Effect of pH on Conversion of Proinsulin to Insulin by a Subcellular Fraction of Rat Islets[1] (36207)

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(Introduced by P. K. Dixit)

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Previous studies on insulin biosynthesis in rats indicate that insulin is formed by way of a precursor (proinsulin) and one or more intermediates (1). A delay of 10–20 min. between proinsulin synthesis and onset of conversion has been demonstrated. Within this same period of time proinsulin is rapidly accumulated into the secretion granule containing fraction of the cell. The $T_{1/2}$ for conversion of proinsulin to insulin is approximately 1 hr and the newly synthesized insulin appears to be secreted after 1 hr (2).

Cell fractionation studies (3, 4) and electron microscopic radioautography indicate that proinsulin is synthesized on the rough endoplasmic reticulum and sequestered into the secretion granules presumably by way of the Golgi apparatus. These findings have suggested that the conversion of proinsulin to insulin occurs within the Golgi apparatus and/or the developing secretion granules. The distribution of labeled proinsulin and insulin in subcellular fractions of isolated rat islets is consistent with this hypothesis (4). Also several inhibitors of energy metabolism have demonstrated an inhibition of the conversion of proinsulin to insulin, when added prior to the presumed transport of the proinsulin from the endoplasmic reticulum to the Golgi region (5).

It has been suggested that the enzyme necessary for conversion of proinsulin to insulin is trypsin-like, possibly acting in conjunction with a protease having a specificity similar to carboxypeptidase B (6). Studies with thiol inhibitors indicate that the transforming enzyme may belong to a group of proteases having a thiol in the active center, such as cathepsin B. We report here studies on the proteolysis of endogenous and exogenous proinsulin as a function of pH and on the concentration of proteolytic activity in the secretion granule-containing fraction.

Methods and Materials. Islets were isolated from (350–400 g) male Holtzman rats by a modification of the method of Moskalewski (7) developed by Lacy and Kostianovsky (8). This method has previously been reported (9).

Endogenously labeled proinsulin was prepared by preincubating the isolated islets for 30 min in Krebs' solution containing 300 mg/100 ml of glucose, 20 mg/100 ml of natural amino acids (except leucine), and 100 μCi of leucine-4,5-^{3}H. Incubation was carried out in a shaking water bath at 37° under an atmosphere of 95% O_2/5% CO_2. The islets were then washed 3 times in cold 0.25 *M* sucrose and homogenized at 4° for 5 min, using a Teflon pestle attached to a low speed motor.

Subcellular fractionation of this original homogenate (OH) was carried out in the following manner:

F I (intact cells and nuclei): sediment from 2500*g* for 5 min;

F II (secretory granules and mitochondria): sediment from 15,000*g* for 20 min.

Samples of OH and F II, containing endogenously labeled proinsulin, were incubated with 1 mg/ml of pancreatic trypsin inhibitor [(PISF), Worthington Biochemicals] and 0.05 *M* HEPES (*N*-2-hydroxyethylpiperazine-μ'-2-ethanesulfanic acid) succinate buffer adjusted to various pH values. Incubation was carried out as before for 3 hr. Exogenously

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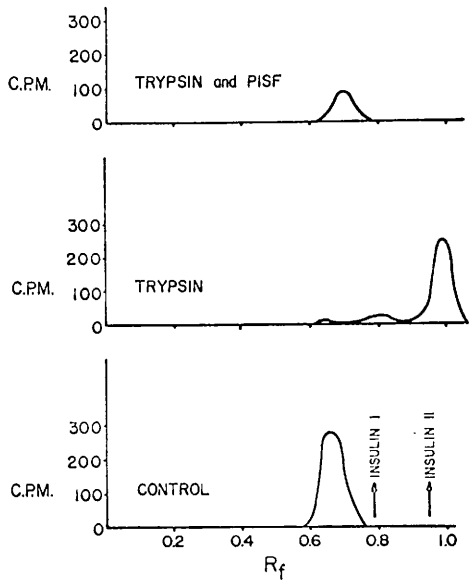

FIG. 1. Islets were pulse-labeled for 30 min with tritiated leucine, TCA-precipitated, and extracted with acid alcohol. The extracted protein was then incubated in 0.04 *M* Tris buffer (pH 7.5) with trypsin (0.0008 mg/ml) or trypsin with an excess of PISF for 1 hr at 37°. Disc electrophoresis with 7% of polyacrylamide gels was performed on the original material and the treated proteins. (control) The labeled electrophoresis pattern of the original protein. The other panels present the electrophoresis patterns of the same material treated with trypsin and with trypsin and PISF.

labeled proinsulin was obtained by preincubating isolated islets, as above, with tritiated leucine. After homogenization, the acid alcohol-soluble components were extracted, dried in a flash evaporator, and taken up in Tris buffer. Aliquots of this solution were incubated with F II suspensions as in the endogenous procedure. The effects of freezing and thawing were tested, using endogenously and exogenously labeled F II samples which were frozen and thawed three times prior to incubation.

The effect of PISF on proteolysis of proinsulin by trypsin was also tested. Intact islets were pulse-labeled for 30 min with tritiated leucine as above; precipitated with trichloracetic acid (TCA); extracted with acid alcohol; and the extracted protein was incubated in 0.04 *M* Tris buffer (pH 7.5) with trypsin (0.008 mg/ml) or trypsin with an excess of PISF for 1 hr at 37°. Disc electrophoresis in 7% polyacrylamide gels were performed on the original material and on the treated proteins.

Following incubation, 50 mg of carrier bovine serum albumin (BSA) was added to the samples, and the total sample was precipitated with an equal volume of 10% TCA. The TCA precipitate was washed three times and extracted with 0.5 ml of acid alcohol. The acid alcohol extract was brought to a pH of 7.5–8.5; and the sediment was removed. The supernatant was dried and taken up in 75 µl of Tris buffer (pH 8.3). All samples were disc electrophoresed at pH 8.9 in 7% polyacrylamide gels. Following electrophoresis the gels were sliced into 20–25 sections; and each slice was eluted at 4° with 0.5 ml of 1% BSA for 24 hr. The eluate was dissolved in 10 ml of Bio-Solv + TLA (Beckman Instruments Inc.) and counted in a Beckman LS-250 scintillation counter.

Discussion and Results. The relative migration rates of proinsulin, intermediates, and insulins I and II have been reported (3). Analysis of proinsulin conversion was made by identifying the proinsulin

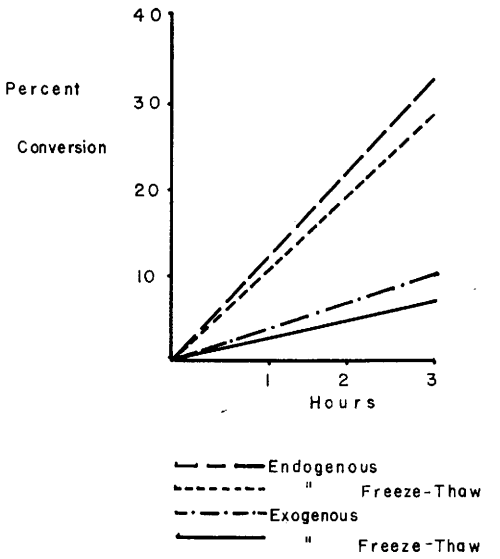

FIG. 2. Endogenously labeled fraction II from isolated islets and fraction II with an exogenous source of proinsulin were incubated for 3 hr at 37°, pH 6.5. In addition, duplicate samples were frozen and thawed 3 times prior to incubation. Conversion was notably diminished in the exogenous system.

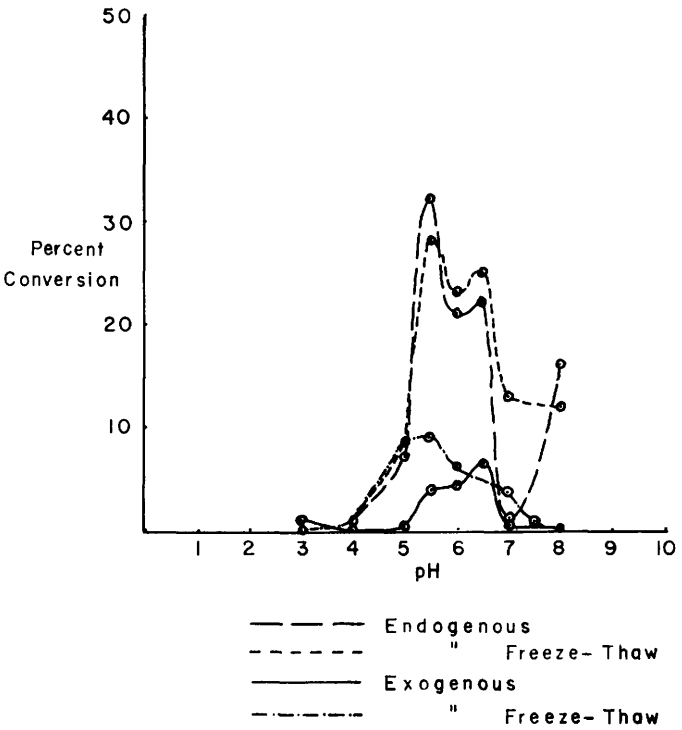

FIG. 3. Endogenously labeled fraction II, fraction II in the presence of an exogenous source of labeled proinsulin plus duplicate freeze–thaw-treated samples were incubated for 3 hr at 37° in media at various pH values. In all samples, conversion optima occurred at pH 5.5 and 6.5.

peak in each instance and totaling the counts obtained beyond this peak, *i.e.*, in the insulin and intermediate range. These counts were presumed to represent insulins I and II, in addition to a number of intermediates. Percentage conversion was determined by dividing the total counts in the insulin and intermediate range by the total counts including proinsulin.

After 1 hr of incubation of labeled protein with trypsin, all of the labeled protein was converted to a substance migrating in the range of desalinated insulin. Addition of PISF completely inhibited proteolysis by trypsin (Fig. 1). Since PISF was utilized in all of the experiments, it appears that the observed proteolysis was not due to the presence of active trypsin.

Conversion of exogenous proinsulin by FII was markedly lower than that observed with the endogenous system (Fig. 2). In both cases, freeze–thaw treatment indicated a loss in conversion activity; however, this reduction appears minor relative to that reported by Kemmler *et al.* (6). The source of this difference is not obvious and a direct comparison of each of the methods used to determine conversion may be necessary to resolve this difference.

Although conversion of proinsulin by FII was low in the exogenous system relative to the endogenous system, qualitatively similar results were obtained (Fig. 3). The pH optima for the endogenous system were pH 5.5 and 6.5, both in the case of untreated and freeze–thaw-treated samples. In the exogenous system, the untreated sample had a shoulder at pH 5.5 and a peak at pH 6.5; whereas the freeze–thaw-treated sample had a peak at pH 5.5 and shoulder at pH 6.5. Whether the optima represent two enzymes or an enzyme with two activities is not certain; and analysis of the profile of counts on the various disc electrophoresis gels did not substantially clarify this point.

A question arising in these experiments is whether the observed proteolytic activity is specific conversion of proinsulin to its inter-

mediates and insulin rather than activity of a nonspecific tissue protease. Further analysis of the products of proteolysis is necessary to answer this question.

The original homogenate and fraction II were analyzed to determine whether a relative concentration of conversion activity could be counts produced by proteolysis of the proinsulin was used as the substrate; and the net counts produced by proteolysis of the proinsulin were determined. The net count (cpm/hr/mg of protein) was determined for the samples as an index of the activity. When the proteolytic activity in fraction II was compared with the original homogenate, a 4.6-fold concentration of enzyme activity was revealed. When insulin was used as a reference (cpm/hr/mg of insulin), a ratio closer to unity (1.7) was observed. This seems to indicate that the proteolytic activity tends to parallel the insulin distribution during fractionation. These data are compatible with the hypothesis that conversion takes place in the Golgi apparatus or developing secretory granules; and the enzyme(s) is associated with these structures. In this experiment as with the others, conversion rates reported represent minimum values since leucine-^{3}H labeled insulin has a third less activity than full labeled proinsulin. The *C*-peptide fragment was lost in the TCA supernatant during extraction of the samples; and, consequently, these data show a lower apparent conversion than expected.

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