Mapping the S′ Subsites of Serine Proteases Using Acyl Transfer to Mixtures of Peptide Nucleophiles†

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ABSTRACT: We have developed a rapid and convenient procedure for the characterization of the S′ subsite specificity of serine proteases. A mixture of peptide nucleophiles is incubated with the enzyme in the presence of excess of a specific ester substrate. The decrease in each nucleophile concentration is monitored by high-performance liquid chromatography analysis of the dansylated mixture. Relative kinetic parameters for each nucleophile in the mixture are then calculated using a new statistical algorithm that relates all pairs of nucleophiles. As a first application, we investigated the S'_1 subsite specificity of chymotrypsin, trypsin, and a recently described trypsin mutant, Tr → Ch[S1 + L1 + L2] with chymotrypsin-like primary specificity [Hedstrom, L., Szilagyi, L., & Rutter, W. J. (1992) Science 255, 1249–1253]. For this purpose 21 peptide nucleophiles of the general structure H-Xaa-Ala-Ala-Ala-Ala-NH_2 were prepared by multiple solid-phase synthesis, where Xaa represents D-alanine, citrulline, and all natural amino acids except cysteine. Relative second-order rate constants for the enzyme-catalyzed acyl transfer to these nucleophiles were determined over a range of 10^2. Chymotrypsin and trypsin have markedly different S'_1 specificities. The order of preference in chymotrypsin-catalyzed acyl transfer reactions is positively charged > aliphatic > aromatic ≫ negatively charged, D-Ala, Pro P'_1 side chain. Trypsin prefers hydrophobic residues, but like chymotrypsin aliphatic residues are better than aromatic residues in P'_1 position. The S'_1 specificity of the mutant Tr → Ch[S1 + L1 + L2] is similar to the specificity of trypsin; however, P'_1 aromatic residues have low reactivity characteristic of chymotrypsin.

The function of a protease is revealed by its substrate specificity. However, the determination of substrate specificity is currently a tedious procedure. Generally, specificity studies have focused on the S_1–S_5 subsites [subsite nomenclature of Schechter and Berger (1967)], while the S′ subsites have been ignored. Usually, protease substrate specificity is characterized by synthesizing a large number of substrates and testing each separately [reviewed in Schellenberger et al. (1991c)]. Clearly, the ability to test mixtures of substrates would streamline this process. Recently, several reports have described the use of peptide libraries to characterize the binding specificity of antibodies or receptors (Devlin et al., 1990; Fodor et al., 1991; Furka et al., 1991; Houghten et al., 1991; Lam et al., 1991; Scott & Smith, 1990; Zuckerman et al., 1992). Peptide mixtures have also been used to characterize protease substrate specificity by monitoring hydrolysis by Edman degradation (Birkett et al., 1991; Petithory et al., 1991) or HPLC/MS (Berman et al., 1992). However, these methods have two major limitations: (i) a specific library has to be synthesized and analysis conditions have to be established for each enzyme of interest, and (ii) all peptides in the substrate mixture may not be hydrolyzed at the same peptide bond. So far, hydrolysis of peptide mixtures was analyzed by applying the kinetics of *pairs* of competing substrates. As a consequence, the kinetic constants calculated for a peptide mixture were within the same order of magnitude.

We have developed an improved facile method for determining the S′ subsite specificity of serine proteases. The S′ subsite specificity of serine and cysteine proteases can be analyzed by studying the reversal of peptide hydrolysis, i.e., acyl transfer to a peptide nucleophile, which yields data very similar to that from peptide hydrolysis (Fersht et al., 1973; Schellenberger & Jakubke, 1991). Acyl transfer to a mixture of peptides can be conveniently monitored by HPLC.[1] This approach has three advantages over the methods developed so far: (i) the specific acyl donor ester is used in large excess to the peptide nucleophiles, which reduces the risk of side reactions, (ii) the same nucleophile library can be used to characterize enzymes with different primary specificity, and (iii) the application of a new statistical method for analysis of multiple substrate kinetics allows the determination of nucleophilic efficiencies varying over 10^2 from the same experiment.

We describe here chymotrypsin- and trypsin-catalyzed acyl transfer reactions using a set of 21 peptide nucleophiles of the general structure H-Xaa-Ala-Ala-Ala-Ala-NH_2.

MATERIALS AND METHODS

Enzymes. Bovine α-chymotrypsin (lyophilized, analytical grade, lot 10860521-39, Boehringer, Mannheim, Germany) and bovine trypsin (lyophilized, analytical grade, lot 26039, Serva, Germany) were used without further purification. Rat trypsin II and the mutant Tr → Ch[S1 + L1 + L2] were expressed in yeast, purified, and activated as described previously (Hedstrom et al., 1992).

Rat chymotrypsin was expressed in yeast culture medium by fusing the chymotrypsinogen-coding sequence to the

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[1] Abbreviations: Bz, benzoyl; BPTI, bovine pancreatic trypsin inhibitor; Cit, citrulline; DMF, dimethylformamide; Dns, dansyl [(dimethylamino)-naphthalene-5-sulfonyl]; Fmoc, 9-fluorenylmethyloxycarbonyl; HPLC, high-performance liquid chromatography; MS, mass spectrometry; OEt, ethyl ester, OMTKY, turkey ovomucoid third domain.

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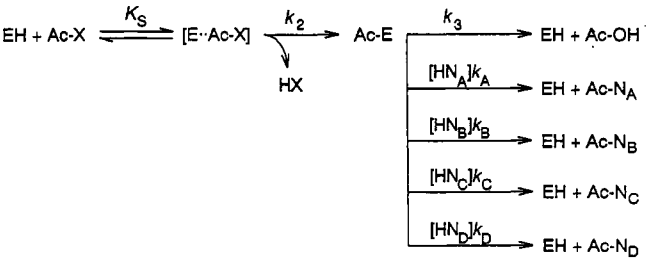
α-factor leader sequence as described for carboxypeptidase A1 (Phillips et al., 1990). The final expression vector pYT contains the inducible alcohol dehydrogenase–glyceraldehydephosphate dehydrogenase (ADH–GADPH) promoter and regulatory regions, the GADPH transcription terminator, and *amp*, *ura3*, and *leu2d* markers. Expression in this vector gives a protein with Glu-Ala added to its N-terminus (Brake & al., 1984). Medium was isolated from a 2-L culture by centrifugation. Chymotrypsinogen was purified by ion-exchange chromatography on Toyopearl SP-650M (Supelco, Bellefonte, PA) using a pH gradient from 0.1 M acetate buffer, pH 3.0, to 0.1 M Tris buffer, pH 8.0. The zymogen was activated overnight at 8 °C in 0.5 M Tris buffer, pH 8.0, and 20 mM $CaCl_2$ using bovine trypsin immobilized to agarose beads (Sigma Chemical Co.). Active chymotrypsin was isolated by affinity chromatography on soybean trypsin inhibitor–agarose (Sigma Chemical Co.). About 4 mg of purified chymotrypsin was obtained.

Peptide Synthesis. The tetrapeptide Ala-Ala-Ala-Ala-NH_2 was synthesized with an ABI peptide synthesizer Model 431 (Applied Biosystems, Foster City, CA) using 1 g (0.54 mmol) of Fmoc-Ala-dimethoxyalkoxybenzylamine resin (Peninsula Laboratories, Belmont, CA) and standard Fmoc chemistry. The tetrapeptide resin was then suspended in 10 mL of DMF and aliquoted into 21 reaction tubes. Coupling of the last amino acid was performed with an Advanced ChemTech MPS 350 multiple peptide synthesizer (Louisville, KY) using double coupling and a 12-fold excess of the appropriate Fmoc amino acid (Bachem, Torrance, CA). Final deprotection and cleavage was performed with 5% anisole and 5% dimethyl sulfide in trifluoroacetic acid for 3 h. After filtration the peptides were precipitated with ethyl ether, dissolved in 1 mL of 50% acetic acid and 10 mL of water, and lyophilized. Liquid secondary ion mass spectrometry (LSIMS) was carried out on a Kratos Analytical (Manchester, U.K.) MS-50S mass spectrometer, equipped with a LSIMS ion source. Spectra were recorded on a Gould (Cleveland, OH) ES-1000 electrostatic recorder. Samples were run in a 1:1 glycerol/thioglycerol matrix acidified with 0.1 M HCl on a coolable sample introduction probe.

Preparation of Peptide Mixtures. The peptide nucleophiles (about 5 μmol) were dissolved in 3 mL of water. Eight microliters of a 10-fold diluted solution of each peptide was dansylated and analyzed as described below. We prepared three peptide mixtures so that all peptides in a mixture were resolved and the peak sizes for all peptides were similar. The following mixtures were prepared (only the N-terminal residues are indicated): mixture I, Glu, Gln, Ser, Thr, Ala, D-Ala, Met, Pro, Trp, His, and Tyr (Dns-Pro-OH, internal standard); mixture II, Asp, Cit, Asn, Ser, Gly, Arg, Val, Ile, Leu, Lys, and Tyr (Dns-Pro-OH, internal standard); mixture III, Ser, Thr, Ala, Met, Phe, and Tyr (Dns-Ile-OH, internal standard). The concentrations of each peptide in the mixtures were approximately 100 μM (individual peptides). Peptide mixtures were stored at −20 °C.

Acyl Transfer. Acyl transfer reactions were performed at 25 °C in a volume of 100 μL containing 0.2 M KCl and 0.1 M borate buffer, pH 9.0, approximately 40 μM peptide nucleophiles, and 10 mM acyl donor ester. The 2-fold-concentrated reaction buffer was prepared once, and aliquots were stored at −70 °C. Stock solutions (400 mM) of Bz-Arg-OEt (Bachem) and Ac-Phe-OEt (Research Plus Inc., Bayonne, NJ) were prepared in water and acetonitrile, respectively. Consequently, 2.5% acetonitrile was present in reactions with Ac-Phe-OEt. The reactions were initiated by addition of enzyme. Samples were taken after 0, 0.5, 1, 2, 4, 8, 16, 32, 64, and 128 min of reaction time.

Scheme I: Serine Protease-Catalyzed Acyl Transfer to a Mixture of Competing Nucleophiles[a]



[a] EH, free enzyme; Ac-X, acyl donor; HX, leaving group; Ac-E, acyl enzyme; Ac-OH, hydrolysis product; HN_x, nucleophiles; Ac-N_x, aminolysis products.

Dansylation and HPLC Analysis. Samples (8 μL) were taken from the reaction mixtures with a microsyringe/pipette (SGE, Australia) and rapidly mixed with 8 μL of 5 mM DnsCl (Sigma Chemical Co.) in acetonitrile by aspirating and releasing the sample 5 times. Eight microliters of 200 mM borate buffer, pH 9.0, was added, and the vials were sealed, vortexed, and kept in the dark at room temperature for a minimum of 5 h. The dansylation reaction was greater than 90% complete under these conditions, and the dansyl peptides were stable for at least 2 days.

HPLC analyses were performed using a Hewlett-Packard 1090 system (Palo Alto, CA) and a 1046A fluorescence detector (333-nm excitation, 522-nm emission) on an AminoQuant C18 column (Hewlett-Packard, 79916AA-572). Samples were eluted at a flow rate of 0.225 mL/min with a step gradient (1 min, 0% B; 3 min, 19% B; 16 min, 37.5% B; 20 min, 62.5% B; 24 min, 100% B), where eluent A was a 4:1 mixture of 4 mM phosphate buffer, pH 6.2, and B, and eluent B was a 1:4 mixture of 100 mM NaAc, pH 7.2, and acetonitrile. Injection volume was 5 μL. The peak area was found to be linearly dependent on the peptide concentration in the sample.

Data Analysis. The peak areas of all peptides were normalized on the basis of the peak area of the internal standard. Nucleophiles whose concentration decreased less than 10% during the reaction were excluded from the calculation. Samples in which no decrease of the nucleophile concentrations was detected (because the acyl donor was used up) were excluded from the calculations. Rate constants were calculated from the peak areas using a statistical algorithm which is described in the accompanying paper (Schellenberger et al., 1993).

RESULTS

Preparation of Peptide Mixtures. Serine proteases catalyze the hydrolysis of a substrate in a three-step mechanism involving an acyl enzyme intermediate as shown in Scheme I. The acyl enzyme intermediate can be attacked by other nucleophiles in addition to water. When those nucleophiles are amines, the reaction is simply the reverse of peptide hydrolysis. Therefore, acyl transfer reactions are a convenient method for analyzing the specificity of the enzyme for the leaving group. We prepared a series of peptides of the general structure H-Xaa-Ala-Ala-Ala-Ala-NH_2, where Xaa represents D-Ala, Cit, and all natural amino acids except Cys. These peptides are nucleophilic acceptors in protease-catalyzed acyl transfer reactions and the variable residue Xaa is analogous to the P'_1 position in substrates R-CO-Xaa-Ala-Ala-Ala-Ala-NH_2. The peptide nucleophiles were synthesized using standard solid-phase methods. A sample of each nucleophile was dansylated and analyzed by HPLC. Only one major peak

was observed for all nucleophiles. Three nucleophile mixtures were prepared such that all of the nucleophiles in a mixture could be resolved by HPLC, and each nucleophile was included in at least one of the mixtures. H-Ser-Ala-Ala-Ala-Ala-NH_2 was included in all mixtures as a reference compound. The amount of this nucleophile decreased to less than 50% of its starting concentration in all acyl transfer reactions, which allows precise quantitative analysis.

Acyl Transfer to Competing Nucleophiles. The kinetics of protease-catalyzed acyl transfer to a mixture of peptide nucleophiles is described by Scheme I. In this mechanism each nucleophile and water compete for the acyl enzyme intermediate Ac-E. The acylation rate k_x for a nucleophile HN_x is given by

$$-d[HN_x]/dt = [Ac\text{-}E][HN_x]k_x \quad (1)$$

where k_x represents a second-order rate constant. Consequently, the ratio of the acylation rates for two nucleophiles is given by

$$\frac{d[HN_x]}{d[HN_y]} = \frac{[HN_x]k_x}{[HN_y]k_y} \quad (2)$$

Equation 2 demonstrates that the kinetics of competing nucleophiles in an acyl transfer reaction can be treated in essentially the same way as the kinetics of competing substrates in normal Michaelis–Menten kinetics (Fersht, 1985). Note that the ratio of acylation rates for two nucleophiles is independent of the concentration of Ac-E; therefore, eq 2 is valid when either acylation or deacylation is rate-determining for the reaction of Ac-X.

Acyl transfer reactions were performed by mixing the peptide nucleophiles, Dns-Ile-OH or Dns-Pro-OH as an internal standard, and a large excess (250-fold) of the acyl donor ester. The reactions were initiated by addition of enzyme, and 6–10 samples were taken between 0 and 128 min of reaction time. The concentrations of the nucleophiles were determined by derivatization with dansyl chloride and subsequent HPLC analysis. Peak areas were very reproducible, exhibiting standard deviations of 1–4% for identical samples. Thus, at each time point we obtained a concentration for each of 6–11 nucleophiles, so that a large matrix of data rapidly accumulated. Relative rate constants for each nucleophile can be calculated from such data using the statistical algorithm described in the accompanying paper (Schellenberger et al., 1993).

Figure 1 shows the time course of a trypsin-catalyzed acyl transfer reaction using a mixture of 11 competing nucleophiles. A comparison of the HPLC profiles shows that the concentration of most of the nucleophiles decreases significantly during the first 32 min of the reaction. The peptide nucleophiles and the aminolysis products are efficiently protected from trypsin-catalyzed hydrolysis in the presence of the acyl donor ester. After 32 min, most of the acyl donor ester is consumed and hydrolysis of the peptide products Bz-Arg-Xaa-Ala-Ala-Ala-Ala-NH_2 can occur. As a result, an increase of the nucleophile concentrations is observed at time 128 min.

S'_1 Subsite Specificity of Chymotrypsin and Trypsin. Acyl transfer reactions were performed using Bz-Arg-OEt for trypsin and Ac-Phe-OEt for reactions with chymotrypsin and the mutant Tr → Ch[S1 + L1 + L2]. Compounds with N-terminal Pro, D-Ala, and Asp (as well as Glu in chymotrypsin-catalyzed reactions) were extremely poor nucleophiles. The concentrations of these nucleophiles decreased by less than 10% during the reaction, and no rate constants could be calculated. The concentrations of all other nucleophiles

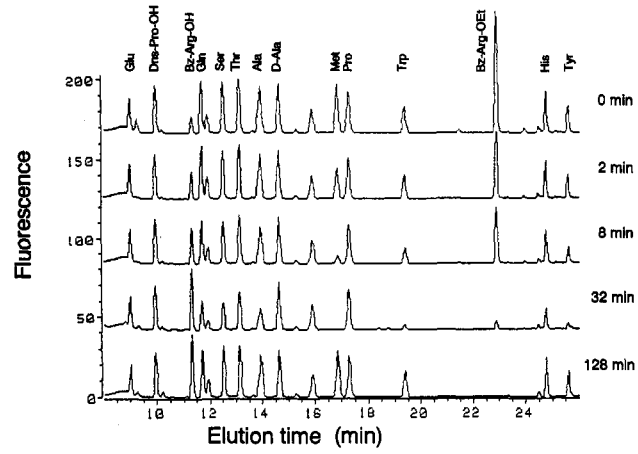


FIGURE 1: Bovine trypsin-catalyzed acyl transfer to a mixture of nucleophiles of the structure H-Xaa-Ala-Ala-Ala-Ala-NH_2. The reaction mixture contained 10 mM Bz-Arg-OEt, about 40 μM nucleophiles, and 10 nM bovine trypsin. Reaction times and N-terminal amino acids of the nucleophiles in the mixture are indicated.

decreased significantly during the reactions. Quantitative analysis of the data using the statistic procedure described in the accompanying paper (Schellenberger et al., 1993) yielded relative rate constants with good reproducibility (see Table I).

Several nucleophiles were included in more than one of the peptide mixtures. In all cases the calculated kinetic parameters were identical within the limit of error. This justifies the conclusion that kinetic parameters of nucleophiles that were not analyzed in the same mixture can be compared and validates the statistical method.

DISCUSSION

None of the enzymes included in this study catalyzed acyl transfer to nucleophiles with P'_1 Pro or D-Ala. This observation suggests that all enzymes require the P'_1 residue to have the L-configuration and an NH_2 group. In addition, there are marked differences in the specificity of the enzymes for the P'_1 side chain. The efficiency of chymotrypsin-catalyzed acyl transfer decreases in the order positively charged > aliphatic > aromatic ≫ negatively charged P'_1 side chain. Trypsin prefers nucleophiles with bulky hydrophobic P'_1 residues, but as with chymotrypsin, nucleophiles with large aliphatic P'_1 residues are preferred over nucleophiles with aromatic P'_1 residues.

The overall low efficiency of nucleophiles with negatively charged P'_1 residues may be caused by a partial protonation of the α-amino group of these compounds at pH 9.0. The pK_a of peptides with N-terminal Asp is significantly higher if compared to peptides with uncharged amino acids in this position (Fasman, 1976). The negative charge of P'_1 Asp could also interfere with the catalytic events during acyl transfer.

Structural Information about the S′ Subsites. High-resolution crystal structures are available for several protease–inhibitor complexes (Bode & Huber, 1991). The structure of these complexes is a reasonable model for enzyme–substrate contacts during peptide hydrolysis. In order to understand S′–P′ interactions we superimposed the structures of bovine trypsin–BPTI complex (Ruhlmann et al., 1973) and bovine chymotrypsin–OMTKY complex (Fujinaga et al., 1987). The α-carbon atoms of residues 189–195, 214–216, and 225–228, which form a major part of the active site and the S_1 binding pocket, were superimposed (rms deviation 0.48 Å). The backbones of both inhibitors were found to be superimposed

Table I: Acyl Transfer to Nucleophiles of the Structure H-Xaa-Ala-Ala-Ala-Ala-NH_2[a]

P'_1 residue	bovine chymotrypsin	rat chymotrypsin	bovine trypsin	rat trypsin	Tr → Ch[S1 + L1 + L2]
D-Ala	<0.05	<0.05	<0.05	<0.05	<0.05
Pro	<0.05	<0.05	<0.05	<0.05	<0.05
Gly	0.34 ± 0.04	0.20 ± 0.02	0.25 ± 0.03	0.34 ± 0.06	0.42 ± 0.05
Ala	1.04 ± 0.07	0.74 ± 0.05	1.24 ± 0.08	1.36 ± 0.12	1.80 ± 0.13
Val	0.88 ± 0.02	0.74 ± 0.01	1.17 ± 0.03	1.27 ± 0.22	1.50 ± 0.05
Ile	0.84 ± 0.04	0.78 ± 0.02	7.65 ± 0.56	7.59 ± 0.65	3.31 ± 0.25
Leu	0.96 ± 0.01	0.79 ± 0.01	5.35 ± 0.35	5.05 ± 0.34	2.10 ± 0.13
Met	1.31 ± 0.09	1.06 ± 0.03	12.1 ± 2.2	12.7 ± 1.2	6.62 ± 0.62
Phe	0.29 ± 0.02	0.37 ± 0.01	3.25 ± 0.39	2.75 ± 0.29	0.50 ± 0.06
Tyr	0.28 ± 0.03	0.30 ± 0.03	2.48 ± 0.17	2.16 ± 0.35	0.39 ± 0.14
Trp	0.19 ± 0.01	0.30 ± 0.01	2.98 ± 0.28	4.04 ± 0.70	0.70 ± 0.11
His	0.67 ± 0.12	0.77 ± 0.03	1.20 ± 0.17	1.56 ± 0.13	0.77 ± 0.12
Ser	1.00	1.00	1.00	1.00	1.00
Thr	0.76 ± 0.04	0.81 ± 0.02	0.63 ± 0.04	0.65 ± 0.09	0.62 ± 0.06
Asn	0.21 ± 0.01	0.22 ± 0.02	0.36 ± 0.05	0.69 ± 0.09	0.42 ± 0.03
Gln	0.54 ± 0.04	0.30 ± 0.02	1.06 ± 0.05	1.49 ± 0.22	1.61 ± 0.04
Asp	<0.05	<0.05	<0.05	<0.05	<0.05
Glu	<0.05	<0.05	0.24 ± 0.02	0.44 ± 0.10	0.41 ± 0.09
Lys	7.83 ± 0.59	2.96 ± 0.35	2.61 ± 0.29	1.76 ± 0.47	1.59 ± 0.08
Arg	29.9 ± 2.5	7.92 ± 0.27	3.62 ± 0.12	2.28 ± 0.24	1.36 ± 0.09
Cit	1.59 ± 0.03	1.21 ± 0.02	5.54 ± 0.58	5.32 ± 0.38	2.60 ± 0.16

[a] Acyl donor was Bz-Arg-OEt for trypsin and Ac-Phe-OEt for chymotrypsin and the mutant Tr → Ch[S1 + L1 + L2]. Rate constants are relative to the constant of H-Ser-Ala-Ala-Ala-Ala-NH_2. Data are mean ± SD calculated from triplicate experiments.

	33	40	58	65
Bovine chymotrypsin	Leu-Gln-Asp-Lys-Thr-Gly-Phe-His		Cys-Gly-Val-Thr-Thr-Ser-Asp-Val-Val	
Rat chymotrypsin	Leu-Gln-Asp-Lys-Thr-Gly-Phe-His		Cys-Gly-Val-Lys-Thr-Ser-Asp-Val-Val	
Bovine trypsin	Leu-Asn---------Ser-Gly-Tyr-His		Cys-----Tyr-Lys-Ser-Gly-Ile-Gln-Val	
Rat trypsin	Leu-Asn---------Ser-Gly-Tyr-His		Cys-----Tyr-Lys-Ser-Arg-Ile-Gln-Val	

FIGURE 2: Comparison of the S′ subsite-forming loops of chymotrypsin and trypsin.

from $N(P_2)$ to $C(P'_2)$. Therefore, the orientation of peptide substrates in the active sites of chymotrypsin and trypsin seems to be very similar.

There are many contacts in the S′ region between the backbones of the inhibitors and the enzymes which are identical in the trypsin–BPTI complex and the chymotrypsin–OMTKY complex. The structures of both protease–inhibitor complexes reveal that P'_1 side chains can interact with two loops of the enzymes, residues 35–39 and 59–64. These loops differ in both the number and character of amino acid residues for chymotrypsin and trypsin (see Figure 2), which may explain the different S'_1 specificities of these enzymes. However, there is little information about the potential conformation of P'_1 side chains because this position is occupied by Ala in the trypsin–BPTI complex, while P'_1 Glu of OMTKY is believed to adopt a conformation which avoids unfavorable electrostatic contacts with Asp-35 and Asp-64 of chymotrypsin (see discussion below).

Comparison of the Chymotrypsin and Trypsin S'_1 Specificity. Chymotrypsin and trypsin differ remarkably in their specificity toward nucleophiles with charged P'_1 side chains. Chymotrypsin prefers positively charged residues; Arg is about 10-fold better than Cit, which has a similar structure but no charge. The specificity of chymotrypsin for P'_1 Arg and Lys has also been observed in a statistical analysis of proteolysis data (Keil, 1987). In a previous paper we attributed this specificity to electrostatic interactions between P'_1 Arg/Lys and Asp-35 and Asp-64 of chymotrypsin (Schellenberger et al., 1990). Both residues are located in the S′ subsites of the enzyme and Asp-64 forms a water-mediated salt bridge with P'_3 Arg in the chymotrypsin–OMTKY complex (Fujinaga et al., 1987). The electrostatic nature of these interactions has been confirmed by studies of their salt dependence (Schellenberger et al., 1991a,b). Interaction of the P'_1 residue with Asp-35 and Asp-64 also explains the failure of H-Glu-Ala-Ala-Ala-Ala-NH_2 to act as a nucleophilic acceptor.

The S′ site-forming loops of trypsin do not contain Asp or Glu (see Figure 2), which explains why trypsin shows no preference for positively charged P'_1 residues. A more positive electrostatic field in the S′ subsites of trypsin can also explain that H-Glu-Ala-Ala-Ala-Ala-NH_2 is a nucleophilic acceptor in trypsin-catalyzed reactions.

Comparison of the data in Table I shows that trypsin significantly prefers P'_1 residues with hydrophobic side chains. This may be caused by interactions with the side chain of Lys-61, which is oriented toward the S'_1 subsite in trypsin. Similar rate constants are observed for nucleophiles with β-branched and unbranched amino acid residues. This is in accordance with the open structure of the S′ subsites.

We measured similar rate constants for nucleophiles with hydrophobic and hydrophilic P'_1 side chains in chymotrypsin-catalyzed reactions, but nucleophiles with aromatic P'_1 residues were significantly less active. Proteolysis data also reveal a very low specificity of chymotrypsin for aromatic residues in the P'_1 position (Keil, 1987). Our data apparently contradict the observation that amides of aromatic amino acids are more efficient nucleophiles than amides of aliphatic amino acids in chymotrypsin-catalyzed acyl transfer (Fersht et al., 1973; Gololobov et al., 1992). This difference in P'_1 specificity observed for amino acid amides could be due to missing P'_2–S'_2 and P'_3–S'_3 contacts. The smaller number of contacts with the enzyme gives amino acid amides a larger conformational freedom during aminolysis, and consequently, aromatic P'_1 residues can be accommodated.

Comparison of Bovine and Rat Enzymes. The S'_1 specificity is very similar for both trypsins and chymotrypsins from rat and cow (see Figure 3). This is in accordance with the almost identical sequences of the S′ site-forming loops of the enzymes from both species (Figure 2). The largest differences were observed for nucleophiles with charged P'_1 residues. The

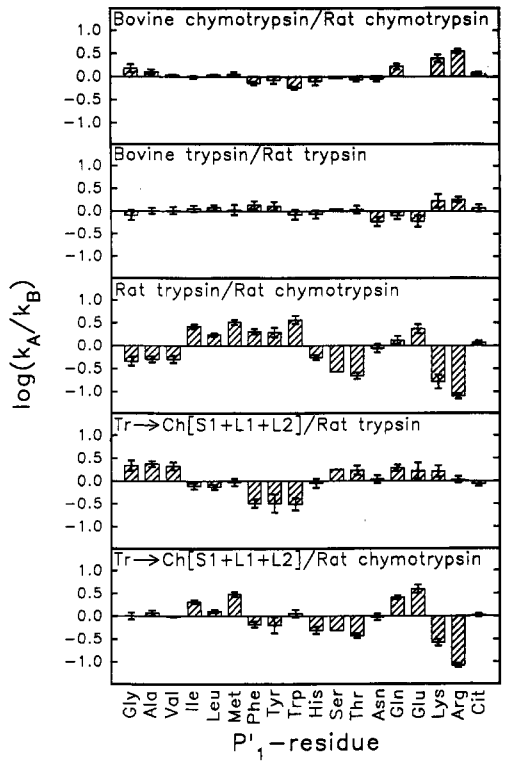


FIGURE 3: Comparison of the S'_1 specificity of various enzymes. Ratios of the rate constants were calculated using data from Table I. The logarithmic data are normalized so that the sum of data for nucleophiles with uncharged P'_1 residues equals zero. Error bars give the sum of standard deviations observed for both enzymes.

specificity for positively charged residues is less pronounced in rat chymotrypsin than in the bovine enzyme. This altered specificity is probably caused by the substitution of Thr-61 for Lys in rat chymotrypsin. Similarly, nucleophiles with positively charged P'_1 residues have higher rate constants with bovine trypsin than with rat trypsin, whereas H-Glu-Ala-Ala-Ala-Ala-NH_2 has a higher rate constant with the rat enzyme. This observation might be explained by the additional positive charge of Arg-63 in the S′ subsites of rat trypsin. The net charge of bovine trypsin is 12.5 units higher than the net charge of rat trypsin. Our data suggest that the charge of Arg-63 causes a local change in the electrostatic field, whereas the effect of many distant negative charges is effectively shielded. Calculations of the electrostatic potentials of rat and bovine trypsin using the finite difference Poisson–Boltzmann method showed that the potentials in the active sites of both enzymes are dominated by local interactions (Soman et al., 1989). This conclusions seems to apply to the S′ subsites as well.

S′ Specificity of Tr → Ch[S1 + L1 + L2]. In the trypsin mutant Tr → Ch[S1 + L1 + L2], residues 138, 189, and 192 and two loops, 185–188 and 221–225, are replaced by the respective chymotrypsin residues. All of these changes occur at locations far away from the S′ subsites of the enzyme. This mutant possesses a chymotrypsin-like primary specificity, especially with long substrates (Hedstrom et al., 1992). Figure 3 shows a comparison of the S′ specificity of Tr → Ch[S1 + L1 + L2] with chymotrypsin and trypsin. The mutant shows a mixed trypsin/chymotrypsin specificity. Like trypsin, the mutant prefers bulky hydrophobic P'_1 residues, and the rate constants for nucleophiles with charged P'_1 residues are not significantly changed by the mutations. However, like chymotrypsin, Tr → Ch[S1 + L1 + L2] has a significantly decreased specificity for aromatic residues in P'_1 position.

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REFERENCES

Berman, J., Green, M., Sugg, E., Anderegg, R., Millington, D. S., Norwood, D. L., McGeehan, J., & Wiseman, J. (1992) *J. Biol. Chem. 267*, 1434–1437.

Birkett, A. J., Soler, D. F., Wolz, R. L., Bond, J. S., Wiseman, J., Berman, J., & Harris, R. B. (1991) *Anal. Biochem. 196*, 137–143.

Bode, W., & Huber, R. (1991) *Curr. Opin. Struct. Biol. 1*, 45–52.

Brake, A. J., et al. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4642–4646.

Devlin, J. J., Panganiban, L. C., & Devlin, P. E. (1990) *Science 249*, 404–406.

Fasman, G., Ed. (1976) pK values of peptides, *Handbook of Biochemistry and Molecular Biology*, CRC Press Inc., Cleveland, OH.

Fersht, A. (1985) *Enzyme structure and mechanism*, p 112, W. H. Freeman and Co., New York.

Fersht, A. R., Blow, D. M., & Fastrez, J. (1973) *Biochemistry 12*, 2035–2041.

Fodor, S. P., Read, J. L., Pirrung, M. C., Stryer, L., Lu, A. T., & Solas, D. (1991) *Science 251*, 767–773.

Fujinaga, M., Sielecki, A. R., Read, R. J., Ardelt, W., Laskowski, M., Jr., & James, M. N. G. (1987) *J. Mol. Biol. 195*, 397–418.

Furka, A., Sebestyen, F., Asgedom, M., & Dibo, G. (1991) *Int. J. Pept. Protein Res. 37*, 487–493.

Gololobov, M. Y., Voyushina, T., Stepanov, V., & Adlercreutz, P. (1992) *FEBS Lett. 307*, 309–312.

Hedstrom, L., Szilagyi, L., & Rutter, W. J. (1992) *Science 255*, 1249–1253.

Houghten, R. A., Pinilla, C., Blondelle, S. E., Appel, J. R., Dooley, C. T., & Cuervo, J. H. (1991) *Nature 354*, 84–86.

Keil, B. (1987) *Protein Seq. Data Anal. 1*, 13–20.

Lam, K. S., Salmon, S. E., Hersh, E. M., Hruby, V. J., Kazmierski, W. M., & Knapp, R. J. (1991) *Nature 354*, 82–84.

Petithory, J. R., Masiarz, F. R., Kirsch, J. F., Santi, D. V., & Malcolm, B. A. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 11510–11514.

Phillips, M. A., Fletterick, R., & Rutter, W. J. (1990) *J. Biol. Chem. 265*, 20692–20698.

Ruhlmann, A., Kukla, D., Schwager, P., Bartels, K., & Huber, R. (1973) *J. Mol. Biol. 77*, 417–436.

Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157–162.

Schellenberger, V., & Jakubke, H.-D. (1991) *Angew. Chem., Int. Ed. Engl. 30*, 1437–1449.

Schellenberger, V., Schellenberger, U., Mitin, Y. V., & Jakubke, H.-D. (1990) *Eur. J. Biochem. 187*, 163–167.

Schellenberger, V., Jakubke, H.-D., & Kasche, V. (1991a) *Biochim. Biophys. Acta 1078*, 8–11.

Schellenberger, V., Kosk, M., Jakubke, H.-D., & Aaviksaar, A. (1991b) *Biochim. Biophys. Acta 1078*, 1–7.

Schellenberger, V., Braune, K., Hoffmann, H.-J., & Jakubke, H.-D. (1991c) *Eur. J. Biochem. 199*, 623–636.

Schellenberger, V., Siegel, R., & Rutter, W. (1993) *Biochemistry* (preceding paper in this issue).

Scott, J. K., & Smith, G. P. (1990) *Science 249*, 386–390.

Soman, K., Yang, A.-S., Honig, B., & Fletterick, R. J. (1989) *Biochemistry 28*, 9918–9926.

Zuckermann, R. N., Kerr, J. M., Siani, M. A., Banville, S. C., & Santi, D. V. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 4505–4509.