Characterization of a nontrypsin cholecystokinin converting enzyme in mammalian brain

(cholecystokinin fragments/hormone specificity/species specificity/gastrin/radioimmunoassay)

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ABSTRACT   An enzyme has been partially purified from canine and porcine cerebral cortical extracts that differs from trypsin in that it manifests some degree of hormone specificity since it converts porcine cholecystokinin to smaller immunoreactive forms, i.e., the COOH-terminal dodecapeptide and octapeptide fragments, but fails to convert big gastrin (34 amino acids) to heptadecapeptide gastrin. This enzyme is distinguishable from trypsin not only in substrate specificity, but also in several physicochemical properties. It is not inhibited in the presence of concentrations of lima bean trypsin inhibitor sufficient to inhibit 1 mg of trypsin per ml of incubation mixture. It is inactivated when incubated with substrate at 45°C for 1 hr, whereas trypsin remains fully active when incubated under the same conditions at 55°C. The enzyme elutes in the void volume on Sephadex G-50 and G-75 gel filtration. On sucrose gradient centrifugation, the proteolytic activity associated with trypsin is recovered above albumin but that of the solubilized brain enzyme is recovered below gamma globulin. The enzyme is not detectable in splenic extracts, which do contain nonspecific proteases capable of completely degrading cholecystokinin. Further investigation is required to determine whether the enzyme in the gut that converts cholecystokinin to the bioactive and immunoactive COOH-terminal fragments resembles or is different from the brain converting enzyme.

Over the past decade it has become evident that many, if not all, peptide hormones are found in their tissues of origin in more than one form (see ref. 1 for review). Following the discovery that proinsulin is the precursor of insulin (2) it has generally been assumed that, if the larger hormonal form contains a peptide with amino acid sequence identical to or if it is convertible by enzymatic conversion to a smaller, well-characterized, and more biologically active form, the larger molecule is a prohormone, whether or not the biosynthetic precursor relationship has been established. Thus, a precursor relationship has been definitely established for several peptide hormones, including proinsulin (2) and proparathyroid hormone (3–5), but it has only been assumed for others, such as big (34-amino-acid) gastrin (gastrin-34) (6, 7) and the 39-amino-acid cholecystokinin variant (CCK-39) (8). Similarly, cholecystokinin (CCK-33) can be considered to be a precursor for the cholecystokinin dodecapeptide (CCK-12) and octapeptide (CCK-8), the COOH-terminal fragments which have biologic potencies greater than that of the presumed parent CCK-33 molecule.

Although several groups have reported on the existence in the tissues of origin of tryptic-like enzymes involved in the conversion of proinsulin (9) and proparathyroid hormone (10, 11), precursors to insulin and parathyroid hormone, respectively, no evidence has as yet been presented to determine whether the same enzyme may be involved in processing more than one hormonal family. In this report we characterize and partially purify an enzyme, obtained from extracts of mam-malian brain, which converts porcine cholecystokinin (pCCK-33) to smaller immunoreactive forms but which fails to convert gastrin-34 to heptadecapeptide gastrin (gastrin-17). We demonstrate that this enzyme is distinguishable from trypsin in a variety of physicochemical systems.

MATERIALS AND METHODS

Preparation and Fractionation of Brain Extracts as Source of Enzyme. Cold water extracts of porcine and canine cerebral cortical tissues were prepared as follows. Animals were killed by injection of lethal doses of sodium pentathol. Immediately after injection the entire brain was removed, placed in a plastic bag, and rapidly frozen on dry ice. Pieces of frozen cerebral cortical tissue were subsequently cut and extracted at 4°C with a Teflon grinder in distilled water, which was added to a concentration of 0.1 g of wet weight tissue per ml. No differences in activity were observed if the extractant was 0.9% NaCl or 0.25 M phosphate buffer (pH 7.5). The extracts were centrifuged at 10,000 × g for 15 min. The supernatants were tested for ability to convert CCK-33 and gastrin-34 to smaller immunoreactive forms as described below. They were also chromatographed on 1 × 50 cm columns containing Sephadex G-50 or G-75. The columns were calibrated by application of radioactive markers to establish the positions of the void volume and the radioiodide peak. After application of brain extract, the columns were eluted with 0.9% NaCl and 1-ml fractions were collected and tested for enzymatic activity.

The active fractions from the Sephadex column were also centrifuged at 190,000 × g for 2 hr. Portions of the supernatant and resuspended pellets were then layered above a continuous 10–40% sucrose gradient in a 5-ml polyethylene tube and centrifuged at 45,000 rpm for 22 hr in a Spinco model L ultracentrifuge fitted with a Beckman swinging bucket rotor (SW50.1). After the centrifuge came to rest without braking, successive 0.4-ml samples were removed from the bottom of each tube and studied for enzyme activity. For comparison, ^{125}I-labeled albumin, ^{125}I-labeled gamma globulin, and pancreatic trypsin were similarly layered in duplicate tubes and centrifuged along with the active fractions of the brain extracts.

Substrates and Enzymatic Assay. Two substrates were used: purified pCCK-33, which was a gift from Victor Mutt (Gastrointestinal Hormone Research Unit, Stockholm) and was received through the NIAMDD Gastrointestinal Hormone Resource, Bethesda, MD, and gastrin-34, which was a gift from R. A. Gregory (University of Liverpool, England) and which had been purified from a human gastrinoma.

Enzymatic activity was tested by incubating the brain extract

Abbreviations: CCK-33, the 33-amino-acid cholecystokinin; CCK-12, COOH-terminal cholecystokinin dodecapeptide; CCK-8, COOH-terminal cholecystokinin octapeptide; gastrin-34, the 34-amino-acid big gastrin; gastrin-17, heptadecapeptide gastrin.

or the various fractions from the separation systems with pCCK or gastrin-34, usually at concentrations of 500 ng/ml in 0.25 M phosphate buffer (pH 7.5), fortified with 1 mg of human serum albumin per ml. The incubation was generally carried out at 25°C for 60 min. At the end of that time the samples were boiled for 5 min to inactivate the enzyme. The substrates, before and after enzymatic digestion, were then fractionated by starch gel electrophoresis or Sephadex gel filtration. Radioimmunoassay of the eluates was then used to determine alterations in substrate induced by enzymatic conversion. The same substrates were labeled with ^{125}I and fractionated similarly as well as on paper chromatoelectrophoresis, and the enzymatic degradation of labeled and unlabeled substrates was compared.

The activity and specificity of the brain enzyme were compared with those of chymotrypsin-free trypsin (Sigma, bovine trypsin, DCC-treated type XI) at a concentration of 1 mg/ml. The active fraction from the Sephadex purification of the brain extract was used at a concentration of 40 μl of active fraction per ml of incubation volume. The effect of temperature on enzymatic activity was tested at 25°, 37°, 45°, and 55°C by preincubating each of the enzymes for 15 min to bring it to temperature, then adding each of the two ^{125}I-labeled substrates and incubating for 60 min longer at the four temperatures. The pH range of activity of both enzymes was evaluated by incubation of enzyme and substrate at pH 4.5–9. For some studies excess lima bean trypsin inhibitor (1 mg/ml, sufficient to inhibit 3.2 mg of trypsin per ml) was added to both enzymes before addition of each of the labeled and unlabeled substrates. In some studies an extract from the spleen prepared similarly to the brain extract was tested for enzymatic activity with both unlabeled substrates. In all cases the samples were boiled for 5 min to inactivate the enzymes before fractionation in the various physicochemical systems to test for enzymatic conversion.

Radioimmunoassay. The gastrin and CCK peptides were measured by radioimmunoassay according to published methods (12–14). ^{125}I-Gastrin-17 (porcine heptadecapeptide gastrin was a gift from R. A. Gregory through the courtesy of Morton Grossman, Wadsworth, VA) was used as tracer for both assay systems. The guinea pig antiserum used for the gastrin assay does not crossreact with CCK or CCK-8 (13). The antiserum obtained at the current bleedings of rabbit B crossreacts identically with CCK and CCK-8 and somewhat more strongly with gastrin-17. The crossreactivity with gastrin-17 does permit use of ^{125}I-gastrin-17 as a tracer but presents no problem since pure CCK substrate is used in the CCK assays. Starch gel and Sephadex eluates were assayed as described (12, 13).

RESULTS

^{125}I-pCCK, in common with many peptide hormones such as insulin, corticotropin, and parathyroid hormone, remains at the site of application when applied to paper for chromatoelectrophoresis while the serum proteins, iodide, and other more acidic peptides such as gastrin, CCK-8, and fragments such as the iodotyrosines migrate anodally (12). Therefore, in this system it is difficult to distinguish total proteolytic degradation from specific cleavage to a fragment of CCK such as CCK-8. However, paper chromatoelectrophoresis does serve as a rapid screening test for degradative activity. Shown in Fig. 1 are intact ^{125}I-pCCK-33 before and after incubation at 25°C for 60 min with a fraction containing active enzyme. After Sephadex G-50 and G-75 gel filtration of the brain extracts, only the void volume region contained active fractions; i.e., those able to fragment the ^{125}I-CCK-33.

The void volume eluate was incubated under the same conditions also with ^{125}I-gastrin-34, gastrin-34 (0.5 μg/ml), and pCCK-33 (0.5 μg/ml) each in 0.25 M phosphate buffer (pH 7.5)
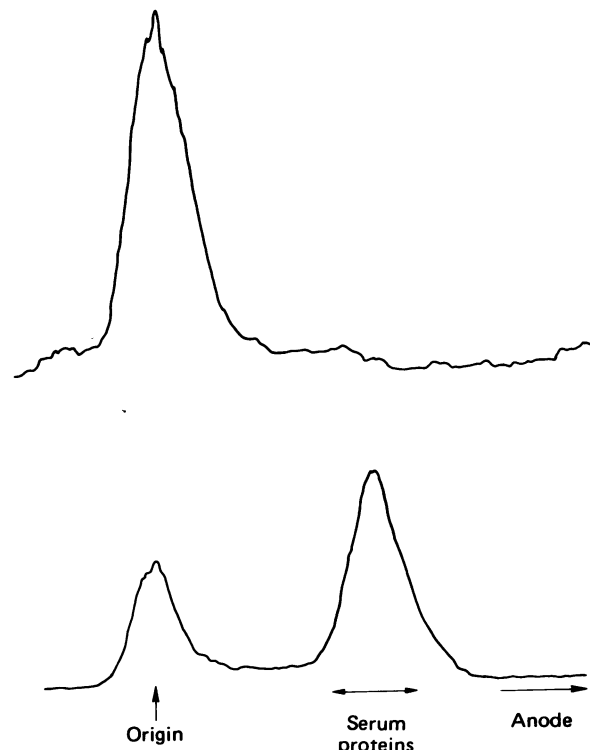

FIG. 1.   Paper chromatoelectrophoresis of ^{125}I-CCK before (*Upper*) and after (*Lower*) treatment with active fraction (void volume eluate after Sephadex G-50 gel filtration) of extract of pig cerebral cortex. Intact ^{125}I-CCK remains at the site of application; the ^{125}I-labeled fragments migrate with the serum proteins.

fortified with 1 mg of human serum albumin per ml. The brain enzyme was used at a concentration of 40 μl of active fraction per ml of incubation mixture. The same four substrates in the same buffer were also incubated with 1 mg of trypsin per ml. The starch gel electrophoretic patterns of the four substrates before and after treatment with the two enzyme preparations is shown in Fig. 2. Trypsin converted labeled and unlabeled gastrin-34 to a peptide having the starch gel electrophoretic behavior of gastrin-17 (Fig. 2 *right, bottom*). It also converted labeled and unlabeled CCK-33 to a peptide resembling CCK-8 (Fig. 2 *left, bottom*). However, the Sephadex G-50 void volume eluate of the brain extract, which converted CCK-33 to smaller immunoreactive peptides resembling CCK-12 and CCK-8 (Fig. 2 *left, middle*), did not alter gastrin-34 (Fig. 2 *right, middle*).

Other experiments over a range of enzyme concentrations (40–800 μl of void volume eluate per ml) and substrate concentrations (0.2–5.0 μg of CCK per ml) confirm the ability of the brain enzyme to convert CCK to smaller immunoreactive fragments and its failure to convert gastrin-34 to gastrin-17. Thus, the substrate specificity for the brain enzyme does not resemble trypsin. Furthermore, lima beam trypsin inhibitor does not inhibit the brain enzyme at concentrations sufficient to inhibit completely 1 mg of trypsin per ml of incubation mixture. The brain enzyme also differs from trypsin in its temperature sensitivity. Trypsin remained fully active in converting CCK-33 to the smaller fragments when maintained for 1 hr at temperatures up to 55°C while the brain enzyme was inactive when incubated with substrate for 1 hr at 45°C.

The proteolytic activity of both the brain enzyme and trypsin was maintained over the pH range from 6.5 to 9. As yet no attempt has been made to determine pH optimum for activity of the brain enzyme; it is inactive at pH 6 and below.

The brain enzyme differs significantly from trypsin not only in substrate specificity and temperature sensitivity, but also in
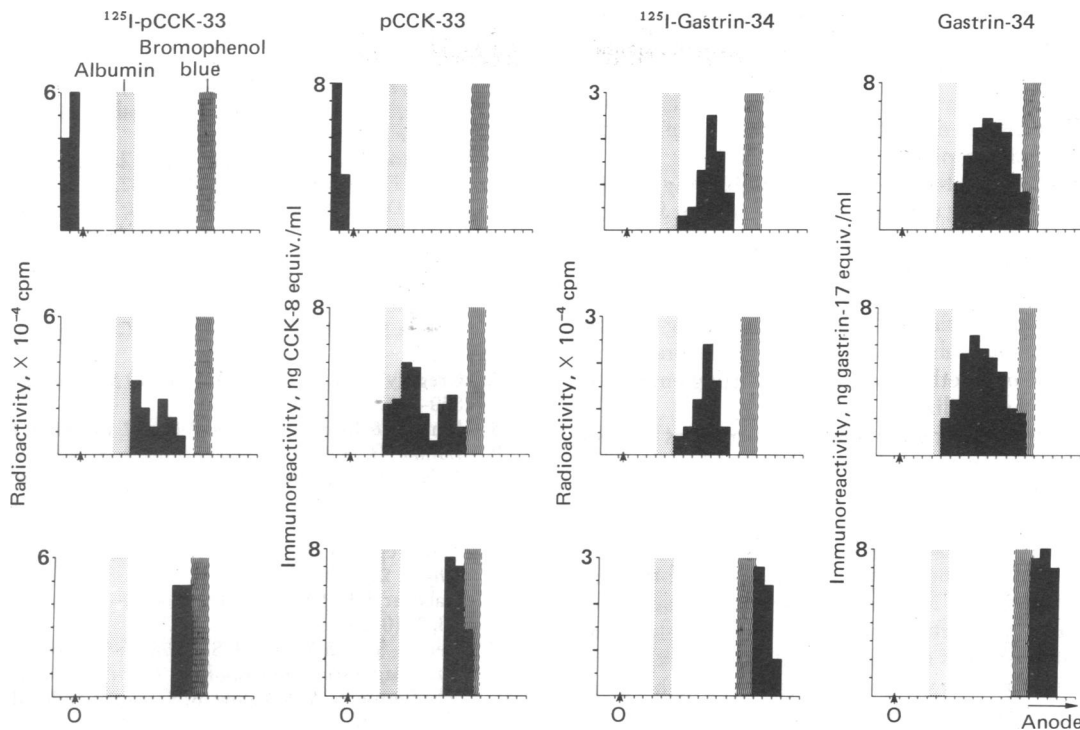
FIG. 2.   Starch gel electrophoresis of ^{125}I-labeled and unlabeled CCK and of ^{125}I-labeled and unlabeled gastrin-34 before (*Top*) and after treatment with brain converting enzyme (*Middle*) or with trypsin (*Bottom*). Trypsin converts labeled and unlabeled CCK to fragments with starch gel electrophoretic mobility resembling CCK-8 and labeled and unlabeled gastrin-34 to fragments resembling gastrin-17. The brain converting enzyme does not alter gastrin-34 but converts CCK to fragments with starch gel electrophoretic mobility resembling CCK-12 and CCK-8. O, origin.

molecular size. Thus, the brain enzyme activity is found in the void volume eluates on Sephadex G-75 fractionation while trypsin appears in eluates at about 34% of the volume between the void volume and the radioiodide peak. The void volume fraction was centrifuged at 190,000 $\times$ *g* for 2 hr. The pellet was resuspended in the original volume of saline. The enzyme activity of the supernatant equalled that of the resuspended pellet. Portions of both fractions were then subjected to density gradient ultracentrifugation and their sedimentation pattern was compared with that of ^{125}I-albumin, ^{125}I-gamma globulin, and trypsin. Consistent with its known molecular size, the tryptic proteolytic activity is recovered above albumin in the ultracentrifuge tube while the brain enzyme activity both of the supernatant and of the resuspended pellet is found below gamma globulin.

The species specificity of the brain enzyme was also investigated. The potency of canine cerebral cortex extracts in converting pCCK to the smaller peptide did not differ significantly from the potency of the porcine cerebral cortex extracts.

An enzyme similar to the brain enzyme could not be found in the spleen. Splenic extracts prepared similarly to the brain extracts completely destroyed the immunoreactivity of both CCK-33 and gastrin-34, suggesting the presence of nonspecific proteolytic enzymes. The void volume eluates of Sephadex G-75 filtration of the spleen extracts, prepared similarly with the brain extracts, did not alter CCK-33.

DISCUSSION

Steiner and associates (9) have hypothesized that trypsin-like and carboxypeptidase B-like proteinases have important roles in the conversion of proinsulin into insulin as well as in the intracellular processing of a variety of other precursor forms and were able to demonstrate the existence of similar enzymatic

activities in the Islets of Langerhans. However, they were unable to implicate those enzymes directly in the endogenous conversion process. MacGregor *et al.* (10) and later Habener *et al.* (11) have demonstrated that subcellular fractions of bovine parathyroid tissue contain enzymatic activity capable of specifically converting the prohormone to intact parathyroid hormone. None of these groups has presented evidence indicating whether the converting enzyme studied was active in converting two or more prohormones to smaller forms.

The present study was undertaken because of the demonstration that the cerebral cortical tissues from several mammalian species contain CCK peptides in amounts comparable to those found in the gastrointestinal mucosa and that the fraction of the immunoreactivity in components smaller than CCK-33 is greater in the brain than in the gut (15). These observations suggested to us that brain extracts might contain an enzyme of high potency for the specific conversion of CCK-33 to the smaller hormonal forms. The question answered by these studies is that the brain enzyme, which is capable of cleaving CCK-33 at the Arg-Ile bond to yield CCK-12 and at the Arg-Asp bond to yield CCK-8, is more specific than simply tryptic-like since, unlike trypsin, it fails to cleave big gastrin at the Lys-Lys-Glu bonds. Furthermore, this enzyme can also be distinguished from trypsin not only in terms of substrate specificity, but also in terms of molecular size, temperature stability, and behavior in the presence of trypsin inhibitor.

Although the brain enzyme appears to manifest hormone specificity, it does seem to effect cleavage at two different sites, as shown by its ability to generate both CCK-12 and CCK-8. This raises the possibility that there are two different but closely related enzymes between which we have been unable to distinguish because our purification methods are as yet relatively crude. There is no evidence that the enzyme is species specific in that both canine and porcine cortical extracts appear to be equally effective in cleaving porcine CCK-33. Thus, there is

no reason to believe that the failure of porcine brain enzyme to convert human gastrin-34 to gastrin-17 was due to species specificity. The enzyme has some degree of organ specificity in that a degradative enzyme of the same molecular size was not detectable in splenic extracts, which contain nonspecific proteases capable of completely degrading CCK.

In our studies we have had no problem solubilizing a major fraction of the converting enzyme. The supernatant fraction, after centrifugation for 2 hr at 190,000 × *g*, contained half the activity and this activity was shown to be associated with a molecule somewhat larger than gamma globulin. We have not yet determined whether the packaging of converting enzyme and the CCK hormonal substrate is different in the brain from in glandular tissue or whether there is a converting enzyme in gut that is as easily solubilized.

It is of interest to consider some possible comparisons between the CCK–converting enzyme system described here and the kidney renin–brain isorenin systems (see ref. 16 for review). The converting enzymes of the renin–angiotensin and the CCK systems do not have the dibasic peptide specificity characteristic of many other prohormone–hormone systems. The brain and kidney renins are similar in molecular size and certain other physical chemical properties, including sufficient structural identity so that antibodies against dog kidney renin also have an inhibitory effect on brain isorenin activity. Furthermore, both renins from one species are active on substrates from another species. Nonetheless, the brain and kidney enzymes are not identical in that rat brain isorenin has high affinity for dog substrate whereas rat kidney renin does not. Thus, brain and kidney renins are similar but not identical. Further investigation is required to determine whether the enzyme in the gut that converts CCK to the bioactive and immunoactive COOH-terminal fragments resembles or is different from that found in the brain.

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