Eur. J. Biochem. **259**, 528–533 (1999) © FEBS 1999

The differential specificity of chymotrypsin A and B is determined by amino acid 226

Péter Hudáky, Gyula Kaslik, István Venekei and László Gráf

Department of Biochemistry, Eötvös University, Budapest, Hungary

The A and B isoforms of the pancreatic serine proteinase, chymotrypsin are known to cleave substrates selectively at peptide bonds formed by some hydrophobic residues, like tryptophan, phenylalanine and tyrosine. We found, however, that the B forms of native bovine and recombinant rat chymotrypsins are two orders of magnitude less active on the tryptophanyl than on the phenylalanyl or tyrosyl substrates, while bovine chymotrypsin A cleaves all these substrates with comparable catalytic efficiency. Analysing the structure of substrate binding pocket of chymotrypsin A prompted us to perform an Ala226Gly substitution in rat chymotrypsin B. The specificity profile of the Ala226Gly rat chymotrypsin B became similar to that of bovine chymotrypsin A suggesting that only the amino acid at sequence position 226 is responsible for the differential specificities of chymotrypsin A and B isoenzymes.

Keywords: chymotrypsin B; site specific mutagenesis; specificity profile.

Proteinases were evolved to be relatively resistant toward self-cleavage (autolysis). The site specificity of the proteinase action may be a means to achieve this goal. This 'invention' offers a simple mechanism to control the rate of autolysis (the half life of proteins in general): the more accessible cleavage sites are on the surface of the proteinase, the faster the rate of (auto)-degradation is. A consequence of the narrow substrate specificity of the proteinases is that the rapid and complete degradation of protein substrates requires simultaneous action of a series of proteinases with different specificities. The pancreatic juice is an example of such a mixture of enzymes (such as, trypsin, chymotrypsin and elastase) to accomplish efficient endoproteolysis in the digestive tract. In addition to the major pancreatic serine proteinases, their isoforms with altered substrate specificities can also contribute to the efficiency of the proteolysis.

Chymotrypsin, the enzyme investigated in the present study, was described as the second major proteinase component (trypsin being the first one) in the pancreatic juice [1]. While trypsin hydrolyses the polypeptide chain at the lysyl and arginyl bonds, chymotrypsin selectively cleaves peptide bonds formed by aromatic residues (Phe, Tyr, Trp) [2,3]. Another molecular form of chymotrypsin was also isolated from beef pancreas and named first as protein B [4], and later as chymotrypsin B [5,6]. The two isoforms of chymotrypsin were shown to have the same molecular weight [7] but different electrophoretic mobilities [8]. Furthermore, their retardation on ion-exchange resins [9] and their sensitivities toward naturally occurring trypsin inhibitors [10] were also different. Studies on the reaction mechanism and substrate specificity, however, were almost exclusively performed with the A isoform of chymotrypsin (for review see [11]). Some early substrate specificity studies on chymotrypsin A and B revealed only minor differences [5,10], except for an observation that chymotrypsin B hydrolyses acyltryptophan ester more slowly than tyrosyl and phenylalanyl esters [12,13]. Some further minor chymotrypsin isoforms were also isolated from pancreas (chymotrypsin C [13] and CTRL-1, a chymotrypsin-like serine proteinase [14]) and were described as chymotrypsins with different cleavage site specificities. The chymotrypsin isoforms have been typified according to their sequence homology [15,16].

While the pancreatic serine proteinases, trypsin, chymotrypsin and elastase specifically cleave protein substrates at different amino acids, they have highly homologous primary and tertiary structures. The cleavage site discrimination is achieved primarily through selective binding of the amino acid residue in the substrate that is N terminal to the scissile bond (P1 position). For efficient catalysis the P1 residue of the substrate must snugly fit into the S1 substrate binding pocket and establish a number of contacts with the enzyme.

To explore the structural basis of substrate discrimination by serine proteinases a large number of chymotrypsin mutants have recently been constructed in our laboratory [17]. Chymotrypsin used in these studies was recombinant rat chymotrypsin, that on the basis of its isoelectric point and amino acid sequence belongs to the B-type chymotrypsins [18]. A comparison of the substrate specificities of bovine A and rat B chymotrypsins has revealed a so far overlooked specificity difference between the A-type and B-type enzymes. Here we report a thorough characterization of the substrate specificities of bovine chymotrypsin A and B, and rat chymotrypsin B, and describe the structural basis for the observed specificity difference.

MATERIALS AND METHODS

Bovine chymotrypsin A was a commercial enzyme, purchased from Sigma Aldrich Kft, Hungary (type II).

Isolation of the bovine chymotrypsin B

Bovine chymotrypsinogen B was prepared from bovine pancreas according to Laskowski [19]. After activation the enzyme was

Correspondence to Prof. László Gráf, Department of Biochemistry, Eötvös University, Puskin u. 3., 1088 Budapest, Hungary. Tel. (36–1)2667858. Fax: (36 1)2667830. E-mail: graf@ludens.elte.hu

Abbreviations: AMC, 7-amino-4-methyl coumarine; pNa, *p*-nitroanilide; PDB, Protein Data Bank; Succ., succinyl.

(Received 6 August 1998, revised 27 October 1998, accepted 2 November 1998)

purified on a Toyopearl SP650M cation-exchange column (a product of Supelco) with a pH gradient, of pH 3.0 (100 mM acetic acid, 3 mM sodium acetate) to pH 9.2 (100 mM Tris/HCl), with a flow rate of 0.5 mL·min^{-1}. One mL fractions were collected, and their chymotryptic activities were measured by adding 100-μL aliquots of the fractions to 900 μL of a solution containing 50 mM Tris/HCl (pH = 8.0), 10 mM $CaCl_2$, 100 mM NaCl and 50 μM benzoyl-tyrosine-pNa. After 1 h incubation at 37 °C the absorbencies were measured at 410 nm.

The cross-contamination of the chymotrypsin isoforms was checked by sodium dodecyl sulphate (SDS) and native polyacrylamide gel electrophoresis. Gel concentrations were 12.5% and 7%, respectively. SDS gel electrophoresis was performed under nonreductive conditions and native gel electrophoresis in a Tris/glycine buffer of pH 9.2.

Preparation of recombinant rat chymotrypsin B and its mutant

In all constructs used in this study the chymotrypsin propeptide region of the rat chymotrypsinogen B clone was replaced by the trypsin propeptide and Cys122 was mutated to serine [20]. Oligonucleotide CTTATGTGGGGCCCTCACC was used to introduce the Ala226Gly mutation into rat chymotrypsinogen B according to the method of Kunkel [21]. The amino acid substitution was verified by DNA sequencing. The wild-type and mutant rat chymotrypsinogens (trypsinogen propeptide constructions) were expressed in a yeast expression system, purified and activated as described [20].

Enzyme assays and computer graphics

The enzyme concentration was determined by active site titration using 4-methylumbeliferyl *p*-(*N*,*N*,*N*-trimethyl ammonium) cinnamate chloride [22]. Initial amide hydrolysis rates were measured with a SPEX Fluoromax fluorometer at 380 nm excitation and 460 nm emission, in 50 mM Hepes buffer of pH 8.0, containing 10 mM $CaCl_2$ and 100 mM NaCl, on 37 °C. Succ-Ala-Ala-Pro-Xaa-AMC (where Xaa were Tyr, Phe, Trp, Lys, Arg, Leu, Met and AMC is the fluorescent leaving group) tetrapeptide substrates were used in a 2–200 μM concentration range. Typical saturation curves were plotted from eight data points, that were averaged from at least two parallel measurements.

The three-dimensional structures of chymotrypsin and its mutant were visualized with the MidasPlus 2.0 software [23–25], on a Silicon Graphics computer. The protein models 6GCH [26] and 8GCH [27] were taken from the PDB database. The amino acid replacement was performed without altering the surrounding structure.

RESULTS

Purification of bovine chymotrypsin B

Chymotrypsins of the same species were used to compare the substrate specificities of the A-type and B-type chymotrypsins. In order to minimize the contamination of chymotrypsin B with the A form we applied an additional purification step on a cation-exchange FPLC column after the isolation procedure according to Laskowski (see Materials and methods). Separation was based on the dissimilar isoelectric points of the A-type (pI = 8.3) and B-type (pI = 4.7) enzymes [19].

The FPLC chromatography patterns of commercial bovine chymotrypsin A and the newly isolated B isoform (Fig. 1) show

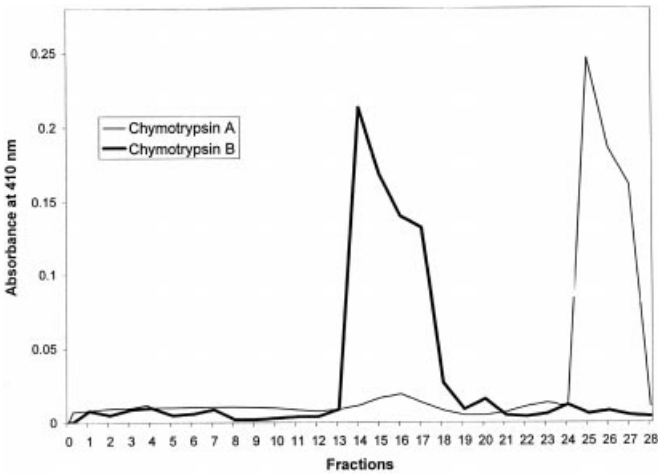


Fig. 1. Cation-exchange chromatography of bovine chymotrypsin A and chymotrypsin B samples. Absorbencies at 410 nm are given as the measure of chymotryptic activity in the fractions (see Materials and methods). Thin line represents chymotrypsin A, and thick line shows chymotrypsin B. Enzyme activities were measured as described in the Materials and methods, and the absorbencies of the liberated pNa are shown.

that under the conditions used the two chymotrypsin isoforms could be separated. When bovine chymotrypsin A was subjected to chromatography, the main activity peak eluted in fractions 24–28, while the enzyme activity of the bovine chymotrypsin B preparation was found in fractions 13–18. Gel electrophoretic patterns (Fig. 2) of chymotrypsin A and B confirmed that the two isoforms did not cross-contaminate each other.

Substrate specificity of the A-type and B-type chymotrypsins

The specificity profiles of native bovine chymotrypsin A and B and recombinant rat chymotrypsin B were determined on seven

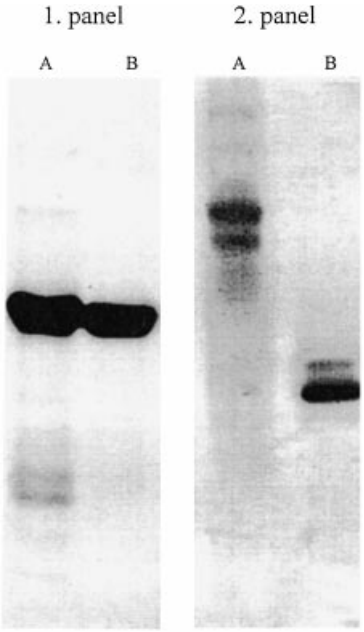


Fig. 2. Gel electrophoresis of the bovine chymotrypsins. Commercial chymotrypsin A (lanes A) and purified chymotrypsin B (lanes B) were analysed on non-reductive SDS (panel 1) and native (panel 2) polyacrylamide gels. Five microlitre samples from 2 mg·mL^{-1} protein solutions were loaded.

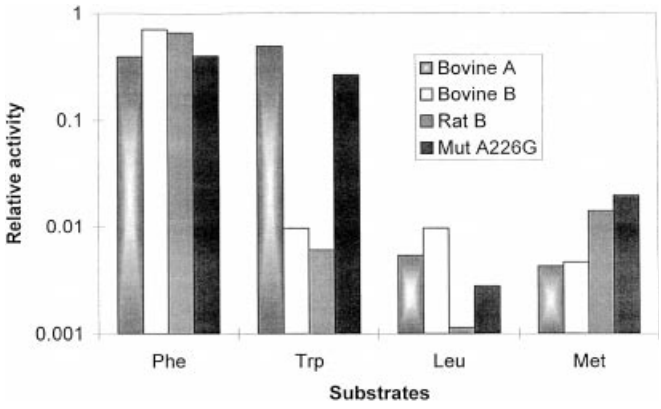


Fig. 3. Specificity profiles of wild-type A and B chymotrypsin and recombinant rat chymotrypsin. The specificity profiles were calculated from kinetic data on hydrophobic substrates in Table 1. The k_{cat}/K_m values of (P1) Phe, Trp, Leu and Met substrates were compared with that of Tyr substrate.

tetrapeptide amide substrates with different amino acids at the P1 position.

The specificity profile for the bovine A-type enzyme was essentially the same as what is known as a chymotrypsin-like substrate specificity: substrates with aromatic amino acids (Phe, Tyr, Trp) at the P1 position were hydrolysed with similar catalytic efficiency, leucyl and methionyl substrates were hydrolysed with two orders of magnitude lower rate, while there was a further two orders of magnitude drop in catalytic efficiency on the classic trypsin substrates containing Lys or Arg at the P1 position (Table 1, Fig. 3).

Our data for the two B-type chymotrypsins revealed a significant specificity difference from the A-type enzyme. Bovine and rat chymotrypsin B showed a significant discrimination against the tryptophanyl substrate as compared with the phenylalanyl and tyrosyl ones: the specific activity of these enzymes was two orders of magnitude smaller on the tryptophanyl than on the other two aromatic substrates. This was largely due to a 50–70-fold reduction in catalytic performance (k_{cat}) on the tryptophanyl substrate. The comparison of kinetic parameters for the chymotrypsin isoforms suggested that the observed activity difference was not a species-specific property of these enzymes, but it was rather due to species-independent structural difference(s) between the two chymotrypsin types.

Enzyme–substrate contacts in chymotrypsin B

We assumed that the discrimination of the B-type enzymes against Trp at P1 site is the result of a local rather than global

```
                          181         191         201         211         221
                           |           |           |           |           |
bovine chymotrypsin A   ICAGASGV-SS CMGDSGGPLV CKKNGAWTLV -GIVSWGS-STC S-TSTPGVYAR
bovine chymotrypsin B   ICAGASGV-SS CMGDSGGPLV CQKNGAWTLA -GIVSWGS-STC S-TSTPAVYAR
rat    chymotrypsin B   TCAGASGV-SS CMGDSGGPLV CQKDGVWTLA -GIVSWGS-GVC S-TSTPAVYSR
human  chymotrypsin B   ICAGASGV-SS CMGDSGGPLV CQKDGAWTLV -GIVSWGS-DTC S-TSSPGVYAR
porcine elastase     1  VCAGGDGVRSG CQGDSGGPLH CLVNGQYAVH -GVTSFVSRLGC NVTRKPTVFTR
human  elastase      2a ICAGGDGVISS CNGDSGGPLN CQASDGRWQV HGIVSFGSRLGC NYYHKPSVFTR
```

Fig. 4. Sequence comparison of A and B chymotrypsins and pancreatic elastases 1 and 2. The amino acids forming the specificity pocket are in bold, the amino acids at site 226 are underlined.

structural effect; therefore, the sequence regions forming the S1 substrate binding pockets (residues 189–195, 214–220 and 225–228) were compared (Fig. 4). Sequence divergence between chymotrypsin A and B occurs only at sites 218, 219 and 226. The chymotrypsin A structures (6GCH [26] and 8GCH [27] from the Protein Data Bank) show that amino acid side chains in positions 218 and 219 are turned away from the substrate and point towards the solvent. We therefore thought it unlikely that they could significantly affect substrate binding. In contrast, the modelled side chain of amino acid 226 appeared to extend into the pocket (see below). The glycine at this site in bovine chymotrypsin A is in a loose fit with the side chain of Phe and in a proper fit with that of Trp at P1 position of the substrate. The distances from the α-carbon of Gly226 are 564 pm and 328 pm to the nearest carbon of the benzyl ring in Phe, and to that of the indole ring in Trp, respectively.

To estimate the effect on tryptophan binding of the methyl group of alanine at site 226 in the bovine and rat B-type enzymes we introduced an alanine at site 226 in the X-ray structures of bovine chymotrypsin A complexed with *N*-acetyl-phenylalanyl-trifluoromethyl ketone [26] and with glycyl-alanyl-tryptophan [27] (the corresponding Protein Data Bank structures are 6GCH and 8GCH, respectively, Fig. 5). We used the A-type enzyme for modelling because no structure of a B-type enzyme is available yet. Reliable information from this modelling can be derived, however, as site 226 has the same conformation in each chymotrypsin-like serine-proteinase known. It is in a conservative β-sheet segment (amino acids 220–229) where the corresponding backbone atoms match each other with an average error of 17 pm RMS when the trypsin (226Gly) [28], chymotrypsin A (226Gly) [26] and elastase 1 (226Thr) [29] models are superimposed.

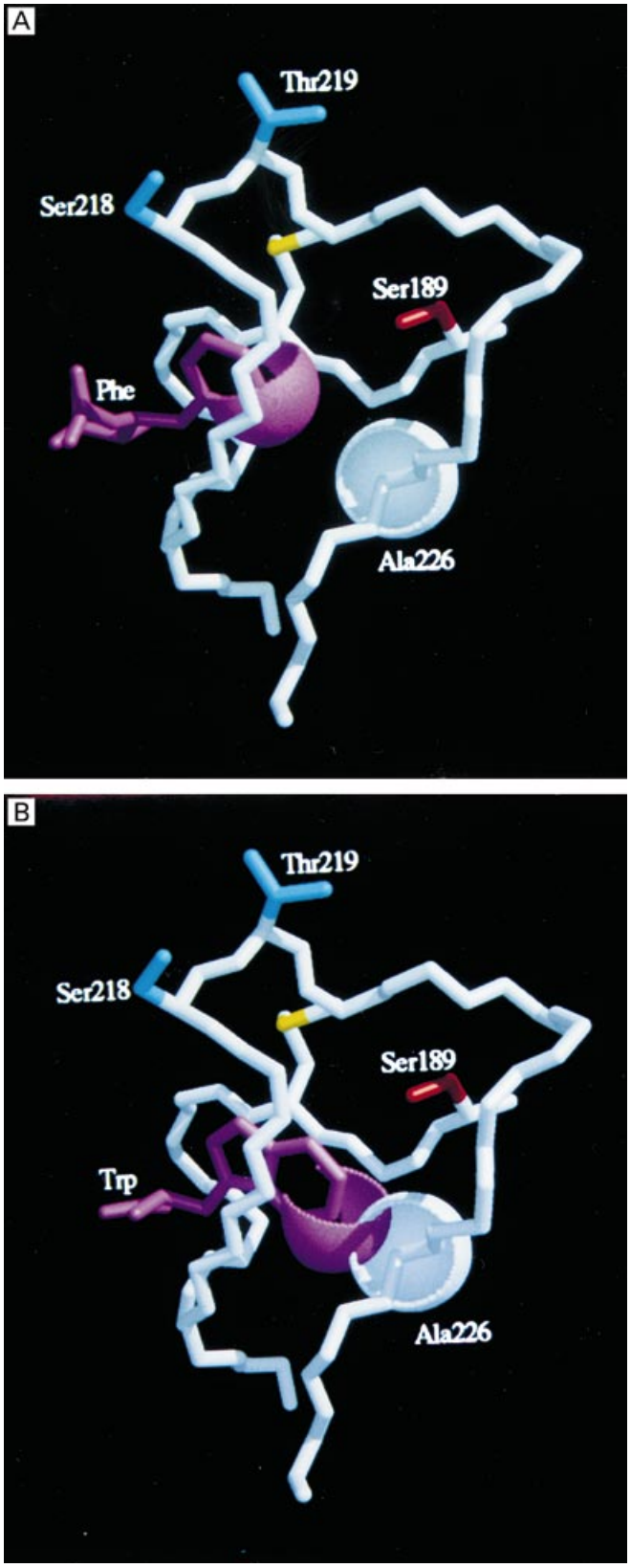
The methyl group introduced into the chymotrypsin A structure by modelling the Gly226Ala substitution did not have steric conflict with the surrounding protein atoms and did not seem to interfere with the Tyr (not shown) and Phe side chains of the bound substrates either (Fig. 5a). Modelled binding of the

Table 1. Kinetic parameters of wild-type and mutant chymotrypsins measured on synthetic substrates. Amide hydrolysis rates were determined on Succ-Ala-Ala-Pro-Xaa-AMC tetrapeptide substrates (see methods).

	Xaa=	Tyr	Phe	Trp	Arg	Lys	Leu	Met
Bovine A	k_{cat}	34.8	18.3	8.50	0.0202	0.0547	0.632	1.13
	K_m	32.1	42.9	15.9	496	589	110	246
	k_{cat}/K_m ($\times 10^{-2}$)	108	42.7	53.5	0.0040	0.0093	0.575	0.458
Bovine B	k_{cat}	13.9	13.7	0.200	0.0630	0.0418	1.10	2.44
	K_m	18.2	25.4	27.2	275	152	148	694
	k_{cat}/K_m ($\times 10^{-2}$)	76.5	54.0	0.738	0.023	0.027	0.742	0.352
Rat B	k_{cat}	37.3	30.0	0.712	0.0017	0.105	0.387	1.44
	K_m	18.7	22.9	59.5	174	451	169	50.8
	k_{cat}/K_m ($\times 10^{-2}$)	200	131	1.20	0.0098	0.023	0.228	1.70
Mut A226G	k_{cat}	52.7	36.7	11.2	0.0418	0.0873	0.693	1.36
	K_m	26.2	45.4	20.9	1239	727	123	33.8
	k_{cat}/K_m ($\times 10^{-2}$)	202	80.8	53.7	0.0033	0.012	0.563	4.02

k_{cat}: [s^{-1}] K_m: [μM] k_{cat}/K_m: [$s^{-1}\cdot\mu M^{-1}$]

side chain of a P1 tryptophan, however, appeared to be sterically hindered by Ala226 (Fig. 5b). Thus, we have assumed that the relatively decreased activity of B-type chymotrypsins towards tryptophanyl substrates may be due to the presence of Ala instead of Gly at sequence position 226.



Specificity conversion by mutagenesis

To prove experimentally that the specificity of chymotrypsin may be modified by the size of residue 226, alanine in this position of rat chymotrypsin B was replaced with glycine. The comparison of kinetic parameters of the wild-type and mutant enzymes on tetrapeptide amide substrates showed that this single amino acid replacement converted the specificity of the B-type enzyme to that of chymotrypsin A (Fig. 3, Table 1). Removal of the methyl group from position 226 led to a 50-fold increase of enzyme activity (resulting from a 16-fold increase of the rate constant and a threefold decrease in K_m) on the tryptophanyl substrate reaching the level of activity on the phenylalanyl and tyrosyl substrates. Activities on other substrates remained the same with minor differences in some individual parameters.

DISCUSSION

It has been the general notion that chymotrypsin A and B cleave the phenylalanyl, tyrosyl and tryptophanyl peptide bonds with about the same catalytic efficiency. Our present study has revealed, however, a so far overlooked specificity difference between chymotrypsin A and B. Accordingly, the B-type enzymes exhibit a two orders of magnitude smaller activity on the tryptophanyl substrate than on the tyrosyl and phenylalanyl ones. These latter activities are about the same as those of chymotrypsin A on the same substrates. Comparative modelling of the structures of bovine chymotrypsin A and B suggested, while the actual substitution of Ala226 in rat chymotrypsin B by glycine provided evidence that the discrimination of chymotrypsin B against tryptophanyl peptide bonds is due only to the steric hindrance between the methyl group of Ala226 in the enzyme and the P1 site tryptophan in the substrate.

The highly homologous S1 specificity sites of chymotrypsin and other pancreatic serine proteinases such as trypsin and elastase are built up from peptide segments 189–195, 213–220 and 226–228. The P1 residue of the substrate is known primarily to contact the main chain atoms of these regions. The reason for this is that the side chains of most residues that form the specificity site are rotated out of the pocket. There are only four residues, at positions 189, 190, 216 and 226, the side chains of which would point toward the substrate binding pocket. Residues at positions 189 and 190 are essential in determination of the trypsin-like and chymotrypsin-like specificities [30,31], while amino acids at sites 216 and 226 are thought to be the specificity determinants of pancreatic elastases [32,33]. The latter enzymes prefer hydrophobic residues of various size at the P1 site of the substrate. Valine at site 216 and threonine at site 226 in porcine elastase 1 (Fig. 4) reduce the depth of the S1 substrate binding pocket so much that alanine becomes the most

Fig. 5. The effect of an alanine residue at site 226 on the binding of phenylalanine (A) and tryptophan (B) substrate residues to the specificity pocket of chymotrypsins. Glycine to alanine substitutions were introduced into the structures of γ-chymotrypsin complexed with *N*-acetyl-L-phenylalanyl-trifluoromethyl-ketone (6GCH, A) and Gly-Ala-Trp peptide (8GCH, B). The backbone atoms of amino acids constituting the specificity pocket are shown. The side chains are displayed only at sites 189, 218, 219, 226 and at the substrate. The van der Waals surfaces are indicated on the methyl group of Ala226, the CP3 atom of phenylalanyl and CZ3 atom of tryptophanyl substrate. On showing the van der Waals surfaces the MIDAS software's default radii were applied. The calculation method assumes that no explicit hydrogens are present in the models [25]. Surface residues 218 and 219 are blue, Ser 189 pointing towards the substrate is red, the Cys 191–220 disulphide bridge is yellow and the substrates are magenta.

preferred P1 site residue for the enzyme [34]. In contrast, human elastase 2a that contains Gly216 and Ser226 (Fig. 4) cleaves peptide bonds formed by larger hydrophobic, aliphatic and aromatic residues but not tryptophan [35]. This similarity of the specificity profile of elastase 2a to that of rat and bovine chymotrypsin B suggests that the S1 substrate binding pockets of these enzymes must also be similar. It follows from the size difference between the side chains of alanine and serine at sequence position 226 of chymotrypsin B and elastase 2 that the latter proteinase may have a shallower specificity pocket.

Our result, that an Ala→Gly substitution at site 226 converted the specificity of a B-type chymotrypsin into that of an A-type one, shows that the amino acid at position 226 is not only one of the determinants of chymotrypsin or elastase-type specificity, but it is also responsible for the specificity difference of chymotrypsin isoenzymes. Furthermore, amino acids at site 226 were shown to play important roles in the specificity of crab collagenolytic serine proteinase 1 [36,37] and mouse granzyme B [38]. An Asp226Gly substitution in crab collagenase, a serine proteinase with a broad specificity profile, reduced by about two orders of magnitude the trypsin-like activity of the enzyme with a full retention of the chymotrypsin-like one [36,37]. In mouse granzyme B the arginine residue at site 208 (this position in granzyme B is analogous to site 226 in chymotrypsin) proved to play a key role in specificity toward P1 Asp substrates [38].

Conventional distinction of chymotrypsin A and B has so far been made on the basis of overall sequence comparison of the different variants. Rat chymotrypsin was named as chymotrypsin B as its structure appeared to be more homologous to bovine chymotrypsin B than to bovine chymotrypsin A. In this case, as our present study shows, sequence homology coincides with the unique substrate specificities of bovine chymotrypsin B and rat chymotrypsin. Discrimination against tryptophanyl substrates, however, appears to be determined by a single amino acid residue at position 226. Based on this criterion, human chymotrypsin that contains glycine at sequence position 226 [39] should have an A-type rather than B-type substrate specificity profile. However, based on its sequence homology with bovine chymotrypsin B, more extended than with the A-type enzyme, it was originally classified as chymotrypsin B [39]. To avoid further confusion, we are proposing here that the specificity profile of the newly discovered forms of chymotrypsin rather than the overall sequence homology to any known variants should be the basis of classification: chymotrypsins with comparable activity on phenylalanyl, tyrosyl and tryptophanyl substrates would belong to group A, while chymotrypsins with relatively low activity on tryptophanyl substrates would be classified as B-type enzymes.

REFERENCES

1. Kunitz, M. & Northrop, J.H. (1935) Crystalline chymotrypsin and chymotrypsinogen. I. Isolation, crystallisation and general properties of a new proteolytic enzyme and its precursor. *J. Gen. Physiol.* **18**, 433–441.
2. Bergmann, M. & Fruton, G.W. (1937) On proteolytic enzymes. XIII. Synthetic substrates for chymotrypsin. *J. Biol. Chem.* **118**, 405–415.
3. Fruton, G.W. & Bergmann, M. (1942) The multiple specificity of chymotrypsin. *J. Biol. Chem.* **145**, 253–265.
4. Keith, C.K., Kazenko, A. & Laskowski, M. (1947) Studies on proteolytic activity of crystalline protein B prepared from beef pancreas. *J. Biol. Chem.* **170**, 227–238.
5. Brown, K.D., Shupe, R.E. & Laskowski, M. (1948) Crystalline activated protein B (chymotrypsin B). *J. Biol. Chem.* **173**, 99–108.
6. Fruton, J.S. (1948) Specificity of chymotrypsin B. *J. Biol. Chem.* **173**, 109–110.
7. Smith, E.L., Brown, D.M. & Laskowski, M. (1951) Molecular weights of chymotrypsinogen and chymotrypsin α and B. *J. Biol. Chem.* **191**, 639–650.
8. Kubacki, V., Brown, K.D. & Laskowski, M. (1949) Electrophoresis and solubility of chymotrypsinogen B and chymotrypsin B. *J. Biol. Chem.* **180**, 73–78.
9. Hirs, C.H.W. (1953) A chromatographic investigation of chymotrypsinogen α. *J. Biol. Chem.* **205**, 93–99.
10. Wu, F., C. & Laskowski, M. (1955) Action of the naturally occurring trypsin inhibitors against chymotrypsin α and B. *J. Biol. Chem.* **213**, 609–619.
11. Schellenberger, V., Braune, K., Hoffman, H.-J. & Jakubke, H.-D. (1991) The specificity of chymotrypsin. A statistical analysis of hydrolysis data. *Eur. J. Biochem.* **199**, 623–636.
12. Keller, P.J., Cohen, E. & Neurath, H. (1958) The proteins of bovine pancreatic juice. *J. Biol. Chem.* **233**, 344–349.
13. Folk, J.E. & Schirmer, W.E. (1965) Chymotrypsin C. I. Isolation of the zymogen and the active enzyme: preliminary structure and specificity studies. *J. Biol. Chem.* **240**, 181–192.
14. Reseland, J.E., Larsen, F., Solheim, J., Eriksen, J.A., Hanssen, L.E. & Prydz, H. (1997) A novel human chymotrypsin-like digestive enzyme. *J. Biol. Chem.* **272**, 8099–8104.
15. Hartley, B.S. (1964) Amino-acid sequence of bovine chymotrypsinogen A. *Nature* **201**, 1284–1291.
16. Smillie, L.B., Furka, A., Nagabhushan, N., Stevenson, K.J. & Parkes, C.O. (1968) Structure of chymotrypsinogen B compared with chymotrypsinogen A and trypsin. *Nature* **218**, 343–346.
17. Venekei, I., Szilágyi, L., Gráf, L. & Rutter, W.J. (1996) Attempts to convert chymotrypsin to trypsin. *FEBS Lett.* **379**, 143–147.
18. Bell, G.I., Qiunto, C., Quiroga, M., Valenzuela, P., Craik, C.S. & Rutter, W.J. (1984) Isolation of a sequence of a rat chymotrypsin B gene. *J. Biol. Chem.* **259**, 14265–14270.
19. Laskowski, M. (1955) Chymotrypsinogens and chymotrypsins. *Methods Enzymol.* **II**, 8–26.
20. Venekei, I., Gráf, L. & Rutter, W.J. (1996) Expression of rat chymotrypsinogen in yeast: a study on the structural and functional significance of the chymotrypsinogen propeptide. *FEBS Lett.* **379**, 139–142.
21. Kunkel, T.A. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl Acad. Sci. USA* **82**, 488–492.
22. Jameson, G.W., Roberts, D.V., Adams, D.V., Kyle, W.S. & Elmore, D.T. (1973) Determination of the operational molarity of solutions of bovine alpha-chymotrypsin, trypsin, thrombin and factor Xa by spectrofluorimetric titration. *Biochem. J.* **131**, 107–117.
23. Ferrin, T.E., Huang, C.C., Jarvis, L.E. & Langridge, R. (1988) The MIDAS display system. *J. Mol. Graphics* **6**, 13–27.
24. Huang, C.C., Pettersen, E.F., Klein, T.E., Ferrin, T.E. & Langridge, R. (1991) Conic: a fast renderer for space-filling molecules with shadows. *J. Mol. Graphics* **9**, 230–236.
25. Bash, P.A., Pattabiraman, N., Huang, C.C., Ferrin, T.E. & ad Langridge, R. (1983) Van der Waals surfaces in molecular modelling: implementation with real-time computer graphics. *Science* **222**, 1325–1327.
26. Brady, K., Wei, A., Ringe, D. & Abeles, R.H. (1990) Structure of chymotrypsin-trifluoromethyl ketone inhibitor complexes. Comparison of slowly and rapidly equilibrating inhibitors. *Biochemistry* **29**, 7600–7607.
27. Harel, M., Su, C.T., Frolow, F., Silman, I. & Sussman, J.L. (1991) Gamma-chymotrypsin is a complex of alpha-chymotrypsin with its own autolysis products. *Biochemistry* **30**, 5217–5225.
28. Marquart, M., Walter, J., Deisenhofer, J., Bode, W. & Huber, R. (1983) The geometry of the reactive site and of the peptide groups in trypsin, trypsinogen and its complexes with inhibitors. *Acta Crystallogr. Sect. B* **39**, 480–484.
29. Takahashi, L.H., Radhakrishnan, R., Rosenfield Junior, R.E., Meyer Junior, E.F. & Trainor, D.A. (1989) Crystal structure of the covalent complex formed by a peptidyl alpha,alpha-difluoro-beta-keto amide with porcine pancreatic elastase at 1.78-angstroms resolution. *J. Am. Chem. Soc.* **111**, 3368–3381.
30. Steitz, T.A., Henderson, R. & Blow, D.M. (1969) Structure of

crystalline α-chymotrypsin. 3. Crystallographic studies of substrates and inhibitors bound to the active site of alpha-chymotrypsin. *J. Mol. Biol.* **46**, 337–348.

31. Bode, W. & Schwager, P. (1975) The refined crystal structure of bovine β-trypsin at 1.8 Å resolution. II. Crystallographic refinement. Calcium binding site, benzamidine binding site and active site at pH 7.0. *J. Mol. Biol.* **98**, 693–717.
32. Schotton, D.M. & Watson (1970) Three-dimensional structure of tosyl-elastase. *Nature* **225**, 811–816.
33. Perona, J.J. & Craik, C.S. (1995) Structural basis of substrate specificity in the serine proteases. *Protein Sci.* **4**, 337–360.
34. Harper, J.W., Cook, R.R., Roberts, C.J., Mclaughlin, B.J. & Powers, J.C. (1984) Active site mapping of the serine proteases, human leukocyte elastase, cathepsin G, porcine pancreatic elastase, rat mast cell proteases I and II, bovine chymotrypsin A alpha and *Staphylococcus aureus* protease V-8 using tripeptide thiobenzyl ester substrates. *Biochemistry* **23**, 2995–3002.
35. Del Mar, E.G., Largman, C., Brodrick, J.W., Fassett, M. & Geokas, M.C. (1980) Substrate specificity of human pancreatic elastase 2. *Biochemistry* **19**, 468–472.
36. Tsu, C.A., Perona, J.J., Schellenberger, V., Truck, C.W. & Craik, C.S. (1994) The substrate specificity of *Uca pugilator* collagenolytic serine proteinase 1 correlates with the bovine type 1 collagen cleavage sites. *J. Biol. Chem.* **269**, 19565–19572.
37. Tsu, C.A., Perona, J.J., Fletterick, R.J. & Craik, C.S. (1997) Structural basis for the broad substrate specificity of fiddler crab collagenolytic serine protease 1. *Biochemistry* **36**, 5393–5401.
38. Caputo, A., James, M.N.G., Powers, J.C., Hudig, D. & Bleackley, R.C. (1994) Conversion of the substrate specificity of mouse proteinase granzyme B. *Nat. Struct. Biol.* **1**, 364–367.
39. Tomita, N., Izumoto, Y., Horii, A., Doi, S., Yokouchi, H., Ogawa, M., Mori, T. & Matsubara, K. (1989) Molecular cloning and nucleotide sequence of human pancreatic prechymotrypsinogen cDNA. *Biochem. Biophys. Res. Commun.* **158**, 569–575.