The catabolism of I-131-insulin by rat lens homogenates

Morris Green and Robert S. Schwartz

The ability of a rat lens homogenate to degrade radioiodinated zinc insulin into fractions which now are TCA soluble was used as a criterion of proteolysis. The assay method is extremely simple, rapid, and sensitive. Proteolytic activity is linearly related to the amount of lens homogenate added. The lens enzyme has a pH optimum of approximately 7.2, a temperature maximum near 50° C., and is inactivated by sulfhydryl inhibitors and heavy metals. Almost all of the enzyme activity is found in the water soluble fraction of the lens. Addition of crystalline zinc insulin competitively inhibits the breakdown of the I-131-insulin. This enzyme system or another enzyme exhibits a pH optimum above pH 10, a temperature maximum near 50° C., and has rather unique reaction kinetics.

A variety of analytical procedures has been employed to study proteolytic enzymes of the lens. The nature of the method varies widely depending on the substrate employed. Many peptides, amino acid esters, and proteins have been used to assay lens proteolytic activity. With protein substrates, trichloroacetic acid (TCA) is added to aliquots of the incubation mixture to precipitate the unreacted proteins and larger peptides. The TCA-soluble fraction can then be analyzed by measuring the nonprotein nitrogen, amino acids, or absorption at 280 μm. This article will report on the degradation of the radioiodinated zinc insulin into fractions which are TCA soluble. The method for the assay of lens proteolytic enzymes is extremely simple, rapid, and probably the most sensitive system yet employed.

Materials and methods

All of the lenses were obtained from Sprague-Dawley rats. The animals were sacrificed by decapitation. The lenses were removed, gently blotted on moist filter paper, and homogenized in the cold with a small Dounce homogenizer. The acetate and glycine buffers were 0.2M; the tris (tris [hydroxymethyl] aminomethane) buffer was 0.22M.

Radioiodinated zinc insulin was purchased from Abbott Laboratories. The iodination procedure involved the substitution of approximately 1 atom of iodide per molecule of insulin, assuming a molecular weight of 6,000 for insulin. Fig. 1 illustrates the amino acid sequence of insulin. Three to five milliliters of the I-131-insulin solution were dialyzed for 24 to 36 hours against 0.006N hydrochloric acid (2,000 ml. changed every 12 hours) to remove nonprotein bound radioactive iodine and phenol. The I-131-insulin was then diluted with deionized water to a convenient specific activity, about 50 to 100,000 counts per minute per 0.5 ml.

In most experiments, 0.5 ml. of I-131-insulin solution was added to 1 ml. of buffer in a small round bottom tube. One-half milliliter of lens homogenate was immediately added and mixed.

From the Department of Research, New York Eye and Ear Infirmary, 218 Second Ave. New York 3, N. Y.

This investigation was supported in part by Research Grant RH-19 from the Division of Radiological Health, Bureau of State Services, United States Public Health Service.

635
Fig. 1. Amino acid sequence of insulin. Each of the four tyrosine groups is marked with an asterisk.

Proteolysis was recorded as the per cent increase in radioactivity above the 3 to 5 per cent "blank" value. It is necessary to add plasma to the incubation mixture before the addition of TCA, since the plasma acts as a protein carrier and facilitates the precipitation of the undegraded I-131-insulin and polypeptides. Preliminary experiments indicate that plasma has no ability to degrade I-131-insulin.

Results and discussion

Rat lens homogenates degrade I-131-insulin to products which are TCA soluble. At pH 7.2, the increase in the TCA-soluble radioactivity is linear over the 150 minutes tested (Fig. 2). The "blank," containing water instead of lens homogenate, illustrates the stability of the I-131-insulin at pH 7.2, since there is no significant change in the per cent of TCA-soluble radioactivity.

The pH optimum for proteolysis is approximately 7.2 (Fig. 3). A steady increase in proteolytic activity is also demonstrable with alkaline glycine buffers, but, because no attempt was made to raise the pH significantly higher than 10.7, it is unknown whether there is a true alkaline pH optimum.

The tubes were incubated in a water bath at 37 ± 1° C. The controls or "blank tubes" contained 0.5 ml. of water instead of lens homogenate. At the end of the incubation, 0.5 ml. of plasma (prepared from outdated human blood) was added to the incubation tubes. The solution was mixed and the proteins were immediately precipitated with 2 ml. of 20 per cent TCA. Thirty minutes later the tubes were centrifuged at 1,000 g for 15 to 20 minutes. One milliliter aliquots of the supernatants were carefully pipetted off without disturbing the protein precipitate. To determine the total radioactivity in the incubation mixture, aliquots of a solution containing 0.5 ml. of the radioactive insulin diluted to 25 ml. in the presence of about 0.5 ml. of plasma were counted. The aliquots were counted in a well-type scintillation counter. All experiments were done in duplicate; appropriate corrections for dilution and decay were made.

Although the control tube contained no lens homogenate, 3 to 5 per cent of the total radioactivity was found in the TCA-soluble fraction.
optimum. Lens homogenates have almost no ability to degrade I-131-insulin at pH 3.5 to 4.0. Generally, intracellular proteinases, cathepsins, exhibit maximal activity at this pH. A report on the insulin-destroying enzyme(s) in pure cultures of Walker carcinoma 256 cells notes that there were two pH optima, one near pH 7 and another near 4. Unpublished observations by the author (M. G.) clearly indicate that liver homogenates contain a neutral and acidic activity peak.

A temperature-activity study has been carried out at both pH 7.2 and 10.6 (Fig. 4). At both pH's, the rate of proteolysis first increases with increasing temperature and then decreases. The decrease in rate at higher temperatures is associated with thermal inactivation of the enzyme by denaturation. The presence of a temperature optimum, about 50° C. for the pH 10.6 activity, mitigates against the idea that this increased breakdown is due to alkaline hydrolysis of the radioactive insulin. However, unlike the reaction at pH 7.2, the blank values at pH 10.6 increase steadily with increasing temperature. Furthermore, it remains to be established whether the pH 7.2 enzyme(s) is the same enzyme(s) as that operative at pH 10.6.

Fig. 5 (Curve A) illustrates the breakdown rate of a reaction mixture at pH 7.2 containing 0.1 µg of I-131-insulin. The addition of 3 µg of crystalline zinc insulin (Curve B) reduces the per cent breakdown of I-131-insulin to one third the original value. The breakdown rate is linear until about 40 per cent of the insulin is degraded, at which point a decreased slope is noted. This decrease in velocity may be related to the fact that the insulin concentration is now limiting. The fact that crystalline insulin lowers the reaction rate indicates that crystalline insulin competes with the radioactive zinc insulin for the pH 7.2 enzyme site(s).

The reaction rates for the enzyme(s) operative at pH 10.6 (Curves C and D) are very different from those obtained at pH 7.2. The presence of added insulin

---

**Fig. 3.** Per cent increase of radioactivity in the TCA-soluble fraction as a function of pH. Glycine buffers (squares), pH 2.7 to 3.3 and pH 8.8 to 10.7; acetate buffers (triangles), pH 3.7 to 5.7; tris buffers (circles), pH 6.9 to 9.2. The dashed portion of the figure is an extrapolation of the acetate buffer curve. The reaction mixture (0.5 ml. of buffer; 0.5 ml. of lens homogenate, 1.3 µg, 63,000 counts per minute; and 0.3 ml. of lens homogenate, 5 µg.) was incubated for 40 minutes at 37 ± 1° C.

---

**Fig. 4.** Per cent increase of radioactivity in the TCA-soluble fraction as a function of temperature. The pH 7.2 reaction mixture (1 ml. of buffer; 0.5 ml. of lens homogenate, 7.6 mg.; and 0.5 ml. of insulin, 1.4 µg, 100,000 counts per minute) was incubated for 70 minutes at 37 ± 1° C.
The pH 10.7 reaction mixture (1 ml. of buffer; 0.5 ml. of lens homogenate, 3.5 mg.; and 0.5 ml. of insulin, 2.2 µg, 31,000 counts per minute) was incubated for 125 minutes at 37 ± 1° C.
Fig. 5. Per cent increase of radioactivity in the TCA-soluble fraction as a function of time in minutes. Curves A and B relate to experiments at pH 7.2, and curves C and D to experiments at pH 10.7. One milliliter of the reaction mixtures contained 0.5 ml. of buffer, 0.25 ml. of lens homogenate (2.5 mg.), and 0.25 ml. of insulin-I-131 (0.1 μg). In addition, 1 ml. of the reaction mixtures for Curves B and D received 3 μg of crystalline zinc insulin. (Curve D) causes no significant change in the reaction kinetics. In both cases the reaction rate is linear for about 40 minutes and then there is no further increase. Further experiments at pH 10.6, in which varying amounts of crystalline zinc insulin (up to 20 μg) are added, demonstrate that the breakdown of I-131-insulin proceeds in dependently of the added insulin.

Almost all of the enzyme activity at pH 7.2 and pH 10.6 is associated with the soluble proteins of the lens. The whole lens homogenate has slightly more proteolytic activity than the soluble fraction of the lens homogenate (prepared by centrifugation at 1,200 g for 20 minutes at 4° C.).

When the enzyme concentration and other conditions are kept constant, the reaction rate depends on the substrate concentration. In many experiments the maximum rate is 0.4 μg of total insulin degraded per hour. The apparent K_m (half saturation concentration) is approximately 8.3 x 10^{-4} M. These results are in complete accord with the hypothesis that the lens enzyme (pH 7.2) cannot distinguish between crystalline zinc insulin and I-131-insulin. A Lineweaver-Burk type plot of the data is illustrated in Fig. 6.

The lens enzyme activity varies linearly with increasing concentration of lens homogenate (Fig. 7). Curve A illustrates the increase of I-131-radioactivity in the TCA-soluble fraction when only 0.2 μg of I-131-insulin is present. The enzyme activity in the lens homogenates to which 6 μg of crystalline zinc insulin are added is shown in Curve B. Curve A reveals that under the conditions used the proteolytic activity of 1 mg. of lens can be determined. By altering the volumes used, the I-131-insulin concentration, and the time, one can probably determine the proteolytic activity of 0.1 mg. of lens or less.

To demonstrate further the versatility and sensitivity of this system, the ability of various concentrations of crystalline chymotrypsin to degrade I-131-insulin was studied (Fig. 8). The rate of breakdown varies linearly with increasing concentra-

---

Fig. 6. Lineweaver-Burk plot of the micrograms of insulin degraded as a function of micrograms of total insulin. The reaction mixtures (1 ml. of pH 7.2 tris buffer; 0.5 ml. of lens homogenate; 5 mg.; 0.5 ml. of insulin-I-131 0.11 μg; and 0.2 ml. of varying concentrations of crystalline zinc insulin) were incubated for 60 minutes at 37 ± 1° C. To calculate the micrograms of insulin degraded, the micrograms of total added insulin were multiplied by the per cent increase of radioactivity in the TCA-soluble fraction.
Catabolism of I-131-insulin by rat lens

Fig. 7. Per cent increase of radioactivity in the TCA-soluble fraction as a function of milligrams of lens homogenate. The reaction mixtures (1 ml. of pH 7.2 buffer; 0.5 ml. of lens, 0.5 to 13.5 mg.; and 0.5 ml. of I-131-insulin containing 0.3 μg) were incubated for 60 minutes at 37 ± 1° C. The reaction mixtures for Curve B are the same except that 6 μg of crystalline zinc insulin were added.

Fig. 8. Per cent increase of radioactivity in the TCA-soluble fraction as a function of micrograms of chymotrypsin. The reaction mixtures (1 ml. of pH 7.2 buffer; 0.5 ml. of chymotrypsin solutions, 0.7 to 22.5 μg; and 0.5 ml. of I-131-insulin, 1.1 μg, 49,500 counts per minute) were incubated for 60 minutes at 37 ± 1° C.

The degradation of radioactive insulin by chymotrypsin was studied at various pH's (pH 4 to 10.6). A single optimum, pH 7 to 8, was found. There was no indication of a pH optima at alkaline pH. Sanger's has established that chymotrypsin can hydrolyze 7 peptide bonds of insulin, 6 of which involve a tyrosine group.

The effect of various compounds at 10⁻⁴ M on the proteolytic activity of lens homogenates is listed in Table I. The fact that N-ethyl maleimide and p-chloromercuribenzoate inhibit enzymatic activity implies that this enzyme requires an intact SH group for activity. Reduced glutathione has a small activating effect; oxidized glutathione inhibits slightly. The metal ions—zinc, cadmium, and mercury—greatly inhibit the reaction rate.

In this report the degradation of insulin-1-131 to TCA-soluble derivatives has been used as a criterion of proteolysis. The validity of this criterion is indicated by the fact that this method may be utilized to assay chymotrypsin. The term "insulinase" has not been used, since there is already much commentary in the literature relating this word to a rather specific enzyme found in mammalian livers and other tissues.9 10

A major advantage in the use of I-131-insulin to study proteolysis is that all of the work is performed with an undenatured protein hormone. Others11 have reported that I-131-labeled insulin may be used as a tracer for insulin metabolism if

### Table I. Per cent activity of lens homogenates in the presence of various compounds (at 10⁻⁴ M)

<table>
<thead>
<tr>
<th>Added compound</th>
<th>Per cent activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water control</td>
<td>100</td>
</tr>
<tr>
<td>Taurine</td>
<td>100</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>80</td>
</tr>
<tr>
<td>N-ethyl maleimide</td>
<td>45</td>
</tr>
<tr>
<td>p-chloromercuribenzoate</td>
<td>35</td>
</tr>
<tr>
<td>Cysteine</td>
<td>100</td>
</tr>
<tr>
<td>Reduced glutathione</td>
<td>110</td>
</tr>
<tr>
<td>Oxidized glutathione</td>
<td>90</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>95</td>
</tr>
<tr>
<td>Mg**</td>
<td>100</td>
</tr>
<tr>
<td>Mn**</td>
<td>90</td>
</tr>
<tr>
<td>Zn**</td>
<td>20</td>
</tr>
<tr>
<td>Cd**</td>
<td>15</td>
</tr>
<tr>
<td>Hg**</td>
<td>10</td>
</tr>
</tbody>
</table>
strict precautions are taken in the experimental procedures. Because the insulin molecule is small (about 6,000 molecular weight), the hydrolysis of even a small number of bonds may yield an increase in radioactivity in the TCA-soluble fraction. There are three possible mechanisms to account for the increase in TCA-soluble radioactivity: (1) deiodination of the insulin, (2) the enzymatic reduction of the disulfide bridges, and (3) hydrolysis of the peptide bonds. Preliminary chromatographic results indicate that the lens enzyme system is not a deiodinase, since no significant increase in free radioactive iodide has been noted. The nature of the other degradation products is currently under investigation.

REFERENCES