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Cleavage of CCK 33 by Recombinant PC2 *in Vitro*

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The specificity and kinetic properties of CCK 33 cleavage by recombinant prohormone convertase 2 (PC2) was investigated using a combination of Sephadex G-50 chromatography, HLPC and RIA methods. It is shown that CCK 33 can be cleaved effectively by PC2 to form CCK 8, the reaction of which has a K_m of 104.8 μM. No CCK 22 or other products were detected and the reaction is completely inhibited by the PC2 inhibitor, p-CMS (p-chloromercuriphenylsulfonic acid), suggesting that the cleavage is PC2 specific. The time course of this reaction shows an initial lag phase followed by a rapid increase in velocity consistent with a previously reported spontaneous transformation from a 71 kDa relatively inactive form into a more active form of 62 kDa (1). PC2 did not cleave recombinant pro CCK or CCK 1-21. These results demonstrate that PC2, an enzyme usually associated with dibasic cleavages, can cleave easily at single basic residues.

Cholecystokinin (CCK), a neuropeptide which exists in both gastrointestinal and central nervous systems, has become increasingly important due to its functional significance in mammalian brain. A large body of evidence has shown that CCK may act as a neurotransmitter and play a role in anxiety, memory and satiety (2). The biosynthesis of pro CCK includes the sulfation of three tyrosine residues, the recognition of pro CCK as secretory protein and its sorting to secretory granules. The processing of pro CCK involves a number of specific endoproteolytic cleavages at mono (arginine or lysine) or dibasic residues, followed by the amidation of the carboxyl-terminal, yielding the active product, CCK 8.

Two aspects of the structure of pro CCK are unusual: the sulfated tyrosine residues and the fact that 3 out of 5 of the cleavage sites contain single Arg or Lys residues. It was originally thought that these single basic cleavages were performed by different enzymes than those which cleaved at dibasic sites. The enzymes which are responsible for the processing of pro CCK have not been found, but a number of candidates have been identified from previous studies. One is the so-called CCK 8 generating enzyme (CGE), an endoprotease purified from rat brain synaptosomes. CGE has been shown to cleave CCK 33 at an Arg-Asp site to generate CCK 8. This enzyme will also make the Asn-Lys cleavage of CCK 33 to generate CCK 22 and cleave CCK 1-21 at the same site (3). It is possible that CGE is involved in the post-translational processing of pro CCK due to its specificity and subcellular location. The cloning of CGE is still in progress.

Another enzyme which will make this cleavage is Yeast Aspartic Protease 3 (YAP3). Previous studies have shown the ability of this enzyme to cleave the paired-basic residues of pro-opiomelanocortin (POMC) to produce adrenocorticotropin (ACTH) (4). A more recent study has shown that YAP3 can also cleave CCK 33 at Lys-Asn bond to yield CCK 22 and at Arg-Asp to generate CCK 8 (5). The mammalian equivalent of YAP3 has not been identified.

Two additional candidates for the processing of pro CCK are the subtilisin-like enzymes PC1 and PC2 (6,7). Both PC1 and PC2 have been found in a number of endocrine tumor cells which express and process pro CCK to CCK 8 (8). The aim of present study is to explore enzymatic reaction of PC2 with CCK 33, so as to aid in better understanding of pro CCK processing as a whole. Availability of both purified recombinant PC2 and synthetic CCK 33 allow us to examine specific *in vitro* cleavage in detail.

MATERIALS AND METHODS

Materials. Synthetic CCK 33 and porcine CCK 1-21 (KAPSGRVSMIKNLQS LDPSHR) was obtained from Peninsula Laboratories, Inc. (Belmont, CA). CCK 22 was made by enzymatic cleavage of CCK 33 by Endoproteinase Lys-C (Sigma) in 0.1 M Tris, pH 8.5 at 37°C overnight. Purified recombinant PC2 (0.0575 mg/ml) was the gener-

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Abbreviations: MCA, methylcoumarin amide; AMC, aminomethylcoumarin; PC2, Prohormone Convertase 2; p-CMS, p-chloromercuriphenylsulfonic acid.

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ous gift of Dr. Iris Lindberg (New Orleans, Louisiana) and CCK 8 was purchased from Bachem (Torrence, CA). HPLC column (Reverse phase C18) was obtained from Vydac (Hesperia, CA).

Cleavage of CCK 33 by PC2. CCK 33 at various concentrations was incubated with PC2 (0.28 μg) in 100 mM sodium acetate buffer (pH 5.0), containing 5 mM $CaCl_2$, 0.1% Brij (Sigma) in 500 μl polypropylene microcentrifuge tubes. Samples were frozen immediately after each incubation period at −20°C for subsequent analysis by Sephadex G-50 chromatography and HPLC. For the inhibition experiment, PC2 was preincubated with PC2 inhibitor (p-CMS) (100 μM) at 37°C for 15 min prior to the addition of CCK 33. PC2 activity was periodically checked by its ability to cleave 100 μM Pyr-Arg-Thr-Lys-Arg-MCA (Peptide Institute, Inc., Japan) at 37°C over night in the incubation buffer mentioned above. The AMC released was measured by spectrofluorophotometry with excitation of 380 nm and emission of 460 nm.

Sephadex column chromatography. Frozen samples of the incubation mixtures were thawed at 4°C and an equal volume of the elution buffer were added to the samples prior to applying to the column. A 2.5 × 44 cm G-50 Sephadex column was eluted with 50 mM Tris, pH 7.8, containing 200 mM NaCl, 0.02% Sodium Azide and 0.1 % BSA. Fractions of 1.5 ml were collected at 4°C at a flow rate of 0.4 ml/min and analyzed for CCK by RIA.

HPLC. CCK 33 samples incubated with PC2 along with some CCK standards were analyzed by HPLC (Waters 600, multisolvent Delivery System from Millipore) with elution buffer A, 0.09% trifluoroacetic acid (TFA) and buffer B, 90% acetonitrile in 0.09% TFA. Fifty five 1 ml fractions were collected and 25 μl aliquots were taken from each fraction and dried in a Speedvac concentrator for about 30 min. The pellets were then assayed by RIA CCK. The elution from HPLC were also monitored by absorption at 214 nm and was compared with the RIA measurements.

Radioimmunoassay (RIA). The CCK RIA was performed as previously described (9).

RESULTS AND DISCUSSION

Samples of CCK 33 incubated with PC2 were analyzed by Sephadex G-50 column chromatography and the results are shown in Figure 1. The arrows indicate the positions of elution of CCK 33, CCK 22 and CCK 8 standards. The G-50 column chromatography profile of CCK 33 shows a single peak at fraction 40, while, the CCK 33 incubated with PC2 reveals an additional peak at fraction number 60, which coelutes with CCK 8. The amount of CCK 8 generated is about 1/8 of the total CCK 33 present and no detectable CCK 22 was produced. To verify the specificity of PC2 for CCK 33, a sample of CCK 33 incubated with PC2 and PC2 inhibitor was also analyzed and reveals a nearly identical pattern as that of CCK 33, indicating that the cleavage of CCK 33 is indeed by PC2. To test the effect of Brij content in the incubation buffer on the elution profile of the samples, CCK 33 with and without Brij were applied to the G-50 column under identical conditions. No significant difference in terms of the elution pattern were observed, indicating a minimal effect, if any, of Brij on the experimental results.

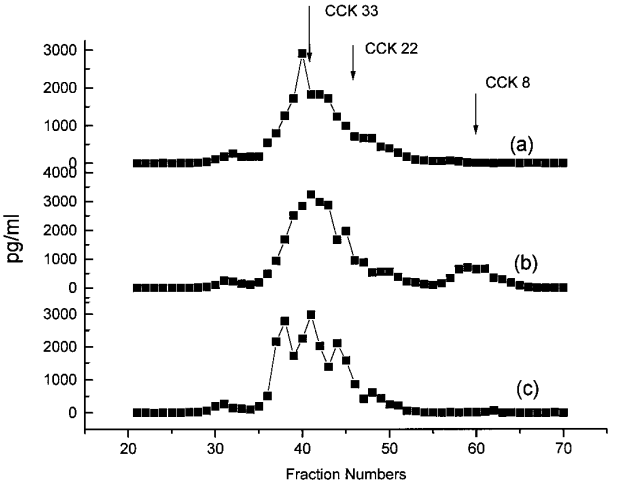

FIG. 1. Sephadex G-50 chromatography of (a). CCK 33, (b). CCK 33 incubated with PC2, (c). CCK 33 incubated with PC2 and PC2 inhibitor (100 μM). The incubation time for all the samples was 8 hours.

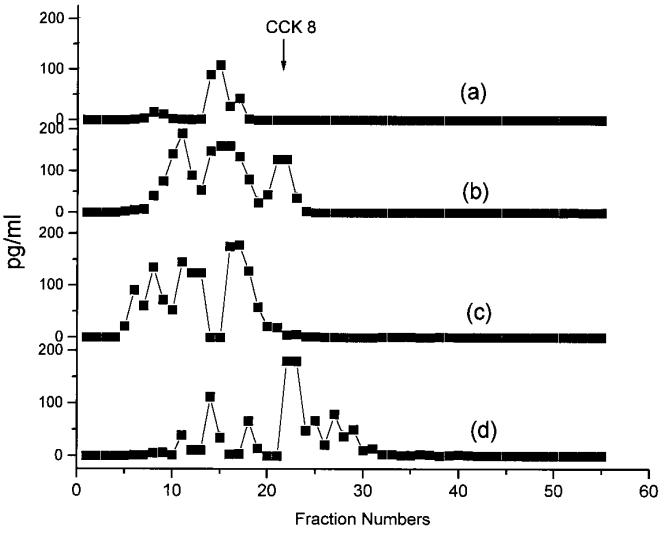

FIG. 2. HPLC chromatography of (a). CCK 33, (b). CCK 33 incubated with PC2, (c). CCK 33 incubated with PC2 and PC2 inhibitor and (d). CCK 8 in the assay buffer as described above except without Brij. Elution gradient was 30 to 60 % B in 50 min.

In addition, we also analyzed the same set of samples for the G-50 column chromatography on HPLC and the results are shown in Figure 2. The incubation conditions are the same as those for the G-50 column chromatography except that no Brij was present. The Brij content in the incubation buffer caused the samples to elute much earlier, resulting in a lower resolution. Again, the HPLC results show clearly the cleavage of CCK 33 by PC2 and the fact that the additional peak observed in the sample of CCK 33 with PC2 appears at the same position as that of CCK 8 standard, strongly supports the formation of CCK 8. The additional peaks that appear earlier are presumably due to the oxidized peptide impurities which elute early on this HPLC system. Judging from the results from G-50 and HPLC,

we can conclude with reasonable certainty that PC2 cleaves CCK 33 to generate CCK 8.

To find the optimum incubation time for the kinetic studies, we conducted a time course study of CCK 33 cleavage with PC2. Figure 3 shows the formation of CCK 8 as a function of incubation time, which exhibits a lag phase at earlier time points (less than 0.5 hr). There is a rapid increase in velocity between the 0.5 hr and 2 hr incubation time, followed by a saturation after 2 hrs. This result is similar to that observed in the study of the hydrolysis of Cbz-Arg-Ser-Lys-Arg-AMC by PC2 (1). They demonstrated that most of the purified PC2 was initially in the 71 kDa form, which is not very active. However, after incubation at 37°C, all the PC2 is transformed to the active 66-kDa form within a hour, the mechanism of which is thought to be spontaneous cleavage. We assume that this is the case for the cleavage of CCK 33 by PC2 as well.

To examine the kinetic properties of CCK 33 and PC2 reaction, various concentrations of CCK 33 were incubated with PC2 under the same conditions mentioned above. PC2 was incubated in the incubation buffer for one hour before CCK 33 was added. Then the samples were incubated for another hour at 37°C. One hour is chosen for the incubation time because the reaction rate at this time period is in the linear range of the time course (Fig. 3). The formation of CCK 8 is calculated by the summation of three maximum RIA values in the elution fractions from the G-50 column for each reaction and the reciprocal of the reaction rate is plotted versus that of the substrate concentration, as is shown in Figure 4. An apparent linear fit allowed estimation of the K_m of 104.8 μM, comparable to the K_m of 124 and 131 obtained with substrate Cbz-Arg-Ser-Lys-Arg-AMC and Pyr-Arg-Thr-Lys-Arg-AMC, respectively (1), indicating a similar mechanism for these enzymatic reactions.

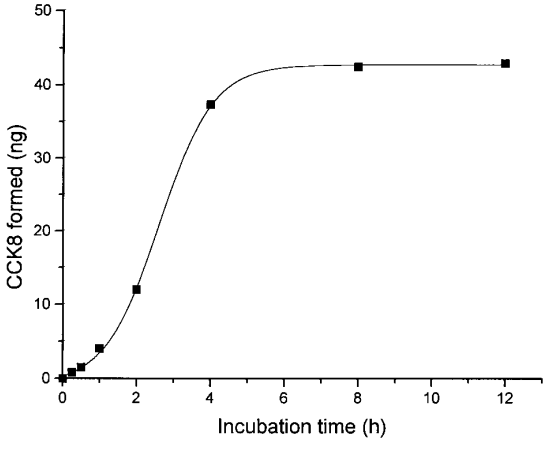

FIG. 3. Time course of the cleavage of CCK 33 by PC2. PC2 (0.288 μg) was incubated with 20 μM CCK 33 at 37°C for different time period followed by G-50 column chromatography. The amount of CCK 8 formed for each sample in the elution fractions is determined by RIA.

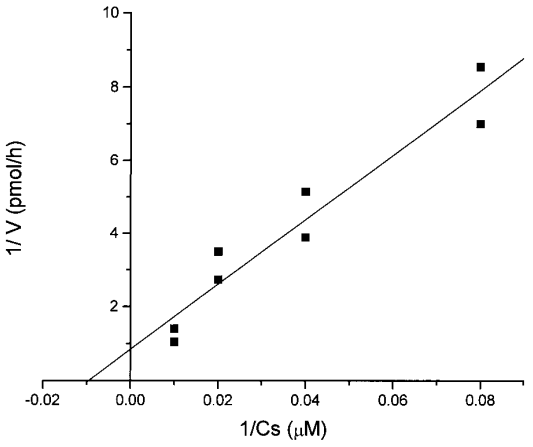

FIG. 4. Plot of the reciprocal relation between substrate concentration, Cs, and initial velocities, v. for the cleavage of CCK 33 by PC2. Purified PC2 was preincubated in incubation buffer for 1h at 37°C followed by incubation with various concentrations of CCK 33 for an additional hour. The Michaelis constant, K_m, was then obtained.

We also extended our effort to explore the possibility of cleavage of pro CCK by PC2. We incubated PC2 with purified recombinant pro CCK produced in bacteria and by *baculovirus* cells. We did not detect measurable cleavage under conditions where PC2 cleaves CCK 33. The inability of PC2 to cleave pro CCK could be due to impurities remaining in the pro CCK preparations or failure to closely enough simulate *in vitro* cleavage conditions. It is also possible that PC2 did not cleave because PC1 must cleave before PC2 as has been suggested in previous studies (7,10). A reasonable approach to test this hypothesis would be to incubate Pro CCK with both PC1 and PC2. Unfortunately, due to lack of purified PC1, we could not conduct this experiment. PC2 did not cleave CCK 33 at the Lys-Asn bond to produce CCK 22. It was also unable to cleave the synthetic peptide CCK 1-21 at the same site.

Three enzymes have been identified which will cleave CCK 33 to produce CCK 8: CGE, YAP3 and now PC2. CGE and YAP3 will also produce CCK 22 and cleave CCK 1-21. Which of these enzymes perform this cleavage *in vivo* is still unclear. The fact that three different enzymes, two of which were originally mainly associated with cleavage at double basic sites indicates that there is nothing unique about the single basic cleavage sites in pro CCK and they may not have specific functional significance in processing.

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