Bull. Chem. Soc. Jpn., 73, 2587—2590 (2000)

Interaction Mode of H397A Mutant Carboxypeptidase Y with Protein Substrates Analyzed by the Surface Plasmon Resonance

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(Received July 3, 2000)

In order to study the substrate binding mode and subsite properties of carboxypeptidase Y (CPY), the dissociation and association constants, K_D and K_A, of its catalytically inactive mutant, H397A, were determined by surface plasmon resonance with protein substrates, i.e., α-casein, RCM-RNase A, RCM-lysozyme, and RCM-BSA. K_D values obtained for four substrates were in a range of 10^{-8} to 10^{-7} M, being equal to K_m values of catalytically active wild-type CPY for the same protein substrates. These results suggest that the acylation step is a rate-limiting step in the CPY-catalyzed hydrolysis of protein substrates. Small substrates like *N*-acylated dipeptides gave K_m values in a range of 10^{-4} to 10^{-2} M, considerably smaller than those for protein substrates, suggesting the presence of additional subsites in addition to S'_1 and S_1 in the substrate binding pocket of CPY.

Carboxypeptidase Y from bakers' yeast (CPY) catalyzes a stepwise removal of C-terminal amino acid from peptide or protein substrates. The preference of this enzyme toward amino acid placed at the C-terminal (P'_1) and penultimate (P_1) positions of the substrates and the properties of the corresponding S'_1 and S_1 subsites are studied.[1,2] Recently it has been shown that CPY hydrolyzes heptapeptide substrates at a rate 52,000-fold faster than the dipeptides,[3] suggesting that extra subsites, S_2 to S_5, might be responsible for such a drastic increase in rate constant. Crystallographic study on a complex of chymostatin and carboxypeptidase WII, which is classified to serine carboxypeptidase group and homologous to CPY with respect to structure and function, indicates that the enzyme binds P_2 through P_4 positions in addition to P_1 and P'_1.[4]

Metallo-carboxypeptidases, i.e., carboxypeptidase A (CPA) and carboxypeptidase B (CPB), also interact with oligopeptides by five and six subsites, respectively.[5] However, S'_1 subsite of them strongly binds the C-terminal carboxylate group of the peptide substrate via salt linkage with Arg145 of CPA and CPB, so that any contribution of additional subsites (S_1—S_4 or S_5) to bind the peptide substrate is weak.

The present study focuses on the evaluation of the affinity of CPY to protein substrates by means of surface plasmon resonance (SPR) instruments, such as BIAcore, in order to analyze the detailed size and properties of subsites in CPY. Dissociation constants (K_D) for protein substrates are determined in the following strategy. A catalytically inactive mutant CPY, H397A, in which an essential component of the catalytic triad, His397,[6] is replaced to alanine residue by site-directed mutagenesis, keeping its gross structure identical with those of the wild-type CPY as demonstrated previously,[7] is immobilized on a sensor chip via carbohydrate moieties. Since all carbohydrate moieties locate at the one side of the protein molecule, opposite from the active site cavity,[8] the substrate-binding pocket is considered fully available to incoming substrates in the BIAcore instrument to perform protein-substrate interaction.

Material and Methods

Materials. Wild-type CPY and a mutant CPY, H397A, were prepared as described previously.[1,7] Bovine serum albumin (BSA), bovine pancreatic ribonuclease A (RNase A), and egg white lysozyme were purchased from Sigma (Missouri, USA) and reduced and carboxymethylated (RCM) according to Crestfield et al.[9] α-Casein was obtained from Sigma. 6-[6-(Biotinoylamino)-hexanoylamino]hexanoic acid hydrazide (biotin-XX hydrazide) was the product of Molecular Probes (Oregon, USA). BIAcore X instrument, its Sensor Chip SA5, and HEPES buffer saline (HBS), containing 10 mM HEPES pH 7.4, 150 mM NaCl, 3.4 mM EDTA ($M = mol\,dm^{-3}$), and 0.005%(v/v) Surfactant P20, were obtained from Pharmacia Biosensor AB (Uppsala, Sweden). CPY-specific inhibitor (I_c)[10] was kindly supplied by J. Mima, Kyoto University. Fmoc–$(Glu)_5$Ala–OH was synthesized by a solid phase synthesizer of Pioneer (PerSeptive Biosystems, Massachusetts, U.S.A.) and purified by an HPLC using a Shimadzu C18 column (4.6×150 mm). Amino acid composition and the purity of this Fmoc–$(Glu)_5$Ala–OH were confirmed by amino acid analysis.

Biotinylation of Wild-Type and H397A CPY. Carbohydrate moieties of wild-type CPY and H397A mutant were biotinylated according to Hsiao and Royer[11] as follows: Enzyme (200 μg) was incubated in 200 μl of 18 mM sodium periodate at 4 °C for 1 h in the dark. After excess reagent was removed by ultrafiltration with Centricon 30 (Amicon, Beverly, U.S.A.), the solvent was replaced by 190 μM biotin-XX hydrazide and the mixture was incubated at room temperature for 1 day. Finally the solvent was replaced by water with ultrafiltration. The degree of biotinylation was determined

by the published procedure.[12] Enzyme activities were measured toward *N*-benzoyl-L-tyrosine-*p*-nitroanilide by the method described previously.[1]

Immobilization of H397A on the Sensor Chip Surface. Biotinylated H397A CPY was immobilized in a BIAcore X chamber as follows: Thirty-five micro liters of the biotinylated H397A CPY (200 μg ml^{-1} of 0.3 M NaCl) was introduced to the SA5 Sensor Chip surface, on which streptavidin was pre-immobilized, while the flow was maintained at 5 μl min^{-1}. Finally, the sensor chip surface was washed by injecting 20 μl of 0.5 M NaCl.

Interaction of Immobilized CPY with Protein Substrates. To analyze association phase, concentrations of α-casein, RCM-BSA, RCM-RNase A, RCM-lysozyme, and RCM-insulin β-chain were adjusted to 10 to 60 μg ml^{-1} in HBS, and the solutions were loaded onto the H397A CPY-immobilized sensor chip surface at a flow rate of 10 μl min^{-1} for 4 min. Then, to analyze the dissociation phase, HBS was injected at a flow rate of 10 μl min^{-1} for 6 min or more. These operations were conducted at 25 °C. At the end of the dissociation procedure, the sensor chip surface was regenerated by being exposed to 0.5 M NaCl for 10 min.

Association and dissociation rates (k_{ass} and k_{diss}, respectively) and association and dissociation constants (K_A and K_D, respectively) between H397A CPY and protein substrates were calculated by using BIA-evaluation software (Pharmacia Biosensor AB). To estimate these parameters for Fmoc-(Glu)$_5$Ala-OH, the mixture of RCM-protein (RCM-BSA or RCM-lysozyme) and Fmoc-peptide were loaded as described above and changes in these parameters were analyzed by the same software using a heterogeneous analyte model which is designed to analyze the competitive interaction of two analytes.

Kinetics of RCM-Protein Hydrolysis with Wild-Type CPY. RCM-RNase A or RCM-lysozyme (0.1 to 5.5 μM) was incubated with wild-type CPY (1.6 nM) in 50 mM MES buffer (pH 6.5) at 25 °C for 2 min. Reaction was stopped by an addition of 10% 5-sulfosulicylic acid to make a final concentration of 2.5%. After a brief centrifugation, the supernatant was adjusted to pH 2.2 with 5 M NaOH and subjected to amino acid analysis with a JEOL JLC-500/V amino acid analyzer. Under assay conditions, only the C-terminal amino acid was released from the substrate with a linear increase for 2-min incubation. K_m and k_{cat} were estimated from the rate of C-terminal amino acid release according to Lineweaver-Burk plots.

Results

Biotinylation and Immobilization of CPY. The biotinylated wild-type CPY was fully active. This shows that biotinylation step does not damage any function of CPY. The biotinylated H397A CPY (1.26 mol biotin/mol CPY) was immobilized on a sensor chip surface to yield a sufficient level of immobilization (2,200 resonance units in 7-min introduction).

Sensorgrams of H397A CPY with Protein and Peptide Substrates. Sensorgrams showed that immobilized H397A CPY interacted with native α-casein, RCM-BSA, RCM-RNase A, and RCM-lysozyme, whereas the native forms of these three proteins showed no interaction (see Fig. 1, line 1—5 for an example). When RCM-insulin β-chain was applied there were no change of the sensorgram, although wild-type CPY releases its C-terminal amino acid.[13] This is probably because the molecular mass of RCM-insulin β-chain, 3.5 kDa, is too small to be differentiated on the SPR sensorgram (Da = u).

These changes of sensorgram were diminished when I_c was injected prior to the injection of protein substrates (Fig. 1, line 6). These changes were weakened in the presence of the Fmoc-(Glu)$_5$Ala-OH, toward which wild-type CPY showed K_m of 1.3×10^{-7} M, and they were lost in the presence of 100-fold excess amount of the Fmoc-peptide (Fig. 1, line 7,

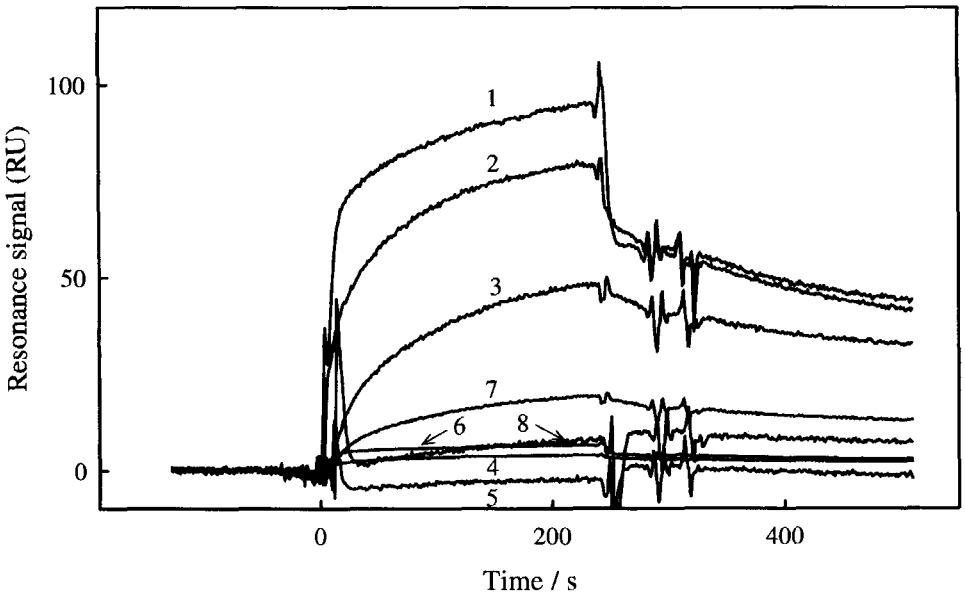

Fig. 1. Superimposed sensorgrams with immobilized H397A CPY obtained at several concentration of RCM-BSA and Fmoc-(Glu)$_5$Ala-OH. After initial equilibration of the sensor chip surface with HBS, RCM-BSA solution (40 μl) was injected in the presence or absence of Fmoc-(Glu)$_5$Ala-OH. At 240 s HBS was injected and the dissociation phase was monitored for 260 s. Lines 1, 2, 3, and 4 show 10, 4, 1, and 0 μM RCM-BSA, respectively; line 5, 10 μM native BSA; line 6, 10 μM RCM-BSA after 25 μM I_c injection; line 7and 8, 1 μM RCM-BSA in the presence of 1 and 100 μM Fmoc-peptide, respectively.

8).

Association and Dissociation Constants of H397A CPY with Protein and Peptide Substrates. Table 1 summarizes kinetic and thermodynamic parameters of the interaction of immobilized H397A CPY with protein and Fmoc-peptide substrates. K_D values for α-casein, RCM-BSA, RCM-lysozyme, and Fmoc–$(Glu)_5$Ala–OH were nearly equal in the order of 10^{-7} M in spite of the differences of their C-terminal amino acid sequences. K_D value for RCM-RNase A (7.4×10^{-8} M) was smaller by one order than those for other substrates, reflecting its high k_{ass} value.

Thermodynamic and kinetic parameters for RCM-proteins in the presence of Fmoc–$(Glu)_5$Ala–OH obtained by the heterogeneous analyte model were the same as those for RCM-proteins in the absence of Fmoc-peptide. This result shows that the Fmoc-peptide interacts competitively with protein substrates and the immobilized H397A CPY correctly recognizes the C-terminal region of protein substrates.

Kinetics of RCM-Protein Hydrolysis by Wild-Type CPY. Table 2 shows K_m and k_{cat} in the wild-type CPY-catalyzed release of C-terminal amino acid from RCM-proteins. k_{cat} values for RCM-lysozyme and RCM-RNase A are similar to those for *N*-blocked dipeptide substrates (1 to 20 s^{-1})[1,14] in the range of 10-times difference. However, K_m values for RCM-lysozyme and RCM-RNase A are much smaller by approximately 10^{-3} to 10^{-2} times than those for the *N*-blocked dipeptide substrates (10^{-4} to 10^{-3} M),[1,14] although they showed similar values with K_D values obtained in the present SPR measurement in the range of 20-times difference.

Discussion

The gross structure of H397A CPY is identical with that of the wild-type CPY as demonstrated previously.[7] The present results showed that Fmoc–$(Glu)_5$Ala–OH interacted competitively with immobilized H397A CPY. Immobilization process caused neither damages of functionalities nor effects on substrate binding of CPY, because H397A was immobilized by the use of carbohydrate moiety, which locates far from the active site, with a long linker. Our previous results also showed that the deletion of carbohydrate moiety did not affect the structure and function of CPY.[15] Therefore, we conclude that the subsite and substrate-binding mode of wild-type CPY are preserved in the immobilized mutant CPY. This judgment is also supported by the fact that biotinylated wild-type CPY was fully active. Therefore, K_D values should reflect the true dissociation constant between immobilized H397A and protein substrates.

Table 2. Kinetic Parameters for CPY-Catalyzed Release of C-Terminal Amino Acid from RCM-Proteins

The reaction was performed in 0.05 M MES, pH 6.5, at 25 °C for 2 min and release of Val and Leu in RCM-RNase A and RCM-lysozyme, respectively, was determined with an amino acid analyzer. The k_{cat} and K_m were determined at substrate concentrations from 0.1 to 5.5 μM using 1.6 nM CPY.

Protein	k_{cat}/s^{-1}	K_m/M
RCM-RNase A	3.2 ± 0.2	$1.5 \pm 0.2 \times 10^{-6}$
RCM-lysozyme	1.4 ± 0.1	$4.3 \pm 0.3 \times 10^{-7}$

CPY follows a typical catalytic mechanism of serine protease, which hydrolyzes peptide or ester substrates in the following three steps[16]:

$$E+S \underset{k_{diss}}{\overset{k_{ass}}{\rightleftharpoons}} ES \xrightarrow{k_2} ES_1 \xrightarrow{k_3} E+P_2 \qquad (1)$$
$$\downarrow P_1$$

where E, S, P, and ES_1 represent enzyme, substrate, product, and acyl-enzyme intermediate, respectively. In this equation, K_m, K_D, and k_{cat} are expressed as follows:

$$K_m = (k_{diss} + k_2)k_3/\{k_{ass}(k_2 + k_3)\} \qquad (2)$$

$$K_D = k_{diss}/k_{ass} \qquad (3)$$

$$k_{cat} = k_2k_3/(k_2 + k_3) \qquad (4)$$

Table 1. Thermodynamic and Kinetic Parameters of H397A CPY for Protein and Fmoc–$(Glu)_5$Ala–OH Substrates Obtained by SPR Analysis

The reaction was performed in HBS at pH 7.4, 25 °C. The association and dissociation constants (K_A and K_D, respectively) were obtained by the following equations; $K_A = k_{ass}/k_{diss}$ and $K_D = k_{diss}/k_{ass}$. k_{ass} and k_{diss} values for protein substrates were determined at the concentration from 10 to 60 μg $ml.^{-1}$ k_{ass} and k_{diss} values for Fmoc-peptide were estimated by heterogeneous analyte model at the concentration from 1.0 to 20 μM in the presence of 60 μg ml^{-1} protein substrates.

Analyte (C-terminal sequence)	k_{ass} $M^{-1}s^{-1}\times10^3$	k_{diss} $s^{-1}\times10^{-4}$	K_A $M^{-1}\times10^6$	K_D $M\times10^{-7}$
α-Casein (–Thr–Thr–Met–Pro–Leu–Trp–OH)	3.5 ± 0.3	9.5 ± 1.0	3.7 ± 0.7	2.6 ± 0.5
RCM-BSA (–Thr–Gln–Thr–Ala–Leu–Ala–OH)	1.3 ± 0.2	7.6 ± 0.9	1.7 ± 0.5	5.8 ± 1.3
RCM-RNase A (–His–Phe–Asp–Ala–Ser–Val–OH)	9.7 ± 0.8	7.2 ± 0.7	13 ± 2.7	0.74 ± 0.12
RCM-lysozyme (–Ile–Arg–Gly–CM·Cys–Arg–Leu–OH)	2.0 ± 0.1	8.2 ± 0.9	2.4 ± 0.3	4.1 ± 0.6
RCM-BSA + Fmoc–$(Glu)_5$Ala–OH				
RCM-BSA	1.5 ± 0.3	7.5 ± 0.8	2.0 ± 0.5	5.0 ± 1.3
Fmoc-peptide	1.2 ± 0.2	6.2 ± 0.5	2.0 ± 0.4	5.2 ± 1.1
RCM-lysozyme + Fmoc–$(Glu)_5$Ala–OH				
RCM-lysozyme	1.9 ± 0.3	8.0 ± 1.0	2.4 ± 0.6	4.2 ± 1.0
Fmoc-peptide	1.6 ± 0.2	6.0 ± 0.7	2.7 ± 0.6	3.8 ± 0.8

The present result demonstrates that K_D and K_m values derived from two independent methods agree well for the protein substrates. This agreement indicates that acylation step (k_2) is a rate-limiting step in the CPY-catalyzed hydrolysis of protein substrates (see Eqs. 2 and 3).

CPY shows higher affinity for RCM-proteins and Fmoc-hexapeptide than for *N*-acylated dipeptide substrates, while the k_{cat} values for protein and *N*-acylated dipeptide substrates are nearly the same. This suggests that CPY has extended subsites in addition to S_1' and S_1 subsites. Agreement of K_D values for RCM-proteins and Fmoc-hexapeptide indicates that CPY has at least six subsites (S_1' and S_1—S_5).

It is of interest to note that K_m values in CPY-catalyzed hydrolyses of peptides are highly dependent on the length of peptide, while those in metallo-carboxypeptidases, CPA and CPB, catalyzed hydrolyses of di- and oligopeptides are nearly equal although they have five and six subsites, respectively.[5] This is due to the difference in the C-terminal carboxylate-binding mode between metallo-carboxypeptidases and serine carboxypeptidases. The former CPA and CPB show a strong salt linkage between the C-terminal carboxylate ion of the substrate and the side chain of Arg145 which functions as primary subsite,[5a,17] so additional subsites play a minor role in the substrate recognition. On the other hand, the latter CPY utilizes hydrogen bonds, which has a weaker interaction than salt linkage, to recognize C-terminal carboxylate: the backbone amide of Gly52 and the side chain of Asn51 and Glu145 in CPY donate hydrogen bonds to the carboxylate of the substrate.[18] Therefore, CPY needs additional subsites in substrate binding to compensate for the weak interaction of C-terminal carboxylate group in the substrate to the enzyme.

The multi-subsite which produces high affinity to protein substrates, being independent of the kind of C-terminal amino acid residues, makes the specificity of CPY broad enough to degrade unneeded proteins in vacuole.

References

1 R. Hayashi, "Methods in Enzymology," ed by L. Lorand, Academic Press, New York (1976), Vol. 45, p. 568.

2 a) K. Olesen, U. H. Mortensen, S. Aasmul-Olsen, M. C. Kielland-Brandt, M. S. J. Remington, and K. Breddam, *Biochemistry*, **33**, 11121 (1994). b) S. B. Sørensen, M. Raaschou-Nielsen, U. H. Mortensen, S. J. Remington, and K. Breddam, *J. Am. Chem. Soc.*, **117**, 5944 (1995).

3 K. Olesen, M. Meldal, and K. Breddam, *Protein Pep. Lett.*, **3**, 67 (1996).

4 T. L. Bullock, K. Breddam, and S. J. Remington, *J. Mol. Biol.*, **255**, 714 (1996).

5 a) J. A. Hartsuck and W. N. Lipscomb, "The Enzymes," ed by P. D. Boyer, Academic Press, New York (1971), Vol. 3, p. 1. b) Y. Sukegawa, H. Akanuma, and M. Yamasaki, *J. Biochem.*, **87**, 1691 (1980).

6 G. Jung, H. Ueno, R. Hayashi, and T.-H. Liao, *Protein Sci.*, **4**, 2433 (1995).

7 G. Jung, H. Ueno, and R. Hayashi, *J. Biochem.*, **124**, 446 (1998).

8 J. A. Endrizzi, K. Breddam, and S. J. Remington, *Biochemistry*, **33**, 11106 (1994).

9 A. M. Crestfield, S. Moore, and W. H. Stein, *J. Biol. Chem.*, **238**, 622 (1963).

10 A. W. Bruun, Ib. Svendsen, S. O. Sørensen, M. C. Kielland-Brandt, and J. R. Winther, *Biochemistry*, **37**, 3351 (1998).

11 H.-Y. Hsiao and G. P. Royer, *Arch. Biochem. Biophys.*, **198**, 379 (1979).

12 J. A. Cifonelli, *Carbohyd. Res.*, **8**, 233 (1968).

13 R. Hayashi, S. Moore, and W. H. Stein, *J. Biol. Chem.*, **248**, 2296 (1973).

14 R. Hayashi, Y. Bai, and T. Hata, *J. Biochem.*, **77**, 69 (1975).

15 H. Shimizu, H. Ueno, and R. Hayashi, *Biosci. Biotechnol. Biochem.*, **63**, 1045 (1999).

16 Y. Bai, R. Hayashi, and T. Hata, *J. Biochem.*, **78**, 617 (1975).

17 M. F. Schmid and J. R. Herriott, *J. Mol. Biol.*, **103**, 175 (1976).

18 a) U. H. Mortensen, S. J. Remington, and K. Breddam, *Biochemistry*, **33**, 508 (1994). b) D. I. Liao, K. Breddam, R. M. Sweet, T. Bullock, and S. J. Remington, *Biochemistry*, **31**, 9796 (1992).