Cholecystokinin-converting enzymes in brain
(cholecystokinin fragments/Michaelis constant/enzyme specificity/radioimmunoassay)

ALBERTO MALESCI, EUGENE STRAUS, AND ROSALYN S. YALOW

Solomon A. Berson Research Laboratory, Veterans Administration Medical Center, Bronx, New York 10468

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ABSTRACT  Crude extracts of porcine cerebral cortical tissue convert cholecystokinin (CCK) to its COOH-terminal fragments, the dodecapeptide (CCK-12) and the octapeptide (CCK-8). The Sephadex G-75 void volume eluate of the crude extract cleaves the arginine-isoleucine bond and effects conversion only to CCK-12; the Sephadex G-50 void volume eluate of the same extract cleaves the arginine-aspartate bond as well, so that both CCK-12 and CCK-8 are end products. Thus, there are at least two enzymes; the one involved in the conversion to CCK-12 is of larger molecular radius than the other. The \( K_m \) for the cleavage of CCK at the arginine-isoleucine bond by the Sephadex G-75 void volume eluate enzyme is 1.1 \( \times 10^{-4} \) M; the \( K_m \) for trypsin cleavage of the same bond is 4.7 \( \times 10^{-5} \) M. The lower \( V_{max} \) for the brain enzyme (1.5 \( \times 10^{-11} \) mol/min per g of extract) compared with trypsin (86 \( \times 10^{-11} \) mol/min per g of trypsin) simply reflects the lesser degree of purity of the brain extract than of the highly purified trypsin.

We have previously partially purified an enzyme that is readily solubilized from extracts of mammalian brain and that converts porcine cholecystokinin (CCK-33) to its COOH-terminal fragments, the dodecapeptide (CCK-12) and the octapeptide (CCK-8), yet fails to convert "big" gastrin (G-34) to heptadecapeptide gastrin (G-17) although in each case the bond hydrolyzed is one whose carboxyl residue is donated by either an arginine or a lysine residue (1). Thus, the enzyme is distinguishable from trypsin in substrate specificity. It also differs from trypsin in size, temperature sensitivity, and other physicochemical properties. The enzyme does not appear to be species specific in that extracts from the brains of pig, dog, rabbit, rat, and mouse convert CCK to the COOH-terminal fragments.

The finding that there are two end products of the conversion, CCK-12 and CCK-8, suggested that there might be two enzymes involved (1). In this report we demonstrate that there are at least two enzymes which differ in molecular size and substrate specificity. The kinetic constants for the enzyme that converts CCK-33 to CCK-12 are compared with those of trypsin for the same conversion.

MATERIALS AND METHODS

Preparation of Partially Purified Enzyme. Extracts of porcine cerebral cortical tissue were prepared by a modification of a previously published method (1). Two grams of frozen porcine cerebral cortex were extracted at 4°C in 0.1 M barbitral buffer at pH 8.6 with a Teflon grinder; the final concentration was 0.1 g (wet weight) of tissue per ml. The extract was then centrifuged at 10,000 \( \times g \) for 15 min. For some studies, crude brain extract was used. For others, 10 ml of the supernatant was chromatographed on a Sephadex G-75 column (2.5 \( \times 85 \) cm) previously calibrated by application of \( ^{125}I \)-labeled gamma globulin and \( ^{131}I \) to mark the positions of the void volume and iodide peak. The column was eluted with the same buffer at a flow rate of 0.5 ml/min. The void volume fractions were pooled and diluted to a final protein concentration of 1 mg/ml; 1-ml portions were lyophilized and stored at \(-20^\circ C\). Immediately before use, the lyophilized material was reconstituted with 1.0 ml of 0.02 M barbital. On occasion, the pooled void volume eluates of a Sephadex G-50 column were also tested. The protein concentrations of the crude brain extract and of the void volume eluates were determined by the method of Henry (2) and all were adjusted to 1 mg/ml.

Experimental Procedure for Kinetic Study. Purified porcine CCK-33, a gift from Victor Mutt (Karolinska Institute, Stockholm) received through the Gastrointestinal Hormone Research Service of the National Institute of Arthritis, Metabolism, and Digestive Diseases (Bethesda, MD), was used as a substrate in concentrations ranging from 0.13 to 36 \( \mu g \) in standard diluent (0.5 g of bovine serum albumin per 100 ml of 0.02 M barbital buffer at pH 8.6). Substrate and enzyme solutions were placed separately in a constant-temperature bath at 37°C and allowed to reach temperature equilibrium. Then, 0.2 ml of enzyme solution was added to 0.3 ml of substrate solution and the mixture was vortexed. At various times between 5 and 60 min, 10- to 100-\( \mu l \) portions were removed from each incubation mixture and pipetted into 2 ml of standard diluent. The diluted samples were immediately boiled for 5 min to inactivate the enzyme. Fractionation of immunoreactive CCK peptides was then carried out by differential absorption to QUSO G32 (Philadelphia Quartz, Philadelphia) as described (3); 10 mg of QUSO was added to 2-ml samples. After vortexing and centrifugation at 3000 rpm (2300 \( \times g \)) for 15 min, the concentration of immunoreactive COOH-terminal fragments was measured by radioimmunoassay of the supernatant. This procedure permits distinction between CCK and the COOH-terminal fragments but does not distinguish between CCK-12 and CCK-8.

For control studies, similar enzyme preparations were boiled and then incubated under identical conditions. Converting enzyme activity in the whole brain extract is inactivated by exposure to temperatures above 45°C (1).

Similar studies were performed with chymotrypsin-free trypsin (Sigma, bovine trypsin, DPCC-treated* type XI) at final concentrations ranging up to 1 mg of trypsin per ml.

Additional studies were performed with synthetic sulfated CCK-8 (a gift from Squibb Research Institute, through the courtesy of S. J. Lucania) and synthetic sulfated CCK-12 (prepared by M. A. Ondetti and received through the courtesy of V. Mutt) as substrates.

The hormonal form of the final reaction product was determined from starch gel electrophoretic patterns (1).

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

* Diphenylcarbamoyl chloride-treated to inactivate chymotrypsin.
Radioimmunoassays. The CCK peptides were measured by radioimmunoassay according to published methods (4, 5). The rabbit B antiserum used crossreacts identically on a molar basis with CCK-33, CCK-8, and CCK-12. 125I-Labeled G-17 was used as tracer. The minimal detectable concentration of CCK-8 in this system was 5 pg/ml.

RESULTS

The distribution of immunoreactive CCK peptides on starch gel electrophoresis is shown in Fig. 1. The position of CCK-12 is just anodal to albumin (Top Left); CCK-8 is just cathodal to bromphenol blue (Top Right). In this system, intact CCK-33, which is a very basic peptide, migrates slightly cathodally from the origin.

Treatment of CCK-33 for up to 6 hr at 37°C with the Sephadex G-75 void volume pooled eluate of the brain extract in final concentrations ranging from 0.4 to 4.0 mg/ml produced only an immunoreactive peptide with starch gel electrophoretic properties of CCK-12 (Fig. 1 Middle Left). Thus, irrespective of the concentration of this converting enzyme or the time of exposure to it, the end product is always CCK-12. Furthermore, exposure of synthetic CCK-12 to 4 mg of this pooled eluate per ml for 1 hr at 37°C resulted in no loss of immunoreactivity and no alteration in the starch gel electrophoretic pattern (Fig. 1 Bottom Left). The existence of a second converting enzyme is shown by the observation that, after incubation of CCK-33 for 1 hr at 37°C with only 0.4 mg of crude brain extract per ml, there is conversion to CCK-8 (Fig. 1 Middle Right) and that synthetic CCK-12 is also about 50% converted to CCK-8 by incubation with 0.4 mg of crude brain extract per ml for 2 hr (Fig. 1 Bottom Right).

We have again verified our previous finding (1) that the Sephadex G-50 void volume eluates convert CCK to both CCK-12 and CCK-8. This confirmation was necessary because the current study was performed at pH 8.6 in barbital buffer and the earlier one was performed at pH 7.5 in phosphate buffer, and one could not rule out a priori that the differences in products of conversion with Sephadex G-75 and G-50 void volume fractions might have been related to pH. Because the Sephadex G-50 void volume fraction effects conversion in part to CCK-8 but the Sephadex G-75 fraction does not, we conclude that the conversion to CCK-8 is effected by an enzyme likely to be smaller in molecular radius than the one involved in conversion to CCK-12.

Conversion to CCK-12 or CCK-8 involves cleavage of bonds between arginine and isoleucine or between arginine and as-
has obtained for Michaelis-Kolmogorov analysis of enzyme activity.

Fig. 3. Lineweaver-Burk plot of the conversion of CCK-33 to CCK-12 by the Sephadex G-75 void volume fraction of the brain converting enzyme (■), and by a low (0.125 mg/ml) concentration of trypsin (▲) so that the final products were the same with both enzymes. For brain enzyme: \( V_{max} = 4.6 \times 10^{-12} \text{ mol/min}; K_m = 1.1 \times 10^{-6} \text{ M}. \) For trypsin: \( V_{max} = 8.3 \times 10^{-11} \text{ mol/min}; K_m = 4.7 \times 10^{-6} \text{ M}. \) The lower \( K_m \) for brain enzyme than for trypsin indicates a higher affinity; the lower \( V_{max} \) for brain enzyme reflects its lesser degree of purification.

Neither whole brain extract nor trypsin effects any alteration in CCK-8, whether synthetic or derived enzymatically from CCK-33 or CCK-12.

We have not as yet purified from the brain an enzyme preparation for which the final product of CCK-33 conversion is CCK-8. However, because the final product of treatment with Sephadex G-75 void volume fraction is CCK-12, we compared the kinetics of reaction with CCK-33 of this enzyme (at 0.4 mg/ml) with that of trypsin at a concentration (0.125 mg/ml) such that the end product is also CCK-12. The rate of accumulation of CCK-12 was determined over a wide range of substrate concentrations (0.15–36.0 \( \mu \text{g/ml} \)). Fig. 3 shows data obtained during the period of linear accumulation plotted according to the reciprocal method of Lineweaver and Burk (6). The Michaelis constants \( (K_m) \) obtained from these plots, 1.1 \( \times 10^{-6} \) and 4.7 \( \times 10^{-6} \) M for brain enzyme and trypsin, respectively, indicate that, under these conditions, the brain enzyme has a greater affinity for CCK-33 than does trypsin. The lower \( V_{max} \) obtained for brain enzyme \( (1.5 \times 10^{-11} \text{ mol/min per g of extract}) \) compared with trypsin \( (66 \times 10^{-11} \text{ mol/min per g of trypsin}) \) simply reflects the lesser purity of the brain preparation compared to trypsin.

**DISCUSSION**

In this report we demonstrate that there are at least two enzymes involved in the conversion of porcine CCK to CCK-12 and CCK-8. The enzyme involved in the conversion to CCK-12 (Sephadex G-75 void volume fraction) cannot effect conversion to CCK-8 either from intact hormone or from synthetic or enzymatically produced CCK-12. The CCK-12 generated by the enzyme has biologic activity considerably greater than that of intact CCK and comparable to that of synthetic CCK-8 (unpublished observations).

Until further purification of the enzyme(s), it is not possible to determine whether CCK-8 is generated by conversion from intact hormone or from CCK-12 or both or whether more than one enzyme is involved in this conversion. At the present state of purification, all that is known is that the enzyme(s) that generates CCK-8 has a smaller molecular radius than the one involved in generating CCK-12.

The converting enzyme in the Sephadex G-75 void volume fraction has greater affinity and therefore is more specific than trypsin for cleaving the arginine-isoleucine bond of CCK. unanswered at this time is the question of whether this enzyme hydrolyzes the arginine-isoleucine bond of any peptide or whether it is hormone specific. The enzyme certainly is not like trypsin in that the arginine-asparagin bond of CCK does not serve as substrate.

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