Proc. Natl. Acad. Sci. USA
Vol. 76, No. 8, pp. 3612–3616, August 1979
Biochemistry

Sequential cleavage of proinsulin by human pancreatic kallikrein and a human pancreatic kininase

(β cell endopeptidase/aprotinin inhibition)

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Contributed by K. Frank Austen, March 28, 1979

ABSTRACT **A pancreatic endopeptidase localized to the β-cells of the pancreas by immunohistochemical techniques has been purified to homogeneity by following its functional and antigenic characteristics as a glandular kallikrein (EC 3.4.21.8). The enzyme gave a single stained band on alkaline disc gel electrophoresis which corresponded in location with the kinin-generating activity eluted from a replicate gel, was of 54,000 molecular weight by gel filtration, was devoid of caseinolytic activity, elicited a monospecific antiserum in a rabbit, and gave a line of complete identity with a single constituent in pancreatic extract, crude urine, and purified urokallikrein when analyzed with monospecific antibody to urokallikrein. The pancreatic glandular kallikrein generated three cleavage products of increasing anodal mobility from bovine and porcine proinsulin, and the presence of pancreatic kininase or bovine carboxypeptidase B increased the quantity of these products. Although the conversion products did not correspond to diarginyl- and monoarginylinsulin, the product of intermediate mobility was also obtained when proinsulin was treated with a low concentration of trypsin in the presence of kininase. The most rapidly migrating product did correspond to desalanylinsulin in the reference standard. Kininase alone had no action on proinsulin, and aprotinin prevented cleavage by kallikrein alone or in combination with kininase. Although the chemical structure of the proinsulin cleavage products has not been established, human pancreatic kallikrein is considered a putative activator of proinsulin because of its location in the β-cell, its preferential action on proinsulin and kininogen as compared to azocasein, and its capacity to generate insulin intermediate products that are further modified by human pancreatic kininase or bovine carboxypeptidase B.**

Kallikreins or kininogenases (EC 3.4.21.8) are endopeptidases that generate vasoactive kinin polypeptides from plasma α_2-globulin substrates, kininogens (1, 2). The glandular kallikreins, which have been identified in pancreas (3, 4), salivary glands (5), and urine (6, 7), are antigenically related and distinct from plasma kallikrein (8, 9). The IgG fraction of a monospecific antiserum to human urinary kallikrein, which gave a line of identity with a single constituent in crude urine, whole pancreatic extract, and purified kallikreins from each of these sources, was used for the specific immunohistochemical localization of pancreatic kallikrein to the β-cells of the islets (10). The immunohistochemical cellular distribution of glandular kallikrein antigen was identical to that of insulin in normal human pancreas and in islet cell tumors with β-cells, whereas both antigens were absent in tissues lacking β-cells, such as other islet cell tumors and the pancreas of a patient with juvenile onset diabetes. That the antigen recognized by antiurokallikrein was a glandular kallikrein and not insulin was established by the failure of absorption with insulin to diminish the recognition of a β-cell antigen by antiurokallikrein, whereas absorption with purified urinary or pancreatic kallikrein abolished the localization. The immunohistochemical localization of a human glandular kallikrein to the β-cell of pancreatic islets introduces the possibility that this endopeptidase may play a role in the biosynthetic pathways of insulin.

Proinsulin can be converted to native insulin by the action of appropriate mixtures of commercial trypsin and carboxypeptidase B (11). Trypsin cleavage initially yields a polypeptide fragment, C-peptide with Lys-Arg residues at its carboxy terminus, and diarginylinsulin (11–13). The basic residues are sequentially removed from the carboxy terminus of the B chain of diarginylinsulin by carboxypeptidase B. The cleavage of proinsulin in intact rat islet cells (13, 14), islet cell homogenates (15, 16), and crude secretion granule fractions (17–20) demonstrated the presence of tryptic endopeptidase activities distinct by location from the exocrine enzyme, trypsin (13, 14, 20). Low amounts of carboxypeptidase B-like activities have also been detected in these preparations, and the sequential action of a tryptic endopeptidase and a carboxypeptidase B-like enzyme could constitute the insulin-activating mechanism of the endocrine pancreas (13, 19, 20). A β-cell endopeptidase, pancreatic glandular kallikrein, has been purified to homogeneity and shown to cleave proinsulin, and a pancreatic carboxypeptidase B-like enzyme, kininase, acted in concert to augment proinsulin cleavage.

MATERIALS AND METHODS

Reagents. Hexadimethrine bromide (Polybrene; Aldrich); PM-10 Diaflo membranes (Amicon, Lexington, MA); sodium dodecyl sulfate, acrylamide, *N,N'*-methylenebisacrylamide, ammonium persulfate, and high grade reagent urea (Bio-Rad); Kunitz bovine basic pancreatic trypsin inhibitor, aprotinin (Boehringer Mannheim); 4,5-dihydroxy-2,7-naphthalene disulfonic acid, chromotropic acid, and glacial acetic acid (Eastman); Ampholine carrier ampholytes (LKB, Hicksville, NY); 99.9% pure methyl alcohol (Fisher); synthetic bradykinin (New England Nuclear Corp.); activated CH-Sepharose 4B, Sephadex G-100, Sephacryl S-200, and sizing standards (Pharmacia); bovine insulin and *p*-tosyl-L-arginine methyl ester (TAME) (Sigma); DEAE (DE-52)-cellulose (Whatman); diisopropyl phosphorofluoridate-treated bovine carboxypeptidase B and soybean trypsin inhibitor (SBTI) (Worthington) were obtained as indicated. Bovine and porcine proinsulin and a tryptic digest of porcine proinsulin were gifts of Ronald E. Chance (Eli Lilly). The IgG fraction of rabbit anti-human urinary kallikrein (urokallikrein) serum was that described (21). Commercial trypsin was further purified by affinity chromatography (22).

Isolation of Kallikrein and a Kininase from Human Pancreas. The pancreas of a patient without a history of diabetes was obtained at 3-hr post mortem. The peripancreatic fat and capsule were removed and the tissue was stored at −70°C. Frozen, sliced pancreas (47 g) was homogenized at 4°C in 0.75

Abbreviations: TAME, *p*-tosyl-L-arginine methyl ester; SBTI, soybean trypsin inhibitor.

M NaCl containing 70 μM SBTI. The homogenate was freeze–thawed six times and centrifuged at 25,000 × *g* for 20 min at 4°C. The supernatant was removed, dialyzed for 12 hr at 4°C against 0.05 M Tris·HCl (pH 8.5), and centrifuged at 25,000 × *g* for 20 min at 4°C. This supernatant was concentrated at 4°C to 40 ml by positive pressure ultrafiltration, dialyzed for 12 hr at 4°C against 0.05 M Tris·HCl (pH 8.0), made 0.5 M in NaCl, and filtered through a 5 × 100 cm column containing Sephacryl S-200 previously equilibrated against the dialysis buffer. The column was run at a flow rate of 100 ml/hr and 16-ml fractions were collected. The fractions were screened for: protein, A_{280}; kinin-generating activity, micrograms of kinin released from heat-inactivated plasma per ml of fraction (9, 23); kallikrein antigen by Ouchterlony analysis with anti-urokallikrein IgG (21); TAME hydrolyzing activity, micromoles of methanol released per ml (21, 24); caseinolytic activity, microgram trypsin equivalents per ml (25); and kininase activity, micrograms of bradykinin inactivated per ml (26). For the last assay 100 or 200 ng of synthetic bradykinin was incubated with 2- to 50-μl samples of column fractions for 5 min at 37°C, and the residual kinin was bioassayed on the guinea pig terminal ileum. Fractions containing kinin-generating activity, glandular kallikrein antigen, or kininase were combined, concentrated from 730 to 15 ml by positive pressure ultrafiltration, dialyzed against 0.1 M $NaHCO_3$, pH 7.9/0.5 M NaCl for 12 hr at 4°C, and applied to an aprotinin-Sepharose 4B affinity column (21) equilibrated with the dialysis solution at 24°C. After 60 min the column was transferred to 4°C and washed with the cold dialysis solution; 4-ml fractions were collected at a flow rate of 60 ml/hr until the absorbance at 280 nm decreased to zero. The column was eluted with 0.05 M Tris·HCl, pH 6.5/0.5 M NaCl until the absorbance again fell to zero. Elution with 0.1 M sodium acetate–acetic acid solution, pH 3.2/1 M NaCl was carried out at a flow rate of 80 ml/hr, and 2-ml fractions were collected in tubes containing 2 ml of 2 M Tris·HCl, pH 8.5. Approximately 2% of the protein, 2% of the TAME hydrolyzing activity, 3% of the caseinolytic activity, and all detectable kinin-generating activity and glandular kallikrein antigen appeared in the last elution step (Fig. 1).

Fractions 91–105 from the affinity chromatography column were pooled, concentrated by positive pressure ultrafiltration to 9.7 ml, dialyzed against 0.05 M Tris·HCl (pH 8.0), made 0.2 M in NaCl, and applied to a 2.6 × 90 cm column containing Sephadex G-100 previously equilibrated with the dialysis buffer. The column was calibrated with human serum albumin, ovalbumin, chymotrypsinogen A, and ribonuclease A. Kinin-generating activity and glandular kallikrein antigen filtered together at an approximate molecular weight of 54,000 with approximately one-half of the applied TAME hydrolyzing activity. The caseinolytic activity filtered at a molecular weight of 26,000 and was coincident with the rest of the applied TAME hydrolyzing activity. Alkaline disc gel electrophoresis of 20 μg of purified enzyme showed a single stained protein band, which corresponded to the kinin-generating activity recovered in 88% yield from a replicate gel prepared with 10 μg of purified enzyme (Fig. 2). The three-step-purification procedure yielded a pancreatic kallikrein that generated 1730 μg of kinin per mg of enzyme per min when incubated with heat-inactivated plasma.

Kininase was separated from kallikrein at the aprotinin-Sepharose affinity column step where it appeared at the front in association with 98% of the applied protein. Fractions 1–32 (Fig. 1) were pooled, concentrated to 16 ml, dialyzed for 12 hr at 4°C against 0.05 M Tris·HCl (pH 7.2), and applied to a 2.5 × 60 cm column containing DE-52 cellulose previously equilibrated with the dialysis buffer. Eighty percent of the applied

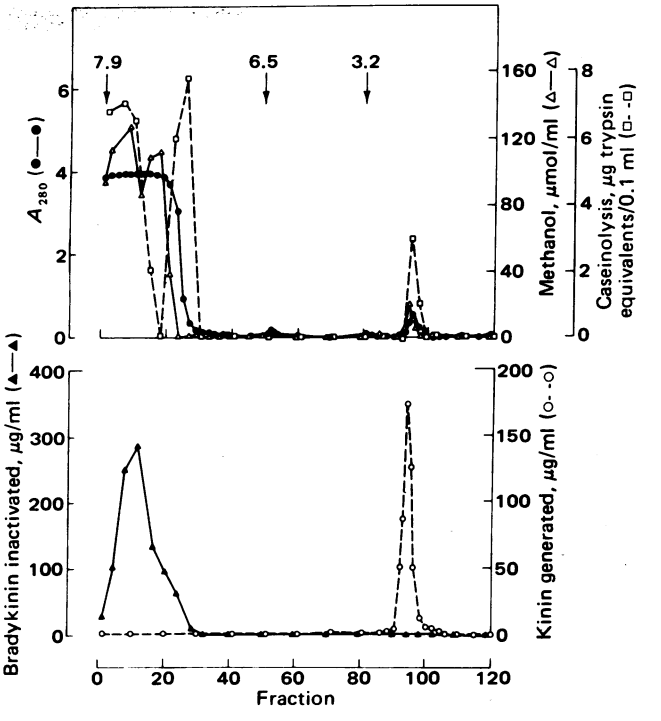

FIG. 1. Aprotinin affinity chromatography of pancreatic kallikrein obtained after Sephacryl S-200 gel filtration of the supernatant recovered from isoelectric precipitation of the tissue extract. The arrows indicate the application of the equilibrating solution, a pH 6.5 buffer, and a pH 3.2 buffer. Glandular kallikrein antigen was detected only in fractions 92–96.

bradykinin-inactivating activity was recovered in the effluent in association with less than 5% of the applied protein. The effluent fractions were pooled, concentrated from 145 to 7 ml by positive pressure ultrafiltration, dialyzed against 0.05 M Tris·HCl, pH 8.0/0.2 M NaCl, and filtered through a Sephadex G-100 column under the same conditions described for pan-

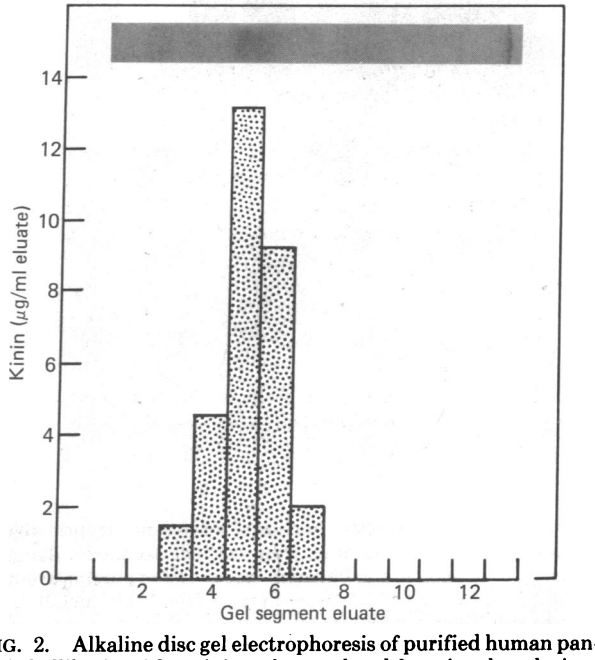

FIG. 2. Alkaline disc gel electrophoresis of purified human pancreatic kallikrein with staining of one gel and functional analysis of eluates of a replicate gel. The anode was at the left.

creatic kallikrein. Kininase filtered with 30% of the applied protein at an approximate molecular weight of 24,100. The four-step purification procedure yielded a kininase capable of destroying 1.04 mg of synthetic bradykinin per mg of enzyme per min at 37°C. Acid disc gel electrophoresis of 30 μg of purified kininase gave a single stained protein band. The anionic characteristics of this kininase on ion exchange chromatography and acid disc gel electrophoresis, the apparent molecular weight of 24,100 by gel filtration, and the capacity to inactivate bradykinin and to cleave hippuryl-arginine are compatible with the characteristics of mammalian pancreatic carboxypeptidase B (27).

RESULTS

The proteolysis of proinsulin by low and high concentrations of trypsin was used to develop cleavage products for comparison with those obtained when kallikrein acted on the same substrate. Forty-microgram portions of porcine proinsulin were incubated at 37°C with 2.0 μg of trypsin with and without 1.9 μg of kininase for 1 min, with 0.04 μg of trypsin with and without 2.7 μg of kininase for 5 min, with 2.7 μg of kininase, or with buffer alone. All reactions were carried out in 0.2 M Tris·HCl, pH 8.0, in a 100-μl final volume. The reaction products were characterized by their relative electrophoretic mobilities (R_F) in alkaline disc gels (28) containing 7 M urea (29). The 17.5% acrylamide separating gel, the 5% acrylamide stacking gel, the electrophoresis buffers, and the 100-μl samples were all made 7 M in urea. The gels were pre-electrophoresed for 20 min at 1.25 mA per gel, and the cathodic buffer was replenished before the samples were applied. Electrophoresis was carried out until the tracking dye reached the anodal end of the gels. The gels were then stained with 0.1% Coomassie brilliant blue.

Urea/polyacrylamide gel electrophoresis of porcine proinsulin gave a single major stained band with $R_F = 0.31$, and a reference tryptic digest known to contain diarginylinsulin, monoarginylinsulin and desalanylinsulin demonstrated three bands of $R_F = 0.34$, 0.42, and 0.54, respectively (Fig. 3) (12). Two micrograms of trypsin generated cleavage products after 1 min of incubation that corresponded in mobility to the three products in the reference tryptic digest, and the presence of kininase in the reaction mixture gave more complete conversion to the desalanylinsulin. After incubation of proinsulin with 0.04 μg of trypsin for 5 min, there was no appreciable formation of the monoarginyl and desalanyl forms, whereas the addition of kininase to the incubation mixture resulted in the generation of a product with an R_F of 0.45 which is intermediate between those of monoarginyl- and desalanylinsulin. The exposure of proinsulin to 0.04 μg of trypsin and kininase for 2 hr resulted in a product with an R_F of 0.55 corresponding to that of desalanylinsulin. When kininase was incubated with proinsulin in the absence of trypsin, no cleavage products were observed.

The capacity of purified human pancreatic kallikrein to cleave porcine proinsulin was also examined with or without kininase. Forty-microgram portions of porcine proinsulin were incubated for 2 hr at 37°C with buffer, with 0.88 μg of kallikrein alone, and with kallikrein and 1.9 μg of kininase or 0.72 μg of commercial bovine pancreatic carboxypeptidase B. Incubation of porcine proinsulin with kallikrein alone generated a small amount of material ($R_F = 0.38$) that moved just anodally to proinsulin as well as a product with an R_F of 0.45 (Fig. 4). The

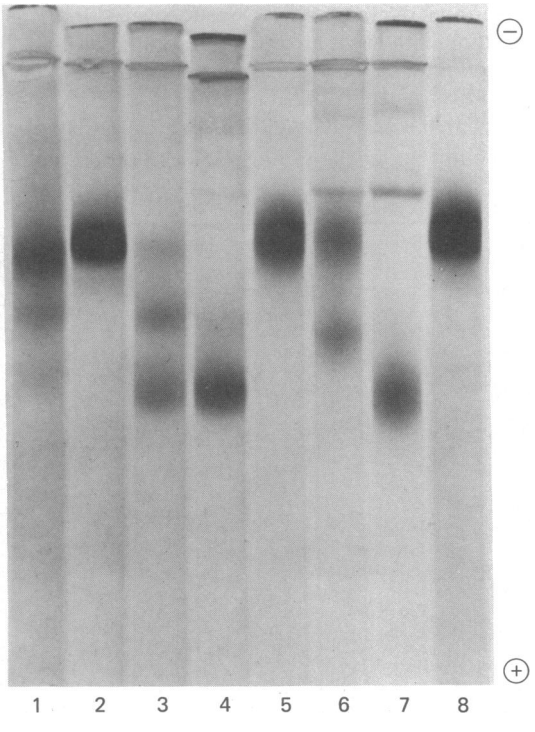

FIG. 3. Urea gel electrophoresis of a reference tryptic digest of porcine proinsulin containing, from cathode to anode, diarginyl-, monoarginyl-, and desalanylinsulin (gel 1) and of porcine proinsulin treated with buffer (gel 2), 2 μg of trypsin for 1 min (gel 3), 2 μg of trypsin and 1.9 μg of kininase for 1 min (gel 4), 0.04 μg of trypsin for 5 min (gel 5), 0.04 μg of trypsin and 2.7 μg of kininase for 5 min (gel 6), 0.04 μg of trypsin and 2.7 μg of kininase for 2 hr (gel 7), and 2.7 μg of kininase alone for 2 hr (gel 8).

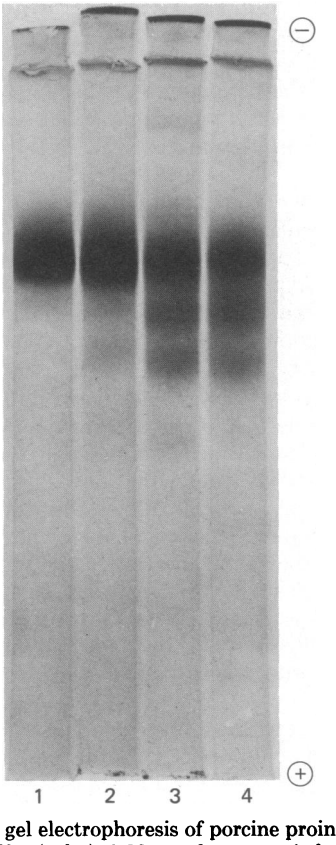

FIG. 4. Urea gel electrophoresis of porcine proinsulin incubated for 2 hr with buffer (gel 1), 0.88 μg of pancreatic kallikrein (gel 2), kallikrein and 1.9 μg of human pancreatic kininase (gel 3), and kallikrein and 0.72 μg of bovine carboxypeptidase B (gel 4).

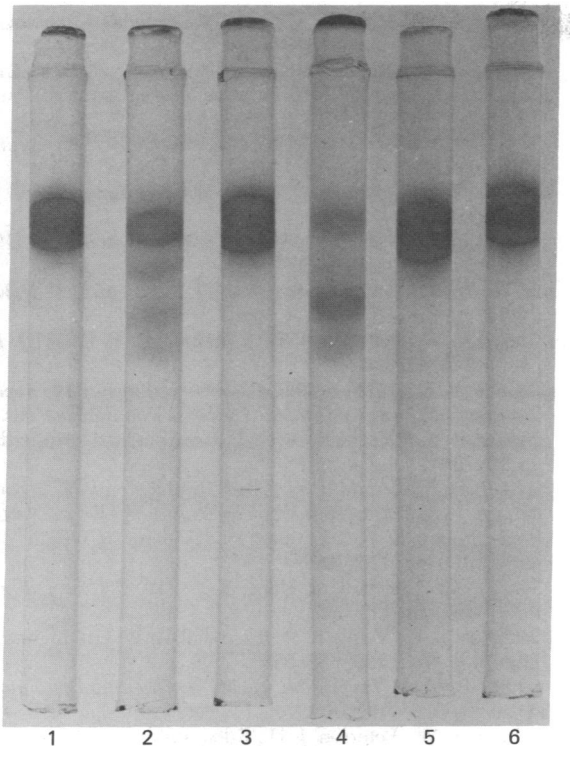

FIG. 5. Urea gel electrophoresis of 30 μg of bovine proinsulin incubated for 2 hr at 37°C with buffer (gel 1), 3 μg of human pancreatic kallikrein (gel 2), 0.9 μg of human pancreatic kininase (gel 3), 3 μg of pancreatic kallikrein and 0.9 μg of kininase (gel 4), 3 μg of pancreatic kallikrein and 10 μg of aprotinin (gel 5), and 3 μg of kallikrein, 10 μg of aprotinin, and 0.9 μg of pancreatic kininase (gel 6). The anode was at the bottom.

addition of 1.9 μg of kininase to the kallikrein and proinsulin reaction mixture led to the increased formation of these bands (R_F = 0.38 and 0.45) and to the appearance of a band with a mobility of R_F = 0.55. Substitution of bovine pancreatic carboxypeptidase B for the human pancreatic kininase resulted in the formation of reaction products with the same mobilities as those generated in the presence of kininase (Fig. 4).

Bovine proinsulin was also a substrate for human pancreatic kallikrein. Thirty-microgram portions of proinsulin were incubated with 3 μg of purified kallikrein, 0.9 μg of pancreatic kininase, human pancreatic kallikrein and kininase, human pancreatic kallikrein and 10 μg of aprotinin, both enzymes with aprotinin, or buffer alone at 37°C for 2 hr. Pancreatic kallikrein diminished the proinsulin band and generated three products with R_F values of 0.33, 0.40, and 0.45 (Fig. 5). The kininase alone had no effect on proinsulin but, together with kallikrein, cleaved the majority of proinsulin and increased the amount of the second product band with an R_F of 0.40. In the presence of aprotinin neither pancreatic kallikrein alone nor kallikrein and kininase cleaved proinsulin (Fig. 5). When the reaction was carried out for 2 hr at pH 6.4, rather than at pH 8.0, there was no detectable proinsulin cleavage by kallikrein or kininase alone, whereas the combination cleaved about one-third of the proinsulin to yield the cleavage product with an R_F of 0.40.

DISCUSSION

A pancreatic endopeptidase localized to the β-cells of the islets by immunohistochemical techniques (10) has been isolated by following its functional and antigenic characteristics as a glandular kallikrein. The initial pancreatic extraction was carried out in the presence of SBTI to inhibit trypsin; and chymotrypsinogen, trypsinogen, trypsin, and elastases were depleted by isoelectric precipitation (30). The pancreatic kallikrein was then isolated by Sephacryl S-200, aprotinin-Sepharose affinity chromatography (Fig. 1), and Sephadex G-100 gel filtration. Twenty micrograms of the purified pancreatic kallikrein gave a single stained band on alkaline disc gel electrophoresis in a region that corresponded to kinin-generating activity eluted from a replicate gel (Fig. 2). The kinin-generating activity expressed as μg of kinin per mg of enzyme per min using excess heat-inactivated plasma substrate was comparable to that observed for purified urinary kallikrein (21), whereas the TAME esterase activity of the pancreatic enzyme expressed as μmol of methanol generated per mg of enzyme per min was only 1/10th that observed with urokallikrein. Two micrograms of glandular pancreatic kallikrein had no caseinolytic activity in an assay capable of detecting 0.2 μg of trypsin. The caseinolytic activity in the pancreatic extract that was not inhibitable by SBTI or susceptible to isoelectric precipitation appeared almost entirely in the equilibrating buffer in the aprotinin-Sepharose affinity step. The caseinolytic activity that bound to the affinity ligand was separated from the glandular kallikrein by gel filtration in which it exhibited a molecular weight of 26,000 compared to 54,000 for the pancreatic glandular kallikrein. The purified pancreatic kallikrein elicited a monospecific antibody in a rabbit and gave a line of complete identity with a single constituent in crude urine, pancreatic extract, and purified urokallikrein when analyzed with a monospecific antibody to urokallikrein (21). Based upon the concordance of the antigenic and functional properties of pancreatic glandular kallikrein and the absence of caseinolytic activity, it was concluded that the endopeptidase isolated was that localized to the β-cells of the islets by immunohistochemical techniques.

The action of pancreatic glandular kallikrein on proinsulin was compared to that of high and low concentrations of trypsin. Porcine proinsulin exposed to 2 μg of trypsin for 1 min presented three bands on urea gel electrophoresis that corresponded to diarginylinsulin, monoarginylinsulin, and desalanylinsulin when compared to a reference standard (12). As expected, the additional presence of a kininase, with carboxypeptidase B-like activity, augmented the conversion of diarginyl and monoarginyl intermediates to desalanylinsulin (Fig. 3). One-fiftieth the concentration of trypsin produced no detectable cleavage of proinsulin during a 5-min interaction. That some cleavage of proinsulin may have occurred was suggested by the fact that the introduction of kininase to the reaction mixture converted approximately one-half of the proinsulin to a cleavage product intermediate in anodal mobility (R_F = 0.45) to monoarginylinsulin (R_F = 0.42) and desalanylinsulin (R_F = 0.55). Prolongation of the interaction time to 2 hr resulted in the complete conversion of proinsulin (R_F = 0.31) to a product with the mobility of desalanylinsulin (R_F = 0.55). That kininase alone had no effect on proinsulin indicated that the proinsulin did not already contain cleavages critical to the action of kininase.

Treatment of porcine proinsulin with 0.88 μg of human pancreatic kallikrein generated two distinct products, one of which had an R_F of 0.45 and was intermediate in mobility to monoarginyl- and desalanylinsulin and similar in mobility to the product observed with the low concentration of trypsin; the other (R_F = 0.38) moved between proinsulin and monoarginylinsulin and could either be diarginylinsulin or a split form of proinsulin (12) with increased anodal mobility (Fig. 4). The presence of either kininase or commercial carboxypeptidase B augmented the conversion of proinsulin to the two cleavage products observed with kallikrein alone and permitted generation of a band corresponding in position to desalanylinsulin

(R_F = 0.55). In the absence of amino acid analysis, it is not yet possible to assign definitive structures to the cleavage products, but it is clearly evident that human pancreatic kallikrein and kininase have a concerted action on proinsulin.

When the concentration of pancreatic kallikrein was increased 4-fold and the substrate was bovine proinsulin, the 2-hr incubation yielded three cleavage products of increasing anodal mobility (Fig. 5). These could be diarginylinsulin or a split form of proinsulin, a product analogous to the major intermediate obtained with the lower concentration of trypsin and kininase and either desalanylinsulin or native insulin. The presence of kininase resulted in almost complete cleavage of proinsulin and increased the prominence of the two products with greater anodal mobility. The presence of aprotinin in the reaction mixture prevented any detectable proinsulin cleavage by either pancreatic kallikrein or pancreatic kallikrein and kininase, indicating that the action of the endopeptidase precedes that of a carboxypeptidase B-like enzyme.

The prevailing view that the mechanism of proinsulin activation involves two internal cleavages adjacent to basic amino acid residues is based upon three lines of evidence: the known structures of sequential cleavage products of proinsulin obtained with isolated islet cells (13, 17, 20); the identification of comparable cleavage products by treatment of proinsulin with bovine trypsin and carboxypeptidase B (11); and the detection of tryptic and carboxypeptidase B-like activities in whole islet cell homogenates or their subcellular fractions (20). The tryptic activity detected with different assays differed in pH optima and susceptibility to inhibition by SBTI and could well have been of lysosomal origin or from non-β islet cells. An endopeptidase capable of converting proinsulin to insulin in the absence of carboxypeptidase B-like activity has been isolated from bovine pancreas (31) and characterized by a molecular weight of 70,000, an isoelectric point of 4.8, and susceptibility to inhibition by SBTI, diisopropyl phosphorofluoridate, and aprotinin. Human pancreatic kallikrein appears as a logical candidate for the activation of proinsulin because of its localization to the β-cell by histochemical techniques, its relative preference for proinsulin and kininogen as substrates as compared to casein, and its capacity to generate proinsulin cleavage products that are further modified by human and bovine carboxypeptidase B.

This work was supported in part by Grants AI-07722, AI-10356, HL-22939, and RR-05669 from the National Institutes of Health. O.o.-MY. was supported in part by private grants from Robert G. Stone and Royal Little, Esq.

1. Webster, M. E. & Pierce, J. V. (1963) *Science* **104,** 91–105.
2. Habal, F. M., Movat, H. Z. & Burrowes, C. E. (1974) *Biochem. Pharmacol.* **23,** 2291–2303.
3. Kraut, H., Frey, E. K. & Werle, E. (1930) *Z. Physiol. Chem.* **189,** 97–106.
4. Webster, M. E. & Prado, E. S. (1970) *Methods Enzymol.* **19,** 681–699.
5. Werle, E. & von Roden, P. (1936) *Biochem. Z.* **286,** 213–219.
6. Frey, E. K. & Kraut, H. (1926) *Langenbecks Arch. Exp. Pathol. Pharmakol.* **157,** 32–61.
7. Frey, E. K. & Kraut, H. (1928) *Langenbecks Arch. Exp. Pathol. Pharmakol.* **133,** 1–56.
8. Ørstavik, T. B. & Glenner, G. G. (1978) *Acta Physiol. Scand.* **103,** 384–393.
9. ole-MoiYoi, O., Spragg, J., Halbert, S. P. & Austen, K. F. (1977) *J. Immunol.* **118,** 667–672.
10. ole-MoiYoi, O., Pinkus, G. S., Spragg, J. & Austen, K. F. *N. Engl. J. Med.*, in press.
11. Kemmler, W., Peterson, J. D. & Steiner, D. F. (1971) *J. Biol. Chem.* **246,** 6786–6791.
12. Chance, R. E. (1971) in *Diabetes Proceedings of the Seventh Congress of the International Diabetes Foundation,* eds. Rodriguez, R. R. & Vallance-Owen, J. (Excerpta Med., Amsterdam), pp. 292–304.
13. Steiner, D. F., Kemmler, W., Clark, J. L., Oyer, P. E. & Rubenstein, A. H. (1972) in *Handbook of Physiology-Endrocrinology I,* eds. Steiner, D. F. & Freinkel, N. (Williams & Wilkins, Baltimore, MD), pp. 175–198.
14. Sun, A. M., Lin, B. J. & Haist, R. E. (1973) *Can J. Physiol. Pharmacol.* **51,** 175–182.
15. Sorensen, R. L., Shank, R. D. & Lindall, A. W. (1972) *Proc. Soc. Exp. Biol. Med.* **139,** 652–655.
16. Smith, R. E. & Van Frank, R. M. (1974) *Endocrinology* **94,** A190.
17. Kemmler, W., Peterson, J. D., Rubenstein, A. H. & Steiner, D. F. (1972) *Diabetes* **21,** Suppl. 2, 572–581.
18. Kemmler, W., Steiner, D. F. & Borg, J. (1973) *J. Biol. Chem.* **248,** 4544–4551.
19. Steiner, D. F., Kemmler, W., Tager, H. S. & Peterson, J. D. (1974) *Fed Proc. Fed. Am. Soc. Exp. Biol.* **33,** 2105–2115.
20. Zühlke, H., Steiner, D. F., Lenmark, Å. & Lipsey, C. (1976) in *Polypeptide Hormones: Molecular and Cellular Aspects, Ciba Foundation Symposium 41* (Elsevier, Amsterdam), pp. 183–195.
21. ole-MoiYoi, O., Spragg, J. & Austen, K. F. (1978) *J. Immunol.* **121,** 66–71.
22. Czop, J. K., Fearon, D. T. & Austen, K. F. (1978) *J. Immunol.* **120,** 1132–1138.
23. Orange, R. P. & Austen, K. F. (1976) in *Methods in Immunology and Immunochemistry,* eds. Williams, C. A. & Chase, M. W. (Academic, New York), pp. 145–149.
24. Siegelman, A. M., Carlson, A. S. & Robertson, T. (1962) *Arch. Biochem. Biophys.* **97,** 159–163.
25. Laskowski, M. (1955) *Methods Enzymol.* **2,** 26–36.
26. Yang, H. Y. T., Erdös, E. G. & Levin, Y. (1970) *Biochim. Biophys. Acta* **214,** 374–376.
27. Reeck, G. R., Walsh, K. A., Hermodson, M. A. & Neurath, H. (1971) *Proc. Natl. Acad. Sci. USA* **68,** 1226–1230.
28. Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.* **121,** 404–427.
29. Tung, A. K. & Yip, C. C. (1969) *Proc. Natl. Acad. Sci. USA* **63,** 442–449.
30. Baugh, R. J. & Travis, J. (1976) *Biochemistry* **15,** 836–841.
31. Yip, C. C. (1971) *Proc. Natl. Acad. Sci. USA* **68,** 1312–1315.