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Isolation of Brain Endopeptidases: Influence of Size and Sequence of Substrates Structurally Related to Bradykinin†

Eduardo B. Oliveira, Antonio R. Martins, and Antonio C. M. Camargo*

ABSTRACT: Two thiol-activated endopeptidases with pH optima near pH 7.5 were isolated from the supernatant fraction of rabbit brain homogenates by DEAE-cellulose chromatography, gel filtration and isoelectrofocusing. Peptide bond hydrolysis was measured quantitatively by ion-exchange chromatography with an amino acid analyzer. Brain kininase A hydrolyzes the Phe^5-Ser^6 peptide bond in bradykinin (Bk), Arg^1-Pro^2-Pro^3-Gly^4-Phe^5-Ser^6-Pro^7-Phe^8-Arg^9. It is isoelectric near pH 5.2 and has a molecular weight of approximately 71 000. The enzyme also hydrolyzes the Phe–Ser peptide bond in Lys-Bk, Met-Lys-Bk, des-Arg^1-Bk, Lys^9-Bk, Pro-Gly-Phe-Ser-Pro-Phe-Arg, and Gly-Pro-Phe-Ser-Pro-Phe-Arg, but does not hydrolyze (0.1%) this bond in des-Phe^8-Arg^9-Bk. Brain kininase B hydrolyzes the Pro^7–Phe^8 peptide bond in Bk. It is isoelectric at pH 4.9 and has a molecular weight of approximately 68 000. Brain kininase B also hydrolyzes the Pro–Phe bond in Lys-Bk, Met-Lys-Bk, Lys^9-Bk, Ser-Pro-Phe-Arg, and Phe-Ser-Pro-Phe-Arg. Pretreatment of denatured kininogen with brain kininase A or B did not reduce the amount of trypsin-releasable Bk from this precursor protein, indicating that the Bk sequence, when part of a large protein, is not a substrate for either enzyme. However, kininase A and B hydrolyze the octadecapeptide Gly-Leu-Met-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Ser-Val-Gln-Val. The data show that a large part of the C-terminal portion of bradykinin is important for the brain kininase A activity and, for both enzymes, the size of the peptide and presumably the residues adjacent to the scissile bond are important in determining the rate of peptide bond hydrolysis by these endopeptidases.

The continuous breakdown and renewal of proteins in the brain of mature animals occurs despite the stability of nervous tissue with respect to cell division and differentiation (Leblond and Walker, 1956; Messier et al., 1958; cf. also Droz, 1969). This high turnover of brain proteins is probably related to the functional activity of neurons. Although this relationship has long been postulated (Hydén, 1943, and cf. Droz, 1969), some of the brain proteolytic enzymes that may participate in the process of intracellular catabolism have only been identified in the last decade (cf. Marks and Lajtha, 1971). The difficulties of isolating the enzymes that regulate intracellular protein degradation reflect the fundamental problem of finding

† From the Departments of Pharmacology and Biochemistry and the Protein Chemistry Laboratory, Faculdade de Medicina de Ribeirão Preto, U.S.P., São Paulo, Brazil. *Received November 18, 1975.* Research was carried out under the auspices of the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

appropriate substrates for peptidases whose specificity is not known. In addition, intracellular peptidases appear to be extremely labile during isolation (Bohley et al., 1971).

The mechanisms that control intracellular proteolysis have not been defined. This process should occur in several steps starting from the native proteins, through peptides of various sizes, and finally to free amino acids (cf. Marks and Lajtha, 1971; Segal and Dunaway, Jr., 1975). Recently, information about the initial and probably rate-limiting step of protein breakdown has come from the studies of Katunuma et al. (1975) and Schimke (1975). Katunuma et al. (1975) have shown that the initial step of pyridoxal enzyme degradation is carried out by a "group-specific" protease acting on the apoenzyme. In contrast, Schimke (1975) has presented evidence that intracellular proteins become susceptible to proteases, and the basis of selectivity is related to the size and/or conformation of the substrate protein. However, the enzymes that participate in the hydrolysis of intermediate and small peptides as well as the basis of regulation of their activity, if regulation does occur, are essentially unknown.

In previous reports, we described the use of the peptide hormone bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) as a substrate for brain peptidases (Camargo et al., 1973). In this paper we report the preparation of an enzyme that hydrolyzes the Pro^7–Phe^8 bond of bradykinin and extend the description of another enzyme which hydrolyzes the Phe^5–Ser^6 bond (Camargo et al., 1973). The rate of hydrolysis of bradykinin, kininogen (plasma precursor of bradykinin), and related peptides was used to examine the effects of substrate size and sequence on the activity of the enzymes.

Experimental Procedure

Materials. Aminex A-5 and A-6 resins were purchased from Bio-Rad Laboratories. DEAE-Cellulose, Whatman DE-52, was obtained from Reeve Angel Co. Ampholine (pH 3–10) is a product of LKB-Produkter. Ovalbumin, blue dextran, cytochrome *c*, iodoacetamide, and trypsin were purchased from Sigma Chemical Co. Chymotrypsinogen and α-chymotrypsin were obtained from Worthington. Kininogen prepared from dog plasma was a gift from Dr. C. R. Diniz of the Department of Biochemistry, Faculdade de Medicina de Ribeirão Preto, Brazil.

Bradykinin, Lys-bradykinin, and Met-Lys-bradykinin were purchased from Schwarz/Mann. The other synthetic peptides used in this study were generously provided to us by Dr. A. C. M. Paiva (Escola Paulista de Medicina, São Paulo, Brazil), Dr. John M. Stewart (University of Colorado Medical School, Denver, Colo.) and Drs. E. Flanigan, L. Hohberger, and L. J. Greene (Brookhaven National Laboratory, Upton, N.Y.). The peptides had integral molar ratios of constituent amino acids after acid hydrolysis and were homogeneous by high voltage electrophoresis at pH 3.5.

Methods. Determination of Bradykinin: Kininase Assay (Camargo et al., 1973). A bioassay with the isolated guinea pig ileum was used to measure the concentration of bradykinin. The kininase assay consists of the determination of residual bradykinin activity by a matching technique. The isolated guinea pig ileum was bathed at 37 °C in 10 ml of tyrode buffer containing 3.5×10^{-7} M atropine and 1.7×10^{-6} M diphenylhydramine. Bradykinin, 5–10 nmol, was incubated with enzyme in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl and 3×10^{-4} M dithiothreitol for 10–20 min at 37 °C. The concentration of enzyme was selected to give 40 to 60% inactivation in 15 min.

Enzyme Preparation. Preparation of pH 5.0 Supernatant Fraction from Rabbit Brain. Mature rabbits were anesthetized with pentobarbitol (40 mg/kg) and both carotid arteries were cannulated. After the jugular veins were excised, cold saline solution was infused to eliminate blood. The brain was separated from the brain stem and cerebellum, homogenized with a Potter-Elvehjem homogenizer in four volumes (w/v) of 0.25 M sucrose, and centrifuged at 25 000*g* for 60 min. The pH of the supernatant was adjusted to 5.0 by the dropwise addition of 0.5 M acetic acid and held at 4 °C for 2 h. The suspension was centrifuged at 900*g* for 15 min and the precipitate discarded. The pH 5.0 supernatant fraction accounted for more than 90% of the bradykinin inactivating enzyme activity present in the 0.25 M sucrose homogenate supernatant. The activity of the pH 5.0 supernatant fraction was stable after lyophilization and storage at −22 °C, but frozen solutions of this fraction rapidly lost activity. When it was convenient to store frozen solutions, the pH was adjusted to 7.5. The activity was stable for at least 3 months at −22 °C in either the lyophilized powder or the frozen pH 7.5 solution.

DEAE-Cellulose Chromatography. The pH 5.0 supernatant fraction was concentrated to one-third of the original volume under reduced pressure at 4 °C in $^8/_{32}$ Nojax Visking Casing (Berggard, 1961). The solution was equilibrated with the starting buffer used for DEAE-cellulose chromatography, 0.05 M Tris-HCl buffer, pH 7.5, containing 0.03 M NaCl, by dialysis against 100 volumes of the buffer for 4 h at 4 °C. Fresh buffer was added after 4 h and dialysis was repeated twice. The pH and conductivity of the sample solution were the same as the starting buffer after dialysis. The DEAE-cellulose column was developed by stepwise increase of NaCl concentration in 0.05 M Tris-HCl buffer, pH 7.5. The experimental conditions are given in the legend to Figure 1.

Gel Filtration on Sephadex G-100 Fine. Fractions A (320 to 530 ml) and B (560 to 650 ml) from the DEAE-cellulose column (Figure 1) were concentrated to 5 to 10% of the original volume under reduced pressure at 4 °C and were submitted individually to gel filtration under the conditions given in the legend to Figure 2.

The molecular weight of enzyme preparations was determined by gel filtration after the electrofocusing step (Andrews, 1970). The Sephadex G-100-F column (22 × 100 cm) was developed at 4 °C with 0.05 *M* Tris-HCl buffer, pH 7.5, 0.1 M NaCl at 10 ml/hr. Fractions of 2.4 ml were collected. Void and total column volumes were determined with blue dextran and glucose, respectively. Brain kininase A (0.60 mg of protein), brain kininase B (0.32 mg), and 5 mg each of serum albumin, ovalbumin, chymotrypsinogen, and cytochrome *c* were run separately.

Preparative Isoelectrofocusing. The apparatus employed the U-tube principle described by Valmet (1969). We have used coiled polyethylene tubing to provide discrete fractions rather than a two-part plastic assembly (Macko and Stagemann, 1970). Sucrose is used in our experiments to stabilize the activity of the enzyme preparations.

The electrofocusing cell consists of 3 m of PE 280 tubing (Clay-Adams) coiled around a hollow glass support tube (2.2 cm in diameter). Forty-five to fifty complete turns are obtained. The sample (~10 ml) almost fills the coil. The coil terminals are connected to two glass reservoirs (~10 ml) which are filled with 0.02 N NaOH (cathode) and 0.02 N H_2SO_4 (anode). The coil assembly is suspended in a Lucite compartment which is cooled by circulating water at 0–3 °C. Electrofocusing is carried out at 4500 V for 60 to 72 h until the current is stabilized (cf. Figures 3 and 4). After the run, one

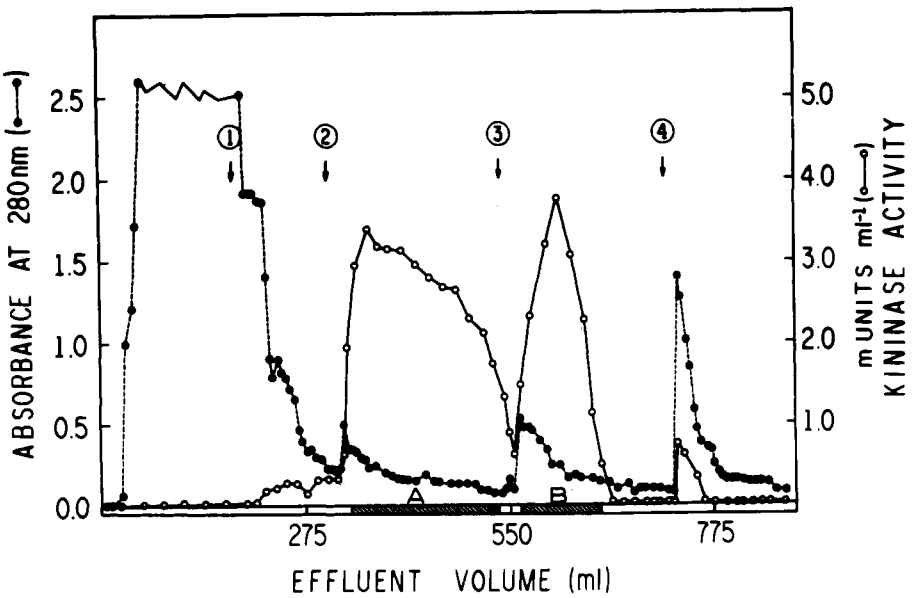

FIGURE 1: Chromatography on DEAE-cellulose of pH 5.0 supernatant fraction from rabbit brain. The column (0.9 × 40 cm) was equilibrated with 0.05 M Tris-HCl buffer (pH 7.5), 0.03 M NaCl. The sample contained 4.5 kininase units and 238 mg of protein in 190 ml of 0.05 M Tris-HCl (pH 7.5), 0.03 M NaCl. After sample application, the column was developed by stepwise gradient of NaCl using 130 ml of 0.05 M Tris-HCl (pH 7.5), 0.05 *M* NaCl (arrow 1), 230 ml of 0.05 M Tris-HCl (pH 7.5), 0.07 M NaCl (arrow 2), 225 ml of 0.05 M Tris-HCl (pH 7.5), 0.10 M NaCl (arrow 3), and 155 ml of 0.05 M Tris-HCl (pH 7.5), 0.15 *M* NaCl (arrow 4). The column was operated at 12 ml/h, 4 °C, and fractions of 5.5 ml were collected. Kininase activity was determined with the isolated guinea pig ileum. (● - - ●) Absorbance at 280 nm; (○—○) kininase activity. The bars represent the fractions that were pooled for further purification.

end of the tubing is clamped just below entry to the reservoir to prevent liquid flow. The other end is disconnected and the tubing is cut with a razor blade at the top of each turn. The contents of each segment are transferred with 2.5 ml of H_2O to a separate test tube to provide the fractions indicated in Figures 3 and 4. The pH was measured at 4 °C.

Before electrofocusing, the sample is concentrated to less than 10 ml at 4 °C under reduced pressure and dialyzed against 10% (w/v) sucrose. Ampholine, pH 3–10, is added to provide a final concentration of 2.0% and the volume adjusted to 10 ml with 10% sucrose.

Determination of Peptides and Amino Acids Derived from Bradykinin. An automatic amino acid analyzer (Alonzo and Hirs, 1968) was operated as previously described (Camargo et al., 1973). Samples were analyzed with the two column system of Spackman et al. (1958) and with the buffer systems described in the legend to Figure 5 and by Camargo et al. (1973). The relative color yields (area/concentration) for Arg-Pro-Pro-Gly, Arg-Pro-Pro-Gly-Phe, Arg-Pro-Pro-Gly-Phe-Ser, Arg-Pro-Pro-Gly-Phe-Ser-Pro, Ser-Pro-Phe-Arg, Ser-Pro, Phe-Arg, Phe, and Arg were 0.25, 0.23, 0.16, 0.26, 0.80, 0.68, 0.84, 0.96, and 1.0, respectively.

Enzymatic hydrolysis was carried out in 1 ml of 0.05 *M* Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl and 3×10^{-4} M DTT[1] at 37 °C. The substrate concentrations are indicated in the tables and figures. The reaction was stopped by acidification with 0.05 N HCl and 0.2 M sodium citrate containing 15% (v/v) polyethylene glycol and applied directly to the amino acid analyzer without further treatment.

Reduction and Alkylation of Canine Kininogen. Kininogen, 10 mg/ml in 0.5 M Tris-HCl buffer, pH 8.1, 0.002 M EDTA, 6 M guanidine, was reduced with DTT (20 mM) for 4 h at 50 °C and alkylated with iodoacetamide (40 mM) as described by Konigsberg (1972). After alkylation, the solution was exhaustively dialyzed against water.

Heat Denatured Kininogen. A solution containing 10 mg/ml was kept at 100 °C for 5 min.

Proteolytic Activity of Brain Kininase A and B on Kininogen, Heat-Denatured Kininogen, S-Carboxymethyl-Kininogen and Gly-Leu-Met-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Ser-Val-Gln-Val-Ser. In order to determine if kininases hydrolyze the bradykinin portion of kininogen or of a synthetic octadecapeptide containing bradykinin, the substrate was first incubated with enzyme and then treated with trypsin. The amount of trypsin-releasable "kinin" activity was taken as a measure of the integrity of the bradykinin portion of substrate. The kininogen (0.2 mg/ml) or the octadecapeptide (5 nmol/ml) in 0.05 M Tris-HCl buffer, pH 7.5, 0.1 M NaCl, was treated with kininase A (0.02 mg/ml) or B (0.015 mg/ml) at 37 °C. The incubation mixture was boiled for 2 min and then trypsin was added to a final concentration of 0.1 mg/ml. After incubation for 30 min at 37 °C, kinin activity was determined by bioassay. Chymotrypsin, 0.025 mg/ml, or no enzyme addition was used in control experiments in place of kininase A or B. The quantities of chymotrypsin, kininase A or B were selected to give equivalent rates of inactivation of bradykinin (0.4 nmol/min) with 10 nmol of substrate/ml.

Results

Preparation of Rabbit Brain Kininase A and B. DEAE-Cellulose Chromatography of Supernatant Fraction. Ninety to ninety-five percent of the kininase activity and 30% of the protein is soluble when the pH of the supernatant fraction prepared from rabbit brain and homogenized in 0.25 M sucrose is adjusted to pH 5.0. Figure 1 gives the elution diagram obtained when the pH 5.0 supernatant fraction is submitted to chromatography on DEAE-cellulose. The column was developed with step increases in sodium chloride at pH 7.5, as indicated in the legend to Figure 1. All of the kininase activity was adsorbed by the ion exchange resin and subsequently

[1] Abbreviations used are: DTT, dithiothreitol; PCMB, *p*-chloromercuribenzoate.

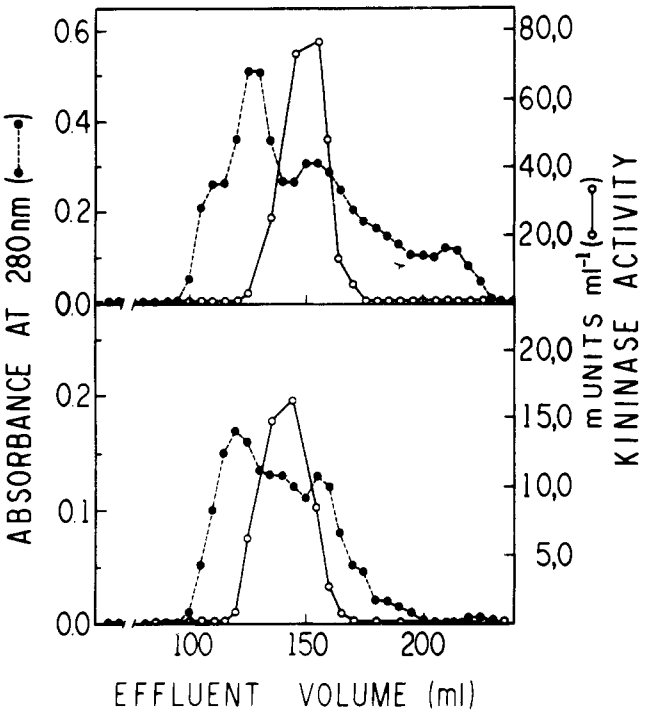

FIGURE 2: Gel filtration of DEAE-A (top) and DEAE-B (bottom) on Sephadex G-100. The column (2.2 × 110 cm) was equilibrated and developed at 4 °C with 0.05 *M* Tris-HCl buffer (pH 7.5), 0.1 M NaCl, at a flow rate of 12 ml/h, and 6-ml fractions were collected. Kininase activity was determined with the isolated guinea pig ileum. The samples, in 9 ml of 0.05 M Tris-HCl (pH 7.5), 0.1 *M* NaCl, contained 0.6 kininase unit and 8.5 mg of protein for the DEAE-A fraction, and 0.2 kininase unit and 5.4 mg of protein for the DEAE-B fraction, respectively. (● - - ●) Absorbance at 280 nm; (o—o) kininase activity.

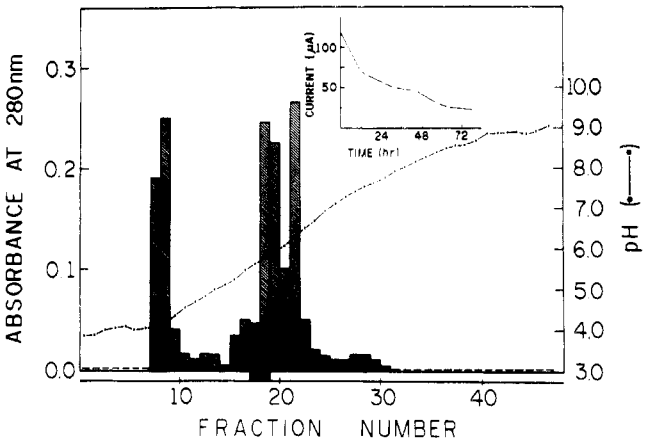

FIGURE 3: Isoelectric focusing of kininase A. The sample contained 0.18 kininase unit and 2.7 mg of protein in 10 ml of 10% sucrose containing 2% ampholine (pH 3–10). Focusing was carried out in 3 m PE 280 tubing at 4500 V for 72 h. The current is given in the inset. Kininase activity was present only in fractions 18 and 19 corresponding to pH 5.1 and pH 5.3. (● — ●) pH; bars, absorbance at 280 nm.

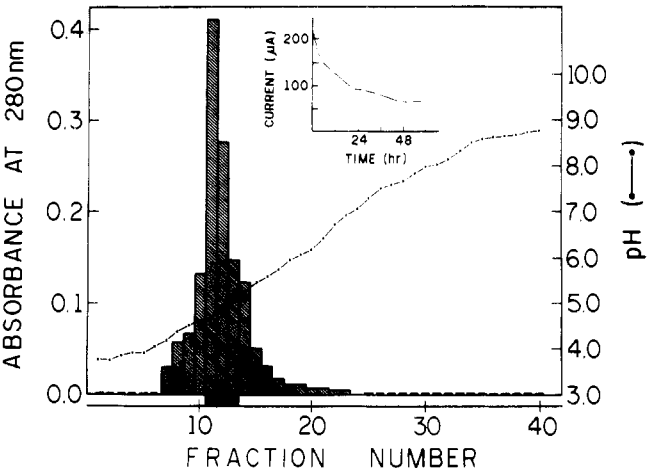

FIGURE 4: Isoelectric focusing of kininase B. The sample contained 0.05 kininase unit and 1.5 mg of protein. The conditions are the same as given in the legend to Figure 3. Fraction 12 (pH 4.8) contained 80% of the activity. (●—●) pH; bars, absorbance at 280 nm.

eluted in two major fractions with 0.07 M and 0.1 M NaCl, respectively. Smaller amounts of activity were also eluted with 0.05 and 0.15 M NaCl. The recovery of kininase activity was 50–65%.

The effluent corresponding to fractions A and B (indicated by the bars in Figure 1) was combined. Analysis of the peptide products formed by the action of A and B on bradykinin showed that fraction A contained a small amount of kininase B and fraction B had appreciable quantities of kininase A activity.

Gel Filtration on Sephadex G-100. Fractions A and B were submitted to gel filtration on Sephadex G-100 (Fine) (2.2 × 110 cm) developed with 0.05 M Tris-HCl buffer, pH 7.5, 0.1 M NaCl. Some purification of the kininase activity (Figure 2) was achieved. The recovery of activity for this step (relative to 0.25 M sucrose supernatant) was 15% for pool A and 8% for pool B. In separate experiments not documented here it was shown that kininase B still was contaminated with kininase A.

Isoelectrofocusing. The data for preparative electrofocusing for brain kininase A and brain kininase B are given in Figures 3 and 4. Kininase activity was detected only in the fractions indicated by the solid bars below each figure. These correspond to pH 5.2 and 4.9 for A and B, respectively. Some protein had precipitated during electrofocusing. After determining the pH of the fractions, the pH was adjusted to 7.5 with concentrated Tris-HCl buffer. All of the precipitated material was soluble. The kininase activity was more stable at the higher pH. The diagrams show that isoelectric focusing provides an effective step for purification.

Evidence presented below demonstrates that each enzyme preparation hydrolyzes bradykinin at only one peptide bond: brain kininase A at the Phe^5–Ser^6 and brain kininase B at the Pro^7–Phe^8, to release the corresponding peptide fragments. In our original description of the enzymes from brain homogenate, A was designated I and B as II. We have changed these names to avoid confusion with plasma kininase I which hydrolyzes Phe^8–Arg^9, and plasma (Yang and Erdös, 1967) and kidney (Erdös and Yang, 1967) kininase II which hydrolyzes the Pro^7–Phe^8 bonds of bradykinin. The procedure described here differs from our previous report by reversing the order of the DEAE-cellulose and Sephadex G-100 steps. The use of DEAE-cellulose as the first step permits us to apply larger amounts of material to the column. However, the specific activity of the product after gel filtration is somewhat lower than previously achieved. The enzyme preparations must be considered to be partially purified, for we have no data on chemical homogeneity.

Properties of Kininase A and B: Isoelectric Point. Kininase A and B are isoelectric at 4.9 and 5.2, respectively, as determined by electrofocusing with ampholine in the presence of 10% sucrose (cf. Figures 3 and 4).

Molecular Weight Determined by Gel Filtration on Sephadex G-100. Kininase A activity was constantly eluted

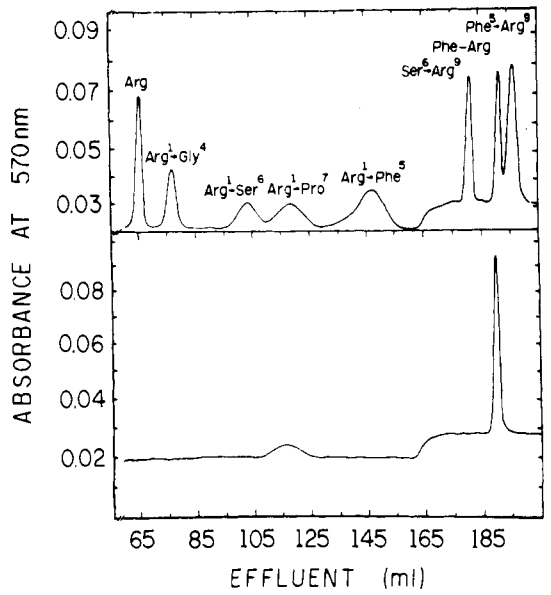

FIGURE 5: Chromatography of Arg, Arg-Pro-Pro-Gly, Arg-Pro-Pro-Gly-Phe, Arg-Pro-Pro-Gly-Phe-Ser, Arg-Pro-Pro-Gly-Phe-Ser-Pro, Phe-Ser-Pro-Phe-Arg, Ser-Pro-Phe-Arg, and Phe-Arg (top) and products derived from bradykinin by hydrolysis with brain kininase B (bottom). The chromatograms were obtained with Aminex A-5 resin (0.9 × 12.5 cm) equilibrated and developed at 80 °C with 0.65 M sodium citrate buffer (pH 4.20) and 0.388 M sodium citrate, pH 7.5, after 151 min. Top: The sample contained 12 nmol of arginine, 46 nmol of Arg-Pro-Pro-Gly, 49 nmol of Arg-Pro-Pro-Gly-Phe-Ser, 42 nmol of Arg-Pro-Pro-Gly-Phe-Ser-Pro, 60 nmol of Arg-Pro-Pro-Gly-Phe, 15 nmol of Ser-Pro-Phe-Arg, 15 nmol of Phe-Arg, and 22 nmol of Phe-Ser-Pro-Phe-Arg. Bottom: Bradykinin (60 nmol) and brain kininase B (0.025 μg of protein) were incubated in 0.5 ml of 0.05 M Tris-HCl buffer (pH 8.5), 0.1 *M* NaCl for 30 min at 37 °C. The reaction was stopped by addition of 0.7 ml of 0.2 M sodium citrate buffer (pH 2.2), 0.12 M HCl containing 30% (v/v) polyethylene glycol; 0.5 ml of this solution, containing the equivalent of 25 nmol of bradykinin, was applied to the column.

somewhat more rapidly from the column than kininase B in both preparative (Figure 2) and analytical experiments. Apparent molecular weights of 71 000 and 69 000 were obtained for A and B, respectively.

Site of Peptide Bond Hydrolysis in Bradykinin. Brain kininase A hydrolyzes bradykinin at the Phe[5]–Ser[6] peptide bond (Camargo et al., 1973). The products of the hydrolysis of bradykinin by brain kininase B were determined and quantitated by chromatography on Aminex A-5 resin, as shown in Figure 5. The elution diagram for arginine and peptide fragments of bradykinin corresponding to hydrolysis at the Gly[4]–Phe[5], Phe[5]–Ser[6], Ser[6]–Pro[7], and Pro[7]–Phe[8] bonds is given in Figure 5, top panel. The tripeptide Pro-Phe-Arg, not shown in the figure, is eluted 120 min after Phe-Ser-Pro-Phe-Arg. The color yields for these peptides and arginine varied over a sixfold range (cf. Experimental Procedure). The bottom panel shows the products obtained by complete hydrolysis of 23 nmol of bradykinin by brain kininase B. The only peptide products detected were Arg-Pro-Pro-Gly-Phe-Ser-Pro (24 nmol) and Phe-Arg (23 nmol). No other fragments were detected in this elution diagram and when twice the amount of hydrolysate was applied to the column. An aliquot of the digestion mixture was also analyzed on the long column of the amino acid analyzer, and no free amino acids or neutral peptides were detectable.

pH Optimum. The pH optimum for the hydrolysis of the Phe[5]–Ser[6] peptide bond by kininase A in bradykinin is near pH 7.0 (Carmargo et al., 1973). Figure 6 shows that the pH optimum for the hydrolysis of the Pro[7]–Phe[8] bond in bradykinin by brain kininase B is near 8.5. The data given in Figure

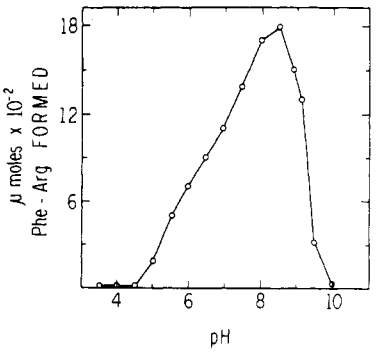

FIGURE 6: Effect of pH on the rate of hydrolysis of bradykinin by brain kininase B. The pH of the enzyme solution in 0.05 M Tris-HCl (pH 7.5), 0.1 *M* NaCl was adjusted to the appropriate pH with 0.1 M acetic acid or 0.1 M NaOH. The incubation mixture containing bradykinin (210 nmol/ml) and 0.02 mg of enzyme was held at 37 °C for 30 min. The products of the reaction were determined with the amino acid analyzer as indicated in the legend to Figure 5.

6 are for the release of Phe-Arg. The amino-terminal peptide Arg[1]-Pro[7] was released in equimolar proportions over the entire pH range studied.

Effect of Peptide Length on the Relative Rate of Peptide Bond Hydrolysis. The data given in Table I for bradykinin and related peptides by brain kininase A hydrolysis show that addition of amino acid residues to the amino terminus (peptides 4–6) or deletion of the amino-terminal Arg (peptide 7), Arg-Pro (peptide 8), or Arg-Pro-Pro (peptide 9) of bradykinin has a small or no effect on the rate of hydrolysis of the Phe–Ser bond when compared to bradykinin (peptide 1). Similarly, substitution of phenylalanine with tyrosine at the scissile bond (peptide 2) or arginine with lysine at the carboxyl terminus (peptide 3) has only a small effect on the rate of hydrolysis by brain kininase A. However, deletion of the Arg-Pro-Pro-Gly (peptide 10) from the aminoterminus, or Arg (peptide 11), Phe-Arg (peptide 12), Pro-Phe-Arg (peptide 13) from the carboxy terminus of bradykinin reduces the rate of hydrolysis by one to two orders of magnitude. These data show that the activity of brain kininase A is extremely sensitive to deletion of amino acid residues up to four residues distant from the scissile bond.

The relative rates of hydrolysis of bradykinin and related peptides by brain kininase B at the Pro–Phe peptide bond (Table II) appear to be much less sensitive to peptide length. The addition of amino acids to the amino terminus (peptides 2–4) has little effect on the rate of hydrolysis. The tetrapeptide Ser-Pro-Phe-Arg (peptide 5) is a better substrate than bradykinin (peptide 1), where some hydrolysis of the Ser–Pro bond was also observed. However, the tripeptide Pro-Phe-Arg (peptide 6) was cleaved at only 30% of the rate of bradykinin. In our previous report, we compared the properties of brain kininase B with angiotensin converting enzyme which releases Phe-Arg followed by Ser-Pro from bradykinin (Erdös and Yang, 1967; Dorer et al., 1974; Soffer et al., 1974). However, the absence of activity of brain kininase B on peptide 7, Arg-Pro-Pro-Gly-Phe-Ser-Pro, demonstrates that, although both enzymes can release Phe-Arg from bradykinin, the angiotensin converting enzyme, but not kininase B, can release Ser-Pro from the heptapeptide. The data presented in Tables I and II were derived from experiments carried out at one substrate concentration at or above the K_m of the enzymes for bradykinin. Thus, they do not permit us to evaluate the contribution of differences in K_m and V_{max} to the observed rates.

Proteolytic Activity on Kininogen and an Octadecapeptide Containing Bradykinin as an Internal Segment. Kininase A

TABLE I: Relative Rates of Peptide Bond Hydrolysis by Brain Kininase A.[a]

Peptide		Phe^5-Ser^6
1	Arg-Pro-Pro-Gly-Phe↓Ser-Pro-Phe-Arg	*1.0*
2	Arg-Pro-Pro-Gly-Tyr↓Ser-Pro-Phe-Arg	0.75
3	Arg-Pro-Pro-Gly-Phe↓Ser-Pro-Phe-Lys	1.2
4	Lys-Arg-Pro-Pro-Gly-Phe↓Ser-Pro-Phe-Arg	1.3
5	Met-Lys-Arg-Pro-Pro-Gly-Phe↓Ser-Pro-Phe-Arg	1.3
6	Gly-Arg-Met-Lys-Arg-Pro-Pro-Gly-Phe↓Ser-Pro-Phe-Arg	0.6
7	Pro-Pro-Gly-Phe↓Ser-Pro-Phe-Arg	1.5
8	Pro-Gly-Phe↓Ser-Pro-Phe-Arg	0.8
9	Gly-Phe↓Ser-Pro-Phe-Arg	0.9
10	Phe↓Ser-Pro-Phe-Arg	0.03
11	Arg-Pro-Pro-Gly-Phe↓Ser-Pro-Phe	0.50
12	Arg-Pro-Pro-Gly-Phe↓Ser-Pro	0.02
13	Arg-Pro-Pro-Gly-Phe↓Ser	0.03

[a] Peptides ($1.8–2.2 \times 10^{-4}$ *M*) were incubated with 30 μg of enzyme in 10 ml of 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl, 0.3 mM DTT for 30 min at 37 °C. The reaction mixture was analyzed with an amino acid analyzer as described in the legend to Figure 5. Hydrolyses of peptides 2 and 4–9 were evaluated by measuring the amount of Ser-Pro-Phe-Arg formed; peptides 3 and 11 by measuring Arg-Pro-Pro-Gly-Phe; and for peptides 1, 10, 12, and 13 by measuring both products.

TABLE II: Relative Rates of Peptide Bond Hydrolysis by Brain Kininase B.[a]

Peptide		Pro-Phe
1	Arg-Pro-Pro-Gly-Phe-Ser-Pro↓Phe-Arg	*1.0*
2	Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro↓Phe-Arg	1.1
3	Met-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro↓Phe-Arg	0.9
4	Gly-Arg-Met-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro↓Phe-Arg	1.2
5	Ser-Pro↓Phe-Arg	2.8
6	Pro↓Phe-Arg	0.3
7	Arg-Pro-Pro-Gly-Phe-Ser-Pro	0.0
8	Arg-Pro-Pro-Gly-Phe-Ser-Pro↓Phe	0.78

[a] Peptides ($2.0–2.5 \times 10^{-4}$ *M*) were incubated with 20 μg of enzyme in 1.0 ml of 0.05 M Tris-HCl buffer, pH 8.5, containing 0.1 M NaCl, 0.3 mM DTT for 30 min at 37 °C. The reaction mixture was analyzed with an amino acid analyzer as described in the legend to Figure 5. Hydrolysis of peptides 2–4 was evaluated by measuring the amount of Phe-Arg formed. Both products were quantitated for the remaining peptides.

and B do not hydrolyze denatured hemoglobin nor is the rate of hydrolysis of bradykinin by the enzyme affected by a 12-fold molar excess of hemoglobin (Camargo et al., 1973). In order to determine if the absence of proteolytic activity on a protein substrate is due to the absence of a sensitive peptide bond (or appropriate amino acid sequence) we have tested dog plasma kininogen as a substrate in the native, heat-denatured, and reduced carboxymethylated forms. The design of the experiment presented in Figure 7 was pretreatment of the protein with kininase A or B, followed by treatment with trypsin to determine the "releasable" kinin activity (cf. Methods). Pretreatment with chymotrypsin was used as a control for it hydrolyzes bradykinin at the Phe^5–Ser^6 and Phe^8–Arg^9 peptide bonds.

The data presented in Figure 7 show that pretreatment of carboxymethylated kininogen with kininase A did not reduce the amount of kinin activity which could be later released by trypsin. The amount of kininase A used was sufficient to completely inactivate the nonapeptide bradykinin. In the control experiments, chymotrypsin completely inactivated the kinin segment of carboxymethylated kininogen. The concentrations of chymotrypsin and kininase A were selected to provide equivalent rates of inactivation of the nonapeptide bradykinin. In contrast to the inability of kininase A to act on the kinin fragment within kininogen, kininase A does hydrolyze the octadecapeptide containing bradykinin as residues 5 through 13. In separate experiments not documented here, it was shown that reduced or alkylated kininogen containing 1 nmol of bradykinin equivalents has no demonstrable effect on the rate of hydrolysis of bradykinin by kininase A. This series of experiments was also carried out with native and heat-denatured kininogen and with kininase B for the three substrates. The results were the same as illustrated in Figure 7.

Discussion

The use of the nonapeptide bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) as substrate has made it possible to identify and monitor the purification of two neutral endopeptidases which we call by the trivial names brain kininase A (hydrolysis of the Phe^5–Ser^6 bond) and brain kininase B (hydrolysis of Pro^7–Phe^8 bond). A and B have molecular weights near 70 000 and are isoelectric at pH 5.2 and 4.9, respectively. Both have been shown (Camargo et al., 1973) to be activated by DTT and inhibited by PCMB. Each enzyme hydrolyzes bradykinin at a single peptide bond over the range pH 6 to 9. Brain kininase A has a pH optimum at 7 and B near 8.5. Neither enzyme hydrolyzes the bradykinin fragment within kininogen, but they do hydrolyze an octadecapeptide containing bradykinin as an internal segment. The properties of brain kininase A and B documented here and previously

(Camargo et al., 1973) indicate that these enzymes have not been described before. The site of peptide bond hydrolysis in bradykinin,[2] the inability to hydrolyze denatured hemoglobin, and the absence of a $CaCl_2$ requirement distinguish both enzymes from the neutral and alkaline proteinase isolated from brain by Marks and collaborators (Marks and Lajtha, 1963, 1965; Marks and Pirotta, 1971; Marks and Stern, 1974).

Brain kininase B hydrolyzes the Pro^7–Phe^8 peptide bond of bradykinin to release the dipeptide Phe-Arg. Enzymes from other tissues that hydrolyze the same peptide bond in bradykinin also convert angiotensin I to angiotensin II by releasing the dipeptide His-Leu from the carboxyl terminus of angiotensin I (Yang et al., 1970; Soffer et al., 1974; and Dorer et al., 1974). The enzyme referred to in the literature as peptidyl-peptide hydrolase (EC 3.4.15.1) is also able to sequentially release a second dipeptide, Ser-Pro, from bradykinin (Yang et al., 1971). Preliminary studies (Spadaro and Greene, unpublished result) indicate that brain kininase B hydrolyzes angiotensin I without converting it to angiotensin II. The inactivation of angiotensin I and the absence of hydrolysis of the Phe–Ser bond of des-Phe-Arg-bradykinin peptide (Table I) show that brain kininase B and the kininase II from plasma, kidney, or lung are different in their enzymatic properties.

Many tissues contain enzymes capable of metabolizing peptide hormones such as bradykinin, angiotensin (Erdös, 1975), glucagon, and insulin (Bohley et al., 1971). Apart from the important question of the physiological inactivation of these peptides which is related to the modulation of hormone activity, the use of peptide hormones as substrates has contributed to the isolation and characterization of several proteolytic enzymes (cf. Bohley et al., 1971). Indeed, the peptidases described here would not be easily detected and distinguished using classical substrates such as peptide amides, esters, or denatured proteins. Even peptides structurally related to bradykinin might not have been substrates, as one would have expected on the basis of the properties of classical endopeptidases such as chymotrypsin or trypsin. This more restricted specificity is illustrated with brain kininase A by the reduced rate of hydrolysis of des-Arg^9-bradykinin (50% relative to bradykinin) and des-Phe^8-Arg^9-bradykinin (2% relative to bradykinin), and also by the inability of either enzyme to hydrolyze the bradykinin fragment located within the peptide chain of a reduced and alkylated, or denatured protein, kininogen. In spite of the fact that the amino acid sequence adjacent to the sensitive bond is identical, the size and perhaps the conformation of the substrate appear to be important for hydrolysis by brain endopeptidases A and B. The contribution of the interactions of amino acids adjacent to the scissile bond in the substrates with subsites on the proteolytic enzyme has recently been documented for papain (Schechter and Berger, 1967, 1968), elastase (Thompson and Blout, 1973), and pepsin (Sachdev and Fruton, 1970). Thus, the sequence of amino acids immediately preceding the sensitive peptide bond may be required for specific cleavage by an endopeptidase leading important metabolic processes as it occurs with the activation of plasminogen by the factor Xa (Sottrup-Jensen et al., 1975).

[2] Marks and Pirotta (1971) reported that brain neutral proteinase preparations completely hydrolyze bradykinin to free amino acids upon treatment for 25 h. They also concluded that the Phe^5–Ser^6 peptide bond of bradykinin had been specifically hydrolyzed on the basis of the rates of release of the free amino acids serine, proline, phenylalanine, and arginine at shorter time intervals. We suggest that conclusions concerning the specificity of an endopeptidase, based on the release of amino acids, are not justifiable.

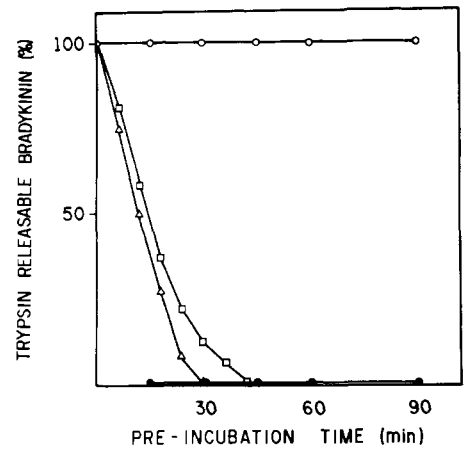

FIGURE 7: Hydrolysis of bradykinin portion of kininogen and Gly-Leu-Met-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Ser-Val-Gln-Val. Carboxymethylated kininogen (0.2 mg/ml), the octadecapeptide containing bradykinin (5 nmol/ml), or bradykinin (5 nmol/ml), in Tris-HCl buffer, pH 7.5, 0.1 M NaCl and 0.5 mM DTT, was treated with kininase A (0.02 mg/ml) for 15, 30, 45, 60, and 90 min at 37 °C. After this preincubation step, the reaction mixture was boiled and treated with trypsin (0.1 mg/ml) at 37 °C for 30 min. The amount of residual bradykinin was estimated by bioassay using the isolated guinea pig ileum. (○—○) Carboxymethylated kininogen + brain kininase A; (△—△) bradykinin + brain kininase A; (□—□) octadecapeptide + brain kininase A; (●—●) carboxymethylated kininogen + chymotrypsin.

The relationship of substrate conformation to susceptibility to proteolysis is well known for denatured proteins (cf. Rupley, 1967) and has recently been shown for abnormal proteins (Goldberg et al., 1975). The view that not only amino acids adjacent to the sensitive bond but also a large portion of the peptide molecule are important to achieve the mutual conformation adjustments required for the interaction of large protein substrates and proteases has been suggested by Berger et al. (1969) and Fruton (1973). According to Schimke (1975), the properties of the proteolytic enzymes associated with conformational changes in intracellular proteins may explain part of the control mechanism for intracellular protein degradation. It is possible that in the process of protein breakdown to free amino acids, endopeptidases like those described here participate only in the hydrolysis of oligopeptides formed by the action of specific proteases on native enzymes.

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