INACTIVATION OF CARBOXYPEPTIDASE Y BY MUTATIONAL REMOVAL OF THE PUTATIVE ESSENTIAL HISTIDYL RESIDUE

by

LENE M. BECH and KLAUS BREDDAM

Department of Chemistry, Carlsberg Laboratory,
Gamle Carlsberg Vej 10, DK-2500 Copenhagen Valby

Keywords: Carboxypeptidase, homology, catalytic triad, histidine, cysteine, site-directed mutagenesis

Carboxypeptidase Y is a serine carboxypeptidase assumed to contain a catalytic triad similar to the serine endopeptidases. On the basis of the homology between various serine carboxypeptidases His-397 is suspected to be part of the catalytic triad. To test this it was exchanged with Ala and Arg by site-directed mutagenesis of the cloned PRC1 gene. The catalytic efficiency of the mutant enzymes were reduced by a factor of $2 \cdot 10^4$ and $7 \cdot 10^2$, respectively, confirming the key role of His-397 in catalysis. Treatment of Ala-397-CPD-Y with Hg^{++} or CNBr , hence modifying Cys-341 located in the vicinity of the active site abolished the residual activity of the enzyme, indicating an additional involvement of this residue in catalysis.

1. INTRODUCTION

All the serine endopeptidases, both the "subtilisin-like" and the "trypsin-like", have evolved the same catalytic triad, consisting of Ser, His and Asp, although from two entirely different families of genes (17). A category of carboxypeptidases isolated from fungi, plants and mammals also contains an essential seryl residue and are consequently termed serine carboxypeptidases (8). No three-dimensional structures of such enzymes have yet been determined but it is probable that they also contain a

catalytic triad similar to the serine endopeptidases (8). In the serine carboxypeptidase from yeast (CPD-Y) Ser-146 is the essential residue (27) and chloromethylketones, which in the endopeptidases have been demonstrated to specifically modify the essential histidyl residue (24, 26), also react with a single histidyl residue in this enzyme and cause inactivation. However, the position of the modified residue has not been identified.

The amino acid sequence of five different serine carboxypeptidases has been determined,

Abbreviations: Ala-397-CPD-Y = CPD-Y with an alanyl residue at position 397; Arg-397-CPD-Y = CPD-Y with an arginyl residue at position 397; Bicine = N,N-bis(2-hydroxyethyl)glycine; CABS-Sepharose = [N-(ϵ -aminocaproyl)-p-aminobenzyl]succinyl-Sepharose 4B; CD = circular dichroism; CPD-Y = carboxypeptidase Y; DFP = diisopropylphosphorofluoridate; EDTA = ethylenediamine tetraacetic acid, sodium salt; FA = furylacryloyl; GYBS-Sepharose = [(N-glycyl-L-tyrosine)-p-azo-benzyl]succinyl-Sepharose 4B; Hepes = N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; HPLC = high performance liquid chromatography; Mes = 2(N-morpholino)ethanesulfonic acid; SDS = sodium dodecylsulfate. Abbreviations of amino acids and peptides are according to the guidelines of IUPAC-IUB Commission on Biochemical Nomenclature.

		146												397											
CPD-Y	-	A	G	E	S	Y	A	G	H	-		-	N	G	G	H	M	V	P	F	D	V	P	-	
CPD-MI	-	A	G	E	S	Y	A	G	V	-		-	G	A	G	H	T	V	P	E	Y	K	P	-	
CPD-MII	-	A	G	E	S	Y	A	G	H	-		-	G	A	G	H	E	V	P	L	H	R	P	-	
CPD-MIII	-	T	G	E	S	Y	A	G	H	-		-	N	A	G	H	M	V	P	M	D	Q	P	-	
CPD-WII	-	A	G	E	S	Y	A	G	H	-		-	G	A	G	H	E	V	P	L	H	R	P	-	

Figure 1. Homology of catalytical important regions of carboxypeptidase Y (7), carboxypeptidases I (28), II (29) and III (30) from malted barley and carboxypeptidase-WII from wheat bran (10). The numbering is that of carboxypeptidase Y.

and they show significant homology (7, 10, 28, 29, 30). The essential seryl residue is located in a highly conserved region and it is likely that other catalytically important residues similarly are located in well conserved regions. A number of aspartyl residues appear to be conserved, and it is difficult to predict which one might be the essential, while His-397 is the only conserved histidine (Fig. 1). Hence, it is the most probable candidate for a histidyl residue functioning as a general acid/base catalyst in the active site of serine carboxypeptidases. In the present paper this residue is replaced with alanine and arginine, using site-directed mutagenesis of PRC1, the structural gene for CPP-Y, and it is demonstrated that these mutations greatly reduce k_{cat} and have only minor effect on K_m .

2. MATERIALS AND METHODS

2.1. Materials

The plasmid pTSY3 containing the PRC1 gene, coding for CPD-Y, and *Saccharomyces cerevisiae* SEY2202 (Mata $\Delta prc1-\Delta 1$ (LEU2) leu2-3 leu2-112 ura3-52 his4-519) were kindly provided by Dr. TOM STEVENS, University of Oregon, USA. The *E. coli* strain RZ1032 was a generous gift from Dr. TOMAS A. KUNKEL, National Institute of Environmental Health Sciences, USA. The *E. coli* strains JM101 (20) and DH5 α (14) and the M13mp8-derivative G42 (35) were from house collections. The *E. coli* strain TG1 was provided in the oligonucleotide-directed in vitro mutagenesis system version 2

kit from Amersham, GB. Restriction endonucleases, T4 polynucleotide kinase, adenosine triphosphate were obtained from Boehringer Mannheim, FRG. HPLC-purified 2' deoxyribonucleic acids were from Pharmacia, Sweden. The Sequenase kit for DNA-sequencing was from United States Biochemical Corp., USA and the GeneClean-kit for purification of DNA fragments was from Bio101, USA. [α - 32 P]dATP (>800 Ci/mmol) and [γ - 32 P]ATP (>7000 Ci/mmol) were from New England Nuclear, USA. Trizma base, Mes, Hepes, DTT, agarose and L-amino acids were from Sigma, USA. HgCl₂ was from Ferak Berlin, FRG and CNBr was from Fluka, Switzerland. FA-Phe-OMe was from Bachem, Switzerland and FA-Phe-Leu-OH was prepared as previously described (6). The synthetic oligonucleotides were synthesized on an Applied Biosystem 380 DNA synthesizer according to the procedures provided by the manufacturer. All other reagents and solvents were of analytical purity and obtained from Merck, FRG.

2.2. Methods

2.2.1. Preparation of mutant CPD-Y

In vitro mutagenesis was performed using a M13mp8 subclone of PRC1 (35). High frequencies of mutants were obtained using either a uracil containing template as described by KUNKEL (18) (Arg-397-CPD-Y) or the "oligonucleotide-directed in vitro mutagenesis system version 2" kit from Amersham, based on the

method of ECKSTEIN and coworkers (23, 25, 31, 32) (Ala-397-CPD-Y).

Autolysis of yeast cells harbouring mutant plasmids and activation of mutant enzymes were carried out as described in ref. 16. The extract was adjusted to pH 4.5 by addition of 1 M HCl and applied to a 50 ml GYBS-Sepharose column, equilibrated with 0.02 M NaAc, pH 4.5. The column was washed with the same buffer until A_{280} was 0.03. The enzyme was eluted with 0.1 M NaH_2PO_4 , pH 7.5 and the eluate was dialysed against 0.01 M NaH_2PO_4 , pH 7.0 and applied to a DE 52 cellulose column (50 ml), equilibrated with the same buffer. After washing of the column with buffer until A_{280} was 0.02 the elution was performed with a gradient from 0 to 0.5 M NaCl in 0.01 M NaH_2PO_4 , pH 7.0 using a total of 9 column volumes. The enzyme eluted at approximately 0.3 M NaCl, and the eluate was dialysed against water and concentrated by ultrafiltration using equipment and YM 10 membrane from Amicon. The concentration of NaCl was adjusted to 0.1 M by addition of 5 M NaCl, and the sample was chromatographed on a Sephadex G 100 column (2.6×87 cm) equilibrated with 0.02 M NaAc, 0.1 M NaCl, pH 4.5. Finally, the enzyme solution was concentrated and dialysed against water. The purification was monitored by Ouchterlony immuno diffusion assays, using 5 μl of an antiserum raised against wild type CPD-Y. The diffusions were performed in 1% agarose gels containing 24.3 mM 5,5-diethyl barbituric acid, 73.2 mM Tris, 0.8 mM calcium lactate, 2 mM NaN_3 , pH 8.66 using well diameters of 4 mm. The purity of the resulting enzymes was ascertained by SDS polyacrylamide gel electrophoresis on 10-15% gradient gels using the Phast system from Pharmacia (34), and by N-terminal sequencing on an Applied Biosystems Model 470 A gas-phase sequencer using the program provided by the company. The phenylthiohydantoin amino acid derivatives were identified on-line by reverse phase HPLC using equipment from Applied Biosystems.

2.2.2. Characterization of mutant CPD-Y

The kinetic constants for the hydrolysis of FA-Phe-OMe and FA-Phe-Leu-OH were deter-

mined from Lineweaver-Burk plots. Assays were performed in 0.05 M Hepes, 1 mM EDTA pH 7.5 and 0.05 M Mes, 1 mM EDTA, pH 6.5, respectively, and hydrolysis rates were determined spectrophotometrically at 329-342 nm and 25 °C, using a Perkin Elmer lambda 7 spectrophotometer. The pH-optimum for the hydrolysis of FA-Phe-OMe was investigated by assays in 25 mM buffer, 0.1 M NaCl, 1 mM EDTA. The following buffers were used: acetic acid, pH 4-5.23; Mes, pH 5.62-6.72; Hepes, pH 6.75-7.80; Bicine, pH 8.28-8.93. Using FA-Phe-OMe as a substrate some background (nonenzymatic) hydrolysis was observed at pH values above 7 due to long reaction times, and the activities measured were therefore corrected for this spontaneous hydrolysis.

Circular dichroism measurements were made using a Dichrographe II instrument from Rous-sel Jouan, Paris, using quartz cuvettes with length of 1 cm or 0.2 cm. The window never exceeded 1 mm. Affinity chromatography on CABS-Sepharose was performed as previously described (6).

2.2.3. Modifications of mutant enzymes

Modification of Ala-397-CPD-Y with HgCl_2 was performed in 0.05 M Hepes, pH 7.5 using an enzyme concentration of 2.33 mg/ml and 1.5 equivalents of Hg^{++} . After 15 min incubation at room temperature 10 μl of the reaction mixture was assayed against 0.2 mM FA-Phe-Leu-OH, 0.05 M Mes, 2.5% CH_3OH , pH 6.5. The effect of KI was tested by including 10^{-3} M KI in the assay mixture.

Modification of Ala-397-CPD-Y with CNBr was performed in 0.05 M NaH_2PO_4 , 1.5 mM CNBr, pH 7.5, using an enzyme concentration of 2.33 mg/ml. After two hours of incubation at room temperature 10 μl of the reaction mixture was assayed against 0.2 mM FA-Phe-Leu-OH, 0.05 M Mes, 1 mM EDTA, 2.5% MeOH, pH 6.5.

3. RESULTS AND DISCUSSION

3.1. Formation of mutant enzymes

The two mutations His-397 to Arg and His-397 to Ala were accomplished in the structural gene for CPD-Y (PRC1) by standard procedures

Table I. Kinetic parameters for the hydrolysis of FA-Phe-Leu-OH and FA-Phe-OMe with CPD-Y, Arg-397-CPD-Y and Ala-397-CPD-Y.

Substrate	Enzyme	k_{cat} (min^{-1})	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ ($\text{min}^{-1} \cdot \text{mM}^{-1}$)	$\frac{k_{\text{cat}} (\text{wt})}{k_{\text{cat}} (\text{mutant})}$
FA-Phe-Leu-OH	CPD-Y	4,900	0.021	230,000	-
	Arg-397-CPD-Y	7.3	0.024	300	$7.7 \cdot 10^2$
	Ala-397-CPD-Y	0.33	0.048	6.8	$3.4 \cdot 10^4$
FA-Phe-OMe	CPD-Y	11,000	0.39	28,000	-
	Arg-397-CPD-Y	15	0.36	41	$6.8 \cdot 10^2$
	Ala-397-CPD-Y	1.0	0.38	2.7	$1.0 \cdot 10^4$

Assay conditions as described in section 2.2.2.

(18, 23, 25, 31, 32), and the presence of the mutations were confirmed by chain termination sequencing. The mutated sequences were reintroduced into the *E. coli*/yeast shuttle-vector pTSY3 replacing the original wild type sequence, and plasmids containing the mutations were used to transform *S. cerevisiae* strain SEY2202, which contain a deletion in the *PRC1*-gene. Plasmid bearing yeast cells were grown to saturation in a synthetic medium free of uracil. Mutant enzymes were isolated by affinity chromatography, ion-exchange chromatography and gel filtration as described in section 2.2.1. 7.2 mg Ala-397-CPD-Y and 4.7 mg Arg-397-CPD-Y were isolated from 10 l culture medium. The specific activities towards FA-Phe-OMe were 0.004 $\mu\text{mol}/\text{min}/\text{mg}$ and 0.13 $\mu\text{mol}/\text{min}/\text{mg}$, respectively, compared to 70 $\mu\text{mol}/\text{min}/\text{mg}$ for wildtype CPD-Y. SDS-gel electrophoresis and N-terminal sequence determinations demonstrated that the enzyme preparations were homogeneous with molecular weights identical to that of CPD-Y (64,000).

Another mutation, His-397- to Phe, was accomplished too, but upon expression of the mutated gene, no mutant enzyme was produced, as judged by the immuno diffusion assay of the yeast extract.

3.2. Characterization of mutant enzymes

The low specific activity of the preparation of mutant enzyme could potentially be due to the presence of a small fraction of native (folded) enzyme with relatively high activity among a population of denatured enzyme molecules.

Therefore, CD spectra of Ala-397-CPD-Y and wild type CPD-Y were recorded between 265 and 210 nm. The spectra were identical, indicating that the secondary structure of the two enzymes are clearly related, and that no significant amount of denatured enzyme is present.

In order to rule out that the residual activities of Ala-397-CPD-Y and Arg-397-CPD-Y are due to contamination of the preparations with wild type CPD-Y the mutant enzymes were rechromatographed on a CABS-Sepharose affinity resin as described in reference 6. This resin has previously been employed to separate active site modified CPD-Y from unmodified CPD-Y (6): at pH 4.8 both enzymes were bound to the CABS-Sepharose, but only the modified derivative was eluted by an increase of the ionic strength of the buffer; the unmodified enzyme remained bound to the column, but could subsequently be eluted by increasing the pH to 7.5. Using identical conditions Ala-397-CPD-Y and Arg-397-CPD-Y were bound to this resin, and could subsequently be eluted as active, mutant enzymes by including 0.5 M NaCl in the buffer. Increasing pH to 7.5 did not elute any measurable activity, confirming that no wild type enzymes was present.

In modified subtilisin BPN' it has previously been shown that replacement of the catalytic seryl, histidyl or aspartyl residues reduce k_{cat} by $1.7 \cdot 10^6$, $1.6 \cdot 10^6$ and $2.6 \cdot 10^4$, respectively, while K_{m} remains approximately unchanged (11, 12). The more pronounced reduction resulting from substitution of Ser-221 and His-64, as compared with Asp-32, are consistent with the central role of these residues in catalysis (17).

In order to investigate whether the mutant carboxypeptidases behaved similarly they were investigated kinetically using the substrates FA-Phe-OMe and FA-Phe-Leu-OH (Table I). Replacement of His-397 with an alanyl residue resulted in reduction of k_{cat} for both substrates by approximately a factor of 2×10^4 , whereas K_m remained almost unchanged. Introduction of an arginyl residue similarly had no effect on K_m , while k_{cat} was decreased by a factor of 7×10^2 (Table I). Thus it is clear that the catalytic apparatus of CPD-Y is drastically damaged by removal of His-397, while the ability of the enzyme to bind the substrate remains essentially unaffected. This is consistent with His-397 being the essential histidyl residue. The 25 fold higher value of k_{cat} of Arg-397-CPD-Y as compared with Ala-397-CPD-Y suggest that the arginyl residue to a small extent may replace the histidyl residue as a general acid/base catalyst. It is remarkable that the His→Ala mutation in CPD-Y has a smaller effect than the corresponding mutation in subtilisin BPN'. This indicates that His-397 in CPD-Y is less essential for catalysis than His-64 of subtilisin BPN', perhaps because other residues of relatively greater importance are involved in catalysis as well.

The pH profiles for the esterase activity of wild type CPD-Y, Arg-397-CPD-Y and Ala-397-CPD-Y towards FA-Phe-OMe are shown in Figure 2. These pH profiles reflect k_{cat}/K_m since the hydrolysis reactions were carried out at a substrate concentration below K_m . For wild type CPD-Y the activity increases 50 times between pH 4 to 7, reflecting deprotonation of a group with pK_a of 5.8 - 6.0 - presumably the essential histidyl residue.

The activities of Arg-397-CPD-Y and Ala-397-CPD-Y are also influenced by pH, but the increase in activity in the pH range 4 to 7 is only 10 and 3 times, respectively. This indicates that His-397 is not the only group influencing the pH-profile.

3.3. Chemical modifications of mutant enzymes

Studies in this laboratory have shown that Hg^{++} , in the absence of halides, i.e. Cl^- , Br^- , SCN^- and CN^- , reacts with Cys-341 and causes complete inactivation of wild type CPD-Y regardless of the nature of the substrate (5). This total lack

Figure 2. pH profiles for the hydrolysis of FA-Phe-OMe catalyzed by CPD-Y (○), Arg-397-CPD-Y (□) and Ala-397-CPD-Y (△). The ordinate indicates the logarithm to activities in $\mu\text{mol}/\text{min}/\text{mg}$.

of activity of the Cys-341 modified enzyme is possibly due to the mercuric ion forming a -S-Hg-X bridge between the sulfhydryl group of Cys-341 and some other amino acid side chain X capable of forming a rather tight complex with Hg^{++} , e.g. X = His, Asp or Glu (33). This explanation is supported by the observation that addition of halides, which form tight complexes with Hg^{++} (33), partially reactivates the enzyme without removing the metal from the enzyme (5) presumably by displacing the side-chain X from the mercuric ion bound to the sulfhydryl group thus forming a partially active enzyme species. The total loss of activity of the Cys-341

modified enzyme with no halide present suggest that the side chain X may be essential for catalysis, and thus the essential histidyl residue is a probable candidate as side chain X.

Modification of Ala-397-CPD-Y with 1.5 equivalents of HgCl_2 , as described in section 2.2.3, abolished more than 95% of the activity towards FA-Phe-Leu-OH, and addition of 10^{-3} M KI did not reactivate the enzyme. As addition of KI to the assay mixture has no detectable effect on the activity of the Hg^{++} derivative, the loss of activity presumably is due to the modification per se - and not to formation of an -S-Hg-X bridge. This indicates that His-397 is the residue partitioning in the bridge in wild type enzyme. Similarly, cyanilation of Cys-341 of Ala-397-CPD-Y with CNBr as previously described for wild type enzyme (9) abolished more than 95% of the activity towards FA-Phe-Leu-OH.

These results indicate that Cys-341 to a small extent may replace His-397, and thus presumably is responsible for the residual activity of Ala-397-CPD-Y. Assuming that the depletion of activity upon modification of this residue is due mainly to decreases in k_{cat} , the Hg^{++} - and the cyanylated derivatives both have k_{cat} values below $0.34 \text{ min}^{-1} \cdot \text{mM}^{-1}$, and this is a reduction by a factor of $7 \cdot 10^5$ or more as compared to wild type enzyme. This decrease is of the same order of magnitude as that observed with the His→Ala mutation of subtilisin (12), and the results are consistent with Cys-341 being involved in catalysis (1, 5, 9, 36). A number of endopeptidases belonging to the subtilisin-like type of enzymes: thermitase from *Thermoactinomyces vulgaris* (2, 19), proteinase K from *Tritirachium album* (15), proteinase B from yeast (22) and the yeast *KEX-2* encoded protein (21) all contain a free cysteinyl residue at the same position in the sequence. The three-dimensional structures of proteinase K and thermitase have been solved (3, 4, 13) and in both cases the free cysteinyl residue is buried behind the essential histidyl of the catalytic triad, such that it is practically inaccessible to solvent. HgCl_2 is known to react with the conserved cysteinyl residue of these two enzymes and cause inactivation, and thus it is possible that it has a functional resemblance to Cys-341 of CPD-Y.

The results above demonstrate that His-397 of

CPD-Y is highly involved in catalysis. Chemical modification of Cys-341 of the mutant enzyme Ala-397-CPD-Y almost depletes the activity of the enzyme, indicating that this residue presumably is responsible for the residual activity of Ala-397-CPD-Y.

ACKNOWLEDGEMENTS

The authors acknowledge Professor MARTIN OTTESEN for critically revising the manuscript. Ms. LILLIAN ABILDGAARD is acknowledged for excellent technical assistance.

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Accepted by H. KLENOW