Insulin Interactions with Liver Plasma Membranes

INDEPENDENCE OF BINDING OF THE HORMONE AND ITS DEGRADATION

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PIERRE FREYCHET,* RONALD KAHN, JESSE ROTH, AND DAVID M. NEVILLE, JR.

From the Section on Diabetes and Intermediary Metabolism, Clinical Endocrinology Branch, National Institute of Arthritis and Metabolic Diseases, and the Section on Physical Chemistry, Laboratory of Neurochemistry, National Institute of Mental Health, National Institutes of Health, Bethesda, Maryland 20014

SUMMARY

Insulin interactions with purified plasma membranes of rat liver were studied with respect to insulin degradation and specific binding to receptors. $^{125}$I-insulin was rapidly degraded upon exposure to liver membranes. After only 5 min of incubation at 30° of $^{125}$I-insulin (0.3 nM) and liver membranes (1 to 2 mg of protein per ml), 40 to 60% of the labeled hormone was degraded as measured by its ability to specifically bind to a second aliquot of membranes. After 90 min of exposure, less than 10% of the $^{125}$I-insulin was intact when measured by its ability to bind specifically to membranes. Binding by anti-insulin antibody, precipitation by trichloroacetic acid, and adsorption by talc were less sensitive methods of measuring degradation. Degradation of $^{125}$I-insulin was dramatically reduced at 1°.

No significant deiodination was associated with the degradation process. Gel filtration patterns suggested that $^{125}$I-insulin degradation products are composed mainly of small peptide fragments that loosely adsorb to the gel and are eluted after the salt peak.

The independence of binding to receptors and degradation is strongly suggested by the following findings. (a) $^{125}$I-desalanine-desaparagine insulin, which has an affinity for receptors that is only 2% that of insulin, is degraded to the same extent as $^{125}$I-insulin. (b) There is no relationship between the bioactivity of an insulin analogue and its ability to prevent the degradation of $^{125}$I-insulin. (c) The apparent $K_m$ for insulin degradation is $1.7 \times 10^{-7}$ M, which is 40-fold more than that concentration of insulin that produces half-maximal inhibition of specific binding of $^{125}$I-insulin to receptors in the liver membrane. (d) Insulin that is recovered from membranes upon dissociation of the hormone-receptor complex is undisagregated.

Proinsulin is very slowly degraded by the liver membranes, but may act as a competitive inhibitor of insulin degradation. This is similar to the findings by others of the insulin-specific protease of muscle and liver.

These studies provide evidence that interaction of insulin with liver plasma membranes is a complex phenomenon that involves at least two processes, degradation and binding to receptors. These two processes are largely independent and unrelated under a variety of conditions. Degradation of insulin must be accounted for in making precise quantitative measurements of the interaction of insulin with its receptors in the plasma membrane.

The liver is the major organ that removes insulin from the circulation (1, 2). It is also clear that insulin directly alters various metabolic pathways in the liver (3-6), while at the same time it is being inactivated by the liver. This degradative phenomenon has been attributed to proteolysis (7, 8) or reductive cleavage (9, 10). Until now it has been impossible to separate the interaction of insulin with its biologically active site and its degradation.

In previous studies (11), we described and characterized a site of specific binding of $^{125}$I-insulin to purified liver plasma membranes. The data presented strongly suggested that this site is of specific biological importance for several reasons. (a) The insulin concentration for binding and displacement was in the range of hepatic portal blood concentrations; (b) the binding was inhibited only by insulins that are biologically active; and (c) in all cases inhibition of $^{125}$I-insulin binding was proportional to the biological activity of the insulin preparation. We have furthermore shown that monoiiodoinsulin retains its full biological activity and that it is an appropriate tracer for studying the reactions between insulin and liver plasma membranes (12).

In the present work we have studied another aspect of the interaction of insulin with liver, the degradation or inactivation of hormone upon exposure to purified liver plasma membranes. The purpose of this paper is to describe some pertinent features of this degrading system and to establish that specific binding of the hormone to its biologically important receptors and degradative processes are largely independent phenomena. This is demonstrated by comparing the degradation of insulin analogues which have a wide variety in their affinity for the biologically important receptor. Furthermore, it will become apparent that the measured degree of inactivation of the insulin molecule

* Permanent address, Unite de Recherche de Diabetologie (I.N.S.E.R.M.), Hopital Saint-Antoine, Paris 12, France.
will vary with the sensitivity of the method used to estimate degradation, and by using multiple parameters, it is possible to distinguish both subtle and gross alterations in insulin.

**Experimental Procedure**

**Materials**

Porcine insulin (P. J. 5589) and proinsulin (615-984B-99-C) were gifts of Eli Lilly. Desalanine-desasparagine (JG-II-21-10) bovine insulin was kindly supplied by Dr. H. Carpenter. Carboxymethylated A and B chains were purchased from Mann Chemical Company. Synthetic oxytocin was a gift of Sandos, Dr. R. Bates (NIH B-16). Guinea pig anti-porcine insulin was kindly supplied by Dr. H. Carpenter. Carboxymethylated A and B chains were gifts of Eli Lilly. Desalanine-desasparagine (JG-11-21-10) bovine insulin was kindly supplied by Dr. H. Carpenter. Genuine Whatman cellulose powder and Whatman No. 3MM chromatography paper from W. & R. Bolstorm Ltd., Dowex AG 1 X 10 (200-400 mesh, chloride form) from Bio-Rad, and Sephadex (1-50) (fine) from Pharmacia. Glutathione, L-tryptophan, ATP, N-ethylmaleimide, and p-chloromercuribenzoic acid were purchased from Sigma Chemical Company. Other chemicals were of reagent grade.

**Methods**

**Iodination of Insulin and Analogues**

\(^{125}\text{I}-\text{insulin}, \ 125\text{I}-\text{desalanine-desasparagine-insulin (DAA-insulin)},^1 \text{ and } 125\text{I}-\text{proinsulin were prepared at specific activities of 100 to 200 } \mu\text{Ci per } \mu\text{g (600 to 1200 } \mu\text{Ci per mole). Iodinations were performed with the following modifications of the method previously described (12). (a) The proportion of hormone: iodine:chloramine T was 1:1:0.5 to 1, in molar equivalents; (b) the iodination volume was kept small (up to 50 } \mu\text{l); (c) 20 to 30 } \text{s after exposure of hormone and } 125\text{I to chloramine T, sodium metabisulphite was added in 3-fold excess, and bovine serum albumin was then added to give a final albumin concentration of 1.5%; (d) the iodination mixture was then immediately purified by chromatography on cellulose according to the procedure of Yalow and Berson (13); (e) after elution from the cellulose, the labeled hormone was diluted in Krebs-Ringer phosphate buffer (pH 7.5) that contained 1% bovine serum albumin and was then stored at \(-20^\circ\). These modifications were introduced in order to shorten the chromatographic purification of \(^{125}\text{I}rnonoiodoinsulin previously described (12) while retaining the stoichiometric conditions required to minimize the introduction of more than 1 atom of iodine per insulin molecule and the possible deleterious effects of the oxidizing agent. \(^{125}\text{I}-\text{insulin that had been prepared following this modified method was } 97\% \text{ precipitated by } 5\% \text{ trichloroacetic acid, } 95\% \text{ adsorbed to talc, and } 91 \text{ to } 95\% \text{ bindable to anti-insulin antibody. It exhibited the same ability to bind specifically to liver plasma membranes as the preparation previously described (12). Labeled hormones were stored in small aliquots at \(-20^\circ\) and thawed as needed. There was some gradual damage to hormones during storage with time, but the effect of this in each experiment was minimized by use of appropriate controls for each experiment as described later.**

1 The abbreviations used are: DAA-insulin, desalanine-desasparagine insulin.

**Liver Cell Membranes**

Plasma membranes were prepared from rat livers as previously described (15). The fully purified plasma membrane fraction (Step 15 of Reference 15) was used in all studies except where specifically stated. Protein concentrations were determined by the method of Lowry (16) using bovine serum albumin as the standard.

**Incubation Procedure**

Labeled hormone and liver membranes were mixed in the Krebs-Ringer phosphate buffer to give a final volume of 0.5 or 1 ml with final concentrations of 40 mM NaCl, 1.7 mM KCl, 0.4 mM MgSO\(_4\), 0.4 mM KH\(_2\)PO\(_4\), pH 7.5. The incubation mixture also contained 1% bovine serum albumin and concentrations of hormone and membranes as indicated in legends to the figures. The reaction was stopped by cooling the mixture and immediately centrifuging for 5 min at 10,000 \( \times g \) in a Sorvall centrifuge or Beckman-Spinco microfuge in a 4\(^\circ\) refrigerated room. The supernates were rapidly transferred to chilled tubes and kept at 4\(^\circ\). Analytical procedures were then immediately performed.

**Methods of Measuring Degradation and Inactivation of Hormone**

**Precipitation by 5% Trichloroacetic Acid—Aliquots (10 to 20 } \mu\text{l} \text{ of the supernates were transferred to 1 ml of chilled medium containing } 0.25\% \text{ human serum albumin in } 0.05 \text{ M Veronal buffer, pH 8.6. One milliliter of } 10\% \text{ trichloroacetic acid was immediately added, and the tubes were centrifuged for 5 min at } 2500 \text{ rpm. Radioactivity was then counted in each precipitate and supernate.**

**Adsorption to Talc—The procedure was as above except that one 50-ng talc tablet was added to the medium containing each supernate aliquot, vigorously mixed on a Vortex mixer, then centrifuged for 5 min at } 2500 \text{ rpm. Under these conditions, the radioactivity adsorbed to talc is a measure of the undamaged hormone (17).**

**Paper Chromatoelectrophoresis—Aliquots (50 to 100 } \mu\text{l} \text{ of the supernates were mixed with } 20 \mu\text{l} \text{ of plasma containing bromphenol blue and } 0.05 \text{ M KI and immediately applied to Whatman No. 3MM paper strips in a chromatoelectrophoresis apparatus. Undamaged hormone is estimated by its adsorption to the point of application, whereas damaged components migrate with serum proteins and free iodide migrates further toward the anode (18).**

**Binding to Anti-Insulin Antibody—Aliquots (10 to 20 } \mu\text{l} \text{ of each supernate were transferred to 0.5 ml of chilled medium that contained } 0.25\% \text{ human serum albumin, } 0.01\% \text{ rabbit Fraction II, } 5 \text{ mM EDTA, } 0.25\% \text{ guinea pig serum, and guinea pig anti-insulin serum in a final dilution of } 1:1000 \text{ in } 0.05 \text{ M Veronal buffer, pH 8.6. After } 4 \text{ hours at } 4^\circ, \text{ the antibody-bound hormone was precipitated by addition of a rabbit anti-guinea pig antiserum (14). After an additional } 15 \text{ hours at } 4^\circ, 0.5 \text{ ml of cold medium was added and the mixture was centrifuged for 20 min at } 2900 \text{ rpm in a } 4^\circ \text{ refrigerated centrifuge. The radioactivity of both the supernate and the precipitate was counted.**

**Specific Binding to Liver Membranes—The binding activity of the } ^{125}\text{I-insulin remaining at the end of the experiment was determined by mixing duplicate } 100-\mu\text{l aliquots of the supernate-**
tant containing the 125I-insulin with 100-μl aliquots of 1 mM KHCO₃ containing 200 to 300 μg of liver plasma membranes in the absence and presence of 10 μM unlabeled insulin. After 30 min at 30°C, membrane-bound radioactivity was determined by centrifuging duplicate 100-μl aliquots of each mixture through 250 μl of Krebs-Ringer phosphate that contained 1% bovine serum albumin as previously described (11). For each pair, the specific binding was calculated by subtracting the percentage of 125I radioactivity bound to the membrane pellet in the presence of 10 μM unlabeled insulin from the percentage bound in the absence of unlabeled insulin. This procedure is useful in eliminating the nonspecific adsorption or trapping since the specific binding sites of insulin are primarily occupied by unlabeled insulin. The presence of 10 μM unlabeled insulin, and the residual radioactivity bound by the membranes is accounted for by these nonspecific factors (11).

Expression of Results In each experiment, appropriate controls were prepared which were identical with respect to temperature, time, pH, and composition of the buffer, except that membranes were omitted. These controls represented 100% of the substrate available for degradation. For most procedures used to evaluate hormone degradation, results were calculated as percentage of control remaining as follows.

\[
\text{% Hormone remaining} = \frac{\text{% Intact hormone in experimental tube}}{\text{% Intact hormone in control tube}} \times 100
\]

In all experiments there was less than 5% degradation of the control hormone.

RESULTS

Time and Temperature Dependents of Degradation—After only 10 min of exposure to purified liver membranes at 30°C (Fig. 1), approximately 40% of the 125I-insulin was degraded as measured by binding to anti-insulin antibodies or binding to fresh liver membranes. This percentage increased to 80% by 90 min when measured by binding to liver membranes. The parameters of t alc adsorption and trichloroacetic acid precipitation also revealed degradation but were significantly less sensitive in all experiments. When the same experiment was performed at 1°C (Fig. 1) using the same membrane and insulin preparations and concentrations, the degradation of 125I-insulin was dramatically reduced to less than 15% after 90 min. This latter finding was in sharp contrast to the persistence of significant specific binding of insulin to liver membranes at this temperature (11).

Gel Filtration Studies—The nature of the degradation products was studied by gel filtration on a Sephadex G-50 (fine) column which was carefully calibrated with 131I-albumin, 131I-proinsulin, 125I-insulin, and [251I]NaI. After exposure of 131I-insulin to liver membranes for 90 min at 30°C, the majority of the radioactivity was recovered in a peak which elutes slightly after the 131I marker (Fig. 2, left). The fraction is probably composed of iodotyrosyl peptide fragments which absorb to the Sephadex, accounting for their delayed elution (19). The fact that this was not just deiodination and free 125I was confirmed by chro- matoelectrophoresis on Whatman No. 3MM paper and by lack of adsorption to Dowex 1 × 10 anion exchange resin at pH 1. The possibility that the degradation results in A and B chains is excluded by the elution volume of the products.

The estimate of intact insulin by gel filtration closely approximated that obtained by the antibody binding method (Table I).

![Fig. 1. Time course of degradation of 125I-insulin by purified liver membranes.](http://www.jbc.org/)
FIG. 2. Gel filtration patterns of 125I-insulin (left) and 125I-proinsulin (right) under control (top) and experimental (bottom) conditions. The percentage of total 125I radioactivity recovered is expressed for each eluted fraction (0.8 ml). Recoveries were 75 to 85% of the radioactivity applied. Controls consisted of 125I-insulin or 125I-proinsulin incubated in buffer (see "Methods") without membranes for 90 min at 30°. Experiments consisted of 1.8 x 10^-10 M 125I-insulin or 1.5 x 10^-10 M 125I-proinsulin exposed to 1.9 mg per ml of liver membranes. After 90 min at 30°, 0.1-ml aliquots of each supernatant (see "Methods") were mixed with 0.7 ml of medium (0.5 M Veronal, 0.25% human serum albumin, 0.01% rabbit Fraction II, pH 8.6) and applied to a Sephadex G-50 (fine) column (1 X 50 cm) that had been equilibrated and was eluted with the same medium at 4°. In both control and experimental conditions, 131I-albumin and [131I]NaI markers were applied simultaneously to the sample of 125I-insulin. In addition to 131I-albumin and [131I]NaI markers, the 125I-proinsulin sample also contained a 131I-insulin marker. The final volume of each sample applied was 0.9 ml. Vertical arrows denote, from left to right, the 131I-albumin peak (V), the proinsulin peak (PRO), the insulin peak (INS), and the salt peak 131I.

Table I

Comparison of methods used to measure intact insulin and proinsulin

Experimental conditions were the same as those described in the legend to Fig. 2. Aliquots of the supernatant obtained after 90 min incubation at 30° were analyzed for intact insulin, as described under "Methods."

| Substrate          | Percentage of initial substrate remaining after incubation with membranes estimated by the following methods |
|--------------------|-----------------------------------------------------------------------------------------------------------|
|                    | Talc                      | Chromatofocusing                        | Antibody binding | Gel filtration | Membrane binding |
| 125I-Insulin       | 48                        | 43                                     | 32              | 21            | 17              | 9                |
| 125I-Proinsulin    | 90                        | 92                                     | 86              | 80            | 78              | 120              |

The initial velocity of degradation of insulin as a function of substrate concentration is shown in Fig. 4. The amount of degradation in this experiment could be determined only by antibody binding and trichloroacetic acid precipitation, since at these high insulin concentrations there is saturation of the specific membrane receptors, making precise measurements of binding to fresh membranes impractical. When 1/α was plotted against 1/θ, a straight line was obtained (Fig. 5). From these data, the $K_m$ for insulin degradation is $1.7 \times 10^{-7}$ M. This is in close agreement with the $K_m$ obtained by Brush (21) with the insulin-specific protease of muscle. When the velocity of degradation of insulin was determined in the presence and absence of $10^{-6}$ M proinsulin and the results were plotted by the method of Lineweaver and Burk (22), two straight lines were obtained which intersected at the vertical axis, indicating that proinsulin behaves as a competitive inhibitor for the liver membrane insulin degrading system (Fig. 5). The $K_i$ for proinsulin was calculated to be $1.4 \times 10^{-7}$ M.
Inhibition of Degradation by Insulin Analogues—Our earlier studies (11) have shown that proinsulin and DAA-insulin, on a molar basis, have about 20% and 2%, respectively, of the ability of insulin to compete with ¹²⁵I-insulin for specific binding to liver membranes. In order to investigate the possible relationship between the binding of insulin to its specific receptors and the degradation process, we compared the inhibition of ¹²⁵I-insulin binding and degradation by insulin, proinsulin, and DAA-insulin. To study this question, the degradation of ¹²⁵I-insulin at $2.5 \times 10^{-10}$ M was measured in the absence and in the presence of unlabeled insulin, proinsulin, and DAA-insulin at concentrations of $2.5 \times 10^{-7}$ M. At this concentration insulin, proinsulin, and DAA-insulin inhibit the specific binding of ¹²⁵I-insulin to liver membranes by 100, 70, and 40%, respectively (11). As

Fig. 5. Lineweaver-Burk (22) plot of insulin degradation (as measured by 5% trichloroacetic acid precipitation). Conditions were as described in Fig. 4. Incubations were carried out with the indicated total concentrations of insulin in the presence and absence of 1.0 μM porcine proinsulin. Velocity is expressed as picomoles of insulin degraded over 10 min exposure to 0.5 mg of membrane protein at 30°. Note that proinsulin behaves as a competitive inhibitor of insulin degradation.

Fig. 6. Degradation of ¹²⁵I-insulin in the absence and in the presence of unlabeled insulin, unlabeled DAA-insulin, and unlabeled proinsulin. Insulin remaining intact with respect to precipitation by trichloroacetic acid (TCA), adsorption to talc, and binding to anti-insulin antibody is plotted (percentage of controls) as a function of time of exposure to 2 mg of membrane of protein per ml.

Fig. 7. Left, the degradation of ¹²⁵I-proinsulin at $7 \times 10^{-10}$ M and of ¹²⁵I-insulin at $4 \times 10^{-10}$ M are compared within the same experiment. Labeled hormone remaining intact (expressed as percentage of controls) is plotted against duration of its exposure to 1.6 mg of membrane protein per ml. In control tubes, specific (see "Methods") binding of ¹²⁵I-proinsulin to membranes was 0.009 pmole bound per mg of membrane protein; specific binding of ¹²⁵I-insulin was 0.01 pmole bound per mg. The molar ratio of bound proinsulin/bound insulin = 0.18 which is close to the value (0.20) reported in our earlier studies (11) with respect to the ability of proinsulin and insulin to inhibit the specific binding of ¹²⁵I-insulin to liver membranes. Note that ¹²⁵I-proinsulin is degraded to a much smaller extent than ¹²⁵I-insulin. Right, the degradation of ¹²⁵I-DAA-insulin at $3.6 \times 10^{-10}$ M (-----) and of ¹²⁵I-insulin (------) at $5 \times 10^{-10}$ M are compared within the same experiment. Data are expressed as in the left panel. Membrane protein concentration was 1.99 mg per ml. In control tubes, specific (see "Methods") binding of ¹²⁵I-DAA-insulin to membranes was 0.004 pmole bound per mg of membrane protein; specific binding of ¹²⁵I-insulin was 0.06 pmole per mg. Note that ¹²⁵I-DAA-insulin and ¹²⁵I-insulin were degraded to a similar extent with respect to each procedure employed.
shown in Fig. 6, unlabeled insulin and DAA-insulin were both equally effective in inhibiting the degradation of $^{125}$I-insulin. This was in striking contrast to their ability to inhibit the specific binding of $^{125}$I-insulin to liver membranes at this concentration (11). Unlabeled proinsulin which has an intermediate affinity for the liver receptor, was slightly less effective in inhibiting degradation than insulin and DAA-insulin.

Degradation of Proinsulin and DAA-Insulin—To further substantiate this difference between specific binding to insulin receptors and degradation by liver membranes, the degradation of $^{125}$I-proinsulin and $^{125}$I-DAA-insulin was studied directly. When $^{125}$I-proinsulin at $7 \times 10^{-10}$ M was exposed to liver membranes at 30°, very little degradation occurred (Fig. 7, left). Even after 90 min of exposure, 85% of the radioactivity was still precipitated by trichloroacetic acid and adsorbed to tale, 90% was bindable to anti-insulin antibody, and 80% was capable of binding to fresh liver membranes. The degradation of $^{125}$I-insulin was determined simultaneously with the same membrane preparation for comparison (Fig. 7, left).

The degradation of $^{125}$I-proinsulin was also studied by gel filtration (Fig. 2, right, and Table I). There was a good agreement between this method and other methods of measuring intact $^{125}$I-proinsulin. It is of particular interest that there was no conversion of proinsulin to insulin.

On the other hand, $^{125}$I-DAA-insulin was degraded to almost the same extent as $^{125}$I-insulin when exposed to the same membrane preparation in parallel experiments (Fig. 7, right). Thus, insulin and DAA-insulin, which differ by almost two orders of magnitude in their biological activity in fat cells and in their ability to compete with $^{125}$I-insulin for specific binding to insulin receptors in the liver membranes, are equally degraded. Proinsulin, on the other hand, which has an intermediate efficacy between insulin and DAA-insulin in competing with $^{125}$I-insulin for specific binding to liver membranes (11), is degraded to a much lesser extent than both insulin and DAA-insulin.

Membrane-bound Insulin—Thus far, all degradation studies were performed on the insulin in the supernatant of the incubation mixture. To investigate the possibility of degradation of insulin bound to the membrane, $^{125}$I-insulin which had been bound was eluted with acid and tested for degradation. As can be seen in Table II, even after 30 min exposure to membranes, there was no degradation of the $^{125}$I-insulin bound to and eluted from the membranes, and, in fact, there was actually a purification of the insulin toward the characteristics which it had on the day of iodination. This is in contrast to the insulin in the supernatant of the same experiment which was 75% degraded as measured by binding to fresh membrane (Table II).

Degradation in Other Liver Fractions and Fat Cells—We attempted to study the various fractions of liver and fat cells to

| TABLE II

| Properties of $^{125}$I-insulin eluted from liver membranes |
|----------------------------------------------------------|
|                                                        |
| Experimental conditions were as described in the legend  |
| to Fig. 1 using liver membranes at 1.2 mg per ml in a    |
| total volume of 6 ml at 30° with $10^{-2}$ M $^{125}$I-    |
| insulin. After 30 min the mixture was centrifuged at     |
| 12,500 X g in a Sorvall centrifuge at 4° for 5 min.      |
| The supernatant was removed and the membrane pellet     |
| was washed twice in 10 ml of buffer. The membranes were  |
| then resuspended in 0.1 M HCl containing 2% bovine serum  |
| albumin, allowed to incubate for 10 min, and then        |
| centrifuged at 12,500 X g at 4°. The supernatant        |
| contained 72% of the radioactivity that was initially     |
| bound to the membrane pellet. The supernatant           |
| was neutralized with 1 N NaOH then diluted in an equal    |
| volume of Krebs-Ringer phosphate with 1% bovine serum    |
| albumin and assayed by the analytical procedures described |
| under "Methods." The supernatant from the initial       |
| incubation was treated in the same manner for            |
| comparison. Two control values are given—                |
| one for the $^{125}$I-insulin on the day of its           |
| preparation and the other for the day of use in this      |
| experiment 3 weeks later.                                 |

| $^{125}$I Insulin | Not exposed to membranes | Eluted from membranes | Supernatant of initial incubation |
|-------------------|--------------------------|----------------------|----------------------------------|
|                   | Day 1                    | Day 21               |                                  |
| Precipitable by 5% trichloroacetic acid.               | 98                      | 80                    | 94                               |
| Adsorbed by tale.                                      | 94                      | 84                    | 94                               |
| Bound to anti-insulin antibody.                        | 91.5                    | 80                    | 90.1                             |
| Bound to fresh membranes.                              | 9.9                     | 12.9                  | 2.5                              |

* This indicates percentage bound under the conditions of this experiment only. Recently prepared $^{125}$I-insulin is 80% or more bindable by the membranes.

Experimental conditions for incubation of liver membranes were as described in the legend to Fig. 1. A volume of 600 μl containing 600 to 100 μg of membrane protein and 840 pg $^{125}$I-insulin was incubated for 30 min at 30°. A duplicate set of samples also containing 40 μg per ml of unlabeled insulin was prepared and incubated under identical conditions. The specific binding was determined in duplicate 100-μl aliquots, as described under "Methods." The remainder of the incubation mixture was centrifuged and the supernatant saved for determination of degradation.

The isolated fat cells were prepared by the method of Rodbell (23) and the homogenate and particulate fraction as described by Custraceas (34). The intact cells (10⁶ per ml) or equivalent concentrations of homogenate or 23,000 X g fraction were incubated in 1.2 ml containing 1,100 pg of $^{125}$I-insulin for 30 min at 30°. Duplicate sets were prepared with 40 μg per ml of unlabeled insulin. After incubation, aliquots of each were filtered on 0.8 μ Millipore filters and were washed with 3 ml of Krebs-Ringer phosphate with 1% bovine serum albumin. The remainder of the incubation mixture was filtered and the filtrate saved to test for degradation.

Degradation in both cases was determined by loss of binding to anti-insulin antibodies.

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Table III

**Insulin binding and degradation with purification of the plasma membranes**

Experimental conditions for incubation of liver membranes were as described in the legend to Fig. 1. A volume of 600 μl containing 600 to 100 μg of membrane protein and 840 pg of $^{125}$I-insulin was incubated for 30 min at 30°. A duplicate set of samples also containing 40 μg per ml of unlabeled insulin was prepared and incubated under identical conditions. The specific binding was determined in duplicate 100-μl aliquots, as described under "Methods." The remainder of the incubation mixture was centrifuged and the supernatant saved for determination of degradation.

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Degradation in both cases was determined by loss of binding to anti-insulin antibodies.

| Cellular fraction | Purification | $^{125}$I Insulin bound | $^{125}$I Insulin degraded |
|-------------------|--------------|-------------------------|---------------------------|
| Liver             | Step 2 (Reference 18) | 15                      | 1008                      |
| Homogenate...     | Step 3 (Reference 18) | 35                      | 812                       |
| 1500 X g spin.    | Step 11 (Reference 18)| 70                      | 798                       |
| Float.            | Step 15 (Reference 18)| 127                     | 728                       |
| Purified membrane | Step 15 (Reference 18)| 127                     | 728                       |
| Fat cells         | Reference 23  | 11                      | 125                       |
| Isolated cells... | Reference 24  | 2.4                     | 504                       |
| Homogenate...     | Reference 24  | 2.4                     | 504                       |
| 20,000 X g spin.  | Reference 31  | 9.5                     | 192                       |
findings, other investigators (33-35) have shown that insulin 
prepared from fat cells and rat liver under conditions where no 
bind to liver plasma membranes.

Degradation of glucagon by partially purified liver membranes 
brates and extracts (7, 8, 24-26), by liver slices (25), and by the 
membranes will obviously affect any quantitative calculations 
and insulin receptors. We also found insulin-degrading activity 
can be seen in Table III, there is an over-all decrease in the 
increases with purification of the insulin-specific receptor. As 
have previously described a site of specific binding of 
insulin in highly purified rat liver plasma membranes which has 
the characteristics of a biologically important receptor (11). 
In the studies presented here we have demonstrated insulin 
degradation by these purified plasma membranes and character-
ized its specificity in contrast to that of the site of specific 

Studies of insulin degradation are complex. Not only is 
degradation a function of time, temperature, and membrane 
concentration, it is also a function of the criteria used to evaluate 
the degradation. In this respect, the ability of insulin to bind 
specifically to fresh membrane receptors appears to be the most 
sensitive measure of structural integrity since this correlates 
well with bioactivity. Binding by anti-insulin antibody provides 
useful information on the general integrity of the hormone 
molecule, but, as shown by our data in the present and other 
studies (11), antibody binding often poorly discriminates changes 
that affect bioactivity of specific binding to receptors. Gel 
filtration, chromatoelectrophoresis, precipitation by trichloro-
aetic acid, and adsorption to talc consistently underestimate the 
actual extent of degradation of inactivation of the hormone 
such as suggested by Tomizawa (9).

Degradation of insulin upon exposure to the purified liver 
membranes and specific binding of the hormone to its receptors 
in the same membranes appear to be largely independent 
phenomena. This is strongly suggested by several findings.

1. 125I-DAA insulin whose affinity for receptors is only 2% 
that of insulin (11) is degraded to the same extent as 125I-insulin. 
Conversely, 125I-proinsulin whose affinity for insulin receptors is 
30% that of insulin on a molar basis (11) is degraded to a much 
smaller extent by the membranes than either insulin or DAA-
insulin.

2. There is no relationship between the bioactivity of insulin 
analogue and its ability to prevent the degradation of 125I-insulin. 
Thus, DAA-insulin is as effective as insulin in inhibiting 125I-
insulin degradation but has only 2% of the binding affinity and 
bioactivity of insulin (Fig. 6).

3. The apparent $K_m$ (half-maximal concentration) of insulin 
for the degradative process ($1.7 \times 10^{-4} M$) is about 50 times 
higher than the concentration of insulin that produces 50% 
inhibition of the 125I-insulin specific binding to the membranes 
(11). This difference might be even higher if the latter value 
were corrected by accounting for insulin degradation during 
exposure to membranes. 

Inhibitors of Degradation—The degradation of insulin by liver 
membrane will obviously affect any quantitative calculations 
link sites and affinity constants. For subsequent studies, it 
was therefore important to attempt to find a suitable inhibitor of 
degradation. $N$-ethylmaleimide and $p$-chloromercuribenzoic 
acid appear to markedly inhibit insulin degradation, while 
Trasylol, zinc, and high sodium chloride concentrations are 
markedly less effective (Table IV). Concentrations of albumin 
from 0.1 to 4% have no effect on degradation. Further studies 
are being conducted to determine the effects of these inhibitors 
on binding of insulin to liver plasma membranes.

**DISCUSSION**

The degradation or inactivation of insulin by liver homoge-
nates and extracts (7, 8, 24-26), by liver slices (27), and by the 
isolated perfused liver (28-31) have been well documented. 
Degradation of glucagon by partially purified liver membranes 
(32) has also been recently reported. In contrast to these 
findings, other investigators (33-35) have shown that insulin 
binding may occur to intact fat cells and to particulate fractions 
prepared from fat cells and rat liver under conditions where no 

degradation can be demonstrated. These latter studies, how-
ever, were performed at 24° and with low concentrations of 
receptor preparation, both conditions which minimize degrada-
tion.

*Contamination with insulin 1/1000 moles accounts for small 
decrease in activity seen.

1. Konttinen, P. Freychet, J. Roth, and D. M. Neville, Jr., manu-
script in preparation.

P. Freychet and B. Perlman, unpublished observations.

A. H. Rubenstein, personal communication.
4. Finally, serial purification of the plasma membrane and specific insulin binding site is associated with a decrease, rather than increase, in insulin degrading activity. There is, therefore, substantial evidence that specific binding to receptors and degradation are separate phenomena whose occurrences appear to be unrelated under a variety of conditions.

The labeled hormone that is actually bound to the membranes appears to be protected from degradation (Table II). Similar findings have been reported with glucagon (32) and insulin (33-35). This does not exclude the possibility that insulin which has bound to the receptor and activated cellular processes is inactivated and rapidly released into the medium. Further studies are required to investigate this hypothesis.

It is of interest that no significant conversion of proinsulin to insulin occurs during exposure of proinsulin to the liver membranes. No conversion of proinsulin to insulin has also been observed in vivo (38) or with the isolated perfused rat liver or with rat hemidiaphragm (39). Although gel filtration data do not allow us to exclude the conversion of intact single chain proinsulin to two chain intermediate forms between proinsulin and insulin, this possibility appears unlikely. The absence of significant conversion of proinsulin to insulin complements other observations which strongly suggest that proinsulin has intrinsic biological activity (38-40) that may be entirely accounted for by its lower affinity for receptors (11).

The insulin degradation process is not restricted to liver. Degradation of insulin has also been observed with kidney (26, 41), muscle (21), adipose tissue (42), isolated fat cells (42, 43) and both homogenates and particulate fractions of fat cells (36, 44). Our data on fat cells and their fractions (Table III) confirm these findings. Serial fractions obtained during the purification procedure of the liver plasma membranes show a progressive decrease in degrading activity with a progressive increase in specific binding.

Another interesting aspect of the work presented here is the remarkable similarity, if not identity, between the insulin degrading system of the purified liver membranes and the partially purified soluble enzymes from rat muscle (21) and liver (25). Both systems show a relative specificity for insulin when compared to proinsulin, whereas the latter behaves as a competitive inhibitor of insulin degradation. The $K_m$ for insulin is remarkably close and both systems show inhibition by sulphydryl inhibitors and stimulation by reduced glutathione. Other polypeptide hormones do not significantly affect the degrading activity. A discrepancy between the degradation of insulin and proinsulin has been observed with isolated perfused rat liver (61, kidney (41), and rat adipose tissue (21, 42).

The possible physiological significance of these findings deserves comment. Significant insulin degradation was observed at concentrations close to those in hepatic portal blood (45). The possibility that an "insulin-specific enzyme" is located in the plasma membrane is suggested by the similarity between the liver perfusion studies and the studies presented here. Hemmington and Dunn (46) have separated a discrete peak of insulin immunoreactivity from liver homogenates which appears to be bound to a 300,000 molecular weight fraction. This fraction elutes coincident with the insulinase activity. The liver plasma membranes which we use have been shown to be rich in protease activity (47). These findings all suggest that the insulin degradation by the liver plays a major role in allowing more of the secreted proinsulin to reach the peripheral circulation, thus accounting for the longer half-life of proinsulin (48) and the reduced anteriovenous difference (49) when compared to insulin.

In summary, the interaction of insulin with purified plasma membranes of rat liver involves at least two largely independent processes: specific binding to receptors and degradation. Insulin is not degraded when bound to the membranes. In contrast to insulin and DAA-insulin which, despite the wide difference in their affinity for receptors, are degraded to the same extent, proinsulin is much more slowly degraded and is not converted to insulin. Moreover, proinsulin is a competitive inhibitor of insulin degradation.

Further studies are in progress to quantitate the specific interaction of insulin with its receptors by accounting for the insulin degradation process.

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