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Isolation of a Glucagon-containing Peptide: Primary Structure of a Possible Fragment of Proglucagon

(hormones/precursors)

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ABSTRACT **The heterogeneity of crystalline bovine (ox)/porcine glucagon has been examined by gel filtration and ion-exchange chromatography. A strongly basic peptide that reacted well with antibodies to bovine/porcine glucagon was isolated and its primary structure was determined. The amino-acid sequence of the NH_2-terminal 29 residues of the 4500-dalton peptide is identical with that of intact bovine or porcine glucagon. The remaining eight residues at its COOH-terminus are Lys-Arg-Asn-Asn-Lys-Asn-Ile-Ala. Small amounts of other glucagon-immunoreactive peptides having molecular weights ranging from 3700 to 9000 were also detected in crystals of bovine/porcine glucagon. We propose that the 37-residue peptide is a fragment of bovine or porcine proglucagon.**

Accumulated evidence has suggested the existence of higher molecular weight precursor forms for several polypeptide hormones including insulin (1, 2), parathyroid hormone (3, 4), gastrin (5, 6), and glucagon (7–10). In 1970, Rigopoulou *et al.* reported that extracts of canine pancreas contain two components with glucagon-like immunoreactivity, the larger having a molecular weight of about 9000 (7). Studies of the biosynthesis of glucagon in islets of the anglerfish by Noe and Bauer showed that a 11,400-dalton component possessed several of the characteristics required for a biosynthetic precursor to glucagon (8, 11). Other workers also have suggested the existence of biosynthetically labeled glucagon precursors in islets of pigeon (9) and guinea pig (10). None of these high molecular weight, glucagon-like substances, however, has been isolated or characterized chemically.

Since proinsulin and several of its partially converted intermediate forms cocrystallize with insulin during the commercial preparation of that hormone (12, 13), we considered the possibility that small amounts of a glucagon precursor might be present in crystalline preparations of glucagon. Bromer *et al.* recently examined the heterogeneity of crystalline glucagon by ion-exchange chromatography (14), but limited their attention to the isolation and characterization of desamidoglucagon. Our studies proceeded from gel-filtration of crystalline bovine/porcine glucagon so that we might examine only those components larger than glucagon itself.

MATERIALS AND METHODS

Materials. The glucagon used during these studies was a mixture of the bovine and porcine hormones. The crystalline preparation was purchased from ELANCO Products Co., Indianapolis, Ind. (lot no. 6PK72B) and is of pharmaceutical quality.

Abbreviation: Dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.

Isolation of Peptides. 1 g of crystalline glucagon was filtered through Bio-Gel P-30 with 3 M propionic acid as the eluting solvent. Two early-eluting peaks of UV absorbance were separately pooled, and the fractions were concentrated by rotary evaporation. Each of the pools was then chromatographed on a column of SP-Sephadex C-25 using 5 mM sodium acetate buffer (pH 4.0), 8 M in freshly deionized urea, with a series of salt gradients eventually reaching 1 M in NaCl. Appropriate peaks of UV absorbance were pooled, the proteins were desalted by passage through a column of Bio-Gel P-2 equilibrated with 3 M acetic acid, and the resulting solutions were concentrated by rotary evaporation.

Molecular Weight Determination. A column of Bio-Gel P-30 equilibrated with 0.1 M Tris·HCl buffer (pH 7.5) containing 8 M urea was calibrated with several proteins oxidized with performic acid (15) beforehand to obtain products free from *tertiary* structure. Samples of glucagon-like peptides were applied to the column, and aliquots of the fraction were removed for immunoassay.

Immunoassay for Glucagon. The assay system used the double-antibody technique described by Morgan and Lazarow (16). Anti-glucagon serum was the generous gift of Dr. Ann Lawrence and was prepared in guinea pigs with bovine/procine glucagon. Antibody against guinea pig globulin prepared in rabbits and [^{124}I]- glucagon were obtained from commercial sources. The efficiency of antigen–antibody binding was not affected by 2.5 mM urea, the concentration of that substance present in some of the immunoassays.

Sequence Techniques. The intact peptide was subjected to Edman degradation according to the method described by Peterson *et al.* (17). Phenylthiohydantoin derivatives were identified by thin-layer chromatography (18). About 20 nmol of peptide B7 (see below) was treated with an excess of dansyl (5-dimethylaminonaphthalene-1-sulfonyl) chloride (19) to block the NH_2-terminal residue and to derivatize the ϵ-amino groups of lysine residues. The dansyl peptide was then incubated with a 300-fold excess of BrCN dissolved in 0.4 ml of 70% formic acid for 24 hr at room temperature (23°) (20) to cleave on the carboxyl side of the single residue of methionine. The resulting peptide was subjected, without purification, to the dansyl-Edman degradation described by Gray (21). Sequence determination from the COOH-terminus was accomplished by digestion of the peptide with the diisopropylflurophosphate-treated carboxypeptidases A and B (EC 3.4.2.1. and 3.4.2.2., respectively) in 0.2 M *N*-ethylmorpholine acetate buffer (pH 8.0). Free amino acids were

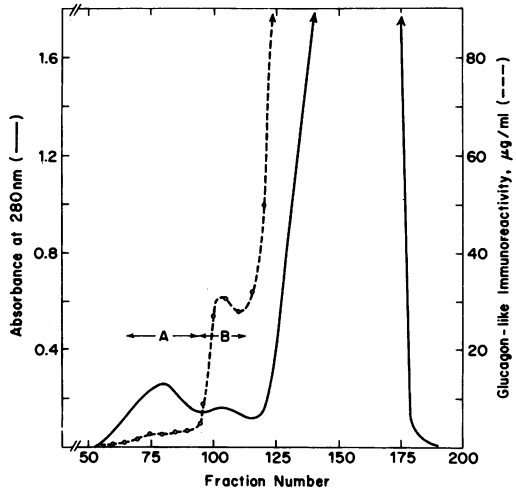

FIG. 1. Elution profile of a 1-g sample of crystalline glucagon from a column of Bio-Gel P-30. The gel column (5 × 100 cm) was eluted with 3 M propionic acid at the rate of 90 ml/hr, each fraction containing 10 ml of column effluent. The large peak of absorbance is due to glucagon and desamidoglucagon. The early elution peptides were collected in two pools labeled *A* and *B*, as indicated by the *two-headed arrows* above.

quantitated by the amino-acid analyzer by use of, in some cases, the lithium citrate buffers described by Benson *et al.* (22).

RESULTS

Gel-filtration of crystalline glucagon on Bio-Gel P-30 with 3 M propionic acid results in the separation of two small peaks of high molecular weight material from the large peak containing both glucagon and desamidoglucagon (Fig. 1). Only the peak containing peptides of intermediate size and the peak of glucagon itself exhibit significant glucagon-like immunoreactivity. Disc-gel electrophoresis of the pools labeled *A* and *B* showed that both fractions were markedly heterogeneous.

Fig. 2 shows the elution profile of pool B from a column of the strong cation exchanger SP-Sephadex. Disc-gel electrophoresis at pH 8.7 (23) of fractions B1–B7 showed multiple peptide bands for samples B1–B6. Sample B7 did not yield any band at all. The gel electrophoresis of the same samples at pH 4.4 (24) showed, once again, heterogeneity in B1–B6, but showed a single peptide band for B7. Pool A from Fig. 1 was also chromatographed on SP-Sephadex and was heterogeneous (not shown). Since none of these fractions contained significant glucagon-like immunoreactivity, they were not examined further.

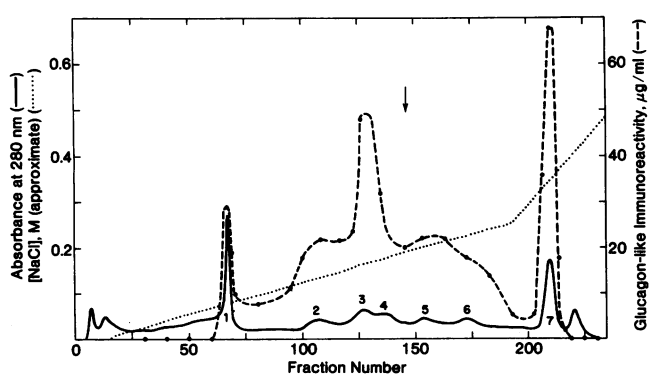

FIG. 2. Elution profile of pool *B* from a column of SP-Sephadex. The column (1.5 × 25 cm) was eluted with a linear gradient formed from 375 ml of 5 mM sodium acetate (pH 4.0) and 375 ml of 0.3 M NaCl in the same buffer. After 600 ml of effluent had been collected, the gradient was changed to 375 ml of acetate buffer 0.25 M in NaCl and 375 ml of buffer 1.0 M in NaCl. The rate of flow was 30 ml/hr and 3-ml fractions were collected. Seven pools of UV-absorbing and glucagon-immunoreactive fractions were formed as indicated above. The *vertical arrow* shows the elution position of glucagon from a similar column.

As shown in Fig. 3, the immunoreactive peptides appearing in fractions B1–B7 possessed molecular weights ranging from about 3700 to about 9000. Several of the pools also showed multiple peaks of glucagon-like immunoreactivity. Most of our further experiments were devoted to characterizing the approximately 4500-dalton peptide labeled B7. After B7 was purified and desalted, the yield of material was about 600 μg.

The amino-acid composition of the purified fraction B7 is presented in Table 1. The peptide is notably similar in composition to that of glucagon, but contains eight additional amino-acid residues. The very basic nature of this peptide, as indicated by paper and acrylamide electrophoresis and by ion-exchange chromatography, is confirmed by the addition of three basic residues to those found in glucagon itself.

Treatment of peptide B7 with dansyl chloride and chromatography of the hydrolyzed product identified histidine at the NH_2-terminus of the peptide. Sequential Edman degradation of about 100 nmol of the peptide permitted the identification of the amino-acid sequence of its NH_2-terminal 23 residues (Fig. 4). The 24th and subsequent residues could not be identified due to a generally high background and to low yields of the phenylthiohydantoin derivatives. The 23 residues identified correspond, however, to the NH_2-terminal sequence of bovine or porcine glucagon (25, 26).

Preliminary results showed that peptide B7 was inert to carboxypeptidase B but was partially degraded by digestion with carboxypeptidase A. The results shown in Fig. 5 identify

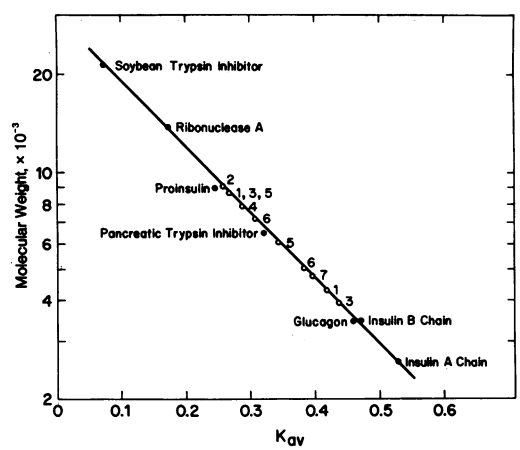

FIG. 3. Molecular weight determination of glucagon-immunoreactive components in pools B1–B7. The column was Bio-Gel P-10 (0.9 × 100 cm) and the eluant was 0.1 M Tris·HCl (pH 7.5), 8 M in urea. The flow rate was 6 ml/hr and the fraction size was 1 ml. Aliquots of each fraction were assayed for immunoreactivity with anti-glucagon serum. The column was standardized with the indicated proteins, which had been oxidized with performic acid.

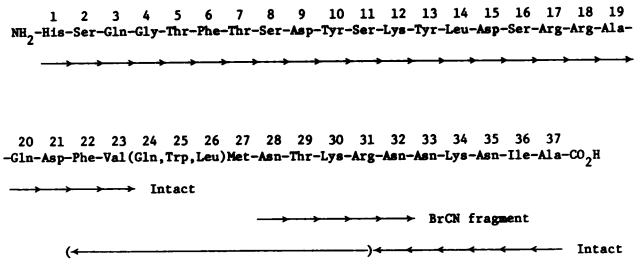

FIG. 4. Primary structure of peptide B7. The amino-acid sequence 1–23 was determined by identification of phenylthiohydantoin derivatives during Edman degradation; the sequence 28–32 was determined by the dansyl-Edman method described by Gray (21); the sequence 32–37 was determined by carboxypeptidase digestion. Residues 24–26, which were shown to be present by exhaustive carboxypeptidase digestion, were placed by homology with glucagon.

the COOH-terminal sequence as Asn-Ile-Ala. Digestion of B7 with a mixture of carboxypeptidases A and B resulted in the release of various amino acids, including those present in the COOH-terminal sequence of glucagon itself.

The addition of carboxypeptidase B to the carboxypeptidase A-digested peptide resulted in a rapid and quantitative release of 1 mol of lysine per mol of peptide, as shown in Table 2. The further release of almost two residues of asparagine before the release of other amino acids identified the COOH-terminal sequence of B7 as Asn-Asn-Lys-Asn-Ile-Ala. The partial release of another lysine residue and of an arginine residue before the release of other amino acids present in the COOH-terminal region of glucagon suggested that a Lys-Arg or an Arg-Lys sequence preceded the Asn-Asn sequence in peptide B7.

The methionine known to be present at position 27 in glucagon (25, 26) and known to be present in the COOH-terminal

TABLE 1. *Amino-acid composition of peptide B7*

Amino acid	Found	Theory	Excess over glucagon
Aspartic acid	6.8	7	3
Threonine	2.8	3	0
Serine	3.8	4	0
Glutamic acid	3.3	3	0
Proline	0	0	0
Glycine	1.6	1	0
Alanine	1.9	2	1
Valine	1.1	1	0
Methionine	0.9	1	0
Isoleucine	1.0	1	1
Leucine	2.2	2	0
Tyrosine	1.9	2	0
Phenylalanine	1.9	2	0
Lysine	2.9	3	2
Histidine	0.9	1	0
Arginine	3.0	3	1
Tryptophan	—	1	0
Total residues		37	8

All results are expressed as mol of amino acid per mol of peptide. Hydrolysis proceeded in 6 N HCl at 110° for 20 hr. Tryptophan was not determined by amino-acid analysis.

TABLE 2. *Release of amino acids from peptide B7 by sequential degradation with carboxypeptidases A and B*

Amino acid	Conditions for digestion: 2.5 min 21°	30 min 21°	60 min 21°	+ 60 min 37°
	nmol of amino-acid released per nmol of peptide			
Lysine	1.0	1.2	1.5	1.9
Asparagine	0	1.7	1.9	2.6
Arginine	0	0	0.5	1.0
Threonine	0	0	Trace	0.9
Methionine	0	0	Trace	0.8
Leucine	0	0	Trace	0.8
Tryptophan	0	0	0	0.5
Glutamine	0	0	0	0.7
Valine	0	0	0	0.8
Phenylalanine	0	0	0	0.8

Portions (1.4 nmol) of the proglucagon fragment were incubated with carboxypeptidase A for 60 min at 37°. The solutions were then cooled to 21° and carboxypeptidase B was added. The digestion was stopped after selected periods by the addition of glacial acetic acid, and the samples were applied to the columns of the amino-acid analyzer. The values reported above have been corrected for the release of 1 nmol each of alanine, isoleucine, and asparagine by carboxypeptidase A alone (Fig. 5).

portion of peptide B7 (Table 2) provided a convenient site for chemical cleavage. We were able to identify the NH_2-terminal five residues of the BrCN fragment of B7 as Asx-Thr-Lys-Arg-Asx. Although a constant background of dansyl histidine was observed, that contaminant did not interfere with the identification of further dansyl derivatives. The carboxypeptidase digestions shown in Table 2 are consistent with the sequence obtained from the BrCN fragment and identified Asx as asparagine.

Our sequence data for peptide B7 are summarized in Fig. 4. Edman degradation of the intact peptide and its BrCN fragment show sequences corresponding to those in bovine or porcine glucagon. By inference, methionine can be placed at position 27 of the peptide, this residue being amino to the NH_2-terminal residue identified in the BrCN fragment. Residues 24–26 of B7 were not placed in sequence due to the

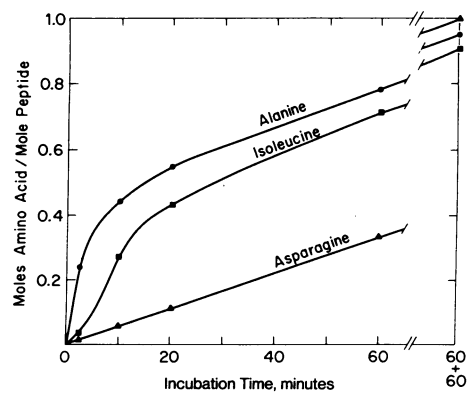

FIG. 5. Release of amino acids from peptide B7 by carboxypeptidase A. The temperature of incubation was 21°. The data points on the *right ordinate* were obtained after incubation at 21° for 60 min followed by incubation at 37° for another 60 min.

lack of sufficient amounts of material for further studies. Those three residues, however, can reasonably be placed as shown in Fig. 4 since they, as well as residues 22–23 and residues 27–36, are released from B7 by a mixture of carboxypeptidases A and B (Table 2).

DISCUSSION

High molecular weight components possessing glucagon-like immunoreactivity have been identified in pancreatic extracts of dogs, oxen, ducks, rats, and humans (7) and in islet extracts of anglerfish (8) and guinea pigs (10). Furthermore, biosynthetic studies using [^{3}H]tryptophan have suggested that a precursor to glucagon is synthesized in islets of anglerfish (8), pigeons (9), and guinea pigs (10). The biosynthesis of these largely uncharacterized proteins appears to be under a control similar to that serving for glucagon secretion since high glucose concentrations inhibit both processes (8–10). Based on reports in the literature, the precursor appears to have a molecular weight of at least 9000.

Figs. 1–3 demonstrate that various peptides having glucagon-like immunoreactivity with molecular weights between 3700 and 9000 occur as contaminants in commercially prepared crystalline glucagon. Although most of the glucagon-like peptides are present in very small amounts, one highly basic peptide, corresponding to less than 0.1% of the starting material, was isolated in a quantity sufficient for chemical characterization. The primary structure of this peptide (B7) corresponds to that of glucagon with an eight-residue extension at its COOH-terminus (Fig. 4), suggesting the existence of a larger glucagon precursor. Since the peptide was isolated from a mixture of bovine and porcine glucagons, we cannot be sure if this peptide occurs in both species notwithstanding the identity of bovine and porcine glucagons (25, 26). The multiple forms with glucagon-like immunoreactivity indicated in Fig. 3 may represent intermediates in the pathway of conversion of proglucagon to glucagon *in vivo*, or they may arise artifactually from chemical or enzymatic hydrolysis during the isolation of the native hormone. Nevertheless, we can discuss in chemical terms the consequences of the primary structure of peptide B7 in relation to the structure of the unisolated intact precursor.

Recent structural studies have shown that the nonhormonal portions of proparathyroid hormone (27) and progastrin (6) occur at the NH_2-termini of the respective circulating hormones. The unique *quarternary* structure of insulin requires, of course, that the nonhormonal portion of proinsulin occurs in the central portion of the hormone precursor (2). We have no evidence concerning the possibility of an NH_2-terminal extension of glucagon in its precursor form. The trypsin-sensitive sequence at the connecting region between glucagon and the COOH-terminal portion of peptide B7, however, is not unlike connecting sequences for various other polypeptide hormone precursors. Trypsin-like enzymes can account at least partially for conversion of proparathyroid hormone to parathyroid hormone (27), large gastrin to gastrin (6), β-lipotrophin to β-melanocyte-stimulating hormone (28), and proinsulin to insulin (2). The potential tryptic cleavage between residues 30 and 31 of peptide B7, however, would result in the production of glucagon with a COOH-terminal lysine residue. Although no direct evidence is available, we suspect that, as appears to be true for the conversion of proinsulin (2, 29), a mixture of trypsin-like and carboxypeptidase B-like activities would be necessary for the enzymatic conversion of a peptide similar to B7 in native glucagon *in vivo*. Several studies indicating that the tryptic products of glucagon and those of its precursor are of similar size (8–10) and immunoreactivity (7) are consistent with the occurrence of trypsin-sensitive sites proximal to the termini of the native hormone within proglucagon. The suggested trypsin-like activity, however, must certainly be under close control since glucagon itself is highly sensitive to hydrolysis by pancreatic trypsin (30).

Why glucagon is synthesized initially in a larger precursor form is an interesting question. The existence of proinsulin can be related to the necessity for disulfide bond formation in the native hormone (31), but this explanation clearly does not hold for the parathyroid hormone, gastrin, and glucagon precursors. Although glucagon in dilute, aqueous solution possesses only small amounts of α-helix (32), the hormone is known to exist in a compact, stable conformation (33, 34). Nevertheless, since the urea-induced denaturation of glucagon is spontaneously reversible (34), it seems unlikely that the precursor form would be necessary for the initial attainment of correct secondary structure. Other explanations for the existence of nonenzyme protein precursors include possible limitations on the size or structure of mRNA and possible peptide signals indicating that a protein is to be packaged by the cell for secretion (35). It is indeed possible that the glucagon precursor represents a single copy of an array of linearly associated genes. Any constraints placed on the biosynthesis of a small, secreted peptide might then be overcome by selective mechanisms for the conversion of larger proteins.

The results reported here provide convincing evidence for the existence of a glucagon precursor and suggest possible modes for its conversion to the native hormone. Further studies on the nature of proglucagon must certainly include the isolation and full structure determination of the intact prohormone.

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