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Altered Amidation of Pancreatic Polypeptide in Cultured Dog Islet Tissue†

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ABSTRACT: Three forms of immunoreactive pancreatic polypeptide (PPI) were detected in extracts of cultured dog pancreatic PP cells: PPI of (1) larger apparent molecular weight than PP, (2) similar apparent molecular weight but different isoelectric point than PP, and (3) identical apparent molecular weight and isoelectric point with PP. Dog pancreatic endocrine cells in culture were labeled biosynthetically with tritiated amino acids, and extracted proteins were fractionated by sodium dodecyl sulfate gel electrophoresis. A total of 97% of the PPI migrated like PP itself while about 3% of the PPI migrated like proteins up to of 7200 molecular weight. PPI migrating like PP was analyzed further by isoelectric focusing and was found to occur in a neutral form like PP and a more acidic form. Peptide mapping of neutral and acidic PPI forms showed that both were like PP with the exception that the C-terminal [^{3}H]tyrosine-containing peptide was a peptide with a net negative charge of 1 arising from a peptide extension of one or a few amino acids. The acidic form of PP was also shown to occur in pancreas extracts. However, neutral PPI was 90% of the total PPI in the pancreas extracts while the converse was true of culture extracts. We conclude that culturing the PP cell affects the efficiency of the process of amidation, that acidic PP could be either biosynthetic precursor or end product, and that the existence of the larger PP form(s) signals (signal) the possible production of yet other peptides by the PP cell.

Pancreatic polypeptide (PP)[1] was originally discovered as a minor component of purified insulin (Lin & Chance, 1972; Kimmel et al., 1975). It appears to be a hormone in that it is found in the pancreas in high concentrations (Gersell et al., 1979) and derives from a unique pancreatic endocrine cell type (Greider et al., 1978). Furthermore, it has an amidated C-terminal like many biologically active peptides and exhibits plasma concentration variations suggestive of a role in the postprandial state (Floyd et al., 1977). However, PP falls short of qualification as a hormone since no physiological role for it has been identified. The principal potential target tissue appears to be the pancreas itself where PP can inhibit sec-

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[1] Abbreviations used: PP, pancreatic polypeptide; pPP, porcine pancreatic polypeptide; PPI, pancreatic polypeptide immunoactivity measured by radioimmunoassay and expressed in porcine pancreatic polypeptide equivalents; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane. A major form of pancreatic polypeptide immunoactivity in culture extracts which migrated to near pH 5 in isoelectric focusing is referred to as acidic PPI to distinguish it from immunoactivity which migrated to near pH 7 as did pPP.

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retin–cholecystokinin-stimulated pancreatic exocrine secretion, an action whose significance is not well understood (Lin et al., 1976; Taylor et al., 1979). Precedent suggests that if PP were a hormone, then it should be the major secretory product of the PP cell. However, in view of the apparent lack of a physiological role for PP and in view of the fact that other peptides may arise during the biosynthesis of PP, much as C peptide arises during insulin biosynthesis, the possibility must be considered that other biologically active peptides are secreted from the PP cell in addition to PP. The development of dog endocrine cell cultures has made it possible to investigate the biosynthesis of PP. The first step in this process was to ascertain whether or not the PP produced by such cultures is in fact identical with PP first found in purified insulin.

The results described here show that more than one molecular form of PP is synthesized by cultured PP cells and that a form different than PP itself was the principal form of PP stored in cultured PP cells.

Experimental Procedures

Immunoassay. Pancreatic-polypeptide-like immunoactivity (PPI) was measured with a guinea pig antiporcine pancreatic polypeptide antibody. This antibody cross-reacted with the following hormones to the degree indicated: porcine insulin and glucagon, 0.001; somatostatin, gastric inhibitory polypeptide, and secretin, 0.0001. This antiserum failed to cross-react with either tryptic or chymotryptic peptides of pPP and therefore must require either the entire pPP sequence or must recognize a site in the C-terminal of pPP where most of the tryptic and chymotryptic cleavages occur. The midpoint of the assay was 100 pg, and the sensitivity was 20 pg/tube. pPP was iodinated with sodium hypochlorite as the oxidant (Redshaw & Lynch, 1974). Bound and free fractions were separated with dextran-coated charcoal containing 1.5 g of neutral Norit A, 0.15 g of dextran T-70 (Pharmacia), 50 mg of bovine γ-globulins (Miles), and 0.5% sodium azide in 0.05 M sodium phosphate, pH 7.5. Immunoassay tube contents were as follows: 0.1 mL of sample and 0.1 mL of tracer premixed with antibody. Tubes were incubated for 16–24 h after which time 0.2 mL of charcoal solution was added, incubated 10 min further, and centrifuged; 0.30 mL of supernatant was withdrawn and counted. pPP 615 D63-158-4, a gift of R. Chance, was used as standard. The amino acid sequences of porcine and canine PPs have been shown to be identical (Chance et al., 1979).

Cultures and Labeling. The culturing procedure has been described (Scharp et al., 1980). Briefly, it involves digestion of minced dog pancreas with 1.5% trypsin, followed by dispersion into cells by shearing in a syringe needle. The endocrine cells in the dispersed cell suspension are enriched on a Ficoll gradient and then put into culture in flasks in a gyrotatory water bath. After a week of culturing, the endocrine cells reaggregate into islet-like structures referred to as pseudoislets. Cultures were labeled after 1–2 weeks of culturing. Labeling was for 16–18 h in a deficient medium so that the specific activity of the amino acids was near that provided by the manufacturer but reduced slightly by the presence of 5% serum. Labels were [3,5-$^3H_2$]tyrosine and [4,5-$^3H_2$]leucine. Pseudoislets contained about 12 ng of PPI and about 30 μg of PPI/mg of protein (Coomassie blue protein assay).

Extractions. Pseudoislets were washed twice with cold unlabeled medium and frozen at −80 °C prior to dissolving in $NaDodSO_4$ gel loading solution (see below).

Uncinate process of dog pancreas was frozen on dry ice. Pieces were broken off, weighed, homogenized as a 10% (w/v) solution, and centrifuged at 10000g for 10 min. In some cases, the tissue was boiled in water for 10 min prior to homogenization. All homogenization solvents contained 0.2 μg/mL phenylmethanesulfonyl fluoride added just prior to homogenization. Various extractions produced the following amounts of PPI in micrograms per gram wet weight: for unboiled tissue, 0.1 M HCl/80% ethanol, 46; for boiled tissue, 0.1 M HCl/80% ethanol, 51; for boiled tissue, 1% acetic acid, 31; for boiled tissue, 0.1 M ammonium bicarbonate, 12. pPP recoveries assessed at the time of assay by doubling the amounts of PPI in each tube by adding pPP standard were 92%, 88%, 125%, and 92%, respectively.

Gel Electrophoresis. (*A*) *$NaDodSO_4$ Gels.* Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed in 5.5 × 90 mm gels containing 17% acrylamide–0.34% bis(acrylamide). Gel buffer and running buffer were 0.2 M Tris–acetate, pH 6.4, and 0.1% $NaDodSO_4$. Gel loading solution was 8 M urea, 2% $NaDodSO_4$, 4% mercaptoethanol, and 0.04 M Tris–acetate, pH 6.4. Samples were prepared for running by heating for 3 min at 100 °C in loading solution, cooling, and adding bromophenol blue tracking dye. Gels were run for 16 h at 40 V at room temperature. Gels were sliced into 2-mm slices which were crushed and eluted for 24 h by shaking in immunoassay buffer. Aliquots of the eluates were either subjected to immunoassay or counted in a scintillation counter. The mobilities of various proteins relative to the center of the dye band were the following: pPP, 0.92; aprotinin, 0.78; cytochrome *c*, 0.63; myoglobin, 0.53; yeast alcohol dehydrogenase, 0.28.

(*B*) *Isoelectric Focusing Gels.* Focusing was performed in a gel system designed to be used as a second dimension after $NaDodSO_4$ gel electrophoresis (Tuszynski et al., 1979). Gels were 8.5% acrylamide–0.17% bis(acrylamide) and were polymerized with riboflavin 5-phosphate (100 μg/30 mL) and tetramethylethylenediamine (TEMED) (5 μL/30 mL). The gel buffer was 2% Bio-Lyte 3/10 ampholyte (Bio-Rad), 6 M urea, and 2% Triton X-100. Electrode buffers were 0.05 M ethanolamine, pH 10.5, and 0.05 M glutamic acid, pH 2.5. Gels were prerun 30 min at 4 °C at 160 V, and samples were mixed in running buffer prepared with recrystallized urea. Samples were run from cathode to anode for 16 h at 4 °C at 160 V. Gels were sliced, eluted, and sampled as for the $NaDodSO_4$ gels. Parallel gels containing myoglobin (pI = 7.0–7.3), cytochrome *c* (pI = 9.3), soybean trypsin inhibitor (pI = 4.5), and ovalbumin (pI = 4.8) were run as standards.

Trypsin and Chymotrypsin Digestions. Isoelectric focusing gel eluates containing 200–400 cpm were made to 0.1 mL with water. A 2-μg sample of bovine serum albumin was added, followed by 22 μL of 100% (w/v) trichloroacetic acid. After 5 min on ice, the samples were centrifuged 5 min at 10000g. The supernatant was withdrawn and counted, and the pellet was washed with 1:1 acetone–ether. The recovery of counts in the pellet at this step was 85%.

Trichloroacetic acid precipitates were taken up in 0.1 mL of 0.1 M ammonium bicarbonate, and 1 μg of tosyllysyl chloromethyl ketone (TLCK) chymotrypsin (Sigma) or diphenylcarbamoyl chloride (DPCC) trypsin and 2 μg of pPP were added. Digestion was complete in 2 h at 37 °C and was stopped by acidification and lyophilization. Chymotryptic peptides were determined by digestion of pPP under the conditions given above followed by thin-layer high-voltage electrophoresis (see below), elution, and amino acid analysis. The peptides so identified were pPP (1–20) or Ala-Pro-Leu-Glu-Pro-Val-Tyr-Pro-Gly-Asp-Asp-Ala-Thr-Pro-Glu-Gln-Met-Ala-Gln-Tyr, pPP (21–24) or Ala-Ala-Glu-Leu, pPP

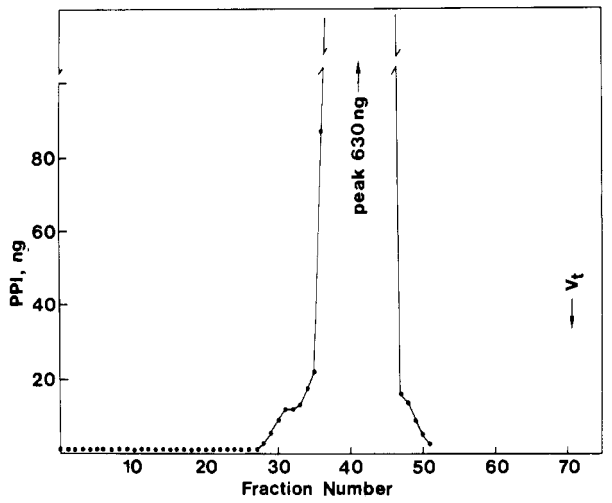

FIGURE 1: Guanidine hydrochloride Bio-Gel P-100 chromatography of a pseudoislet extract. Approximately 200 pseudoislets were dissolved in 6 M guanidine hydrochloride and 4% mercaptoethanol at 100 °C for 3 min and applied to a 0.5 × 60 cm column of Bio-Gel P-100 (fine) and eluted with 5 M guanidine hydrochloride, 0.1 M mercaptoethanol, and 0.2 μg/mL bovine serum albumin. The flow rate was 2 mL/h. Fractions were diluted and immunoassayed; 2.3 μg of PPI was applied and 1.6 μg was recovered.

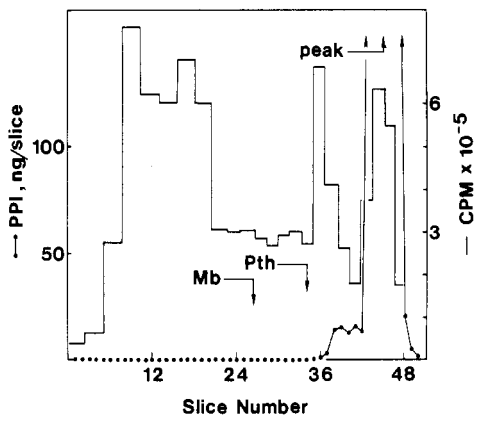

FIGURE 2: $NaDodSO_4$ gel analysis of a pseudoislet extract. Approximately 200 pseudoislets were extracted by heating in gel loading solution (see Experimental Procedures) at 100 °C for 3 min. The gel was run 16 h at 40 V and sliced, and the slices were eluted and the eluates immunoassayed. A 2.1-μg sample of PPI was applied and 1.9 μg was recovered; the peak slice contained 1.25 μg. In a separate experiment, [^{3}H]tyrosine-labeled pseudoislets were extracted and electrophoresed under the same conditions as described for immunoassay except that the gel was sliced by hand without the aid of a slicing block. The location of the PPI on the gel was determined, and the ^{3}H counts were plotted by using the PPI peak as a reference point. Mb is myoglobin and Pth is ^{125}I-labeled parathyroid hormone.

(25–27) or Arg-Arg-Tyr, pPP (28–31) or Ile-Asn-Met-Leu, and pPP (32–36) or Thr-Arg-Pro-Arg-Tyr(NH_2). Tryptic peptides were predicted to be pPP (1–25 or 26), pPP (27–35) and pPP (36). Peptides for amino acid analysis were prepared by scraping the cellulose thin-layer electrophoresis medium into a Pasteur pipet column and eluted with 1% acetic acid followed by water and then 1% pyridine. For counting, peptides were eluted into 1% acetic acid containing 0.1% Triton X-100 and 10 μg/mL tyrosinamide.

Thin-Layer High-Voltage Electrophoresis. Thin-layer high-voltage electrophoresis was performed on a cooled flat plate on 20 × 20 cm cellulose thin-layer sheets by using 0.1% triethylamine–carbonate at pH 9.5 as a buffer. Electrophoresis runs were for 40 min at 50 V/cm. Spots were visualized with fluorescamine (Schiltz et al., 1977). pPP itself was not visible with fluorescamine but could be located with phenanthrenequinone (Reed & MacKay, 1978). pPP did not move from the origin. The mobility of the basic chymotryptic peptides was both temperature and pH sensitive, and they did not always migrate toward the cathode. Peptides were identified by scraping the cellulose into scintillation vials and counting.

Gel Filtration. Gel filtration was in a 0.5 × 60 cm column containing Bio-Gel P-100 (fine). Elution was with 5 M guanidine hydrochloride containing 0.2 mg/mL bovine serum albumin and 0.1 M mercaptoethanol at a rate of 2 mL/h. Samples were prepared by heating 3 min at 100 °C in 5 M guanidine hydrochloride and 4.0% mercaptoethanol. Gel filtration of peptides was in a 0.8 × 15.0 cm glass column of Sephadex G-25 eluted with 1:1:1 pyridine–acetic acid–water.

Results

Fractionation of Pseudoislet and Pancreas Extract PPI by Gel Filtration and $NaDodSO_4$ Gel Electrophoresis. Pseudoislet extracts were fractionated under conditions which would prevent proteolytic artifacts and give the best estimates of molecular weights for the PPI detected. Gel filtration of a batch of about 200 pseudoislets in 5 M guanidine hydrochloride is shown in Figure 1. The bulk of the PPI elutes in the position of the ^{125}I-labeled pPP internal marker while a few percent elutes in a position (fraction 31) corresponding to about M_r 7000. $NaDodSO_4$ gel analysis of a pseudoislet extract was performed with results similar to those obtained by gel filtration. Figure 2 shows that pseudoislets labeled with [^{3}H]tyrosine fractionated by $NaDodSO_4$ gel have 3–5% of the total PPI in fractions corresponding to a larger molecular weight than pPP and 95–97% of the PPI in the same fractions as pPP would have been found. The largest apparent molecular weight PPI (mobility 0.79) was 7200.

For determination of whether or not extracts of dog pancreas contained different relative proportions of larger and smaller molecular weight PPIs, an acid alcohol extract of boiled dog uncinate process was analyzed by $NaDodSO_4$ gel electrophoresis. The extract contained 200 μg of protein and 200 ng of PPI. The results were indistinguishable from those shown in Figure 2.

Isoelectric Focusing Analysis of PPI Comigrating with pPP on $NaDodSO_4$ Gels. Isoelectric focusing was performed in order to ascertain whether or not PPI from the pseudoislets was identical with pPP in charge, as would be expected since porcine and canine PPs share the same amino acid sequence. Figure 3 shows the results of focusing pPP, an acid alcohol extract of dog uncinate process, and an aliquot of the eluate of the pPP-like (mobility 0.92) fractions from $NaDodSO_4$ gels of [^{3}H]tyrosine- or [^{3}H]leucine-labeled pseudoislets. The isoelectric point of pPP was near 7, in agreement with calculation (Edsall, 1943). Most of the PPI in pancreas extracts focuses like pPP while about 10% of the PPI focuses near pH 5. While similarly focusing bands were found in pseudoislet extracts, surprisingly most of the PPI focused near pH 5 and was subsequently referred to as acidic PPI. Less than 0.5% of the PPI in the pPP standard was found in the position of acidic PPI.

Acidic PPI was very close to pPP in apparent molecular weight by guanidine hydrochloride chromatography as shown in Figure 4. The point in the $NaDodSO_4$ gel to which pPP and acidic PPI migrated is a region in which mobility and molecular weight are poorly related. Therefore, ^{3}H-labeled neutral and acidic PPIs eluted from focusing gels were subjected to gel filtration on the same columns as shown in Figure 1. An excess of pPP was mixed with the sample prior to loading the column so that pPP could be detected by immunoassay independently from the ^{3}H-labeled neutral and acidic forms of PPI. Two components were detected in the pPP

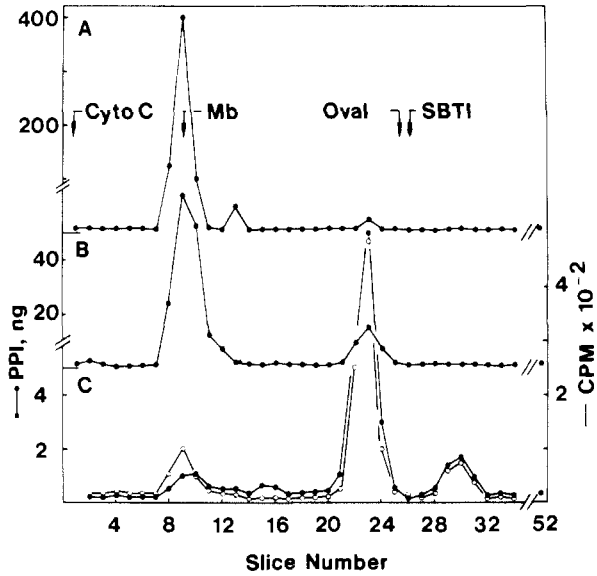

FIGURE 3: Isoelectric focusing of pPP, pancreas extract, and [^{3}H]-tyrosine-labeled PPI fractions from an NaDodSO$_4$ gel. Samples were focused on 5.5 × 100 mm tube gels containing 6 M urea, 1% Triton X-100, and 2% Bio-Lyte 3/10 ampholyte. Gels were sliced, eluted, and aliquoted for immunoassay or scintillation counting. Mb = myoglobin (pI = 7.0–7.3); SBTI = soybean trypsin inhibitor (pI = 4.5); and Oval = ovalbumin (pI = 4.7). (A) pPP standard, 1.0 μg, was applied and 0.6 μg was recovered. (B) Acid alcohol extract of dog pancreas, 220 ng, was applied and 173 ng was recovered. (C) [^{3}H]Tyrosine PPI from the mobility 0.93 (pPP-like) fractions eluted from an NaDodSO$_4$ gel. PPI recovery was 60%.

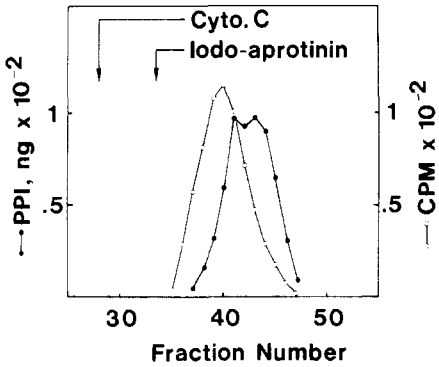

FIGURE 4: Guanidine hydrochloride Bio-Gel P-100 chromatography of ^{3}H-labeled acidic PPI. ^{3}H-Labeled PPI eluted from the acidic PPI peak of the focusing gel (fraction 23, Figure 3C) was mixed with 1.0 μg of pPP and loaded onto and eluted from the same column as in Figure 1.

standard, and acidic PPI eluted one fraction ahead of the first pPP component. Neutral PPI coeluted with the first pPP component (results not shown). It was concluded from this experiment that acidic PPI was very close in molecular weight to pPP.

It was necessary to show that neither acidic PPI nor the striking difference in the relative proportions of acidic and neutral PPIs in pseudoislet and pancreas extracts was an artifact of analysis. Three possible artifacts were considered. The combination of NaDodSO$_4$ gel and focusing gels might have been generating acidic PPI from neutral. However, as shown in Figure 3, acidic PPI was not generated from pPP upon focusing, and NaDodSO$_4$ gel electrophoresis prior to focusing did not change this result. The second and third possible artifacts were that acidic PPI might have been changed to neutral under the conditions of pancreas extraction or insoluble in the acid alcohol extraction solvent. Interconversion or insolubility of neutral or acidic PPIs was monitored by mixing ^{3}H-labeled PPI eluted from an NaDodSO$_4$ gel with

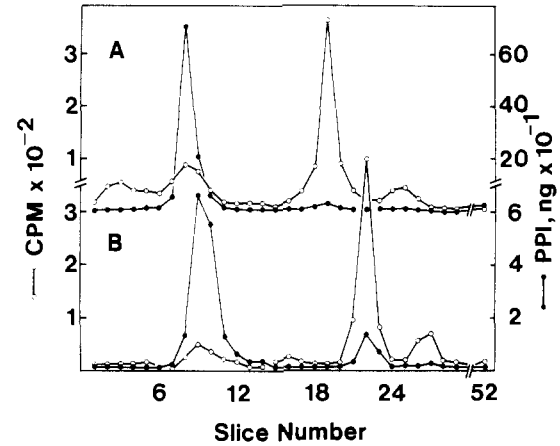

FIGURE 5: Focusing of NaDodSO$_4$ gel purified [^{3}H]tyrosine PPI (3.0 ng of PPI) mixed with pPP or pancreas extract. pPP (2.0 μg) in (A) or pancreas homogenate (240 ng of PPI) in (B) were incubated 3 min with ^{3}H-labeled PPI from eluates of the pPP-like (mobility = 0.92) fractions of an NaDodSO$_4$ gel. The homogenate was centrifuged, and the supernatant was lyophilized and focused. The recovery of ^{3}H was 90%, pPP 0.6 μg, and homogenate PPI 195 ng.

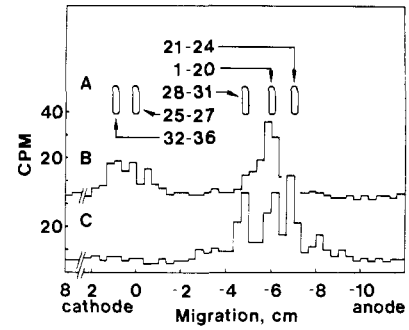

FIGURE 6: Thin-layer high-voltage electrophoresis of chymotryptic peptides of acidic PPI purified by NaDodSO$_4$ gel and focusing. ^{3}H-Labeled PPI from the major focusing peak near pH 5 was analyzed by chymotryptic digestion as described in methods. (A) pPP peptides; (B) [^{3}H]tyrosine label; (C) [^{3}H]leucine label. The recovery of ^{3}H was 70%.

pancreas homogenate or clarified supernatant from such a homogenate. When homogenate was incubated with labeled PPI, fewer than 10% of the counts were lost into the pellet upon centrifugation, and the distribution of counts after focusing was unaltered as shown in Figure 5. The results of focusing the incubated clarified homogenate focusing were the same (not shown).

Figure 5 also shows that neutral and acidic PPIs from both pancreas extracts and pseudoislet extracts focus at the same point in the gel and probably, therefore, are identical.

In conclusion, the experiments in Figure 5 show that neither the NaDodSO$_4$ gel nor the focusing gel could generate acidic PPI from pPP. Furthermore, pancreas extracts contained no more than 10% acidic PPI while pseudoislet extracts contained 90% acidic PPI. Finally, acidic PPI was neither partitioned into the insoluble portion of a pancreas homogenate nor converted by the homogenate or the supernatant into neutral PPI. Therefore neither extraction nor analysis appeared responsible for the existence of acidic PPI nor the difference in relative proportions of neutral and acidic PPIs in pseudoislet or pancreas extracts.

Therefore it remained to show the degree of structural similarity between neutral PPI and pPP and the structural basis for the difference between neutral and acidic PPIs in the pseudoislet extracts. Peptide mapping of [^{3}H]tyrosine- and [^{3}H]leucine-labeled PPIs was consequently performed.

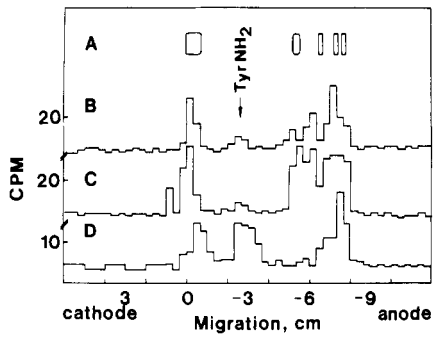

FIGURE 7: Thin-layer high-voltage electrophoresis of tryptic peptides of neutral and acidic PPIs. ^{3}H-Labeled neutral and acidic PPIs purifed by $NaDodSO_4$ gel followed by focusing were digested with trypsin as described under Experimental Procedures. (A) Peptides of pPP; (B) digest of [^{3}H]leucine-labeled acidic PPI; (C) digest of [^{3}H]-tyrosine-labeled acidic PPI; (D) digest of [^{3}H]tyrosine-labeled neutral PPI. The recovery of ^{3}H was 70%.

Peptide Mapping. Peptide mapping showed that both neutral and acidic PPIs exhibited peptide labeling patterns in accordance with the known sequence of pPP with the exception that the acidic PPI had a peptide extension at the C-terminal. Figure 6 shows the distribution of counts among thin-layer electrophoresis fractions after chymotrypsin digestion of either [^{3}H]tyrosine or [^{3}H]leucine acidic PPI. This distribution was what would be expected of pPP. pPP contains three leucines, and a 60:73:60 ratio of counts was found among the peptides when a 1:1:1 ratio was expected. It was reasoned therefore that acidic PPI must contain an extra charge in a C-terminal extension since (a) all pPP peptides in which an artifactual charge change might have occurred (methionine and asparagine or glutamine containing peptides) were also leucine containing peptides, and (b) chymotrypsin would remove any extension at the C-terminal of pPP. Trypsin peptides of [^{3}H]tyrosine-labeled acidic PPI could reveal modifications at the C-terminal since the C-terminal tryptic peptide of PP contains tyrosine. The results of [^{3}H]tyrosine labeling and tryptic peptide analysis are shown in Figure 7C. A 62:219:8 ratio of counts in the basic–acidic–tyrosinamide regions of the thin-layer sheet was found, near 1:3:0 instead of the 1:2:1 ratio expected of pPP itself. The recovery of a small quantity of apparent tyrosinamide suggested either poor recovery of counts of one or more peptides (since overall recovery was only about 75%) or a minor tryptic cleavage. Tryptic digestion of [^{3}H]leucine-labeled acidic PPI, however, also revealed counts in the region of tyrosinamide (Figure 7B), suggesting that a minor cleavage of the PPI was producing the tyrosinamide-like counts. Figure 7B also revealed fewer counts in the basic edge of the group of acidic peptides, a region of the thin-layer sheet where peptides with one net negative charge run. As shown in Figure 7D, when neutral PPI labeled with [^{3}H]tyrosine was digested with trypsin basic–acidic–tyrosinamide, counts appeared in a 23:40:23 ratio near the 1:2:1 ratio expected from the structure of pPP.

For confirmation of the finding that the counts associated with the terminal tyrosine of acidic PPI was at least a dipeptide bearing one net negative charge, the [^{3}H]tyrosine-labeled peptides of acidic PPI were fractionated on Sephadex G-25 as shown in Figure 8B. It was reasoned that the peptide running near the column volume had to be C-terminal acidic PPI since an extension on PP as large as either of the other two labeled peptides eluting near the void volume would almost certainly have caused a noteworthy separation between pPP and acidic PPI upon gel filtration in Bio-Gel P-100 in the experiment shown in in Figure 5. This column volume peptide was electrophoresed and was found to migrate exactly as had

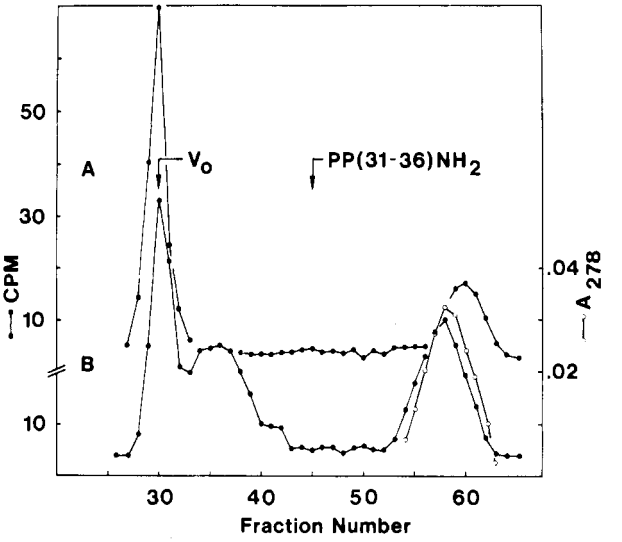

FIGURE 8: Gel filtration of tryptic and carboxypeptidase A digests of [^{3}H]tyrosine-labeled acidic PPI. Tryptic and carboxypeptidase A digests were prepared as described under Experimental Procedures, applied to a 0.8 × 15 cm column of Sephadex G-25 and eluted with 1:1:1 pyridine–acetic acid–water. (A) Carboxypeptidase A digest; (B) tryptic digest mixed with 250 μg of tyrosinamide. Half of the digest was subjected to thin-layer high-voltage electrophoresis as shown in Figure 9C.

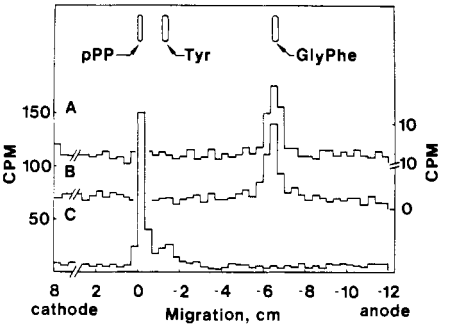

FIGURE 9: Thin-layer high-voltage electrophoresis of the C-terminal peptide and carboxypeptidase digest of [^{3}H]tyrosine-labeled acidic PPI. (A) A tryptic digest of acidic PPI was purified further by Sephadex G-25 gel filtration as shown in Figure 8. No tyrosinamide carrier was added, and the column volume ^{3}H-labeled fractions were pooled, halved, lyophilized, and applied to the thin-layer sheet. (B) The other half of the column pool was lyophilized, resuspended, and hydrolyzed with 3 M HCl for 90 min at 37 °C, dried under stream of nitrogen, and applied to the thin-layer sheet. (C) A carboxypeptidase A digest was lyophilized, resuspended, and applied to the thin-layer sheet. Half the sample was subjected to gel filtration as shown in Figure 8A.

been predicted, that is, migration like peptides with a single net negative charge (i.e., like Gly-Phe). These results are shown in Figure 9A.

In view of the possibility that that this extra negative charge was due to a tyrosine sulfate as occurs in gastrin II, the column volume peptide from the G-25 column was subjected to the same mild acid hydrolysis used to remove the sulfate from gastrin II (Gregory et al., 1964). Migration of the acid-treated peptide is shown in Figure 9B; no counts appeared in the region of tyrosine or tyrosinamide.

In order to show that the counts in this peptide were actually contained in a tyrosine moiety, labeled acidic PPI was incubated with carboxypeptidase A which would remove the C-terminal amino acids and stop at the proline in position 34. When aliquots of the carboxypeptidase digestion mixture were applied to thin-layer electrophoresis as shown in Figure 9C, the counts appeared in the position of tyrosine and at the origin in a 87:340 ratio. Similarly, when an aliquot of the carbox-

ypeptidase digestion mixture was applied to the Sephadex G-25 column, as shown in Figure 8A, the only counts appeared in the void volume and at the column volume in a fashion consistent with the removal of the most C-terminal tyrosine from the acidic PPI.

Discussion

Two immunoreactive forms of PP of similar molecular weight were extracted from dog islet cultures and dog pancreas. These two forms were separated by isoelectric focusing; one form focused near neutrality like pPP and the other focused near pH 5. Both neutral and acidic forms had tryptic and chymotryptic peptide fragments expected of pPP with one exception, namely, that the more acidic form appeared to have a peptide extension of one or more amino acids at the C-terminal. A single negative charge such as a free carboxyl group would account for the difference in isoelectric points of neutral and acidic PP (Edsall, 1943). This extra negative charge did not result from removal of the terminal tyrosinamide, from hydrolysis of the amide, nor from sulfation of the tyrosine hydroxyl. The number and identity of the amino acids in the extension was not determined; however, the results are consistent with the possibility that the extension is a glycine residue. There are three instances where amidated peptides are known or predicted to exist in variant molecular forms where the next amino acid is a glycine. Such forms of α-melanocyte stimulating hormone (namely, corticotropin) and melittin (Suchanek et al., 1978) are known, and the mRNA for calcitonin contains the codon for glycine in the position adjacent to that of the C-terminal amino acid (Amara et al., 1980; Jacobs et al., 1981).

A small amount of larger molecular weight PPI was detected in culture extracts by electrophoresis. A similar large molecular weight form of PP has been detected in culture extracts by immunoprecipitation and column chromatography; this large form of PP has been shown to be a biosynthetic precursor to the acidic PP form and has been further shown to be extended at the C-terminal (Schwartz et al., 1980). It has not been established whether the acidic PP found in these culture extracts is a biosynthetic precursor to the neutral form or whether a separate precursor to the neutral form is also present in the extracts. It will be interesting to know why the acidic PP accumulates in culture and at what point in the biosynthetic pathway processing is altered to cause the acidic PP to accumulate. This might occur as easily by a decrease in amidating capacity in culture as by a change in precursor processing to produce a proform which is more readily converted to the acidic PP than amidated to PP itself.

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