M	20	2.6	1.8	12
	k_{cat}	3.5	15	41	4.3
	k_{cat}/K_M	0.17	5.9	23	0.47
Boc-Ala-Ala-Nle-SBzl	K_M	22	4.3	5.4	20
	k_{cat}	8.2	24	63	11
	k_{cat}/K_M	0.37	5.5	12	0.57
Boc-Ala-Ala-Ile-SBzl	K_M	NR	NR	NR	NR
	k_{cat}				
	k_{cat}/K_M				
Boc-Ala-Ala-Phe-SBzl	K_M	43	9.1	6.9	48
	k_{cat}	44	135	320	100
	k_{cat}/K_M	1.0	15	46	2.1
Boc-Ala-Ala-Met-SBzl	K_M	34	6.0	6.2	25
	k_{cat}	7.0	22	190	21
	k_{cat}/K_M	0.20	3.7	31	0.84
Boc-Ala-Ala-Met(O)-SBzl	K_M	400	180	380	
	k_{cat}	4.9	130	140	
	k_{cat}/K_M	0.013	0.72	0.36	0.013[d]
Boc-Ala-Ala-Ser-SBzl	K_M	900	270	370	1090
	k_{cat}	3.3	16	78	30
	k_{cat}/K_M	0.0037	0.059	0.21	0.027
Boc-Ala-Ala-Asn-SBzl	K_M	270	250	*e*	650
	k_{cat}	5.5	35		43
	k_{cat}/K_M	0.020	0.14		0.066
Boc-Ala-Ala-Glu-SBzl	K_M	NR	1500	NR	NR
	k_{cat}		6.5		
	k_{cat}/K_M		0.0043		
Boc-Ala-Ala-Pro-SBzl	K_M	NR	NR	NR	NR
	k_{cat}				
	k_{cat}/K_M				

[a] Hepes buffer (0.1 M), pH 7.5, 0.5 M NaCl, and 10.2% Me_2SO at 25 °C. Enzyme concentrations: 5.5–20.0 nM cathepsin G; 0.45–5.5 nM chymotrypsin A_α; 0.16–0.40 nM RMCP I; 2.6–9.3 nM RMCP II. [b] The units of k_{cat}, K_M, and k_{cat}/K_M are s^{-1}, μM, and 10^{-6} M^{-1} s^{-1}, respectively. [c] NR, no reaction (hydrolysis rate less than 5×10^{-12} M s^{-1} at substrate concentration from 100 to 200 μM. [d] Only k_{cat}/K_M could be calculated since the Hanes plot had a slope near zero. [e] Nonlinear Hanes plot.

Porcine Pancreatic Elastase. Studies of peptide 4-nitroanilides and peptide chloromethyl ketones with PP elastase indicate that this enzyme prefers substrates with small amino acids such as Ala in P_1 (Cs.-Szabo et al., 1980; Powers et al., 1977; Zimmerman & Ashe, 1977). As shown in Table II, several of the substrates reported here are significantly better than MeO-Suc-Ala-Ala-Pro-Val-SBzl, the most reactive PP elastase substrate previously reported ($k_{cat}/K_M = 0.74 \times 10^6$ M^{-1} s^{-1}) (Castillo et al., 1979). The best substrate, Boc-Ala-Ala-Nva-SBzl, is 9.2-fold more reactive than the tetrapeptide thio ester. Substitution of Leu or Nle for Nva caused a 1.5–2.3-fold decrease in k_{cat}/K_M while substitution of methoxysuccinyl and succinyl at P_4 resulted in a 1.4–3.1-fold decrease. Substrates with Ala, Val, Ile, and Met in P_1 were 4–10-fold less reactive than the Nva derivative. Interestingly, peptide thio esters with Val and Ala in P_1 had similar re-

Table IV: Relative k_{cat}/K_M Values for the Hydrolysis of Thio Ester Substrates[a]

substrate	HL elastase	PP elastase	HL cathepsin G	RMCP I	RMCP II	chymotrypsin A_α	protease V-8
Boc-Ala-Ala-Ala-SBzl	0.46	0.46	0	0	0	0.002	0
Boc-Ala-Ala-Val-SBzl	1.88	0.41	0	0	0	0	
Boc-Ala-Ala-Nva-SBzl	5.75	4.25	0.13	7.50	0.47	1.00	0
MeO-Suc-Ala-Ala-Nva-SBzl	2.06	3.06	0.08	9.38	0.44	2.31	
Suc-Ala-Ala-Nva-SBzl	0.11	2.31	0.16	7.94	0.62	1.50	
Boc-Ala-Pro-Nva-SBzl(Cl)	81.25	1.38	0.16	15.00	2.69	2.00	
Boc-Ala-Ala-Leu-SBzl	1.38	2.75	0.14	5.00	0.14	3.13	
Boc-Ala-Ala-Leu-SBzl(NO_2)	2.50	2.00	0.16	35.00	0.34	2.88	
Boc-Ala-Ala-Leu-SBzl(Cl)	4.63	2.06	0.11	18.13	0.54	4.63	
Boc-Ala-Ala-Leu-SBzl(OCH_3)	1.88	1.82	0.11	14.38	0.29	3.69	0
Boc-Ala-Ala-Nle-SBzl	1.13	1.88	0.23	7.5	0.36	3.44	
Boc-Ala-Ala-Ile-SBzl	2.44	0.61	0	0	0	0	
Boc-Ala-Ala-Phe-SBzl	0	0	0.63	28.75	1.31	9.38	
Boc-Ala-Ala-Met-SBzl	0.54	1.06	0.13	19.38	0.53	2.31	
Boc-Ala-Ala-Met(O)-SBzl	0.003	0.09	0.008	0.36	0.008	0.45	
Boc-Ala-Ala-Ser-SBzl	0.14	0.27	0.002	0.13	0.02	0.04	0
Boc-Ala-Ala-Asn-SBzl	0	0	0.01		0.04	0.09	0
Boc-Ala-Ala-Glu-SBzl	0	0	0	0	0	0.003	0.26
Boc-Ala-Ala-Pro-SBzl	0	0	0	0	0	0	

[a] k_{cat}/K_M value for the hydrolysis of Boc-Ala-Ala-Nva-SBzl by chymotrypsin A_α is set at 1.00. The best value for each enzyme is underlined.

activities, while with amide substrates, Ala is 11.3-fold more reactive than the corresponding Val substrate (McRae et al., 1980). Oxidation of the Met side chain of Boc-Ala-Ala-Met-SBzl resulted in a 215-fold drop in reactivity, which is in agreement with the trends observed with HL elastase.

Human Leukocyte Cathepsin G. Cathepsin G, like HL elastase, is localized in the granule fraction of human leukocytes. Although its function is not clear, it appears that this enzyme is involved in elastin turnover and may act synergistically with HL elastase in the elastin degradation process (Boudier et al., 1981). Studies with peptide 4-nitroanilides (Yoshida et al., 1980; Nakajima et al., 1979; Zimmerman & Ashe, 1977), peptide amides (McRae et al., 1980), and peptide chloromethyl ketones (Powers et al., 1977) have shown that this enzyme prefers aromatic and bulky amino acids in P_1. The most reactive substrates reported thus far for cathepsin G are the thio ester substrates Suc-Phe-Leu-Phe-SBzl (k_{cat}/K_M = 0.78×10^6 M^{-1} s^{-1}) and Suc-Ala-Ala-Pro-Phe-SBzl (k_{cat}/K_M = 0.30×10^6 M^{-1} s^{-1}) (Harper et al., 1981), and the most reactive peptide 4-nitroanilide, Suc-Phe-Pro-Phe-NA, is 223-fold less reactive than Suc-Phe-Leu-Phe-SBzl (Yoshida et al., 1980). The best substrate reported here is Boc-Ala-Ala-Phe-SBzl, which is slightly more reactive than Suc-Phe-Leu-Phe-SBzl (Table III). Substitution of Nva, Leu, Met, or Nle for Phe resulted in a 2.7–7.6-fold decrease in reactivity. Substrates containing a P_1 methionine sulfoxide, Asn, or Ser were hydrolyzed slowly while no hydrolysis was observed when Ala, Val, Ile, Glu, or Pro occupied P_1. The finding that the methionine sulfoxide peptide is 15-fold less reactive than the corresponding Met substrate is consistent with previous studies with peptide 4–nitroanilides. MeO-Suc-Ala-Ala-Pro-Met-NA has a k_{cat}/K_M of 1700 M^{-1} s^{-1} while the corresponding sulfoxide is not hydrolyzed by cathepsin G (Nakajima et al., 1979).

Chymotrypsin A_α. Chymotrypsin is known to prefer aromatic or bulky amino acids in the P_1 position of peptide substrates. One of the best peptide thiobenzyl ester substrates reported thus far for chymotrypsin is Suc-Ala-Ala-Pro-Phe-SBzl (k_{cat}/K_M = 15×10^6 M^{-1} s^{-1}) (Harper et al., 1981) and is 40 times more reactive than the corresponding 4-nitroanilide substrate (Nakajima et al., 1979). As shown in Table III, Boc-Ala-Ala-Phe-SBzl is as susceptible to hydrolysis as the most reactive thio ester substrates yet reported for chymotrypsin. Substrates with Nva, Leu, Nle, or Met in P_1 were 2–9-fold less reactive while substrates with Ala, methionine sulfoxide, Ser, Asn, and Glu in P_1 were hydrolyzed very slowly.

Rat Mast Cell Proteases I and II. RMCPs I and II have been isolated from typical and atypical mast cells, respectively. While the primary function of these proteases is not known, they have been implicated in degradation of basement membrane collagen (Sage et al., 1979) and may also play a role in the inflammatory reactions associated with mast cell degranulation (Woodbury & Neurath, 1980).

Studies with small peptides and peptide 4-nitroanilides have shown that these enzymes have primary specificities similar to that of chymotrypsin and cathepsin G but the secondary interactions are quite different (Kobayashi & Katunuma, 1978; Yoshida et al., 1980). The best peptide thio ester substrate for RMCPs I and II yet reported is Suc-Ala-Ala-Pro-Phe-SBzl, which has a k_{cat}/K_M of 4.7×10^6 M^{-1} s^{-1} for both enzymes, while the best nitroanilide substrates, Suc-Phe-Leu-Phe-NA and Suc-Phe-Pro-Phe-NA respectively are from 6- to 130-fold less reactive (Yoshida et al., 1980).

The best substrate for RMCP I reported here, Boc-Ala-Ala-Leu-SBzl(NO_2), is only slightly better than Boc-Ala-Ala-Phe-SBzl but is 12-fold more reactive than Suc-Ala-Ala-Pro-Phe-SBzl. Boc-Ala-Ala-Met-SBzl and Boc-Ala-Pro-Nva-SBzl(Cl) were also found to be highly reactive.

The most reactive substrate for RMCP II, Boc-Ala-Pro-Nva-SBzl(Cl), is just slightly less reactive than Suc-Ala-Ala-Pro-Phe-SBzl. This high reactivity, as compared to other substrates in this series, can be attributed to the placement of Pro in P_2. Substrates with Leu, Nle, Phe, and Met in P_1 were 2–10-fold less reactive than Boc-Ala-Pro-Nva-SBzl(Cl). The substitution of succinyl for Boc in Boc-Ala-Ala-Nva-SBzl has a favorable effect on the reactivity. The favorable effect of anionic groups at P_4 has also be noted with peptide 4-nitroanilides (Yoshida et al., 1980) and is thought to be due to the presence of a protonated His residue near S_4, which can interact with the succinyl group. As with RMCP I, peptides containing Ala, Val, Ile, Glu, and Pro were not hydrolyzed.

S. aureus Protease V-8. The protease, which is isolated from strain V-8 of *S. aureus*, has been used quite frequently in protein sequencing studies due to its specificity. Studies using proteins as substrates have shown that this enzyme cleaves only after Glu and Asp residues, and cleavage after

Asp is observed only under special conditions of pH (Drapeau, 1976). The only synthetic substrate reported thus far for this enzyme is *N*-CBZ-glutamic acid phenyl ester, which has a K_M of 78 μM, a k_{cat} of 230 s^{-1},and a k_{cat}/K_M of 2.95×10^6 M^{-1} s^{-1}. Boc-Ala-Ala-Glu-SBzl was found to be 7.2-fold less reactive than the phenyl ester substrate and has a K_M of 0.33 mM, a k_{cat} of 135 s^{-1}, and a k_{cat}/K_M of 410 000 M^{-1} s^{-1}. This enzyme did not cleave peptide thio esters containing Ser, Asn, Leu, Nva, or Ala in P_1, which is in agreement with its strict primary specificity.

Effects of Substituents on the Thiobenzyl Ester Group. In an attempt to introduce specificity and/or reactivity into the thio ester substrate Boc-Ala-Ala-Leu-SBzl(X), we synthesized the 4-nitro-, 4-chloro-, and 4-methoxythiobenzyl ester derivatives. Leu in P_1 was chosen because it was a reasonably good substrate for every enzyme except protease V-8. In general, these substitutions affected HL elastase and RMCP I more favorably than the other enzymes. The 4-nitro group increased the reactivity toward RMCP I by 7-fold over that of the unsubstituted derivative. The 4-chloro compound was found to be 3.3-fold more reactive than the unsubstituted derivative toward HL elastase. The increase in reactivity can be attributed to a decrease in K_M. The value of k_{cat} was insensitive to the substituent with all the enzymes tested, which is consistent with deacylation being the rate-limiting step in thio ester hydrolysis (Hirohara et al., 1977).

Summary. Table IV shows the relative reactivities of chymotrypsin-like and elastase-like enzymes toward several tripeptide thiobenzyl ester substrates. In general, RMCP I was the most reactive enzyme with these substrates while HL elastase, PP elastase, and chymotrypsin A_α showed intermediate reactivity, except for Boc-Ala-Pro-Nva-SBzl(Cl) with HL elastase which is the most reactive serine protease substrate reported here. Cathepsin G and RMCP II are much less reactive than the other enzymes tested. The comparatively low reactivity of cathepsin G and RMCP II toward peptide 4-nitroanilides has been noted (Yoshida et al., 1980). The most specific protease tested was *S. aureus* protease V-8, which only reacted with Boc-Ala-Ala-Glu-SBzl.

Several of the substrates reported here are the most reactive substrates yet reported for the enzymes tested. The HL elastase substrate, Boc-Ala-Pro-Nva-SBzl(Cl), is 110-fold more reactive than the previous best substrate MeO-Suc-Ala-Ala-Pro-Val-SBzl. The best RMCP I substrate is 12-fold more reactive than the best substrate reported thus far, Suc-Ala-Ala-Pro-Phe-SBzl. Studies by Brouwer & Kirsch (1982) on diffusion-limited rates of chymotrypsin-catalyzed hydrolysis of *N*-(methoxycarbonyl)-L-tryptophan *4*-nitrophenyl ester ($k_{cat}/K_M = 35 \times 10^6$ M^{-1} s^{-1}) indicate that this reaction is as much as 40% diffusion controlled. Several of the substrates reported here have k_{cat}/K_M values of the same magnitude as the 4-nitrophenyl ester substrate and, therefore, may be at least partially diffusion controlled. This is especially true of the reaction of Boc-Ala-Pro-Nva-SBzl(Cl) with HL elastase, which has a k_{cat}/K_M value 4-fold higher than the 4-nitrophenyl ester substrate.

In conclusion, we have reported a series of highly reactive thio ester substrates for serine proteases. This series could prove useful in mapping the S_1 subsite of new serine proteases and would allow small quantities of enzyme to be used due to the high sensitivity of these substrates. In addition, the sequences reported here could be used to develop more effective inhibitors of these proteases.

Acknowledgments

We are grateful to Dr. James Travis and his group at the University of Georgia for supplying the human leukocyte elastase and the human leukocyte cathepsin G used in this study. Rat mast cell proteases I and II were kindly provided by Dr. Richard Woodbury and Dr. Hans Neurath of the University of Washington. We are thankful to Thomas Seifferman for preparing Boc-Ala-Ala-Ala-SBzl.

Registry No. RMCP I, 82599-73-3; RMCP II, 82599-74-4; Boc-Ala-Ala-Ala-SBzl, 90171-45-2; Boc-Ala-Ala-Val-SBzl, 90171-46-3; Boc-Ala-Ala-Nva-SBzl, 90171-47-4; MeO-Suc-Ala-Ala-Nva-SBzl, 90171-48-5; Suc-Ala-Ala-Nva-SBzl, 90171-49-6; Boc-Ala-Pro-Nva-SBzl(Cl), 90171-50-9; Boc-Ala-Ala-Leu-SBzl, 90171-51-0; Boc-Ala-Ala-Leu-SBzl(NO_2), 90171-52-1; Boc-Ala-Ala-Leu-SBzl(Cl), 90171-53-2; Boc-Ala-Ala-Leu-SBzl(OCH_3), 90171-54-3; Boc-Ala-Ala-Nle-SBzl, 90171-55-4; Boc-Ala-Ala-Ile-SBzl, 90171-56-5; Boc-Ala-Ala-Phe-SBzl, 90171-57-6; Boc-Ala-Ala-Met-SBzl, 90171-58-7; Boc-Ala-Ala-Met(O)-SBzl, 90171-59-8; Boc-Ala-Ala-Ser-SBzl, 90171-60-1; Boc-Ala-Ala-Asn-SBzl, 90171-61-2; Boc-Ala-Ala-Glu-SBzl, 90171-62-3; Boc-Ala-Ala-Pro-SBzl, 90171-63-4; *tert*-butyloxycarbonylnorvaline benzyl thio ester, 90171-64-5; *tert*-butyloxycarbonylserine benzyl thio ester, 90171-65-6; norvaline benzyl thio ester hydrochloride, 90194-62-0; *tert*-butyloxycarbonylnorvaline, 53308-95-5; *tert*-butyloxycarbonylserine, 3262-72-4; *tert*-butyloxycarbonylalanylalanine, 27317-69-7; glutamic acid γ-*tert*-butyl α-benzyl thio ester, 90171-66-7; *tert*-butyloxycarbonylalanine hydroxysuccinimide ester, 3392-05-0; *tert*-butyloxycarbonylalanylglutamic acid γ-*tert*-butyl α-benzyl thio ester, 90171-67-8; alanylglutamic acid α-benzyl thio ester trifluoroacetate, 90171-69-0; elastase, 9004-06-2; cathepsin G, 56645-49-9; chymotrypsin, 9004-07-3; *Staphylococcus aureus* protease V-8, 66676-43-5; serine proteinase, 37259-58-8.

References

Baugh, R. J., & Travis, J. (1976) *Biochemistry 15*, 836–841.
Boudier, C., Holle, C., & Bieth, J. G. (1981) *J. Biol. Chem. 256*, 10256–10258.
Brouwer, A. C., & Kirsch, J. F. (1982) *Biochemistry 21*, 1302–1307.
Castillo, M. J., Nakajima, K., Zimmerman, M., & Powers, J. C. (1979) *Anal. Biochem. 99*, 53–64.
Cho, K., Tanaka, T., Cook, R. R., Kisiel, W., Fujikawa, K., Kurachi, K., & Powers, J. C. (1984) *Biochemistry 23*, 644–650.
Cs.-Szabo, G., Pozsgay, M., Gaspar, R., & Elodi, P. (1980) *Acta Biochim. Biophys. Acad. Sci. Hung. 15*, 263–273.
Drapeau, G. R. (1976) *Methods Enzymol. 45*, 469–475.
Drapeau, G. R. (1978) *Can. J. Biochem. 56*, 534–544.
Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70–77.
Farmer, D. A., & Hageman, J. H. (1975) *J. Biol. Chem. 250*, 7366–7371.
Grassetti, D. R., & Murray, J. F., Jr. (1967) *Arch. Biochem. Biophys. 119*, 41–49.
Green, G. D. J., & Shaw, E. (1979) *Anal. Biochem. 93*, 223–226.
Hanes, C. S. (1932) *Biochem. J. 26*, 1406–1421.
Harper, J. W., Ramirez, G., & Powers, J. C. (1981) *Anal. Biochem. 118*, 382–387.
Hirohara, H., Phillip, M., & Bender, M. L. (1977) *Biochemistry 16*, 1573–1580.
Kobayashi, K., & Katunuma, N. (1978) *J. Biochem. (Tokyo) 84*, 65–74.
Marossy, K., Cs.-Szabo, G., Pozsgay, M., & Elodi, P. (1980) *Biochem. Biophys. Res. Commun. 96*, 762–769.
McRae, B., Nakajima, K., Travis, J., & Powers, J. C. (1980) *Biochemistry 19*, 3973–3978.
McRae, B. J., Lin, T. Y., & Powers, J. C. (1981a) *J. Biol. Chem. 256*, 12362–12366.
McRae, B. J., Kurachi, K., Heimark, R. L., Fujikawa, K., Davie, E. W., & Powers, J. C. (1981b) *Biochemistry 20*, 7196–7206.
Metrione, R. M. (1972) *Biochim. Biophys. Acta 268*, 518–522.

Nakajima, K., Powers, J. C., Ashe, B. M., & Zimmerman, M. (1979) *J. Biol. Chem. 254*, 4027–4032.
Powers, J. C. (1983) *Am. Rev. Respir. Dis. 127*, S54–S58.
Powers, J. C. Gupton, B. F., Harley, A. D., Nishino, N., & Whitley, R. J. (1977) *Biochim. Biophys. Acta 485*, 156–166.
Renaud, A., Lestienne, P., Hughes, D. L., Bieth, J. G., & Dimicoli, J. L. (1983) *J. Biol. Chem. 258*, 8312–8316.
Sage, H., Woodbury, R. G., & Borstein, P. (1979) *J. Biol. Chem. 254*, 9893–9900.
Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157–162.
Tanaka, T., McRae, B. J., Cho, K., Cook, R., Fraki, J. E., Johnson, D. A., & Powers, J. C. (1983) *J. Biol. Chem. 258*, 13552–13557.
Travis, J., Bowen, J., & Baugh, R. (1978) *Biochemistry 17*, 5651–5656.
Woodbury, R. G., & Neurath, H. (1980) *FEBS Lett. 114*, 189–196.
Woodbury, R. G., Everitt, M. T., & Neurath, H. (1981) *Methods Enzymol. 80*, 588–609.
Yoshida, N., Everitt, M. T., Neurath, H., Woodbury, R. G., & Powers, J. C. (1980) *Biochemistry 19*, 5799–5804.
Zimmerman, M., & Ashe, B. M. (1977) *Biochim. Biophys. Acta 480*, 241–245.

Tryptophan Emission from Myosin Subfragment 1: Acrylamide and Nucleotide Effect Monitored by Decay-Associated Spectra†

Peter M. Torgerson

ABSTRACT: The intrinsic emission due to the tryptophan residues of the heavy chain of myosin subfragment 1 can be divided into three classes, on the basis of spectra associated with lifetimes of 0.72, 4.5, and 8.8 ns. The percentage contribution of each component to the total emission is 9%, 45%, and 46%, respectively. Low concentrations of acrylamide quench the long component with a quenching constant of 14.9 ± 2.9 M^{-1}, while the intermediate and short components are unaffected. Upon addition of ATP the total intensity increases by 17%. The bulk of this increase is in the intermediate lifetime component. Quenching by acrylamide in the presence of ATP again quenches only the long lifetime component, indicating that the tryptophan residues affected by ATP binding are not accessible to the solvent.

The absorption and emission behavior of the tryptophan residues of myosin subfragment 1 (S1)[1] has been utilized in several ways to investigate the structure of S1 and its role in contraction. The degree of polarization of the intrinsic fluorescence was employed in several studies (Dos Remedios et al., 1972; Kishi & Noda, 1983). The effect of nucleotide binding was first examined by Morita (1967), who observed the difference absorption spectrum. Werber et al. (1972) examined the structural features of the nucleotide required for maximal fluorescence enhancement. They also concluded that ATP binding protects two (out of the approximately 20) tryptophan residues of heavy meromyosin from quenching by iodide. Onishi et al. (1973) examined the binding of the fluorescent ATP analogue 1,N^6-ethenoadenosine triphosphate to heavy meromyosin. On the basis of iodide quenching experiments they were able to divide the tryptophan emission into two classes, accessible and inaccessible, and showed that energy transfer to the ATP analogue occurs from the accessible residues.

Advantage has been taken of this nucleotide-induced fluorescence enhancement in the investigation of the kinetics of myosin and actomyosin ATPase activity (Bagshaw & Trentham, 1974; Trybus & Taylor, 1982; Stein et al., 1979; Chock & Eisenberg, 1979). In particular, the fluorescence increase induced by nucleotide has been shown to correspond to more than one step in the hydrolysis cycle (Chock et al., 1979; Johnson & Taylor, 1978). A rapid initial enhancement is produced by ATP binding, followed by a further increase upon hydrolysis. It would be useful if these effects could be linked to alterations in the environment of specific S1 tryptophans.

Recent advances in instrumentation and data analysis have resulted in more thorough analyses of the intrinsic emission characteristics of several proteins (Ross et al., 1981a,b; Privat et al., 1980; Torikata et al., 1979). In particular, more detailed information on the behavior of the emission spectrum during the fluorescence lifetime is not obtainable. Such information includes time-resolved emission spectra, which are the spectra obtained at given times after excitation, and decay-associated spectra, which are those associated with individual components in a multicomponent fluorescence decay. This enables the resolution of the emission into the contributions due to individual tryptophans or groups of tryptophans. An elegant example of such a resolution was obtained with horse liver alcohol dehydrogenase, which contains two tryptophans (Ross et al., 1981b; Knutson et al., 1982). To date, most investigations have employed proteins with small numbers of tryptophan residues and with known sequences and X-ray crystallographic structures. The aim of the present paper is to extend this approach to S1. This is a less tractable problem since S1 contains several tryptophan residues in its heavy chain, about which there is relatively little auxiliary structural information. There is some evidence for segregation of their

† From the Cardiovascular Research Institute, University of California, San Francisco, California 94143. *Received October 4, 1983.* This work was supported by Grants HL-16683, PCM-7922174, and CI-8. This research was carried out during the tenure of a Postdoctoral Fellowship from the Muscular Dystrophy Association.

[1] Abbreviations: S1, chymotryptic subfragment 1 of myosin; TMAC, tetramethylammonium chloride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; TES, 2-[[tris(hydroxymethyl)-methyl]amino]ethanesulfonic acid; DAS, decay-associated spectra; ATPase, adenosinetriphosphatase; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

0006-2960/84/0423-3002$01.50/0