Studies on the Conversion of Proinsulin to Insulin

II. EVIDENCE FOR A CHYMOTRYPsin-LIKE CLEAVAGE IN THE CONNECTING PEPTIDE REGION OF INSULIN PRECURSORS IN THE RAT

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SUMMARY

A chymotryptic-like fragment of the rat proinsulin connecting peptide (C-peptide) has been isolated from whole rat pancreas. Amino acid analysis and partial sequence determination showed the fragment to contain the NH₂-terminal 22 residues of C-peptide I. A radioactive fragment of similar electrophoretic mobility was also isolated from rat pancreatic islets which had been incubated with [³H]-leucine. Stepwise Edman degradation of the radioactive fragment showed the positions of its leucine residues to be identical with those of the unlabeled material. Homogenation of [³H]leucine-labeled rat C-peptide with whole pancreas and extraction under conditions used for the isolation of the fragment did not result in cleavage of the C-peptide to the fragment. Furthermore, the fractional ratio of fragment to C-peptide (normally about 0.2) did not increase when rat islets were incubated with [³H]leucine for 1 hour and then incubated with unlabeled leucine for 1, 2, or 3 hours. Treatment of a mixture of [³H]leucine-labeled proinsulin and proinsulin intermediates with high amounts of trypsin resulted in the release of the C-peptide fragment as well as desglutamine (residue 31) C-peptide. It is concluded that a chymotryptsin-like cleavage in the C-peptide region of proinsulin or in one of its intermediates occurs normally during the conversion of proinsulin to insulin in the rat.

Steiner et al. (3) first noted that the conversion of proinsulin to an insulin-like substance (desalanine insulin) proceeded readily during the digestion of the radioactively-labeled precursor with trypsin. Although Grant and Coombs (4) pointed out that a trypsin-like enzyme may well serve to convert proinsulin to insulin in many fishes, enzymes having only trypsinic specificity will not account for all aspects of the conversion of proinsulin to insulin in higher animals. The determination of the primary structures of bovine (5) and porcine (6) proinsulins suggested that enzymes with trypsin-like and carboxypeptidase B-like activities would be required for the conversion of proinsulin to intact insulin. Kemmler et al. (7), in fact, showed that bovine proinsulin is readily and quantitatively converted to intact insulin and C-peptide when the precursor is treated with trypsin and carboxypeptidase B under conditions where the trypsin activity is rate-limiting.

Partially converted forms of bovine proinsulin have been isolated and identified (5, 8) corresponding to the structures

\[
\text{NH₂-B chain-Arg-Arg-C-peptide-COOH} \quad \text{NH₂-A chain-COOH}
\]

and

\[
\text{NH₂-B chain-COOH} \quad \text{NH₂-C-peptide-Lys-Arg-B chain-COOH}
\]

A partially cleaved form of porcine proinsulin corresponding to the first of the above structures has also been isolated (9). Evidence accumulated from studies of the incorporation of radioactive amino acids into proinsulin and from the conversion of the precursor into insulin in several in vitro preparations of islet tissue (10–12) has indicated the likelihood that these structures represent intermediates in the conversion of proinsulin to insulin.

A second type of proinsulin intermediate showing a chymotryptsin-like cleavage in the C-peptide region of the molecule has been isolated from commercial preparations of crystalline porcine insulin (9). The existence of such an intermediate should result in the appearance of a chymotryptsin-like fragment of the C-peptide as well as the intact C-peptide among the final products of the conversion reactions. Studies of proinsulin conversion in

The abbreviation used is: C-peptide, connecting peptide.
the rat, especially with regard to the condition of the C-peptide, provide an excellent probe for such a chymotrypsin-like cleavage. Since the two C-peptides found in the rat contain penultimate arginine residues (13, 14), cleavage at any of several possible sites by a chymotrypsin-like enzyme would give rise to more acidic peptides. These peptides should be easily separated from the undegraded material because of their charge difference at low pH.

The existence of fragment of the rat C-peptide was suggested by Clark (15) during his studies of the biosynthesis of insulin in isolated pancreatic islets and his studies of the isolation of C-peptide from whole pancreas. We report here the identification of an NH₂-terminal fragment of the rat C-peptide isolated from whole rat pancreas. Control studies have indicated that the fragment is not an artifact of isolation. Biosynthetic evidence is also presented suggesting that the fragment arises within the pancreatic β-cell from a proinsulin intermediate which has undergone a chymotrypsin-like cleavage in its C-peptide region.

METHODS

Isolation of Unlabeled C-peptide Fragment—The isolation of the C-peptide fragment (peptide A) was carried out as previously described for the isolation of the intact C-peptide (14). An ether-acetone-insoluble protein fraction was isolated from an acid-acetone extract of whole pancreas and the resulting material was gel-filtered in 3 M acetic acid on Bio-Gel P-30 (14). Paper electrophoresis in 30% formic acid of the protein peak containing insulin resulted in the isolation of a fraction containing the fragment as well as the two C-peptides. The fragment migrated less rapidly on paper than the intact C-peptide, but complete separation was not possible on the preparative scale.

The mixture of peptide and fragment was applied to a QAE Sephadex column in 0.05 M pyridine formate buffer at pH 5.0 and the peptide material eluted with a stopped pH gradient as previously described (14). After the pH of the column effluent had decreased to about 2.5, the column was eluted with 50% acetic acid to remove even the most acidic peptide material. The C-peptide fragment eluted with the acetic acid front and was detected by spotting aliquots of each fraction on paper followed by visualization with ninhydrin spray. The fragment was then further purified by paper electrophoresis in 10% pyridine brought to pH 6.5 with acetic acid. The yield of material from 400 g of pancreas was about 0.5 mg.

Isolation and Incubation of Rat Islets—The rats used in these studies were 400 to 500 g males of the Sprague-Dawley strain, which were injected with cortisone-acetate (10 mg per kg body weight every 2nd day) for 5 to 15 days prior to sacrifice in order to obtain higher yields of pancreatic islets (3). Treatment with cortisone-acetate causes some hyperplasia of the islets (16) and seems to enhance their insulin biosynthetic activity without altering the over-all kinetics of proinsulin processing (3). Pancreatic islets were isolated from minced tissue essentially as described elsewhere (17). Crude collagenase was obtained from the Worthington Biochemical Company. Hanks’ salt solution and the incubation medium were prepared fresh and adjusted to pH 7.4 by gassing with 95% O₂-5% CO₂. The tracer used in all experiments was [3H]leucine (Amersham Radiochemical Center) having a specific activity of 46.9 Ci per mmole. During each experiment, 75 to 600 islets were incubated in 150 to 300 ml of medium containing 0.1 μCi per ml of [3H]leucine. When the incubation medium was to be analyzed, extreme care was taken to obtain islets free from contamination by acinar tissue. After incubation at 37° under the appropriate conditions, the islets and sometimes the incubation medium were extracted in acid-ethanol by a modification of the Davoren procedure (18), using 1 mg of insulin or proinsulin and 0.1 mg of leucine as carriers.

Islet extracts were gel-filtered on a column (1 × 50 cm) of Bio-Gel P-30 equilibrated with 3 M acetic acid, and 1.5 ml fractions were collected. Ultraviolet absorbance of the carrier protein was measured at 275 nm and aliquots from each fraction were counted in 15 ml of Triton-toluene scintillation fluid (17) in a Packard model 527 liquid scintillation spectrometer. Various fractions were then pooled and brought to dryness by a rotary evaporator. Insulin- and C-peptide-containing fractions were dissolved in 30% formic acid and were subjected to paper electrophoresis in that solvent for 5 hours at 7.5 volts per cm.

Isolation of [3H]Leucine-labeled C-peptide and C-peptide Fragment—After electrophoresis in 30% formic acid of the material isolated from the incubation of rat islets with [3H]leucine and after localization of the radioactive peptides on the electrophoretograms (see later), the appropriate bands were cut from the paper and the peptides eluted using 50% acetic acid. The acetic acid was then removed by rotary evaporation. In one control experiment, the radioactive intact C-peptide was homogenized with 1 g of whole rat pancreas in acid-acetone and the homogenate further treated as for the isolation of the unlabeled material (14). These isolation steps included precipitation of contaminating proteins at pH 8.5, precipitation of insulin and C-peptide by the addition of absolute ethanol and ether at pH 5.3, gel filtration in 3 M acetic acid, and electrophoresis in 30% formic acid.

Tryptic Digestion of [3H]Proinsulin-like Material—Pooled proinsulin-containing fractions obtained from the Bio-Gel P-30 column were subjected to paper electrophoresis for 6 hours, the paper electrophoretograms were scanned on a Vanguard model 880 Chromatogram Scanner at a speed of 6 inches per hour. When lesser amounts of radioactivity were present, we obtained improved results by counting paper strips in the liquid scintillation spectrometer. The electrophoretogram was cut in 2-mm strips perpendicular to the direction of migration and each strip was counted for radioactivity in 10 ml of Triton-toluene scintillation fluid. The recovery of radioactivity was approximately 30%.

Other Procedures—Amino acid analysis was performed on a model 120C or 121 Beckman amino acid analyzer using standard methods. Steunwise Edman degradation was performed by the semi-micro procedure recently described by this laboratory (19). Phenylthiohydantoin derivatives of the amino acid residues were identified by thin layer chromatography on silica gel sheets (20). During the degradation of the [3H]leucine-labeled peptidase, aliquots of the phenylthiocarbamyl amino acids were counted for radioactivity in 10 ml of Triton-toluene scintillation fluid. Car-
bovine carboxypeptidase A digestion were carried out at a 1:100 ratio of enzyme to substrate in 0.1 M Tris-HCl buffer, pH 8.5 containing 0.5% NaCl. After incubation for 0, 3, 3, 10, 20, or 60 min at 21°C, or for 60 min at 21°C followed by 60 min at 37°C, the reaction was stopped by the addition of glacial acetic acid and the samples were applied to the long column of the amino acid analyzer for the determination of free amino acids released during the enzyme digestion.

**RESULTS**

Suitable methods for the isolation of insulin and C-peptide from either whole pancreas or isolated pancreatic islets are now well established (8, 10, 14, 18, 19). The purification procedure used here, which included acid-ethanol extraction, ethanol-ether precipitation, and gel-filtration, results in a highly purified mixture of C-peptide and insulin. Fig. 1 shows the pattern of radioactivity obtained when the insulin- and C-peptide-containing fraction resulting from the incubation of rat pancreatic islets with [3H]leucine is submitted to paper electrophoresis in 30% formic acid. The sizable amount of radioactivity occurring in a peptide migrating to a position slightly nearer the origin than the C-peptide led us to suppose that this peptide might be significant in normal β-cell metabolism. A peptide with identical electrophoretic mobility also appears in the insulin- and C-peptide-containing fraction isolated from whole rat pancreas (see legend Fig. 1). Each of the two rat C-peptides maintains a net charge of +2 in 30% formic acid, pH 0.9, and both peptides migrate to the same position after paper electrophoresis (14). At that pH, rat insulins 1 and 2 maintain net charges of +7 and +6 (al), respectively, resulting in their partial separation as shown in Fig. 1. Each of the two rat C-peptides maintains a net charge of +2 in 30% formic acid, pH 0.9, and both peptides migrate to the same position after paper electrophoresis (14). At that pH, rat insulins 1 and 2 maintain net charges of +7 and +6 (al), respectively, resulting in their partial separation as shown in Fig. 1.

The amino acid composition of the slower migrating peptide A obtained from whole rat pancreas is presented in Table I. The peptide notably lacks arginine, lysine, histidine, isoleucine, phenylalanine, and tyrosine. Furthermore, the composition of peptide A corresponds rather well to that expected from the NH₂-terminal 22 residues of rat C-peptide I (Table I), notwithstanding the relatively high alanine content which may arise from a minor contaminant.

We purified the [3H]leucine-labeled peptides by paper electrophoresis in 30% formic acid in order to avoid possible loss of the very small amount of material due to nonspecific binding to QAE-Sephadex in dilute, aqueous buffers. Fig. 2 (left) shows the electrophoretic patterns obtained separately from the [3H]labeled C-peptide and the [3H]labeled peptide A after the earlier purification of the peptides by paper electrophoresis. We were able to obtain peptide A in a purity of about 97%. The labeled, principal C-peptide fraction was contaminated with approximately 12% peptide A, but was considered to be of sufficient purity to be used in a probe for possible artifacts arising from the isolation procedures. A portion of the [3H]labeled C-peptide was homogenized with 1 g of rat pancreas and the homogenate treated to obtain the fraction containing peptide A, C-peptide, and insulin. Electrophoresis in 30% formic acid revealed that the residual [3H]labeled C-peptide-like material contained 13% peptide A (Fig. 2, right) indicating that peptide A did not arise from the degradation of the C-peptide during isolation.

The [3H]leucine-labeled peptide A and the unlabeled peptide A were separately subjected to 15 cycles of Edman degradation, with the results shown in Fig. 3. The partial sequence of the unlabeled material is identical to that of the NH₂-terminal 15 residues of intact rat C-peptide I. Furthermore, the positions of the leucine residues in the labeled material are identical to those in peptide A obtained from whole pancreas. Treatment of unlabeled peptide A with carboxypeptidase A revealed the COOH-terminal sequence of the peptide to be Leu-Glu-(Thr) (Table II). Since the approximately 0.5 mole of threonine present in 1 mole of peptide A (Table I) appeared early in the carboxypeptidase A digest, it may well occur as the COOH terminus in a portion of the peptide A molecules. It is presumed that free amino acids other than leucine, glutamine, and threonine were not detected in the carboxypeptidase A digest because the Gly-Asp sequence preceding Leu-Glu in the rat C-peptides blocks against further carboxypeptidase action (14).

Fig. 4 shows the results of a pulse-chase experiment designed to confirm the identity of peptide A (Table I) as the peptide leading us to suppose that this peptide might be significant in normal β-cell metabolism. A peptide with identical electrophoretic mobility also appears in the insulin- and C-peptide-containing fraction isolated from whole rat pancreas (see legend Fig. 1). Each of the two rat C-peptides maintains a net charge of +2 in 30% formic acid, pH 0.9, and both peptides migrate to the same position after paper electrophoresis (14). At that pH, rat insulins 1 and 2 maintain net charges of +7 and +6 (al), respectively, resulting in their partial separation as shown in Fig. 1.

**Table I**

| Amino acid | NH₂-terminal fragment (residues found) | C peptide 1° (residues 1-22) | C peptide II° (residues 1-22) |
|------------|--------------------------------------|-----------------------------|-------------------------------|
| Aspartic acid | 2.2 | 2 | 2 |
| Threonine | 0.5 | 0 | 0 |
| Serine | (Trace) | 0 | 0 |
| Glutamic acid | 6.8 | 7 | 6 |
| Glycine | 3.8 | 4 | 5 |
| Alanine | 1.6 | 1 | 2 |
| Proline | 2.8 | 3 | 2 |
| Valine | 2.1 | 2 | 2 |
| Leucine | 3.0 | 3 | 3 |

* These amino acid compositions are abstracted from the primary structures of the rat C-peptides presented in Reference 14.
The fragment (24 nmoles by amino acid analysis) was incubated in 0.30 ml of 0.1 M Tris-HCl buffer, pH 8.5, containing 0.24 n mole of carboxypeptidase A. After selected periods of incubation, aliquots of the reaction mixture were removed for amino acid analysis as described under "Methods." A blank digestion, quenched with acid before the addition of enzyme, revealed only trace amounts of glycine and alanine.

| Incubation conditions | Threonine | Glutamine | Leucine |
|-----------------------|-----------|-----------|---------|
| 3.3 min, 21°          | 0.19      | 0.21      | 0.21    |
| 10 min, 21°           | 0.32      | 0.43      | 0.26    |
| 60 min, 21°           | 0.46      | 0.95      | 0.48    |
| 60 min, 21° + 60 min, 37° | 0.52 | 0.98      | 0.95    |

We also considered the possibility that peptide A might arise biosynthetically from the proinsulin and proinsulin intermediate fraction present in β-cells of the rat pancreatic islets. The digestion of bovine proinsulin with trypsin results in the liberation of C-peptide with an additional lysine residue at its COOH terminus (8). Digestion of rat proinsulin with trypsin, however, would result in the formation of the desglutamine C-peptide since the COOH-terminal Arg-Gln sequences of the two rat C-peptides are cleaved by trypsin (13). Thus, the C-peptide-like material obtained from trypsin digestion of rat proinsulin should retain the electrophoretic mobility of the unmodified C-peptide.

As shown in Fig. 5, trypsin digestion of a fraction containing proinsulin and proinsulin intermediates obtained after a 6-hour incubation of rat islets with [3H]leucine resulted in the liberation of peptide A as well as C-peptide or desglutamine C-peptide or both. The control digestion of an unlabeled mixture of C-peptide, peptide A, and insulin 1 and 2 under similar conditions showed that the migrations of peptide A and C-peptide were unchanged, although the migration of the insulin-like components was slowed considerably (cf. Fig. 1) due to cleavage carboxyl to Lys 3 and Arg 22 in the rat insulin A chains (21). We also studied the products resulting from the trypsin digestion of proinsulin obtained from rat islets incubated with [3H]leucine for 3 hr chase, 2 hr chase, and 1 hr chase.

S. O. Emdin and D. F. Steiner, unpublished results.
Although smaller and more acidic than the C-peptide, it apparently maintains many of the chemical and physical characteristics of the intact material. The finding that intact [3H]leucine-labeled rat C-peptide was not degraded to the NH2-terminal fragment during homogenization with whole pancreas and subsequent resinolation eliminates the possibility that peptide A is an artifact of isolation, arising either from residual enzymatic activity in the exocrine pancreas or from acid-catalyzed cleavage.

The stoichiometry of amino acid residues in peptide A (Table I) suggests that the process resulting in its formation is specific. In assigning a likely catalytic source for the formation of the NH2-terminal fragment, it is pertinent to note that two sites in the C-peptide in the region distal to Leu 21 are sensitive to chymotrypsin. We previously reported that chymotrypsin cleaved the intact rat C-peptides between Glu-Thr (residues 22 and 23) (14), whereas Markussen and Sundby (13) found chymotryptic cleavage to occur between Leu-Ala (residues 24 and 25). The results of the digestion of peptide A with carboxypeptidase (Table II) suggest that the peptide arises from a chymotryptic-like cleavage at one or the other of these sites.

Thus, the early release of a small amount of leucine and the release of approximately 0.5 mole % of threonine by carboxypeptidase A may indicate heterogeneity in the COOH terminus of peptide A. It is possible that an initial chymotryptic-like cleavage occurred between residues 24 and 25 yielding a peptide with the COOH terminal sequence Leu-Glu-Thr-Leu. Carboxypeptidase activity within the β-cell might slowly degrade the material to a mixture of several peptides lacking 1 or 2 neutral amino acid residues. On the other hand, the threonine present in the carboxypeptidase digest may arise from a minor contaminant. The unequivocal COOH-terminal sequence Leu-Gln might then result from a chymotryptic-like cleavage between residues 22 and 23. In either case, we cannot exclude the possibility that the principal cleavage enzyme is similar to papain, cathepsin B, or cathepsin D, since these enzymes and a-chymotrypsin can show overlapping substrate specificities (22-24).

Since the proportion of the NH2-terminal fragment to the C-peptide does not increase as the C-peptide ages within the β-cell (Fig. 4), we can exclude the possibility that the fragment arises directly from a chymotryptic-like cleavage of the C-peptide itself. Had such an enzymatic cleavage occurred, the ratio of fragment to C-peptide would have increased during the longer periods of chase, following the reaction course, C-peptide → NH2-terminal fragment. The lack of increase in the ratio of fragment to peptide, however, might also be explained by the existence of the following sequence of reactions: C-peptide → NH2-terminal fragment → small peptides. If the first reaction in this sequence were rate-limiting, the ratio of NH2-terminal fragment to C-peptide might not rise until most of the C-peptide had been degraded. This reaction sequence apparently does not play a major role in the formation of the fragment since the ratio of C-peptide-like material to insulin-like material remains constant under a wide variety of incubation conditions (25).

The results shown in Fig. 5 clarify the origin of the NH2-terminal fragment of the rat C-peptide. Trypsin treatment of a mixture of rat proinsulin and proinsulin intermediates released the NH2-terminal fragment as well as the desglutaminate C-peptide. These results suggest that in endocrine tissue this fragment arises from a proinsulin intermediate which has been cleaved in its C-peptide region, rather than from secondary cleavage of the free C-peptide. The release of the chymotryptic-like fragment from this precursor in vivo may then result from the conversion of the proinsulin intermediate to insulin. Furthermore, the retention of the fragment within the secretory granules of the β-cell results in its
pears as the NH₂-terminal fragment of the C-peptide. The NI-Iz-terminal fragment of the C-pcptide. The rat. The chymotrypsill-like cleavage of proinsulin or of insulin intermediates similar in structure to Intermediates 3 or 4 do occur during the normal course of proinsulin conversion in pancreatic islets incubated with [3H]phenylalanine. The intermediates were not fully characterized and may have consisted of small amounts of such cleaved forms.

The biological significance of the chymotrypsin-like cleavage of rat proinsulin I since cleavage apparently does not occur in the same position of the free C-peptide during the biosynthesis and storage of insulin. Such selectivity may also explain the apparent lack of a chymotrypsin-like cleavage of proinsulin or proinsulin intermediates to yield NH₂-terminal fragments of the C-peptide containing residues 1 to 10 or residues 1 to 12 (14). We cannot exclude, however, the existence of small amounts of such cleaved forms.

As mentioned previously, our studies on the conversion of proinsulin to insulin in the rat were aided by the charge difference between the intact C-peptide and its NH₂-terminal fragment. The isolation of requisite intermediates for a similar chymotrypsin-like cleavage in porcine proinsulin (9) suggests that the occurrence of such activities in the conversion of proinsulin to insulin is not restricted to one species. The apparent lack of such intermediates in the case of bovine proinsulin may simply relate to the lack of a site sufficiently sensitive to chymotrypsin in the C-peptide region of that molecule (5, 8). It may be anticipated, however, that fragments of the C-peptide will be found in extracts of pancreas from other species.

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Fig. 6. Diagrammatic representation of the pathways for the conversion of proinsulin to insulin. Structures 1 to 5 represent possible proinsulin-like intermediates. The major pathway for proinsulin conversion is shown in heavy lines. The sites on either side of the C-peptide filled by pairs of basic amino acid residues are indicated by circles and squares. Appropriate cleavage at these sites to yield insulin and C-peptide requires both trypsin-like and carboxypeptidase B-like activities. The slash represents a chymotrypsin-sensitive site in the C-peptide region of proinsulin. The NH₂ termini of the A and B chains of insulin are indicated by dots.
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