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Leu254 Residue and Calcium Ions as New Structural Determinants of Carboxypeptidase T Substrate Specificity

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Abstract—New determinants of *Thermoactinomyces vulgaris* carboxypeptidase T (CPT) substrate specificity—structural calcium ions and Leu254 residue—were found by means of steady-state kinetics and site-directed mutagenesis. The removal of calcium ions shifted the selectivity profile of hydrolysis of tripeptide substrates with C-terminal Leu, Glu, and Arg from 64/1.7/1 to 162/1.3/1. Substitution of the hydrophobic Leu254 in CPT for polar Asn did not change hydrolysis efficiency of substrates with C-terminal Leu and Arg, but resulted in more than 28-fold decrease in activity towards the substrate with C-terminal Glu. It is shown that the His68 residue is not a structural determinant of CPT specificity.

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The main goal of structural enzymology is to study how the structure of enzymes determines their functions. *Thermoactinomyces vulgaris* metallocarboxypeptidase T (CPT) is one of the best models for this study [1]. This enzyme has a high space homology of the active site structure with pancreatic carboxypeptidases A (CPA) and B (CPB), which are among the best-studied enzymes [2, 3]. For these three enzymes, high-resolution X-ray structures have been determined [4-6]. The specificity of carboxypeptidase T is between those of carboxypeptidases A and B; it splits off hydrophobic as well as positively charged residues from the C-terminus of the substrate.

Earlier a wealth of experimental data on metallocarboxypeptidases (MCPs) was summarized into the theory of MCP substrate specificity, which can be briefly described as follows [4, 7-9]. Upon binding to a metallocarboxypeptidase the side chain of the C-terminal residue of the substrate enters a pocket called the S1′-subsite of the binding area (according to Schechter and Berger [10]) or the enzyme primary specificity pocket. Interaction between the seven residues lining the surface of the primary specificity pocket and the side chain of the C-terminal residue of the substrate determines the substrate specificity of MCP; these are residues 203, 207, 243, 250, 253, 255, and 268 (CPA numeration) [4, 7-9]. Residue 255 plays a key role in discrimination of hydrophobic and positively charged substrates [7].

CPA, possessing a hydrophobic S1′-subsite, in which Leu203, Ile243, Ala250, and Ile255 are incorporated (Table 1), splits off mainly hydrophobic C-terminal amino acid residues [2, 8]. CPB bears a negative charge at the bottom of the primary specificity pocket (Asp255) and hydrolyses substrates with C-terminal Arg and Lys [3, 11]. When Asp255 in CPB was replaced by Arg or Lys, the resulting mutants lost CPB-like specificity and gained the capacity to split off dicarboxylic amino acid residues from the C-terminus [12].

Study of CPT selectivity revealed contradictions with the classical theory of MCP substrate specificity. CPT possessing negatively charged Asp260 at the primary specificity pocket is able to split off C-terminal positively charged residues, though by two orders of magnitude slower than CPB does (Table 2). CPT exhibits the maxi-

Abbreviations: CPA) carboxypeptidase A; CPB) carboxypeptidase B; CPT) carboxypeptidase T; CHAPS) 3-[(3-cholamidopropyl)dimethylammonium]-1-propane sulfate; DnpAAR) 2,4-dinitrophenyl-alanyl-alanyl-arginine; MCP) metallocarboxypeptidase; ZAAL) benzyloxycarbonyl-alanyl-alanyl-leucine; ZAAE) benzyloxycarbonyl-alanyl-alanyl-glutamic acid; ZAALpNA) benzyloxycarbonyl-alanyl-alanyl-leucine *p*-nitroanilide.

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mal activity towards hydrophobic substrates, which are hydrolyzed by CPT only one order of magnitude slower than by CPA (Table 2), although only Ala250 and Leu211, the latter being conservative for all three MCPs, are the hydrophobic residues in the CPT primary specificity pocket. In spite of the negatively charged Asp260, CPT can split off Glu at the C-terminus more efficiently than Arg. For substrates with C-terminal Leu, Glu, and Arg, the profile of CPT substrate specificity is 64/1.7/1, respectively [13].

Neither the placement of Asp in the key for CPT 262 position [14] nor complete reconstruction of primary structure of CPT S1′-subsite by analogy with that of CPB [13] (CPT5 variant, Table 1) resulted in decreased CPT specificity towards hydrophobic substrates. Upon comparison of the 3D structure of CPB and molecular model of CPT5 [13], new structural differences were revealed in addition to the well-known seven residues of the primary specificity pocket; these new structural differences might explain such a great difference in selectivity of these enzymes.

In this work, we obtained corresponding CPT mutants and studied their kinetic properties.

MATERIALS AND METHODS

Materials. Reagents for gene engineering were from Fermentas (Lithuania); primers encoding mutations from Sintol (Russia); pet23a vector and *Escherichia coli* BL21(DE3)pLysS strain [15] from Novagen (USA). The initial pET-CPTwt construct contains the wild type procarboxypeptidase T *cpT* gene [16] having a T7 Tag instead of the prepeptide, cloned in pet23a vector via *Xho*I and *Eco*RI restriction endonucleases sites. Peptide substrates ZAALpNA (benzyloxycarbonyl-alanyl-alanyl-leucine *p*-nitroanilide) [17], DnpAAR (2,4-dinitrophenyl-alanyl-alanyl-arginine) [18], ZAAE (benzyloxycarbonyl-alanyl-alanyl-glutamic acid), and ZAAL (benzyloxycarbonyl-alanyl-alanyl-leucine) [19] were obtained earlier in our laboratory. In this study, we also used Superdex-75 and SP-Sephadex C-25 from Pharmacia LKB Biotechnology (Sweden) and ultrafiltration cells and PM10 membranes from Amicon (USA). Affinity sorbent ([N-(ε-aminocaproyl)-*p*-aminobenzyl]succinyl-Sepharose 4B (CABS-Sepharose)) was prepared according to [20].

Mutants of gene *cpT* were obtained by overlap extension PCR mutagenesis [21] using T7-terminator and T7-

Table 1. Amino acid residues determining the substrate specificity of MCPs [4, 7-9]

Enzyme	Homologous residues*						
CPA	Leu203	Gly207	**Ile243**	Ala250	Gly253	**Ile255**	Thr268
CPB	Leu203	Ser207	Gly243	Ala250	Gly253	**Asp255**	Thr268
CPT	Leu211	Gly215	Ala250	Thr257	**Asp260**	Thr262	Thr275
CPT5	Leu211	Ser215	Gly250	Ala257	Gly260	**Asp262**	Thr275

* Residues playing a key role in MCP substrate specificity are given in bold.

Table 2. Kinetic parameters of CPT and its variants*

Enzyme	DnpAAR			ZAAL			ZAAE
	K_m, mM	k_{cat}, sec^{-1}	k_{cat}/K_m, $mM^{-1}\cdot sec^{-1}$	K_m, mM	k_{cat}, sec^{-1}	k_{cat}/K_m, $mM^{-1}\cdot sec^{-1}$	k_{cat}/K_m**, $mM^{-1}\cdot sec^{-1}$
CPA [35]	–	–	–	0.012	35	2900	–
CPB [35]	0.07	35	500	–	–	–	–
CPT_{wt} [13]	4.4 ± 1.3	15 ± 3	3.6 ± 0.6	0.046 ± 0.003	10 ± 2	230 ± 65	6.1 ± 2.6
CPT H68N	5.9 ± 0.1	18 ± 1	2.9 ± 0.2	0.020 ± 0.002	8.4 ± 0.8	420 ± 40	2.3 ± 0.2
CPT L254N	4.0 ± 0.3	14 ± 1	3.6 ± 0.1	0.063 ± 0.003	5.6 ± 0.5	87 ± 4	< 0.2***

Note: –, no data available.

* Expression, renaturation, activation, and purification procedures and determination of kinetic parameters were repeated thrice. The average values with the standard errors of mean are given.

** There was no saturation with substrate under the experimental conditions.

*** $p < 0.05$ according to Student's t-test.

promoter flanking primers and also direct and reverse mutagenic primers. Mutant genes were again cloned in pet23a vector [22]. Variant CPT H68N had AAT Asn codon instead of CAT His codon; variant CPT L254N had AAC Asn codon instead of CTC Leu codon. Correctness of site-directed mutagenesis was proved by complete sequencing of the CPT variant genes.

Enzyme expression as inclusion bodies and subsequent renaturation *in vitro*. Expression of the wild type CPT gene and its variants in *E. coli* BL21(DE3)pLysS was induced by addition of 0.5 mM isopropyl-β-D-thiogalactoside [15]. Wild type proCPT and its mutants were expressed in the form of inclusion bodies with ~50% yield of total cell protein.

After overnight incubation of the culture at 28°C, cells were precipitated and ultrasonically disintegrated. Inclusion bodies thus obtained were washed with 0.05% CHAPS (w/v), 2 M NaCl, and water. Renaturation was performed as described in [23] but with some modifications that increased protein yield. Briefly, the inclusion body precipitate was dissolved in 8 M urea, rapidly diluted 10 times with 50 mM Tris-HCl, 30% glycerol (v/v), 0.5 M NaCl, 10 mM $CaCl_2$ (from here on the percentage composition is given in volume fractions), pH 8.0, and incubated overnight at 37°C. Then the solution was diluted twofold with 50 mM Tris-HCl, 0.5 M NaCl, 10 mM $CaCl_2$, pH 8.0, and concentrated by ultrafiltration to the volume of about 20 ml. To remove propeptide, subtilisin in molar ratio subtilisin/CPT = 1 : 200 was added, and the mixture was incubated for 30 min at 37°C. The subtilisin was then inhibited with diisopropylfluorophosphate. Denatured enzyme formed at each stage was removed by centrifugation.

Enzyme purification. The wild type CPT and its variants were purified by molecular exclusion chromatography on a column with Superdex-75 pre-equilibrated with 10 mM Tris-HCl, 0.5 M NaCl, 10 mM $CaCl_2$, pH 7.5. After gel filtration the pH was adjusted to 6.0 by addition of 100 mM MES-NaOH buffer, pH 6.0, and then the solution was applied on a column with CABS-Sepharose [20], washed with 10 mM MES-NaOH, pH 6.0, 0.5 M NaCl, 10 mM $CaCl_2$ and eluted with 10 mM Tris-HCl, 0.5 M NaCl, 10 mM $CaCl_2$, pH 8.0.

Protein concentrations were determined according to Bradford [24] and by optical absorption at 280 nm using ε_{280} = 64,000 $M^{-1}\cdot cm^{-1}$ predicted for CPT [25]. SDS-PAGE was performed according to Laemmli [26].

Efficiency of refolding and purification was about 1.5% of protein mass taken for renaturation and about 3-5 mg/(liter of culture medium) for the wild type enzyme as well as its mutants. After cleavage of propeptide by subtilisin and purification, enzyme purity exceeded 95% according to SDS-PAGE.

Kinetic studies. The enzyme activity towards DnpAAR substrate was tested in 25 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.5, at substrate concentrations in the range 0.4-4 mM at 37°C. The reaction was stopped by addition of 50% CH_3COOH. The reaction product DnpAA was separated from the positively charged substrate using SP Sephadex C-25 and its amount was determined spectrophotometrically (ε_{360} = 15,000 $M^{-1}\cdot cm^{-1}$). The activity towards ZAAL and ZAAE substrates was tested in 25 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.5, at 40-200 μM ZAAL and 0.1-1 mM ZAAE concentrations at 25°C. Optical absorption decrease at 225 nm was converted to the product concentration using ε_{225} = 376 $M^{-1}\cdot cm^{-1}$. The enzyme concentration was chosen based on its activity against each substrate.

The reaction rates were measured four times for the four substrate concentrations within the given range. The data were processed by linear or nonlinear regression using the Origin 6.1 program.

Expression, renaturation, activation, and purification procedures and also determination of kinetic parameters were independently repeated thrice for each CPT variant studied in this work.

To investigate the role of Ca^{2+}, we used buffers based on 25 mM Tris-HCl, pH 7.5, with $CaCl_2$ concentration range from 1 μM (dilution of the enzyme preparation containing 10 mM $CaCl_2$) to 10 mM. Three nanomolar Ca^{2+} concentration in the reaction buffer was obtained by addition of 10 μM EGTA to $CaCl_2$-free buffer. The enzyme activity was evaluated at the following substrate concentrations: 0.4 mM DnpAAR, 0.4 mM ZAAE, and 0.05 mM ZAAL. A mixture of the reaction buffer and enzyme was preincubated for 15 min at 37°C, and then the substrate solution was added and activity was determined.

A control test of the absence of subtilisin in the CPT preparation was performed in 50 mM Tris-HCl, 10 mM $CaCl_2$, pH 8.5, 0.1 mM chromogenic substrate ZAALpNA at 37°C. When the reaction mixture turned light yellow, the reaction was stopped by addition of 0.5 M HCl. Subtilisin concentration was evaluated using ε_{410} = 8200 $M^{-1}\cdot cm^{-1}$ (molar extinction coefficient for *p*-nitroaniline), and specific enzyme activity of 10 units per optical unit. Subtilisin admixture in the enzyme preparations was less than 0.00015%.

Thermal stability measurements. Reaction buffer containing the enzyme at operating concentrations was heated for 15 min at 50-70°C and then cooled on ice. The enzyme activity was evaluated under the standard conditions by addition of DnpAAR substrate to concentration 0.4 mM.

RESULTS AND DISCUSSION

Comparing the CPT5 molecular model with the CPB structure, we revealed some specific features in CPT organization. In a previous article of this group [13] upon analysis of their possible role in determination of the wide substrate specificity of CPT, we considered that the difference in residue 68 nature and in residue 254 nature and

position is significant. Also, CPT possesses structural calcium ions that CPB lacks [4].

CPT H68N variant. Many differences in positions of the charged residues were found in the two enzymes. Most of these residues are localized on the surface of the molecule, where the presence of water molecules and counter ions significantly reduces their electrostatic field. Of the residues in the protein core, His68 is present in CPT, while in CPB its place is occupied by Phe68 [13]. Considering that His68 is in the neighborhood of the active site (9 Å from the guanidino group of the substrate according to the CPT5 model [13]) and that this amino acid residue might be charged, this structural difference can decrease seven-fold the binding of the positively charged substrate and equally increase binding of the negatively charged substrate by CPT as compared with CPB [13]. To check the possible role of His68 as a determinant of CPT substrate specificity, this residue was replaced by uncharged Asn (CPT H68N variant).

Considering that His68 is located inside the protein, the replacement strategy chosen by us was based on the principle of minimal structural rearrangements. Analysis of CPT structure [4] showed that on replacement of His68 by Asn, the hydrogen bond formed by the N^{δ} atom of the imidazole ring with the structurally bound water molecule was retained. Besides this, the Asn side chain fits well in place of His and does not form sterically unfavorable contacts with other atoms of the enzyme.

Efficiency of hydrolysis of all the three substrates by CPT H68N was the same as by the wild type enzyme. Kinetic parameters of the hydrolysis of the substrate with C-terminal Arg were almost unchanged. On ZAAL hydrolysis, decrease in K_m was accompanied by decrease in k_{cat}, but this had practically no effect on the specificity constant k_{cat}/K_m. CPT H68N variant as well as the wild type enzyme hydrolyzed the substrate with C-terminal Glu without substrate saturation within the concentration range used by us. The substrate specificity profile of CPT H68N remained the same as that of CPT_{wt} (183/1/1.3 and 64/1.7/1, respectively), demonstrating the maximal affinity to the hydrophobic substrates. The absence of effect of His68Asn replacement on CPT selectivity to the positively and negatively charged substrates means that His68 is uncharged.

CPT L254N variant. Comparing the structures of the S1′-subsites of CPT5 and CPB, we found different spatial positions of the homologous residues Leu254 of CPT and Ile247 of CPB [13]. To elucidate the role of CPT Leu254 in binding to hydrophobic substrates, we replaced it by isosteric polar Asn. We expected that selectivity of this variant towards the hydrophobic substrates would decrease. Nonetheless, efficiency of hydrolysis of the substrate with C-terminal Leu by the L254N mutant remained the same as that of the substrate with C-terminal Arg. However, kinetic characteristics of hydrolysis of the negatively charged substrate were changed drastically. The dependence of reaction rate on the substrate concentration did not obey the Michaelis–Menten equation because of the substrate activation in the ZAAE concentration range used by us. In this case, we managed to determine only the upper limit of the k_{cat}/K_m parameter at the minimal ZAAE concentration used. Under these conditions, catalytic activity of the variant decreased more than 28-fold. Thus, for the substrates with C-terminal Leu, Glu, and Arg, the enzyme specificity profile changed from 64/1.7/1 to 24/<0.06/1 (for wild type CPT and CPT L254N, respectively).

It is most likely that activity decrease towards the negatively charged substrate is caused by removal of the hydrophobic contact between Leu254 and the aliphatic part of Glu of the substrate on replacement of the hydrophobic Leu by polar Asn. Since activity of hydrolysis of two other substrates with a significant hydrophobic component remained the same, the participation of Leu254 in binding of them is not so pronounced. In contrast to the side chain of the C-terminal Glu, side chains of C-terminal Arg and Leu additionally have one CH_2^{δ}- and two CH_3^{δ}-groups, respectively; these groups will form hydrophobic contacts with Leu211 and stabilize binding of the substrates.

For CPA and CPB, the effect of substrate activation was found only on hydrolysis of short di- and monopeptide substrates [27, 28]. These data were rationalized by the possibility of the second substrate molecule to bind to the "activation" site [27]. In the context of present day data, this phenomenon might be rationalized by binding of short substrates in the active site regions more distant from the S1′- and S1-subsites, as it was found for the complex of CPA with the products of hydrolysis [29]. This is also evidenced by disappearance of the substrate activation when the more extended substrates are used [28, 30].

In our case, the origin of kinetically anomalous behavior of CPT L254N in relation to the more extended tripeptide substrate ZAAE is unclear. Moreover, there were no deviations from the Michaelis–Menten kinetics on hydrolysis of ZAAL and DnpAAR. Recently it has been shown that the CPA4 residue 247 homologous to CPT 254 residue is a constituent of the S5-subsite along with participation in the S1′-subsite formation [31]. The data suggest that replacement of the hydrophobic Leu for polar Asn changes the properties of this subsite, promoting binding to the second ZAAE molecule.

Effect of calcium ions. Four calcium ions located about 30 Å from the enzyme active site are present in CPT and are absent in CPB. Due to the large net charge (+8), this difference might result in decrease in binding of substrate Arg and increase in binding of substrate Glu up to tenfold, in spite of the distant position of the calcium ions. Therefore, the kinetic properties of Ca^{2+}-free CPT were studied.

To decrease Ca^{2+} concentration in the reaction mixture to 3 nM, EGTA chelating agent was added to the final

concentration 10 μM to 35 nM enzyme solution containing 1 μM $CaCl_2$. In this case, the concentration of the protein-accessible calcium was one order of magnitude lower than the enzyme concentration. Removal of Ca^{2+} from the enzyme structure was evidenced by decrease in CPT thermal stability (Fig. 1). Thus, after preincubation for 15 min at 70°C, the enzyme with Ca^{2+} losses ~30% of its activity, whereas the Ca^{2+}-free enzyme is fully deactivated.

Dependence of the enzyme activity towards DnpAAR on Ca^{2+} concentration (Fig. 2) was sigmoid with bending at $CaCl_2$ concentration in the range 10-100 μM, whereas the rate of activity change is maximal. At the lower Ca^{2+} concentrations, the activity curve became flat. Similar dependence was interpreted as evidence for the role of structurally bound calcium in selectivity of *Streptomyces griseus* aminopeptidase [32].

On removal of Ca^{2+} from CPT, the enzyme activity changed significantly (Table 3). For the charged substrates DnpAAR and ZAAE, the activity of the Ca^{2+}-free enzyme decreased 1.7- and 2.2-fold, respectively. In contrast, the enzyme activity towards the hydrophobic substrate ZAAL increased 1.5-fold. Thus, specificity of the Ca^{2+}-free enzyme towards hydrophobic substrates increased threefold.

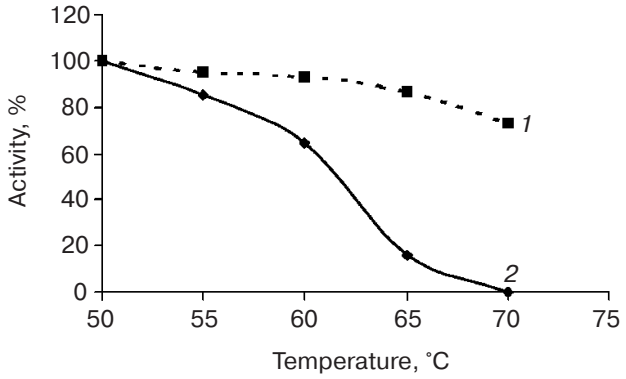

Fig. 1. Thermal stability of CPT. *1*, *2*) Activities of the enzyme preincubated in buffer containing 1 mM and 3 nM $CaCl_2$, respectively. Standard deviations are less than 10%.

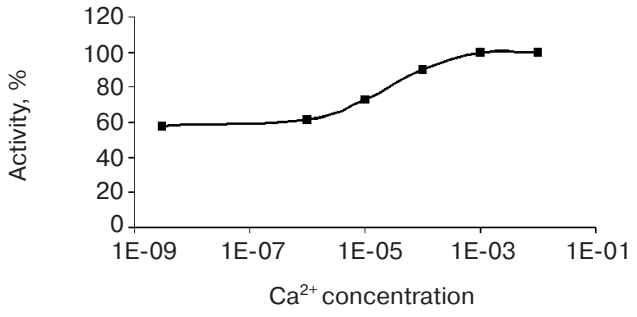

Fig. 2. Activity of CPT against DnpAAR versus Ca^{2+} concentration. Standard deviations are less than 10%.

Table 3. Activity of CPT* in buffer containing 1 mM and 3 nM Ca^{2+}

Substrate	Activity, %	
	1 mM Ca^{2+}	3 nM Ca^{2+}
DnpAAR	100 ± 19	58 ± 11**
ZAAL	100 ± 6	147 ± 3**
ZAAE	100 ± 4	45 ± 6**

*CPT activity in buffer containing 1 mM $CaCl_2$ was taken as 100%. Average values and their standard deviations are given.
** $p < 0.05$ according to Student's t-test.

The effect of Ca^{2+} removal was fully reversible. If $CaCl_2$ was added to 1 mM concentration to the enzyme preincubated in the EGTA-containing buffer, activity of the latter towards DnpAAR increased to that of the enzyme preincubated in buffer containing 1 mM $CaCl_2$.

The effect of structurally bound calcium on the activity of carboxypeptidases is known from the literature. The rate of hydrolysis of a substrate with the C-terminal Arg by carboxypeptidase E increased on removal of Ca^{2+} [33]. This is rationalized by electrostatic interaction between the substrate and metal ions. However, in our case calcium ions most probably influence the enzyme activity via another mechanism. Increased efficiency of hydrolysis of hydrophobic substrates and decreased efficiency of hydrolysis of positively charged substrates suggest that Ca^{2+} effects via conformational changes in the region of the Ca^{2+}-binding sites, which are transferred to the region of the enzyme active site. The effect of structurally bound calcium on the substrate specificity of metal carboxypeptidases has not been described earlier.

The absence of change in CPT selectivity on insertion of mutations that restore the CPB binding site indicates that along with the well-known seven residues of the S1′-subsite, there are additional substrate specificity determinants, including some that are not constituents of the primary specificity pocket. The search for the latter revealed the role of residue 254, a constituent of the primary specificity pocket, and calcium ions distant from the active site. Along with works on redesign of trypsin substrate specificity into that of chymotrypsin [34], the data indicate that the enzyme selectivity mechanism is more complex than was considered earlier.

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