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Permalink
https://escholarship.org/uc/item/85q75574

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Publication Date
2015-11-24

Peer reviewed
The Insulin Receptor

STRUCTURAL BASIS FOR HIGH AFFINITY LIGAND BINDING*

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(Received for publication, December 19, 1986)

The insulin receptor has a heterotetrameric subunit structure of the type (α2β2), and all subunits are linked into a complex by disulfide bonds (1–4). Based on their susceptibility to reductants, these bonds have been grouped into class I disulfides and dissociation of the tetrameric receptor into the dimeric αβ form. The αβ receptor halves exhibit a reduced affinity for insulin binding (Boni-Schnetzler, M., Rubins, J. B., and Pilch, P. F. (1988) J. Biol. Chem. 261, 15281–15287). Kinetic experiments reveal that reduction of class I disulfides is a faster process than the loss of affinity for ligand, indicating that events subsequent to reduction of interhalf disulfides are responsible for the affinity change. We show that a third class of α subunit intrachain disulfides is more susceptible to reduction at pH 7.6 than at pH 8.5 and appears to form part of the ligand binding domain. Reduction of the intrachain disulfide bonds in this part of the α subunit leads to a loss of insulin binding. Modification of this putative binding domain by dithiothreitol can be minimized if reduction is carried out at pH 8.5. When the insulin receptor in placental membranes is reduced at pH 8.5, the receptor's affinity for insulin is not changed when binding is measured in the membrane. However, the Kᵢ for insulin binding is reduced 10-fold when αβ receptor halves are subsequently solubilized. Scatchard analysis of insulin binding to reduced or intact receptors in the membrane and in soluble form together with sucrose density gradient analysis of soluble receptors suggests that αβ receptor halves remain associated in the membrane after reduction, but they are dissociated upon solubilization. We interpret these results to mean that the association of two ligand binding domains, 2 αβ receptor halves, is required for the formation of an insulin receptor with high affinity for ligand.

The insulin receptor has a heterotetrameric subunit structure of the type (α2β2), and all subunits are linked into a complex by disulfide bonds (1–4). Based on their susceptibility to reductants, these bonds have been grouped into class I and 2 disulfides (5). Class 1 disulfides link one αβ to another αβ and are readily accessible to reduction, whereas class 2 disulfides joining the α to the β subunit are virtually impossible to reduce without denaturing the protein. Several studies have suggested that the reduction of the class 1 disulfides is accompanied by a modulation of the binding function (5–9). The majority of these studies used receptors on plasma membranes. Depending on the origin of the membrane, either the binding affinity, the number of binding sites, or both change upon treatment with dithiothreitol (DTT) (5–8). More recently it has been shown for solubilized receptors from human placenta that conditions leading to reduction of class 1 disulfides also result in a loss of the number of binding sites (9). However, a loss of binding sites under these conditions could be indicative of a major structural perturbation of the binding domain. Thus, due to the variable results of these previous studies and the possibility that lipids (10) and/or receptor-associated membrane proteins (11, 12) may also modulate ligand binding, it has not been possible to determine unequivocally how class 1 disulfides participate in the ligand binding function of the receptor.

It has been shown that when the insulin receptor is cross-linked to [125I]insulin and then electrophoresed in the presence of various concentrations of DTT, the tetrameric holoceptor begins to migrate slower concomitant with the appearance of individual subunits (3). This decrease in receptor mobility in SDS gels as a function of DTT concentration is an indication of the unfolding of a domain rich in intrachain disulfides, as exists in albumin for example. The protein sequence of the insulin receptor predicted from the cDNA clone verifies the existence of such a domain in the α subunit (13, 14), and it seems highly likely that this domain participates in the ligand-recognizing function of the receptor. However, the existence of such a disulfide-rich region is potentially complicating with regard to employing chemical reduction protocols to probe receptor structure/function relationships.

We have recently utilized a mild reduction protocol employing low concentrations of DTT at mildly alkaline pH, conditions that favor the reoxidation of intrachain disulfides (15–17). In contrast, interchain disulfides such as the class 1 disulfides of the insulin receptor remain reduced, and functional receptor halves can be isolated (17). Scatchard analysis reveals that detergent-solubilized receptors treated with DTT in this protocol exhibit a lower binding affinity for insulin, but the total number of binding sites remains unchanged (17). These data show that there is no causal relationship between the reduction of class I disulfides and the loss of binding sites. In order to further define the role of the various classes of disulfide bonds in the modulation of insulin binding, we extended our use of mild reducing conditions. We find that the disulfide-rich region of the α subunit does indeed appear to comprise part of the ligand binding domain. On the other

* This work was supported in part by Grant DK 36424 from the United States Public Health Service. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† Recipient of a postdoctoral fellowship from the Juvenile Diabetes Foundation.
‡ Supported as a postdoctoral fellow by Grant AG-000115 from the United States Public Health Service.
¶ Recipient of a research career development award from the United States Public Health Service.

1 The abbreviations used are: DTT, dithiothreitol; EGF, epidermal growth factor; SDS, sodium dodecyl sulfate.
hand, the principal role of the class 1 disulfides appears to be to maintain the physical proximity of the two potential binding sites that are necessary for high affinity insulin binding.

**EXPERIMENTAL PROCEDURES**

**Preparation of Soluble Insulin Receptor**—Insulin receptor was purified from human placental microsomes as published previously (18, 19). Briefly, receptors were solubilized from membranes with 1% Triton X-100 in the presence of 1 mM phenylmethylsulfonyl fluoride for 1 h at 4°C. The soluble fraction was obtained upon centrifugation at 100,000 × g for 1 h and separated on a Sephacryl S-400 column. From the insulin binding peak, the glycoprotein fraction was isolated by adsorption to and elution from wheat germ agglutinin-agarose. The receptor preparations were finally equilibrated with 30 mM Hepes, pH 7.6, 0.1% Triton X-100, and 0.02% azide (buffer A) by dialysis. For the experiments described in Fig. 5, affinity purified insulin receptor was used. This receptor was obtained essentially as described previously (20) except that the receptor was eluted from the affinity resin with 50 mM sodium acetate, 1 mM NaCl, 0.1% Triton X-100, pH 5.0 (21). Fractions from the column were collected into tubes containing 0.5 mM Hepes, 0.1% Triton X-100, pH 7.6, which neutralizes the column eluate. The receptor preparation was desalted and concentrated on a small wheat germ agglutinin-agarose column. After washing the column with buffer A, the receptor was eluted with buffer A containing 0.3 M N-acetylglucosamine. Finally, the concentrated receptor was dialyzed overnight against buffer A.

**Insulin Binding and Receptor Cross-linking**—Insulin binding to soluble receptor was determined with a polyethylene glycol precipitation assay exactly as described (22). Insulin binding to receptors in membranes was done as follows: 50 μg of membrane protein was dispensed in a total volume of 200 μl of 30 mM Hepes buffer, pH 7.6, containing 0.1% bovine serum albumin and 60 fmol of [125I]-insulin. After a 1-h incubation at room temperature, 1 ml of ice-cold Hepes/bovine serum albumin buffer was added, and the samples were immediately centrifuged in a Microfuge for 5 min at 15,000 × g. The pellets were counted in a gamma counter. Non-specific binding was determined by adding 3.5 × 10−7 M unlabeled insulin and was less than 10% of the total binding under all assay conditions. [125I]-labeled insulin was cross-linked to receptor preparations according to Pilch and Czech (23) using 0.2 mM disuccinimidyl suberate.

**Reduction of Insulin Receptors into αβ Dimers**—Reduction of soluble insulin receptor was performed with 1.25 mM DTT and 75 mM Tris, pH 8.5 (17), or 20 mM DTT at pH 7.6 for the times indicated. In all protocols, the reaction was stopped by centrifuging the samples through 3 ml of a desalting gel (Bio-Gel P-60DG, Bio-Rad) which had been equilibrated with 30 mM Hepes, pH 7.6, 0.1% Triton X-100, and 0.05 M NaCl. The supernatant was aspirated for 5 min, and the samples added to the prespun columns were recovered upon centrifugation at 1000 × g for 4 min. To ensure that this procedure was effective in removing DTT, a mock reduction protocol was carried out in the absence of receptor and was spun as described above. The eluate from these incubations was either labeled insulin, and a binding assay was conducted. The eluate was without effect on insulin binding, thus confirming that insufficient DTT was present to reduce the disulfides of insulin and hence influence our data and its interpretation.

**Reduction of Insulin Receptors in Membranes**—Membranes of human placenta at a concentration of 3 mg/ml protein were treated with 1.25 mM DTT and 75 mM Tris, pH 8.5, for 30 min. The reaction was stopped by adding a 10-fold excess of cold Hepes buffer, pH 7.6. The membranes were centrifuged at 30,000 × g for 20 min, and the pellet was washed once. In some experiments, receptor reduction was performed in the presence of 1% Triton X-100 and 1 mM PMSF for 1 h on ice. The soluble fraction was added to wheat germ agglutinin-agarose and allowed to bind for 1 h on a shaker. The wheat germ agglutinin beads were subsequently washed and equilibrated with 30 mM Hepes, pH 7.6, 0.1% Triton X-100, and 0.02% azide. The sample was solubilized in the presence of 0.3 M N-acetylglucosamine. [125I]Protein A was used to elute the receptor from the wheat germ agglutinin-agarose, and the samples were immediately used for binding assays.

**Cleave of the Insulin Receptor with Chymotrypsin**—Affinity purified insulin receptor, cross-linked to 0.5 mM [B26-(3-[125I])-iodotyrosine]-iodotyrosine-containing myosin substate (0.2 mM), was chymotrypsin-digested in the buffer system of Cleveland et al. (24). The hormone-receptor complex was dissolved in 125 mM Tris, 0.5% SDS, 10% glycerol, pH 6.8, and heated at 100°C for 2 min. The chymotryptic digestion was performed at 37°C for 30 min with 50 μg/ml chymotrypsin and 8 μg/ml receptor. Laemml sample buffer was then added to give a final SDS concentration of 2% and proteolysis stopped by heating the sample at 100°C for 2 min. The sample was then divided into several aliquots which were treated with different DTT concentrations. After boiling an additional 2 min, these samples were electrophoresed on 13-15% polyacrylamide gels.

**Polycrylamide Gel Electrophoresis and Autoradiography**—All samples were treated for 5 min with 20 mM N-ethylmaleimide prior to addition of Laemml sample buffer (25). Electrophoresis in 3-10% SDS polyacrylamide gels was performed according to Laemmli (25). Autoradiographs were obtained upon exposure of the dried gels to Kodak X-Omat AR film in the presence of a Cronex Lightning Plus intensifying screen. 32P incorporation into receptor was quantitated by excising the bands and counting their Cerenkov radiation.

**Materials**—Monocomponent porcine insulin was a gift of the Eli Lilly Co., and iodinated to a specific activity of 100-150 μCi/μg using Na151I (from Amersham Corp. and [B26-(3-[125I])-iodotyrosine]- and [B26-(3-(1261))-iodotyrosine]-insulin were isolated from an iodination mixture by high performance liquid chromatography (26). The isomers were eluted isocratically from a Vydac C4 column (25 × 0.46 cm) with triethylammonium formate buffer, pH 6.0, containing 27.2% acetonitrile. The B26-moniodoinsulin was used in experiments involving cross-linking and the A14 isomer for all other experiments. [γ-32P]ATP was prepared from [32P]orthophosphate (Du Pont-New England Nuclear) using a Gammaprep kit from Porgena Biotech. Hepes and dithiothreitol were purchased from Research Organics. Electrophoresis reagents were obtained from Bio-Rad and disuccinimidyl suberate from Pierce Chemical Co. Chymotrypsin (Type VII, 1-chloro-3-tosylamido-7-amino-2-heptanone-treated) and all other reagents were purchased from Sigma.

**RESULTS**

**Measurement of Class 1 Disulfide Reduction**—We have previously shown that treatment of pure and/or partially pure soluble insulin receptor with 1.25 mM DTT and 75 mM Tris at pH 8.5 for 30 min results in the formation of αβ receptor h. We also show in the 5-6-fold higher affinity for insulin, while the total number of binding sites is maintained (17). If the reduction of class 1 disulfides is the sole cause for these affinity changes, reduction of these bonds should have the same kinetics as loss of ligand binding. In order to monitor the structure of the receptor during reduction protocols, we labeled the β subunit of the receptor by adding [32P]ATP in the absence of insulin as described under “Experimental Procedures,” and then reduced the receptor for various times. After blocking all free sulfhydryl groups with N-ethylmaleimide, we separated the receptor forms with nonreducing SDS-gel electrophoresis. The autoradiogram in Fig. 1 shows that essentially all the tetrameric receptor is reduced to the αβ form after 20 min, and there are no free αs and βs generated (see also Ref. 17). Therefore, this procedure does not lead to cleavage of all class 2 disulfides, and the determination of the relative amounts of tetrameric and dimeric receptor is a measurement of class 1 disulfide reduction. The same pattern of reduction shown in Fig. 1 is also obtained when affinity purified receptor is used and then analyzed by silver staining (data not shown). Thus, there was no indication that the class 1 disulfides of the phosphorylated form would have a different susceptibility to DTT than the nonphosphorylated form. The same finding was recently obtained by Wilden et al. (27).

Thus, the use of phosphorylated receptors to monitor the structure allows an accurate quantitation of the relative amounts of tetrameric and dimeric receptor in a given sample and is a more convenient alternative to silver staining.

The receptor preparations shown in Fig. 1 also contain insulin-like growth factor 1 receptors, and we use the ligand independent kinase function to label the β subunit of both receptor types. We therefore compared the reduction profiles of receptor samples which had been labeled in the presence of insulin to those labeled in the absence of insulin. Fig. 2

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shows that the kinetics of reduction are the same if we use insulin-induced autoposphorylation, which under the present conditions is specific for the insulin receptor, or if we use basal phosphorylation, which also labels the insulin-like growth factor 1 receptor. Using the basal kinase activity to follow the structural change during reduction therefore allows us to simultaneously monitor tracer ligand binding and to compare directly whether loss of binding and $\alpha\beta$ dimer formation occur with the same kinetics.

Comparison of the Kinetics of Reduction of Class 1 Disulfides with the Kinetics of Tracer Binding Loss—When soluble receptor is reduced with 1.25 mM DTT, 75 mM Tris at pH 8.5 for various times, the kinetics of the change in insulin binding reveal a characteristic profile (Fig. 3A, closed circles). During the first 5 min, tracer binding slightly increases and then steadily decreases. As previously shown by Scatchard analysis, the decreased binding after 30 min of reduction is the result of an affinity change (17). Fig. 3B shows the effect of these reduction conditions (closed circles) on the reduction of class 1 disulfides. These kinetics are characterized by a rapid decrease of the tetrameric receptor form (dashed line) during the first 10 min (see also Fig. 2) which then starts to plateau. The appearance of the $\alpha\beta$ form (solid line) mirrors the decrease of the tetramer. A comparison of the kinetics of binding loss to that of class 1 disulfide reduction reveals that the reduction of class 1 disulfides is a faster process than the loss of affinity, and these data suggest that events subsequent to class 1 disulfide reduction contribute to the loss of affinity for ligand. Two experimentally testable explanations come to mind for these results. The first is that we are observing effects of DTT on disulfides which are different from class 1 disulfides, i.e. on the intrachain disulfide cluster of the $\alpha$ subunit. The second possibility is that dissociation of two $\alpha\beta$ receptor halves subsequent to class 1 disulfide reduction is responsible for the reduction of binding. Of course, both
phenomena might occur simultaneously.

Effect of pH during Reduction on the Kinetic Parameters—From our previous studies we know that for complete reduction of the tetrameric to the dimeric receptor form at pH 7.6, 20 mM DTT is required, and this treatment in our hands results in a substantial loss of binding (17). Kinetic analysis under these reduction conditions (Fig. 3, open circles) shows that the kinetics of class 1 disulfide reduction are the same as if 1.25 mM DTT, 75 mM Tris at pH 8.5 is used (Fig. 3B). The apparent decrease of αβ halves with prolonged incubation in 20 mM DTT is the result of loss of 32P label rather than loss of receptor (9, 17). However, the kinetics of binding loss is substantially different if the reduction is carried out at the high rather than at the low pH (Fig. 3A). At the lower pH, insulin binding is diminished faster and is at all time points lower than if reduction is carried out at the higher pH. The finding that two different reduction conditions can be employed that reduce class 1 disulfides with the same efficiency and kinetics while having distinct effects on ligand binding support the notion that there is a third class of disulfides structurally involved in insulin binding. We think this third class of disulfides represents the intrachain cluster previously discussed, and we show (see below and Fig. 5) that a disulfide-containing receptor fragment, covalently linked to bound insulin, can be isolated following proteolysis and may therefore be close to or part of the receptor’s ligand binding region.

Migration of αβ Receptor Halves in SDS—We always observe that αβ receptor halves that have been reduced with 20 mM DTT at pH 7.6 migrate with less mobility than the small amounts of the αβ species that are present in unreduced receptor preparations. Soluble receptor reduced with 1.25 mM DTT and 75 mM Tris, pH 8.5, shows, in a minority of experiments, a different migration profile than the αβ species isolated from nonreduced receptor preparations. This mobility shift is illustrated in Fig. 4, where soluble receptor has been reduced (lane 4) or left intact (lane 3) and, following removal of the reductant, cross-linked to 125I-labeled insulin and separated by nonreducing gel electrophoresis. The αβ half receptor and its proteolytically derived form obtained upon reduction show a shift to a less mobile position when compared to the native αβ form seen in untreated receptor preparations. As noted above, such changes in SDS gel migration patterns are commonly observed with disulfide-rich proteins upon reduction and are the result of unfolding the three-dimensional structure of the protein. We do not know with certainty which receptor disulfide(s) are responsible for this mobility shift, but at least some may be on the α subunit (see below). Note that when the reduction of the receptor is carried out in the membrane at pH 8.5, 1.25 mM DTT (Fig. 4, lane 2), there is no increase in the apparent molecular weight of the receptor, indicating that most of the intrachain disulfides are unaffected in this experiment.

Insulin Binds in Proximity to a Proteolytically Derived Region of the α Subunit that Unfolds upon Exposure to Reducing Agents—Our postulate is that a third class of disulfides functions to form some part of the insulin binding domain. It is reduction of these disulfides that leads to a loss in ligand binding (Fig. 3) and to the mobility shift just described for the αβ half receptor under certain conditions. In an attempt to isolate the ligand binding region of the insulin receptor, we cross-linked 125I-insulin to affinity purified receptor using disuccinimidyl suberate and cleaved the complex with chymotrypsin. The resultant proteolytically derived receptor-ligand fragment, M, 50,000, was electrophoresed in the presence of the DTT concentrations indicated in Fig. 5. As depicted in the autoradiogram, we observe that the insulin-binding fragment does in fact substantially increase in mobility as the DTT concentration is increased. Since the reduction is performed in SDS, lower concentrations of DTT than those used to reduce native receptor change the migration profile of this fragment. The shift in mobility from about 50 to 46 kDa (compare lanes E and F) is most probably a result of the loss of the A chain of insulin because >80% of the cross-linking of insulin occurs via B chain reactions.2 However, both the residual 50-kDa fragment and the resultant 46-kDa piece migrate, as a function of DTT concentration, in a fashion very similar to albumin, a protein with 17 intrachain disulfides (28). It is also theoretically possible that this large mobility change could be a function of a single disulfide. In any case, our data suggest that insulin can be cross-linked to a part of its receptor that, like the αβ receptor half, shows a substantial mobility shift after reduction. These data are consistent with

2 S. Waugh, E. DiBella, and P. Pilch, unpublished data.
one or more disulfides being present in some proximity to the ligand binding region of the receptor. Note that in lane K, which shows the undigested insulin receptor, we observe a substantial amount of α-α cross-linking. This result is commonly observed (23, 29), and it does not interfere with our interpretation of the data.

Reduction of Insulin Receptors in the Membrane Does Not Result in Binding Loss, but This Loss Does Occur upon Receptor Solubilization—As shown in lanes 1 and 2 in Fig. 4, and in contrast to the results shown in lanes 3 and 4, αβ receptor halves obtained by reduction in membranes with 1.25 mM DTT at pH 8.5 do not show a shift in SDS gel mobility and typically run at the position of the native αβ. This result suggested to us that we might be able to separate the effects of DTT on class 1 disulfides from the effects on intrachain disulfides since the latter seem minimal under the conditions of Fig. 4, lane 2. Thus, we examined the effects of the milder reduction conditions (1.25 mM DTT, 75 mM Tris, pH 8.5) on insulin binding to receptors in placental membranes. Under conditions that give at least 75–80% reduction to receptor halves, but no measurable intrachain disulfide reduction (Fig. 4, lane 2), we see no alteration in insulin binding to reduced receptor as compared to intact receptor (Fig. 6A).

However, when receptor reduced in membranes was solubilized in Triton X-100, concentrated on wheat germ agglutinin-agarose, eluted, and subjected to insulin binding and Scatchard analysis, the high affinity insulin binding component was completely abolished when compared to unreduced receptor treated in the same fashion (Fig. 6B). The remaining binding is all low affinity, corresponding to a 10-fold reduction in the $K_d$ for insulin as compared to the high affinity component in the intact solubilized receptors (Fig. 6B). Note that the Scatchard plots performed on receptors in membranes are curvilinear, whereas after reduction and solubilization, the Scatchard plot is linear. One interpretation of this data (see “Discussion”) is that there is no interaction between the αβ halves once they are solubilized and hence no curvilinearity in the binding plot.

αβ Receptor Halves Are Dissociated upon Solubilization—We verified that reduction of receptors in membranes followed by solubilization leads to dissociation of receptor halves by using sucrose density gradient analysis. Receptors reduced in the membrane or left intact were solubilized with 1% Triton X-100 and bound to wheat germ agglutinin-agarose to lower the Triton X-100 concentration to 0.1%. 200 μl of eluates were centrifuged over 5-25% sucrose velocity density gradients exactly as described previously (17). The fractions were assayed by measuring tracer insulin binding. The dashed line in Fig. 6 shows the profile of the tetrameric receptor, which elutes in fractions 14–16. The small peak in fractions 20–22 corresponds to the αβ form. If reduced receptor is applied to a gradient, most of the binding activity is found in the fraction of the αβ form, and therefore αβ receptor halves are not associated by noncovalent interactions upon solubilization. The total tracer insulin binding to the reduced receptor sample is, as expected from the previous data, reduced. These findings support the notion that αβ receptor halves dissociate upon solubilization and as a result exhibit a lower affinity for ligand. Because it is essentially impossible to employ chemical
reduction protocols to achieve 100% reduction, the exact quantitation of affinity constants is problematic, but our qualitative results are consistent.

**DISCUSSION**

The primary translation product of the insulin receptor is a protein of approximate Mr, 210,000 (after glycosylation) that is subsequently cleaved into individual α and β subunits, and each αβ half is further assembled into a tetrameric holoreceptor form (αβββ) by disulfide linkage (30, 31). Thus, the insulin receptor contains duplicates of its two functional domains, those required for ligand binding and for kinase activity, respectively. In order to determine what significance this covalent duplication of domains has for the ability of insulin to enhance the receptor’s kinase activity, we developed a protocol for the gentle reduction of the disulfide bonds that link the receptor halves together (17). We found that this protocol had no apparent effect on the kinase domain of the receptor, and it diminished the receptor’s affinity for insulin by 3-6-fold, but it completely abolished the ability of insulin to stimulate kinase activity. In the present report, we have extended our studies using DTTeX to reduce two classes of disulfide bonds within the insulin receptor (17). We find that the lowered affinity for ligand seen after gentle reduction of interhalf disulfides reflects the intrinsically lower affinity of αβ receptor monomers for insulin. We also suggest that additional α subunit intrachain disulfides play a part in the ligand recognition function of the receptor. An insulin-receptor fragment can be isolated following proteolysis of the complex that may represent at least part of a ligand binding region.

We can rule out the possibility that it is simply chemical reduction of class 1 disulfides that is causative for the receptor’s reduction in affinity for insulin following DTTeX exposure based on several lines of evidence. First, the kinetics of class 1 disulfide reduction is faster than the kinetics of affinity loss. Therefore, events subsequent to reduction of class 1 disulfides are largely responsible for losses in binding affinity. Second, different reduction conditions can be used which result in the same kinetics of class 1 disulfide reduction but have profoundly different effects on the kinetics of the affinity decrease (Fig. 3). Third, when mild reduction conditions are applied to placental membranes, virtually complete reduction of class 1 disulfides is obtained with decreased binding affinity (Figs. 4 and 6). The last point shows that maintenance of the equivalent linkage between two αβ receptor halves is not an absolute requirement to form a receptor with high affinity insulin binding.

After exposure to DTTeX in the membrane, the receptor binds insulin in a manner indistinguishable from untreated receptor (Fig. 6A). The Scatchard plot of ligand binding to reduced receptor in the membrane is nonlinear and shows a high affinity ligand binding component (Fig. 6A), whereas binding to the solubilized αβ β half receptor exhibits a linear Scatchard plot and has no high affinity binding component (Fig. 6B). Based on kinetic experiments (32, 33) as well as determination of the stoichiometry of insulin binding (34), it has been suggested that the observed curvilinearity of insulin binding can be explained as a result of the negative cooperation between the two potential ligand binding sites of the holoreceptor. Binding isotherms to purified or partially purified receptors appear to show high affinity binding to half of the sites, consistent with the double label experiments of Pang and Shafer (34), demonstrating that there is only one high affinity insulin binding site/tetramer. Our data also support this model (Fig. 6B), and we postulate that the reduced receptors in the membrane remain associated and are therefore able to cooperate and exhibit nonlinear binding plots. After solubilization, the αβ halves are dissociated (Fig. 7) and exhibit linear Scatchard plots indicating no cooperativity between binding sites. Since we have not used affinity purified receptor in the studies of Fig. 6, we cannot determine unequivocally the ligand binding stoichiometry of αβ dimers and tetramers (see below, however).

It is not technically feasible to determine if reduced receptor halves remain physically contiguous in a membrane environment. We therefore have no direct data showing that the bulk of receptor halves remain noncovalently associated after reduction in the membrane, although we can observe some cross-linking of the reduced receptors into α-α complexes (data not shown). Previous work demonstrated that αβ receptor halves, reduced in cell membranes, can be reoxidized to the tetrameric form (5). This reoxidation process most certainly requires close proximity of two reaction partners. Furthermore, recent evaluations on the association state of proteins in the membrane revealed that if the excluded volume effects and the preoriented state is taken into account, the likelihood to form dimers in the membrane is 10^6 times higher than in solution and therefore a thermodynamically favored process (35). Finally, it has been shown by Sweet et al. (36) that the insulin receptor halves can indeed remain associated in low detergent concentrations under their experimental conditions (36). These investigators have more recently shown that the stoichiometry of insulin binding to dissociated and purified αβ dimers is 1:1 and that the dimers have a reduced affinity for insulin compared to intact receptor, consistent with our results presented here.

![Graph of cpm vs. fraction number](https://example.com/figure.png)

**Fig. 7.** αβ Dimers are dissociated upon solubilization. Dimeric (solid line) or tetrameric receptor (dashed line) was obtained upon solubilization of reduced or control membranes as described for Fig. 6B. 200 µl of samples were layered over 4 ml of a 5-25% sucrose density gradient containing 30 mM Hepes, pH 7.6, 0.1% Triton X-100, 0.02% azide, and 10% glycerol. Velocity sedimentation was carried out at 2 °C for 18 h at 190,000 × g. 100-µl fractions were collected. Specific binding of A14-labeled moniodoinsulin at tracer concentration, as determined by subtracting the binding in the presence of 3.5 × 10^{-9} M unlabeled insulin from the binding in the absence of unlabeled insulin, was plotted against the fraction number. The low fraction numbers correspond to the bottom of the gradient and the high numbers to the top.

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3 J. Pessin, personal communication.
The association of two identical ligand binding domains has been proposed as a mechanism for high affinity binding to nerve growth factor receptors (37, 38), and the existence of nerve growth factor receptor dimers has been demonstrated by chemical cross-linking (39, 40), which is in its overall structure analogous to the insulin receptor αβ half. We have recently isolated noncovalently associated EGF receptor dimers and analogues to the insulin receptor of cooperativity for insulin binding to native affinity for ligand than do EGF receptor monomers. Since subunit association may be a common regulatory mechanism for these proteins, as well as for others of this type.

Acknowledgment—We thank Aram Kilagian for his technical assistance.

Note Added in Proof—Since this manuscript was submitted, the data referred to in Footnote 3 (J. Pessin, personal communication) has been accepted for publication (Sweet, L. J., Morrison, B. D., and Pessin, J. E. (1987) J. Biol. Chem. 262, 6039–6046). The insulin receptor is not a proteolytically generated, disulfide-containing ligand-receptor fragment, and a useful tool with which to ascertain that ligand binding occurs in such a domain. The location of this fragment within the α subunit can be determined by microsequencing. Overall, considering the common structural and functional characteristics of the receptors noted above, it appears likely that the modulation of ligand affinity by receptor subunit association may be a common regulatory mechanism for these proteins, as well as for others of this type.

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