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Alkyl Alkanethiolsulfonate Sulfhydryl Reagents: β-Sulfhydryl-Modified Derivatives of L-Cysteine As Substrates for Trypsin and α-Chymotrypsin

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Derivatives of L-cysteine and the A chain of bovine insulin have been chemically modified at the cysteinyl β-sulfhydryl by certain sulfhydryl-specific alkyl alkanethiolsulfonate reagents. The alkanethiolation products possess mixed-disulfide side chains structurally similar to the side chains of lysine and phenylalanine and hence were studied here as substrates for trypsin and α-chymotrypsin, respectively. Kinetic parameters were obtained for the enzyme-catalyzed hydrolyses of the modified L-cysteine analogs and of specific reference amino acids which were derivatized analogously at both the α-amino and α-carboxyl groups and assayed identically. For both enzymes it was found that the specificity constants, k_{cat}/K_m, for analog esters compare favorably with those for specific reference esters, whereas specificity constants for analog amides compare much less favorably with those for specific reference amides. This discrepancy is largely a consequence of the k_{cat} values for the analog amides being relatively much lower than the corresponding values for the reference amides. Consistent with this trend, no detectable enzyme-catalyzed hydrolysis of the amide bonds at the sites of modified cysteine residues in the A chain of bovine insulin was observed. It is proposed that the predominant kinetic consequence of the mixed-disulfide side chains of the alkanethiolated cysteine moieties is a decrease in the acylation rate constants, k_2, arising from an increase in the transition-state free energies of acylation.

INTRODUCTION

Previous studies (*1–5*) have shown that alkanethiolation reactions of alkyl alkanethiolsulfonate ($RSSO_2R'$)[3] reagents with sulfhydryl (-SH) groups proceed (i) independently of the nature (size, charge, or alkyl or aryl) of both the R′ and, most

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[3] Abbreviations used: $RSSO_2R'$, alkyl alkanethiolsulfonate; BzArgOEt, *N*-benzoyl-L-arginine ethyl ester; BzTyrOEt, *N*-benzoyl-L-tyrosine ethyl ester; BzArgNan, *N*-benzoyl-DL-arginine-*p*-nitroanilide; BzPheNan, *N*-benzoyl-DL-phenylalanine-*p*-nitroanilide; AEtTosCysOPr, β-*S*-(β-aminoethyl)-*N*-*p*-toluenesulfonyl-L-cysteine *n*-propyl ester; DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid); NEM, *N*-ethylmaleimide; $TosLysCOCH_2Cl$, *N*-*p*-toluenesulfonyl-L-lysine-chloromethyl ketone; $TosPheCOCH_2Cl$, *N*-*p*-toluenesulfonyl-L-phenylalanine-chloromethyl ketone; NMM, *N*-methylmorpholine; DTT, dithiothreitol; TLE, thin layer electrophoresis; λ, wavelength; S_0, initial substrate concentration; E_0, initial enzyme concentration; v_i, initial velocity; Me_2SO, dimethyl sulfoxide.

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importantly, the R groups (within steric limitations); (ii) under mild conditions, nondestructive to proteins; (iii) with high selectivity for cysteinyl sulfhydryls; (iv) extremely rapidly; (v) quantitatively to complete conversion to the mixed-disulfide without large excesses of reagents; and (vi) reversibly to regenerate free sulfhydryl groups upon addition of large molar excesses of exogenous thiols. These results suggest that new alkyl alkanethiolsulfonate reagents can be designed to deliver "customized" RS- groups for a wide range of highly specialized biochemical purposes.

Therefore, we have pursued a novel application of two new $RSSO_2R'$ reagents as a simple demonstration of this untapped potential. Methyl aminoethanethiolsulfonate hydrobromide (**1**) and methyl benzylthiolsulfonate (**2**) react with the β-SH of L-cysteine to give products with mixed-disulfide side chains (*1*) which are identical, with respect to their distal portions, to the side chains of lysine and phenylalanine, respectively. Thus, it was expected that α-amino blocked L-cysteine esters and amides modified with **1** and **2** would be substrates for trypsin and α-chymotrypsin, respectively.

We have confirmed this speculation. In addition, we have investigated the effects on substrate specificity of having *both* large and disulfide-containing side chains. It is known, for example, that lysine derivatives are better substrates for trypsin than the corresponding homolysine derivative with a larger side chain (*6, 7*). Also, one sulfur heteroatom in the side chain of aminoethylated derivatives of L-cysteine results in substrates that are somewhat less kinetically specific for trypsin than analogously derivatized L-lysine, for which the side chain is isosteric (*8, 9*). The present results indicate that in comparison with specific reference substrates, our analog substrates show not so much poorer binding as slower subsequent catalytic steps, particularly acylation. This relative localization of effect can explain the observed discrepancies in specificity constants for the ester vs. the amide analog substrates.

EXPERIMENTAL PROCEDURES

Materials. Materials used were of the highest purity commercially available. Specifically, BzArgOEt, BzArgNan (99+%, gold label), and BzPheNan were from Aldrich; BrArgOEt · HCl, BzTyrOEt, $TosLysCOCH_2Cl$, and $TosPheCOCH_2Cl$ were from Sigma; and spectrograde Me_2SO was from Matheson, Coleman and Bell. Micropolyamide sheets were from Schleicher and Schuell. Reacti-Vials with Mini-Nert caps were from Pierce. Trypsin used was Type I, 1,200 BzArgOEt units/mg at 25°C (Sigma) or A grade, 3,700 National Formulary units/mg at 25°C (Calbiochem). α-Chymotrypsin used was Type II, 43 International Units/mg at 25°C (Sigma).

Methods. Proton nuclear magnetic resonance spectra were obtained using a Perkin–Elmer R12-B, 60-MHz spectrometer. Ultraviolet and visible spectra and absorbance measurements were taken on either Gilford 6040 or 2000 recorders equipped with Beckman DU or DUR monochrometers, respectively, or on a Cary

118-C recording spectrophotometer. Static measurements of pH and pH*[4] (*10–12*) were made using Radiometer PHM 26 meters and glass electrodes, and kinetics were carried out on a Radiometer TTT 2 pH-stat using NaOH solutions standardized against reference solutions of potassium acid phthalate. Computer analyses were performed on a Hewlett–Packard Model 9821 A. Systems for paper chromatography, TLC, TLE, and combined TLC–TLE were as previously described (*1*).

The alkanethiolation products β-*S*(β-aminoethanethiol)-*N*-acetyl-L-cysteine ethyl ester (**3**), β-*S*-(benzylthiol)-*N*-acetyl-L-cysteine ethyl ester (**4**), β-*S*-(β-aminoethanethiol)-*N*-acetyl-L-cysteine-*p*-nitroanilide hydrobromide (**5**), β-*S*-(benzylthiol)-*N*-acetyl-L-cysteine-*p*-nitroanilide (**6**), tetra-[β-*S*-(β-aminoethanethiol)]-A chain insulin (bovine) (**7**), and tetra-[β-*S*-(benzylthiol)]-A chain insulin (bovine) (**8**) were prepared as previously described (*1*).

Ethyl ester hydrolyses. A pH-stat holding 2.0-ml solutions of the desired substrate concentrations and 75.0 mM $CaCl_2$, 0.75 mM EDTA at 30 ± 0.5°C with endpoint pH's (±0.05) of 7.60 for tryptic and 7.80 for chymotryptic assays was used. Kinetic experiments were repeated at least twice.

p-Nitroanilide hydrolyses. In solutions of approximately 1.0 absorbance unit, made as for assays below, the largest value for the difference in absorbances for *p*-nitroaniline (recrystallized from water, mp 147.7–149.5°C) and for each *p*-nitroanilide substrate was seen at 389 nm: *p*-nitroaniline, $\varepsilon_{389} = 15{,}562$; BzArgNan, $\varepsilon_{389} = 485$; BzPheNan, $\varepsilon_{389} = 485$; **5**, $\varepsilon_{389} = 780$; **6**, $\varepsilon_{389} = 494$ (where ε = molar extinction coefficient).

Kinetic assays (*12–14*) performed with 1.5 ml total volume solutions, maintained at 10.0 ± 0.8°C in the thermostated cell compartment of a Cary 118-C containing 33.3% Me_2SO (v/v), 40.0 mM NMM-HCl, and 50.0 mM $CaCl_2$, at pH* 7.89 ± 0.03 followed the rate of differential absorbance changes at 389 nm after addition of enzyme. Because of the limited substrate solubilities and very slow observed rates, kinetic reactions were followed in the region between pseudo-first-order [$K_m < (8.4\text{–}57.7)\ S_0$] and initial velocity [$S_0 < (0.4\text{–}1.4)\ K_m$] conditions and, where possible, with $S_0 \gg E_0$, but always with $S_0 > 2 \cdot E_0$. Kinetic experiments were repeated at least twice.

Enzyme digests and peptide mapping. Solutions of *in situ* alkanethiolated A chain of insulin (*1*), **7**, and **8**, pH 8.0, were incubated in sealed Reacti-Vials at 37°C in the dark for 24 to 52 h with 1 : 3.8 to 100 [E_0: modified peptide (w/w)] trypsin or α-chymotrypsin (6×10^{-7} to 8×10^{-5} M). Aliquots were removed periodically and assayed spectrophotometrically by the method of Mitz and Schleuter (*15*). Following incubations, enzyme activity was verified by assaying aliquots of the digests by the pH-stat method using saturating concentrations of BzArgOEt (for trypsin) or BzTyrOEt (for α-chymotrypsin). Enzyme digests were lyophilized, treated with performic acid by the method of Hirs (*16*), lyophilized three times (two water washes) and redissolved in doubly distilled water (to approximately 0.5 nmol/μl). Controls omitted the enzyme digestion or alkanethiolation steps.

[4] pH* is the experimentally observed apparent protonic activity in an aqueous–organic solvent. Values of pH* were used to give rough estimations of true protonic activities and, more importantly, to indicate any changes in protonic activities during kinetic assays.

TABLE 1

KINETIC DATA FOR TRYPSIN-AND α-CHYMOTRYPSIN-CATALYZED HYDROLYSES OF REFERENCE AND ANALOG SUBSTRATES[a]

Substrate	K_m ($\times 10^4$, M)	k_{cat} ($\times 10^2$, s^{-1})	k_{cat}/K_m[b] ($\times 10^{-1}$, s^{-1} M^{-1})	(k_{cat}/K_m)analogue/ (k_{cat}/K_m)reference[c] ($\times 10^2$)
Trypsin				
3	8.3 ± 1.1	780 ± 24	940 ± 96	31 ± 3.5
BzArgOEt	7.2 ± 2.9	2,200 ± 64	3,100 ± 70	
5	12 ± 0.39	0.0099 ± 0.000095	0.0081 ± 0.00018	0.020 ± 0.00012
BzArgNan	15 ± 2.2	59 ± 7.9	40 ± 0.63	
α-Chymotrypsin				
4	7.3 ± 1.5	300 ± 0.8	410 ± 65	7.9 ± 0.39
BzTyrOEt	5.2 ± 0.25	2,700 ± 170	5,200 ± 150	
6	6.8 ± 0.93	0.0013 ± 0.00011	0.0019 ± 0.00017	0.17 ± 0.015
BzPheNan	1.8 ± 0.0067	0.20 ± 0.00072	1.1 ± 0.0085	

[a] Kinetic parameters (and standard errors) were obtained by computer curve fitting of the data points to the Michaelis–Menten equation. Parameters for ethyl ester substrates were obtained by the pH-stat assay described in the text. Enzyme solutions were made in 10^{-3} N HCl. Stock solutions of **3** and **4** were made in absolute ethanol dried over 3-Å molecular sieves. Parameters for *p*-nitroanilide substrates were obtained by spectrophotometric assay described in the text, where the actual $\Delta A_{obsd} = A_{p\text{-nitroaniline released}} - A_{substrate\text{-}p\text{-nitroaniline consumed}}$. Knowing values for ε_{389} for the substrates and *p*-nitroaniline, correction factors could be calculated as $A_{p\text{-nitroaniline released}}/\Delta A_{obsd}$ and were BzArgNan, 1.032; BzPheNan, 1.032; **5**, 1.053; and **6**, 1.033. Finally, true $A_{p\text{-nitroaniline released}} = \Delta A_{obsd} \times$ correction factor. All *p*-nitroanilide substrate stock solutions were made at near-saturating concentrations in N_2-saturated, spectrograde Me_2SO, stored under N_2 at 0–4°C (frozen) in the dark.

[b] Apparent specificity constant.

[c] Apparent relative specificity constant.

Characterizations of digestion products were performed by any of the paper chromatography, TLC, TLE, or combined TLC–TLE systems previously described (*1*). Digests also were subjected to N-terminal analyses by the semi-micro (1/10 scale; 1 nmol/application to a 5 × 5-cm micropolyamide sheet) dansyl chloride method of Woods and Wang (*17*).

RESULTS

Experiments with ethyl ester substrate analogs. The enzyme-catalyzed rate of ester hydrolysis was calculated as the difference between pH-stat-determined initial rates in the absence (if significant) and then in the presence of enzyme (*18*). For *in situ* assayed **3** and **4** rates were monitored after rapid neutralization of methanesulfinic acid [see (*1*)]. Michaelis–Menten kinetic parameters are shown in Table 1.

Control experiments were performed under conditions of $S_0 \gg K_m \gg E_0$, where appropriate. Over much greater than v_i periods of time no significant rates were

observed (i) with enzymes alone at high concentrations ($>10^{-5}$ M) (*18*); (ii) in assays of enzymes with *N*-acetyl-L-cysteine ethyl ester; (iii) in assays of trypsin with **4** or α-chymotrypsin with **3** (even with $E_0 > 5.0 \times 10^{-6}$ M); and (iv) in assay solutions containing only **1** or **2**. Rates of enzyme-catalyzed hydrolyses of BzArgOEt or BzTyrOEt were the same in the absence or presence of large molar excesses of **1** or **2**. Rates were identical for **3** and **4** either generated *in situ* or column-purified (minus excess **1** or **2** and methanesulfinic acid) (*1*). Large molar excesses of β-mercaptoethanol had no effect on v_i's for BzArgOEt or BzTyrOEt, but abolished the rates of **3** and **4**; for $S_0 > [\beta\text{-mercaptoethanol}]_0$, rates immediately decreased proportionately to the amount of exogenous thiol added.

Product analysis for the assumed enzyme-catalyzed hydrolysis was performed as follows. Solvent was removed from the pool of column-purified **3** (*1*) (>200 mg; limited solubility of **4** prevented an analogous analysis), and an NMR spectrum (D_2O; DSS standard; taken immediately to avoid significant hydrolysis) of greater than 100 mg showed major peaks at δ 1.27 (methyl, t, J = 7 Hz), 2.07 (methyl, s), 3.70 (methylene, q, J = 7 Hz), 4.30 (methylene, t, J = 6 Hz). After addition of 4 drops of absolute ethanol, new methyl triplet (δ 1.17) and methylene quartet (δ 3.63) peaks appeared shifted 7 Hz upfield from the analogous peaks of the ethyl ester of **3**, showing that these latter peaks were not due to solvent. Another approximately 100 mg of **3** was incubated in a 22 mM assay solution at pH 7.6 (pH-stat), 30°C, for 45 min with 2×10^{-6} M trypsin [<1/100, E_0/**3** (w/w)]. Solvent was removed, and the sample was alternately washed with D_2O and dried *in vacuo* (two times). Its NMR spectrum (as before) was superimposable on that for **3** from controls incubated in the absence of enzyme, except for the complete absence of ethyl ester peaks at δ 1.27 (methyl, t) and 3.70 (methylene, q).

Experiments with p-nitroanilide substrate analogs. In aqueous–organic assay solutions of 1/3 (v/v) Me_2SO, the *p*-nitroanilide substrates had minimally satisfactory solubilities on the order of 1 mM at 10°C. The enzymes still retained substantial relative specific activities (trypsin, 131%; and α-chymotrypsin, 55%; versus 100% specific activity as defined in the absence of Me_2SO and determined with specific reference substrates). Michaelis–Menten kinetic parameters are shown in Table 1.

Wherever appropriate, control experiments were performed under the most rapid velocity conditions possible ($S_0 \gg E_0$). In substrate assay solutions lacking enzyme, no significant lyate species-catalyzed *p*-nitroanilide hydrolysis was observed even at 30°C for >42 h. Enzyme activities did not diminish noticeably over much greater than v_i periods of time. Neither DTT_{ox} alone or equimolar amounts of both DTT_{red} and **1** or **2** (*1*) added in large molar excess over S_0 had any effects on the rates of trypsin- or α-chymotrypsin-catalyzed hydrolyses of BzArgNan or BzPheNan, respectively. A 100-fold excess over S_0 of β-mercaptoethanol had no immediate effect on initial velocities with BzArgNan or BzPheNan but with **5** or **6** there were immediate, irreversible cessations of rates. When trypsin or α-chymotrypsin were preincubated at 10°C for 30–120 min in the presence of a 10–100 molar excess of the active-site-specific alkylating inhibitors, $TosLysCOCH_2Cl$ or $TosPheCOCH_2Cl$, respectively, no rates were observed in later assays of any of the reference or analog substrates.

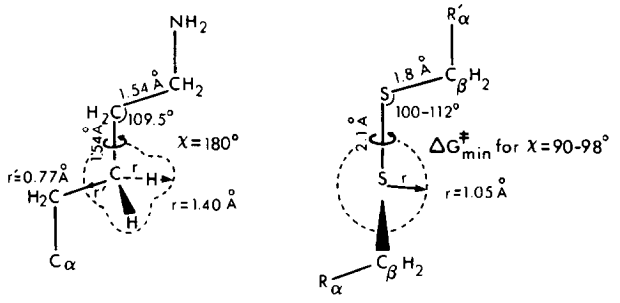

FIG. 1. Schematic representation of mixed-disulfide side chains of β-*S*-(alkanethiol)-cysteine compared to the side chain of lysine. Notation and parameters were obtained from Papas (*41*), Kice and Ekman (*42*), Van Wart *et al.* (*22*), and Cotton and Wilkinson (*43*).

For **5** and **6**, obtaining product analyses was made difficult by the slow enzyme-catalyzed rates which precluded following of reactions to completion. Spectra taken during the reactions, however, showed the appearance of free *p*-nitroaniline concomitant with disappearance of the substrate, to extents dependent on both E_0 and the time period of the reactions.

Experiments with alkanethiolated peptides. No detectable trypsin-catalyzed hydrolyses of **7** or α-chymotrypsin-catalyzed hydrolyses of **8** under a variety of conditions of concentrations, ratios of enzyme to modified peptide, and incubation times were observed using several methods of analyses. These included a direct spectrophotometric technique (*15*), a dansyl chloride N-terminal analysis (*17*), and various systems of paper chromatography and cellulose TLC and TLE. All methods unambiguously gave parallel results for both alkanethiolated and unmodified peptide digests which were incubated for prolonged periods (>2 days, 37°C, optimal pH); afterward, significant enzyme activities were demonstrated independently.

DISCUSSION

Structures of the mixed-disulfide side chains of alkanethiolated cysteine. Both the β-*S*-(β-aminoethanethiol) and β-*S*-(benzylthiol) side chains are longer than the corresponding side chains of Lys or Phe, with which they are structurally identical at their distal ends. However, existing evidence suggested that the steric tolerances of the substrate side chain binding sites of the respective enzymes (*18–21*) would be sufficient to accommodate binding of the former.

Furthermore, as depicted in Fig. 1, the covalent radius of a sulfide sulfur atom is no larger than that of a methylene unit. Also, the C–C, S–S, and S–C bond lengths, and the CH_2–CH_2–CH_2 and CH_2–S–S bond angles, are similar for the juxtaposed side chains. For unstrained CCSSCC structural units in which the disulfide bond is β-methylene substituted and both R′ and R are so small as not to interact sterically, there will exist a wide range of conformations about the C–S bond. And, the C–S–S bond angles will vary with changes in the dihedral angle,

$_{\chi}$(CS-SC), within the approximate range shown in Fig. 1. Most importantly, at ambient temperatures values for χ of ± 90–$98°$ are only slightly preferred; χ rotational free energy barriers may be estimated to range from about 7.0–9.0 kcal/mol (*22–24*) and so at least nearly-rotational isomeric equilibrium should exist.

From these considerations **3**, **4**, **5**, and **6**, at least and, somewhat less likely, **7** and **8**, were expected to be substrates for trypsin or α-chymotrypsin. However, as mentioned earlier, the kinetic consequences of having either abnormally large or monosulfide-containing side chains alone are detectable, and so it was not unexpected that disulfide-containing side chains would have noticeable effects.

Alkanethiol-modified cysteine esters and amides as substrates for trypsin and α-chymotrypsin. The apparent relative specificity constants[5] (*20*) of Table 1 indicate that for the same analog acyl amino acids the ester substrates, **3** and **4**, appear to be much more specific than the very poor amide substrates[6] **5** and **6**. This is especially true for substrates of trypsin. In view of the latter results it is not surprising that no detectable enzyme-catalyzed cleavage of peptide bonds at sites of alkanethiolation of the A chain of bovine insulin for **7** and **8** was observed. It follows from the prior discussion that, in comparison with the amino acid analogs, the peptide chain (R_α in Fig. 1) of **7** and **8** may be expected to impose additional limitations on the range of freely allowed conformations of the mixed disulfide side chains and on the interactions with the enzymes.

Assuming that the apparent specificity differences between ester and amide substrate analogs are not a result of changes in reaction pathway[7] (*13, 27*), they then can be attributed predominantly to differences in values of k_{cat} rather than K_m; (k_{cat} analog ester/k_{cat} reference ester) > (k_{cat} analog amide/k_{cat} reference amide), whereas (K_m analog ester/K_m reference ester) ≅ (K_m analog amide/K_m reference amide) (Table 1). The binding constants (K_s) for these juxtaposed substrates are probably also similar, since it is unlikely that highly differing K_s values could be mutually offset in every case by compensatory differences in rate terms to give

[5] These constants are sensitive only to structural differences in the side chains of *N*-acyl-L-amino acid substrates with the same leaving group when assayed identically. They are largely independent of the nature of the *N*-acyl moieties (*20, 25, 27–29*).

[6] Convenient syntheses for the reference substrates, BzPheNan and BzArgNan, yield racemates. Since it is known that the binding constants $\left(K\frac{D}{n}\right)$ for the D isomers, which are at best extremely poor substrates (*25, 26*), are less than or equal to those $\left(K\frac{L}{p}\right)$ for the L isomers (*25*), the maximal possible deviations of the observed maximum velocities (V_{obsd}; and hence K_{cat}) and binding constants (K_{obsd}) from the true values $\left(V\frac{L}{p}\right.$ and $\left.K\frac{L}{p}\right)$ for the L isomers will be as shown [assuming $K\frac{L}{p} = K\frac{D}{n}$ (*26*)]: $[V_{obsd} = V\frac{L}{p}\left[K\frac{L}{p}\Big/\left(K\frac{L}{p} + K\frac{D}{n}\right)\right] = \frac{1}{2}V\frac{L}{p}$ and $K_{obsd} = K\frac{L}{p} + K\frac{D}{n} = 2K\frac{L}{p}$ where n = nonproductive and p = productive binding modes]. As can be seen, the errors introduced are not large, and only differences much greater than twofold in the K_m and k_{cat} values for **5** and **6** versus BzArgNan or BzPheNan, respectively, are considered significant.

[7] It is noteworthy that concentrations of Me_2SO even greater than the 33.3% employed here in all assays of *p*-nitroanilide substrates only alter neither the kinetic schemes or mechanisms of action of either trypsin or α-chymotrypsin (*12–14*).

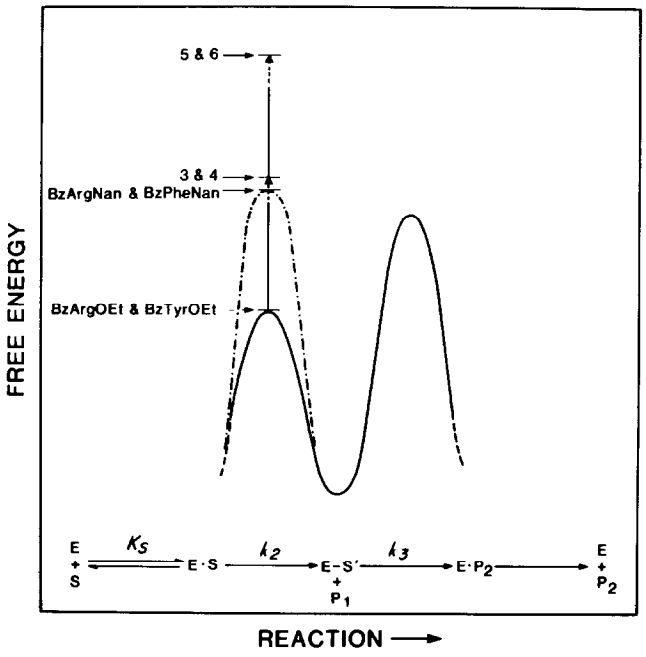

FIG. 2. Reaction coordinate–free energy diagram showing proposed relationships of transition-state free energies of individual catalytic steps for the trypsin- and α-chymotrypsin-catalyzed hydrolyses of reference and β-*S*-derivatized cysteinyl analog substrates. The standard free energy values have been arbitrarily chosen on an undefined scale since they have not been quantitatively determined and are not expected to be the same for ester and amide substrates. Shown below is the general equation for reactions normally catalyzed by serine proteases. Tetrahedral intermediates in both acylation (with rate constant k_2) and deacylation (with rate constant k_3) have not been depicted; their omission does not affect the overall conclusions (*28*). (—) = free energy profile for highly specific reference esters and (–·–·–) = free energy profile for highly specific reference amides. See text for an explanation of the significance of the vertical arrows pertaining to the acylation step.

the similar K_m values observed in Table 1. These observations and interpretations are consistent with the knowledge that large differences in kinetic specificities of substrates for serine proteases usually reflects overriding variations in catalytic versus binding ability (*25, 26, 28, 30*).

From the k_{cat} values of Table 1, the most plausible relationships of transition-state free energies, $\Delta G^{\ddagger}$, of individual catalytic steps for reference and analog substrates can be assigned as in Fig. 2. As shown, the higher $\Delta G^{\ddagger}_{acylation}$ values for **5** and **6** than for BzArgNan and BzPheNan reflect the large observed differences in k_{cat} values, because acylation generally is rate-limiting for amides (*28*). However, the equivalently higher $\Delta G^{\ddagger}_{acylation}$ values expected for **3** and **4** versus BzArgOEt and BzTyrOEt[8] (Fig. 2) are (largely) not reflected in values of k_{cat} since deacylation normally is rate-limiting for esters.

The latter interpretation is consistent with the results of the Eadie plot (Fig. 3)

[8] In other words, the origin of the specificity difference of reference vs. analog substrates should reside in the differences in side chain structure and not in the nature of the leaving groups.

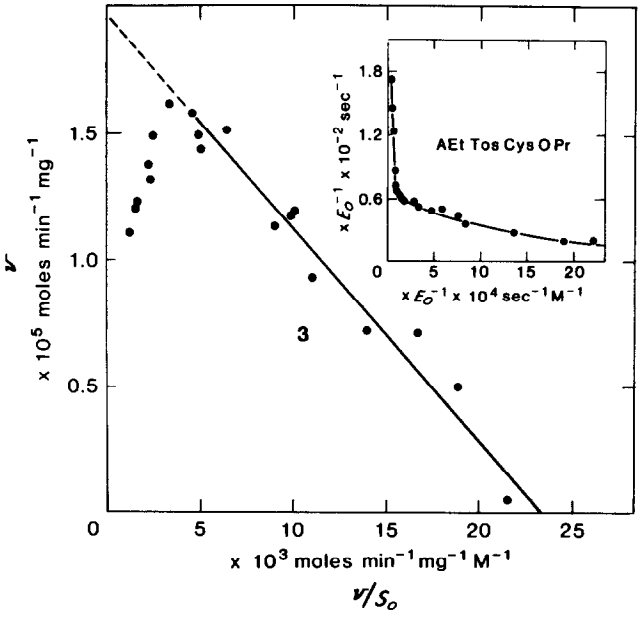

FIG. 3. Comparison of Eadie plots for trypsin-catalyzed hydrolyses of β-*S*-(β-aminoethanethiol) (**3**; pH-stat assay as in text)- and β-*S*-(β-aminoethyl) [inset; pH 8.0, 25°C, from Elmore *et al.* (*44*)]-derivatized *N*-acyl-L-cysteine ester nonspecific substrates. AEtTosCysOPr = β-*S*-(β-aminoethyl)-*N*-*p*-toluenesulfonyl-L-cysteine *n*-propyl ester.

for the tryptic hydrolysis of **3**. It shows a curve of apparent substrate inhibition at high concentrations characteristically seen for acyl amino acid amide substrates, and not a curve of presumed substrate activation (*31*) at high concentrations characteristically seen with nonspecific acyl amino acid ester substrates like AEt-TosCysOPr (*32*). That is, for **3**, at least, acylation most probably is rate-limiting as it is for most amides, $k_2 < k_3$, due to a large increase in the value for $\Delta G^{\ddagger}_{\text{acylation}}$ over that for BzArgOEt. Amino acid and peptide ester substrates of certain proteases for which $k_2 \leq k_3$, that is, for which there is a change from the normally rate-controlling step, have been reported previously (*33, 34*). Alternatively, that our results may be attributed to possible differences in mechanism for ester and amide analog substrates cannot be entirely discounted (*35, 36*).

It is a reasonable supposition that the combination of abnormally large and disulfide-containing side chains of the alkanethiolated analogs creates severe constraints on enzyme–substrate conformations during acylation (*14, 37*). Those which presumably are important in the initial stabilization of tetrahedral intermediates may be allowed, but the ones that facilitate their subsequent decomposition may be hindered (*38–40*).

CONCLUSION

The mixed-disulfide *N*-acyl-L-cysteine analogs of this study, **3**, **4**, **5**, and **6**, obviously tax the limitations of the active sites of trypsin and α-chymotrypsin. As

a result, interesting and unexpected kinetics of enzyme catalyzed hydrolyses are observed.

This work supports the contention that $RSSO_2R'$ reagents can be used for novel applications.

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