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The Role of Disulfide Bond C191-C220 in Trypsin and Chymotrypsin

Éva Várallyay,* Zsolt Lengyel,†,[1] László Gráf,*,† and László Szilágyi†,[2]
**Department of Biochemistry, Eötvös University, Budapest, Hungary; and *Institute for Biochemistry and Protein Research, Agricultural Biotechnology Center, Gödöllő, Hungary*

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Serine proteases of the chymotrypsin family contain three conserved disulfide bonds: C42-C58, C168-C182, and C191-C220. C191-C220 connects the loops around the substrate binding pocket. Using site directed mutagenesis, cysteines of this disulfide bridge were replaced by alanines in trypsin, in chymotrypsin, and in Tr→Ch-[S1+L1+L2+Y172W], a mutant trypsin with high chymotrypsin like activity. The functional role of this "active site" disulfide was assessed by comparing the catalytic properties of wild-type and mutant enzymes. Its removal from all three proteases caused a decrease in k_{cat}/K_M of two to three orders of magnitude, mainly as a consequence of a dramatic increase in K_M. The pH dependence of the activity also changed: the rather wide pH optimum, characteristic of the wild-type enzymes (especially trypsin), narrowed since the pK_a in the alkaline region shifted downwards. Results show that C191-C220 is necessary for the high activity of both trypsin and chymotrypsin. By contrast, elimination of this disulfide bridge greatly decreased the specificity of trypsin and of Tr→Ch-[S1+L1+L2+Y172W], but had no significant change on that of chymotrypsin.

Trypsin and chymotrypsin catalyzes peptide bond cleavage by identical mechanisms (1) and possess similar tertiary structures (2, 3), but strikingly different substrate specificity. Trypsin hydrolyses substrates with arginine or lysine residue at P1 position, while chymotrypsin prefers large hydrophobic residues. Because of the similarity of structures of the substrate binding pocket the different specificity was thought to be due to a difference in the charge of residue 189. However, replacement of Asp189 of trypsin with the analogous Ser189 of chymotrypsin fails to convert specificity and resulted in a poor, non-specific protease (4). Conversion of trypsin to a chymotrypsin-like protease requires substitution of non conservative residues in the S1 site together with the exchange of two adjacent surface loops, which do not directly contact the substrate (5). This mutant, Tr→Ch[S1+L1+L2], has been improved to a protease with about 20% of the activity of chymotrypsin by mutating Tyr172 to Trp (6) (Fig 1). In addition to these primary effects, it has been proposed that the different conformational flexibilities of the activation domains (7) and different arrangements of water molecules in the specificity pockets (8) contribute to the substrate specificity in trypsin and chymotrypsin.

Enzymes of the chymotrypsin family of serine proteases contain three conserved disulfide bonds: C42-C58, C168-C182, C191-C220. In addition, in rat chymotrypsin there are two (C1-C122 and C136-C201), in rat trypsin three (C22-C157, C127-C232, C136-C201) more disulfide bonds. The conservative disulfide bridges connect short loops and are probably important for structural stability as they appeared early in the evolution. The elimination of C1-C122 from chymotrypsin (replacing chymotrypsin activation propeptide sequence by that of trypsin and mutating C122 to Ser) has no influence on the activity and specificity of chymotrypsin (9). The disulfide bridge between Cys191 and Cys220 is one of the ancient ones. It connects Loop 1 and Loop 2 which were shown to be key elements involved in the substrate discrimination of trypsin and chymotrypsin (5,6,10,11). The effect of the elimination of this disulfide bond was investigated in order to study its functional role. Cysteines of this disulfide bond were replaced by alanines in trypsin, in chymotrypsin and in Tr→Ch[S1+L1+L2+Y172W]. To test the effect of the elimination we measured the activity, pH dependence of activity, and specificity of these enzymes.

MATERIALS AND METHODS

Site directed mutagenesis/construction of mutant enzymes. Site-directed mutagenesis was performed according to Kunkel as de-

[1] Present address: Navix, Inc. Lincoln Technology Park, 542 Flynn Road, Camarillo, CA 93012.

[2] To whom correspondence should be addressed at Department of Biochemistry, Eötvös University, Puskin u.3., H-1088, Budapest, Hungary. Fax: (36) 1 266 7830. E-mail: szilagyi@ludens.elte.hu.

Abbreviations used: ϵ-ACA, ϵ-amino-capronic-acid; AMC, 7-amino-4-methylcoumarin; pNA, p-nitroanilide; MUGB, 4-methylumbelliferyl p-guanidinobenzoate; MUTMAC, 4-methyl umbelliferyl p-(N,N,N-trimethyl ammonium)cinnamate chloride; CBZ, carbobenzoxy.

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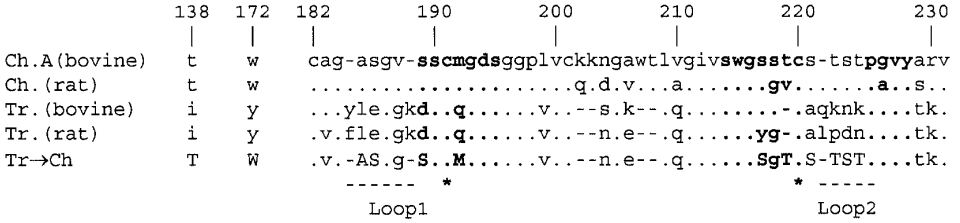

FIG. 1. Sequences of chymotrypsins, trypsins and mutant trypsin. Chymotrypsin numbering is used. Ch, chymotrypsin; Tr, trypsin; Tr→Ch, trypsin[S1+L1+L2+Y172W]. Periods denote residues which are identical to cow chymotrypsin, boldface lettering identifies residues that form the S1 binding pocket, and capital letters denote mutations. Loop1 and Loop2 are identified by dashed lines; Cys191 and Cys220 are marked with asterisks.

scribed previously (5,12) using M13 template containing the coding sequence of rat trypsinogen or chymo-trypsinogen. The following oligonucleotides were used: Trypsin mutants: *C191A:* GGC-AAG-GAT-TCC-GCC-CAG-GGT-GAC; *C220A:* TGG-GGC-TAT-GGC-GCT-GCC-CTG-CCA; *C191V:* GC-AAG-GAT-TCC-GTC-CAG-GGT-GAC-TC; *S1+L1+L2+Y172W+C191A:* A-GGC-TCT-TCC-GCC-ATG-GGT-GAC; *S1+L1+L2+Y172W+C220A:* GGC-TCT-GGC-ACT-GCT-TCC-ACT-TCG; Chymotrypsin mutants: *C191A:* C-GTC-TCT-TCC-GCC-ATG-GGT-GAC; *C220A:* C-AGT-GGC-GTC-GCT-TCC-ACT-TCC. Mutations were confirmed by dideoxy sequencing.

Expression and isolation of mutant enzymes. Wild type and mutant trypsins were produced in *E.coli*, purified and activated as described (4). Chymotrypsin and Tr→Ch[S1+L1+L2+Y172W] as well as their C191A/C220A mutants were expressed in yeast, according to (8). Activation of chymotrypsin was accomplished using (a) bovine trypsin 1/1000 (w/w) ratio in 50 mM ϵ-ACA 10 mM $CaCl_2$ pH:6.0 buffer. When the activation was complete, bovine trypsin was removed from the reaction mixture by two rounds of affinity chromatography on a p-aminobenzamidine-affinity column. Active chymotrypsin was further purified by affinity chromatography on a soybean trypsin-inhibitor column as described (4). The purity of the enzymes was analyzed by SDS polyacrylamide gel electrophoresis, and we used only homogenous preparates for measurements.

Determination of kinetic parameter. Enzyme assays were carried out in 20 mM Tris, 0.1M NaCl, 5 mM $CaCl_2$, pH 8.0 at 37° C. Fluorogenic substrates Suc-Ala-Ala-Pro-X-AMC (X=Arg,Lys,Phe or Tyr) or photometric substrates Z-Gly-Pro-Arg-pNA and Suc-Ala-Ala-Pro-Phe-pNA were used, and the resulting AMC or pNA concentration was measured as described (6). Active trypsin and chymotrypsin concentration were determined spectrofluorimetrically by active site titration with MUGB or with MUTMAC, respectively (13). As the last step of the purification was realized by affinity chromatography, and we obtained good correlation between active enzyme and protein concentration, we assumed that the proteins were correctly folded. Kinetic parameters were calculated from this data using the computer program ENZFITTER.

pH dependence of enzyme activity. Enzyme-catalyzed cleavage of Z-Gly-Pro-Arg-pNA or Suc-Ala-Ala-Pro-Phe-pNA was conducted in 0.1 M NaCl, 5 mM $CaCl_2$, buffered to pH values from 5.5 to 12. (pH was maintained using 20 mM of one of the following buffers within the range recommended by the manufacturers: MES, MOPS, TRIS, TAPS, CHES, or CAPS.) k_{cat}/K_M was calculated from the first order rate constant of the reactions, where $[S] \ll K_M$.

RESULTS AND DISCUSSION

Elimination of Disulfide Bridge C191-C220 Impairs Both k_{cat} and K_M

When we eliminated the conservative C191-C220 from trypsin, the k_{cat} decreased to 10-20 %, K_M increased by at least one order of magnitude, so the k_{cat}/K_M decreased by about two orders of magnitude both on ester (Na-CBZ-L-lysine thiobenzyl ester) and amide substrates (see Table 1). Similar effects were observed on both long and short pNA substrates. The nature of the leaving group was also indifferent in this respect. These results indicate that the utilization of the extended substrate binding interactions (14) as well as the interactions at P1′ site (15) were not influenced by the structural changes induced when the disulfide bridge was eliminated. Almost the same result was obtained when we mutated both cysteines to alanines, or C191 to valine and C220 to alanine. In this latter case, k_{cat} was higher, and K_M was greater than that of C191A/C220A trypsin.

Wild type trypsin prefers Arg substrates over Lys substrates by a factor of about 5-8, because Arg and Lys interact with the substrate binding pocket in a different mode (16). In the case of C191A/C220A, the K_M on Arg tetrapeptide substrate increased by about 20-fold while on Lys only by 5 fold. The decrease in k_{cat} values was independent on the P1 side-chain. Thus, the direct cyclic H-bonded interaction of the guanidino group in the arginine substrate with Asp189 seems to be more susceptible to the structural changes induced by the elimination of disulfide bridge than the water mediated interactions of the lysyl side-chain. When we changed Cys 191 to Val instead of Ala, the larger volume of it's side-chain disturbed the binding of arginine to a larger extent, so the preference toward arginine decreased further.

Elimination of the same (C191-C220) disulfide bridge in chymotrypsin caused more dramatic changes in both k_{cat} and K_M and resulted in an enzyme with almost three orders of magnitude lower catalytic efficiency both on pNA and AMC substrates.

The effect of the same change in Tr→Ch[S1+L1+L2 +Y172W] is again a drastic decrease of k_{cat}, but only a slight increase in K_M. The changes in the structures of these enzymes resulted both in the lowering of ground state binding seen as an increase in K_M, and also in the reduction of transition state stabilization seen as a decrease in the catalytic rate constants, especially in the acylation reaction.

TABLE 1

Kinetic Parameters of Wild Type Trypsin, Chymotrypsin, and Mutant Enzymes on Different Substrates

Substrate	Enzyme	k_{cat} (min^{-1})	K_M (M^{-6})	k_{cat}/K_M (M^{-1} min^{-1})
N-CBZ-L-Lys-SBzl	Tr.(rat)	42320 ± 3174	10.21 ± 0.14	4.14×10^9
	Tr.C191A/C220A	6180 ± 348	44.82 ± 1.25	1.37×10^8
	Tr.C191V/C220A	2220 ± 179	53.64 ± 2.57	4.15×10^7
ZGlyProArgpNA	Tr.(rat)	4400 ± 115	8.62 ± 0.76	5.12×10^8
	Tr.C191A/C220A	1780 ± 130	266.5 ± 45.6	6.68×10^6
	Tr.C191V/C220A	2270 ± 174	608.3 ± 100	3.73×10^6
ZArgpNA	Tr.(rat)	303 ± 27	169.2 ± 42.3	1.79×10^6
	Tr.C191A/C220A	74 ± 6.1	2185 ± 302	3.4×10^4
SucAlaAlaProArgAMC	Tr.(rat)	599 ± 34.7	6 ± 0.42	9.9×10^7
	Tr.C191A/C220A	77.5 ± 6.2	125.8 ± 6.29	6.16×10^5
	Tr.C191V/C220A	257 ± 15.4	1077 ± 143	2.39×10^5
SucAlaAlaProLysAMC	Tr.(rat)	601 ± 48.1	48.5 ± 2.33	1.24×10^7
	Tr.C191A/C220A	74.4 ± 5.06	254.3 ± 15.29	2.93×10^5
	Tr.C191V/C220A	203 ± 24.7	1130 ± 66	1.80×10^5
SucAlaAlaProPhepNA	Ch.	3180 ± 96	64.7 ± 5.3	4.91×10^7
	Ch.C191A/C220A	330 ± 29.8	8527 ± 1270	3.87×10^4
SucAlaAlaProPheAMC	Ch.	780 ± 72	73 ± 13	1.07×10^7
	Ch.C191A/C220A	53 ± 9.4	2450 ± 680	2.16×10^4
	Tr → Ch	1393 ± 80	649 ± 70	2.15×10^6
	Tr → Ch C191A/C220A	3.74 ± 0.21	615 ± 77	6.1×10^3
SucAlaAlaProTyrAMC	Ch.	700 ± 11.5	40.7 ± 1.7	1.72×10^7
	Ch.C191A/C220A	38.9 ± 2.1	2054 ± 205	1.89×10^4
	Tr → Ch	2480 ± 180	429 ± 66	5.78×10^6
	Tr → C191A/C220A	9.17 ± 1.02	1427 ± 34	6.43×10^3

Note. Tr. stands for trypsin, Ch. stands for chymotrypsin, and Tr → Ch stands for trypsin [S1 + L1 + L2 + Y172W].

High resolution X-ray studies show that the C191-C220 disulfide bridge moves about 0.7A upon binding of benzamidine to trypsin. This movement results in a "squeezing" of the substrate binding pocket leading to subtle reorientation of the catalytic side-chains His57 and Asp102 (17). Critical comparison of the structures of free and tripeptide-boronate inhibited trypsin showed that the dihedral angle χ of Cys 220 changes by about 10 degrees. (18). Similar changes, although not yet demonstrated, are probable in chymotrypsin as well.

There is no general rule to predict the structural consequences of the elimination of a disulfide bridge. There are reports about the increase in the flexibility of the polypeptide chain around the site of the mutation (19). In the case of C77A/C95A lysozyme, the dynamic structure determined by normal mode refinement of high resolution X-ray data showed that - contrary to an earlier report (20) - there was no increase in the internal flexibility of the polypeptide chain as a consequence of the mutation. The structural changes were characteristic of a cavity creating mutation since the volume of two alanine side-chains are smaller than that of a cystine (21). We suppose that similar effect may also occur in our work.

We compared the relative accessible surface areas of the eliminated disulfide bridges in these proteins. This value was 18% both in rat and bovine trypsin (2trm and 2ptn) and only 10% in bovine chymotrypsin (2cha). Greater effects observed in the change of activity of chymotrypsin can therefore, at least partly, be a consequence of the more deeply buried nature of this disulfide bridge. The coordinates for Tr→Ch[S1+L1+L2+Y172W] are not available yet, but it is unlikely that this correlation holds for the mutation of this enzyme i.e. the disulfide is even more deeply buried. Here, we suppose the structure which has been engineered to exert high chymotryptic activity within a trypsin-like framework, but is far from being "evolutionarily refined", certainly collapses when such an important structural element as a disulfide bridge is removed.

pH Profile of the Mutant Enzymes Is Narrowed

The pH dependence of k_{cat}/K_M for the hydrolysis of substrates follows a bell shaped curve for both wild-type trypsin and chymotrypsin. The curve is characterized by two ionization constants pK_{a1} and pK_{a2}. pK_{a1} represents the ionization of the catalytically important base (His 57) at the active site whereas the high pH ionization (pK_{a2}) is due to the α-amino group of Ile 16 which holds the enzyme in the catalytically active conformation (22,23).

Our data on rat chymotrypsin are in good agreement with data published for bovine chymotrypsin (Table 2). Similarly, pK_{a1} of rat trypsin is identical to that of bo-

TABLE 2

pK_a Values of Wild Type and Mutant Trypsins and Chymotrypsins

Enzyme	pK_{a1}	pK_{a2}
Tr.(bovine)	6.97 ± 0.13	10.11 ± 0.11
Tr.(rat)	6.93 ± 0.12	11.50 ± 0.13
Tr.C191A/C220A	6.99 ± 0.05	10.03 ± 0.04
Ch.(bovine)[a]	6.80	9.1
Ch.(rat)	6.81 ± 0.12	9.90 ± 0.11
Ch.C191A/C220A	7.07 ± 0.14	8.49 ± 0.17

Note. Tr. stands for trypsin; Ch. stands for chymotrypsin.
[a] Data from (23).

vine trypsin while the higher alkaline stability of rat trypsin (24) is reflected in its pK_{a2} value of 11.50. This extremely high value might even indicate the deprotonation of the arginyl side-chain of the substrate.

The elimination of disulfide bride C191-C220 does not change pK_{a1} in either trypsin or in chymotrypsin. The alkaline pK_a value in C191A/C220A mutant trypsin and chymotrypsin decreased by 1.5 pH units. It is interesting to note that the pK_a value obtained for mutant chymotrypsin is very close to the theoretical ionization constant of an unperturbed α-amino group (pK_a 6.8-7.9). In mutant rat trypsin pK_{a2} is 10.08, still considerably higher than the unperturbed value.

Cysteines C191 and C220 are components of the activation domain, the part of the molecule which undergoes characteristic changes when an ion-pair is formed between Ile16 and Asp 194. On the basis of NMR studies on peptidyl boronate inhibited trypsinogen and trypsin, it has been suggested that the structural transition leading to activation can be regarded as a continuos shift in the equilibrium between the inactive and active forms of trypsin (25). Our results shows that elimination of disulfide bridge C191-C220 changes this equilibrium by destabilizing the ion pair between Ile16 and Asp194. The elimination of this disulfide bridge probably distorts the isoleucyl side-chain binding pocket. This destabilization eventually leads to the decrease of apparent pK_{a2}. It was shown recently (26) that hydrophobic interactions in the isoleucine binding pocket are more important than the ionic interaction between Asp194 and Ile16 in the stabilization of active conformation of trypsin.

Change in Substrate Specificity Is Different in Trypsin and Chymotrypsin

In order to characterize substrate discrimination we compared k_{cat}/K_M values measured on substrate pairs Arg vs. Tyr and Lys vs. Phe (see Table 3). We calculated a ratio in such a manner that the nominator was always the value corresponding the primary specificity of the enzyme. The ratio gives a comparable measure of the specificity or substrate discrimination: the larger value always means higher specificity. This ratio is 10^4-10^5 for trypsin, while only 100-200 for chymotrypsin, indicates that substrate specificity of chymotrypsin is less stringent. The substrate discrimination in chymotrypsin did not significantly change upon removal of disulfide bridge C191-C220. The Phe/Lys ratio was unchanged; the Tyr/Arg ratio was only 10 times lower in the mutant compared to the wild type. On the other hand, in mutant trypsin there was a 10^2 to 10^4 fold drop in the specificity, with the decrease in tryptic activity, there was a significant increase in the activity on chymotryptic substrates.

Similar changes were observed in Tr→Ch-[S1+L1+L2+Y172W]. Specificity of its C191A/C220A mutant decreased by a factor of 10^3-10^4 because of a drastic decrease in the chymotryptic activity and increased catalytic efficiency both on Arg and Lys substrates. This mutant is only a 3 to 6 times worse trypsin than the C191A/C220A trypsin mutant where Asp is present in the substrate binding pocket. It appears that the overall trypsin structure - evolved to maintain higher specificity - responds to the removal of disulfide bridge with greater loss in substrate discrimination, irrespective of the actual structure of the substrate binding pocket. Trypsin-like activity requires a negatively charged group, preferentially aspartic acid in the substrate binding pocket (4,18): but it seems to be true only for those mutants where the substrate binding pocket and the loops surrounding it have a trypsin-like structure. Wild type chymotrypsin is about 100 times more effective on trypsin substrates than D189S and other trypsin mutants lacking the negative charge in the substrate binding pocket. In all probability, the structural basis of the medium level tryptic activity (about 1% of that of wild type trypsin) are different in chymotrypsin and in Tr→Ch[S1+L1+L2+Y172W] C191A/C220A since in the case of chymotrypsin it coincides with a high chymotryptic activity while in the later case tryptic activity is actually higher than chymotryptic activity.

Recent comparative studies on the structure of several members of the chymotrypsin family of serine proteases revealed that, in addition to the identity of residue 189, the backbone conformation around Gly216 is a critical specificity determinant of these enzymes (10). It is far from being understood, however, which intramolecular interactions determine and stabilize the substrate discriminating conformation of this latter site in trypsin and chymotrypsin. Our present results suggest that disulfide bridge C191-C220 is one of these stabilizing interactions, the elimination of which results in differential effects on the substrate specificity of trypsin and chymotrypsin. Therefore, we believe that these pairs of trypsin and chymotrypsin mutants may serve as appropriate models to investigate further the mech-

TABLE 3

Specificity of Wild Type and Mutant Trypsins and Chymotrypsins

Enzyme	k_{cat}/K_M (M^{-1} min^{-1})				Substrate discrimination	
	Arg	Tyr	Lys	Phe	Arg/Tyr	Lys/Phe
Tr.(rat)	9.9×10^7	4.4×10^2	1.2×10^7	5.2×10^2	2.3×10^5	2.3×10^4
Tr.C191A/C220A	6.2×10^5	2.8×10^4	2.9×10^5	1.4×10^3	22	2.1×10^2
Tr.C191V/C220A	7.4×10^5	6.9×10^2	1.8×10^5	6.9×10^2	1.1×10^3	2.6×10^2
Ch.(rat)	9.1×10^4	1.7×10^7	1.1×10^5	1.1×10^7	1.9×10^2	10^2
Ch.C191A/C220A	9.0×10^2	2.2×10^4	2.0×10^2	1.9×10^4	24	95
Tr → Ch	1.9×10^4	5.7×10^6	3.3×10^3	2.1×10^6	3×10^2	6.3×10^2
Tr → Ch C191A/C220A	1.8×10^5	6.4×10^3	4.8×10^4	6.4×10^3	3.6×10^{-2}	1.3×10^{-1}
Tr.D189S[a]	1.4×10^3	1.1×10^4	9.7×10^2	3.7×10^3		

Note. Tr. stands for trypsin, Ch. stands for chymotrypsin, and Tr → Ch stands for trypsin [S1 + L1 + L2 + Y172W]. k_{cat}/K_M were determined on SucAlaAlaPro-X-AMC substrates (X denotes Arg, Lys, Phe, or Tyr).

[a] Data from Gráf et al. (4).

anism by which a large fraction of the global architecture of these proteases influence substrate specificity.

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