

The Interactions of Proinsulin with Insulin Receptors on the Plasma Membrane of the Liver

PIERRE FREYCHET

From the Unité de Recherche de Diabétologie et d'Etudes Radio-Immunologiques des Hormones Protéiques, Institut National de la Santé et de la Recherche Médicale, Hôpital Saint-Antoine, 75012-Paris, France

ABSTRACT The interactions of proinsulin with the insulin-specific receptors were investigated in purified rat liver plasma membranes. These studies were designed to characterize the binding of proinsulin to the insulin receptors, to search for proinsulin-specific receptor sites, and to examine the possibility of proinsulin conversion at the insulin receptor site. Proinsulin was only 3-5% as potent as insulin in binding to insulin receptors. Proinsulin reacted with all of the insulin-specific receptors, and direct binding studies of [125 I]-porcine proinsulin and [125 I]-rat proinsulin did not reveal proinsulin-specific receptor sites other than the insulin receptors in rat liver membranes.

Quantitative data derived from steady-state and transient-state comparative binding studies of both [125 I]-proinsulin and [125 I]-insulin indicated that a 20-fold lower association rate constant essentially accounts for the reduced affinity of proinsulin for the insulin receptors. The possibility of proinsulin conversion at the insulin receptor sites was investigated. Material recovered from the membranes upon dissociation of the proinsulin-receptor complex was intact proinsulin and did not exhibit any conversion by a variety of analytical methods.

These results indicate that the lower affinity of proinsulin for the insulin receptor in the liver is an intrinsic property of the proinsulin molecule. The lower uptake of proinsulin by the insulin receptor represents, in addition to a slower degradation of the prohormone, a further mechanism by which proinsulin exerts prolonged, albeit reduced, action *in vivo*.

INTRODUCTION

Since the demonstration of proinsulin as the precursor of insulin (1) and the elucidation of its amino acid sequence (2) investigators have performed studies both

in vivo and *in vitro* to decide whether proinsulin possessed intrinsic biological activity or whether it was converted to insulin or some intermediate which was, in fact, the active species. These studies have been recently reviewed (3, 4). In the circulation, there appears to be negligible conversion of proinsulin to insulin (5-9), and proinsulin degradation in the liver appears to be independent of any conversion to insulin (10, 11). However, such processes operating at the plasma membrane of target cells were specifically not excluded by these studies.

Recently, polypeptide hormone-receptor interactions have been studied extensively (for a comprehensive review see reference 12). In one of these studies (13), we observed that proinsulin competed with [125 I]-insulin for binding to insulin receptors and that the potency of proinsulin in competing with insulin was in direct proportion to its biological potency *in vitro*. This strongly suggested that proinsulin indeed had intrinsic, albeit reduced, biological activity, but possible conversion on the receptor was not examined. It was also found that insulin binding to the receptor sites in liver plasma membranes was independent of the hormone degradation, though both processes were present in the same subcellular fraction (14). Proinsulin was found to bind well to the insulin degrading enzyme(s) but was itself a much poorer substrate; i.e., it behaved as an inhibitor of insulin degradation. No significant conversion of proinsulin to insulin occurred during exposure to the liver membranes (14).

In the present study, we have investigated directly the binding of [125 I]-proinsulin to the liver plasma membrane and characterized further the interactions of proinsulin with the insulin receptors in the liver. The possibility of proinsulin conversion at the insulin receptor site has been examined. We have also searched for proinsulin-specific receptor sites; i.e., binding sites that have an affinity for proinsulin that is equal to or exceeds their affinity for insulin.

Received for publication 8 June 1973 and in revised form 25 July 1974.

METHODS

Materials. Purified porcine proinsulin (lot 615-1112 B-84-C and 615-1082 B-108-C), desdipeptide proinsulin (lot 615-1112 B-32), desnonapeptide proinsulin (lot 615-1112 B-31), diarginine insulin (lot 615-1070 B-16), and mono-arginine insulin (lot 615-1039 B-180-4) were generously supplied by Dr. R. E. Chance (The Lilly Research Laboratories, Indianapolis, Ind.), who has described the preparation and properties of these porcine proinsulin-like and insulin-like intermediates (3, 15). Rat proinsulin was a generous gift of Dr. D. F. Steiner. Purified porcine "mono-component" (16) insulin (lot MCS 970, 27.2 IU/mg) was generously supplied by Dr. J. Schlichtkrull (The Novo Research Institute, Copenhagen, Denmark). The proinsulins and porcine insulin were used for iodination as well as unlabeled standards in binding studies to the membrane receptors.

Carrier free Na^{125}I (I-S₄) was purchased from Commissariat à l'Energie Atomique (Saclay, France). A guinea pig anti-insulin serum (17) and talc tablets (Silicosorbe, 50 mg, Dreyfus Herschel, Paris) were used in experiments measuring degradation (14) of the [^{125}I]proinsulin and [^{125}I]insulin. Sephadex G-50(fine) was purchased from Pharmacia Fine Chemicals, Inc. (Le Chesnay, France), DEAE-cellulose (microgranular DE 52), Whatman, from Reeve Angel Co. (Rungis, France), and bovine albumin (fraction V) from Pentex Biochemical (Kankakee, Ill.). Other chemicals were of reagent grade.

Iodination of proinsulin and insulin. Proinsulin and insulin were iodinated directly with 0.7–0.8 I atoms/molecule by the use of chloramine T under conditions that yield largely monoiodo-hormone that is biologically fully active (18). Before use in these experiments the iodinated products were chromatographed on DEAE-cellulose, as described previously (18), except that urea was omitted because of the small quantity (microgram amounts) of polypeptide applied to the ion exchanger. The chromatographic procedure removed free iodide as well as damaged and diiodinated peptides, and excluded possible contamination of labeled proinsulin with any labeled insulin. This method also separates noniodinated hormone from I-hormone, thereby enriching slightly the specific activity of the hormone (18). Specific activities of 200–250 $\mu\text{Ci}/\mu\text{g}$ and 300–380 $\mu\text{Ci}/\mu\text{g}$ were obtained with [^{125}I]proinsulin and [^{125}I]insulin, corresponding to approximately 1,800–2,300 Ci¹/mmol² or about 0.8–1.0 iodine atom/mol of polypeptide.

[^{125}I]Proinsulin and [^{125}I]insulin displayed clearly different elution patterns on DEAE-cellulose chromatography (Fig. 1). The first narrow peaks represent damaged component(s). [^{125}I]Proinsulin and [^{125}I]insulin elute as major broad peaks, the [^{125}I]proinsulin being eluted before the [^{125}I]insulin. The main peaks of [^{125}I]proinsulin and [^{125}I]insulin radioactivity, which are about 200 ml apart in the effluent volume from the DEAE-cellulose column (Fig. 1), were selected for these studies. The [^{125}I]proinsulin and [^{125}I]insulin were free of any insulin and proinsulin contaminant, respectively, as demonstrated by their chromatographic pattern on polyacrylamide gel electrophoresis (Fig. 10). The main peak of [^{125}I]insulin eluted at the same ionic strength as did biologically active monoiodoinsulin in earlier studies (18). Increasing amounts of [^{125}I]insulin exhibited the same kinetics of binding as that observed with a fixed

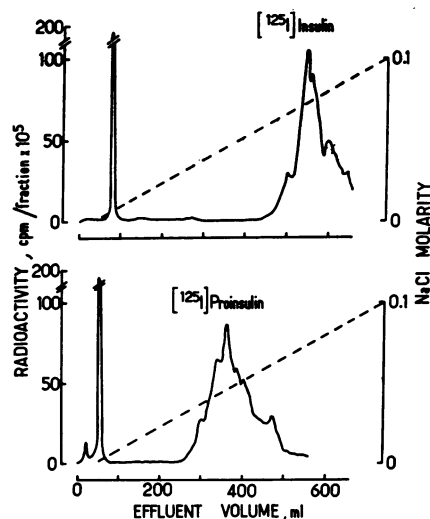


FIGURE 1 Elution profiles for [^{125}I]insulin (top) and [^{125}I]proinsulin (bottom) on DEAE-cellulose chromatography. Iodination mixtures (5 μg of polypeptide with an average of 0.7–0.8 I atom/mol) were applied to a $0.9 \times 20\text{-cm}$ column of DEAE-cellulose that had been equilibrated in 50 mM Tris-HCl, pH 9.4, at 4°C (starting buffer). After 100 ml of starting buffer had passed over the column, a gradient to 0.1 M NaCl at constant pH was applied. The first narrow peaks represent damaged components. The main peaks of [^{125}I]insulin and [^{125}I]proinsulin, which are about 200 ml apart in the effluent volume, were selected for the present studies.

dose of [^{125}I]insulin diluted in increasing amounts of unlabeled insulin (data not shown), thus indicating that the [^{125}I]insulin possesses the same affinity as the unlabeled insulin for binding to the receptor sites. Since the ratio of binding affinity between the [^{125}I]proinsulin and [^{125}I]insulin was close to the ratio of biological potency between the unlabeled proinsulin and insulin (see Results), we assume that the [^{125}I]proinsulin retains the affinity of unlabeled proinsulin for binding to the receptor sites.

Liver plasma membranes. Plasma membranes were prepared from rat livers according to Neville (19). The fully purified plasma membrane fraction (step 15 of reference 19) was used in all studies. Protein concentrations were determined by the method of Lowry, Rosebrough, Farr, and Randall (20) using bovine serum albumin as the standard.

Binding studies. Studies of binding of [^{125}I]proinsulin and [^{125}I]insulin to membranes were conducted at 30°C for the time indicated in the legends in Krebs-Ringer phosphate buffer, pH 7.5, in a final volume of 0.5 ml/incubation tube that contained [^{125}I]polypeptide at 0.15–0.25 nM, unlabeled polypeptide at various concentrations from 0–20 μM , membrane protein at 0.2–0.4 mg/ml incubation medium, and bovine albumin at 15 mg/ml. At the times indicated, duplicate samples from each incubation tube were transferred to microfuge tubes and the membrane-bound [^{125}I]polypeptide was isolated by rapid centrifugation as described previously (13). In studies of the time-course of association and dissociation of the [^{125}I]proinsulin and [^{125}I]insulin, the incubation mixture was distributed into 100–200- μl samples (in triplicate for each incubation time) that were filtered

¹ Specific activity of carrier-free $^{125}\text{I} \approx 18 \text{ mCi}/\mu\text{g}$.

² Molecular weight assigned to proinsulin and insulin: 9,000 and 6,000, respectively.

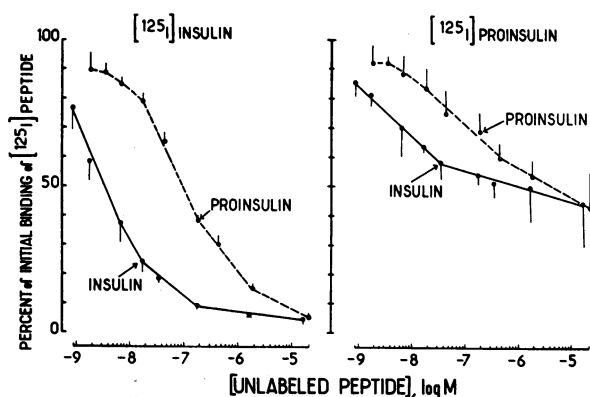


FIGURE 2 Inhibiting effect of unlabeled insulin and proinsulin on the binding of [125 I]insulin (left) and [125 I]proinsulin (right) to liver plasma membranes. Binding is expressed as percent of initial binding of [125 I]polypeptide, i.e., the binding of [125 I]polypeptide in the absence of unlabeled polypeptide. Initial binding was 20–25% of the total [125 I]insulin and 2–3% of the total [125 I]proinsulin. Both [125 I]polypeptides were at 0.15–0.20 nM and were incubated with membranes at 0.3 mg protein/ml incubation medium for 60 min at 30°C. Each point is the mean \pm one-half of the range for three separate experiments; in each of the experiments determinations were done in duplicate.

on cellulose acetate EGWP (0.2 μ m) Millipore filters as described in the legend to Fig. 7. Although both the centrifugation and filtration gave identical results, the latter procedure was preferred in time-course experiments since it was more convenient for the rapid assay of large numbers of samples.

Other analytical studies. Methods to measure the degradation and inactivation of insulin and proinsulin have been described in detail elsewhere (14). Briefly, precipitation in 5% trichloroacetic acid, adsorption to talc, binding to anti-insulin antibody, and ability to rebinding to specific receptors in liver membranes were used to measure the degree of alteration of the [125 I]proinsulin and [125 I]insulin, whether recovered free in the medium or recovered from the membrane upon dissociation of the hormone-receptor complex. These methods are not equally sensitive in measuring degradation; although each method reflects, to a certain extent, the integrity of the polypeptide, the ability to bind to fresh membranes is the most sensitive method to measure alteration of the hormone, as shown in previous studies (14).

Biological activity was assayed by measuring the insulin-stimulated conversion of [U - 14 C]glucose to 14 CO $_2$ by isolated fat cells (21), as described previously (18), except that the fat cell concentration was reduced to 3–5 mg/ml of incubation medium.

The polyacrylamide gel electrophoresis, performed with 20% gels (2), is described in the legend to Fig. 10.

RESULTS

Steady-state studies

Comparison of proinsulin and insulin in inhibiting the [125 I]insulin binding. When unlabeled proinsulin was compared to unlabeled insulin in its ability to com-

pete with [125 I]insulin for binding to the specific receptors on the membrane under steady-state conditions, the biosynthetic precursor had only 3.5–4% of the affinity³ of insulin for the insulin receptors (Figs. 2 and 3). This reduced affinity corresponded closely to the lower potency of proinsulin⁴ in stimulating glucose oxidation by isolated fat cells in vitro (Table I).

Since high concentrations of proinsulin, as well as insulin, displaced 95% of the [125 I]insulin (Figs. 2 and 3, left), we concluded that proinsulin interacts with all of the insulin-specific receptor sites. This is in contrast, for example, to the glucagon receptor sites in liver and fat where the gut glucagon-like material reacts with some but not all of the sites for pancreatic glucagon (22, 23).

[125 I]Proinsulin binding to receptors. To investigate the possibility that proinsulin might also interact with specific sites distinct from the insulin receptors, we studied directly the binding of [125 I]proinsulin to the membranes. Under steady-state conditions of binding (Figs. 2 and 3, right) as well as in the time-course experiments (Fig. 7), the binding of [125 I]proinsulin was much less than the binding of [125 I]insulin. Within the same experiments and under identical quantitative conditions, 2–3% of the total [125 I]proinsulin was bound in contrast to binding of 20–25% of the total [125 I]insulin. The “nonspecific” binding, defined as 125 I radioactivity bound to the membranes in the presence of a great excess (15–20 μ M) of unlabeled hormone, was the same in absolute counts for both of the labeled hormones (Fig. 3) but represented about 40% of the total binding of the [125 I]proinsulin (Fig. 2, right), whereas it accounted for only 5% of the total binding of [125 I]insulin (Fig. 2, left). As a result, the specific binding of [125 I]proinsulin was only 5–7% that of [125 I]insulin. This reduced degree of direct binding of [125 I]proinsulin agrees well with the reduced affinity of unlabeled proinsulin in competing with [125 I]insulin for the insulin receptor sites (Figs. 2 and 3, left) and does not favor the presence of proinsulin-specific receptor sites on the liver plasma membrane. Accordingly, unlabeled proinsulin was only 5–6% as potent as unlabeled insulin in competing with the binding of [125 I]proinsulin and very large amounts of proinsulin failed to displace the [125 I]-

³ The relative affinity of proinsulin for the insulin receptors was determined from data in Fig. 2 (left) and calculated as $100 \times (\text{molar concentration of insulin that inhibits 50\% of } [^{125}\text{I}] \text{ insulin binding}) / (\text{molar concentration of proinsulin that inhibits 50\% of } [^{125}\text{I}] \text{ insulin binding})$.

⁴ In earlier studies (13) we had observed that a proinsulin preparation was 20% as potent as insulin in binding to the insulin receptors and in stimulating glucose oxidation by isolated fat cells. Since all subsequent proinsulin preparations that we have studied have 2–5% of the potency of insulin, we presume that the first preparation consisted of partially converted intermediate(s) (see Table I).

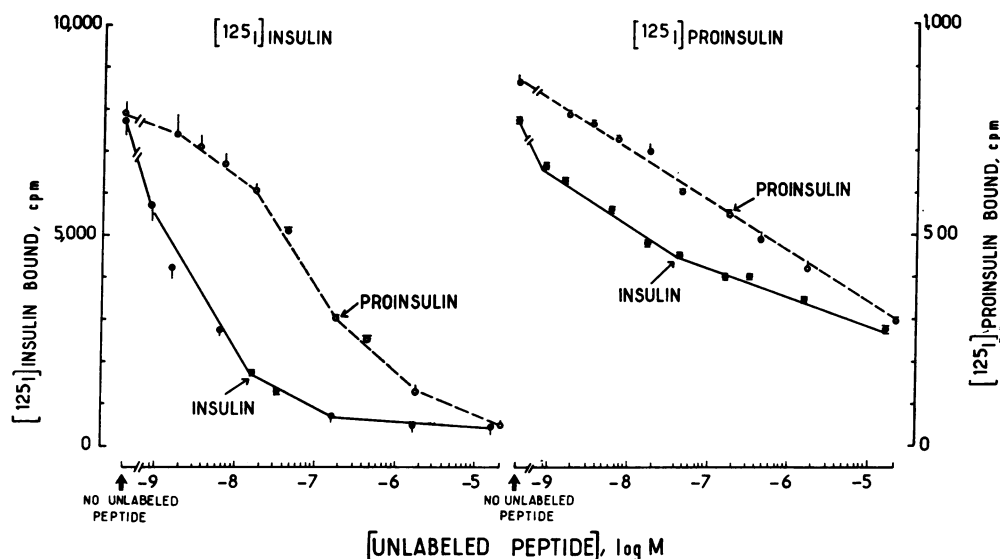


FIGURE 3 Binding and displacement of [125 I]insulin and [125 I]proinsulin by unlabeled insulin and proinsulin. Data are expressed as absolute numbers of counts of [125 I]insulin and [125 I]proinsulin bound under identical experimental conditions (see legend to Fig. 2). Each point is the mean \pm one-half of the range for two separate experiments; in each of the experiments determinations were done in duplicate. Note that vertical scales are different.

proinsulin to a significantly greater extent than that achieved with high concentrations of insulin (Figs. 2 and 3, right).

Because the amino acid sequences of rat and porcine connecting peptides (C-peptides) differ markedly (24, 25), we studied the binding of [125 I]rat proinsulin to

TABLE I
Relative Potencies* of Proinsulin and Proinsulin Intermediates in Binding to Liver Membranes and in Stimulating Glucose Oxidation by Fat Cells

	Liver membranes‡	Fat cells§
Proinsulin-like		
Proinsulin	3.3 \pm 0.7	2.1
Split proinsulin	3.7 \pm 0.3	2.8
Desdipeptide proinsulin	14.0 \pm 1.3	19
Desnonapeptide proinsulin	14.3 \pm 0.7	20
Insulin-like		
Diarginine insulin	88.9 \pm 9.8	73¶
Monoarginine insulin	79.1 \pm 3.8	70

* The potency of each preparation was compared to the potency of insulin in each system. The numbers in the table = $100 \times (\text{molar concentration of insulin to achieve 50\% effect}) / (\text{molar concentration of proinsulin or intermediate to achieve 50\% effect})$.

‡ Mean \pm SEM of four separate experiments in the [125 I]-insulin-membrane binding system.

§ Mean of two separate experiments.

|| See footnote 4 in the text.

¶ 86% with another preparation of porcine diarginine insulin (lot 615-D 63-49).

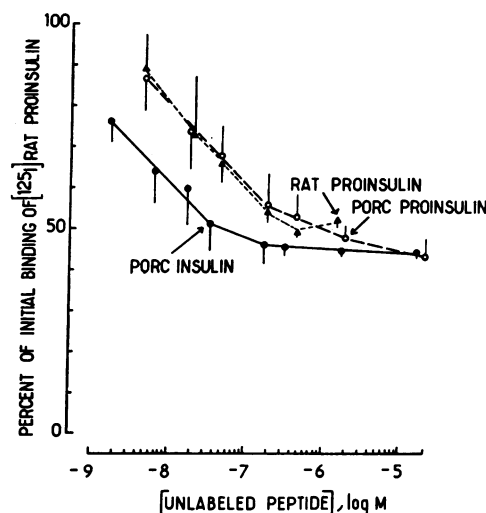


FIGURE 4 Inhibiting effect of unlabeled porcine insulin, porcine proinsulin, and rat proinsulin on the binding of [125 I]rat proinsulin to rat liver plasma membranes. The data are expressed as indicated in the legend to Fig. 2. Initial binding was 3.8% of the total [125 I]rat proinsulin; 0.2 nM [125 I]rat proinsulin was incubated with membranes at 0.2 mg protein/ml for 120 min at 30°C. Each point is the mean \pm one-half of the range for two separate experiments; in each of the experiments determinations were done in duplicate.

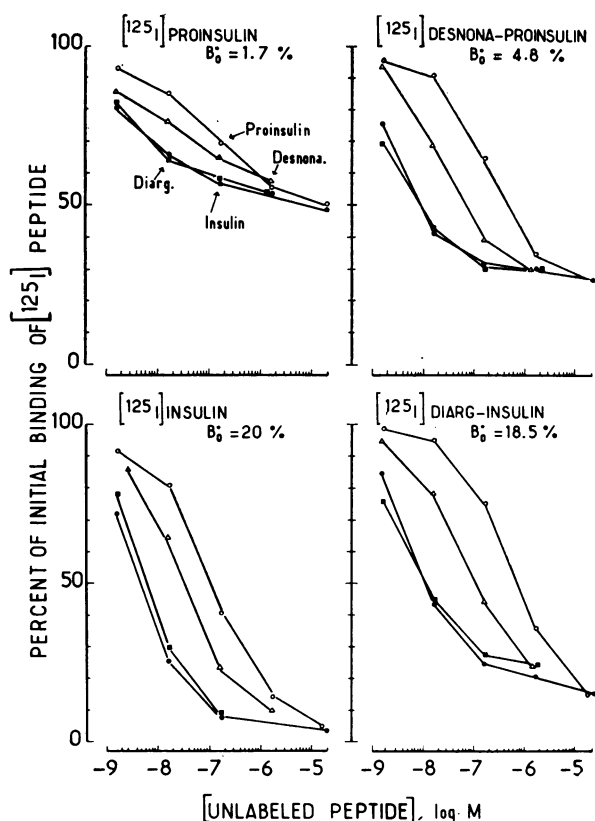


FIGURE 5 Binding and displacement of [125 I]proinsulin (upper left), [125 I]desnapeptide proinsulin (upper right), [125 I]diarginine insulin (lower right), and [125 I]insulin (lower left) by unlabeled proinsulin (open circles), desnapeptide proinsulin (open triangles), diarginine insulin (closed squares), and insulin (closed circles). The data are expressed as indicated in the legend to Fig. 2. The percentage of total radioactivity initially bound (B_0) is indicated for each [125 I]peptide. Experimental conditions were as indicated in the legend to Fig. 2. Each point is the mean of duplicate determinations in a single experiment.

the rat liver membranes. Rat proinsulin was not more potent than porcine proinsulin in competing with the binding of [125 I]rat proinsulin (Fig. 4). This indicates the absence of proinsulin-specific receptors in the liver.

Binding studies with proinsulin intermediates. To investigate further the interactions of proinsulin with the insulin-specific receptors in liver membranes, we studied directly the binding of proinsulin-like components that lack portions of the connecting peptide segment and that possess intermediate biological activities between proinsulin and insulin (3, 15). Proinsulin, the desnapeptide proinsulin, the diarginine insulin, and insulin exhibited the same order of relative potency in all of the binding systems studied (Fig. 5). The specific binding of each [125 I]peptide was in direct proportion to the relative potency of the unlabeled homologous peptide in the competition experiments (Fig. 5). These results

suggest again that porcine proinsulin and intermediates do not react with receptor sites other than the insulin specific receptors on the rat liver plasma membrane, i.e., receptors whose affinity for proinsulin or intermediate would be equal to or greater than their affinity for insulin. As observed in our other studies with a number of insulin analogues and derivatives (13, 26), we found a good agreement between the binding affinity in liver membranes and the biological potency in vitro (Table I).

Quantitative aspects of insulin and proinsulin binding. With all systems for the study of polypeptide hormone interactions with specific receptors, there are several problems⁵ that are encountered in the quantitative analysis of the kinetic and thermodynamic properties of the interaction. It is difficult to be certain that the steady-states observed are truly equilibria. Also, the numbers of sites exposed to the medium, even in highly purified sheet-like plasma membranes such as those used in these studies, may change with time, albeit rather slowly (28). The proper handling in the calculation of the nonspecific binding is as yet uncertain. Further, virtually all preparations that contain specific hormone receptors also contain independent systems that degrade hormone and that degrade receptor (14, 28, 30). The present studies were undertaken with full awareness of these problems, and experimental designs were selected to minimize them.

If we assume that the steady state of binding that is approached by 30–60 min at 30°C with both [125 I]peptides (Fig. 7) approximated equilibrium conditions,⁶ then the data in Fig. 6 give the best approximation for the amounts of proinsulin and insulin bound over a wide range of peptide concentrations and allow calculation of the apparent dissociation constants (K_d) of the insulin and proinsulin-receptor complexes. These constants differ by 25–30-fold (Table II), as might be expected from the respective ability of proinsulin and insulin to inhibit the binding of [125 I]proinsulin and [125 I]insulin. The data in Fig. 6 also give estimates of the proinsulin and insulin binding capacities per unit of plasma membrane protein (Table II).

Time-course studies

Time-course of association. At 30°C, the specific binding of [125 I]proinsulin and [125 I]insulin was a rapid

⁵ These have been analyzed by Berson and Yalow in the study of insulin-antibody interactions (27). They have been studied in detail (28) and reviewed elsewhere (12, 29) for the study of insulin-receptor interactions.

⁶ Steady-state measurements (Figs. 2, 3, and 6) were performed at 30°C after 60 min of incubation of [125 I]peptide with membranes in the absence and in the presence of unlabeled peptide at various concentrations. Similar data were obtained when [125 I]insulin and [125 I]proinsulin were incubated for 90 and 180 min, respectively.

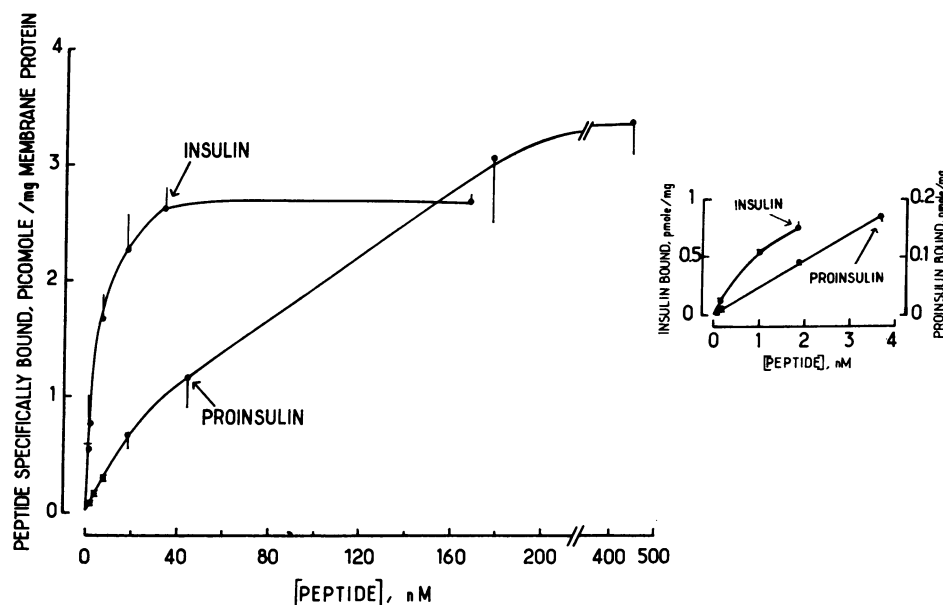


FIGURE 6 Binding of insulin and proinsulin as a function of the peptide concentration. The absolute amounts of peptide bound were calculated from data shown in Fig. 2, left, for insulin (i.e., [125 I]insulin and increasing concentrations of unlabeled insulin) and in Fig. 2, right, for proinsulin (i.e., [125 I]proinsulin and increasing concentrations of unlabeled proinsulin). The amount of peptide nonspecifically bound (i.e., [the percent of 125 I-radioactivity bound in the presence of 20 μ M unlabeled peptide] \times the peptide concentration) has been subtracted from each point. The inset to the right represents the amounts of insulin (closed circles) and proinsulin (open circles) bound at low peptide concentrations. Each point is the mean \pm one-half of the range for three separate experiments; in each of the experiments determinations were done in duplicate. Note that vertical scales are different in the inset.

reaction: by 15 min, the binding had reached 70–80% of its average maximum value (Fig. 7). The binding reached a maximum by 30–60 min and remained stable for at least 120 min with a slight (but significant) increase in the [125 I]proinsulin binding observed at 180 min (Fig. 7). The binding of [125 I]proinsulin remained considerably lower than that of [125 I]insulin throughout the entire time-course, within the same experiments and under identical quantitative conditions for both labeled peptides (Fig. 7). From the initial slopes of the binding curves (Fig. 7, inset) together with the concentrations of binding sites derived from steady-state studies (Fig. 6), we approximated the association (forward) rate constants (k_1) for the hormone- and the prohormone-receptor interaction (Table II). The association rate constant of proinsulin binding was 20-fold lower than that of insulin and the data agree well with the values calculated from steady-state and dissociation experiments (Table II). Thus it is apparent that a 20-fold lower association rate constant mainly accounts for the 25–30-fold lower affinity of proinsulin as compared to insulin. This was confirmed by direct studies of the dissociation of both [125 I]peptides.

Time-course of dissociation. Dissociations of [125 I]-proinsulin and [125 I]insulin were simultaneously measured under identical conditions (Fig. 8). The binding of [125 I]proinsulin and [125 I]insulin was largely reversible: after 60 min at 30°C, 75–80% dissociation was achieved (Fig. 8). With both [125 I]peptides, dissociation did not follow simple first-order kinetics (28); this was observed even after subtraction of the nonspecific binding throughout the experiment. However, assuming mean half-times of dissociation of 5–7 min and 8–10 min (Fig. 8) for proinsulin and insulin, respectively, one can calculate overall dissociation rate constants of approximately $11 \times 10^{-3} \text{ min}^{-1}$ for proinsulin and $8 \times 10^{-3} \text{ min}^{-1}$ for insulin.⁷ Such a slight difference between the dissociation rate constants is largely insufficient to explain the reduced binding affinity of proinsulin and con-

⁷ Similar data were observed when the dissociation of [125 I]proinsulin and [125 I]insulin from liver membranes was obtained by dilution of the [125 I]peptide-receptor complex, except that the dissociation rate constants were slightly slower ($\sim 7 \times 10^{-3} \text{ min}^{-1}$ for proinsulin and $\sim 5 \times 10^{-3} \text{ min}^{-1}$ for insulin) than those observed in the presence of unlabeled peptide (see text and Fig. 8).

trasts sharply with the great difference between the association rate constants.

As indicated in Table II, there is a good agreement between numbers derived from steady-state experiments (Figs. 2, 3, and 6) and those obtained from time-course studies (Figs. 7 and 8). There is also a close correspondence for the association rate constants between the measured values (from the initial velocities of the forward reactions in Fig. 7) and the calculated values (from the dissociation rate constants in Fig. 8 together with the dissociation constants at steady state) (Table II). Again, these numbers must be regarded as approximate, at least in absolute terms, because of the complexity of the insulin- (28) and proinsulin-receptor interactions. However, they allow a quantitative comparison between the respective binding of proinsulin and of insulin to the insulin receptor sites since they derive from experiments in which the two peptides were simul-

taneously studied under identical quantitative conditions. Moreover, insulin degradation by the membranes (14), another important factor of discrepancy between the respective fates of insulin and proinsulin exposed to the membrane under certain experimental conditions (14), was minimized in the present binding studies by the use of low membrane protein concentrations (28) and was less than 15% after 60 min exposure of [125 I]insulin to membranes at 30°C. Thus it is clear from both the steady-state and the time-course studies that proinsulin has a 20–30-fold lower affinity for receptor than insulin and that this is mainly accounted for by a decreased ability of proinsulin to associate with receptor since, in contrast to the association rates, the dissociation rates do not appear to differ to a very large extent. The only thing that would alter this conclusion would be if conversion of proinsulin to insulin occurred on the receptor.

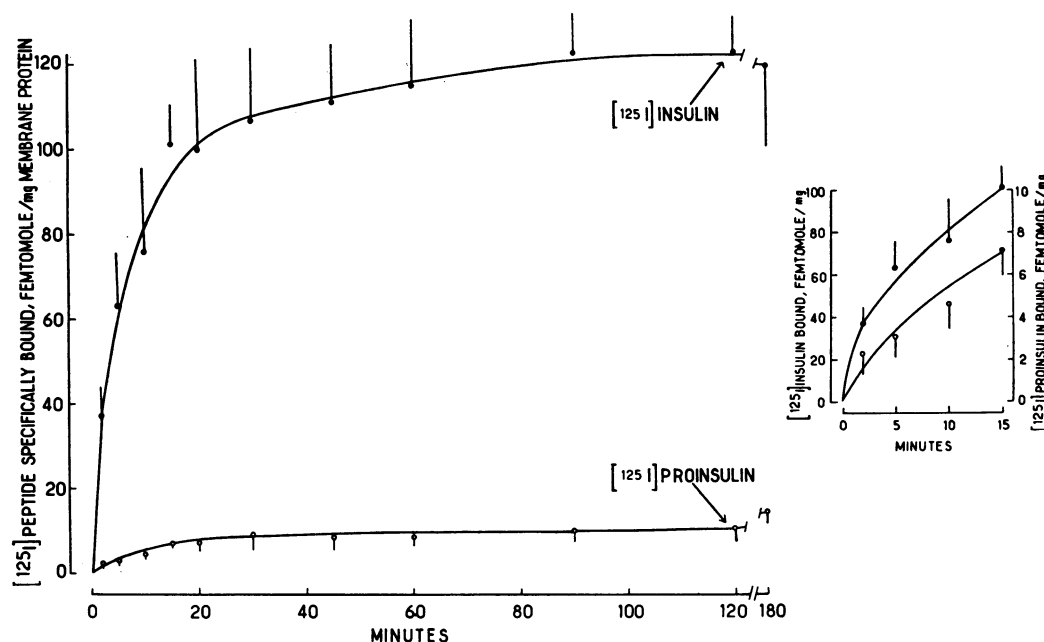


FIGURE 7 Time-course of binding of [125 I]insulin and [125 I]proinsulin to liver membranes at 30°C. The binding is expressed as absolute amounts of [125 I]peptide specifically bound. Within the same experiments, [125 I]insulin and [125 I]proinsulin, both at 0.15 nM, were mixed with membranes at 0.3 mg protein/ml incubation medium (time 0) in the absence and in the presence of a large excess (20 μ M) of unlabeled homologous polypeptide. The latter was used to determine the proportion of nonspecific binding which has been subtracted from each experimental point. Incubation mixtures were immediately distributed into 100- or 200- μ l samples. At the indicated times, 2 ml of ice-cold buffer were rapidly added to duplicate or triplicate samples that were immediately filtered on cellulose acetate (EGWP, 0.2 μ m) Millipore filters. Further washing of membranes on the filters was performed with the rapid filtration of 8 ml of cold buffer through the filters (the filtration and washing procedure did not consume more than 30–45 s). Each point is the mean \pm one-half of the range for three separate experiments; determinations were done in triplicate in two of the experiments and in duplicate in one of the experiments. The inset to the right represents the amounts of [125 I]insulin (closed circles) and [125 I]proinsulin (open circles) bound at the early time points. Note that vertical scales are different in the inset.

TABLE II
Apparent Dissociation Constants, Rate Constants, and Binding Capacities for the Specific Interaction of Proinsulin and Insulin with the Insulin Receptors in Liver Plasma Membranes at 30°C

Constant	Proinsulin	Insulin
Dissociation constant, K_d , M		
From steady state data*	80×10^{-9}	3×10^{-9}
From k_{-1}/k_1	69×10^{-9}	2.4×10^{-9}
Association rate constant, k_1 , $M^{-1} \text{ min}^{-1}$		
Measured†	1.6×10^8	3.3×10^7
Calculated‡	1.4×10^8	2.7×10^7
Dissociation rate constant, k_{-1} , min^{-1}	11×10^{-2}	8×10^{-2}
Binding capacity, ¶ mol/mg	3.4×10^{-12}	2.7×10^{-12}

* Dissociation constants, K_d , were measured as the concentration of peptide that produces half-maximal inhibition of the specific binding of the corresponding [125 I]peptide (from data in Fig. 2); this value corresponded closely to the concentration of peptide that gives half-maximal occupancy of the binding sites as determined in Fig. 6.

† The association rate constants, k_1 , were approximated from the initial velocity v of binding in Fig. 7, as $v = k_1 [H] [Ro]$, where $[H]$ is the concentration of peptide and $[Ro]$ is the concentration of binding sites (determined from data in Fig. 6).

‡ These were calculated from the rate constants of dissociation k_{-1} and from the dissociation constants, K_d , as $k_1 = k_{-1}/K_d$.

¶ Overall dissociation rate constants, k_{-1} , were calculated from measured half-times of dissociation (Fig. 8) according to the equation $t_{1/2} \cong \ln 2/k_{-1}$. See also footnote 7 in the text.

¶ Binding capacity is expressed as moles of peptide bound per milligram of plasma membrane protein and was determined at saturating concentrations of peptide as shown in Fig. 6.

Properties of proinsulin recovered from the membrane receptor

To examine the possibility of proinsulin conversion at the receptor site, we studied the properties of the [125 I] radioactivity recovered from the membrane pellet. After [125 I]proinsulin had been bound to the membranes, a high concentration (30 μ M) of unlabeled proinsulin was added so that the [125 I]proinsulin was dissociated from the receptor. In all of these experiments, the proportion of radioactivity recovered from the membrane pellet agreed well with the extent of dissociation of [125 I]proinsulin from membranes as measured by the time-course experiments (Fig. 8); i.e., 70–75% was dissociated after 60 min at 30°C (see legends to Figs. 9 and 10). Radioactivity thus recovered from the membranes eluted as a single major peak in a position consistent with unmodified proinsulin when submitted to gel filtration on Sephadex G-50 (fine) (Fig. 9). Similar results were obtained with dissociation of the proinsulin-membrane receptor complex by acid treatment of the membrane pellet.

On polyacrylamide gel electrophoresis (Fig. 10), [125 I] radioactivity recovered from the membranes (gel D) was indistinguishable from control [125 I]proinsulin (gel C). Further, it was completely distinct not only from control [125 I]insulin (gel A) but also from the proin-

sulin-like intermediate, [125 I]desnonapeptide proinsulin (gel B), which moved in a position between proinsulin and insulin (Fig. 10). This provides direct evidence that [125 I]proinsulin which is dissociated from the insulin receptor in liver plasma membranes is unconverted proinsulin.

Other properties of the [125 I]proinsulin recovered from the membranes also indicate the lack of conversion. The radioactive material recovered from the membranes showed little or no enhancement of binding to anti-insulin antibody or to liver membranes (Table III). If the membrane-bound [125 I]proinsulin (or a significant amount of it) had been converted to insulin, the recovered material would have exhibited a much higher binding ability to the membranes since the binding of [125 I]insulin is much higher than that of [125 I]proinsulin. Even a partial conversion to intermediate(s) such as the desdi-peptide and/or the desnonapeptide proinsulin should have caused at least a fourfold increase in the binding ability of the recovered radioactivity due to the higher binding affinity of these intermediates (Fig. 5 and Table I).

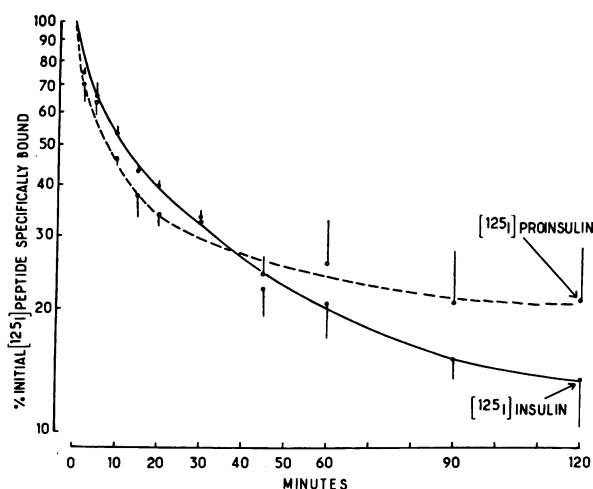


FIGURE 8 Time-course of dissociation of [125 I]insulin and [125 I]proinsulin from liver membranes at 30°C. Membranes (0.4 mg protein/ml incubation mixture) were incubated at 30°C in two steps. During the first step, [125 I]polypeptide at 0.20 nM was allowed to bind to membranes for 60 min; the percent of [125 I]polypeptide bound (initial binding) was determined on triplicate samples of each incubation mixture. The second step was then initiated immediately (time 0 on figure) by addition of homologous unlabeled polypeptide to give final concentrations of 7 μ M insulin and 9 μ M proinsulin. Membrane-bound radioactivity was measured at the indicated times as described in the legend to Fig. 7. The nonspecific binding, which has been subtracted from each experimental point, was determined throughout in a simultaneous experiment where unlabeled peptide at 20 μ M was added to the incubation medium at the very beginning of the experimental procedure. Each point is the mean \pm one-half of the range for two separate experiments; in each of the experiments determinations were done in triplicate.

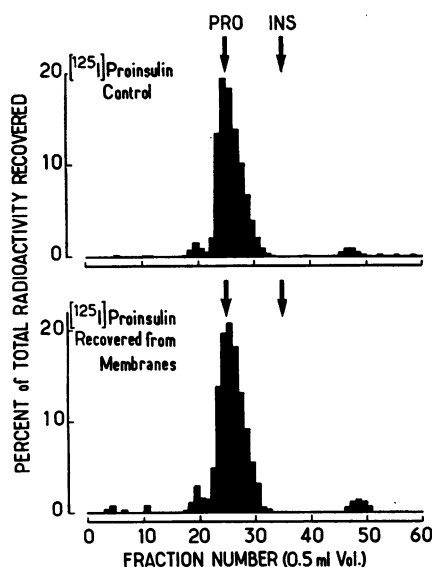


FIGURE 9 Gel filtration patterns of [125 I]proinsulin: control (top) and recovered from membranes by dissociation of the [125 I]proinsulin-membrane receptor complex (bottom). The percent of total radioactivity recovered from Sephadex G-50 (fine) columns (0.9×40 cm) is indicated for each eluted fraction (0.5 ml). The columns were equilibrated in Krebs-Ringer phosphate buffer containing 10 mg/ml bovine serum albumin, pH 7.5, and were eluted with the same buffer at 4°C. In both control (top) and experimental (bottom) conditions, the sample of [125 I]proinsulin that was applied to the column contained [125 I]insulin as marker. The final volume of sample applied was 0.5 ml. Vertical arrows denote the proinsulin peak (PRO) and the insulin peak (INS). The recovery of total radioactivity applied to the columns was 85%. Experimental conditions were as described in the legend to Fig. 8, except that membranes were collected by rapid centrifugation (13) at the end of the first incubation and washed once with cold buffer. The second step (dissociation of [125 I]proinsulin from the membrane pellet) was then started immediately by resuspension of the pellet in buffer containing 30 μ M unlabeled proinsulin. After 60 min at 30°C, the mixture was centrifuged and a sample of the supernate was applied to the column. The supernate contained 72% of the radioactivity that was initially bound to the membrane pellet. In the control experiment, [125 I]proinsulin was incubated in buffer for 60 min at 30°C and then applied to the column.

The apparent slight increase in binding ability to fresh membranes that was observed with the recovered [125 I]-proinsulin (Table III) probably reflects some "purification" of the receptor-bound polypeptide similar to that previously (14) observed with receptor-bound [125 I]insulin. The possibility that conversion of proinsulin to insulin could have been masked by rapid degradation of newly formed insulin can also be ruled out. Indeed, receptor-bound insulin is not degraded and is even protected from degradation (14). Other data in Table III confirm our previous observation that proinsulin which is recovered free in the incubation medium ex-

hibits very little degradation when compared to insulin under similar conditions (14).

DISCUSSION

The observations reported here provide evidence that proinsulin interacts with the specific insulin receptors

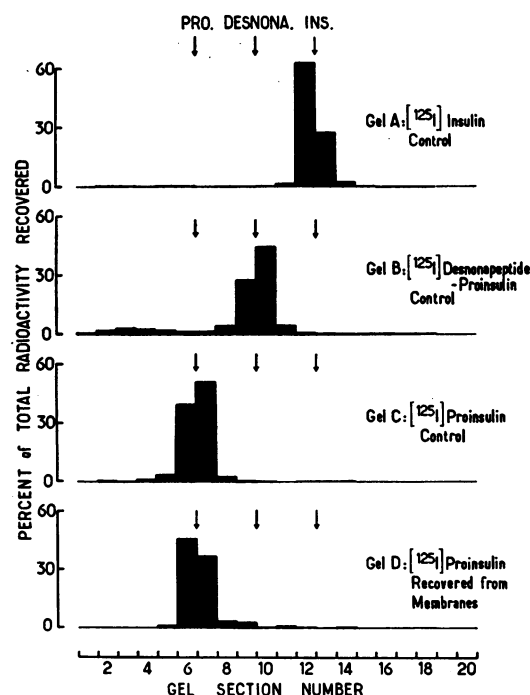


FIGURE 10 Polyacrylamide gel patterns of (from top to bottom): [125 I]insulin (control, gel A), [125 I]desnonapeptide proinsulin (control, gel B), [125 I]proinsulin (control, gel C), and [125 I]proinsulin recovered from membranes (gel D) by dissociation of the [125 I]proinsulin-membrane receptor complex. The percent of total radioactivity recovered from each gel is indicated for 5-mm sections taken from the origin of the small pore gel (left) to the gel front (right). Small pore gel was prepared from 20% acrylamide (2) and 0.6% *N,N'*-methylenebisacrylamide; large pore gel was prepared from 2.5% acrylamide. The gel size was 0.8×20 cm and 0.8×2 cm for the small pore and the large pore gel, respectively. The amount of protein applied to each gel, along with the [125 I]polypeptide used as marker, was 400 μ g bovine serum albumin. Unlabeled proinsulin (about 100 μ g) that had been used to dissociate the [125 I]-proinsulin-receptor complex was also contained in the sample applied to gel D; the same amount of proinsulin was added to [125 I]proinsulin (control) immediately before application to gel C. All samples (600 μ l containing 20% sucrose) were applied to the large pore gel and subjected to electrophoresis at pH 8.8 with 1 mA/tube for 1 h and 2 mA/tube for the subsequent 12 h at 4°C. Each gel was run in duplicate. Experimental conditions to dissociate [125 I]proinsulin from the membrane receptor were as described in the legend to Fig. 9, except that the buffer used here was 20 mM Tris-HCl, pH 7.5, that contained 1 mg/ml bovine serum albumin. The radioactivity recovered from the membrane pellet was 70% of that initially bound.

in rat liver plasma membranes. Such interaction was demonstrated both indirectly, with the inhibition of the [125 I]insulin binding by unlabeled proinsulin, and directly, with studies of the [125 I]proinsulin binding. In both studies, proinsulin exhibited a similar low binding affinity. This low affinity is an intrinsic property of the proinsulin molecule. Indeed, purification and selection of the [125 I]proinsulin on DEAE-cellulose (Fig. 1) excluded possible contamination of labeled proinsulin with any labeled insulin or proinsulin-like intermediate as confirmed by the polyacrylamide gel patterns (Fig. 10). Since under identical experimental conditions the membranes specifically bind 20–25% and 1.5–2% of the total [125 I]insulin and [125 I]proinsulin, respectively, one can calculate that a 6–10% contamination of the [125 I]-proinsulin with [125 I]insulin would be necessary to explain the observed binding only by virtue of contamination with insulin. Similar calculations indicate that a 30–40% contamination of the [125 I]proinsulin with [125 I]-desdipeptide or desnonapeptide proinsulin would be required. Such levels of contamination can be ruled out by the polyacrylamide gel data which showed that 96, 3, and 0.4% of the total radioactivity were recovered in the proinsulin, the desnonapeptide proinsulin, and the insulin areas, respectively (gel C in Fig. 10).

The mechanism by which proinsulin interacts with the insulin receptor sites appears to be a direct one in that no evidence for conversion of [125 I]proinsulin to insulin was observed after proinsulin had bound to the liver plasma membranes. In previous studies (14), we observed that proinsulin, recovered free in the medium after it had been exposed to liver membranes, showed no appearance of conversion to insulin. In the present work, [125 I]proinsulin that was recovered from membranes upon dissociation of the [125 I]proinsulin-receptor complex was not altered in its chromatographic pattern on polyacrylamide gel electrophoresis (Fig. 10) or on Sephadex G-50 (Fig. 9) when compared to control [125 I]proinsulin. Further, partial conversion leading to intermediates (3, 15, 31) such as the desdipeptide and/or the desnonapeptide proinsulin(s), that would not have been detected by the gel filtration technique (8, 32), can be ruled out from the polyacrylamide gel experiments and from other properties of the recovered [125 I]-material, such as its binding ability to fresh membranes, which remains very low and is consistent with that of unmodified proinsulin.^a These results substantiate, at

^a Accordingly, the slight increase of [125 I]proinsulin binding observed at 180 min in the time-course experiment (Fig. 7) cannot be explained by any substantial conversion of the [125 I]proinsulin since it represents only a 1.5-fold increase above the steady-state level values observed between 30 and 120 min. However, conversion to split proinsulin or a very partial conversion to the desdipeptide- or the desnonapeptide-proinsulin (Table I) cannot be excluded after such a long period of exposure to membranes.

TABLE III
Properties of (a) [125 I]Proinsulin Recovered from Liver Membranes by Dissociation of the [125 I]Proinsulin-Membrane Receptor Complex and (b) [125 I]-Proinsulin Recovered Free in the Medium after Exposure to Liver Membranes

Property	[125 I]Proinsulin	
	(a) Recovered from membranes	(b) Free in medium
	% of controls	
Bound to fresh membranes	135	93
Bound to anti-insulin antibody	106	94
Adsorbed by talc	104	97
Precipitable by 5% trichloroacetic acid	100	98

After 0.9 nM [125 I]proinsulin had been exposed to 0.8 mg membrane protein/ml for 30 min at 30°C in a total volume of 3 ml, membranes were collected by centrifugation. Samples of the supernate were analyzed to measure the properties of free [125 I]proinsulin (14). Membranes were immediately washed once with cold buffer and then resuspended in 1 ml of 0.1 N HCl containing 20 mg/ml bovine serum albumin. This procedure was used to dissociate the [125 I]proinsulin-membrane receptor complex and allowed recovery, after 30 min at room temperature, of 60–70% of the radioactivity that was initially bound to the membrane pellet. After centrifugation of the mixture, the supernate was neutralized with 1 N NaOH and then diluted with buffer, and samples were analyzed to measure the properties of the radioactivity thus recovered from membranes. Control [125 I]proinsulin was treated similarly except that membranes were omitted. The data are expressed as percent of controls. Each value is the mean of two separate experiments.

the level of the insulin receptor in the liver plasma membrane, other observations which indicate that proinsulin injected in vivo or incubated in vitro remains essentially unconverted (6, 8).

From the comparative data obtained here with time-course studies of [125 I]proinsulin and [125 I]insulin binding, it appears that a lower forward rate constant essentially accounts for the lower affinity of the proinsulin molecule. Although the insulin moiety in proinsulin appears to be in the same conformation as that of native insulin (33) and although proinsulin can form hexamers in a way similar to insulin (34), the connecting peptide (C-peptide) may interfere with the molecular arrangement in crystal formation (3). It is possible that in a like manner the C-peptide alters interaction with the receptor. The C-peptide could cause a steric hindrance that impedes the interaction between the insulin moiety in the proinsulin molecule and the receptor. It may also prevent a conformational adaptation possibly required for binding to the receptor. In

this regard, it is of interest that chemically modified insulins with an intramolecular crosslink between Gly A₁ and Lys B₂₉ behave like proinsulin: they have very reduced binding affinity and biological potency *in vitro* (26), and they readopt their original conformation after reduction and reoxidation (35). Also of interest is the observation that the desdipeptide and the desnonapeptide proinsulins possess a higher binding affinity (Fig. 5 and Table I) and a higher biological potency (Table I; reference 36) than intact proinsulin, indicating that a free NH₂-terminal glycine on the A chain is an important requirement for binding to the receptor. However, the remaining portion of the C-peptide still alters interaction of both intermediates with the receptor since their binding affinity and their biological potency are substantially lower than that of native insulin. This is in contrast to the diarginine and the monoarginine insulins, which have binding affinities and biological potencies much closer to that of insulin (Fig. 5 and Table I). From these observations, we can conclude that the C-peptide hinders the binding process for proinsulin and proinsulin-like molecules; however, once it is formed, the proinsulin-receptor complex appears to be nearly as stable as an insulin-receptor complex.

Our results indicate that proinsulin interacts with all of the insulin receptor sites. They do not reveal, at least in the liver plasma membrane, the presence of receptor sites that would have an affinity for proinsulin (or for proinsulin intermediates) equal to or in excess of their affinity for insulin and thus would be specific for proinsulin (or for proinsulin intermediates). The present studies cannot exclude the possibility that the C-peptide itself, and not the proinsulin molecule as a whole, may interact with a specific receptor. However, the lack of biological effect of C-peptides in homologous fat tissues (36) does not favor such possibility.

Finally, our data have direct physiological and clinical implications. It is evident that the lower uptake of proinsulin by the liver insulin receptors, as well as its much slower degradation by the liver (10, 11, 14, 37), allows more of the secreted proinsulin to reach the peripheral circulation and may account for the longer half-life of proinsulin (38) and its prolonged activity *in vivo* (3, 4, 39) when compared to insulin. There are several clinical states in which the proportion and/or the properties of the circulating proinsulin component(s) deviate from the normal pattern (40). Studies of these components at the membrane receptor level such as those described here would represent a direct measurement of their respective affinity for the insulin receptors and should give some insight into the pathophysiology of the observed syndromes.

ACKNOWLEDGMENTS

The author would like to thank Drs. J. Roth and P. de Meyts for their helpful advice during this study; Drs. R. E. Chance, J. Schlichtkrull, and D. F. Steiner for their generous gifts of highly purified proinsulins and insulins; F. Rançon for her kind assistance in the polyacrylamide gel studies; N. Grenier-Brossette and M. C. Chamblier for their skillful technical assistance; and D. Lhenry and D. Lemnoury for their careful preparation of the manuscript. The author would also like to acknowledge the referees and the editors of the Journal of Clinical Investigation whose rigorous and thorough reviews were most helpful in preparing the final version of this article.

This study was supported in part by a grant from the Institut National de la Santé et de la Recherche Médicale (I. N. S. E. R. M.).

REFERENCES

1. Steiner, D. F., D. Cunningham, L. Spigelman, and B. Aten. 1967. Insulin biosynthesis: evidence for a precursor. *Science (Wash. D. C.)* **157**: 697-700.
2. Chance, R. E., R. M. Ellis, and W. W. Bromer. 1968. Porcine proinsulin: characterization and amino acid sequence. *Science (Wash. D. C.)* **161**: 165-167.
3. Chance, R. E. 1971. Chemical, physical, biological, and immunological studies on porcine proinsulin and related polypeptides. In *Diabetes*. R. R. Rodriguez and J. Valance-Owen, editors. Excerpta Medica, Amsterdam, 292-305.
4. Kitabchi, A. E., W. C. Duckworth, F. B. Stentz, and S. Yu. 1972. Properties of proinsulin and related polypeptides. In *C. R. C. Critical Reviews in Biochemistry*. R. A. Harte, editor. Chemical Rubber Company, Cleveland. **1**: 59-94.
5. Rubenstein, A. H., S. Cho, and D. F. Steiner. 1968. Evidence for proinsulin in human urine and serum. *Lancet*. **1**: 1353-1355.
6. Steiner, D. F. 1969. Proinsulin and the biosynthesis of insulin. *N. Engl. J. Med.* **280**: 1106-1112.
7. Rubenstein, A. H., F. Melani, S. Pilakis, and D. F. Steiner. 1969. Proinsulin. Secretion, metabolism, immunological and biological properties. *Postgrad. Med. Suppl. J.* **45**: 476-481.
8. Lazarus, N. R., J. C. Penhos, T. Tanese, L. Michaels, R. Gutman, and L. Recant. 1970. Studies on the biological activity of porcine proinsulin. *J. Clin. Invest.* **49**: 487-496.
9. Shaw, W. N. 1970. The effect of porcine proinsulin *in vivo* in the rat. In *Proceedings of the 7th Congress of the International Diabetes Federation*. *Excerpta Med. Int. Congr. Ser.* **209**: Abstr. n. 9.
10. Stoll, R. W., J. L. Touber, L. A. Menahan, and R. H. Williams. 1970. Clearance of porcine insulin, proinsulin and connecting peptide by the isolated rat liver. *Proc. Soc. Exp. Biol. Med.* **133**: 894-896.
11. Rubenstein, A. H., L. A. Pottenger, M. Mako, G. S. Getz, and D. F. Steiner. 1972. The metabolism of proinsulin and insulin by the liver. *J. Clin. Invest.* **51**: 912-921.
12. Roth, J. 1973. Peptide hormone binding to receptors: a review of direct studies *in vitro*. *Metab. (Clin. Exp.)*. **22**: 1059-1073.
13. Freychet, P., J. Roth, and D. M. Neville, Jr. 1971. Insulin receptors in the liver: specific binding of ¹²⁵I-insulin to the plasma membrane and its relation to insulin

- bioactivity. *Proc. Natl. Acad. Sci. U. S. A.* **68**: 1833-1837.
14. Freychet, P., C. R. Kahn, J. Roth, and D. M. Neville, Jr. 1972. Insulin interactions with liver plasma membranes. Independence of binding of the hormone and its degradation. *J. Biol. Chem.* **247**: 3953-3961.
15. Chance, R. E. 1972. Amino acid sequences of proinsulins and intermediates. *Diabetes* **21** (Suppl. 2): 461-467.
16. Schlichtkrull, J., J. Brange, A. H. Christiansen, O. Hallund, L. G. Heding, and K. H. Jorgensen. 1972. Clinical aspects of insulin antigenicity. *Diabetes* **21** (Suppl. 2): 649-656.
17. Rosselin, P. G., and J. Dolais. 1968. Application de la méthode radio-immunologique au dosage de l'insuline humaine et au dosage de l'hormone folliculo-stimulante humaine (H F.S.H.). *Ann. Biol. Clin.* **26**: 763-791.
18. Freychet, P., J. Roth, and D. M. Neville, Jr. 1971. Mono-iodo-insulin: demonstration of its biological activity and binding to fat cells and liver membranes. *Biochem. Biophys. Res. Commun.* **43**: 400-408.
19. Neville, D. M., Jr. 1968. Isolation of an organ specific protein antigen from cell-surface membrane of rat liver. *Biochim. Biophys. Acta.* **154**: 540-552.
20. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265-275.
21. Rodbell, M. 1964. Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. *J. Biol. Chem.* **239**: 375-380.
22. Bataille, D. P., P. Freychet, P. E. Kitabgi, and G. E. Rosselin. 1973. Gut glucagon: a common receptor site with pancreatic glucagon in liver cell plasma membranes. *FEBS Fed. Eur. Biochem. Soc. Lett.* **30**: 215-218.
23. Bataille, D. P., P. Freychet, P. Kitabgi, and G. E. Rosselin. 1973. Gut glucagon: common receptor sites with pancreatic glucagon in membranes of target cells. In Proceedings of the 8th Congress of the International Diabetes Federation. *Excerpta Med. Int. Congr. Ser.* **280**: Abstr. no. 114.
24. Markussen, J., and F. Sundby. 1972. Rat-proinsulin C-peptides. Amino acid sequences. *Eur. J. Biochem.* **25**: 153-162.
25. Tager, H. S., and D. F. Steiner. 1972. Primary structures of the proinsulin connecting peptides of the rat and the horse. *J. Biol. Chem.* **247**: 7936-7940.
26. Freychet, P., D. Brandenburg, and A. Wollmer. 1974. Receptor-binding assay of chemically modified insulins. Comparison with *in vitro* and *in vivo* bioassays. *Diabetologia.* **10**: 1-5.
27. Berson, S. A., and R. S. Yalow. 1959. Quantitative aspects of the reaction between insulin and insulin-binding antibody. *J. Clin. Invest.* **38**: 1996-2016.
28. Kahn, C. R., P. Freychet, J. Roth, and D. M. Neville, Jr. 1974. Quantitative aspects of the insulin-receptor interaction in liver membranes. *J. Biol. Chem.* **249**: 2249-2257.
29. Neville, D. M., Jr., C. R. Kahn, A. Soll, and J. Roth. 1973. Plasma membrane insulin receptor regulation by genetic, metabolic and hormonal factors. In *Protides of the Biological Fluids: 21st Colloquium*. H. Peeters, editor. Pergamon Press Ltd., New York. 269-273.
30. Freychet, P., C. R. Kahn, J. Roth, and D. M. Neville, Jr. 1973. Insulin receptors in liver cell plasma membranes. In Proceedings of the 4th International Congress of Endocrinology. *Excerpta Med. Int. Congr. Ser.* **273**: 335-340.
31. Steiner, D. F., O. Hallund, A. Rubenstein, S. Cho, and C. Bayliss. 1968. Isolation and properties of proinsulin, intermediate forms, and other minor components from crystalline bovine insulin. *Diabetes.* **17**: 725-736.
32. Gutman, R. A., N. R. Lazarus, and L. Recant. 1972. Electrophoretic characterization of circulating human proinsulin and insulin. *Diabetologia.* **8**: 136-140.
33. Frank, B. H., and A. J. Veros. 1968. Physical studies on proinsulin-association behavior and conformation in solution. *Biochem. Biophys. Res. Commun.* **32**: 155-160.
34. Frank, B. H., and A. J. Veros. 1970. Interaction of zinc with proinsulin. *Biochem. Biophys. Res. Commun.* **38**: 284-289.
35. Brandenburg, D., and A. Wollmer. 1973. The effect of a nonpeptide interchain crosslink on the reoxidation of reduced insulin. *Hoppe-Seyler's Z. Physiol. Chem.* **354**: 613-627.
36. Yu, S. S., and A. E. Kitabchi. 1973. Biological activity of proinsulin and related polypeptides in the fat tissue. *J. Biol. Chem.* **248**: 3753-3761.
37. Burghen, G. A., A. E. Kitabchi, and J. S. Brush. 1972. Characterization of a rat liver protease with specificity for insulin. *Endocrinology.* **91**: 633-642.
38. Stoll, R. W., J. L. Touber, L. C. Winterscheid, J. W. Ensink, and R. H. Williams. 1971. Hypoglycemic activity and immunological half-life of porcine insulin and proinsulin in baboons and swine. *Endocrinology.* **88**: 714-717.
39. Galloway, J. A., M. A. Root, R. E. Chance, R. P. Rathmacher, D. R. Challoner, and W. N. Shaw. 1969. *In vivo* studies of hypoglycemic activity of porcine proinsulin. *Diabetes* **18** (Suppl. 1): 341. (Abstr.)
40. Gorden, P., J. Roth, P. Freychet, and C. R. Kahn. 1972. The circulating proinsulin-like components. *Diabetes* **21** (Suppl. 2): 673-677.