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Attempts to convert chymotrypsin to trypsin

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Abstract Trypsin and chymotrypsin have specificity pockets of essentially the same geometry, yet trypsin is specific for basic while chymotrypsin for bulky hydrophobic residues at the P1 site of the substrate. A model by Steitz, Henderson and Blow suggested the presence of a negative charge at site 189 as the major specificity determinant: Asp189 results in tryptic, while the lack of it chymotryptic specificity. However, recent mutagenesis studies have shown that a successful conversion of the specificity of trypsin to that of chymotrypsin requires the substitution of amino acids at sites 138, 172 and at thirteen other positions in two surface loops, that do not directly contact the substrate. For further testing the significance of these sites in substrate discrimination in trypsin and chymotrypsin, we tried to change the chymotrypsin specificity to trypsin-like specificity by introducing reverse substitutions in rat chymotrypsin. We report here that the specificity conversion is poor: the Ser189Asp mutation reduced the activity but the specificity remained chymotrypsin-like; on further substitutions the activity decreased further on both tryptic and chymotryptic substrates and the specificity was lost or became slightly trypsin-like. Our results indicate that in addition to structural elements already studied, further (chymotrypsin) specific sites have to be mutated to accomplish a chymotrypsin → trypsin specificity conversion.

Key words: Chymotrypsin; Substrate discrimination; Specificity conversion

1. Introduction

The great achievement of enzymes is the enormous acceleration of chemical reactions accompanied by a strict discrimination between substrates. There is a ~ 10^4 fold difference between trypsin and chymotrypsin activities if they are compared on polypeptide amide substrates with Phe and Lys side chains at the P1 position[1] despite the fact that the main chain atom positions are almost identical in the two enzymes in the S1 specificity site. (They are superimposable with a root-mean square deviation of less than 0.6 Å [2]). Since the S1 site is the primary specificity determinant, the specificity difference has been ascribed mainly to the presence of the negative charge of the Asp^{189} carboxylate in trypsin (matching the positive charge of the P1 side chain in the substrate) and its absence in chymotrypsin [3]. Two other differences were also thought to have some role. One of these is the position of Ser190 side chain. It is rotated out of the pocket in chymotrypsin while in trypsin it is in the pocket and can form a hydrogen bond with a P1-arginine side chain [4–7]. The other difference is at site 192: methionine in chymotrypsin can provide a slightly more hydrophobic environment for the substrate than glutamine, the amino acid at this position in trypsin [8].

Suprisingly enough, the corresponding mutations in trypsin did not transform the specificity: although the trypsin activity measured on polypeptide amide substrates were already completely gone on an Asp189Ser substitution [9], no significant chymotrypsin-like activity was observed. Moreover, the tryptic activity of this mutant returned when non-covalently bound acetate ion was present in the pocket [10]. Further substitutions at sites 138 and 192 did not significantly change the chymotryptic activity of the Ser189 trypsin [2]. Partial replacement in a surface loop (later referred to as loop2, sites 217–224) raised the chymotrypsin-like activity 2–40 fold (Depending on the P1 side chain) as compared to Ser189 trypsin [12]. Further substitutions to modify the amino acid sequence to a chymotrypsin like one in loop2 and in an other surface loop (loop1, sites 184a through 195), along with a $Ile^{138}Thr$ mutation, incrased the activity to one percent of wild type chymotrypsin (Tr → Ch[S1 + L1 + L2] trypsin mutant in ref. [2]). A Tyr172Trp substitution in the latter mutant brought the activity of the mutant enzyme into the range of wild-type chymotrypsin [13,14]. From these findings it was concluded that sites 138, 172, 184a–189, 192, 217–224 have a significant role in determining the substrate specificity of trypsin [11].

It appeared to be likely that the sites listed above have a general role in determining both chymotrypsin and trypsin-like specificities. If this were the case, the reverse amino acid substitutions in chymotrypsin would convert its specificity to that of trypsin, similarly to the specificity conversion in trypsin upon substitutions at the same sites. We examined this possibility by generating and kinetically characterizing a series of rat chymotrypsin mutants.

2. Materials and methods

2.1. Materials

Highly purified enterokinase was the product of Biozyme (EK-3). TSK-Toyopearl SP-650M cation exchanger was obtained from Supelco. SBTI-Sepharose, MUTMAC, MUGB, AMC and 7-methylumbelliferon were from Sigma Chemical Co. The tetrapeptide substrates with AMC fluorogenic leaving group were prepared as described [9].

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Abbreviations: SBTI, soybean trypsin inhibitor; BPTI, bovine pancreatic trypsin inhibitor; AMC, 7-amino-4-methylcoumarin; MUGB, 4-methylumbelliferyl *p*-guanidinobenzoate; MUTMAC, 4-methylumbelliferyl *p*-(*N*,*N*,*N*-trimethyl ammonium) cinnamate chloride.

[1]P1 denotes the amino acid residue of the substrate that interacts with the S1 site of the enzyme. The scissile bond is between sites P1 and P1′ [1].

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2.2. Cloning of rat chymotrypsinogen

A lambda gt11 cDNA library of the rat cell line, AR425 (from Chris Nelson) was amplified by polymerase chain reaction using two oligonucleotides corresponding to the 5′ and 3′ ends of the chymotrypsinogen coding region. At the same time the oligonucleotides (5′-GCCACCC-AAGCTTGTGGAGTCCCT-3′ and 5′-GCGAGCTCAGGTGTCTT-CCAAGAT-3′) added a *Hin*dIII site to the 5′ end and a *Sac*I site to the 3′ end.

2.3. Mutagenesis

Asp189 chymotrypsin and its mutant derivatives, mutant 12, 13, 19, 21 and 43 were constructed according to Kunkel [15] using the following oligonucleotides: 5′-GCTAGCGGCGTCGATTCCTGCATG-GGT-3′ (to construct the Asp189 chymotrypsin from the wild-type enzyme); 5′-ACTGTGTGCGCAATCACTGGCTGGGGC-3′ (to introduce a Thr138Ile mutation into the Asp189 chymotrypsin); 5′-GT-GATGACCTGCGCAGGCTTCCTCGAGGGCGGCAAGGATTC-CTGCCAGGGTGACTCCGGTC-3′ (to introduce mutations in loop1 of Asp189 chymotrypsin); 5′-GTGTCCTGGGGCTATGGCTGTGC-CCTTCCGGACAATCCTGCTGTGTATCC-3′ and 5′-TGTGCCCT-TCCGAGTACTCCTGCTGTGTATTCACGCGTCACAGCC-3′ (to introduce substitutions in loop2 of Asp189 chymotrypsin); 5′-CAGC-AGGCCGCGGCGCCCATCGTCTCCGAGGCTGACTGCAAGA-AGTCGTACGGTTCCAAGATCACCGATGTGATGATCTGCGC-AGGC-3′ (to introduce mutations Leu160Ala and Trp172Phe). The mutations in chymotrypsin at site 138 and in the two loops were introduced in a single mutagenesis step using three oligos simultaneously on the same template. Mutants 21 and 49 were generated from the combination of the other chymotrypsin mutants.

Throughout this study we expressed that forms of wild-type and mutant chymotrypsinogens in which the chymotrypsin propeptide was substituted for trypsin propeptide and site Cys122 was mutated to Ser for two reasons: (i) the chimeras could be activated with enterokinase, which minimized the contamination of the enzyme preparations with trypsin (in this way the accurate determination of very low tryptic activities of the wild-type and mutant enzymes became possible); (ii) the chimeric form of zymogen chymotrypsin proved to be much more stable than the natural wild-type (chymotrypsin propeptide containing) form, resulting in a higher yield during enzyme preparation. (For construction characterization of these chimeras see the accompanying paper.) The mutations were verified by DNA sequencing.

2.4. Expression

The mutant and wild-type chymotrypsinogens (in propeptide chimera form – see above) and wild-type trypsinogen were expressed in yeast by fusing the enzyme coding sequences to an α-factor leader peptide containing sequence as described for carboxypeptidase [16]. The culture medium was separated by centrifugation and the proteins were isolated on TSK-Toyopearl SP-650 cation exchanger [17]. The zymogens were activated by highly purified enterokinase at a 100:1 zymogen/enterokinase ratio, then the active forms were purified by affinity chromatography on SBTI-Sepharose column. The mutant enzymes were always purified on unused affinity gels. The purity of the preparates was analyzed by SDS polyacrylamide gel electrophoresis. The enzyme concentration was determined by BioRad protein assay for the mutants of low activity and by active site titration with MUTMAC and MUGB [18] for the wild-type enzymes.

2.5. Enzyme assays

Amide hydrolysis was measured on Succinyl-AlaAlaProXaa-AMC substrates (Xaa was Phe, Tyr, Leu, Trp, Lys and Arg) in a 50 mM HEPES, 10 mM $CaCl_2$, 0.1 M NaCl (pH 8.0) buffer at 37°C using a Perkin Elmer LS5 spectrofluorimeter. The data were analyzed with KinetAsyst software.

2.6. Computer graphics

The structure of several bovine trypsin and chymotrypsin molecules were compared on the models deposited in the Brookhaven Protein Data Bank with the MidasPlus software from the Computer Graphics Laboratory of University of California, San Francisco. For studying the differences in the atom positions the models were aligned at the α-carbon atoms of the residues 44, 140, 160, and 200. Located in very rigid β-sheet segments both the residue types and their main chain conformations are conserved at these sites in trypsins and chymotrypsins. The r.m.s. error of the alignments ranged from 0.07 to 0.22 Å.

3. Results

3.1. Description of the mutants

First the Asp189 chymotrypsin mutant was constructed and then other mutants as derivatives, with further substitutions in various combinations at sites 138, 160, 172 and at a number of sites in loop1 and loop2 (see Fig. 1). Mutants 12 and 13 were designed to be the chymotrypsin counterparts of the Tr → Ch[S1 + L1 + L2] trypsin mutant [2]. The difference between mutants 12 and 13, is that mutant 12 does have the Ile138Thr replacement. Mutant 43 is like mutant 13, but it contains a Leu160Ala and a Trp172Tyr substitutions as well. Apart from the mutation at site 160, this mutant is the chymotrypsin counterpart of the Tr → Ch[S1 + L1 + L2 + Y172W] trypsin mutant [13,14]. The Leu160Ala substitution was introduced to stabilize the environment of Ile138 and Ile16 (Leu vs. Ala is a conservative difference at site 160 between mammalian chymotrypsins and trypsins). Variants of mutants 12, 13 and 43 were also made in which the amino acids at sites 223 and 224 in loop2 were not replaced (mutants 19, 21 and 49 – Fig. 1).

3.2. Kinetic characterization of the mutants

We determined the kinetic constants from hydrolysis rates measured on four polypeptide amide substrates that were different at the P1 position (Table 1A,B).

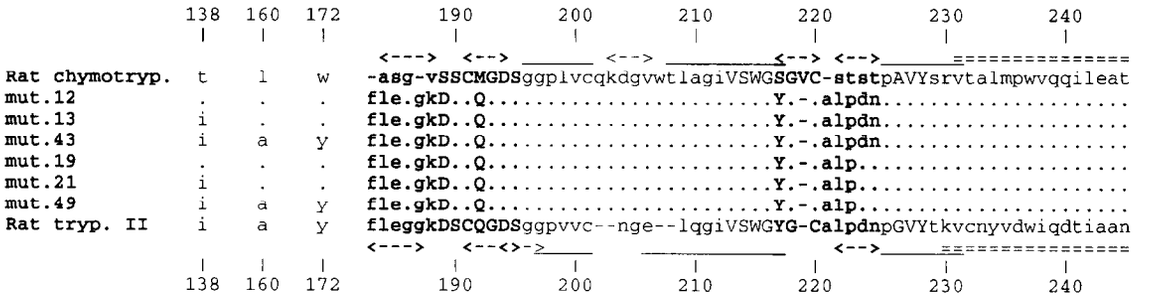

Fig. 1. The aligned amino acid sequences and the position of secondary structure elements around the S1 specificity site of wild-type chymotrypsin, trypsin and chymotrypsin mutants. The amino acids in loop1 (sites 185(a) through 198) and loop2 (sites 217 through 224) are in bold. (The loop assignments are different from those by Hedstrom et al. [2] Here they are defined as structures between the helices and sheet segments.) The amino acids that form the specificity pocket are in capital letters. These amino acids have at least one atom that is not further from the substrate than 5 Å. The position of the secondary structure elements is as determined by the hydrogen bonds in the bovine enzymes [19–21]. Sheet segment: ____; helix: ====; b-turn <-->.

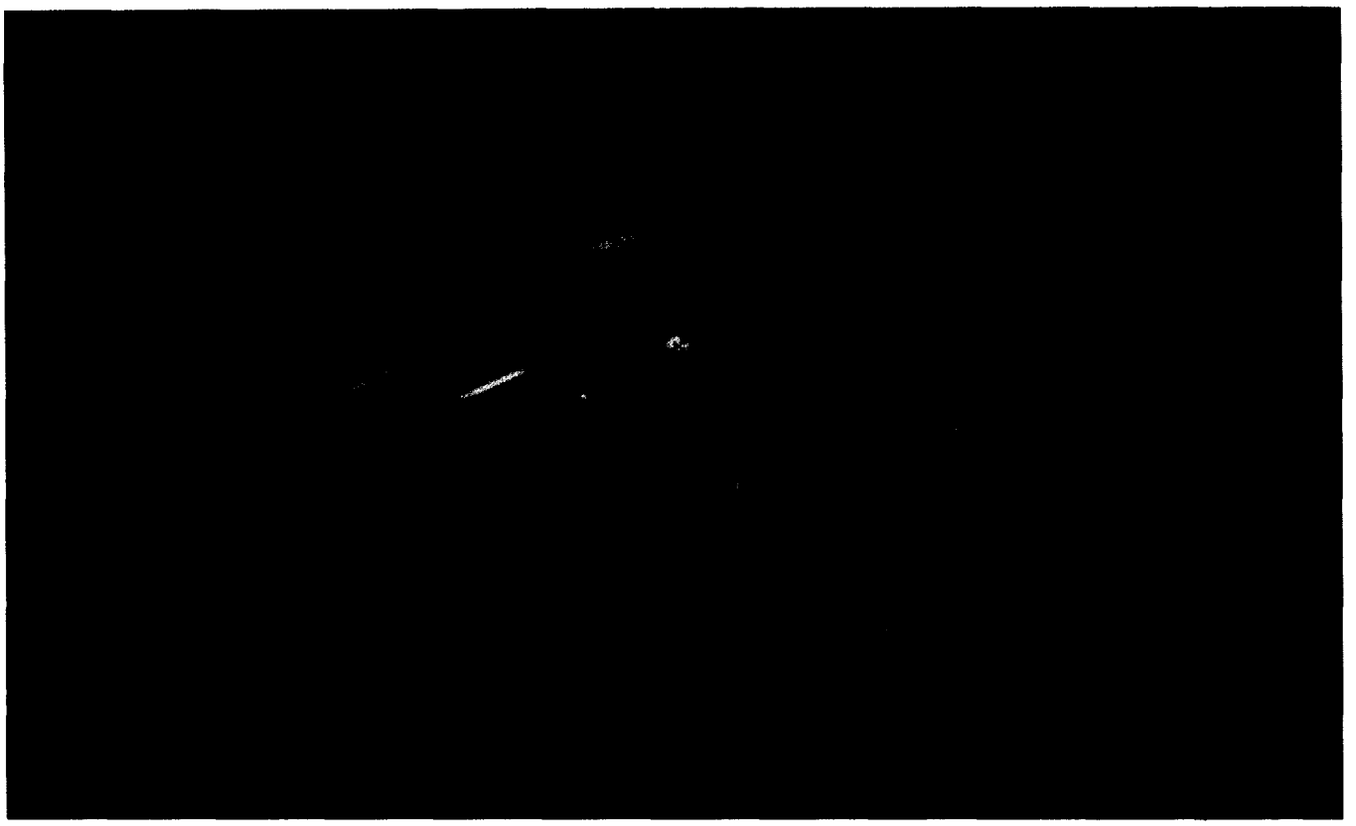

Fig. 2. H-bonding interaction possibilities between loops 1 and 2 in bovine trypsin (A) and bovine chymotrypsin (B). (A) the structure of free trypsin (Brookhaven Protein Data Bank identification number 1TPO) and a trypsin-pancreatic trypsin inhibitor complex (2PTC [7]) were aligned. Only the lysine side chain of the inhibitor (yellow) is shown from the trypsin–inhibitor complex. (B) the structure of free α-chymotrypsin (4CHA [22]) and the *N*-acetyl-L-phenylalanyl-trifluormethyl ketone complexed α-chymotrypsin (6CHA [23]) were aligned. Only the P1-Phe residue is shown from the latter (magenta). Besides the C, Cα and N atoms in the main chain and the Cys191–Cys220 disulphide bridge, only those residues and atoms are displayed that can take part in hydrogen bonds. (Residues 196–198 in loop1 are omitted.) The structure is orange where the main chain atom positions are different in trypsin and chymotrypsin. Dotted lines are the hydrogen bonds.

The introduction of trypsin specific aspartate residue at the bottom of the substrate binding pocket (site 189) reduced the activity on chymotrypsin substrates (Phe or Tyr at the P1 site) by three orders of magnitude but did not change the intrinsic tryptic activity of chymotrypsin on lysyl substrates. The fall in chymotryptic activity was entirely due to a decrease in the catalytic rate constant. Though the Ser189Asp mutation reduced the specificity, the enzyme remained basically chymotrypsin-like (Table 1).

The further substitutions in mutants 12, 13, 19, 21, 43 and 49 reduced the chymotryptic activity by 1–4 orders of magnitude relative to Asp189 chymotrypsin as a result of decrease in catalytic rate constants and of elevation in K_m values (Table 1A,B). Again, the activity of most of these mutants on lysyl substrate remained essentially the same as that of wild type chymotrypsin. However, on arginyl substrate the activities of mutants 24 and 49 dropped even below that of wild-type chymotrypsin due to fall in k_{cat} values. Despite the numerous differences in the amino acid replacements among chymotrypsin mutants 12, 13, 19, 21, 43 and 49, their catalytic properties on Phe, Tyr and Lys substrates were not significantly different, and they were essentially non-specific enzymes (Table 1B).

4. Discussion

The kinetic data of our chymotrypsin mutants show that the trypsin → chymotrypsin and chymotrypsin → trypsin specificity conversions are not 'symmetric' cases despite the almost identical architectures of the two proteins: reverse substitutions in chymotrypsin at sites that were sufficient to be mutated to change the specificity of trypsin, yielded only non-specific enzymes of very low activity. This failure of specificity conversion was unexpected, and indicates that the sites influencing the specificity must be at least partially different in trypsin and chymotrypsin.

Trypsin and chymotrypsin take part by main chain atoms in the interaction with the P1 substrate residue, therefore, through side chain interactions, numerous amino acids can indirectly influence the substrate binding by cooperatively determining the position of atoms that interact with the substrate. All these interactions are part of a hydrogen bonding network intertwining loop1 and loop2. These contain different number of bonds, and have a dissimilar arrangement in the two enzymes at sites where the loops are divergent (Figs. 1 and 2). The trypsin and chymotrypsin type stabilization of this hydrogen bonding net-

Table 1A
Kinetic constants determined on chymotrypsin (A) polypeptide amide substrates, Succinyl-AlaAlaProXaa-

Enzyme	Xaa = Phe			Xaa = Tyr		
	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m
wild-type chymotrypsin	5.2×10^3	2.3×10^1	2.3×10^2	7.1×10^3	1.1×10^1	6.5×10^2
S189D	6.2×10^0	4.1×10^1	1.5×10^{-1}	1.1×10^0	4.0×10^1	2.8×10^{-2}
mut. 12	2.3×10^0	8.9×10^1	2.5×10^{-2}	3.5×10^0	1.7×10^2	2.0×10^{-2}
mut. 13	5.0×10^{-2}	9.7×10^1	5.2×10^{-4}	6.6×10^{-2}	9.2×10^1	7.1×10^{-4}
mut. 43	9.6×10^{-1}	2.4×10^2	4.0×10^{-3}	ND	ND	ND
mut. 19	2.6×10^{-2}	3.8×10^2	6.8×10^{-5}	4.1×10^{-2}	2.4×10^2	1.7×10^{-4}
mut. 21	2.8×10^{-1}	3.4×10^2	8.2×10^{-4}	5.8×10^{-1}	3.4×10^2	1.7×10^{-3}
mut. 49	2.2×10^{-1}	6.8×10^2	3.2×10^{-4}	2.5×10^{-1}	2.3×10^2	1.0×10^{-3}
wild-type trypsin	6.2×10^0	1.3×10^2	4.8×10^{-2}	4.7×10^0	1.5×10^2	3.1×10^{-2}

$[k_{cat}] = s^{-1}$; $[K_m] = \mu M$; $[k_{cat}/K_m] = s^{-1} \cdot \mu M^{-1}$.

Table 1B
Kinetic constants determined on trypsin (B) polypeptide amide substrates, Succinyl-AlaAlaPro**Xaa**-AMC

Enzyme	Xaa = Lys			Xaa = Arg			specificity*
	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m	
wild-type chymotrypsin	1.1×10^0	3.5×10^2	3.1×10^{-3}	7.2×10^0	1.1×10^2	6.9×10^{-2}	7.4×10^4
S189D	3.4×10^{-1}	1.7×10^2	2.0×10^{-3}	1.1×10^{-1}	8.6×10^1	1.3×10^{-3}	7.5×10^1
mut. 12	ND	ND	ND	7.5×10^1	1.1×10^3	6.8×10^{-2}	2.1×10^1
mut. 13	4.6×10^{-1}	3.0×10^2	1.5×10^{-3}	1.6×10^0	7.9×10^2	7.8×10^{-2}	3.5×10^{-1}
mut. 43	4.8×10^0	4.2×10^2	1.1×10^{-2}	ND	ND	ND	3.6×10^0
mut. 19	6.9×10^{-1}	1.3×10^2	5.3×10^{-3}	6.5×10^{-2}	3.0×10^1	2.2×10^{-3}	1.3×10^{-1}
mut. 21	4.0×10^{-1}	1.5×10^2	2.7×10^{-3}	5.8×10^{-2}	1.4×10^2	4.1×10^{-4}	3.0×10^0
mut. 49	2.2×10^{-1}	1.2×10^2	1.8×10^{-3}	6.4×10^{-2}	8.0×10^1	8.0×10^{-4}	1.8×10^0
wild-type trypsin	4.2×10^3	3.2×10^0	1.3×10^3	1.1×10^4	1.3×10^0	8.4×10^3	3.7×10^{-5}

$[k_{cat}] = s^{-1}$; $[K_m] = \mu M$; $[k_{cat}/K_m] = s^{-1} \cdot \mu M^{-1}$; *specificity = $[k_{cat}/K_m]_{Phe}/[k_{cat}/K_m]_{Lys}$.

work defines the structure of the specificity determinant sites (e.g. 189 and 216 in trypsin [11]), gives a distinct structure to the water molecules located in the specificity pockets and probably lends to the substrate binding sites a dissimilar deformability [24]. It has been proposed that different conformational flexibilities rather than evident differences in the tertiary structures of the specificity sites of trypsin and chymotrypsin may represent the true structural basis for substrate discrimination [24,25].

The poor activity and the non-specific character of our chymotrypsin mutants might be the consequence of a missing network stabilization, i.e. of a (partial) disorder in the H-bonding of the two loops, similarly to the case of Tr → Cy[S1 + L1 + L2] trypsin mutant [11,14]. Concerning the origin of disorder we suppose, that the modified, trypsin-like loops cannot pack well in the chymotrypsin scaffold. Further studies are needed to eliminate the possible structural incompatibiliti(es) between the loops and the other parts of the protein, and to reveal additional site(s) that are involved in the substrate discrimination of trypsin and chymotrypsin.

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