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DEGRADATION OF PROINSULIN AND ISOLATED C-PEPTIDE BY RAT KIDNEY NEUTRAL METALLO-ENDOPEPTIDASE *

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Previous studies have shown that a neutral metallo-endopeptidase purified from rat kidney degrades the B chain of insulin, glucagon, ACTH and, at a markedly slower rate, the A chain of insulin. In contrast the enzyme does not attack native insulin, oxytocin, vasopressin, ribonuclease, albumin or denatured hemoglobin. The current studies demonstrate that the neutral peptidase also degrades the isolated C-peptide of proinsulin and cleaves certain peptide bonds in and near the C-peptide moiety of native proinsulin. Time courses of the formation of fluorescamine-reactive material during digestion of proinsulin and isolated C-peptide with the peptidase were identical. However, structural analysis of the peptidase-digested proinsulin showed that the enzyme does not convert proinsulin to insulin but that the peptidase cleaves one bond, Tyr_{26}-Thr_{27}, in the B chain moiety and five bonds in the C-peptide moiety, producing four split proinsulins. One of the split proinsulins is des-octacosapeptide (27-54) porcine proinsulin or des-tetracosapeptide (27-50) bovine proinsulin. Each is a derivative of the insulin molecule having an extension of nine residues (ten residues in the case of the derivative from bovine proinsulin) at the N-terminus of A chain and lacking four residues at the C-terminus of B chain. This two-chain derivative retains full immunoreactivity with insulin antibodies and exhibits 2.4-times more biological activity (promotion of glycogenesis in primary cultured hepatocytes) than proinsulin and about two-thirds the activity of insulin.

Introduction

A number of neutral proteinases with relative specificity for the degradation of collagen [1], histones [2,3], mast cells [4] and intracellular enzymes [5] have been identified. This group of enzymes (i.e., neutral proteinases) seems to be involved in important physiological processes. For example, the angiotensin-converting enzyme is important in the regulation of blood pressure [6]. Recent work from this laboratory reported the purification of a neutral metallo-endopeptidase from rat kidney [7] and demonstrated that a similar enzymatic activity occurs widely in other rat tissues [8]. This enzyme apparently contains a metal ion (most likely Zn^{2+}) essential for its enzymatic activity, and is inhibited by chelating agents, such as EDTA and phenanthroline, and by thiols, such as dithiothreitol, GSH and mercaptoethanol. The purified enzyme degrades the B chain of insulin, glucagon,

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Abbreviations: DOC-PI, des-octacosapeptide (27-54) porcine proinsulin which is a modified insulin derivative having an extension of nine amino acid residues at N-terminus of A chain and lacking four amino acid residues at C-terminus of B chain; DTC-PI, des-tetracosapeptide (27-50) bovine proinsulin which is a modified insulin derivative having an extension of ten amino acid residues at N-terminus of A chain and lacking four amino acid residues at C-terminus of B chain; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; ACTH, adrenocorticotropin.

ACTH and, at a markedly slower rate, the A chain of insulin [7]. In contrast, the enzyme does not degrade native insulin, oxytocin, vasopressin, ribonuclease, albumin or denatured hemoglobin [7].

As part of a study on the characterization of the enzyme and in search of biological functions of the rat kidney neutral peptidase, we investigated the action of the purified enzyme on native proinsulin and the isolated C-peptide of proinsulin and found them to be substrates. We have identified one major proinsulin derivative produced by the action of the enzyme and have determined the immunological and biological activity of the derivative. An abstract of this work has appeared [9].

Materials

[1-^{14}C]Dimethylaminonaphthalene-5-sulphonyl-chloride ([^{14}C]dansyl chloride), specific activity 117–163 μCi/mg, was purchased from Amersham Corporation. Dansyl chloride and Schleicher and Schuell polyamide sheets (F1700) were purchased from Pierce. Amorphous insulin and proinsulin, both from pork, and crystalline insulin and proinsulin from beef were gifts from Drs. W.W. Bromer and R. Chance, Eli Lilly Company. Bovine proinsulin C-peptide was a gift from Novo Co. Sephadex G-25 and G-75 were from Pharmacia. Buffers for amino acid analysis (Pico-Buffer® System II and Femto-Buffer® System I), and Fluoropa® (*o*-phthaladehyde) were obtained from Durrum Chemical (Dionex) Corporation. The reagents for the Edman degradation (phenyl isothiocyanate, *n*-propanol, trifluoracetic acid, *N,N*-dimethyl-*N*-allylamine, benzene and ethyl acetate) were all sequanal grade and were purchased from Pierce.

Methods

The preparations of the cysteic acid derivative of B chain of insulin [7,10], hereafter referred to as B chain, and ^{125}I-labeled B chain and proinsulin [11,12] have been described in the cited references.

Neutral metallo-peptidase from rat kidney was purified through the step of affinity chromatography on Sepharose 4B coupled with insulin B chain as described previously [7]. It consisted of a single component as judged by chromatography on the B chain affinity column and on DEAE-cellulose and by gel filtration on a Sephadex G-200 column. Two preparations of purified enzyme, containing 46 000 and 60 000 enzyme units/mg protein were used. The difference in the specific activities is not due to the degree of purity but reflects different preparations of ^{125}I-labeled B chain used as the substrate during the purification.

Neutral peptidase digestion. Proinsulin (approx. 720 μM) was incubated in 0.05 M $NaHCO_3$, pH 7.2, at 37°C for up to 2 h with purified neutral peptidase (1 463 or 2 000 units/ml). The incubations were terminated at the indicated times by adding EDTA (final concentration, 10 mM) and/or by heating for 2 min in a water bath at 100°C. For identification of susceptible peptide bonds, the polypeptide digests were divided into several portions and utilized for amino-terminal determination, amino acid sequence determination and peptide fragment isolation for subsequent amino acid analysis.

Amino acid sequence determination. The enzyme digest of porcine proinsulin (100 nmol) was subjected to Edman degradation [13]. The Edman degradation was monitored by removing aliquots (approx. 2 nmol) for dansylation at the beginning of each Edman cycle. The aliquot in the coupling buffer was evaporated, redissolved in 20 μl 0.1 M $NaHCO_3$ and subjected to dansylation.

Amino-terminal determination. N-Terminal residues were determined by the dansylation technique according to Gray [14] with some modifications. When the Edman degradation was monitored, 2 nmol of a polypeptide digest (removed prior to each Edman cycle) in 20 μl 0.1 M $NaHCO_3$ were reacted at 37°C for 60 min with 20 μl dansyl chloride (5 mg/ml in acetone). The dansyl peptides were acid-hydrolyzed. The hydrolysate, after evaporation, was extracted with water-saturated ethyl acetate. The ethyl acetate extract was examined for the dansylated neutral and acidic amino acids, and the ethyl acetate residue for dansyl-Cys(O_3H). When dansyl-His or dansyl-Arg was examined, the hydrolysate was not extracted with ethyl acetate. Samples (hydrolysate, ethyl acetate extract or its residue), after drying, were taken up in acetone/acetic acid (3 : 2, v/v) and aliquots were chromatographed on 7.5 × 7.5 cm polyamide sheets for the separation of dansyl amino acids in the solvent system described by Gray [14].

When the formation of new N-terminal groups was quantitated, 14.7 nmol of a polypeptide digest were used and the reaction mixture also contained 1 μCi [^{14}C]dansyl chloride. Fluorescent [^{14}C]dansyl amino acid spots were cut from the sheets, placed in 10 ml Insta-Gel scintillation fluid and counted in a Packard beta counter. For brevity, this procedure is referred to as the quantitative ^{14}C-dansylation technique. This quantitative ^{14}C-dansylation procedure was used only for the estimation of the number of peptides generated by the neutral peptidase in the original enzyme digest. Radioactivity significantly above background indicated the presence of the amino acid at that spot. The number of moles of each [^{14}C]-dansyl amino acid was calculated relative to the radioactivity incorporated in the [^{14}C]dansyl amino acid at the N-terminus of the parent molecule, which served as an internal standard. In all instances, repetitive analysis showed the same yield of radioactivity levels and assignments were confirmed by the finding of expected residues in further sequence analysis as described below.

Amino acid analyses. Peptides were hydrolyzed with constant boiling HCl at 110°C for 22 h in evacuated glass test tubes. Analyses were performed on a Durrum (Dionex) amino acid analyzer (Kit MBF) with a fluorescence detection system using Fluoropa® reagent. Sample loads of 1 nmol amino acid were employed in order to obtain more reliable analyses.

Isolation of modified insulin derivative from proinsulin digest. This was carried out by gel filtration of the neutral peptidase digest of proinsulin on a Sephadex G-75 column (0.9 X 47 cm) previously equilibrated with 3 M acetic acid; additional details are provided in the figure legend.

Polyacrylamide gel electrophoresis. Samples of proinsulin which had been digested with neutral peptidase at 37°C for 5 min were electrophoresed on 15% polyacrylamide cylindrical gels (70 X 6 mm) of pH 9.5 according to Davis [15]. The gels were stained with Coomassie brilliant blue and destained by diffusion against methanol/acetic acid as described previously [16].

Results

Because of the limited amount of materials available, some experiments have been performed with porcine proinsulin and others with bovine proinsulin in arriving at the overall conclusions regarding the action of neutral peptidase on proinsulin.

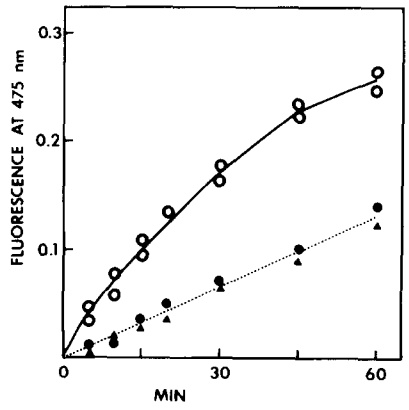

Fig. 1. Degradation of porcine proinsulin, isolated bovine C-peptide and isolated B chain by purified neutral peptidase as measured by the formation of fluorescamine-reactive material. Each polypeptide (15 μM) was incubated at 37°C with neutral peptidase (18.56 units/ml) in 10 mM cacodylate buffer, pH 7.2, and aliquots were withdrawn at the times indicated. The reaction was stopped by the addition of EDTA and fluorescamine-reactive material was measured as described previously [7]. •, proinsulin; ▲, C-peptide; ○, oxidized B chain. The data on the degradation of oxidized B chain, which were determined simultaneously, are included for comparison.

As previously reported [7], the enzyme does not degrade insulin using the same assay conditions in which it readily attacks the other substrates, e.g., insulin B chain and glucagon; the non-susceptibility of insulin was confirmed using the structural analysis techniques employed in the current studies. However, as shown by the results in Fig. 1, the enzyme is able to degrade proinsulin. Since the time-courses of digestion of porcine proinsulin and isolated C-peptide of bovine proinsulin, as measured by the formation of fluorescamine-reactive material, were superimposable (Fig. 1) and since insulin is not susceptible to the enzyme [7], these results raised the possibility that neutral peptidase might be converting proinsulin to insulin by degrading the C-peptide moiety in the proinsulin.

In order to obtain insight into the nature of proinsulin product(s), the enzyme digests of porcine and bovine proinsulin were subjected to structural analysis. Application of the quantitative ^{14}C-dansyla-

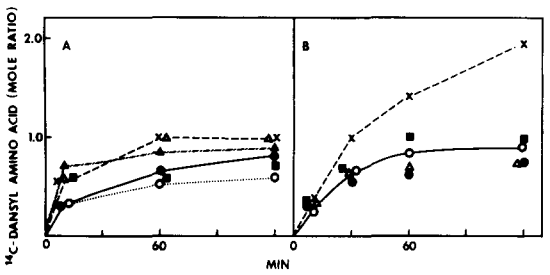

Fig. 2. Time courses of generation of N-terminal groups from porcine and bovine proinsulins as a function of digestion with neutral peptidase. Porcine proinsulin (A) or bovine proinsulin (B), each 200 μM, and 1 463 units/ml of neutral peptidase in a total volume of 100 μl 0.1 M $NaHCO_3$, pH 7.5, were incubated at 37°C and 20 μl aliquots were withdrawn at the indicated times and subjected to the quantitative ^{14}C-dansylation procedure. Results are presented as relative molar quantities based on the radioactivity incorporated at each time point in dansyl phenylalanine which is the N-terminal of the parent molecule. •, dansyl-Leu; X, dansyl-Gly; ○, dansyl-Thr; ▪, dansyl-Glx, ▲, dansyl-Asx; △, dansyl-Ala.

tion technique (Fig. 2) showed, during the 2-h incubation of proinsulin with the enzyme, N-terminal Phe of the parent molecules and the formation of six new N-terminal groups from each proinsulin: about 1 mol each of Leu, Gly, Ala, Asx, Thr and Glu for porcine proinsulin, and about 1 mol each of Leu, Ala, Thr and Glu along with 2 mol Gly for bovine proinsulin. It is interesting that N-terminal Asx is generated from porcine but not from bovine proinsulin, probably because the porcine C-peptide contains one residue of Asn, whereas the bovine C-peptide contains neither Asn nor Asp. Also, the formation of 2 mol dansyl-Gly in the case of bovine proinsulin and 1 mol in the case of porcine proinsulin is consistent with the bovine C-peptide content of one more residue of Gly than the porcine C-peptide. Thus, these results suggest that the susceptibility of proinsulin to neutral peptidase may lie within the C-peptide moiety. However, since there is no Thr in either C-peptide segment, proteolysis within the insulin moiety must occur; therefore, the studies described below were undertaken.

Results of four cycles of dansyl-monitored Edman degradation on the 2-h unfractionated digest of porcine proinsulin are given in Table I. These data were aligned, in the manner previously described [17], with the known proinsulin structure using the

TABLE I

SEQUENCE ANALYSIS OF AN UNFRACTIONATED NEUTRAL PEPTIDASE DIGEST OF PORCINE PROINSULIN

Prorcine proinsulin (715 μM) was digested with neutral peptidase (2 000 units/ml) in 50 mM $NaHCO_3$, pH 7.2, at 37°C for 2 h. Sequences of the peptides present in the unfractionated digest were determined by the Edman degradation technique monitored by dansulation. For brevity, residues in each cycle are arranged with the peptides to which they were finally assigned. Numerals refer to residue numbers in the sequence of porcine proinsulin which is shown in Fig. 3.

Edman cycle				Residue assignment (residue number)	Neutral peptidase susceptible bond
1	2	3	4		
Dansyl-amino acids					
Phe	Val	Asx	Glx	1– 4	(N-terminal segment of the parent molecule)
Thr	Pro	[a]	Ala	27–30	Tyr_{26}-Thr_{27}
Glu	Asx	Pro	Glx	35–38	Ala_{34}-Glu_{35}
Asx	Pro	Glx	Ala	36–39	Glu_{35}-Asn_{36}
Gly	Ala	Val	Glx	40–43	Ala_{39}-Gly_{40}
Leu	Glx	Ala	Leu	51–54	Gly_{50}-Leu_{51}
Ala	Leu	Glx	Gly	55–58	Leu_{54}-Ala_{55}

[a] For reasons as yet unexplained, lysine residue of the sequence, Thr_{27}-Pro_{28}-Lys_{29}-Ala_{30}, was not found in the third cycle; instead leucine was found which remains as yet unassigned.

following criteria. The residues for the N-terminal sequence of the parent molecule, Phe_1, Val_2, Asn_3 and Gln_4, are first assigned in the four cycles. There are two threonine residues; Thr_{A8} is ruled out because Ser and Ile were not found in the second and third cycles, respectively. Therefore, dansylated threonine found in the first Edman cycle of the digest probably represents the sequence Thr_{27}-Pro_{28}-Lys_{29}-Ala_{30}, and indicates a cleavage site between Tyr_{26} and Thr_{27} residues. Although the lysine residue of this sequence (Thr_{27}-Pro_{28}-Lys_{29}-Ala_{30}) was not detected in the third cycle (Table I, footnote a), the amino acid compositions of the isolated products formed from proinsulin (Table II) are also consistent with a cleavage between Tyr_{26} and Thr_{27} residues. The presence of leucine in the third cycle (Table I, footnote a) cannot be assigned to any probable sequence, and may

represent a carry-over of leucine from the second cycle. There are 11 glycine residues in porcine proinsulin. However, all glycines, based on the succeeding Edman cycles, can be ruled out except Gly_{40} since Val was found in the third cycle. Therefore, the peptide with Gly N-terminal represents the sequence Gly_{40}-Ala_{41}-Val_{42}-Glu_{43}, and indicates a cleavage site between Ala_{39} and Gly_{40} residues. Of the seven Ala residues in the proinsulin molecule, the two Ala residues contained in the B chain portion are ruled out since neither Tyr nor Arg was found in the third cycle. The remaining five Ala residues are in the C-peptide segment and the sequence Ala_{55}-Leu_{56}-Glu_{57}-Gly_{58} seems best to fit the data of succeeding cycles, and therefore indicates a cleavage site between Leu_{54} and Ala_{55}. Of the 11 Leu residues in the proinsulin molecule, both Leu residues in the A chain segment and Leu_{B15} are ruled out because Tyr was not found in succeeding cycles. Leu_{B6} and Leu_{B17} are unlikely contributors since the cystine residues should have halted the Edman degradation and therefore should have produced a lesser number of residues in the succeeding cycles. Leu_{C44} and Leu_{C48} can be ruled out because Gly was not found in the second cycle. Thus, Leu found in the first cycle seems to represent the sequence Leu_{51}-Gln_{52}-Ala_{53}-Leu_{54} and a cleavage between Gly_{50} and Leu_{51} seems to be indicated. There are seven Glu and seven Gln residues in porcine proinsulin; from the relative position distribution of various residues in the four Edman cycles the sequence Glu_{35}-Asn_{36}-Pro_{37}-Gln_{38} seems to fit the data, indicating a cleavage site between Ala_{34} and Glu_{35}. Finally, of the four Asn residues, the sequence

TABLE II

N-TERMINAL AND AMINO ACID COMPOSITION DATA OF FRACTIONS OF BOVINE PROINSULIN PRODUCED FOLLOWING DIGESTION WITH NEUTRAL PEPTIDASE

Fractions 26 and 32 of Fig. 4. Amino acid compositions were determined on 22-h hydrolysates and are given in relative mol ratios. Expected residues (fraction 26) for the large insulin derivative having a ten residue extension on the N-terminus of A chain and four residues short on the C-terminus of B chain. Although there is some cross-contamination, the major derivative can be clearly identified. Expected residues (fraction 32) = proinsulin minus the insulin derivative as described for fraction 26.

	Fraction 26		Fraction 32	
	Residues found	Residues expected	Residues found	Residues expected
N-terminal	Phe, Gly	Phe, Gly	Leu, Gly, Ala, Thr Glx	Leu, Gly Ala, Thr Glx
Amino acid composition				
$Cys(O_3H)$	6.55	6	0.54	0
Asp	2.97	3	0.45	0
Thr	0.27	0	0.72	1
Ser	2.83	3	trace	0
Glu	9.05	9	5.37	4
Pro	1.81	2	2.31	3
Gly	7.03	7	5.92	5
Ala	2.90	2	3.37	4
Val	4.70 [a]	5	2.15	2
Ile	0.88 [a]	1	trace	0
Leu	6.67	7	2.96	2
Tyr	2.57	4	0.25	0
Phe	2.79	3	0.33	0
His	2.29	2	0.38	0
Lys	1.25	1	1.07	1
Arg	2.10	2	2.01	2

[a] Values from 72-h hydrolysate.

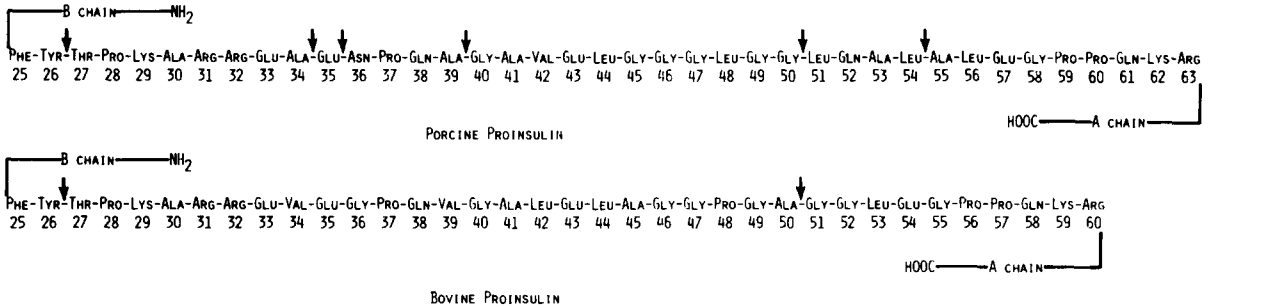

Fig. 3. Amino acid sequence of porcine proinsulin and bovine proinsulin. Arrows denote the neutral peptidase susceptible bonds. In bovine proinsulin, two susceptible bonds are indicated; the other four susceptible bonds as indicated by NH_2-terminal group studies were not determined.

Asn_{36}-Pro_{37}-Gln_{38}-Ala_{39} seems to be the most likely contributor of the Asx N-terminal as judged from the remaining residues found in the succeeding cycles and therefore indicates a cleavage site between Glu_{35} and Asn_{36}. In summary (Fig. 3), neutral peptidase appears to cleave the following six bonds in the porcine proinsulin: Tyr_{B26}-Thr_{B27}, Ala_{C39}-Gly_{C40}, Leu_{C54}-Ala_{C55}, Gly_{C50}-Leu_{C51}, Ala_{C34}-Glu_{C35} and Glu_{C35}-Asn_{C36}. Thus, the enzyme cleaves one bond in the B chain moiety and five bonds in the C-peptide segment, and produces a large molecular weight insulin derivative (DOC-PI) which has an extension of nine residues at the N-terminus of A chain and lacks four residues at the C-terminus of B chain.

When a 2-h digest of bovine proinsulin was subjected to gel filtration on Sephadex G-75, it was separated into two fluorescamine-reactive fractions (Fig. 4). Amino acid analysis and NH_2-terminal determination (Table II) showed that the major product present in fraction 26 of the first peak is DTC-PI which is an insulin derivative having a ten-residue extension (instead of nine residue as in the case of the porcine derivative) at the N-terminus of A chain and lacking four residues at the C-terminus (see Fig. 3 for structure). These findings together with the dansylation data (Fig. 2B) indicate a cleavage at Ala_{C50}-Gly_{C51} bond in the C-peptide moiety of bovine proinsulin (Fig. 3). Fraction 32 of the second peak contained the remaining five amino terminals and the remaining amino acids of the unfractionated digest.

When porcine proinsulin (1 μM) containing a trancer amount of ^{125}I-labeled proinsulin was incubated at 37°C with neutral peptidase (44 units/ml) in 0.1 M Tris-HCl/0.2% bovine serum albumin, pH 7.2, and subjected to gel filtration on Sephadex G-75 in 50% acetic acid as described previously [10,18], there was no radioactivity in the location of the low-molecular weight components even after 5 h

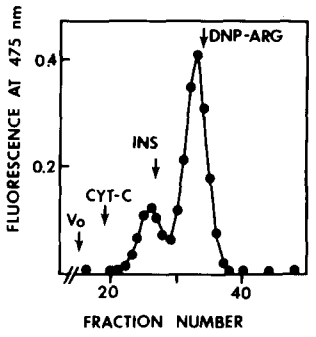

Fig. 4. Elution profile of fluorescamine-reactive material on Sephadex G-75 gel filtration of bovine proinsulin following incubation with neutral peptidase. Bovine proinsulin (107.5 nmol) was incubated at 37°C with neutral peptidase (203 units) in a total of 150 μl 50 mM $NaHCO_3$, pH 7.2. After 2 h, the reaction was stopped by the addition of EDTA (10 mM final concentration). The volume was brought to 300 μl with 6 M acetic acid and the entire mixture was applied to a 0.9 X 47 cm column of Sephadex G-75. The column was eluted with 3 M acetic acid at a flow rate of 8.5 ml/h and 1.0 ml/fraction was collected. Aliquots (50 μl) of each fraction were monitored for fluorescamine-reactive material as described previously [7]. Blue dextran (as a marker for void volume, V_0), cytochrome *c* (CYT-C) (M_r 12 300), insulin (INS) (M_r 6 000) and DNP-Arg (as a marker for low molecular weight components) were used to calibrate the column.

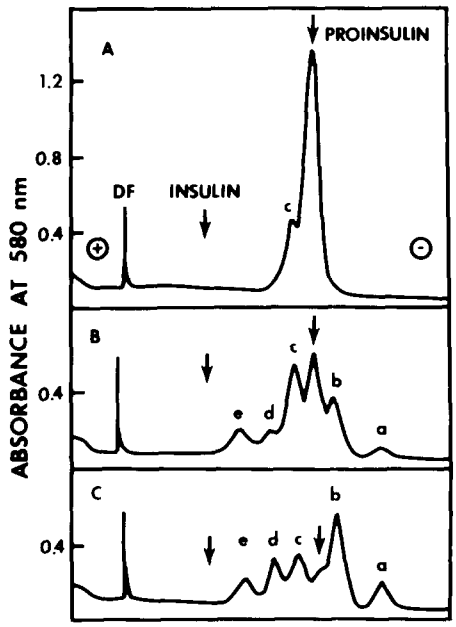

Fig. 5. Polyacrylamide gel electrophoresis analysis of porcine proinsulin previously digested with neutral peptidase. Porcine proinsulin (10 μM) was incubated at 37°C for 5 min with 2.2 (A), 11.2 (B) or 22.3 (C) units of neutral peptidase in a total volume of 100 μl 0.1 M Tris-HCl, pH 7.2. Following termination of the reaction by heating, 20 μl aliquots of each sample were electrophoresed on 7-cm long, 15% polyacrylamide gels according to the procedure of Davis [15]. The gels were stained with Coomassie blue and destained as described previously [16], and scanned at 580 nm. Arrows indicate positions of standard porcine insulin and proinsulin.

incubation (data not shown); these results further support the results obtained with fluorescamine reaction (Fig. 4) and with structural analysis since the small molecular weight fragments originating from and near the C-peptide region do not contain tyrosine residues and therefore would be devoid of radioactivity. There was one radioactive peak that eluted at zero time at the position of standard proinsulin; as the incubation period was increased from 0 to 5 h, the position of the radioactive peak moved towards insulin, eventually eluting at or near the position of standard insulin (data not shown).

When porcine proinsulin was digested for a short time period (5 min) with increasing concentrations of neutral peptidase and the digests subjected to polyacrylamide gel electrophoresis (Fig. 5), five products were observed. None of the products had the mobility of insulin and three products (bands c, d and e) might be the split forms of proinsulin (representing different cleavages in the C-peptide region during the course of digestion with neutral peptidase) since split proinsulins have been reported to appear between proinsulin and insulin under similar conditions of electrophoresis [19].

Thus, summarizing the data of structural analysis, Sephadex gel filtration and polyacrylamide gel electrophoresis, it is concluded that the digestion of proinsulin with the neutral peptidase causes cleavage of peptide bonds only in and near the C-peptide region. During the 2 h incubation period, four split forms of porcine proinsulin are formed; one of these is DOC-PI which is the derivative of insulin having an extension of nine residues at the N-terminus of A chain and lacking four residues at the C-terminus of B chain. From bovine proinsulin the enzyme generates DTC-PI which is an insulin derivative extended by ten residues at the N-terminus of A chain and shortened by four residues at the C-terminus. The neutral peptidase does not convert proinsulin to insulin.

Radioimmunoassay using insulin antibody [20] of porcine proinsulin following incubation with neutral peptidase showed that there was no change or a slight increase in the immunoreactivity during the 1 h incubation with the enzyme (data not shown). The two-chain derivative from DTC-PI, partially purified by gel filtration (Fig. 4) was tested for biological activity, i.e., its ability to promote glycogenesis in primary cultured rat liver cells (Table III). The derivative exhibited 2.4-times more biological activity than standard proinsulin and about two-thirds the activity of insulin.

TABLE III

DETERMINATION OF BIOACTIVITY (GLYCOGENESIS) OF THE TWO-CHAIN DERIVATIVE OF BOVINE PROINSULIN WITH PRIMARY HEPATOCYTE MONOLAYERS

The primary cultured rat hepatocytes received 3 ml basal medium alone or medium containing the indicated concentrations of insulin, proinsulin or the neutral peptidase-produced two-chain derivative from bovine proinsulin (DTC-PI). After 24 h incubation at 37°C in 95% air/5% CO_2 in a humidified incubator, medium was removed and cell glycogen was determined [34]. The culture procedure employed was a modification of previously described procedures [35,36]. Each value is the mean ± S.D. of four culture dishes; the basal value is based on five culture dishes of three different experiments.

Polypeptide	Concentration (nM)	Glycogen μg glucose/mg protein
Basal	–	37 ± 3
Insulin	0.1	227 ± 2
Insulin	1.0	350 ± 62
Insulin	10.0	350 ± 51
Proinsulin	0.1	39 ± 7
Proinsulin	1.0	77 ± 15
Proinsulin	10.0	289 ± 37
Derivative, DTC-PI	0.1	54 ± 6
Derivative, DTC-PI	1.0	137 ± 15
Detivative, DTC-PI	10.0	265 ± 27

Discussion

The preceding data indicate that the conformation of the polypeptide substrate probably influences the actual bonds attacked by the neutral peptidase. While the Tyr_{26}-Thr_{27} bond in the B chain moiety is not attacked as part of the insulin molecule, the same bond is readily cleaved as a component of the proinsulin molecule. These observations suggest that the overall conformation of polypeptide substrates probably imparts some selectivity to the bond susceptibility. The production of essentially the same modified insulin derivatives (consisting of an extended A chain and shortened B chain) from porcine and bovine proinsulin even though the C-pep-

tides of the two proinsulins have quite different primary structures further emphasizes the role of the conformation, which has been found to be the same in the two proinsulins [21]. The susceptibility of the C-peptide-containing region in the proinsulin molecule is consistent with reports that the connecting peptide is folded over the A chain on the surface [22]. That a native conformation alters or restricts the bond susceptibility in the substrate has been previously reported with other proteases. For example, pepsin attacks several bonds (Phe · Phe, Phe · Tyr, Leu · Glu, Glu · Asn and one of the two Leu · Val bonds) in oxidized insulin B chain; however, of the bonds attacked only the Phe · Phe and Phe · Tyr linkages have been shown to be susceptible in synthetic substrates [23]. Despite the ease with which cathepsin B cleaves the arginyl bond in the synthetic substrates, it does not remove the nearly homologous bond in proparathormone [24]. Our findings and those cited in Refs. 23 and 24 are consistent with the suggestion that the reactivity of a susceptible bond in a protein depends not only on the two residues which form the bond but also on the nature of other residues distant from the susceptible bond [25,26].

Based on the present information that insulin B chain, glucagon, ACTH, proinsulin C-peptide and proinsulin are good substrates, whereas oxytocin, vasopressin, insulin, ribonuclease, bovine serum albumin and denatured hemoglobin are resistant to the enzyme action, the following inferences can be made: the functional role of the enzyme seems to be distinctly the degradation of simple peptides. It is unlikely that the neutral peptidase plays any role in the degradation of larger plasma proteins. If this enzyme has any role in the degradation of insulin, it must be dependent upon prior reduction of insulin by glutathione-insulin transhydrogenase [18,27,28]. The fact that the neutral peptidase produces from proinsulin a two-chain derivative having 2.4-times more activity than proinsulin (Table III) indicates two possible physiological functions for the enzyme: (1) to provide a relatively more active form of insulin-like activity particularly in hyperproinsulinemia [29,30], and (2) to facilitate the disposal of proinsulin. The two-chain derivative can either be disposed of via reduction at the disulfide bonds by glutathione-insulin transhydrogenase and proteolysis of resultant chains since the derivative would be more susceptible than proinsulin, or it may be reabsorbed and may then contribute to the circulating pool of split proinsulins [19,31–33]. It is of interest to note that families with hyperproinsulinemia, characterized by elevated levels of split proinsulins, have been recently identified [29,30]. Whether the split proinsulins found in hyperproinsulinemia are related to any of the neutral peptidase-catalyzed products from proinsulin and, if related, whether the neutral peptidase activity is altered in these subjects remains to be seen. Clearly, fluctuations in response to alteration of the phsyiological state of the animal and the wide tissue distribution of neutral peptidase activity [8] suggest that neutral peptidase activity performs an important function in the metabolism of simple peptide hormones and other peptides.

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