IDENTIFICATION OF METHIONYL AND CYSTEINYL RESIDUES IN THE SUBSTRATE BINDING SITE OF CARBOXYPEPTIDASE Y

by

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Chemical modification studies have previously indicated that the S_1 binding site of carboxypeptidase Y contains a cysteinyl residue and that the S'_1 binding site contains a methionyl residue (Carlsberg Res. Commun. 48, 9-19 (1983); 49, 535-554 (1984); 49, 627-638 (1984)). These studies also indicated that the active site contained an additional methionyl residue situated in an as yet unidentified position. In the present paper the positions of these amino acid residues has been identified in the amino acid sequence which has been revised on the basis of the nucleotide sequence (T. STEVENS, personal communications). The cysteinyl residue was identified as Cys-341 after alkylation with ^{14}C -iodoacetic acid followed by separation of the peptides produced by cyanogen bromide cleavage and Edman degradation of the radioactive peptide. The methionyl residue situated in the S'_1 binding site was identified as Met-398 after alkylation with ^{14}C -iodoacetamide, separation of the peptides produced by cyanogen bromide cleavage and thermal cleavage of the peptide containing the radioactivity. This assignment was confirmed by the failure of cyanogen bromide to cleave at the position of carboxypeptidase Y in which this particular methionyl residue had been alkylated or oxidized. Using the same method the other methionyl residue in the active site was identified as Met-313.

1. INTRODUCTION

Chemical modification studies have indicated that a seryl and a histidyl residue are essential for the activity of CPD-Y from baker's yeast (8, 10) and consequently, the enzyme has been classified as a "serine carboxypeptidase" (EC 3.4.16.) (9). In addition, the active site contains a cysteinyl and a methionyl residue located at the S_1 and S'_1 binding sites, respectively (1, 3, 6, 7), and an additional methionyl residue is presumably located in an as yet unidentified portion of the

active site (7). Chemical modifications of these amino acid residues have produced active derivatives with specificities which deviate from that of CPD-Y and it has been shown that these derivatives with advantage can be used in place of unmodified CPD-Y in certain processes concerning enzymatic peptide synthesis (4, 5, 6). In the present communication the position of the cysteinyl residue and the two methionyl residues in the amino acid sequence is identified.

Abbreviations: CM-CPD-Y = CPD-Y modified with iodoacetic acid; CPD-Y = carboxypeptidase Y; EDTA = ethylene diamine tetraacetic acid, sodium salt; IAA-CPD-Y = CPD-Y modified with iodoacetamide; Met-SO-CPD-Y = CPD-Y containing a single methionyl sulfoxide residue; (Met-SO)₂-CPD-Y = CPD-Y containing two methionyl sulfoxide residues; PTH = phenylthiohydantoin. The binding site notation for the enzyme is that of SCHECHTER and BERGER (19). Accordingly, the binding site for the C-terminal amino acid residue of the substrate is denoted S'_1 and those for the amino acid residues in the amino-terminal direction away from the scissile bond are denoted S_1 , S_2 , ..., S_n .

2. MATERIALS AND METHODS

2.1. Materials

CPD-Y was obtained from Carlsberg Biotechnology Ltd., Tagensvej 16, DK-2200 Copenhagen N, Biogel P-4 and P-60 were from Biorad, USA, iodoacetic acid and iodoacetamide were from Sigma, USA, phenacylbromide was from Fluka, Switzerland, and ^{14}C -iodoacetamide and ^{14}C -iodoacetic acid were from Amersham, England. Reagents and solvents used in the sequencer were obtained from Rathburn, England, except ethylacetate which was from Merck, W. Germany. All other chemicals and solvents were of analytical purity and obtained from Merck, W. Germany.

2.2. Methods

CM-CPD-Y with the cysteinyl residue located in the S_1 binding site alkylated with ^{14}C -iodoacetic acid was prepared in the following way: 20 mg lyophilized CPD-Y was dissolved in 1 ml 0.1 M- NaH_2PO_4 , 6 M-guanidine hydrochloride, 10 mM-EDTA, pH 6.0 and 40 μl 0.05 M- ^{14}C -iodoacetic acid was added. Reaction time: 4 $\frac{1}{2}$ hours. IAA-CPD-Y with the methionyl residue located in the S_1' binding site alkylated by ^{14}C -iodoacetamide was prepared in the following way (6): 6.2 mg/ml CPD-Y, 0.18 M-iodoacetamide, 0.12 M- NaCl , 0.03 M- NaH_2PO_4 , pH 6.5, 22 °C. Reaction time: 4 hours. Met-SO-CPD-Y with the methionyl residue located in the S_1' binding site oxidized by H_2O_2 was prepared as previously described (7): 5.3 mg/ml CPD-Y, 0.1 M- NaH_2PO_4 , 0.19 M- H_2O_2 , pH 4.75, 22 °C. Reaction time: 2 h 20 min. (Met-SO) $_2$ -CPD-Y with two methionyl residues oxidized was prepared in the following way (7): 5.3 mg/ml CPD-Y, 0.1 M-sodium acetate, 0.19 M- H_2O_2 , pH 4.75, 22 °C. Reaction time: 8 hours. The reactions were terminated by separating the enzyme from rea-

gent by gel filtration on Biogel P-4, equilibrated with water. The bed volume was 20 – 30 fold the volume of the sample applied. IAA-CPD-Y, Met-SO-CPD-Y and (Met-SO) $_2$ -CPD-Y were separated from unmodified enzyme by affinity chromatography as previously described (7) and stored frozen in water.

CPD-Y and IAA-CPD-Y were oxidized with performic acid using the following procedure: 1.9 ml 70% HCOOH was mixed with 0.1 ml 30% H_2O_2 , left for two hours at room temperature and then placed on ice. 0.7 ml of this reagent was then added to 10 mg lyophilized enzyme and after two hours reaction at 0 °C 30 ml water was added followed by lyophilization.

Thiolysis of oxidized IAA-CPD-Y was carried out essentially as described by NAIDER and BOHAK (16): 7.5 mg lyophilized oxidized IAA-CPD-Y was dissolved in 6.3 ml 5 mM- NaB_4O_7 , pH 9.0 and 80 μl mercaptoethanol was added. After five hours reaction the mixture was dialyzed against water and lyophilized.

Unmodified CPD-Y, CM-CPD-Y, Met-SO-CPD-Y, (Met-SO) $_2$ -CPD-Y and IAA-CPD-Y were treated with CNBr in the following way: 7 mg lyophilized enzyme was dissolved in 980 μl 70% formic acid and bubbled with nitrogen for 10 min. 35 mg CNBr was added and the solution was purged with nitrogen. The reaction mixture was stirred in the dark for 20 hours at 22 °C and subsequently, nitrogen was bubbled through the solution for three hours. The reaction mixtures were applied to a Biogel P-60 column (1.6 $\times$ 87 cm) equilibrated with 30% acetic acid.

Thermal cleavage of IAA-CPD-Y was performed by addition of boiling water to 1 mg lyophilized IAA-CPD-Y and incubating the enzyme at 110 °C for 70 min essentially as described by LAWSON et al. (13) and MARIANO et al. (14).

Cyanogen bromide fragments were identified

Figure 1. Complete amino acid sequence of CPD-Y based on amino acid sequence studies (15,22) and nucleotide sequence studies (TOM STEVENS, Institute of Molecular Biology, University of Oregon, USA, personal communications, results to be published). The sequences as determined by the two different methods vary at four positions: His-149, Gly-150, Glu-278 and Asp-418 in the amino acid sequence are Gly-149, His-150, Tyr-278 and Gly-418 in the nucleotide sequence. Methionyl residues are underlined, the unblocked cysteinyl residue is indicated by SH and the reactive seryl residue by an asterix, and the residues to which carbohydrate is attached are marked with a diamond.

Lys-Ile-Lys-Asp-Pro-Lys-Ile-Leu-Gly-Ile-Asp-Pro-Asn-Val-Thr-Gln-Tyr-Thr-Gly-Tyr-²⁰
 Leu-Asp-Val-Glu-Asp-Glu-Asp-Lys-His-Phe-Phe-Phe-Trp-Thr-Phe-Glu-Ser-Arg-Asn-Asp-⁴⁰
 Pro-Ala-Lys-Asp-Pro-Val-Ile-Leu-Trp-Leu-Asn-Gly-Gly-Pro-Gly-Cys-Ser-Ser-Leu-Thr-⁶⁰
 Gly-Leu-Phe-Phe-Glu-Leu-Gly-Pro-Ser-Ser-Ile-Gly-Pro-Asp-Leu-Lys-Pro-Ile-Gly-Asn-⁸⁰
 Pro-Tyr-Ser-Trp-Asn-Ser-Asn-Ala-Thr-Val-Ile-Phe-Leu-Asp-Gln-Pro-Val-Asn-Val-Gly-¹⁰⁰
 Phe-Ser-Tyr-Ser-Gly-Ser-Ser-Gly-Val-Ser-Asn-Thr-Val-Ala-Ala-Gly-Lys-Asp-Val-Tyr-¹²⁰
 Asn-Phe-Leu-Glu-Leu-Phe-Phe-Asp-Gln-Phe-Pro-Glu-Tyr-Val-Asn-Lys-Gly-Gln-Asp-Phe-¹⁴⁰
 His-Ile-Ala-Gly-Glu-Ser-Tyr-Ala-His-Gly-Tyr-Ile-Pro-Val-Phe-Ala-Ser-Glu-Ile-Leu-¹⁶⁰
 Ser-His-Lys-Asp-Arg-Asn-Phe-Asn-Leu-Thr-Ser-Val-Leu-Ile-Gly-Asn-Gly-Leu-Thr-Asp-¹⁸⁰
 Pro-Leu-Thr-Gln-Tyr-Asn-Tyr-Tyr-Glu-Pro-Met-Ala-Cys-Gly-Glu-Gly-Gly-Glu-Pro-Ser-²⁰⁰
 Val-Leu-Pro-Ser-Glu-Glu-Cys-Ser-Ala-Met-Glu-Asp-Ser-Leu-Glu-Arg-Cys-Leu-Gly-Leu-²²⁰
 Ile-Glu-Ser-Cys-Tyr-Asp-Ser-Gln-Ser-Val-Trp-Ser-Cys-Val-Pro-Ala-Thr-Ile-Tyr-Cys-²⁴⁰
 Asn-Asn-Ala-Gln-Leu-Ala-Pro-Tyr-Gln-Arg-Thr-Gly-Arg-Asn-Val-Tyr-Asp-Ile-Arg-Lys-²⁶⁰
 Asp-Cys-Glu-Gly-Gly-Asn-Leu-Cys-Tyr-Pro-Thr-Leu-Gln-Asp-Ile-Asp-Asp-Glu-Leu-Asn-²⁸⁰
 Gln-Asp-Tyr-Val-Lys-Glu-Ala-Val-Gly-Ala-Glu-Val-Asp-His-Tyr-Glu-Ser-Cys-Asn-Phe-³⁰⁰
 Asp-Ile-Asn-Arg-Asn-Phe-Leu-Phe-Ala-Gly-Asp-Trp-Met-Lys-Pro-Tyr-His-Thr-Ala-Val-³²⁰
 Thr-Asp-Leu-Leu-Asn-Gln-Asp-Leu-Pro-Ile-Leu-Val-Tyr-Ala-Gly-Asp-Lys-Asp-Phe-Ile-³⁴⁰
 SH
 Cys-Asn-Trp-Leu-Gly-Asn-Lys-Ala-Trp-Thr-Asp-Val-Leu-Pro-Trp-Lys-Tyr-Asp-Glu-Glu-³⁶⁰
 Phe-Ala-Ser-Gln-Lys-Val-Arg-Asn-Trp-Thr-Ala-Ser-Ile-Thr-Asp-Glu-Val-Ala-Gly-Glu-³⁸⁰
 Val-Lys-Ser-Tyr-Lys-His-Phe-Thr-Tyr-Leu-Arg-Val-Phe-Asn-Gly-Gly-His-Met-Val-Pro-⁴⁰⁰
 Phe-Asp-Val-Pro-Glu-Asn-Ala-Leu-Ser-Met-Val-Asn-Glu-Trp-Ile-His-Gly-Asp-Phe-Ser-⁴²⁰

Figure 2. Gel filtration of the peptides obtained by cyanogen bromide cleavage of unmodified CPD-Y. Column: 1.6×87 cm Biogel P-60, equilibrated with 30% acetic acid. Flow-rate: 9 ml/hour. Fraction size: 1.7 ml. The fractions were pooled as indicated. The absorbance at 280 nm was measured.

by amino acid analysis and by the N-terminal amino acid sequences as determined using a Beckman 890 C liquid phase sequencer as previously described by JOHANSEN et al. (11). Identification of PTH amino acids was accomplished by high performance liquid chromatography as described by SVENDSEN et al. (21). Amino acid analysis was performed after acid hydrolysis for 24 hours in 6 M-HCl using a Durrum D 500 amino acid analyzer and ^{14}C -measurements were performed by liquid scintillation using a Beckman scintillation counter, Model LS 3155 T.

3. RESULTS

3.1. Cyanogen bromide fragments of CPD-Y

CPD-Y contains five methionyl residues and their positions in the complete amino acid sequence as determined by protein sequencing (15, 22) and nucleotide sequencing (T. STEVENS, personal communications) is illustrated in Figure 1. Unmodified CPD-Y was cleaved with cyanogen bromide and the resultant peptide mixture was subjected to automated Edman degradation. The PTH amino acids present in the first five steps were consistent with the existence of equimolar amounts of the six peptides predicted from Figure 1. The six peptides

could be separated in three fractions by chromatography on Biogel P-60 (Figure 2). Fraction I contained two peptides, 1-191 and 211-313 (Figure 1), fraction II only one peptide, 314-398, while fraction III contained three small peptides, 192-210, 399-410 and 411-421. The amino acid analysis of each of the fractions indicated that all the peptides were present in essentially equimolar amounts.

3.2. Cyanogen bromide fragments of Met-SO-CPD-Y

It is known that cyanogen bromide does not cleave peptide chains at methionyl residues which have been converted to methionyl sulfoxide (17). Thus, the peptide mixture obtained from cyanogen bromide cleavage of Met-SO-CPD-Y with a single methionyl residue oxidized by treatment with H_2O_2 would be expected to contain only five peptides. Edman degradation of this mixture of peptides indicated the existence of equimolar amounts of all the peptides produced from unmodified CPD-Y with the exception of peptide 399-410. The peptides were separated in three fractions by chromatography on Biogel P-60 and the elution profile was similar to that in Figure 2. However, Edman degradation of the peptides in fraction III confirmed that peptide 399-410 was absent suggesting that no cleavage at Met-398 had taken place. This is supported by the fact that the amino acid composition of the peptide eluting in fraction II is consistent with the amino acid composition of peptide 314-410 and not with that of 314-398. These results indicate that Met-398 is specifically oxidized by H_2O_2 .

3.3. Cyanogen bromide fragments of $(\text{Met-SO})_2$ -CPD-Y

Edman degradation of the peptide mixture obtained by cyanogen bromide cleavage of $(\text{Met-SO})_2$ -CPD-Y indicated that peptide 399-410 was absent and that peptide 314-398 was present only in small quantities, i.e. below 10% of the amount of the N-terminal fragment of CPD-Y. The peptides were separated in three fractions by chromatography on Biogel P-60 (Figure 3) and it is seen that the peak eluting in

Figure 3. Gel filtration of the peptides obtained by cyanogen bromide cleavage of (Met-SO)₂-CPD-Y. Column and conditions as listed in Figure 2.

fraction II is very small relative to that observed in Figure 2, suggesting that only very little cleavage had taken place at Met-313. Edman degradation of the peptides eluting in fraction III demonstrated the absence of peptide 399-410, suggesting that no cleavage had taken place at Met-398. These results indicate that it is Met-398 and Met-313 which are oxidized in (Met-SO)₂-CPD-Y.

3.4. Cyanogen bromide fragments of IAA-CPD-Y

Since alkylated methionyl residues are resistant towards oxidation (17) ¹⁴C-IAA-CPD-Y with a single methionyl residue alkylated by iodoacetamide (6) was oxidized with performic acid to convert unmodified methionyl residues to methionyl sulfone (see section 2.2). The alkyl group was then removed from the alkylated methionyl residue by treatment with mercaptoethanol (see section 2.2) followed by cyanogen bromide cleavage. Edman degradation of the resulting preparation indicated the N-terminus of the enzyme and in addition, in equimolar amounts, an additional peptide with the N-terminal sequence Val-Pro-Phe-Asp-Val-. When

Figure 4. Gel filtration of the peptides obtained by cyanogen bromide cleavage of ¹⁴C-IAA-CPD-Y. Column and conditions as listed in Figure 2. Absorbance at 280 nm (●-●-) and radioactivity (□-□-) was measured. The radioactivity was expressed as cpm/ml eluate.

unmodified CPD-Y was oxidized and treated with cyanogen bromide the Edman degradation indicated only the N-terminus of the enzyme. These results suggest that it is Met-398 which is alkylated by iodoacetamide.

In a different series of experiments ¹⁴C-IAA-CPD-Y was treated with cyanogen bromide and the resulting peptides were then separated in three fractions by chromatography on Biogel P-60 (Figure 4). It is apparent that the predominant part of the radioactivity eluted in Fraction II indicating that the alkylated methionyl residue is located in the peptide with Lys-Pro-Tyr-His-Thr- as the N-terminal sequence and the amino acid composition indicated that no cleavage had taken place at Met-398. To demonstrate conclusively the location of the alkylated methionyl residue it was utilized that peptides may be cleaved under mild conditions at alkylated methionyl residues by a mechanism similar to that responsible for the cleavage with cyanogen bromide (13, 14). When fraction II was boiled in water cleavage took place at Met-398 producing a new N-terminal sequence, Val-Pro-Phe-Asp-Val-. Thus, Met-398 is the site of reaction with iodoacetamide.

3.5. Cyanogen bromide fragments of CM-CPD-Y

CPD-Y treated with ¹⁴C-iodoacetic acid under denaturing conditions in order to carboxyme-

Fig. 5. Gel filtration of the peptides obtained by cyanogen bromide cleavage of ^{14}C -CM-CPD-Y. Column and conditions as listed in Figure 2. Absorbance at 280 nm ($\bullet$ - $\bullet$ - $\bullet$) and radioactivity ($\square$ - $\square$ - $\square$) was measured. The radioactivity was expressed as cpm/ml eluate.

thylate the single cysteinyl residue was treated with cyanogen bromide and the resulting peptides separated in three fractions by chromatography on Biogel P-60 (Figure 5). The majority of the radioactivity eluted in fraction II and in addition, this fraction was the only one found to contain CM-Cys as determined by amino acid analysis. This shows that the single free cysteinyl residue is located in the cyanogen bromide fragment 314-398. This was already anticipated from the finding that this peptide contained only a single cysteinyl residue and separated from the other cyanogen bromide fragments during gel filtration (15). The cysteinyl residue in this fragment is located at position 341 (see Figure 1) and to confirm that this residue had been modified by iodoacetic acid, fragment 314-398 was subjected to automated Edman degradation and the radioactivity of the PTH amino acid released in each step determined. It was found that all the radioactivity was attached to the PTH amino acid residue in step 28 and the HPLC identification showed a CM-Cys residue. Thus, the single cysteinyl residue of CPD-Y is located at position 341.

4. DISCUSSION

It has previously been shown that chemical modification of two methionyl residues and a single cysteinyl residue in CPD-Y causes alter-

ations in the enzymatic properties of the enzyme (1, 3, 6, 7) and hence, it may be inferred that these amino acid residues are located in the active site of the enzyme. In the present paper they have been identified as Met-313, Met-398 and Cys-341. Since the reactive seryl residue of CPD-Y is located at position 146 (22) it can be concluded that the active site is formed by amino acid residues which are remote from each other in the amino acid sequence.

Chymotrypsin (12, 18) and subtilisin (20) have been shown each to contain a methionyl residue which may be oxidized by H_2O_2 with alterations in the enzymatic properties as a consequence. However, the amino acid sequences around these methionyl residues exhibit no analogies with those around Met-313 and Met-398 in CPD-Y.

Numerous serine carboxypeptidases and two serine endopeptidases are known to contain a cysteinyl residue, suggesting that this group has a function, although apparently not in catalysis as indicated by the studies with CPD-Y (1, 3). The location of the cysteinyl residue has previously been identified only in thermitase from *Thermoactinomyces vulgaris* (2). However, the amino acid sequence around this amino acid residue exhibits no analogies with that around Cys-341 in CPD-Y, suggesting that their presence is due to a convergent evolution.

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