Evidence That Insulin Receptor from Human Placenta Has a High Affinity for Only One Molecule of Insulin*

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Insulin receptor, partially purified from human placenta by chromatography on wheat germ agglutinin, was shown, by means of double probe labeling, to bind only one molecule of insulin with a high affinity. In the double probe labeling protocol used, $^{125}$I-insulin (probe 1) was affinity cross-linked to its receptor in the presence of an excess of unlabeled $N^2$-biotinylinsulin (probe 2). The ability of succinylavidin to bind to receptor-linked probe 2 and alter the electrophoretic mobility of the cross-linked complex (during polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate) was used to determine the amount of receptor which was cross-linked to both probes relative to that which was cross-linked to only probe 1. The fraction of receptor bound to two molecules of insulin prior to cross-linking was estimated from the cross-linking efficiency and the yield of receptor cross-linked to both probes relative to the yield of receptor cross-linked only to probe 1. The low fraction of receptor bound to both probes in the presence of high concentrations of probe 2 indicated that the affinity of the receptor for a second molecule of insulin was $\sim$100 times less than that for the first and that in the range of insulin concentrations ($\leq 20 \text{ nM}$) usually used to determine the stoichiometry for the interaction between receptor and insulin, more than 80% of the receptor molecules should be bound to only one molecule of insulin. This knowledge of how insulin receptor interacts with insulin was shown to be important for proper determination of receptor purity, interpretation of curvilinear Scatchard plots, and interpretation of the insulin-enhanced rate of dissociation of receptor-bound insulin.

An understanding of the molecular basis for the action of insulin is likely to require considerable knowledge of the initial interactions between insulin and its receptor. The most fundamental aspect of this interaction is the reaction stoichiometry. Determination of stoichiometry for the interaction between insulin and its receptor by classical methods of physical chemistry requires knowledge of the concentration of functional receptor. At present, however, there is no way to determine the molar concentration of functional insulin receptor in plasma membranes or in the purest preparations of insulin receptor which are currently available. Recently, we reported a method for determination of stoichiometry (1), which does not require knowledge of the concentration of receptor. This method, DPL, was used to determine the stoichiometry for the interactions between insulin and its receptor in rat adipocyte membranes. In this application of DPL, $^{125}$I-insulin (probe 1) together with a large excess of nonradioactive biotinylinsulin (probe 2) was affinity cross-linked to the receptor. Reaction with succinylavidin (after denaturation of the receptor) followed by SDS-PAGE was used to assay for the presence of receptor linked to both probes. The presence of some molecules would be signaled by the appearance of radioactive bands (due to the presence of probe 1) of increased molecular weight, due to the interaction of receptor-bound biotinylinsulin (probe 2) with succinylavidin. Radioactive bands of increased molecular weight were not observed, although bands of increased molecular weight were observed in control experiments wherein the $^{125}$I and the biotin were attached to the same molecule of insulin. These observations together with the probe concentrations in equilibrium with the receptor prior to addition of the cross-linking agent indicated that insulin receptor molecules in rat adipocyte membranes had only one high affinity site for insulin and that if the receptor were bivalent, the affinity of the receptor for a second molecule of insulin must be substantially less than its affinity for the first.

This result was unexpected, since insulin receptor is generally thought to bind insulin with a 1:2 stoichiometry. Although there is no direct proof that the receptor is bivalent, the kinetic evidence for negative cooperativity in insulin binding and the results of structural studies which suggest that the insulin receptor has a symmetrical $\alpha_2\beta_2$ structure (e.g., Refs. 2-6) are most easily rationalized in terms of a bivalent insulin receptor wherein each $\alpha$-chain contains an insulin-binding site. This view of the insulin receptor taken together with the univalent character of the receptor in rat adipocyte membranes as revealed by DPL suggested the possibility that extreme negative cooperativity or interactions with a membrane component might have prevented receptor molecules from binding two molecules of insulin. We were, therefore, prompted to determine whether solubilized and partially purified receptor from a different source behaved similarly and whether we could demonstrate weak binding of a second molecule of insulin to the receptor using modified DPL protocols of increased sensitivity. In this work we report

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Dedicated to Eraldo Antonini in memory of his outstanding qualities as a scientist and a human being.

† Part of this work is described in a Ph.D. dissertation to be submitted to the Graduate School of The University of Michigan.

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§ The abbreviations used are: DPL, double probe labeling, DSS, disuccinimidyl suberate; GlcNAc, N-acetylgalactosamine; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; SDS-PAGE, polyacrylamide gel electrophoresis done in the presence of SDS; WGA, wheat germ agglutinin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
DPL experiments which show that partially purified (by chromatography on wheat germ agglutinin) insulin receptor from human placental tissue has only one high affinity site for insulin and that the receptor is able to interact with a second molecule of insulin, albeit much more weakly than with the first. The consequences of these findings on determinations of receptor purity, interpretations of curvilinear Scatchard plots, and on interpretations of the kinetic evidence for negative cooperativity are discussed.

MATERIALS AND METHODS

Wheat germ agglutinin agarose was from E-Y Laboratories; guinea pig anti-porcine insulin serum was from Miles-Yeda Ltd.; phenyl-methanesulfonyl fluoride, leupeptin, antipain, pepstatin A, aprotinin, Nα-tosyl-L-lysine chloromethyl ketone, and phosphoribosyl b were from Sigma. Nα,Nβ-Diacetylchitoiose was generously supplied by Dr. Irwin J. Goldstein (University of Michigan). The source of other materials has been previously reported (1).

Preparation of Human Placental Insulin Receptor—Human placental insulin receptor was solubilized and purified by WGA chromatography according to the procedure described by Harrison and Itin (7), but with some modifications. After precipitation, the microsomal membranes were washed 4 times with cold 50 mM Hepes, pH 7.6, and 0.1 mM PMSF and then solubilized by stirring overnight at 4°C in the same buffer, with additions of 1% Triton X-100, 0.1 mM aprotinin, 0.05% leupeptin, antipain, and pepstatin A, and 5 trypsin-inhibitor units/ml of aprotinin. After centrifugation at 130,000 × g for 60 min at 4°C, the supernatant was kept at 4°C before WGA chromatography.

WGA chromatography was performed at 4°C. Crude receptor (20 ml) was mixed with an equal volume of 50 mM Hepes, pH 7.6, 10 mM MgCl2, 0.1% Triton, and 0.1 mM PMSF (buffer A), and then was passed 6 times through a 10-ml WGA-agarose column, which had been washed with 0.1% SDS and equilibrated with buffer A. After washing the column with 5 volumes of buffer A, the receptor was eluted with 15 ml of 0.3 M GlnAc, 2.5 mM of Nα,Nβ-diacetylchitoiose, 50 mM Hepes, pH 7.5, 0.1% Triton, 0.1 mM PMSF, 0.15 mM NaCl. The fractions with binding activity were pooled and stored at −15°C.

Cross-linking of Receptor with Insulin—The cross-linking procedure was as described for membrane-bound receptor (1, 6). WGA-purified receptor (10 μg), in 200 μl, was incubated with insulin in 50 mM Hepes, pH 7.6, 0.1% Triton, and 0.1 mM PMSF (buffer A), and then was passed 6 times through a 10-ml WGA-agarose column, which had been washed with 0.1% SDS and equilibrated with buffer A. After washing the column with 5 volumes of buffer A, the receptor was eluted with 15 ml of 0.3 M GlnAc, 2.5 mM of Nα,Nβ-diacetylchitoiose, 50 mM Hepes, pH 7.5, 0.1% Triton, 0.1 mM PMSF, 0.15 mM NaCl. The fractions with binding activity were pooled and stored at −15°C.

Cross-linking of Insulin with Anti-insulin IgG—Guinea pig anti-porcine insulin (32 ml) in a final volume of 200 ml was incubated with insulin in buffer B without Triton. After 3½ days at 4°C, cross-linking was initiated by addition of 2 μl of 10% Triton and 2 μl of 10% DSS in dimethyl sulfoxide. After 45 min in ice, the reaction was quenched by addition of an equal volume of 2-fold concentrated SDS-PAGE sample buffer (8), containing 1.25% SDS, but no thiols, and heated at 95°C for 5 min. The samples were analyzed by SDS-PAGE in the presence of succinylated avidin, as described previously (1). A Zeineh soft laser scanning densitometer (Biomed Instruments, Inc., model SL-TRRF) was used for determinations of band density in autoradiograms. Control experiments with radioactive standards indicated, that in the range of band densities used, the band density was proportional to radioactivity.

Cross-linking of Insulin with Anti-insulin IgG—Guinea pig anti-porcine insulin (32 ml) in a final volume of 200 ml was incubated with insulin in buffer B without Triton. After 3½ days at 4°C, cross-linking was initiated by addition of 2 μl of 10% Triton and 2 μl of 10% DSS in dimethyl sulfoxide. After 2 min at room temperature, the reaction was quenched by addition of 10 μl of 0.5 M glycylglycine. The mixture was incubated at room temperature for 15 min, and the cross-linked IgG was precipitated by addition of 0.4 ml of 1 mg/ml of bovine γ-globulin (in 0.1 M sodium phosphate, pH 7.4), 0.4 ml of 25% polyethylene glycol (6000), and incubation in ice for 15 min. The supernatant solution was separated by centrifugation, and the radioactivity in the pellet was measured in a Searle automatic gamma system model 1190.

Determination of Protein—The protein content in the receptor preparation was estimated by using the Bio-Rad protein assay dye reagent, which was developed by Bradford (11). Ovalbumin was used as standard.

Induction of Insulin and Biotinylinsulin—The induction was essentially the same as described previously (1, 12) except that some of the buffers were different. The Sephadex G-50 (fine) column was equilibrated with 1% bovine serum albumin, 25 mM Hepes, pH 7.4. The hormone was iodinated in 0.17 M Hepes, pH 7.4, and was eluted from the column with 0.1% bovine serum albumin, 25 mM Hepes, pH 7.4.

RESULTS

The first use of DPL (1) indicated that insulin receptor molecules in rat adipocyte membranes bound only one molecule of insulin with a high affinity. Since this result was unexpected, we wished to demonstrate the ability of DPL to signal the presence of a protein which bound 2 molecules of insulin with a high affinity. To do this, DPL was used to study the interaction between porcine insulin and guinea pig antiporcine insulin as shown in Fig. 1. Insulin derivatives were incubated in the absence (lane A) or presence of 32 nl of antisera at 4°C and treated sequentially with the crosslinking agent DSS, SDS, succinylavindin, and subjected to SDS-PAGE under nonreducing conditions. Samples A and B show the requirement for antisera to obtain radioactive bands at the positions of IgG when 125I-insulin is treated with DSS. Samples C–E, wherein the antibody was incubated with a mixture of 125I-insulin and nonradioactive Nα-Nβ-biotinylinsulin, show bands of increased molecular weight which might be expected for a complex consisting of IgG, 125I-insulin, and nonradioactive Nα-Nβ-biotinylinsulin, complexed with succinylavindin (IgG-1B-A). The appearance of this band reflects the bivalent character of the antibody. Lanes F–H show the effect of incubating the antibody with radioactive 125I-Nα-Nβ-biotinylinsulin. In the presence of 125I-Nα-Nβ-biotinylinsulin and absence of unlabeled insulin (lanes G–H), bands appear which are attributed to IgG complexes containing one and two molecules of 125I-Nα-Nβ-biotinylinsulin complexed with succinylavindin (IgG-1B-A and IgG-1B2-A2). The relative intensity of the band containing two molecules of 125I-Nα-Nβ-biotinylinsulin (IgG-1B2-A2) was decreased by the presence of nonradioactive insulin (lane F), as expected for replacement of one 125I-biotinylinsulin-succinylavindin complex with an insulin at an antibody-combining site. The higher molecular weight bands including those at the top of the gel are attributed to aggregates which formed prior to the cross-linking of antibody-antigen complexes. Aggregation might have occurred because the antisera contained a mixture of antibodies directed to different domains of insulin. Aggregation and the resulting production of bands at >900 kDa were reduced by use of an excess of insulin or insulin derivative over antibody.

DPL protocols similar to those used to determine the bind-
with DSS, denatured, reacted with succinylavidin, and subjected to incubation in the absence or presence of guinea pig antiserum to porcine insulin for 3.5 days, cross-linked insulin were incubated in the absence or presence of nonradioactive N'Rm-biotinylinsulin: 50 nM (C), 100 nM (D), 200 nM (E); F, 44 nM insulin with 86 nM N'Rm-biotinylinsulin (1.77 x 10^6 cpm/ml); G and H, 125I-N'Rm-biotinylinsulin at 133 nM, 1.13 x 10^6 cpm/ml (G), and 43 nM, 1.77 x 10^6 cpm/ml (H). The labels at the right indicate the presumed migration positions for complexes of IgG, insulin (I), biotinylinsulin (IB), and succinylavidin (A). Samples were analyzed by SDS-PAGE (5% separation gel, acrylamide/methylenebisacrylamide = 100:1). Migration positions for rabbit muscle myosin (205 kDa), Escherichia coli β-galactosidase (116 kDa), and rabbit muscle phosphorylase (94 kDa) are indicated at the left of the gel.

Fig. 1. DPL of IgG against insulin. Derivatives of porcine insulin were incubated in the absence (A) or presence (B–H) of 32 nM of guinea pig antiserum to porcine insulin for 3.5 days, cross-linked with DSS, denatured, reacted with succinylavidin, and subjected to SDS-PAGE under nonreducing conditions. The insulin derivatives used and their concentrations were as follows: A and B, 54 nM 125I-insulin (5,56 x 10^6 cpm/ml); C–E, 10 nM 125I-insulin with the following concentrations of nonradioactive N'Rm-biotinylinsulin: 50 nM (C), 100 nM (D), 200 nM (E); F, 44 nM insulin with 86 nM N'Rm-biotinylinsulin (1.77 x 10^6 cpm/ml); G and H, 125I-N'Rm-biotinylinsulin at 133 nM, 1.13 x 10^6 cpm/ml (G), and 43 nM, 1.77 x 10^6 cpm/ml (H). The labels at the right indicate the presumed migration positions for complexes of IgG, insulin (I), biotinylinsulin (IB), and succinylavidin (A). The rationale for this protocol is that binding of the radiochemical label is limited by dissociation of 125I-insulin from the receptor. As expected, a time-dependent decrease in the density of radioactive bands was produced by dilution of radiochemical label, since the rate of dilution of radiochemical label is limited by dissociation of 125I-insulin from the receptor. Comparison of the lanes with and without succinylavidin, however, failed to indicate definitively the presence of substantial amounts of receptor cross-linked to both probes, although treatment with succinylavidin again produced a barely detectable decrease in the density of the 290-kDa band and a slight increase in the density of the 340-kDa band which may be indicative of the presence of a small amount of receptor cross-linked to both probes. Similar studies with solubilized receptor which had been purified on immobilized WGA also failed to yield the molecular weights reported in this work are only nominal values deduced from the mobilities of protein standards. The nominal values obtained for the molecular weights for the three forms of the insulin receptor correspond to previously reported values (see Ref. 2).

D. T. Pang and J. A. Shafer, unpublished results.
definitive evidence for the binding of a second molecule of insulin to the receptor.

It might be argued that our failure to demonstrate the binding of a second molecule of insulin to the receptor was due to a low cross-linking efficiency. For example, if the cross-linking efficiency were only 1%, the intensity of bands of increased molecular weight corresponding to receptor cross-linked to both probes would be at most 1% of that of the bands corresponding to receptor cross-linked to only probe 1, in spite of the fact that all of the receptor might have been complexed to both probes prior to cross-linking. In such a case the bivalent character of the receptor would have been overlooked. To rule out this possibility, cross-linking efficiencies were determined. Cross-linking efficiency (CE) was evaluated from the relationship,

$$CE = 1 - \frac{(A - B)}{(C - D)} \times 100$$  \hspace{1cm} (1)

where $A$ is the total amount of $^{125}$I-N-$^{829}$-biotinylinsulin bound to the receptor (both covalently and noncovalently) after cross-linking and $B$ is the amount of $^{125}$I-N-$^{829}$-biotinylinsulin bound to receptor after incubation of cross-linked receptor with excess insulin (50 ng/ml) so as to displace specifically bound $^{125}$I-N-$^{829}$-biotinylinsulin which was not cross-linked. This incubation with excess insulin was carried out at pH 6 and 37 °C, conditions which should favor efficient displacement of noncovalently bound insulin (13). $C$ and $D$ are the amounts of $^{125}$I-N-$^{829}$-biotinylinsulin bound to the receptor without cross-linking. For determination of $D$, the complex was treated with excess insulin as described for the cross-linked complex, and for determination of $C$ it was not. Thus, the ratio $(A - B)/(C - D)$ is the fraction of specifically bound material which was not cross-linked. Cross-linking efficiency was also estimated using Equation 2,

$$CE = \frac{B - E}{C - F} \times 100$$  \hspace{1cm} (2)

where $E$ and $F$ are the amounts of $^{125}$I-N-$^{829}$-biotinylinsulin bound to receptor when $^{125}$I-N-$^{829}$-biotinylinsulin together with excess unlabeled insulin (10 μg/ml) was incubated with receptor. $E$ and $F$ correspond to determinations made with (E) and without (F) cross-linking. After cross-linking, sample $E$ was treated with excess insulin in a manner similar to sample B. The methods of Equations 1 and 2 gave similar values for cross-linking efficiency (52 ± 6%) which did not vary systematically with insulin concentration (Table I). Similar determinations of cross-linking efficiency wherein $^{125}$I-insulin was used in place of $^{125}$I-N-$^{829}$-biotinylinsulin yielded a somewhat higher average cross-linking efficiency of 59 ± 9% (Table I).

At the high insulin concentrations of 16-38 nM (Table I) almost all (>95%) of the insulin-binding sites ($K_a$ = 0.6 nM) should have been saturated. At the lowest insulin concentrations of 0.06 and 0.2 nM (Table I), the receptor which contained 3.5 nM insulin-combining sites must have been less than 2 and 6% saturated. If the receptor had two high affinity sites for insulin, more than 90% of the receptor should have been complexed with two molecules of insulin at the high insulin concentrations, whereas at the low insulin concentrations more than 97% of the receptor which contained bound insulin should have been bound to a single molecule of insulin.

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<th>Probe concentration</th>
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<td>Probe</td>
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<td>$^{125}$I-N-$^{829}$-Biotinylinsulin</td>
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<td>Equation 1</td>
<td>53</td>
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<td>Equation 2</td>
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<td>Average</td>
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<td>$^{125}$I-Insulin</td>
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The similar cross-linking efficiencies observed at low and high concentrations of insulin (Table I) indicate that if the 1:2 complex between receptor and insulin is the predominant form at the high insulin concentrations, the insulin molecules in this complex must be cross-linked to the receptor just as efficiently as those in the 1:1 complex. Thus, it is difficult to attribute our failure to trap the 1:2 complex to an inability to cross-link both insulin molecules in this complex.

If all of the receptor were bound to both probes prior to cross-linking, the average values of 59 and 52% for the cross-linking efficiency of probes 1 and 2 predict that the fraction of receptor cross-linked to no probe, only probe 1, only probe 2, and both probes 1 and 2 would be 0.20 (0.41 x 0.48), 0.28 (0.59 x 0.48), 0.21 (0.41 x 0.52), and 0.31 (0.59 x 0.52), respectively. The autoradiograms, however, can indicate only the presence of receptor labeled with probe 1 or receptor labeled with both probes 1 and 2. These species should be present in roughly equivalent amounts (0.28 versus 0.31) and should be detectable on SDS-PAGE. To demonstrate that the presence of such a mixture would have been detected, a 50:50 mixture of receptor cross-linked to ¹²⁵I-insulin and receptor cross-linked to ¹²⁵I-N⁶⁸⁵-N₂⁵I-biotinylinulin was subjected to SDS-PAGE in the presence (+) and absence (−) of succinylavidin. In this experiment, receptor cross-linked to ¹²⁵I-N⁶⁸⁵-biotinylinulin was used to model the behavior of receptor cross-linked to both probes. This model appears reasonable, since the absence of a single insulin molecule in the model should not significantly affect its electrophoretic mobility on SDS-PAGE. As shown in Fig. 3, the mixture yielded an autoradiogram which was distinguishable from those shown in Fig. 2 for receptor cross-linked to ¹²⁵I-insulin in the presence or absence of nonradioactive N⁶⁸⁵-biotinylinulin. Most notably, comparison of the (−) and (+) succinylavidin lanes for the model mixture reveals a decrease in density of the 290-kDa band and an increase in density in the region of the 340-kDa band as well as an increase in density in the region above the 340-kDa band upon treatment with succinylavidin.

The slight increase in density in the 340-kDa region and the slight decrease in density in the 290-kDa region of the (+) succinylavidin lanes for receptor cross-linked in the presence of probe 1 and probe 2 (Fig. 2), however, suggest the presence of a small amount of a 1:2 receptor:insulin complex. Unfortunately, the correspondence between the spacing of the 290-, 320-, and 340-kDa bands and the change in electrophoretic mobility which resulted from treatment with succinylavidin made it infeasible to quantify the presence of small amounts of receptor cross-linked to two insulin molecules. To circumvent this problem the 290-, 320-, and 340-kDa bands obtained upon SDS-PAGE of cross-linked material which had not been treated with succinylavidin were removed from the gel, separately treated with succinylavidin, and subjected to SDS-PAGE. Autoradiograms of the gels shown in Fig. 4A indicate the presence of a small amount of receptor which was cross-linked to both probes, as reflected by presence of secondary bands of decreased electrophoretic mobility above each of the primary bands (Fig. 4A). As expected, samples cross-linked in the presence of only ¹²⁵I-insulin (A) yielded only the major band and samples cross-linked in the presence of only ¹²⁵I-N⁶⁸⁵-biotinylinulin (D) yielded only the minor band.

To test our ability to quantify the relative amount of the two bands, receptor cross-linked in the presence of only ¹²⁵I-insulin (sample A of Fig. 2) and receptor cross-linked in the presence of only ¹²⁵I-N⁶⁸⁵-biotinylinulin (sample D of Fig. 2) were mixed (prior to electrophoresis) in various ratios to model the behavior of a mixture containing receptor cross-linked to probe 1 and receptor cross-linked to both probes. Fig. 4B illustrates the autoradiogram obtained by removing the 290-kDa bands after SDS-PAGE in the absence of succinylavidin and subjecting these samples to SDS-PAGE after treatment with succinylavidin. As expected, the relative densities of the two bands reflected the composition of mixture.

When receptor was cross-linked in the presence of both probes, the density of the primary band relative to that of the secondary band was similar for the 290-, 320-, and 340-kDa species. An average value of 11.3 (±1.4) was obtained for the ratio of densities of the primary and secondary bands when receptor was cross-linked after equilibration with 6 nM probe 1 and 20 nM probe 2. Equation 3 can be used to relate the relative band density D to the concentration of receptor cross-linked to one molecule of probe 1 ([(RI*)],), two molecules of probe 1 ([RI*]_2), and one molecule each of probe 1 and probe 2 ([(RI*IB)]).

\[ D = \frac{[RI*]}{[RI*IB]} \]  

(3)

The concentrations of the various complexes prior to cross-linking (as indicated by square brackets without the subscript x) can be related to their concentrations after cross-linking by Equation 4.

\[ \frac{[RI*]}{[RI*IB]} = \frac{CE1(CE1')(CE2')[(RI*)]}{(CE1')(CE2')(RI*IB)} \]  

(4)

where CE1 and CE2 are efficiencies for cross-linking probes 1 and 2 in the 1:1 receptor-insulin complex. The primes denote efficiencies for cross-linking a probe molecule in the 1:2 receptor-insulin complex. Since probe 1 (I') and probe 2 (IB) have the same affinity for the receptor, the following relationships should hold at equilibrium.

\[ \frac{[I']*}{[IB]} = \frac{[RI*]}{[RI*IB]} = \frac{[RI*]}{[RI*IB]} = \frac{[RI*]}{[RI*IB]} \]  

(5)

where the subscript 0 denotes the total concentration of added probe and [RB] represents the concentration of receptor which is complexed to two molecules of probe 2. If CE2' is set equal to 52% (the average value listed in Table I for probe 2) and if CE1' = CE1, Equations 5 and 6 yield a value of 4.8 for the ratio [RI*]/[RI*IB]. This ratio together with Equations 7–9 yielded a value of 80 nM for the equilibrium constant (K_e) for dissociation of insulin from receptor complexed with two molecules of insulin. This value is considered to be only a rough estimate which should be checked by other methods, since nonspecific cross-linking and an error in the assumption that CE1' = CE1 may have introduced an error in our
**Fig. 3 (left).** Model system for the effect of succinylavidin on the electrophoretic mobility of a mixture of 1:1 and 1:2 receptor-insulin complexes. Equal volumes of samples A and D in Fig. 2 were mixed and analyzed without (−) and with (+) treatment with succinylavidin using SDS-PAGE.

**Fig. 4 (right).** Effect of succinylavidin on the electrophoretic mobility of separated 290-, 320-, and 340-kDa forms of cross-linked complexes. A, samples A, B, D, F, and I in Fig. 2 were first run in SDS-PAGE without treatment with succinylavidin. The gel was stained, destained, air-dried between two cellophane membranes, and finally autoradiographed. With the autoradiogram as a guide, the three receptor bands (290, 320, and 340 kDa) were excised from the gel. Each gel band was further cut into smaller strips and placed in a sample well of a second slab gel; then 50 μl of 0.25 mg/ml of succinylavidin in 0.5% SDS nonreducing sample buffer was added to the well. After SDS-PAGE, the gel was stained, destained, dried, and autoradiographed. The autoradiogram of this gel is depicted here. The molecular weights of the isolated receptor forms used are shown at the bottom of the figure. The amounts of samples used were varied to optimize visualization of all samples in one exposure. B, samples A and D of Fig. 2 were mixed (prior to electrophoresis) in the following ratios: 1:0 (1), 0:1 (2), 1:1 (3), 2:1 (4), 4:1 (5), and 8:1 (6) and subjected to SDS-PAGE, as described in A. Following SDS-PAGE in the absence of succinylavidin, the 290-kDa bands were excised and analyzed in the second run in the presence of succinylavidin. The arrow indicates the position of a 290-kDa protein, estimated from standards. Although not shown, the 320- and 340-kDa species gave the same result. The relative densities of the major and minor bands were within 10% of that expected from the ratio of samples A and D used.

**DISCUSSION**

The use and proposed use of biotinylated derivatives of insulin and other hormones to purify and localize hormone receptors are well documented (e.g., Refs. 14-24). The DPL protocols reported here and previously (1) provide a new application of the biotin-avidin interaction in studies of hormone receptors. In the DPL method, ligand derivatives are used as probes to facilitate determination of the presence of 1:1 and 1:2 complexes between a protein and a ligand. The DPL method as presently applied exploits the very strong interaction between biotin and succinylavidin to alter the electrophoretic mobility of receptor on SDS-PAGE and thereby facilitate detection of doubly substituted receptor. It should be possible, however, to devise DPL protocols which do not use biotin and avidin to determine the presence of doubly substituted receptor.

There are a few aspects of the application of the biotin-avidin interaction in the present DPL protocol which should be noted. (a) Succinylavidin was used in place of avidin, because avidin precipitated in the SDS-containing sample buffer and did not enter the gel under the conditions used for the SDS-PAGE, whereas this complication did not occur with succinylavidin. Succinylavidin has been used in place of avidin in other studies where the purpose of the replacement was to minimize nonspecific interactions between cationic avidin and anionic cell surface proteins (e.g., Refs. 19-22). (b) The receptor was denatured by heating in 0.625% SDS prior to its determination of $K_d$.

\[
K_d = \frac{[R^*][IB]}{[R^*][IB]} 
\]

\[
[IB] = [IB]_0 - [RIB] - 2[RIB_2] - [R^*[IB]] 
\]

\[
[IB] = [IB]_0 - [R^*][IB] - [R^*[IB]] \left( 1 + \frac{2[IB]}{[I^*]} \right) 
\]

\[
[R^*] + [RIB] + 2[R^*[IB]] + [R^*][IB] + [RIB_2] = 2.5 
\]

\[
\left( 1 + \frac{[IB]}{[I^*]} \right) [R^*] + [R^*[IB]] \left( 1 + \frac{[IB]}{[I^*]} \right) = 3.5 
\]
reaction with succinylavidin in 0.5% SDS. The 0.5% SDS probably does not denature the succinylavidin, since it does not disrupt the interaction between succinylavidin and N\textsubscript{biotinyl} insulin (1). (c) Interaction with succinylavidin resulted in a change in electrophoretic mobility which was substantially less than that expected from the molecular weight of succinylavidin (M\textsubscript{s} = 63,000). When succinylavidin reacted with the receptor, the decrease in mobility corresponded to that expected for attachment of a 20- to 30-kDa denatured protein to the receptor. In the model system with the insulin antibody, reaction with succinylavidin resulted in a decrease in mobility corresponding to that expected for a denatured protein with a mass of 30-40 kDa. The discrepancy between the observed changes in electrophoretic mobility and those expected for binding of a denatured protein has been attributed in part to the existence of bound succinylavidin in an undenatured form (1).

The results of the DPL experiments indicate that WGA-purified insulin receptor from human placenta binds only one molecule of insulin with a high affinity. The equilibrium constant for dissociation of insulin from the 1:1 receptor-insulin complex is not very different from the value of 1.4 nM reported for the K\textsubscript{d1} for dissociation of insulin from solubilized receptor from human placenta by Kohanski and Lane (10), who obtained linear Scatchard plots. Using their methods we obtained a value of 0.6 nM for K\textsubscript{d1} for the WGA-purified receptor.\textsuperscript{3} Our finding that the equilibrium constant for dissociation of insulin from the 1:2 receptor-insulin complex is about 80 nM indicates that the receptor has a much reduced affinity for a second molecule of insulin. Extreme negative cooperativity between two insulin-binding sites on a receptor molecule or the existence of a second low affinity insulin-binding site could account for reduced affinity of the insulin receptor for a second molecule of insulin. Such effects, however, should not give rise to curvilinear Scatchard plots at insulin concentrations less than 20 nM, since production of a curvilinear Scatchard plot by a negatively cooperative system would require saturation of the second insulin-binding site, and, therefore, insulin concentrations considerably higher than 80 nM. Thus, it is conceivable that the curvilinear Scatchard plots reported for the binding of insulin to insulin receptor (at several states of purity) from human placenta (25) do not reflect interaction between the receptor and a second molecule of insulin, but instead are a consequence of the presence of receptor populations with different equilibrium constants for formation of the 1:1 complex. It is also possible for experimental artifacts to have given rise to curvilinear Scatchard plots. Several of the experimental problems associated with determination of data for Scatchard plots have been systematically investigated by Kohanski and Lane (10) in their studies of the binding of insulin to solubilized insulin receptor from human placenta. Interestingly, these investigators, using proper precautions to minimize experimental artifacts, obtained linear Scatchard plots (K\textsubscript{d1} = 1.4 nM at pH 7.5, 4 °C) at insulin concentrations ≤8.0 nM. Siegel et al. (26) also obtained linear Scatchard plots for highly purified placental receptor when the insulin concentration was in the range of 0.01–5 nM. At supraphysiological insulin concentrations the Scatchard plots displayed marked curvature. Additional information (including a more exact value for K\textsubscript{d1}) will be needed, however, before we will be able to determine the extent to which curvilinear Scatchard plots for the binding of insulin to its receptor from human placenta (25, 26) and other sources areartifactal, reflect the existence of a heterogeneous population of receptors (with different binding constants for the first insulin molecule), or reflect weak binding of a second molecule of insulin to the receptor.

Although it is not feasible at present to determine the relative contribution of each of these factors to the curvilinear character of a Scatchard plot, there is a simple test to determine whether a curvilinear Scatchard plot can be attributed entirely to negatively cooperative interactions between two binding sites of a bivalent receptor. The adsorption isotherm for such a system is given by the equation

\[ \frac{[L]_0}{[R]} = \frac{[R]L + 2[R]L^2}{[R]L + [R]^2 + [R]} = \frac{K_{d1}[L] + 2[L]^2}{K_{d1}[L] + [L]^2 + K_{d1}K_{d2}} \]

where [L\textsubscript{0}] and [L] represent the concentration of bound and free ligand, K\textsubscript{d1} and K\textsubscript{d2} are the formal equilibrium constants for dissociation of an insulin molecule from RL\textsubscript{1} and RL\textsubscript{2}, respectively, and [R] is the total molar concentration of receptor. The concentration of receptor [W] is usually expressed in arbitrary units which are proportional to [R], i.e.

\[ [R] = [W]n_o/2 \]

where n\textsubscript{o} is the molar amount of binding sites per unit amount of receptor. Thus we may write

\[ \frac{[L]_0}{[W]} = r = 0.5 n_o \frac{K_{d1}[L] + 2[L]^2}{K_{d1}[L] + [L]^2 + K_{d1}K_{d2}} \]

Using the approach of Simms (27), the right-hand side of Equation 12 can be replaced by a function which is simpler to use in data analysis, i.e.

\[ r = 0.5 n_o \frac{G_1 + G_2 K^{-1}}{G_1 + [G_1] + n_1[G_1]} \]

where K = G\textsubscript{1} + G\textsubscript{2}, K\textsuperscript{-1} = G\textsuperscript{1} + G\textsuperscript{2}\textsuperscript{-1}, and n\textsubscript{1} = n\textsubscript{2} = 0.5 n\textsubscript{o}. Examination of Equation 13 reveals that the adsorption isotherm of a bivalent negatively cooperative system is mimicked by the adsorption isotherm for an equimolar mixture of independent noninteracting sites with dissociation constants G\textsubscript{1} and G\textsubscript{2}. Although most curvilinear Scatchard plots for the binding of insulin to its receptor can be fit by Equation 13 (e.g. see Ref. 28), we are unaware of any data which yield equivalent values of the number of high and low affinity sites (n\textsubscript{1} and n\textsubscript{2}) as would be required if the curvilinear character of the Scatchard plot were due entirely to negatively cooperative interactions between two binding sites of a bivalent receptor. Experimental artifacts, receptor heterogeneity, the presence of effectors, and the formation of receptor aggregates with interacting insulin-binding sites\textsuperscript{3} are among the possible contributors to the inequality in the number of high and low affinity insulin-binding sites.

The finding that insulin receptor may bind only one molecule of insulin in the range of insulin concentrations used to determine adsorption isotherms and Scatchard plots must be taken into account to estimate correctly the functional purity of preparations of insulin receptor from its M\textsubscript{r}, protein content, and its capacity to bind insulin. For the receptor from human placenta, it would be most reasonable to estimate the molar concentration of receptor from the concentration of tight binding sites as determined from a linear Scatchard plot obtained at insulin concentrations less than 7 nM, since the DPL studies reported here indicate that the receptor from human placenta binds only one molecule of insulin in this concentration range.

It is important to note that although substantial concentra-

\textsuperscript{3} If such aggregates form with the WGA-purified receptor, they are resistant to cross-linking with DSS, since significant quantities of high molecular weight material which could be attributed to cross-linked receptor aggregates were not observed.
tions of a 1:2 receptor:insulin complex must exist for negative cooperative interactions to cause curvature in a Scatchard plot, very low concentrations of a 1:2 receptor:insulin complex could account for the observation (e.g. Refs. 13, 28, and 29) that the rate of dissociation of insulin from its receptor is increased with increasing concentrations of insulin. Insulin-enhanced dissociation of receptor-bound insulin has been presented (13, 29) as kinetic evidence for the existence of negative cooperativity between two discrete insulin-binding sites on a single receptor molecule. In this model negative cooperative interactions in the doubly substituted receptor are envisaged to account for the observed increased rate of dissociation of insulin at high insulin concentrations, where the concentration of a rapidly dissociating 1:2 complex would be increased. The fraction of 1:2 receptor-insulin complex need not be high, however, to produce the observed enhanced rates of dissociation, since rate enhancements could be produced via a reaction pathway which involved as an intermediate a very low steady state concentration of insulin receptor which was bound to two molecules of insulin.

The realization that the concentration of doubly substituted receptor may well be very low at insulin concentrations where interactions between insulin and its receptor are usually studied provides an explanation for the heretofore puzzling observations of Pollet et al. (28) that (a) when complexes between receptor and insulin are diluted into insulin-free medium, the rate of dissociation of insulin from its receptor is independent of the degree of saturation of insulin-binding sites as indicated by the adsorption isotherm, and (b) at insulin concentrations where the experimentally determined adsorption isotherm indicates saturation of the receptor, substantial enhancements in the rate of dissociation of insulin from the receptor can be realized by further increases in insulin concentration. If receptor-bound insulin with a 1:2 stoichiometry in the concentration range where the adsorption isotherm was determined, the adsorption isotherm should reflect the degree of saturation of both insulin-binding sites. If such were the case, the observed rate constant for dissociation of insulin from the receptor should have increased with increasing receptor saturation when receptor-bound insulin was diluted into insulin-free medium. Furthermore, insulin concentrations beyond that necessary to saturate the receptor should have produced further increases in the observed rate constant for dissociation, since the maximum rate constant corresponding to that for dissociation of insulin from the 1:2 complex should have been observed when the receptor was saturated with insulin.

The DPL experiments reveal, however, that the adsorption isotherms (which were determined below 20 nM insulin) could reflect formation of only the 1:1 complex. Thus when complexes between insulin and its receptor are diluted into insulin-free medium, any trace of 1:2 complex which may be present would be expected to rapidly dissociate, leaving the bulk of the insulin to dissociate from the 1:1 complex via the unenhanced first order process. It is, therefore, not surprising that observation of an enhanced rate of dissociation of insulin from its receptor requires the continual presence of a substantial concentration of nonradioactive insulin to ensure formation of catalytic amounts of the 1:2 complex. Furthermore, increasing rates of dissociation with increasing insulin concent-

In summary the results presented here indicate that partially purified insulin receptor from human placenta has a high affinity for one molecule of insulin ($K_d \sim 0.6$ nM) and can bind a second molecule of insulin, but much more weakly than the first ($K_d \sim 80$ nM). Additionally, the results presented in this work appear to remove the inconsistency between the Scatchard plots for insulin binding and the kinetic evidence for negative cooperativity by indicating the existence of a second insulin-binding site whose low affinity for insulin suggests (a) the formation of catalytic amounts of a 1:2 receptor:insulin complex (with negative cooperative site-site interactions) could account for insulin-enhanced dissociation of receptor-bound insulin and (b) that the curvilinear character of the Scatchard plots reported for binding of insulin to its receptor reflects other factors, such as receptor heterogeneity, in addition to negative cooperativity in the 1:2 receptor:insulin complex.

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