Converting Trypsin to Chymotrypsin: Structural Determinants of S1′ Specificity†

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ABSTRACT: Trypsin and chymotrypsin differ strikingly in substrate specificities despite great similarity in their primary and tertiary structures. This work analyzes the role of two surface loops, loop 40 and loop 60, as structural determinants of the specificity of the S1′-subsite in chymotrypsin and trypsin. Chymotrypsin prefers P1′ Arg/Lys residues, while trypsin prefers hydrophobic P1′ residues. We replaced loop 40 and loop 60 in trypsin with their chymotrypsin counterparts. These mutations do not affect the S1 specificity and catalytic activity of trypsin. The S1′ specificity was analyzed by monitoring acyl-transfer reactions to 16 amino acid amides. The exchange of loop 40 does not affect the S1′ specificity. In contrast, the replacement of loop 60 causes a loss of specificity for P1′-Met/Ile/Leu. Combining both mutations reconstitutes a chymotrypsin-like S1′ specificity. The specificity for Arg-NH_2 increases 3-fold while the preferences for Met-NH_2 and Ile-NH_2 decrease 4- and 8-fold, respectively. Therefore, P1′-Arg/Met discrimination changes by factor 12 and P1′-Arg/Ile discrimination changes by factor 24. Thus, loop 40 and loop 60 act synergistically to determine S1′ specificity in trypsin and chymotrypsin.

A major goal of biochemistry is to understand the structural basis of enzyme specificity. The trypsin family of serine proteases is an ideal model system for understanding this problem. Serine proteases are involved in many important physiological processes, including digestion, blood coagulation and fibrinolysis. Thus, they have been studied for decades and are among the best characterized classes of enzymes. The catalytic mechanism of serine proteases is well understood and dozens of X-ray crystal structures are available (Sweet et al., 1974; Stroud et al., 1974; Bode & Schwager, 1975; Huber et al., 1974; Polgar, 1989). Although trypsin and chymotrypsin have a very similar tertiary structures, they differ strikingly in their substrate specificities. Trypsin requires Lys/Arg residues in P1 while chymotrypsin has a strong preference for Phe/Tyr/Trp in P1 [nomenclature from Schechter & Berger (1967), Figure 1). Recently, chymotrypsin-like specificity was transferred into the rat trypsin II framework, thus identifying the structural determinants of S1 specificity (Hedstrom et al., 1992; Hedstrom et al., 1994; Perona et al., 1995). Surprisingly, the reconstitution of chymotrypsin-like activity required the replacement of two surface loops by the analogous loops of chymotrypsin (loop 1 residues 185−188, loop 2 residues 221−225) in addition to the substitutions in the S1 binding pocket (D189S, Q192M, I138T, insert T219) (Figure 2). These loops do not directly contact the substrates, but connect the walls of the S1 site. This enzyme, Tr→Ch[S1+L1+L2],[1] has 1% of the amidase activity of chymotrypsin. The chymotrypsin-like activity is further improved with an additional mutation, Y172W, which is also located outside the S1-site. This mutant, Tr→Ch[S1+L1+L2+Y172W], exhibits 15% of the chymotrypsin activity against amide substrates (Hedstrom et al., 1994). These mutant enzymes do not bind hydrophobic substrates well, but once bound, substrates are processed almost as efficiently as when bound to chymotrypsin. This observation illustrates the importance of the catalytic step of an enzymatic reaction for substrate discrimination.

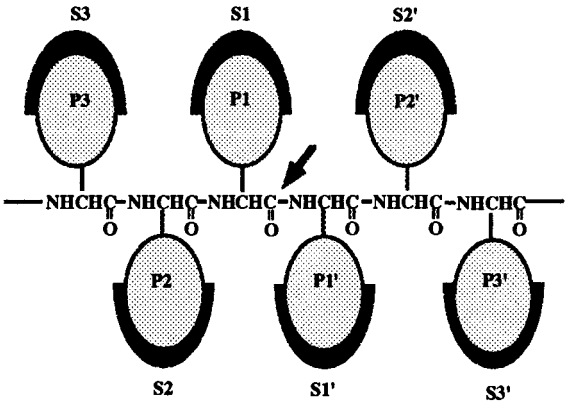

FIGURE 1: The subsites of proteases according to the nomenclature of Schechter and Berger (1967).

Although the substrate specificity of trypsin and chymotrypsin is primarily determined by the S1 site, the other subsites also contribute. In particular, S′ site interactions play a crucial role in the design of highly specific proteases and protease inhibitors and the use of proteases as peptide ligases (Bastos et al., 1995; Willett et al., 1995; Schellenberger & Jakubke, 1991). The S′ subsite specificity has been determined for a number of proteases and the maximal

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[1] Abbreviations: Tr, trypsin; Ch, chymotrypsin; AMC, Aminomethylcoumarin.

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	S1′ site						S1 site													
	33					40	58					66	138	172	185			192	216					225
Rat trypsin II	l	n	-	-	s	g	c	-	y	k	s	r	i	y	f	l	e	g	g	k	d	s	c	q	g	y	g	-	c	a	l	p	d	n	q	v
Cow trypsin	.	.	.	.	.	.	.	.	.	.	.	g	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Rat chymotrypsin A	.	q	d	k	t	.	f	.	.	g	v	.	t	w	-	a	s	.	v	-	s	.	.	m	.	s	s	t	.	s	-	t	s	t	.	.
Cow chymotrypsin A	.	q	d	k	t	.	f	.	.	g	v	t	t	w	-	a	s	.	v	-	s	.	.	m	.	s	s	t	.	s	-	t	s	t	.	.
Cow chymotrypsin B	.	q	d	s	t	.	f	.	.	g	v	t	t	w	-	a	s	.	v	-	s	.	.	m	.	s	s	t	.	s	-	t	s	t	.	.
Tr′->Ch'[L40]	.	q	d	s	t	.	f	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.
Tr'->Ch'[L60]	.	.	.	-	.	.	.	.	.	g	v	.	t	s	d	v	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.
Tr'->Ch'[L40+L60]	.	q	d	s	t	.	f	.	.	g	v	.	t	s	d	v	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.
Tr->Ch[S1+L1+L2+Y172W]	.	.	.	-	.	.	.	.	.	-	.	.	.	.	.	.	.	t	w	-	a	s	.	v	-	s	.	.	m	.	s	.	t	.	s	-	t	s	t
Tr->Ch[S1+L1+L2+Y172W+L40+L60]	.	q	d	s	t	.	f	.	.	g	v	.	t	s	d	v	.	t	w	-	a	s	.	v	-	s	.	.	m	.	s	.	t	.	s	-	t	s	t
	Loop 40						Loop 60								Loop 1																Loop 2							

FIGURE 2: Alignment of trypsin, chymotrypsin, and mutant trypsins. Chymotrypsinogen numbering is used. Periods denote residues which are identical to rat trypsin II, while (−) denotes gaps in the sequences. The designation "S1" refers to the following mutations: D189S, Q192M, I138T, insert T219. L1, L2, L40, and L60 refer to the substitution of the designated regions in rat trypsin II with the regions of chymotrypsin.

discrimination between different residues in P1′−P3′ usually ranges around 10^3-fold (Schellenberger et al., 1993).

Trypsin and chymotrypsin exhibit markedly different preferences for P1′ residues (Schellenberger et al., 1994). Trypsin prefers large hydrophobic residues (Met, Ile), while chymotrypsin favors positively charged residues in the P1′ position. X-ray crystal structures of different trypsin- and chymotrypsin-inhibitor complexes reveal strong similarities in the backbone conformation of P1′−P3′ inhibitor residues (Bode & Huber, 1992). A hydrogen bond is formed between the carbonyl oxygen of Phe 41 and the amide group of the P2′ residue and is probably the most important backbone−backbone interaction in the S′ subsites in these complexes. As a consequence of the extended backbone conformation of the peptide chain, the P1′ and P3′ residues point in the same direction and in the opposite direction to P2′ residue (Figure 1). Moreover, acyl-transfer experiments indicate that large residues in P1′ and P3′ compete for similar regions of the enzyme surface. Therefore, the S1′ and S3′ sites overlap and the specificities are similar (Schellenberger et al., 1994). The structures of rat trypsin complexed with BPTI (Perona et al., 1993) and bovine α-chymotrypsin with OMTKY (Fujinaga et al., 1987) suggest that two surface loops comprise the S1′ and S3′ sites, residues 34−41 (loop 40) and 58−64 (loop 60). These loops differ markedly in the number and character of residues in trypsin and chymotrypsin (Figures 2 and 3), which could explain the differences in their S1′ specificity. Chymotrypsin's preference for P1′ and P3′ Arg/Lys is attributed to electrostatic interactions with Asp 35 and Asp 64. Consistently, Asp 64 forms a water mediated salt bridge with P3′ Arg in the chymotrypsin-OMTKY complex. In contrast, trypsin contains no negatively charged residues in either loop 40 or loop 60. Trypsin's preference for hydrophobic residues in P1′ is probably the result of the hydrophobic character of the S1′ site. X-ray structures of other serine protease inhibitor complexes suggest that loop 40 and loop 60 are general determinants of S′−P′ interactions. These surface loops also contact the P1′−P3′ residues in the kallikrein-BPTI, elastase-OMTKY, and thrombin-hirulog 3 complexes (Chen & Bode, 1983; Bode et al., 1992; Qui et al., 1992).

Trypsin and chymotrypsin also differ in their S2′ specificity. Interestingly, trypsin prefers positively charged P2′ residues while chymotrypsin prefers hydrophobic P2′ residues. The structural determinants of S2′ specificity are located in analogous regions of both enzymes. In the rat trypsin−BPTI complex, Glu 151 forms a salt bridge with P2′ Arg; Asn 143 also contacts P2′ Arg. In chymotrypsin, residue 151 is Thr, while 143 is Leu, thereby accounting for chymotrypsin's preference for hydrophobic P2′ residues.

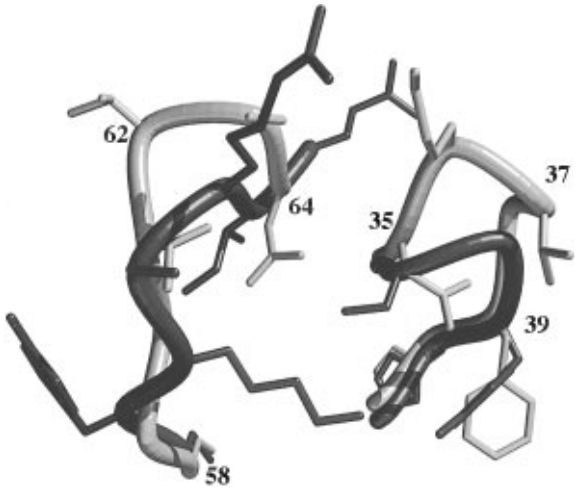


FIGURE 3: The S1′ sites of trypsin and chymotrypsin. The structures of rat trypsin II (dark model, from PDB file 1trm) and bovine chymotrypsin (light model, from PDB file 2gch) are shown. The side chains of residues 35−41 and 58−64 are depicted in sticks, while the main chain is depicted as tubes. This figure was created with RAYSCRIPT (obtained from E. Fonatano, D. Peisach, and E. Peisach, Brandeis University), a program that uses the input of MOLSCRIPT (Kraulis, 1991) to generate input for RAYSHADE (Version 4.0, written by C. Kolb and R. Bogart, Princeton University).

Few studies have addressed the structural basis of S′ specificity. Recent site directed mutagenesis of thrombin identified residues in loop 40 and loop 60 as structural determinants of S2′ and S3′ specificity (LeBonniec et al., 1996). Site directed mutagenesis of carboxypeptidase Y resulted in an altered S1′ specificity (Stennicke et al., 1994). The introduction of negatively charged residues into the S1′ site of carboxypeptidase Y dramatic decreased specificity for P1′ Phe; however, only a modest increase in preference for P1′ Lys was observed with an overall discrimination between Lys and Phe of 1.5-fold. In trypsin, S2′ specificity was changed by creating a metal binding site with the mutation of both Asn 143 and Glu 151 to His. Substrate P2′ His can also coordinate to the metal, thus introducing His specificity in S2′ (Willett et al., 1996). His specificity has also been engineered into the S1′ site of subtilisin and trypsin through substrate-assisted catalysis (Carter & Wells, 1987; Corey et al., 1995). Thus, little is known about the structural features which control S′ specificity.

In this study we describe the engineering of chymotrypsin-like S1′ specificity into the rat trypsin II framework. Loops 40 and 60 were replaced with their chymotrypsin counterparts. Together these substitutions cause a shift of S1′ specificity from Met/Ile to Arg/Lys P1′ residues.

MATERIALS AND METHODS

Materials. Suc-Ala-Ala-Pro-Phe-AMC, D-Val-Leu-Arg-AMC, and D-Val-Leu-Lys-AMC were purchased from Enzyme Systems Products (Livermore CA). Amino acid amides, dipeptides, dipeptide amides, *Z*-Arg-AMC, Bz-Arg-OEt, Ac-Phe-OMe, Tos-Gly-Pro-Arg-AMC, and Tos-Gly-Pro-Lys-AMC were purchased from Bachem. H-Trp-Ala-Ala-Ala-Gly-OH was synthesized according to Ullmann and Jakubke (1994). Rat chymotrypsin was a generous gift of Dr. Laszlo Szilagyi (Eötvös Lorand University, Budapest). If not otherwise stated, all chemicals were of analytical grade.

Construction of Trypsin Mutants. Site-directed mutagenesis was performed by the method of Kunkel (1985) as described previously (Hedstrom et al., 1992). All mutants were completely sequenced in order to ensure that only the desired mutations was introduced. The following oligonucleotides were used (mismatches are underlined):

(1) L40: GTC TCC CTG
CAA GAC TCT ACT GGC TTC CAC TTC TGT

(2) L60: GCA GCT CAC TGC GGT AAG ACC TCC
GAT GTT GTG GTC GCC GGA GAG CAC AAC

Trypsin and trypsin mutants were isolated and purified as described previously (Hedstrom et al., 1994).

CD Spectroscopy. Samples contained 0.25 mg/mL enzyme in 50 mM Na-acetate buffer, pH 4. Activity was measured before and after CD analysis in order to ensure that no degradation of the enzymes occurred. Spectra were measured at the far-UV range (190−250 nm) using a 1 cm path length cell with a sensitivity of 5×10^{-6}, response time of 2 s at 0.2 nm intervals, and 1 cycle on a Jovin-Yvon Mark V autodichrograph.

Activity of Mutant Trypsins. Assay mix contained 50 mM Hepes, pH 8, 10 mM $CaCl_2$, and 0.1 M NaCl. Stock solutions of AMC substrates were prepared in dimethylformamide. The final concentration of dimethylformamide in the assay solution was less than 5%. Hydrolysis of the AMC substrates was monitored fluorometrically, with excitation wavelength of 380 nm and emission wavelength at 460 nm (Zimmerman et al., 1977). Assays were performed using 0.2 mL of assay mix containing substrates (usually 0.5 μM to 2 mM) in a PerSeptive Cyto Fluor spectrofluorimeter at 25 °C. Concentrations of fluorimetric substrates were determined by using a standard curve of AMC fluorescence at 15 different AMC concentrations (0.2−200 μM). Data were analyzed using KinetAsyst software, and reported values are the average of at least two experiments.

Acyl-Transfer Experiments. Reactions were performed at 25°C. A 4 mM stock solution of the acyl donors Bz-Arg-OEt and Ac-Phe-OMe was prepared daily in water. The Ac-Phe-OMe stock contained 1% dimethylformamide. Stock solutions of the amino acid amides (30 mM) were prepared in assay mix readjusted with NaOH to pH 8. The total assay volume was 65 μL. The final acyl donor concentration was 2 mM and the nucleophile concentrations 15 mM, calculated as unprotonated amino acid amide concentration [N] according to the following equation ($[NH]_0$ corresponds to the total nucleophile concentration):

$$[N] = [NH]_0/(1 + 10^{pK-pH}) \quad (1)$$

The acyl-transfer reaction was initiated with 5 μL of enzyme stock solution. Enzyme concentration and reaction time were adjusted to achieve an ester consumption of 50−80% in order to ensure that no secondary hydrolysis of the formed peptide product occurred. The reaction was stopped by diluting 50 μL of the reaction mixture in 0.3 mL 50% aqueous methanol and 1% trifluoroacetic acid. The partition values were determined from three to five independent experiments. Control experiments without the enzyme were performed in order to estimate non enzymatic ester hydrolysis (0−1.5%). HPLC was performed using a Hewlett-Packard 1090LC system (Palo Alto, CA) on a Vydac analytical reversed phase C_{18} column (Vydac 218TP.54). Samples were eluted under isocratic conditions with eluents containing 15−25% acetonitrile (depending on the nucleophile) in 0.1% aqueous trifluoroacetic acid at flow rates of 1.0−1.2 mL min^{-1}. Absorbance was monitored at 254 nm (Bz-Arg-OEt) and 220 nm (Ac-Phe-OMe). The ratio between aminolysis and hydrolysis product was calculated from the corresponding peak areas. The ratio of extinction coefficients of the sample components was obtained by hydrolysis experiments according to Ullmann et al., (1994).

RESULTS AND DISCUSSION

Design of Mutant Trypsins. We constructed four mutant trypsins in order to probe the role of loop 40 and loop 60 in determining the S1′ specificity of trypsin and chymotrypsin (Figure 2). Tr′→Ch′[L40] and Tr′→Ch′[L60] contain the amino acid sequence of chymotrypsin in positions 34−39 and 59−68, respectively (chymotrypsinogen numbering). Tr′→Ch′[L40+L60] combines both mutations. In addition, a fourth mutant was constructed from the chymotrypsin-like mutant Tr→Ch[S1+L1+ L2+Y172W] in order to probe the interdependence of S1 and S1′ specificity (Hedstrom et al., 1994). This mutant, Tr→Ch[S1+L1+ L2+Y172W+ L40+L60], also contains both loop 40 and loop 60. All four mutant trypsins are expressed in a *Saccharomyces cerevisiae* system (Hedstrom et al., 1994). They were purified using standard methods and remained stable in 1 mM HCl.

The Secondary Structures of Tr′→Ch′[L40+L60] and Tr→Ch[S1+L1+L2+ Y172W+L40+L60] Differ from Trypsin. Figure 4 shows the CD spectra of Tr′→ Ch′[L40+L60], Tr→Ch[S1+L1+L2+Y172W+L40+L60], trypsin, and bovine chymotrypsin. The spectra of the mutant enzymes display substantial changes in the 200−220 nm range. The CD spectrum in this wavelength range is determined by the secondary structure of the protein. The minimum of the spectrum of trypsin is 211 nm while that of chymotrypsin is 204 nm. Interestingly, the minimum of the spectra of the mutant enzyme is shifted to lower wavelengths, which may suggest that the structure of these enzymes is more chymotrypsin-like.

Activity of Mutant Trypsins. The hydrolysis of amide substrates by the mutant trypsins was characterized in order to assess the influence of loop 40 and loop 60 on S1

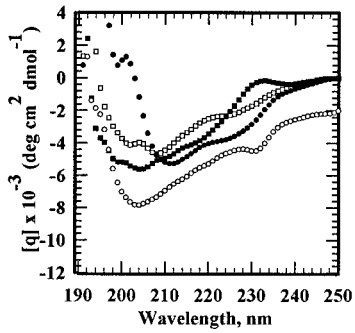


FIGURE 4: CD spectra of trypsin, chymotrypsin, and mutant enzymes. Samples contained 0.25 mg/mL enzyme in 50 mM Na-acetate buffer pH 4. Spectra were measured at the far UV range (190−250 nm) using a 1 cm path length cell with a sensitivity of 5×10^{-6}, response time of 2 s at 0.2 nm intervals, and 1 cycle on a Jovin−Yvon Mark V autodichrograph. Closed circles, rat trypsin S195A; open circles, bovine chymotrypsin; closed boxes, Tr′→Ch′[L40+L60]; open boxes Tr→Ch-[S1+L1+L2+Y172W+L40+L60].

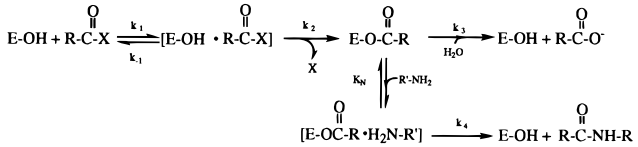
Table 1: Steady-State Kinetic Parameters for the Hydrolysis of Substrates by Trypsin and Trypsin Mutants[a]

substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
	rat trypsin[b]		
Tos-Gly-Pro-Arg-AMC	3.5	29	9.0×10^6
Tos-Gly-Pro-Lys-AMC	12	17	1.4×10^6
D-Val-Leu-Arg-AMC	39	17	4.2×10^5
D-Val-Leu-Lys-AMC	395	13	3.1×10^4
Z-Arg-AMC	55	0.5	9.6×10^3
	Tr′→Ch′[L40]		
Tos-Gly-Pro-Arg-AMC	2	11	8.0×10^6
Tos-Gly-Pro-Lys-AMC	18	28	1.5×10^6
D-Val-Leu-Arg-AMC	13	4	3.6×10^5
D-Val-Leu-Lys-AMC	106	6	5.3×10^4
Z-Arg-AMC	79	0.6	7.0×10^3
	Tr′→Ch′[L60]		
Tos-Gly-Pro-Arg-AMC	5	31	6.3×10^6
Tos-Gly-Pro-Lys-AMC	15	19	1.3×10^6
D-Val-Leu-Arg-AMC	19	7	3.7×10^5
D-Val-Leu-Lys-AMC	215	6	2.7×10^4
Z-Arg-AMC	31	0.3	8.5×10^3
	Tr′→Ch′[L40]+L60]		
Tos-Gly-Pro-Arg-AMC	4	9	2.0×10^6
Tos-Gly-Pro-Lys-AMC	18	10	7.5×10^5
D-Val-Leu-Arg-AMC	45	2	7.8×10^4
D-Val-Leu-Lys-AMC	66	2	3.7×10^4
Z-Arg-AMC	58	0.3	5.2×10^3
	Tr→Ch [S1+L1+L2+Y172W+L40+L60]		
Suc-AlaAlaProPhe-AMC	≥3000[c]	≥30	9.0×10^3
	Tr→Ch [S1+L1+L2+Y172W][d]		
Suc-AlaAlaProPhe-AMC	≥3000	≥30	9.3×10^3

[a] Conditions: 50 mM Hepes, pH 8, 100 mM NaCl, 10 mM $CaCl_2$, 30 °C, λ_{ex} = 380 nm, λ_{em} = 460 nm. [b] Hedstrom et al.; 1994. [c] No saturation with 1 mM substrate, 37 °C; all errors are less than 15%. [d] Hedstrom et al., 1996.

specificity. Tr′→Ch′[L40], Tr′→Ch′[L60], and Tr′→Ch′-[L40+L60] are very similar to trypsin as shown by the Michaelis−Menten parameters in Table 1. Like trypsin, the mutant enzymes hydrolyze oligopeptide substrates much more efficiently than single amino acid substrates (by a factor of 10^3 as measured by k_{cat}/K_m). This observation underlines the role of S2−S4 subsites for specific and efficient substrate processing. As expected, the mutations in loop 40 and loop 60 do not influence the primary specificity and enzymatic activity of trypsin. Similarly, the chymotrypsin-like mutant Tr→Ch[S1+L1+L2+Y172W+L40+L60] exhibits an identical value of k_{cat}/K_M for the hydrolysis of Suc-Ala-Ala-Pro-Phe-AMC as Tr→Ch[S1+L1+L2+Y172W].

Scheme 1: Mechanism for Serine Protease Catalyzed Acyl-Transfer Reactions[a]



[a] Enzyme is denoted E-OH, acyl donor is R-COX, where X is the leaving group, R′-NH_2 is the nucleophile.

Determination of S1′ Specificity. S1′ specificity can be determined by monitoring acyl-transfer reactions with added nucleophiles. This reaction is the reverse of peptide hydrolysis and therefore provides analogous specificity data (Scheme 1; Fersht et al., 1973; Schellenberger & Jakubke, 1991; Schellenberger et al., 1993). This postulate has been validated for a number of serine and cysteine proteases (Stein et al., 1987; Schellenberger et al., 1994; Ullmann & Jakubke, 1994). Since the acyl group of a substrate (R-COX, which is usually an ester) can be transferred both to water and R′-NH_2, two products are formed: R-CO_2H and R-CO-NHR′, respectively. The ratio between hydrolysis and aminolysis is determined by the S′ specificity of the protease. The partition value *p* describes acyl-transfer efficiency and therefore the S1′ specificity of a protease. *p* is defined according to Scheme 1 as

$$p = [\text{R′-NH}_2](v_H/v_A) = k_3K_N/k_4 \qquad (2)$$

where v_H and v_A represent the velocities of hydrolysis and aminolysis, respectively. The partition value can be determined from the product ratios when R′-NH_2 is in excess:

$$p = [\text{R′-NH}_2]_0[\text{R-CO}_2\text{H}]/[\text{R-CONH-R′}] \qquad (3)$$

where $[\text{R′-NH}_2]_0$ is the initial nucleophile concentration and [R-CO_2H] and [R-CONH-R′] represent the product concentrations. In this work, we will utilize the parameter 1/*p* in order to emphasize the aminolysis reaction. Consequently, an increase in the value 1/*p* can be directly correlated with the preference of the protease for a given R′-NH_2. Therefore, the S1′ specificity of trypsin and the mutant enzymes was determined by measuring 1/*p* for 16 amino acid amide nucleophiles, using Bz-Arg-OEt and Ac-Phe-OMe as the acyl donors as appropriate.

S1′ Specificity of Trypsin, Chymotrypsin, and Tr→Ch-[S1+L1+L2+Y172W]. Figure 5, panels a and b, shows the 1/*p* values for the trypsin and chymotrypsin-catalyzed acyl-transfer reactions. These values are consistent with literature data (Fersht et al., 1973; Schellenberger & Jakubke, 1991; Gololobov et al., 1992). In chymotrypsin-catalyzed reactions, 1/*p* for Arg-NH_2 is 3−5-fold higher than 1/*p* for aromatic amino acid amides and 8-fold higher than 1/*p* for aliphatic amino acid amides, while the specificity for small and negatively charged amino acid amides is 50−500-fold lower. This result does not agree with previous observations of Schellenberger et al. (1993), who measured acyl-transfer to pentapeptide nucleophiles and observed a much lower preference for aromatic P1′ residues. Therefore, we performed acyl-transfer reactions to H-Trp-Ala-Ala-Ala-Gly-OH with chymotrypsin. 1/*p* decreases 6-fold for H-Trp-Ala-Ala-Ala-Gly-OH compared to 1/*p* for H-Trp-NH_2 (data not

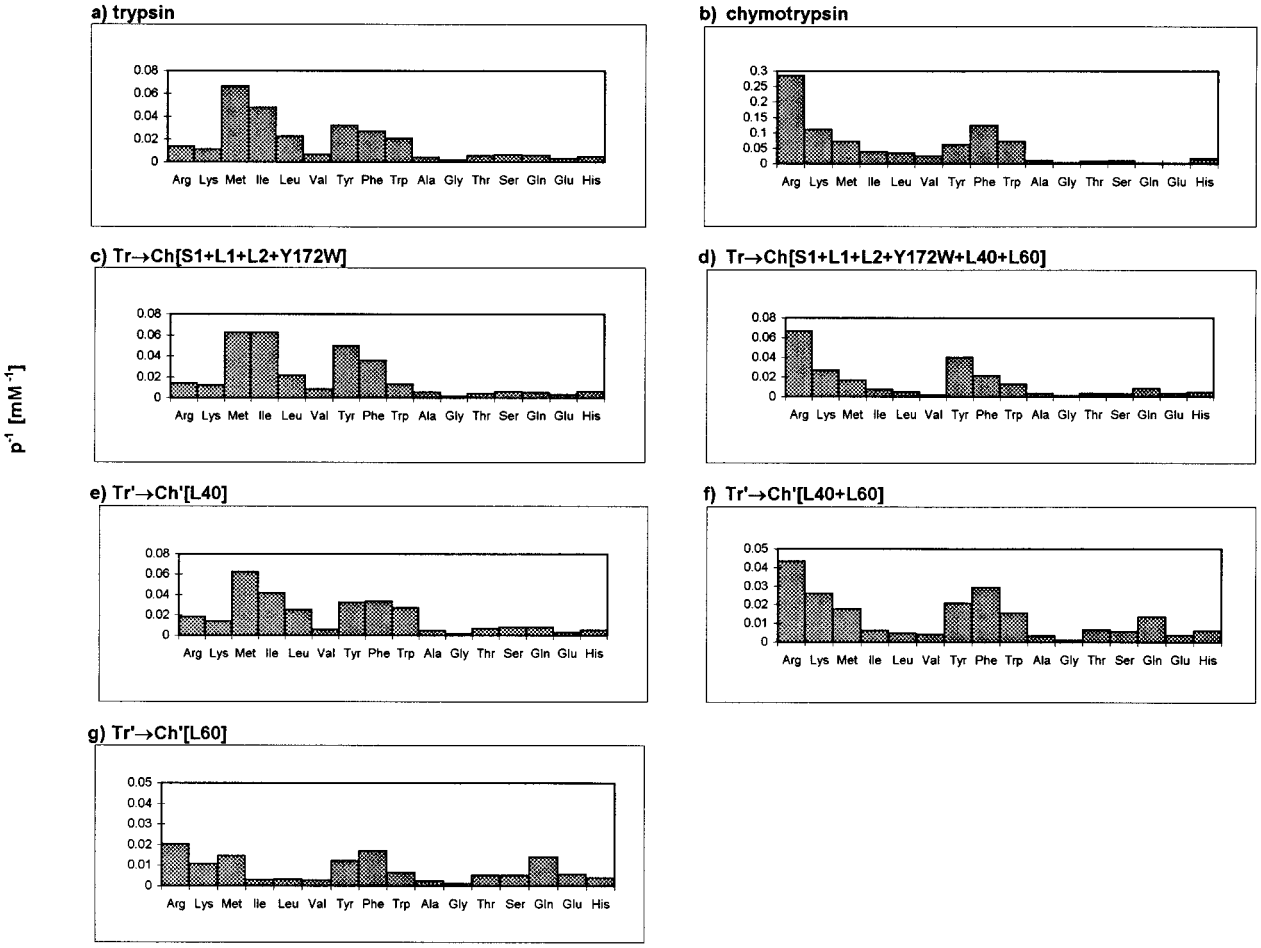


FIGURE 5: S1′ specificity of trypsin, chymotrypsin and mutant enzymes. Assays were performed at 25 °C in assay mix (50 mM Hepes, pH 8, 10 mM $CaCl_2$, 100 mM NaCl, 0.01% Triton) containing 2 mM Bz-Arg-OEt or Ac-Phe-OMe and 15 mM R-NH_2. Reactions were started with enzyme. Reactions were stopped by the addition of 50% methanol and 1%TFA after 50−80% of the ester was consumed. Products were analyzed via reversed phase HPLC (Vydac CAT 218TP54, 12−20% acetonitrile, λ = 254 and 220 nm). The partition value was calculated as p = [Ac-OH][R-NH_2]/[Ac-NH-R]. Since [R-NH_2] ≫ [Bz-Arg-OEt] and Ac-Phe-OMe, it can be assumed that [R-NH_2] remains constant during the course of reaction. (a) Wild-type rat trypsin. The value of 1/p for acyltransfer to Gly-NH_2 is 0.0015. (b) Rat chymotrypsin. The value of 1/p for acyltransfer to Gly-NH_2 is 0.0032, for acyltransfer to Gln-NH_2 is 0.0032, and for acyltransfer to Glu-NH_2 is 0.0010. (c) Tr → Ch[S1+L1+L2+Y172W]. The value of 1/p for acyltransfer to Gly-NH_2 is 0.0018. (d) Tr→Ch[S1+L1+L2+Y172W+L40+L60]. The value of 1/p for acyltransfer to Gly-NH_2 is 0.0009. (e) Tr′→Ch′[L40]. The value of 1/p for acyltransfer to Gly-NH_2 is 0.0017. (f) Tr′→Ch′[L40]. The value of 1/p for acyltransfer to Gly-NH_2 is 0.0012.

shown). This observation implies that P1′ aromatic residues have different binding modes in oligopeptide nucleophiles and amino acid amide nucleophiles in chymotrypsin.

In trypsin-catalyzed acyl-transfer reactions, the values of 1/p for Met-NH_2 and Ile-NH_2 are 1.5−3-fold higher than for Phe-NH_2 and 5-fold higher than for Arg-NH_2. Again, the specificity for small and negatively charged amino acid amides is low, although the specificity for negatively charged residues is much higher than in chymotrypsin. These data are consistent with the values for the analogous pentapeptide nucleophiles (Schellenberger et al., 1993).

S1′ Specificity of Mutant Trypsins. Surprisingly, the exchange of the loop 40 in trypsin has only a modest effect on the S1′ specificity of trypsin. The specificity for positively charged residues in P1′ is only minimally increased and the S1′ specificity for all other nucleophiles remains unchanged (Figure 5e). This observation suggests that loop 40 does not influence the S1′ specificity.

Figure 5g shows that the substitution of loop 60 in trypsin causes a 5−15-fold decrease in specificity for aliphatic amino acid amides. While the preference for most nucleophiles remained constant or dropped, moderate increases in the values of 1/p for Arg-NH_2 and Gln-NH_2 are observed. Consequently, Tr′→Ch′[L60] prefers P1′ Arg and Phe. Nevertheless, the replacement of loop 60 with the analogous chymotrypsin sequence does not create a strong preference for Arg-NH_2 as observed in chymotrypsin.

While alone loop 40 and loop 60 fail to reconstitute chymotrypsin-like S1′ specificity, the mutant combining substitutions in both loops, Tr′→Ch′[L40+L60], has a dramatically changed S_1′ specificity with a strong preference for P1′ Arg. Figure 5f shows that 1/p for acyl-transfer to Arg-NH_2 is increased 3-fold relative to trypsin. The specificity for Lys-NH_2 is 2.5-fold higher than in trypsin. In contrast, the preference for Leu-NH_2, Ile-NH_2, and Met-NH_2 is decreased 4 - 8-fold. Therefore, the exchange of both loop 40 and loop 60 increases the relative P1′ Arg/Met discrimination of trypsin by factor 12 and the P1′ Arg/Ile preference by factor 24. The specificity for the other nucleophiles is similar to trypsin. Surprisingly, Tr′→Ch′-[L40+L60] retains trypsin-like specificity for negatively charged nucleophiles despite the introduction of two Asp residues. Overall the values of 1/p for Tr′→Ch′[L40+L60] remain about 7-fold lower than those observed for chymo-

trypsin-catalyzed acyl-transfer reactions. Nevertheless, the ratio between the values of $1/p$ for Arg-NH_2 and Met-NH_2 are similar for Tr′→Ch′[L40+L60] and chymotrypsin. Thus, our data demonstrate that loop 40 and loop 60 work synergistically to determine the chymotrypsin-like S1′ specificity.

Interdependence of S1 and S1′ subsite. Table 1 and Figure 5 reveal that dramatic changes in the character of the S′ subsite can be introduced without significant influence on the primary specificity of the enzyme. Acyl-transfer experiments were performed with Tr→Ch[S1+L1+L2+Y172W] and Tr→Ch[S1 +L1+L2+Y172W+L40+L60] in order to further assess the influence of the S1 subsite on S1′ specificity. No differences in S1′ specificity of Tr′→Ch′-[S1+L1 +L2+Y172W] and trypsin are observed as measured by acyl-transfer to amino acid amides as nucleophiles (Figure 5, panels a and c). This result is in apparent conflict with Schellenberger et al. (1993), who observed a significant decrease in specificity for P1′ Phe/Tyr/Trp containing pentapeptide nucleophiles in the chymotrypsin-like mutant Tr′→Ch′[S1+L1+L2] compared to trypsin. Therefore, we performed acyl-transfer reactions to H-Trp-Ala-Ala-Ala-Gly-OH with Tr→Ch[S1+L1+L2 +Y172W]. $1/p$ decreases 4-fold for H-Trp-Ala-Ala-Ala-Gly-OH compared to H-Trp-NH_2 (data not shown). As discussed above, similar differences in acyl-transfer to P1′ aromatic amino acid amides and oligopeptides have been observed in chymotrypsin. In contrast, no significant difference in specificity for H-Trp-Ala-Ala-Ala-Gly-OH and H-Trp-NH_2 was obtained for trypsin and Tr′→Ch′[L40+L60]. Thus, P1′ aromatic residues have different binding modes in oligopeptide and amino acid amide nucleophiles in chymotrypsin-like enzymes, but not in trypsin-like enzymes. This observation indicates that the S1 subsite can have modest influence on S1′ specificity.

Figure 5d shows the acyl-transfer data for Tr→Ch-[S1+L1+L2+ Y172W+L40+L60] with Ac-Phe-OMe as the acyl donor. Again the simultaneous substitution of loop 40 and loop 60 strongly affects the S1′ specificity. Since $1/p$ for Arg-NH_2 increases 5-fold while Met-NH_2 and Ile-NH_2 drop 4−8-fold, the P1′ Arg/Met and Arg/Ile discrimination changed 20−40-fold relative to the parent enzyme. As in Tr′→ Ch′[L40+L60], no change is observed in $1/p$ for Phe-NH_2, Tyr-NH_2, and Trp-NH_2 compared to the parent enzyme. The values of $1/p$ for small and negatively charged residues are comparable to Tr→Ch[S1+L1+L2+Y172W]. Tr′→ Ch′[S1+L1+L2+ Y172W+L40 +L60] has a 1.6 fold higher P1′ Arg preference than Tr′→ Ch′[L40+L60]. This observation further indicates that the S1 site can have a modest effect on S1′ specificity.

The Mutation of Loop 40 and Loop 60 Causes a Change in the Kinetic Course of the Acyl-Transfer Reaction. Scheme 1 requires that the product ratio [R-CONH-R′]/ [R-CO_2H] is linearly dependent on the R′-NH_2 concentration. This dependence is observed for trypsin-catalyzed acyl-transfer to Arg-NH_2 , as well as for Tr→Ch[S1+L1+L2+Y172W]-catalyzed acyl-transfer to Arg-NH_2 (Figure 6). However, data for Tr′→Ch′[L40+L60] and Tr→Ch-[S1+L1+L2+Y172W+L40 +L60]-catalyzed acyl-transfer to Arg-NH_2 do not fit this model. Figure 6 shows [R-CONH-R′]/[R-CO_2H] is not linearly dependent on R-NH_2 for both of these mutant enzymes. This deviation occurs at much higher nucleophile concentrations than those used in the previous experiments, and thus does not change the conclusions based on Figure 5. This deviation may result from a change in ionic strength or other solvent properties due to high R′-NH_2 concentrations. However, we do not favor this explanation because this effect is not observed in the parent enzymes. Alternatively, this deviation is consistent with the model shown in Scheme 2, where the acyl enzyme−nucleophile complex can undergo both hydrolysis and aminolysis (Schellenberger & Jakubke, 1991; Gololobov et al., 1993). In this model, [R-CONH-R′]/[R-CO_2H] = k_4/k_5 at infinite R′-NH_2 concentrations. Thus the mutations in loop 40 and loop 60 appear to change the kinetic course of the acyl-transfer reaction. The hydrolysis of the acyl enzyme−nucleophile complex indicates that R′-NH_2 binds nonproductively to the acylenzyme. This nonproductive binding mode suggests that the new S1′ sites are defective.

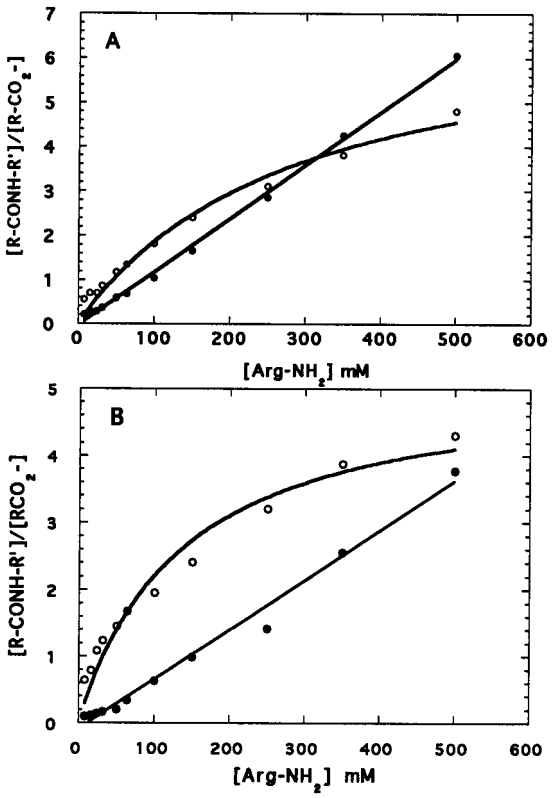
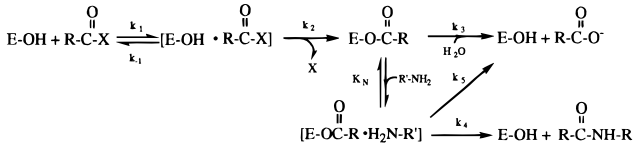


FIGURE 6: Dependence of [R-CO-NH-R′]/[R-COO^-] on [R′-NH_2]. Conditions as described in Figure 5. (a) Open circles, Tr′→ Ch′L40+L60], closed circles, rat trypsin. (b) Open circles, Tr→Ch-[S1+L1+L2+Y172W+L40+L60]; closed circles, Tr→Ch-[S1+L1+L2+Y172W].

Scheme 2: Alternative Mechanism of Serine Protease Catalyzed Acyl-Transfer Reactions[a]



[a] Enzyme is denoted E-OH, acyl donor is R-COX, where X is the leaving group, R′-NH_2 is the nucleophile.

Summary. This work demonstrates that the S1′ specificity of trypsin and chymotrypsin is synergistically determined by two surface loops, loop 40 and loop 60. However, neither Tr′→Ch′[L40+L60] nor Tr′→Ch′[S1+L1+L2+Y172W+L40 +L60] are equivalent to chymotrypsin in the acyl-transfer efficiency, which indicates that additional structural determinants of the S1′ specificity in trypsin and chymotrypsin remain to be identified.

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