ISSN 0006-2979, Biochemistry (Moscow), 2007, Vol. 72, No. 4, pp. 416-423. © Pleiades Publishing, Ltd., 2007.
Original Russian Text © V. Kh. Akparov, A. M. Grishin, M. P. Yusupova, N. M. Ivanova, G. G. Chestukhina, 2007, published in Biokhimiya, 2007, Vol. 72, No. 4, pp. 515-524.

Structural Principles of the Wide Substrate Specificity of *Thermoactinomyces vulgaris* Carboxypeptidase T. Reconstruction of the Carboxypeptidase B Primary Specificity Pocket

V. Kh. Akparov*, A. M. Grishin, M. P. Yusupova, N. M. Ivanova, and G. G. Chestukhina

Stepanov Laboratory of Protein Chemistry, Institute of Genetics and Selection of Industrial Microorganisms, 1-yi Dorozhnyi Proezd 1, 117545 Moscow, Russia; fax: (495) 315-0501; E-mail: vhar@ostrov.net

Received September 21, 2006
Revision received December 5, 2006

Abstract—Site-directed mutagenesis in the active site of *Thermoactinomyces vulgaris* carboxypeptidase T (CpT), which is capable of hydrolyzing both hydrophobic and positively charged substrates, resulted in five mutants: CpT1 (A243G), CpT2 (D253G/T255D), CpT3 (A243G/D253G/T255D), CpT4 (G207S/A243G/D253G/T255D), and CpT5 (G207S/A243G/T250A/D253G/T255D). These mutants step-by-step reconstruct the primary specificity pocket of carboxypeptidase B (CpB), which is capable of cleaving only positively charged C-terminal residues. All of the mutants retained the substrate specificity of the wild-type CpT. Based on comparison of three-dimensional structures of CpB and the CpT5 model, it was suggested that the lower affinity of CpT5 for positively charged substrates than the affinity of CpB could be caused by differences in nature and spatial location of Leu247 and Ile247 and of His68 and Asp65 residues in CpT and CpB, respectively, and also in location of the water molecule bound with Ala250. An additional hydrophobic region was detected in the CpT active site formed by Tyr248, Leu247, Leu203, Ala243, CH_3-group of Thr250, and CO-groups of Tyr248 and Ala243, which could be responsible for binding hydrophobic substrates. Thus, notwithstanding the considerable structural similarity of CpT and pancreatic carboxypeptidases, the mechanisms underlying their substrate specificities are different.

DOI: 10.1134/S0006297907040086

Key words: carboxypeptidase T, protein engineering, substrate specificity, site-directed mutagenesis

The family of Zn-containing metallocarboxypeptidases [1] includes two main subfamilies: digestive and regulatory carboxypeptidases. The first subfamily consists of carboxypeptidase A (CpA) [2], carboxypeptidase B (CpB) [3], and a Glu-specific carboxypeptidase from the corn earworm (HaCA42) [4]. The second subfamily includes carboxypeptidase D (CpD) [5].

Among a few known prokaryotic carboxypeptidases [1], carboxypeptidase T (CpT) from *Thermoactinomyces vulgaris* [6], which is under study in this laboratory, is the best investigated. The primary [7] and tertiary structures [8] of the enzyme have been established. Based on homology of the amino acid sequence and similarity of the three-dimensional structure, this enzyme has been assigned to the same subfamily as CpA and CpB [9]. The structures of the active site and polypeptide backbone surrounding the primary specificity pocket are conservative in CpA, CpB, CpT, and CpD [2, 3, 5, 8]. CpT has wide substrate specificity: the enzyme can cleave C-terminal hydrophobic amino acid residues like CpA and C-terminal positively charged residues like CpB.

Structural studies [2, 3, 5] and gene engineering data [10-12] have shown that the diverse primary specificity of metallocarboxypeptidases is determined by interactions of the substrate C-terminal residue side chain with residues 203, 207, 243, 250, 253, 255, and 268 of the primary specificity pocket (S1′-subsite of the binding zone) of the enzyme (here and further the numbering is after CpA except in "Materials and Methods", where it is given after CpT) (Table 1).

Abbreviations: CHAPS) 3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate; Cp) carboxypeptidase; DnpAAR) 2,4-dinitrophenyl-alanyl-alanyl-arginine; DTT) dithiothreitol; IPTG) isopropyl-β-D-thiogalactoside; RMSD) root mean square deviation; ZAAL) benzyloxycarbonyl-alanyl-alanyl-leucine; ZAALpNA) benzyloxycarbonyl-alanyl-alanyl-leucyl-*p*-nitro-anilide.

* To whom correspondence should be addressed.

The activity of CpA with substrates having a C-terminal hydrophobic residue is due to the presence in the S1′-subsite of two ramified hydrophobic radicals: Ile243 and Ile255 [2]. On the bottom of the primary specificity pocket CpB has a negative charge, which belongs to Asp255 and forms an ionic bond with the C-terminal positively charged residue of a substrate [3]. The functional role of Asp255 was confirmed by its substitution in the structure of human pancreatic CpB by Lys and Arg, which imparted the enzyme the ability to preferentially cleave C-terminal Asp and Glu [10]. There are similar observations in nature: thus, Arg255 was found in the structure of the Glu-specific carboxypeptidase from the corn earworm [4]. The ability of regulatory CpD to cleave C-terminal Arg and Lys is thought to be associated with the presence of negatively charged Asp207 in the binding zone [5].

The ability of CpT to hydrolyze substrates with C-terminal Arg and Lys is explained by presence of Asp253 in the primary specificity pocket. The Asp253 side chain forms in CpT a hydrogen bond with the OH-group of Thr255, and this was thought [8] to facilitate the desolvation of Asp253 upon the binding by CpT of hydrophobic substrate and to be responsible for the high activity of this enzyme with hydrophobic substrates.

The displaced position 253 of the aspartic acid residue in the CpT primary specificity pocket as compared to Asp255 in CpB was thought to be associated with the low affinity of CpT for positively charged substrates [8]. However, the placement of this residue into the 255 position in the CpT mutants Thr255Asp and Asp253Ser Thr255Asp (Table 1) did not change the affinity of the enzyme for positively charged substrates. The affinity for hydrophobic substrates decreased 40-fold in the first mutant and unchanged in the second [13].

To determine residues responsible for the CpA- and CpB-like specificity of CpT, we created in the present work a series of CpT mutants (CpT1-CpT5) with the residues in the CpT primary specificity pocket substituted by similar residues from the CpB S1′-subsite (Table 1). We substituted all five residues in the CpT S1′-subsite by the residues specific for CpB and thus reconstructed in CpT the binding zone of CpB and expected to obtain an enzyme with CpB-like specificity.

MATERIALS AND METHODS

Materials. Restriction endonucleases, T4 DNA ligase, Taq DNA polymerase, and standard primers (T7 promoter and T7 terminator) were from MBI Fermentas (Lithuania); the pET23a vector was from Novagen (USA); primers encoding the mutations introduced into the gene Mut fd and Mut rev were produced by Syntol (Russia). The *E. coli* strains BL21(DE3)pLysS and BMH 71-18 (*mut*S::Tn10, [F′lacZΔM15]) were obtained from Promega (USA). The initial construction pET-proCPTwt

Table 1. Amino acid residues determining the substrate specificity of metallocarboxypeptidases and mutations introduced in the primary specificity pocket of CpT*

Numbering after CpA	Amino acid residue						
	203	207	243	250	253	255	268
CpA	Leu	Gly	**Ile**	Ala	Gly	**Ile**	Thr
CpB	Leu	Ser	Gly	Ala	Gly	**Asp**	Thr
HaCA42	Leu	Gly	Ala	Thr	Gly	**Arg**	Thr
CpD, domain 2	Asn	**Asp**	Gly	Val	Gly	Gln	Thr
CpT	Leu	Gly	Ala	Thr	**Asp**	Thr	Thr
CpT T255D [13]						**Asp**	
CpT D253S T255D [13]					**Ser**	**Asp**	
CpT1			**Gly**				
CpT2					**Gly**	**Asp**	
CpT3			**Gly**		**Gly**	**Asp**	
CpT4		**Ser**	**Gly**		**Gly**	**Asp**	
CpT5		**Ser**	**Gly**	**Ala**	**Gly**	**Asp**	
Numbering after CpT	211	215	251	257	260	262	275

* Amino acid residues playing a major role in determination of the substrate specificity of the enzymes [1, 2, 9] and mutations introduced in the primary specificity pocket of CpT are in bold.

encodes the wild-type carboxypeptidase T proenzyme in the pET23a vector. The sequence of the full-size gene of CpT from *T. vulgaris* was deposited in GeneBank EBI/EMBL (accession number X56901). A Superdex 75 column for molecular exclusion chromatography and SP-Sephadex G-25 cation-exchange resin were from Pharmacia LKB Biotechnology (Sweden); cells and membranes for ultrafiltration were from Amicon (USA). Peptide substrates for kinetic studies were synthesized in this laboratory [14].

Preparation of mutant variants of the *pro-cpT* gene. Mutant variants of the *pro-cpT* gene were prepared by a two-step scheme of directed PCR-mutagenesis using standard primers T7 promoter/T7 terminator, and primers Mut fd and Mut rev, the nucleotide sequence of which was complementary to the gene *pro-cpT* except for sites of introduced mutations. In these sites the nucleotide sequence of primers encoded the desired amino acid replacement. Primers Mut fd and Mut rev were completely complementary to each other. Two PCR amplifications were done in the first step of PCR mutagenesis. The 5′-part of the gene was amplified with a pair of primers T7 promoter/Mut rev at the first one. The 3′-part of the gene was generated with a pair of primers T7 terminator/Mut fd at the second one. In the second step of PCR mutagenesis the two parts of the gene were hybridized with each other due to the complementarity of the Mut primers. The 3′-ends served as primers for completion of the gene. The latter was further amplified with T7 promoter/T7 terminator primers. The mutant variants of the gene obtained in this manner were cloned into the pET23a vector [16]. In the mutant CpT1 (Table 1), the codon GCG of Ala251 (numbering after CpT) was substituted by the codon GGG of Gly. In the mutant CpT2, the codon GAT of Asp260 was substituted by the codon GGA of Gly and the codon ACC of Thr262 was substituted by the codon GAT of Asp. The mutant CpT3 was a combination of the CpT1 and CpT2 mutations. The mutant CpT4, in addition to the mutations of CpT3, had the codon GGC of Gly215 substituted by the AGC codon of Ser. The mutant CpT5, in addition to the mutations of CpT4, had the codon ACC of Thr257 substituted by the codon GCC of Ala. The presence of mutations was confirmed by gene sequencing.

Expression of the CpT proenzyme in *E. coli* as inclusion bodies and the subsequent *in vitro* renaturation of the enzyme. The mutant variants of the *pro-cpT* gene were expressed in the *E. coli* BL21(DE3)pLysS cells according to instructions of the producer Novagen [17]. After the IPTG-induced expression, the cells were precipitated and then broken by ultrasonication. Native CpT was isolated from inclusion bodies as described in [18]. The inclusion bodies were precipitated by centrifugation and washed in 0.05% CHAPS, 4 M NaCl, and water. The precipitate containing the inclusion bodies was dissolved in 8 M urea containing 10 mM dithiothreitol (DTT) to the final protein concentration of 0.5-1.0 mg/ml. Then the protein solution was rapidly diluted 100-fold in 50 mM Tris-HCl (pH 7.5) supplemented with 30% glycerol (v/v, here and further percentages are expressed in volumetric ratio), 0.5 M NaCl, 2 mM DTT, and 10 mM $CaCl_2$, incubated for 24 h, and the non-renatured protein was removed by centrifugation. The protein solution was diluted twofold in 50 mM Tris-HCl (pH 7.5) supplemented with 0.5 M NaCl and 10 mM $CaCl_2$ and concentrated by ultrafiltration through a YM10 membrane to the volume of 6-10 ml. For activation, the solution of proCpT was supplemented with subtilisin 72 at the molar ratio of 1 : 100 and incubated for 30 min at 37°C. The subtilisin was inactivated by addition of diisopropyl fluorophosphate. The solution containing the activated CpT was reconcentrated by ultrafiltration to the volume of 0.5 ml and centrifuged. The activated CpT was purified by molecular exclusion chromatography in an FPLC system on a Superdex TM 75 HR 10/30 column pre-equilibrated with 50 mM Tris-HCl (pH 7.5) containing 10% glycerol, 0.5 M NaCl, and 10 mM $CaCl_2$.

The protein concentration was determined using Bradford's method [19] and by absorption at 280 nm, using $\varepsilon_{280} = 64{,}000\ M^{-1}\cdot cm^{-1}$ for CpT.

SDS-polyacrylamide gel electrophoresis was performed by the Laemmli method [20].

Investigation of enzymatic properties of the CpT mutants. The enzymatic activities of CpT and its mutants were characterized by ability to cleave the arginine residue from 2,4-dinitrophenyl-alanyl-alanyl-arginine (DnpAAR) and leucine from benzyloxycarbonyl-alanyl-alanyl-leucine (ZAAL). Hydrolysis of DnpAAR was performed at 37°C in the presence of 50 mM Tris-HCl (pH 7.5) containing 10 mM $CaCl_2$, 0.35-3.4 mM substrate, and approximately 10 nM CpT. The reaction was stopped by addition of 40 μl of 50% CH_3COOH. The reaction product DnpAA was separated from the charged substrate on SP-Sephadex G-25, and its amount was determined spectrophotometrically ($\varepsilon_{360} = 15{,}000\ M^{-1}\cdot cm^{-1}$). Hydrolysis of ZAAL was performed at 25°C in a cuvette of the spectrophotometer in the presence of 50 mM Tris-HCl (pH 7.5) supplemented with 10 mM $CaCl_2$, 40-200 μM substrate, and 10 nM CpT. The observed decrease in the optical density of the reaction mixture at the wavelength of 225 nm was recalculated with regard to $\varepsilon_{225} = 376\ M^{-1}\cdot cm^{-1}$. The initial rates of the reaction were measured at five concentrations of the substrate in the above-mentioned range. For each concentration of the substrate, the initial rates were measured 3-6 times. Kinetic data were processed by nonlinear and linear regression with the Origin v.6.1 program (www.originlab.com) with regard to all data on the initial rates of the reactions.

Comparison of the CpB and CpT structures. To compare the primary specificity pockets of CpB and CpT, the structure was chosen of the covalent CpB complex with the arginine analog 5-{[amino(imino)methyl]amino}-2-(thiomethyl)pentanoic acid (PBD code 1ZG9) [3] and the activated CpT structure without inhibitor (PDB code

1OBR) [8]. The model of the CpT5 structure was created by substituting the residues Gly215Ser, Ala251Gly, Thr257Ala, Asp260Gly, and Thr262Asp in the CpT structure 1OBR choosing the conformations of these residues closest to those in CpB (according to 1ZG9), and transporting the arginine analog from CpB into CpT5. The CpB and CpT5 structures were superimposed, and the residues were chosen located at the distance of 10 Å or less from the inhibitor and resuperimposed. Among these residues, those were chosen which had deviation lesser than the mean and superimposed again by these residues. Such a superposition was used for further calculations. To analyze electrostatic interactions, the residues were considered located at the distance of 22 Å from the ligand. To analyze the distribution of donors and acceptors of hydrogen bonds, the distance of 7 Å from the ligand was chosen. All these procedures, as well as rotation of side chains of amino acids in the protein, were performed using the Swiss PDB Viewer program [21]. To visualize the molecules and make illustrations, the VMD program was used [22].

Electrostatic interactions were calculated by Coulomb's formula:

$$E = q1 \cdot q2/\varepsilon r, \quad (1)$$

where $q1$ and $q2$ are ion charges, ε is the dielectric permeability of the medium (in all cases taken as 40 [23, 24]), and r is the distance between the ions. Considering the influence of His68 of CpT, two molecules of water were also introduced in the calculation as simple dipoles presumably oriented by the His68 field [23]. Augmentation of His68 potential (Δφ) with each water molecule at the place of substrate guanidino group was calculated by the formula:

$$\Delta\varphi = +e/(r - 0.3) + -e/(r + 0.3), \quad (2)$$

where +e and –e denoted the positive and negative charges in the dipole (equal to 0.66 of the elementary charge) and r is the distance from the dipole center to the point for which the potential is calculated.

Energy of Van der Waals interactions was calculated using the Lennard–Jones potential:

$$U(r) = E_0[(r_0/r)^{12} - 2(r_0/r)^6], \quad (3)$$

where for the CH_2-group $E_0 = 2$ kJ/mol and $r_0 = 4$ Å [23].

RESULTS

The mutant genes of CpT1-CpT5 (Table 1) were prepared by site-directed mutagenesis and then expressed in *E. coli* producing the procarboxypeptidases as inclusion bodies. The renatured proteins were activated with subtilisin and purified by molecular exclusion chromatography (in an FPLC system). The purity of the carboxypeptidase T mutants was proved by SDS-PAGE. The renaturation yield was ≈3%. The absence of subtilisin activity was confirmed using the specific chromogenic substrate ZAALpNA (benzyloxycarbonyl-alanyl-alanyl-leucyl-*p*-nitroanilide).

The successive substitution of five residues in the CpT S1′-subsite by similar residues of CpB resulted in a decrease in the enzyme activity with a positively charged substrate DnpAAR (Table 2). The K_m values of the mutants CpT1 and CpT2 were comparable with that of wild-type CpT, but for CpT3, CpT4, and CpT5 they were higher. The specificity constant k_{cat}/K_m of DnpAAR hydrolysis by CpT1-CpT5 was lower than that of wild-type enzyme. The activity of the CpT5 mutant with the structure closest to the primary structure of CpB was 2.8-fold lower than the activity of CpT.

Introduction of five mutations resulted in increase in both K_m and k_{cat} of hydrolysis of the hydrophobic substrate ZAAL, but the specificity constant k_{cat}/K_m decreased only 1.6-fold. The k_{cat}/K_m values for CpT and CpT5 differed insignificantly; therefore, the activity of CpT5 with ZAAL did not change. Thus, the selectivity of the mutant CpT5 with substrates carrying a C-terminal positively charged amino acid decreased 1.6-fold compared to substrates with a C-terminal hydrophobic amino acid.

Values of k_{cat}/K_m allowed the changes in the binding energy of the transient state to be calculated under the influence of mutations introduced into the enzyme [25, 26]. The binding energy of DnpAAR to CpT5 compared to CpT decreased by 2.6 kJ/mol that could correspond to disappearance of one hydrogen bond (2-6.2 kJ/mol) or appearance of a weakened hydrogen bond (2 kJ/mol) instead of an optimal one [25, 26].

The difference in the energy of DnpAAR binding between CpB and CpT5 was 15.3 kJ/mol. Such a high difference seemed to be associated with the presence in CpB of additional interactions with the substrate compared to CpT. This value could correspond to removal of a component of ionic bond (12-20 kJ/mol) [25, 27], some methylene groups (4-8.3 kJ/mol per CH_2-group) [25, 27], two-three hydrogen bonds between uncharged groups, or one hydrogen bond between charged and uncharged groups of the protein and ligand [26].

DISCUSSION

The reconstruction of the primary specificity pocket of CpT to make it like CpB neither enhanced the CpT5 activity with a substrate possessing C-terminal Arg, nor lowered the activity with a substrate carrying C-terminal Leu. The binding energy of the mutant CpT5 for DnpAAR was decreased compared to that of the wild-type CpT, and this could be caused by removal of the OH-group by the Thr257Ala substitution. The binding energy

Table 2. Kinetic constants of DnpAAR and ZAAL hydrolysis by wild-type carboxypeptidase T and its mutants

Enzyme	DnpAAR			ZAAL			Selectivity $[k_{cat}/K_m]_{Leu}/[k_{cat}/K_m]_{Arg}$
	K_m, mM	k_{cat}, sec^{-1}	k_{cat}/K_m (10^{-3}), $sec^{-1}\cdot M^{-1}$	K_m, mM	k_{cat}, sec^{-1}	k_{cat}/K_m (10^{-3}), $sec^{-1}\cdot M^{-1}$	
CpA [13]	—	—	—	0.012	35	2917	
CpB [13]	0.07	35	500	—	—	—	
CpT[a]	4.4 ± 1.3	14.6 ± 3	3.6 ± 0.6[e]	0.046 ± 0.003[e]	10.3 ± 2.1[e]	230 ± 65	64 ± 21
CpT1	3.6	2	0.55	0.02	6.7	335	609
CpT2	3.6	4.3	1.2	0.04	28	700	583
CpT3[b]	—	—	0.84	0.03	11.1	370	440
CpT4[b]	—	—	0.15	0.09	6.8	76	500
CpT5[b]	—	—	1.3 ± 0.28[e]	0.15 ± 0.01[e]	22.8 ± 3.6[e]	152 ± 21	117 ± 30
$\Delta\Delta G_{CpT5-CpT}$, kJ/mol[c]			2.6			0	
$\Delta\Delta G_{CpT5-CpB}$, kJ/mol[d]			15.3				

Note: –, no data.

[a] The procedure of renaturation, activation, purification, and measurement of kinetic parameters was performed thrice for CpT and CpT5 and once for CpT1-CpT4. For CpT and CpT5 mean values and standard errors of the mean are presented.

[b] No saturation with the DnpAAR substrate was observed for CpT3, CpT4, and CpT5 at the substrate concentrations of 1.8, 1.8, and 3.4 mM, respectively.

[c] Difference in the binding energy of the transient state between CpT5 and CpT: $\Delta\Delta G = -RT\ln[(k_{cat}/K_m)_{CpT5}/(k_{cat}/K_m)_{wt}]$.

[d] Difference in the binding energy of the transient state between CpT5 and CpB: $\Delta\Delta G = -RT\ln[(k_{cat}/K_m)_{CpT5}/(k_{cat}/K_m)_{CpB}]$.

[e] $p < 0.05$ by Student's t-test; values of CpT are taken as the control.

for ZAAL was not changed, and this indicated that these groups failed to interact with the substrate or the introduced mutations did not result in disappearance/appearance of contacts between the enzyme and substrate in the transition state.

The Asp253 side chain in CpT is exposed to the aqueous medium of the primary specificity pocket [8]; therefore, Asp253 more likely forms an ionic bond with the guanidino group of the substrate arginine. Placing Asp into the 255 position did not abolish the ionic bond and did not lead to desolvation of the guanidino group of the substrate arginine, because in this case the decrease in the binding energy would be >12 kJ/mol [25, 27] and not the 2.6 kJ/mol that we found.

To reveal structural differences between CpB and CpT5 that could explain such a significant difference in the specificities of these enzymes, we compared the structures of the CpB and CpT5 S1′-subsites. We substituted five residues discriminating the S1′-subsites of CpT and CpB (Table 1), chose the conformations of these residues the most like those in CpB [3], and, thus, modeled a structure of CpT5. The root mean square deviation (RMSD) of atoms in the polypeptide backbone of the CpT5 and CpB S1′-subsites was no more than 0.6 Å, with an error of 0.3 Å on determination of the atomic coordinates by X-ray crystallography. By analogy with CpB, an inhibitor imitating the C-terminal Arg of the substrate was introduced into CpT5. Changes in the CpB structure upon binding of the inhibitor, which forms a tight bond with zinc ion, were comparable with changes in CpA upon binding of the esterolysis product phenyl lactate, that does not form a covalent bond with the enzyme [28]. Thus, RMSD of atoms in the polypeptide backbone of residues located at the distance to 10 Å from the inhibitor were 0.45 Å for CpB and 0.3 Å for CpA. The comparison was performed with the known structures of proCpB and proCpA [29, 30]. It should be mentioned that the side chain conformation of the substrate arginine could be different from the conformation of the inhibitor (arginine analog), although it is generally considered that the inhibitor position in the active site of CpB is modeling the substrate position [3].

To compare location of charged amino acids in CpB and CpT5 and their roles in substrate binding, we chose the distance of 22 Å from the substrate Arg, because placing of the unit charge at this distance from the charge of arginine (at $\varepsilon = 40$) would only twofold change the binding energy. At this distance, CpB and CpT5 were different in location of approximately 30 charged residues, but the majority of

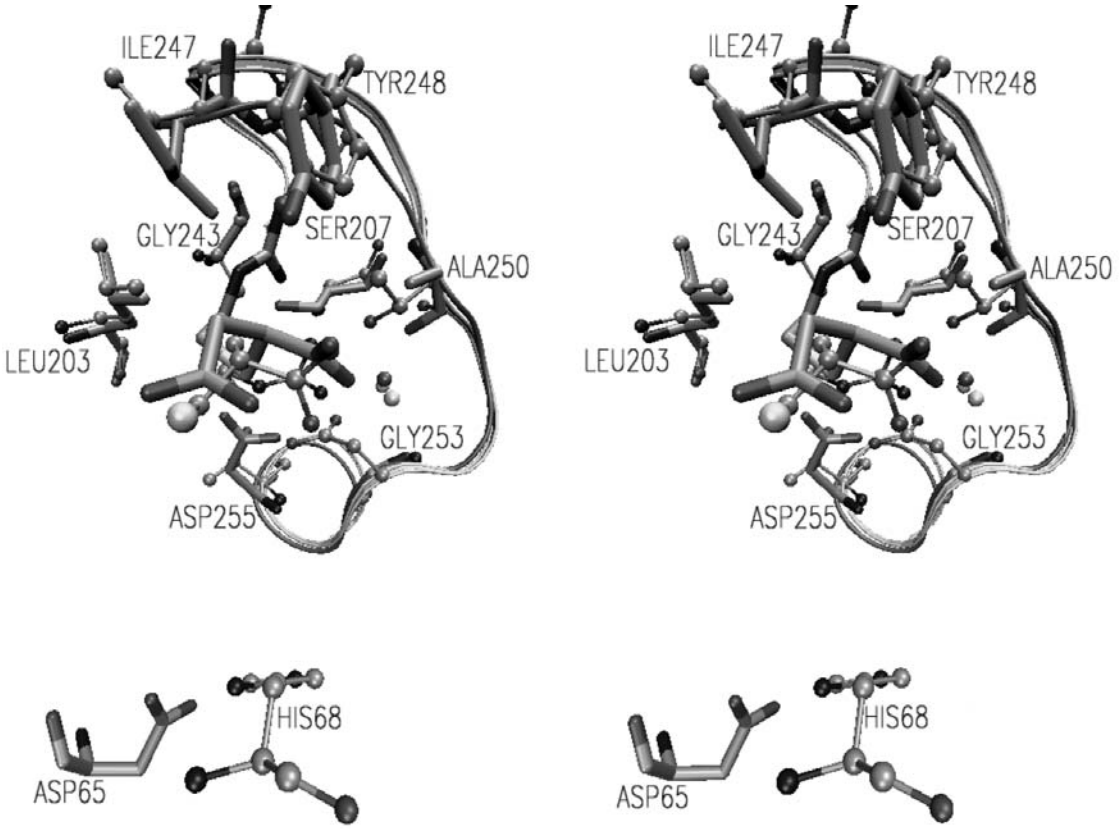
these residues was mainly located on the protein surface where their electrostatic field was significantly weakened in the presence of water and counter-ions, and only one charged residue was inside the protein molecule.

In CpT His68 can carry a positive charge (at the distance of 9.3 Å from the substrate guanidino group) and in CpB a negative charge (11.3 Å from the substrate guanidino group) is carried by Asp65. Both these residues are located inside the protein molecule (figure). Assuming that the carboxyl group of Asp and guanidino group of Arg located at the distance of 3 Å interact with the energy of 12.5 kJ/mol [23], the interaction of the His68 and Asp65 residues with the positively charged guanidino group of the ligand could decrease sevenfold the binding energy of CpT for DnpAAR and increase fourfold the binding energy of CpB for DnpAAR, and this seemed to be one of the factors determining the weak activity of CpT with positively charged substrates.

Considering the additivity of ionic interactions, it is impossible to assess the role of all charged groups in the weak binding by CpT of positively charged substrates without using methods of molecular modeling. The hydrophobic specificity of CpT seems to be unaffected by difference in the charge locations. Note that substitution of two distant negative charges by positive ones in the subtilisin molecule decreased 100-fold its specificity to a substrate with Arg in the P1 position and had no influence on the specificity to a substrate with Phe in P1 [31].

Comparison of the primary specificity pocket surface and polypeptide backbone of CpT5 and CpB [3, 8] reveals differences in the spatial location of amino acid residues Tyr248, Asp255, Asp256, Ala250, and Arg145, in the localization and nature of residue 247, which is Ile247 in CpB and Leu247 in CpT, and also in location of the structurally bound water molecules.

Tyr248 forms a hydrogen bond with the carboxyl group of the substrate C-terminal residue [2]. The hydrogen bond in CpT is 0.27 Å longer and reaches 3.37 Å, as compared to 3.1 Å in CpB. Weakening of the hydrogen bond makes the binding of both hydrophobic and positively charged substrates 2.5-fold weaker. However, this difference cannot explain why CpT and CpT5 display a strong binding of hydrophobic substrates and poorly bind positively charged substrates.



Principal differences in the primary specificity pocket structure of CpB and CpT. The hydrophilic and hydrophobic sites of the primary specificity pocket in CpT. The polypeptide backbone is shown as a narrow ribbon for CpB [3] and a wide ribbon for CpT [8]. Amino acid residues of CpB are shown as lines and labeled, and those of CpT are shown as balls. In the center of the figure the arginine analog 5-{[amino(imino)methyl]amino}-2-(thiomethyl)pentanoic acid is shown (as balls) from the complex with CpB [3] and guanidinoethylmercaptosuccinic acid (as lines) transferred from the complex with CpD [5]. Molecules of crystallization water are shown as light and dark balls in CpB and CpT, respectively

The C_α atom of Leu247 in CpT is 1.8 Å farther from the aliphatic moiety of the substrate arginine than the C_α atom of Ile247 in CpB because of a residue deletion in the Tyr248-containing loop in CpT. As a result, the nearest to the substrate methyl group of the Leu247 side chain in the conformation closest to the substrate is 1 Å farther from the nearest substrate methyl group compared to the same group of the Ile247 side chain in CpB (figure). Calculations of van der Waals interactions using the Lennard–Jones potential indicate that this would lower fivefold the binding energy in the case of substrate with C-terminal arginine.

The carboxyl groups of Asp255 and Asp256 in CpT5 are located 0.17 and 0.5 Å farther, respectively, from the guanidino group of the substrate arginine than in CpB. If the guanidino group position of arginine was unchanged, the substrate binding by the mutant CpT5 would be 22 and 32% weaker, respectively (at $\varepsilon = 40$). Such a difference cannot explain the difference in the specificities of CpT5 and CpB.

The carbonyl group of Ala250 in CpT5 is 1.2 Å farther from the ligand guanidino group than in CpB (3.9 and 5.1 Å in CpB and CpT5, respectively), but in both cases formation of a hydrogen bond is impossible (figure). Most likely, this difference does not cause the different specificities of CpT5 and CpB.

Arg145 is conservative in all metallocarboxypeptidases because it is involved in the binding of the substrate C-terminal residue carboxyl group [1, 2, 9]. The guanidino group of Arg145 in CpT is 0.4 Å nearer to the substrate carboxyl group than in CpB, and this would act as a barrier for penetration of the same charge substrate arginine into the primary specificity pocket. However, analysis of the environments of Arg145 in CpT and CpB revealed that around Arg145 in CpT there were no residue interactions, which could fix this conformation of the Arg145 side chain. The displacement of the Arg145 side chain in CpT compared to CpB seems to be caused by interaction with sulfate anion in the active site of the crystalline CpT used for X-ray crystallography [8] and is not responsible for the lower affinity than that of CpB for positively charged substrates.

Locations of the structurally bound water were analyzed in the primary specificity pockets of CpB and CpT, and, in addition to three conservative sites of hydration, CpB was found to have two sites absent in CpT, whereas CpT had two sites lacking in CpB. In CpB, the guanidino group of arginine forms three hydrogen bonds with two molecules of structurally bound water of the S1′-subsite, and only one of them is present in CpT (figure). The second water molecule can be present in the structure of CpT5; however, the Ala250 carbonyl group, which is one of three acceptors of the hydrogen bond, is 1.4 Å farther from its position in CpB, and this is likely to cause displacement of the water molecule. On the assumption that the energy of a non-optimal hydrogen bond is 2 kJ/mol [26], the weakening of two hydrogen bonds has to nearly fivefold weaken the binding by CpT5 of substrates with C-terminal Arg, and this can be a factor of the low activity of CpT5 with DnpAAR.

The nature and position in CpB and CpT5 of amino acid residues producing the second layer around the substrate were compared, and at the distance nearer than 10 Å from the substrate arginine CpT and CpB were found to differ in positions of residues 148-155, 191-193, 208, 209, 241, 242, 259, and 266 with deviations higher than the mean. The amino acid residues were also different: only 43 of 76 residues were identical in the two enzymes.

The role of the above-listed differences in determination of the CpT substrate specificity is unknown. Nevertheless, today there are many data indicating that the second layer residues, as well as more distant regions of the protein, can sometimes play not a lesser role in determination of the substrate specificity of enzymes than the residues directly forming the binding zone of the enzyme due to influence on the active site plasticity [32-34].

Thus, **the low activity of CpT with positively charged substrates** is determined by some differences in the CpT5 and CpB structures. The difference in nature and position of (possibly) charged His68 in CpT and Asp65 in CpB decreases sevenfold the binding by CpT of a substrate with the C-terminal arginine and increases fourfold the binding by CpB of an arginine-carrying substrate. The difference in nature of residue 247 (Leu247 in CpT and Ile247 in CpB) impairs fivefold the substrate binding by CpT, and a similar decrease in the binding is caused by another position of the structurally bound water molecule at Ala250. Because of the combined influence of these differences, CpT should bind the substrate with the C-terminal Arg 700-fold worse than CpB.

Our findings suggest that the poor activities of CpT and CpT5 with substrates possessing the C-terminal positively charged amino acid, as compared to the CpB activity, could be caused by differences in hydrogen bonds and in electrostatic and van der Waals interactions (figure).

Binding of hydrophobic substrates by CpT. No difference has been revealed in the composition of donors and acceptors of hydrogen bonds at the distance to 7 Å from arginine, and there are only insignificant differences in positions of the CO-group and water molecule at Ala250. Thus, the primary specificity pockets of CpT5 and CpB are similar in hydrophilicity. The hydrophilic area of CpT, surrounding the guanidino group of the arginine analog from the complex with CpB, composed with the side chains of Asp253, Thr255, OH-group of Thr250, is shown on the figure.

The hydrophobic zone of the CpT S1′-subsite interacting with the supposed C-terminal arginine of a substrate is not larger than this zone in CpB (the Ile203 residue, the Thr268 methyl group, and Ile247 in CpB – Leu247 in CpT); however, K_m for ZAAL of CpT is comparable with K_m for ZAAL of CpA (Table 2). How does a hydrophobic substrate bind with CpT?

It was supposed earlier that the CpT activity with hydrophobic substrates could be due to the easier exclusion of water from the contact with Asp253 by a hydrophobic radical because Asp253 forms a hydrogen bond with Thr255 [8]. However, in CpB Asp255 also forms a hydrogen bond with Thr268; nevertheless, this enzyme fails to considerably bind hydrophobic substrates [3].

In the three-dimensional structure of CpT, in addition to the binding site for positively charged substrates, a separate zone was found which seemed to be responsible for the binding of hydrophobic substrates. This zone is delimited by the side chains of Tyr248, Leu247, Leu203, Ala243, the CH_3-group of Thr250, and CO-groups of Tyr248 and Ala243 (figure) and has the dimensions 8.8 × 7.7 × 8.7 Å (length/width/height) sufficient for the accommodation of the leucine side chain. No such site is present in pancreatic carboxypeptidases; however, the regulatory carboxypeptidase D with spatial structure similar to that of CpT has in the same place a pocket for binding positively charged residues [5]. The resemblance of spatial structures of CpT and CpD in this case is based on the primary structure resemblance — both enzymes have a deletion in the Tyr248-containing loop.

Thus, notwithstanding the pronounced structural resemblance of CpA, CpB, and CpT (especially CpT5), mechanisms responsible for the substrate specificity of these enzymes are different in many respects. These mechanisms are provided with involvement of amino acid residues and water molecules, which were neglected earlier in the explanation of the substrate specificity of carboxypeptidases B and T.

The authors are grateful to E. I. Levitin, T. L. Voyushina, and A. M. Bushueva for their technical assistance during the work.

The work was supported in part by the Russian Foundation for Basic Research (project No. 02-04-48755).

REFERENCES

1. Barrett, A. J., Rawlings, N. D., and Woessner, J. F. (eds.) (1997) *Handbook of Proteolytic Enzymes*, Academic Press, Inc., pp. 1318-1350.
2. Christianson, D. W., and Lipscomb, W. N. (1989) *Acc. Chem. Res.*, **22**, 62-69.
3. Adler, M., Bryant, J., Buckman, B., Islam, I., Larsen, B., Finster, S., Kent, L., May, K., Mohan, R., Yuan, S., and Whitlow, M. (2005) *Biochemistry*, **44**, 9339-9347.
4. Bown, D. P., and Gatehouse, J. A. (2004) *Eur. J. Biochem.*, **271**, 2000-2011.
5. Aloy, P., Companys, V., Vendrell, J., Aviles, F., Fricker, L. D., Coll, M., and Gomis-Ruth, F. X. (2001) *J. Biol. Chem.*, **276**, 16177-16184.
6. Stepanov, V. M. (1995) in *Methods in Enzymology*, Vol. 248 (Barrett, A. J., ed.) Academic Press, Inc., pp. 675-683.
7. Smulevitch, S. V., Osterman, A. L., Galperina, O. V., Matz, M. V., Zagnitko, O. P., Kadyrov, R. M., Tsaplina, I. A., Grishin, N. V., Chestukhina, G. G., and Stepanov, V. M. (1991) *FEBS Lett.*, **291**, 75-78.
8. Teplyakov, A., Polyakov, K., Obmolova, G., Strokopytov, B., Kuranova, I., Osterman, A., Grishin, N., Smulevitch, S., Zagnitko, O., Galperina, O., Matz, M., and Stepanov, V. M. (1992) *Eur. J. Biochem.*, **208**, 281-288.
9. Osterman, A. L., Grishin, N. V., Smulevitch, S. V., Matz, M. V., Zagnitko, O. P., Revina, L. P., and Stepanov, V. M. (1992) *J. Protein Chem.*, **11**, 561-570.
10. Edge, M., Forder, C., Hennam, J., Lee, I., Tonge, D., Hardern, I., Fitton, J., Eckersley, K., East, S., Shufflebotham, A., Blakey, D., and Slater, A. (1998) *Protein Eng.*, **11**, 1229-1234.
11. Smith, G. K., Banks, S., Blumenkopf, T. A., Cory, M., Humphreys, J., Laethem, R. M., Miller, J., Moxham, C. P., Mullin, R., Ray, P. H., Walton, L. M., and Wolfe, L. A., III (1997) *J. Biol. Chem.*, **272**, 15804-15816.
12. Zhao, L., Buckman, B., Seto, M., Morser, J., and Nagashima, M. (2003) *J. Biol. Chem.*, **278**, 32359-32366.
13. Trachuk, L. A., Bushueva, A. M., Shevelev, A. B., Novgorodova, S. A., Akparov, V. Kh., and Chestukhina, G. G. (2002) *Vopr. Med. Khim.*, **48**, 577-580.
14. Yusupova, M. P., Kotlova, E. K., Timokhina E. A., and Stepanov, V. M. (1995) *Bioorg. Khim.*, **21**, 33-38.
15. Higuchi, R., Krummel, B., and Saiki, R. K. (1988) *Nucleic Acids Res.*, **16**, 7351-7367.
16. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*, Cold Spring Harbor Press, New York.
17. *Novagen pET System Manual TB055*, 7th Edn. (1997) Novagen Madison, W. I.
18. Trachuk, L., Letarov, A., Kudelina, I. A., Yusupova, M. P., and Chestukhina, G. G. (2005) *Protein Exp. Purif.*, **40**, 51-59.
19. Bradford, M. M. (1976) *Analyt. Biochem.*, **72**, 248-254.
20. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
21. Guex, N., and Peitsch, M. C. (1997) *Electrophoresis*, **18**, 2714-2723.
22. Humphrey, W., Dalke, A., and Schulten, K. (1996) *J. Molec. Graphics*, **14**, 33-38.
23. Finkelstein, A. V., and Ptitsyn, O. B. (2005) *Protein Physics* [in Russian], MGU Publishers, Moscow.
24. Loewenthal, R., Sancho, J., Reinikainen, T., and Fersht, A. R. (1993) *J. Mol. Biol.*, **232**, 574-583.
25. Fersht, A. R. (1999) *Structure and Mechanism in Protein Science*, Freeman, New York, p. 380.
26. Fersht, A. R., Shi, J. P., Knill-Jones, J., Lowe, D. M., Wilkinson, A. J., Blow, D. M., Brick, P., Carter, P., Waye, M. M., and Winter, G. (1985) *Nature*, **314**, 235-238.
27. Serrano, L., Kellis, J. T., Jr., Cann, P., Matouschek, A., and Fersht, A. R. (1992) *J. Mol. Biol.*, **224**, 783-804.
28. Teplyakov, A., Wilson, K. S., Orioli, P., and Mangani, S. (1993) *Acta Crystallogr., D: Biol. Crystallogr.*, **49**, 534-540.
29. Coll, M., Guasch, A., Aviles, F., and Huber, R. (1991) *EMBO J.*, **10**, 1-9.
30. Guasch, A., Coll, M., Aviles, F., and Huber, R. (1992) *J. Mol. Biol.*, **224**, 141-157.
31. Russell, A. J., and Fersht, A. R. (1987) *Nature*, **328**, 496-500.
32. Mace, J. E., Wilk, B. J., and Agard, D. A. (1995) *J. Mol. Biol.*, **251**, 116-134.
33. Hedstrom, L., Szilagyi, L., and Rutter, W. J. (1992) *Science*, **255**, 1249-1253.
34. Hedstrom, L., Perona, J. J., and Rutter, W. J. (1994) *Biochemistry*, **33**, 8757-8563.