Protein Engineering, Design & Selection vol. 17 no. 2 pp. 127–131, 2004
Published online January 20, 2004 DOI: 10.1093/protein/gzh014

Ala226 to Gly and Ser189 to Asp mutations convert rat chymotrypsin B to a trypsin-like protease

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In a previous successful attempt to convert trypsin to a chymotrypsin-like protease, 15 residues of trypsin were replaced with the corresponding ones in chymotrypsin. This suggests a complex mechanism of substrate recognition instead of a relatively simple one that only involves three sites, residues 189, 216 and 226. However, both trypsin→elastase and chymotrypsin→trypsin conversion experiments carried out according to the complex model resulted in non-specific proteases with low catalytic activity. Chymotrypsin used in the latter studies was of B-type, containing an Ala residue at position 226. Trypsins, however, contain a conserved Gly at this site. The substantially decreased trypsin-like activity of the G226A trypsin mutant also suggests a specific role for this site in substrate binding. Here we investigate the role of site 226 by introducing the A226G substitution into chymotrypsin→trypsin mutants which were constructed according to both the simple (S189D mutant) and the complex model (S_1 mutant) of specificity determination. The kinetic parameters show that the A226G substitution in the S_1 mutant increased the chymotrypsin-like activity, while the trypsin-like activity did not change. In contrast, this substitution in the S189D chymotrypsin mutant resulted in a 100-fold increase in trypsin-like activity and a trypsin-like specificity profile as tested on a competing oligopeptide substrate library. Additionally, the S189D+A226G mutant is the first trypsin-like chymotrypsin that undergoes autoactivation, an exclusive property of trypsinogen among pancreatic serine proteases.

Keywords: autoactivation/serine protease/substrate specificity

Introduction

Pancreatic serine proteases have similar tertiary structures, but their specificity profiles are very different. Based on the strict substrate preferences of trypsin (Arg, Lys preference in the P_1 position, nomenclature of Schechter and Berger, 1967), chymotrypsin (Tyr, Phe, Trp) and elastase (Ala, Val, Leu) and the structure comparison of their S_1 regions (residues 185–195, 213–223 and 226–228) it was suggested that residues 189, 216 and 226 might determine the substrate specificity in pancreatic serine proteases (Steitz *et al*., 1969). Asp189 at the base of the substrate binding pocket of trypsin provides a negative electrostatic potential to stabilize the positive charge of P_1 Arg or Lys side chains in the substrates; residues Val216 and Thr226 (both residues are Gly in trypsin) make the S_1 region shallow in elastase-1. Chymotrypsin contains a Ser at position 189, and its less polar pocket is more suitable for accommodating bulky P_1 side chains. Both 216 and 226 positions are Gly in chymotrypsin-A, while in chymotrypsin-B there is an Ala at position 226. This difference was shown to cause the different P_1 specificity profiles of the two isoenzymes (Hudáky *et al*., 1999).

To verify the structural basis of substrate specificity the amino acids at position 189 in trypsin and chymotrypsin were interchanged by site-directed mutagenesis. The D189S mutation failed to confer chymotrypsin-like activity to trypsin (Gráf *et al*., 1988). Apparently, interchanging of all different amino acids in the S_1 regions—including two surface loops (L1, 185–195 and L2, 217–223)—and also two further substitutions at sites 138 and 172 were needed for an almost complete conversion of trypsin to a chymotrypsin-like protease (Hedstrom *et al*., 1992, 1994a,b). This is in line with the view that the differential specificities of trypsin and chymotrypsin may be controlled by extended structural units (Gráf, 1995; Perona *et al*., 1995). To further test this hypothesis, reverse substitutions were introduced into chymotrypsin (Venekei *et al*., 1996b). The S189D mutation greatly reduced the activity, while specificity remained basically chymotrypsin-like. Further substitutions in the S_1 region resulted in non-specific enzymes with even lower activity. Substitutions at the same sites were introduced into trypsin in order to convert it to elastase as well, but the mutants had no measurable amidase activity (Hung and Hedstrom, 1998). These later studies suggest that further sites might be involved in substrate discrimination, and a unique, even more extended structure determines each specificity. Such sites might be components of a co-evolving and mechanically coupled network in the trypsin family that are located outside the S_1 region (Süel *et al*., 2003).

The enzyme in the chymotrypsin→trypsin conversion studies was chymotrypsin-B that contains an Ala at site 226. However, Gly is conserved at this site among trypsins that may indicate its important role in the determination of a trypsin-like substrate specificity. Indeed, the G226A mutation in trypsin reduces the activity on trypsin substrates by three to four orders of magnitude (Craik *et al*., 1985). The crystal structure of the mutant revealed that Ala226 causes misalignment of both Arg and Lys substrates at the active site, and showed the lack of direct electrostatic interaction between the P_1 Arg and Asp189 of trypsin (Wilke *et al*., 1991). To investigate, if residue 226 is important for the chymotrypsin→trypsin specificity conversion as well, we made the A226G replacement in the S189D chymotrypsin-B mutant (S189D+A226G), and in one of our previously constructed chymotrypsin-B mutants with further substitutions in the S_1 region (S_1+A226G) (Figure 1).

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                    190       200       210       220       230
                     |         |         |         |         |
CTRB RAT        -ASG-VSSCMGDSGGPLVCQKDGVWTLAGIVSWGSGVC-STSTPAVYSR
S189D           ......D.....................................
S189D+A226G     ......D.....................................G....
S1              FLE.GKD..Q.....................Y.-.ALP........
S1+A226G        FLE.GKD..Q.....................Y.-.ALP...G....
TRY2 RAT        FLE.GKD..Q......V..--N.E--.Q......Y.-.ALPDN.G..TK
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Fig. 1. Aligned amino acid sequences around the S_1 specificity region of wild-type chymotrypsin-B (CTRB RAT), trypsin (TRY2 RAT) and chymotrypsin-B mutants. Chymotrypsin numbering is used; '.' denote identity with chymotrypsin-B and '–' denote gaps in the sequence.

Materials and methods

Materials

Highly purified enterokinase was the product of Biozyme (EK-3). Bovine chymotrypsinogen, bovine trypsin, SBTI-Sepharose, MUGB, succinyl–Ala–Ala–Pro–Phe–AMC, AMC and 7-methylumbelliferon were from Sigma Chemical Co. Succinyl–Ala–Ala–Pro–Lys–AMC and the oligopeptide substrate library were prepared as described (Gráf *et al*., 1988; Antal *et al*., 2001).

Construction of mutants

Chymotrypsin-B mutants S189D+A226G and S_1+A226G were constructed according to Kunkel (1985), from S189D and a multiple substituted S_1 region mutant, respectively (Figure 1). The mutations were confirmed by DNA sequencing.

Similarly to the previous chymotrypsin→trypsin mutants (Venekei *et al*., 1996a,b) the new mutants were also expressed as a propeptide chimera in which the chymotrypsin propeptide was replaced by the trypsin propeptide and site Cys122 was mutated to Ser, so that they could be activated with enterokinase. This minimized the contamination of the enzyme preparations with trypsin and an accurate determination of even low tryptic activities of the mutant enzymes became possible. The enzyme kinetic parameters of the chimera do not differ from those of wild-type chymotrypsin under physiological conditions (Venekei *et al*., 1996a). On the other hand, the wild-type propeptide increased the stability of the protein as studied under denaturing conditions (non-physiological pH, temperature and denaturing agents) (Kardos *et al*., 1999). To examine if the disulfide-linked chymotrypsin propeptide would still affect the enzymatic properties of our new mutants, the chymotrypsin propeptide-containing forms of the mutants were also expressed and characterized. They did not, however, show any significant difference in their catalytic activities when compared with their chimeric counterparts.

Expression and purification of the mutants

The wild-type and the mutant chymotrypsinogen sequences were cloned into the pET-17b expression vector. These plasmids were transformed into the BL21 (DE3) pLysS *Escherichia coli* strain, the expression was conducted according to the manufacturer's instructions (Novagen, 1997). After induction with IPTG, the cells were collected in a 1/10 volume TE buffer and frozen at –20°C. Then they were thawed and sonicated, and the inclusion body fraction was collected by centrifugation and washed three times with TE buffer. For renaturation of the expressed protein, the inclusion body fraction was solubilized with 6 M GuHCl, 0.1 M Tris–HCl (pH 8.0), 100 mM DTT; the solution contained ~10 mg/ml protein. The solubilized protein was diluted to 200-fold into the refolding buffer containing 1 M GuHCl, 5 mM cysteine, 1 mM cystine, 50 mM Tris–HCl (pH 8.0), 5 mM EDTA. The renaturation process was conducted at 4°C, overnight. The renatured protein solutions were then dialyzed against 2 mM HCl, 10 mM $CaCl_2$ and ultracentrifuged (45 000 r.p.m., 30 min, 4°C). Zymogens were activated by highly purified enterokinase at a 100:1 (w/w) zymogen/enterokinase ratio, and then the active forms were purified by affinity chromatography on an SBTI-Sepharose column. The purity of the preparations was analyzed by SDS–PAGE. The enzyme concentration was determined by Bradford assay for the low-activity mutants, and by active site titration with MUGB and MUTMAC (Jameson *et al*., 1973) for S189D+A226G mutant and wild-type chymotrypsin, respectively.

Enzyme assays

Amide hydrolysis was measured on succinyl–Ala–Ala–Pro–Phe–AMC and succinyl–Ala–Ala–Pro–Lys–AMC substrates in a 50 mM Tris–HCl pH 8.0, 10 mM $CaCl_2$, 0.1 M NaCl reaction buffer at 37°C using a Spex Fluoromax spectrofluorimeter. The data were analyzed with Enzfitter software.

Specificity profiling on a competing oligopeptide substrate library was also performed as described (Antal *et al*., 2001). Briefly, the oligopeptide library has seven members with a sequence HAAPXSADIQIDI, where X represents the different P_1 residues, Lys, Arg, Tyr, Leu, Phe and Trp. X=Pro served as an internal standard. The individual peptide substrates compete for the proteinase during the enzymatic reaction. Enzyme concentrations were optimized to ensure comparable enzyme reaction rates: 0.6 μM for S189D and S189D+A226G mutants, 0.075 μM for chymotrypsin-B and 0.0012 μM for bovine trypsin. The concentration of each substrate mixture component was 40 μM. The reaction was monitored by RP-HPLC separation of the components.

Autoactivation experiments were conducted with incubation at room temperature in the reaction buffer above, and were monitored with SDS–PAGE and activity measurement.

Chymotrypsinogen activation was carried out at 37°C in the reaction buffer above. Chymotrypsinogen (1 μM) was incubated with 16 μM S189D, 14 μM S189D+A226G and 5 nM bovine trypsin for 80 min. Chymotrypsin activity was measured every 10 min on succinyl–Ala–Ala–Pro–Phe–AMC substrate and was expressed as a percentage of the total activity.

For the measurement of benzamidine inhibition of the S189D and S189D+A226G mutants, progress curves were recorded with enzyme concentrations of 80–160 nM and with substrate concentrations of 50–100 μM. Constants were determined by analyzing the progress curves by the DynaFit software (Kuzmic, 1996) according to the Michaelis–Menten equation.

Results and discussion

To study the significance of Gly226 in the specificity determination, A226G mutants were constructed and their kinetic properties were determined on trypsin and chymotrypsin substrates. The A226G substitution was introduced into S189D chymotrypsin-B (S189D+A226G mutant), and into a multiple-substituted chymotrypsin-B→trypsin mutant (S_1+A226G mutant, Figure 1), which has a trypsin-like specificity profile. The kinetic constants were calculated from hydrolysis rates measured on succinyl–Ala–Ala–Pro–Lys–AMC and succinyl–Ala–Ala–Pro–Phe–AMC fluorometric amide substrates.

Table I. Kinetic constants determined on succinyl–Ala–Ala–Pro–Xaa–AMC polypeptide amide substrates

Enzyme	Xaa=Lys			Xaa=Phe			Specificity[b]
	k_{cat} (s^{-1}) (mean ± SEM)	K_M (mM) (mean ± SEM)	k_{cat}/K_M (s^{-1} M^{-1})	k_{cat} (s^{-1}) (mean ± SEM)	K_M (mM) (mean ± SEM)	k_{cat}/K_M (s^{-1} M^{-1})	
Wild-type chymotrypsin[a]	0.018 ± 0.004	0.35 ± 0.07	51	86 ± 2	0.023 ± 0.002	3.7×10^6	4.9
S_1[a]	0.011 ± 0.005	0.13 ± 0.03	84	$(4.3 \pm 2)\times10^{-4}$	0.38 ± 0.06	1.1	–1.9
S_1+A226G	0.031 ± 0.004	1.5 ± 0.8	21	0.18 ± 0.04	0.095 ± 0.003	1900	2.0
S189D[a]	0.0056 ± 0.0011	0.17 ± 0.08	33	0.10 ± 0.03	0.041 ± 0.009	2400	1.9
S189D+A226G	4.1 ± 0.2	0.99 ± 0.04	4100	0.32 ± 0.07	0.39 ± 0.05	820	–0.7
Wild-type trypsin[a]	70 ± 3	0.0032 ± 0.0001	2.2×10^7	0.10 ± 0.02	0.13 ± 0.05	770	–4.4

[a]Data from Venekei *et al*. (1996b).
[b]Specificity = $\log(k_{cat}/K_M^{Phe}/k_{cat}/K_M^{Lys})$.

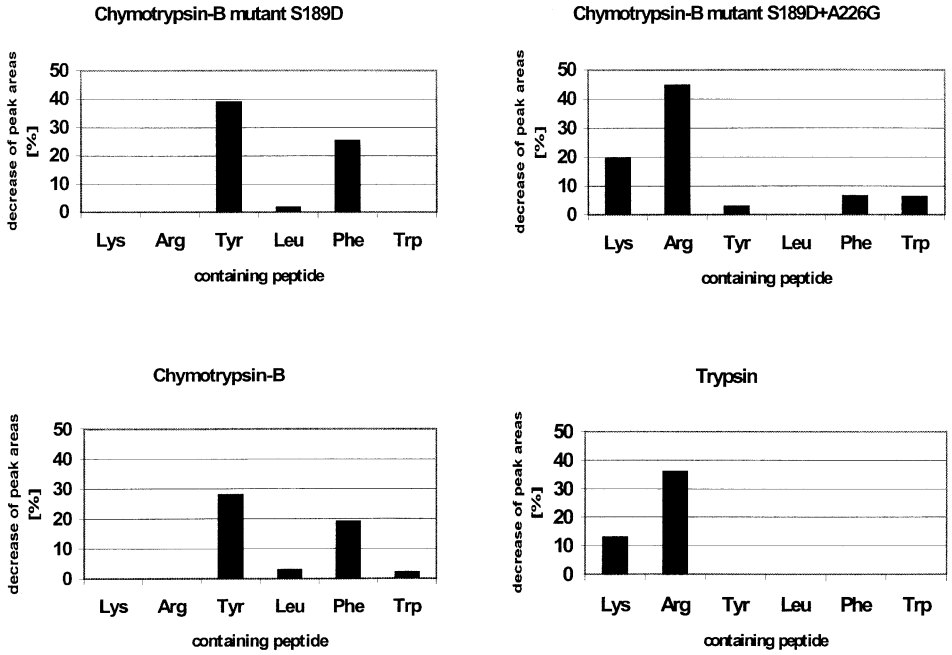

Fig. 2. Graphical presentation of enzyme specificity profiles as determined on a competing oligopeptide substrate library (Antal *et al*., 2001): decrease of peak areas after 16 min digestion. Chymotrypsin-B and trypsin data from Antal *et al*. (2001). Note that the enzyme concentrations in these experiments were different because they were optimized to ensure comparable enzyme reaction rates (see Materials and methods).

The A226G replacement exerted opposite effects on the mutants' specificities (Table I). The S_1 mutant changed to a more chymotrypsin-like protease, with two orders of magnitude higher activity on the chymotrypsin than on the trypsin substrate, while the basically chymotrypsin-like S189D mutant became trypsin-like by the single A226G substitution. The catalytic activity on the Lys-containing substrate was increased by two orders of magnitude and at the same time it was decreased by one order of magnitude on the chymotrypsin substrate. The trypsin-like activity of the mutant allowed the use of active site titration for the measurement of enzyme concentration. It is interesting to note that the increase in activities of both mutants, S189D+A226G and S_1+A226G, resulted mainly from the elevation of the catalytic rate constants.

To further characterize its specificity, the S189D+A226G chymotrypsin mutant was tested on a competing oligopeptide substrate library that we recently developed for specificity profile analysis of serine proteases (see Materials and methods). On this substrate mixture the mutant showed high preference towards trypsin substrates, with a considerable Arg over Lys preference similar to trypsins. Affinities towards Tyr, Phe and Trp substrates were equally low; Leu substrate was not cleaved (Figure 2). The A226G substitution clearly turned the chymotrypsin-like specificity profile of the parent mutant S189D to that of a trypsin-like protease.

The A226G replacement resulted in a chymotrypsinogen mutant (S189D+A226G chymotrypsinogen) that underwent autoactivation that is a typical property of trypsinogen. The same mutation, however, did not turn the zymogen of the S_1 mutant to an autoactivating one. Similarly to trypsinogen, the S189D+A226G zymogen fully activated itself during an overnight incubation. The rate of autoactivation is comparable to those of wild-type trypsinogens (Figure 3). The S189D+A226G chymotrypsin mutant also gained the ability of activating chymotrypsinogen (Figure 4), that is activated by trypsin *in vivo*. Contrary to the autoactivation rates, chymotrypsinogen activation rates of trypsin and the mutant enzyme

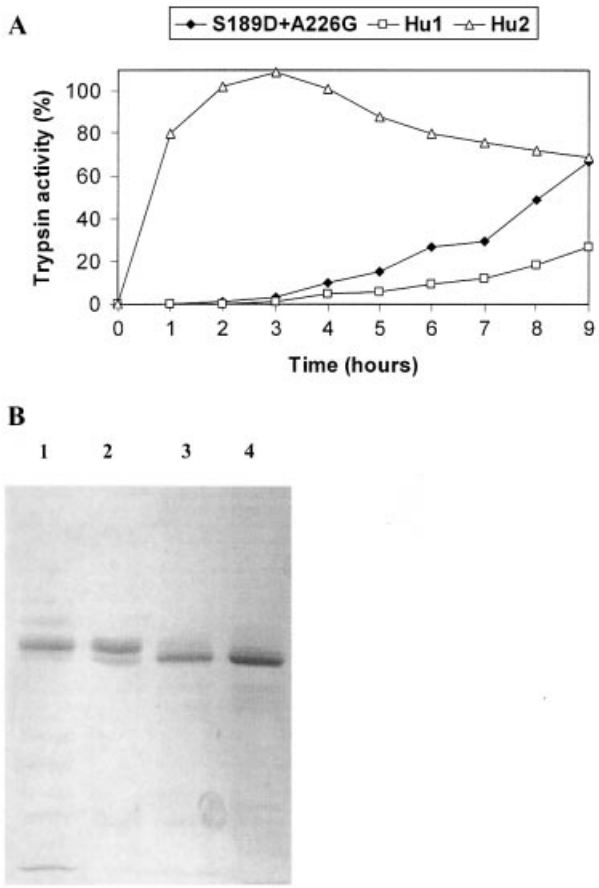

Fig. 3. Autoactivation of mutant chymotrypsinogen S189D+A226G. (**A**) Autoactivation of mutant S189D+A226G (8 μM final zymogen concentration) (diamonds) was compared with that of human recombinant trypsinogen 1 (8 μM) (squares) and human trypsinogen 2 (1.8 μM) (triangles). Zymogens were incubated at 37°C, in the presence of 50 mM Tris–HCl (pH 8.0), 0.1 M NaCl, 10 mM $CaCl_2$. Aliquots were withdrawn from reaction mixtures at the indicated times and trypsin activity was determined with the substrate succinyl–Ala–Ala–Pro–Lys–AMC. Activity was expressed as percentage of the potential total activity, as determined on similar zymogen samples activated with enterokinase. (**B**) SDS–PAGE of the autoactivation of mutant S189D+A226G, reducing conditions, 15% polyacrylamide gel. Lane 1, 0 min; lane 2, 5 h; lane 3, 15 h of incubation; lane 4, activated with enterokinase. Note that the mutant does not autolyse, probably because its chymotryptic activity is not large enough to cleave peptide bonds at the autolytic chymotrypsin cleavage sites.

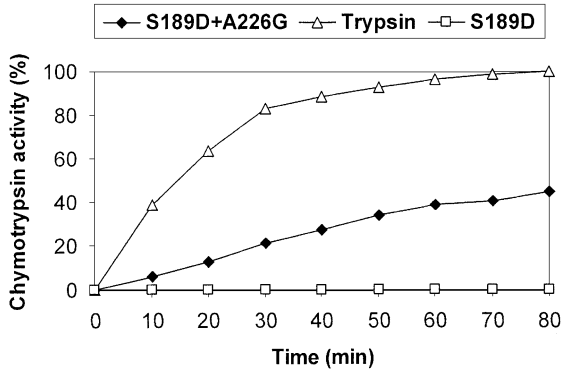

Fig. 4. Activation of chymotrypsinogen. Bovine chymotrypsinogen (1 μM) was incubated at 37°C with the mutant enzymes S189D (16 μM) (squares), S189D+A226G (14 μM) (diamonds) and wild-type trypsin (5 nM) (triangles) for 80 min. Aliquots were assayed every 10 min for chymotrypsin activity that is represented as a percentage of the total activity.

were only comparable when the concentration of the mutant in the reaction mixture was at least 1000-fold higher than that of trypsin. The fact that the S189D+A226G chymotrypsinogen mutant undergoes autoactivation and its activated form activates chymotrypsinogen assumes a certain degree of structural integrity in its substrate binding region. Autolysis of the S189D+A226G mutant was not observed. This may be due to the lack of trypsin-sensitive peptide bonds at the critical autolytic cleavage sites of chymotrypsin (Bódi *et al*., 2001), on the one hand, and to the low chymotryptic activity of the mutant protease on the other.

Benzamidine, a small reversible trypsin inhibitor that forms a direct charge interaction with the Asp189 side chain (Marquart *et al*., 1983), inhibits the S189D+A226G mutant with a K_i value similar to that of trypsin inhibition by benzamidine: $K_i^{S189D+A226G} = 6.9\times10^{-6}$ M, $K_i^{trypsin} = 6.3\times10^{-6}$ M. The wild-type level inhibition by benzamidine might indicate only a shift in the mutant towards a functionally more competent S_1 structure. The K_i value similar to that of wild-type trypsin and the poor K_m value are compatible with an S_1 region of the mutant which forms a stable complex with benzamidine but not with the substrate. Benzamidine of rigid structure and small size can adopt an orientation which is suitable for interactions with the mutant S_1 site. In this respect the S189D+A226G chymotrypsin may be similar to the G226A rat trypsin mutant. The latter, relative to the wild-type enzyme, has a 30-fold higher K_m on oligopeptide substrates while only a 5-fold higher K_i on benzamidine (Craik *et al*., 1985; Wilke *et al*., 1991). X-ray crystallography showed that the mutant bound benzamidine in an orientation very different from that in the wild-type enzyme (Wilke *et al*., 1991).

The crystal structure of the S189D mutant (Szabó *et al*., 2003) shows that the S_1 region contains severe deformations mostly in the loop segments 185–195 and 217–224, including the Cys191–Cys220 disulfide bond. The Asp189 side chain at the bottom of the pocket is turned out to the solvent, presumably because, contrary to trypsin, the S_1 region of chymotrypsin is not suitable for the stabilization of such a partially buried charge. These structural deformations explain the poor activity and the lack of trypsin-like specificity of the S189D mutant, but do not provide any useful information concerning the structural effects of the A226G substitution. From the increased trypsin-like features of the S189D+A226G mutant, however, we can conclude that the negative charge of Asp189 might somehow become more available in the S_1 region. The wild-type level inhibition by benzamidine and the strong preference for the Arg substrate in the case of the oligopeptide mixture suggest that the P_1 Arg and the Asp189 side chains can form even a direct charge interaction. To test this assumption, the three-dimensional structure of the S189D+A226G mutant would be needed, providing further insight into the structural basis of this unexpected specificity conversion.

The A226G substitution can be regarded as a conversion of S189D chymotrypsin-B to S189D chymotrypsin-A mutant. The chymotrypsin-B mutants were not appropriate counterparts of trypsin→chymotrypsin mutants, since site 226 is a conserved Gly in trypsins. The fact that the G226A mutation in trypsin reduces the activity on trypsin substrates by three to four orders of magnitude (Craik *et al*., 1985) also documents

the importance of Gly replacing Ala at site 226. Therefore, the introduction of the S189D mutation into a chymotrypsin-A-like protease can be considered as the first successful attempt to convert chymotrypsin to a trypsin-like protease. Our unexpected finding is that the single mutation S189D in a chymotrypsin-A-like mutant converts the specificity profile of chymotrypsin to that of a trypsin-like protease with enough catalytic potential even for autoactivation. This seems to support the view (also suggested by Hung and Hedstrom, 1998) that there may be different strategies and routes to convert trypsin to a chymotrypsin-like protease and vice versa and that the key residues involved in substrate discrimination and therefore to mutate to interchange the specificities of these proteases may not be identical in the two structures.

Acknowledgements

This work was supported by research grants T26625 and T022376 from OTKA to L.G. and I.V., respectively.

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Received September 4, 2003; revised October 31, 2003; accepted November 4, 2003

Edited by Valerie Daggett