Reoxidation of the Class I Disulfides of the Rat Adipocyte Insulin Receptor Is Dependent upon the Presence of Insulin: The Class I Disulfide of the Insulin Receptor Is Extracellular†

Kenneth B. Chiacchia‡

Department of Biochemistry and Molecular Biology, Harvard University, 7 Divinity Avenue, Cambridge, Massachusetts 02138

Received October 27, 1987; Revised Manuscript Received April 19, 1988

ABSTRACT: Elements of the quaternary structure of the native and dithiothreitol- (DTT) reduced rat adipocyte insulin receptor have been elucidated by vectorial probing and subunit cross-linking. The charged reducing agents glutathione and β-mercaptoethylamine were used to reduce the class I disulfides of the receptor in intact adipocytes, demonstrating the extracellular location of the disulfide directly. This interpretation was confirmed by use of DTT as a reducing agent and the nonpermeant sulfhydryl blocking reagent Thiolyte MQ to prevent the reoxidation of the class I sulfhydryl groups which occurred when they were not blocked. It was found that the above reoxidation of the receptor is dependent on the concentration of insulin in the nanomolar range, not occurring measurably at 4 °C in its absence. Cross-linking studies with ethylene glycol bis(succinimidyl succinate) demonstrated that the α subunits could not be cross-linked to each other after reduction of the class I disulfides, suggesting that the interaction between the receptor heterodimers may be due primarily to the disulfide bonds.

The mammalian cell surface receptor for insulin is a heterotetrameric protein composed of two heterodimers (Massague et al., 1981; Massague & Czech, 1982; Pang et al., 1984). The αβ heterodimer is held together by both noncovalent interactions and disulfide linkages (Massague & Czech, 1982; Grunfeld et al., 1985). These disulfide bonds are highly resistant to reduction in the native state (Massague et al., 1980, 1981). The disulfide bonds which hold the two heterodimers together, on the other hand, are quite susceptible to reduction by dithiothreitol (DTT)[1] in millimolar concentrations (Massague & Czech, 1982), suggesting a more open, less tight interaction between the heterodimers. Reduction of this disulfide interaction in intact rat adipocytes was found not to affect insulin stimulation of [5-^{14}C]glucose uptake as measured by $^{14}CO_2$ release and has no inhibitory effect on the binding of insulin to either human placental, rat liver, or rat adipocyte membranes (Massague & Czech, 1982). Data concerning the effects of reduction of this "class I" disulfide on the receptor's tyrosine kinase activity (Tamura et al., 1984) are contradictory. Some groups have observed autophosphorylation of the receptor after reduction (Shia et al., 1983; Fujita-Yamaguchi & Kathuria, 1985; Pike et al., 1986; Sweet et al., 1986): the inability of the proteolytically cleaved $\alpha_2\beta\beta'$ receptor to autophosphorylate (Chang et al., 1984) and the isolation of inactive αβ heterodimer by Boni-Schnetzler et al. (1986) suggest that the interaction between the two heterodimers is required for kinase activity.

The theoretical importance of this question has been made evident by the recent elucidation of the primary structure of the insulin receptor (Ullrich et al., 1985; Ebina et al., 1985). The amino acid sequence deduced from the cDNA sequence indicates that the β subunit has only one transmembrane domain, and the α subunit has none. The latter prediction is supported by the finding that urea denaturation coupled with reduction will strip the α subunit from the membrane (Grunfeld et al., 1985). Thus, it is important to determine whether or not in the membrane the heterodimers of the reduced receptor (as determined by nonreducing PAGE) remain associated by noncovalent interactions and are still free to interact with each other to transduce the signal of insulin binding across the membrane. If not, the question of how a single transmembrane domain can transduce such a signal across the bilayer must be addressed. In the latter case, the question of why the receptor exists as a dimer in these tissues in the first place must be answered.

In this paper, the structure of the native and reduced rat adipocyte insulin receptor is partially elucidated. Membrane-impermeant reducing agents and sulfhydryl reagents were used to reduce the class I disulfide linkage and block the sulfhydryl groups in intact adipocytes, demonstrating that the class I linkage is extracellular. Of even more interest is the fact that the reoxidation prevented by these sulfhydryl reagents is, under all conditions tested, completely dependent on the presence of insulin in the nanomolar range.

Ethylene glycol bis(succinimidyl succinate) (EGS) was used to cross-link both the native and reduced insulin receptors. While the α subunits are readily cross-linked to form α_2 in the native receptor, no species of molecular weight greater than that of αβ were observed when reduced receptor was cross-linked, suggesting that the $(\alpha\beta)_2$ tetramers may dissociate upon reduction in the membrane. If this is true, then any noncovalent interaction which exists between the heterodimers would be a very weak interaction. These cross-linking experiments also may indicate that the α subunits contain the class I di-

† This research was supported by National Institutes of Health Grant AM27626 to G. Guidotti.

‡ Supported during the course of this work by National Institutes of Health Training Grant GM07598.

[1] Abbreviations: ATP, adenosine triphosphate; DSS, disuccinimidyl suberate; DTT, dithiothreitol; EGS, ethylene glycol bis(succinimidyl succinate); GSH, reduced glutathione; GSSG, oxidized glutathione; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NEM, *N*-ethylmaleimide; PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

0006-2960/88/0427-4894$01.50/0

sulfides, as they were the most readily cross-linked subunits, suggesting proximity.

Materials and Methods

Materials. Rats were obtained from Charles River Laboratories. Collagenase was purchased from Worthington and bovine serum albumin from Armour. Reducing agents, protease inhibitors (including *N*-ethylmaleimide), and SDS–PAGE molecular weight standards were obtained from Sigma. ^{125}I–Insulin, [^{3}H]glutathione, [^{14}C]sucrose, and [^{32}P]orthophosphate were supplied by New England Nuclear. [γ-^{32}P]ATP was synthesized with the Promega Biotech Gammaprep synthesis system. Cross-linking reagents were from Pierce. Thiolyte MQ [a brand name for (monobromotrimethylammonio)bimane] is a product of Calbiochem; other sulfhydryl blocking reagents were from Molecular Probes, Inc. The peroxide-free Triton X-100 used was Pierce Surfact-Amps X-100. Oxidized glutathione was from Mann Research Laboratories, electrophoresis reagents were obtained from Biorad, and X-ray film and intensifying screens were supplied by Kodak. Teflon filters used for the insulin binding assay were Millipore GVWP filters.

Rat Adipocyte Preparation. Adipocytes were isolated from male CD rats: for intact cell isolation 125–150-g rats were used; for plasma membrane preparation 175–250-g rats were used. The adipocytes were isolated in the manner of Resh et al. (1980).

Plasma Membrane Isolation. Plasma membranes were isolated by the method of Resh (1982). The homogenization buffer contained 10 mM *N*-ethylmaleimide (NEM) as a protease inhibitor and sulfhydryl blocking agent, except in the sulfhydryl blocking experiments, in which case proteolysis was inhibited by 10 mM phenylmethanesulfonyl fluoride and 0.5 mg/mL bacitracin, and in the receptor autophosphorylation, in which a cocktail of 300 μM PMSF, 1.5 μg/mL each of chymostatin, pepstatin, and leupeptin, 15 μg/mL benzamidine, and 15 KU/mL aprotinin was used.

Insulin Cross-Linkage. Purified plasma membranes were suspended to 1–3.5 μg/μL membrane protein in 100 μL of HEPES buffer (140 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM HEPES, pH 7.6) in the presence of 3.3–8.3 nM ^{125}I-insulin ($\sim$100 μCi/μg) and 1% (w/v) BSA for 20 h. Nonspecific binding was determined by a control sample containing 1.5 μM unlabeled insulin. Membranes were removed via centrifugation and then resuspended in 100 μL of HEPES buffer containing 91 μM disuccinimidyl suberate (DSS) and incubated for 15 min at 4 °C. The reaction was halted by dilution with HEPES buffer, and the membranes were centrifuged out. Protein determinations were done in the manner of Lowry et al. (1951).

Receptor Autophosphorylation. Membranes treated as described under Results and Discussion were solubilized by treatment at 0 °C for 30 min in a solution containing 3% peroxide-free Triton X-100, 2 mM PMSF, 1000 KU/mL aprotinin, and 50 mM HEPES, pH 7.6 (at approximately 3 μg of membrane protein/μL of solution). Unsolubilized material was removed by centrifugation for 6 min in a Beckman Airfuge, and the supernatants were diluted to 0.2% Triton with 50 mM HEPES, pH 7.6. PMSF and aprotinin were then added to 1 mM and 1000 KU/mL, respectively. Samples were quick frozen in dry ice/acetone and stored at −70 °C. Phosphorylation was carried out by thawing 45-μL aliquots of the frozen samples and adding 5 μL of 50 mM HEPES, pH 7.6, either with or without 1 μM porcine insulin (final insulin concentration 100 nM), incubating for 30 min at 25 °C, and then adding 0.25 volume of a solution containing 50 mM $MgCl_2$, 10 mM $MnCl_2$, 2.5 μM Na_2ATP, and 6 mCi/mL [γ-^{32}P]ATP. Phosphorylation was halted after 3 min by the addition of 5× Laemmli buffer containing 100 mM NEM, and the samples were then boiled for 30 s prior to analysis via nonreducing SDS–PAGE.

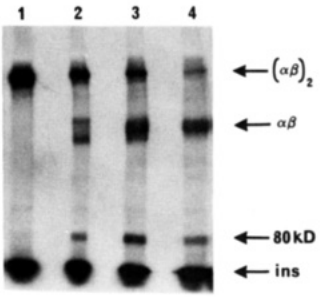

FIGURE 1: Charged reducing reagents can reduce class I disulfides of intact rat adipocytes. Aliquots (1 mL) of packed adipocytes were incubated in 3 mL of KRP/2% BSA buffer [140 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgSO_4$, and 10 mM Na_2HPO_4, pH 7.4, with 2% (w/v) BSA] in the presence of 0, 40, 80, and 160 mM GSH (lanes 1–4, respectively) for 10 min at 37 °C. GSH was removed by washing cells twice in fresh KRP/BSA, and then plasma membranes were isolated from the treated cells. ^{125}I-Insulin was cross-linked to these membranes, and then samples were loaded on a nonreducing 5% gel, 10 000 specific cpm per lane (219, 111, 197, and 309 μg of membrane protein, respectively). Shown is a 3-day autoradiogram of this gel.

Electrophoresis. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed in the manner of Laemmli (1970). For nonreducing conditions, the 2× sample buffer contained 20 mM NEM; for reducing conditions it was 2% (v/v) β-mercaptoethanol. Samples were boiled for 30 s before loading on the gel or storage overnight at −20 °C. NEM was found to be necessary to prevent reduction of the class I disulfides due to small amounts of reducing agents contaminating the sample buffer, a common difficulty in this system (Boyle et al., 1985).

Results and Discussion

Location of Class I Disulfides. The extracellular location of the class I disulfides was tested by reduction of intact adipocytes with the membrane-impermeant reducing agent glutathione (GSH). The ability of GSH to reduce receptor from the tetramer to heterodimer forms (Figure 1) indicates that the class I disulfides are both extracellular and in an aqueous environment. However, the possibility did exist that such large concentrations of GSH are necessary because either the GSH is oxidized to GSSG or the GSH must enter the cytoplasm. The concentration of GSH was tested with 5,5′-dithiobis(2-nitrobenzoate) (Ellman, 1959): it was found to be 94 ± 14% of the expected concentration (n = 2). This showed that the high concentrations of GSH required were not due to oxidation of the reagent.

To address the possibility that such large concentrations of GSH can partition into the cell, it was first useful to determine the ability of GSH to reduce the class I disulfide in isolated adipocyte plasma membranes. This property was tested by reducing plasma membranes isolated as under Materials and Methods with 0, 40, 80, and 160 mM GSH at 37 °C for 10 min. As can be seen in Figure 2, the efficacy of GSH for reducing the class I disulfides in membranes is similar to that in whole cells. This demonstrated that the large concentrations of GSH required are due to its poor ability to reduce the class I disulfides and not to the necessity of crossing the membrane barrier.

To further substantiate this interpretation, intact packed adipocytes were incubated with 100 mM [^{3}H]GSH at 37 °C.

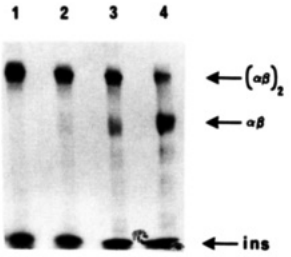

FIGURE 2: GSH reduction of class I disulfides in membrane is no more efficient than in adipocytes. Purified rat adipocyte plasma membranes were susptended at 3.7 μg/mL membrane protein in 100-μL aliquots of HEPES buffer (140 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgSO_4$, and 10 mM HEPES, pH 7.6) in the presence of 1% BSA and 0, 40, 80, or 160 mM GSH for lanes 1–4, respectively. They were incubated for 10 min at 37 °C, excess reducing agent was removed by centrifugation at 4 °C, and the reaction was halted by resuspending in 100 μL of HEPES buffer containing 1 mM NEM. The membranes were then bound and cross-linked to ^{125}I-insulin as described, and a 5% nonreducing gel was used to analyze them, loading 10000 specific cpm per lane. This corresponded to, respectively, 263, 306, 336, and 340 μg of membrane protein per lane: a 3-day autoradiogram is shown.

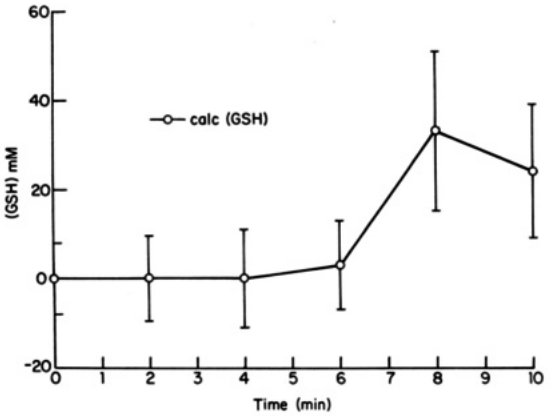

FIGURE 3: GSH leakage into cell is not sufficient to account for reduction of class I disulfides. Aliquots (2 mL) of packed adipocytes were incubated in 4 mL of KRP/2% BSA buffer at 37 °C in the presence of 100 mM [^{3}H]GSH (2.5 μCi/mL) and 10 mM [^{14}C]sucrose (1 μCi/mL). At the indicated times cells were separated from a 100-μL aliquot by centrifugation of the aqueous compartment through dinonyl phthalate, which was then frozen in dry ice/acetone. ^{3}H and ^{14}C radioactivity in the cell layer were simultaneously counted and used to calculate intracellular [GSH] assuming a cytoplasmic volume of 1 pL/cell (Resh et al., 1980). The [^{14}C]sucrose served as an internal control to measure the water volume trapped in the cell layer. Uncertainties shown are equal to ±1 standard error, $n = 4$.

At the times indicated in Figure 3, aliquots of cells were separated from the solution by centrifugation of the aqueous compartment through dinonyl phthalate and then frozen in dry ice/acetone. The cell layer was cut out and counted for ^{3}H radioactivity, which was used to calculate the intracellular [GSH] assuming a cytoplasmic volume of 1 pL/cell (Resh et al., 1980). As can be seen in Figure 3, the concentration of GSH in the cytoplasm for most of the incubation was negligible: even the concentrations of GSH present near the end of the incubation would only be able to reduce less than 20% of the receptor in purified plasma membranes, while 100 mM GSH was found to reduce ~50% of the receptor in both membranes (Figure 2) and intact cells (Figure 1). In addition to the similar efficacy shown by GSH in both membranes and whole cells, the interpretation that sufficient glutathione cannot enter the cell to cause reduction of the receptor is consistent with the findings of May (1985), which showed that glutathione–maleimide cannot cross the bilayer to block the sulfhydryl groups of cytoplasmic glutathione. Also, it was found

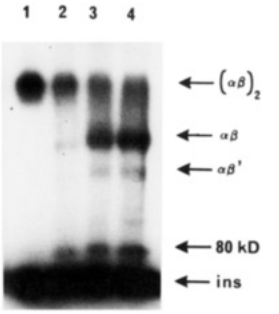

FIGURE 4: Reoxidation of receptor halves in the membrane is prevented by treating with non-membrane-permeant sulfhydryl blocking compound after reduction in adipocytes. Aliquots (1.5 mL) of packed adipocytes were incubated in 2 mL of KRP/2% BSA in the presence of 10 mM DTT for 10 min at 37 °C. Excess DTT was washed away, and then the cells were treated for 15 min at 37 °C in KRP/2% BSA containing 1 mM Thiolyte MQ (lane 3) or 1 mM NEM (lane 4). Lane 2 had no blocking reagent, and lane 1 was untreated. Plasma membranes were isolated as above, and after ^{125}I-insulin binding and cross-linking, they were loaded onto a 5% nonreducing Laemmli gel at 10000 cpm per lane, or 105, 259, 286, of 409 μg of membrane protein per lane (lanes 1–4, respectively). Shown is a 2-day autoradiogram.

that the positively charged reducing reagent β-mercaptoethylamine was able to reduce the class I disulfides at a considerably greater efficacy than GSH (data not shown); however, since it was not possible to obtain a radiolabeled β-mercaptoethylamine derivative, the entry of this compound into adipocytes was not determined.

The extracellular location of the class I disulfide was also demonstrated by showing that Thiolyte MQ, a membrane-impermeant sulfhydryl reagent (Kosower et al., 1979), blocks reoxidation of the reduced receptor (Figure 4). When not treated with either NEM or Thiolyte MQ (lane 3), the receptor was found to have reoxidized to tetramer (compare lane 2 with lanes 3 and 4 in Figure 4). On the other hand, Thiolyte MQ was able to prevent this reoxidation with a similar effectiveness to that of NEM (lane 4). This is in agreement with the hypothesis that the class I disulfides are in an aqueous, extracellular environment. Methods for distinguishing which subunit contains the class I disulfides are now being investigated.

Reoxidation of the Class I Disulfide. The reoxidation of reduced receptor was investigated in more detail. Plasma membranes were reduced with 5 mM DTT and then incubated overnight in the presence of ^{125}I–insulin and 0, 0.5, 5, and 10 mM GSSG (lanes 2–5 of Figure 5). The membranes were then cross-linked as described above. The insulin binding step contained 0.5 mM EDTA, to rule out the possibility that Zn^{2+} present in the insulin preparation (Blundell et al., 1972) may have been the cause of the apparent insulin effect: Zn^{2+} had no effect on reoxidation. Figure 5 shows the autoradiogram of the 5% nonreducing gel with these samples. Including 0.5 mM GSSG (added as free acid in 0.5 M HEPES, pH 7.6) in the overnight incubation at 4 °C with ^{125}I-insulin prior to cross-linking maximized the extent of reoxidation (see lane 3). At higher concentrations, GSSG inhibits dimerization, possibly through the formation of a trapped reoxidative intermediate (compare the apparent molecular weight of $\alpha\beta$ in lanes 4 and 6). Such a species of heterodimer of reduced molecular weight has been observed by Massague and Czech (1982).

Time courses of reoxidation with 0.5 mM GSSG in the presence and absence of ^{125}I-insulin are shown in panels a and b of Figure 6, respectively. Autoradiograms were made of these gels, and then gel slices corresponding to the visible bands

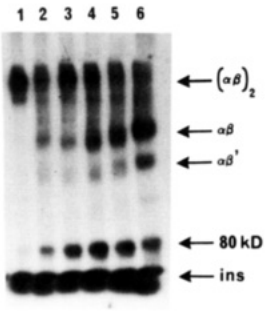

FIGURE 5: Low concentrations of GSSG facilitate reoxidation of receptor halves. Plasma membranes (3.0 μg/μL) in 100-μL aliquots of HEPES were reduced with 5 mM DTT for 10 min at 37 °C and then washed with fresh HEPES to remove excess DTT. They were then incubated overnight in 100 μL of HEPES/1% BSA containing 5.6 nM ^{125}I-insulin and 0, 0.5, 5, and 10 mM GSSG (in lanes 2–5, respectively). EDTA at 0.5 mM was present to chelate both the Zn^{2+} present in the insulin preparation (Blundell et al., 1972) and the Cu^{2+} present in the BSA preparation (Czech & Fain, 1972). Lane 1 is untreated receptor, and lane 6 was treated with 1 mM NEM after washing away DTT, to demonstrate the extent of reduction. After this incubation the membranes were cross-linked with DSS as described, and 6000 specific cpm was loaded into each lane of a 5% nonreducing Laemmli gel, or 66, 60, 97, 149, 178, and 106 μg of membrane protein in lanes 1–6, respectively: shown is a 2-day autoradiogram.

(tetramer, $\alpha\beta$ and $\alpha\beta'$, 80-kDa fragment, insulin, and the top of the gel) were counted for ^{125}I. Shown in Figure 6c are percentages of total counts in the tetramer band (normalized by dividing by the percentage in lane 1 of each autoradiogram, corresponding to untreated receptor) versus time. As can be seen, tetramer reappears with a half-time of about 3 h in the presence of insulin, while only a small decrease, possibly due to degradation, is seen in the absence of insulin. Two points of interest are as follows: (a) even in the absence of insulin (Figure 6b, lanes 2–6), there is a decrease of heterodimer with time, due either to aggregation or degradation; (b) as seen by Massague and Czech (1982), the presence of insulin appears to prevent the cleavage which produces the 80-kDa fragment (compare panel a with panel b of Figure 6). The former observation suggests that the dimer of heterodimers may play a role in preventing degradation of the receptor. The increase seen in material aggregated in the top of the gel at later time points (see lanes 4 and 5, Figure 6a) is balanced by a decrease in the counts found in the insulin band (data not shown), suggesting that almost all, if not all, of the aggregated material is insulin and not the receptor.

The failure of heterodimer to reoxidize in the absence of insulin is notable (Figure 6b). The fact that Massague and Czech (1982) obtained contradictory results is possibly due to the fact that their reoxidation was carried out at 25 °C, while that reported here was at 4 °C: perhaps the heterodimer does have a nominal capacity for reoxidation which is not apparent at 4 °C. Alternatively, the difference may lie in the cocktail of reduced and oxidized glutathione these researchers utilized for reoxidation. In fact when the membranes were treated as described above but at 25 °C, no reoxidation was seen in the absence of insulin. Presumably proteolytic loss of receptor (concurrent with an increase in the intensity of the 80-kDa band) under these conditions despite the addition of 0.2 mM PMSF (Massague & Czech, 1982) may have prevented reoxidation (data not shown). Trace amounts of DTT were unable to elicit reoxidation of the receptor in the absence of insulin, in contrast to the case of RNase (Anfinsen, 1973). It should be noted that reduced receptors that were incubated without insulin overnight and then incubated with insulin without first being treated with NEM were similarly unable to reoxidize (Figure 6b, lane 7, and Figure 6c), indicating that the lower molecular weight heterodimer seen after incubation either is a trapped intermediate in the reoxidative pathway or is otherwise incapable of reoxidation.

The above results in Figure 6c may represent an oversimplification of the reoxidation time course. It was found that, along with a change in binding (as evidenced by counts bound to the membranes), there was also a change in cross-linking efficiency [as evidenced by the ratio of counts in the $(\alpha\beta)_2$, $\alpha\beta$, $\alpha\beta'$, and 80-kDa fragment bands to those in the insulin band]. In the experiment without insulin, the change is relatively straightforward: a decrease in cross-linkage is seen concurrent with the presumably proteolytic loss of $\alpha\beta$. In the presence of insulin, on the other hand, an initial decrease in cross-linkage efficiency after reduction is followed by a partial recovery of cross-linkage efficiency (data not shown). Changes in binding of insulin are easily corrected by adding equal counts of material into each lane of the gel: cross-linkage efficiency, however, must be corrected mathematically. Figure 6d shows the corrected reoxidation curve obtained by dividing the ordinate values in Figure 6c by the cross-linkage efficency (total counts contained in receptor bands/counts in receptor bands in untreated receptor). As can be seen, this correction results in a considerably shorter half-time for the reaction, on the order of 1 h. As will be seen later, this represents a more conservative estimate, and will be used below.

In Figure 7 the possible effects of magnesium ion or ATP on this reoxidation are addressed. Reoxidation was carried out as in Figure 6, with the following alterations: $MnCl_2$ was added to 2 mM, as in the autophosphorylation assay described under Materials and Methods, and EDTA was added to 1 mM. The membranes were then reduced as described and allowed to reoxidize in the presence of 10 nM ^{125}I-insulin, the presence or absence of 5 mM $MgCl_2$, and the presence or absence of 0.5 μM ATP, either present during the initial binding of insulin and the subsequent reduction and reoxidation or added immediately prior to removal of DTT and the initiation of the reoxidation. In the figure, the presence of Mg^{2+} is designated as "+M"; that of ATP is designated by "+Ai" (for ATP present initially) or "+Ar" (for ATP present during reoxidation only). The reaction was stopped with NEM after 1 h, while still in the linear phase of reoxidation (see Figure 6), to maximize any effects that Mg^{2+} or ATP may have had on the rate of reoxidation, and then gel electrophoresis was used as above to separate heterodimer from reoxidized tetramer. Shown in Figure 7 are the percentage counts found in the dimer band in each of the above conditions, as well as in unreduced receptor and receptor treated with NEM immediately after reduction. Since it was not clear which of the two treatments of data (see Figure 6c,d) should be used here, the number of counts both adjusted and not adjusted for cross-linkage efficiency is shown side by side.

In both cases, while ATP added either during the initial insulin binding or only during reoxidation had no statistically significant effect, the absence of Mg^{2+} showed a marked negative effect on heterodimer reoxidation to tetramer. When the effect of Mg^{2+} was compared in the presence or absence of ATP with a paired t test, the effect of Mg^{2+} was shown to be significant to a confidence of better than 95% when the data were adjusted for cross-linkage efficiency and better than 99% when not adjusted: clearly, there is a significant effect of Mg^{2+} on receptor reoxidation.

A possible explanation for the effect of Mg^{2+} on reoxidation is that Mg^{2+} has an effect on insulin binding to the receptor. To address this question, insulin binding to adipocyte mem-

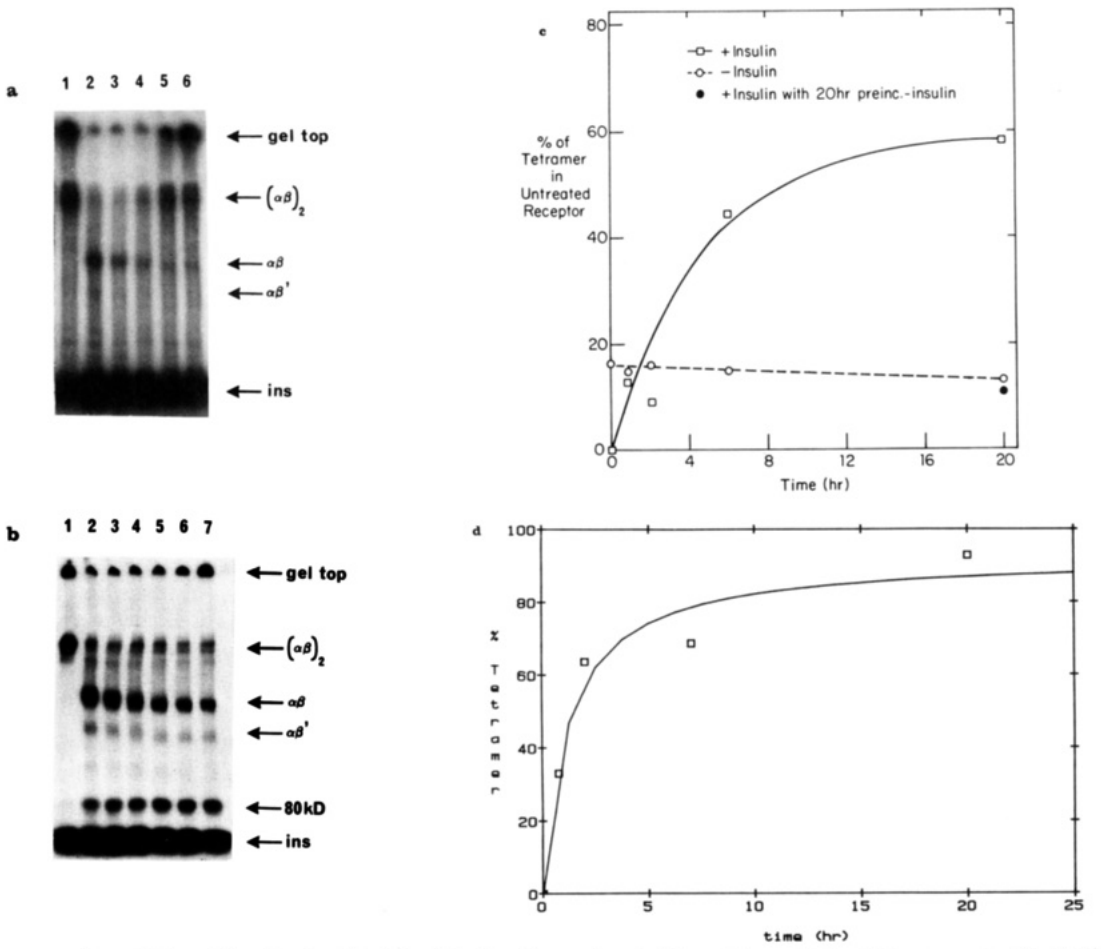

FIGURE 6: Time course of reoxidation of the class I sulfhydryls of the insulin receptor. (a) Receptor reoxidation in the presence of 6 nM ^{125}I-insulin and 0.5 mM GSSG. Membranes (4.3 μg/mL) in 100 μL of HEPES/1% BSA were incubated for 20 h at 4 °C in the presence of 6 nM ^{125}I-insulin; the membranes were centrifuged, and 50 μL of the supernatant was withdrawn and saved. The membranes were resuspended, DTT was added to 5 mM, and the membranes were incubated for 10 min at 37 °C. The reaction was stopped by placing the samples on ice and then washing the membranes once at 4 °C with the supernatant removed earlier, and then they were resuspended in 50 μL of HEPES/1% BSA containing 0.5 mM GSSG and 6 nM ^{125}I-insulin. At the indicated times, NEM was added to 1 mM to halt the reoxidation reaction: the incubation in all tubes was then continued for a total of 20 h. After this incubation, the membranes were centrifuged and resuspended in 50 μL of HEPES buffer and cross-linked as described under Materials and Methods. After cross-linkage the samples were loaded on a 5% nonreducing Laemmli gel: the 3-day autoradiogram of this gel is shown. Lane 1 is untreated receptor, and lanes 2–6 are reduced receptor with NEM added at 0, 0.75, 2, 7, and 20 h, respectively. A total of 9000 specific cpm was loaded per lane, corresponding to 82, 197, 99, 93, 115, and 115 μg of membrane protein in lanes 1–6, respectively. (b) Receptor reoxidation was carried out as above, but in the absence of insulin. Membranes were treated with 1 mM NEM at the indicated times and after that incubation were incubated overnight in HEPES/1% BSA in the presence of 6 nM ^{125}I-insulin prior to DSS cross-linking. A total of 12000 specific cpm was added to each lane of a 5% nonreducing Laemmli gel (139, 171, 211, 202, 194, 182, and 141 μg in lanes 1–7, respectively): lane 1 was untreated, in lanes 2–6 NEM was added at 0, 0.75, 2, 7, and 20 h, respectively, and in the sample in lane 7 NEM was not added before the addition of insulin to determine the reoxidative capability of the reduced molecular weight heterodimer. (c) Gel slices corresponding to the visible bands [gel top, tetramer, $\alpha\beta$ and $\alpha\beta'$, insulin, and in (b) 80-kDa fragment] in the autoradiograms in panels a and b were cut; for the slices of the gel of (a), slices of equal size were cut below the bands corresponding to tetramer and heterodimer to estimate the background counts in that area of the gel. The percentage of these total counts in the tetramer band was calculated, and then these percentages were divided by the percentage of counts in the tetramer band in the untreated lane. This normalized for differences in cross-linking efficiency between the two experiments. Shown are these final normalized percentages plotted vs time. (d) The data from (c) (in the presence of insulin) are shown corrected for cross-linkage efficiency by dividing by the ratio of counts in the various receptor bands at time t and those in the unreduced receptor. Data for reoxidation in the presence of insulin are shown. This normalization corrects for cross-linkage efficiency differences between the different reoxidative intermediates of the receptor.

branes in the presence and absence of 5 mM $MgCl_2$ was measured. Figure 8 shows the Scatchard plot of the data thus obtained. It should be noted that the samples were run in parallel on fractions of the same plasma membrane preparation with the same insulin solutions, to minimize any differences between batches.

Clearly, insulin binds more avidly to the receptor in the presence than in the absence of Mg^{2+}. The K_d of insulin for the receptor in the presence of $MgCl_2$ was found to be (2.08 ± 0.12) × 10^{-9} M, while that in the absence of $MgCl_2$ was either (5.1 ± 1.3) × 10^{-9} M or predominantly (8.6 ± 2.1) × 10^{-9} M, assuming either one or two binding sites, respectively. However, these differences in affinity only cause an approximately 20% (40% for the two-site value) change in binding site occupancy at 10 nM insulin, the concentration used in the reoxidation in Figure 7, compared to a more than 60% reduction in reoxidation at 1 h in the absence of Mg^{2+} (Figure 7). While the effect of Mg^{2+} on insulin binding to the receptor doubtless plays a role in this effect on reoxidation, clearly there is in addition an effect on reoxidation unrelated to insulin binding, which may be due to an allosteric effect of Mg^{2+} on the conformation of the receptor and thus on heterodimer association or reoxidation.

The question of whether the reoxidized receptor represents a species in which reoxidation of the class I disulfides has occurred is addressed in Figure 9, which shows the Scatchard plots of the insulin binding data to untreated, reduced, and reoxidized membranes. Again, the membranes so treated were

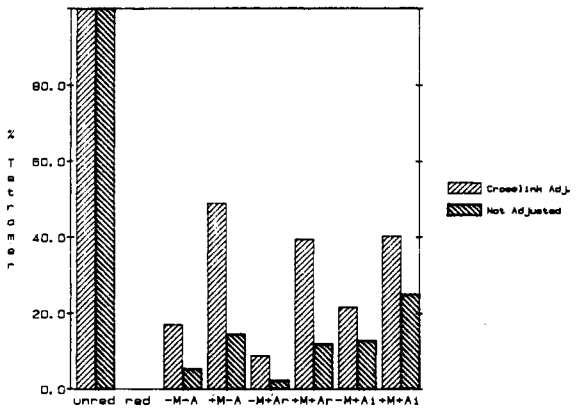

FIGURE 7: Effect on reoxidation of Mg^{2+} and ATP. Membranes were suspended to 3.3 μg/mL and bound to insulin as in Figure 6, but in the presence of 10 nM ^{125}I-insulin and 2 mM $MnCl_2$ and the presence (bars labeled +Ai in the figure) or absence (bars labeled -A and +Ar) of 0.5 μM ATP and the presence (bars labeled +M) or absence (-M) of 5 mM $MgCl_2$. Reduction and reoxidation were carried out as in Figure 6, with the above concentrations of ^{125}I-insulin and $MnCl_2$ and the presence (+Ai, +Ar) or absence (-A) of ATP and the presence (+M) or absence (-M) of $MgCl_2$ at the above concentrations: the bars labeled unred and red represent the values for untreated membranes and membranes reduced and immediately treated with 1 mM NEM, respectively. After 1 h at 4 °C the reaction was stopped with the addition of NEM to 1 mM. The samples were analyzed on a 5% nonreducing Laemmli gel as in Figure 6, and the visible bands in the dried gel were cut out and counted for ^{125}I radioactivity as in Figure 6. Shown are the percentage of counts in the tetramer band either adjusted (left-hand bars) or unadjusted (right-hand bars) for cross-linkage efficiency as in Figure 6.

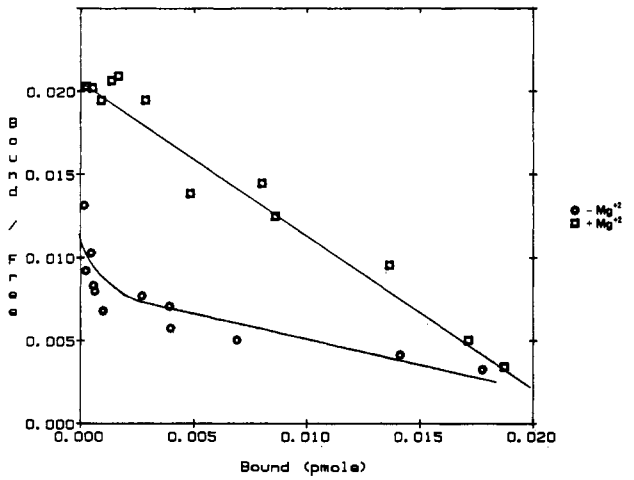

FIGURE 8: Scatchard plots of insulin binding to receptor in the presence and absence of Mg^{2+}. Aliquots of adipocyte plasma membranes were added to a final volume of 1.2 mL in 50 mM HEPES, pH 7.6, 1 mM EDTA, and 0.01% (w/v) BSA at 0.11 μg/mL membrane protein containing 50 pM ^{125}I-insulin at approximately 100 μCi/μg and unlabeled insulin from concentrations of 0 and 27.41 pM to 10.965 nM, either in the presence or absence of 5 mM $MgCl_2$. After an overnight incubation at 4 °C, these samples were filtered through Teflon filters and washed with 50 mM HEPES, pH 7.6, either with or without 5 mM $MgCl_2$, and the ^{125}I radiation retained by the membranes was measured. Shown is a plot of insulin bound (in picomoles) vs the ratio of bound to free insulin.

all from the same plasma membrane preparation to avoid batch heterogeneity. As can be seen, the initial reduction produces a population of high-affinity receptors (approximately 20% of binding sites). The reoxidation does not affect these sites: although some loss of receptor due to proteolysis is seen (i.e., loss of number of binding sites), both the binding constants (i.e., Scatchard slope) and proportion of the two binding sites is unchanged (Figure 7). From this plot can be calculated

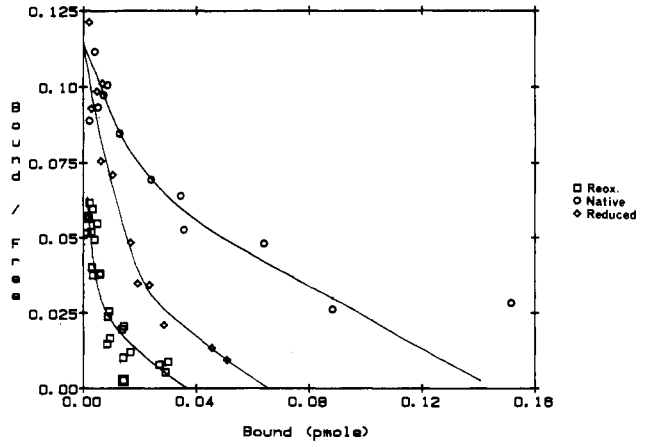

FIGURE 9: Scatchard plots of native, reduced, and reoxidized receptors. Adipocyte plasma membranes were suspended to 5.0 μg/mL membrane protein in 200 μL of HEPES buffer either in the presence (reduced, reoxidized) or absence (native) of 5 mM DTT. When DTT was present, membranes were then incubated for 10 min at 37 °C and then washed with HEPES buffer. For the reoxidation, membranes were resuspended in HEPES buffer containing 1% BSA, 0.5 mM GSSG, and 100 nM insulin and incubated overnight at 4 °C. They were then washed twice and incubated for 30 min at 25 °C in a binding buffer containing 0.01% BSA, 5 mM $MgCl_2$, 1 mM EDTA, and 50 mM HEPES, pH 7.6, to dissociate insulin bound to the receptor. For the reduced material and the native material, the membranes were only washed an equal number of times in the same buffers to normalize the loss of membranes from repeated centrifugation. After this treatment, membranes were resuspended in 100 μL of binding buffer containing 1 mM NEM and incubated for 15 min at 4 °C. Binding buffer (1.3 mL) was then added, and 100-μL aliquots were taken. These aliquots were then diluted to 1.2 mL as in Figure 8, in the presence of 5 mM $MgCl_2$. The insulin bound to the membranes was then determined as in Figure 8: the Scatchard plot thus obtained is shown.

that in the reoxidized receptor the high-affinity site has a K_d of $(2.53 \pm 0.85) \times 10^{-10}$ M and the low-affinity site has a K_d of $(4.8 \pm 1.2) \times 10^{-9}$ M, while the low-affinity site of the native receptor has a K_d of $(3.29 \pm 0.26) \times 10^{-9}$ M (the high-affinity sites were too small a fraction of total sites to be calculated in the native receptor). This result and the insulin dependence of the reoxidation argue against the interpretation that the reoxidation is due to nonspecific reoxidation of the intermolecular sulfhydryls of the heterodimers. If reduction and subsequent aberrant oxidation of the intermolecular disulfides of the heterodimers were occurring, one would expect changes in the tertiary structure, and thus the binding affinity, of the receptor. In fact, the observation that reduction produces, and reoxidation does not remove, a population of high-afinity sites suggests that the "native" receptor is actually a species which has been artifactually oxidized by the membrane isolation procedure and that the reoxidized receptor thus more closely approximates the native structure. Alternatively, this may represent the thiol-sensitive dissociation from the membranes of a regulatory protein, and thus again, the reoxidized receptor is equivalent structurally to the native receptor. The work of Boni-Schnetzler et al. (1987) is also relevant: these workers found that the kinetics for the thiol-induced changes in insulin binding affinity were different from those for reduction of the class I disulfides, suggesting that reduction of these disulfides (and presumably reoxidation) would not be expected to affect insulin binding.

Another interpretation of the above observation is that the proportion of high- to low-affinity receptors is unchanged by reduction and reoxidation but that the low-affinity receptor is more vulnerable to proteolysis. This seems possible, since a rough estimation of the actual number of high-affinity sites

in the native receptor is similar to that in the reduced receptor (see Figure 9). In this case, the affinity of insulin for the receptor is still unchanged by reoxidation, and the differences seen are due entirely to proteolysis. Of course, in the case of reoxidized receptor there would then appear to be equal proteolytic loss of heterodimer and tetramer. This would be explained as a product of the different redox conditions of reduction and reoxidation: presumably two different proteolytic activities, one activated by thiol and one active in an oxidizing environment, would be the cause.

The reoxidized receptor was found not to have regained the auto tyrosine kinase activity seen in the native receptor and lost upon reduction (data not shown). The loss was seen in both the absence and presence of GSSG in the reoxidation step, indicating that it was not merely due to the formation of a mixed disulfide between glutathione and a cysteine residue necessary for kinase function (data not shown). Neither was it possible to recover kinase activity by the addition of 1 mM DTT prior to phosphorylation. This, however, was not entirely unpredictable: that the oxidative environment necessary for the reoxidation of the class I disulfides would cause aberrant oxidation of sulfhydryls in the tyrosine kinase domain is not surprising, in view of the reducing environment of the cytoplasm in which this domain of the β subunit presumably folds together. Indeed, even the failure of 1 mM DTT to free any artifactually oxidized kinase-affecting sulfhydryls is not surprising: it has already been seen in the cases of insulin (Steiner, 1967; Chance et al., 1968) and chymotrypsin (Givol et al., 1965) that reoxidation to a purely native form may be impossible once the peptide linking the subunits has been processed away. Alternatively, the loss in autokinase activity in the heterodimer may be due to a proteolytic event stimulated by the thiol reagent necessary to produce heterodimers: once again, this loss in kinase activity would not be recoverable via reoxidation. Indeed, the interpretation of the lack of kinase activity in the reoxidized tetramer is problematical because, although many groups have investigated the effects of reduction on kinase activity, the relationship between activity and the class I disulfides specifically remains elusive because of the large numbers of other disulfides in the protein (Ullrich et al., 1985; Ebina et al., 1985).

Dimerization State of Heterodimers with Reduced Class I Disulfides. The nature of the insulin-mediated reoxidation of the reduced heterodimers and the degree of noncovalent association between the receptor halves were tested by cross-linking the receptor with the bifunctional cross-linker ethylene glycol bis(succinimidyl succinate) (EGS) and then analyzing the samples thus treated on reducing gels. Since it was found that EGS will cross-link insulin and the receptor, the DSS cross-linkage was unnecessary in this series of experiments. As can be seen in the autoradiogram shown in Figure 10, while reoxidized or native receptor α subunits (lanes 3 and 4, respectively) may be cross-linked to a 249-kDa species by such treatment, the α subunits do not retain the same interaction in the reduced receptor (lanes 1 and 2), suggesting that the reduced receptor halves are no longer in contact, although the possibility exists that the loss in cross-linkage is due to a change in conformation rather than a dissociation between the $\alpha\beta$ subunits. More work will have to be done to distinguish between these two possibilities. It should be noted that the fact that cross-linkage is restored in the reoxidized receptor suggests that the conformation, at least of the α subunits, is the same as in the native receptor, which in turn suggests that the class I disulfides have been properly reformed. The 212-kDa band seen under reducing conditions

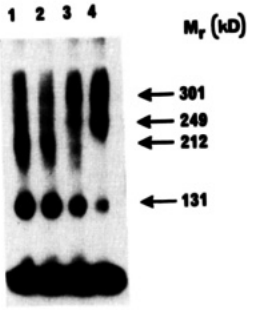

FIGURE 10: Treatment of plasma membranes with EGS causes cross-linking of α subunits to 249-kDa species in native but not reduced receptor. Plasma membranes (3.6 μg/μL) in 100-μL aliquots of HEPES/1% BSA were either untreated (lane 4) or reduced with 5 mM DTT (lanes 1–3) for 10 min at 37 °C. After centrifugation, the material in lane 1 was blocked with 1 mM NEM: that in lane 2 was blocked after incubation in the presence of 6.1 nM ^{125}I-insulin for 15 min, and that in lane 3 was blocked after incubation overnight in the presence of ^{125}I-insulin. After overnight binding to insulin as per Figure 6, all were centrifuged and then resuspended in 100 μL of HEPES containing 5 mM EGS, incubating for 1 h at 4 °C. A total of 12000 specific cpm was loaded into each lane of a 5% reducing Laemmli gel (201, 203, 146, and 144 μg): shown is an overnight autoradiogram.

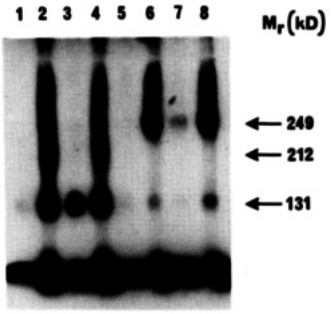

FIGURE 11: The 249-kDa species is a peripheral membrane protein. Plasma membranes (4.7 μg/mL) in 100-μL aliquots of HEPES/1% BSA were treated with (lanes 1–4) or without (lanes 5–8) 5 mM DTT for 10 min at 37 °C and then after centrifugation blocked by resuspending in 100 μL of HEPES containing 1 mM NEM. These membranes were then bound to ^{125}I-insulin as described and cross-linked with 4.5 mM EGS as in Figure 9. After cross-linking, the membranes were incubated in 30 μL of HEPES buffer containing either 4 M urea (lanes 1, 2, 5, and 6) or 4 M urea plus 50 mM DTT (lanes 3, 4, 7, and 8) at 37 °C for 6 min. Membranes were centrifuged, and both membranes (lanes 2, 4, 6, and 8) and supernatants (lanes 1, 3, 5, and 7) from this last centrifugation were loaded onto a 5% reducing Laemmli gel. Samples were loaded such that equal volumes of supernatant and pellet were loaded, at a total of 12000 specific counts per two lanes (1 and 2, 3 and 4, etc). This corresponded to 343, 250, 188, and 168 μg of membrane protein, respectively. Shown is a 3-day autoradiogram.

comigrated with $\alpha\beta$, indicating that it represents cross-linked α and β chains.

The identification of this 249-kDa species as α_2 was accomplished by selective extraction of the α chain from the membrane according to the procedure of Grunfeld et al. (1985) and is shown in Figure 11. In this figure, the odd lanes are the supernatants and the even lanes the pellets from the extraction procedure. In agreement with Grunfeld et al. (1985), while a small amount of α chain was stripped from the membrane by 4 M urea (lane 1 vs lane 2), much greater amounts were released into the supernatant by urea and reduction (lanes 3 and 4). Similarly, while only a very small amount of the 249-kDa species is released by urea (lanes 5 and 6), greater amounts are liberated by urea and reduction (lanes 7 and 8). The lesser extent of release of the 249-kDa species from the membrane compared to the observed extent of release of α

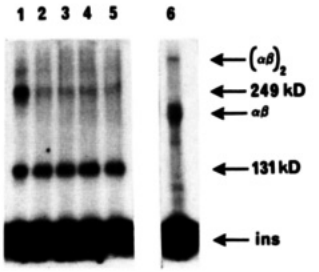

FIGURE 12: Loss of α–α interaction precedes reoxidation. Plasma membranes (7.5 μg/μL) in 100-μL aliquots of HEPES/1% BSA were bound to insulin overnight as described; after this incubation, DTT was added to 4.5 mM to the samples loaded in lanes 2–6, but not added to the sample in lane 1, and the membranes were incubated for 10 min at 37 °C. The reduction was halted by addition of NEM to 9.1 mM in all samples and by placing samples on ice. The membranes were then incubated at 4 °C for 0 min (lanes 1, 2, and 6), 15 min (lane 3), 2 h (lane 4), or 4 h (lane 5) before cross-linkage with 4.5 mM EGS (lanes 1–5) or 0.1 mM DSS (lane 6). After cross-linkage the samples were loaded on a 5% Laemmli gel under reducing (lanes 1–5) or nonreducing (lane 6) conditions. A total of 10 000 specific cpm was loaded per lane, or 237, 438, 495, 548, 575, and 621 μg, respectively. Shown is a 3-day autoradiogram.

subunit may be due to the necessity of breaking up the noncovalent $\alpha\beta$ interactions of two α subunits rather than one. In any case, the similar behavior of α chain and the 249-kDa species as nonintegral proteins, as well as their relative molecular weights, suggests strongly that the 249-kDa fragment is α_2. It should also be noted that the 212-kDa fragment's inability to be stripped from the membrane by urea and reduction further indicates that it is cross-linked $\alpha\beta$.

An attempt to measure the rate of thiol-induced loss of cross-linkage between the α subunits is shown in Figure 12. The autoradiogram shows (Figure 12, lane 2) that at the earliest time point maximum disruption of the α–α interaction necessary for cross-linkage has been achieved, showing that the time required for loss of this interaction is less than that of the first time point, or 15 min. Even assuming that the EGS reaction is relatively slow, this indicates that at least half of the reoxidation events (see Figure 5d) occur between heterodimers which have already lost the α–α interaction characterized by the ability to be cross-linked by EGS. This means either that the receptor halves dissociate more quickly than the reoxidation time course (and thus dissociated heterodimers retain the ability to reoxidize) or that the loss of cross-linkage is due to a conformational change of the α subunits which is concurrent with reduction of the class I disulfides. It should be noted that the lower concentration of EGS used here, in comparison with that used in Figures 10 and 11, resulted in the absence of the 212- and 301-kDa species. this result is readily explained if the 212-kDa species were the result of a small amount of cross-linkage between α and β subunits and the 301-kDa species the result of cross-linkage of 212-kDa with either α or another 212-kD molecule.

Conclusions

The results described above show that the class I disulfides are extracellular. This conclusion, although predictable, required experimental confirmation. Indeed, in the case of more complex transmembrane interactions such as those of the acetylcholine receptor, the inadequacy of predictions based on the cDNA sequence alone in predicting secondary structure is evident [for example, Sumikawa et al. (1982), Young et al. (1985), and Ratnam et al. (1986)]. Furthermore, the paucity of evidence confirming such predictions makes conclusions drawn solely from primary sequence data limited. Similarly, in the case of the insulin receptor, the fact that the class I sulfhydryl groups are oxidized in most tissues would not have been a good indication of their location because of the inability of cytoplasmic concentrations of glutathione to reduce this bond [see May (1985) and Figure 2]. Such intracellular disulfides have been identified in the regulatory subunit of both the cAMP-dependent protein kinase I (Zick & Taylor, 1982) and the cGMP-dependent protein kinase (Monken & Gill, 1980). The direct investigation of protein structures remains a necessity.

That the loss of the α subunits' ability to be cross-linked by EGS upon reduction of the class I disulfides represents dissociation is difficult to determine. Certainly, dissociation of the solubilized receptor halves has been observed upon reduction (Velicelebi & Aiyer, 1984), but the dilution effect of solubilizing the receptor may be the cause. The EGS cross-linkage, while certainly not conclusive, represents at least an indication of what the dimerization state in the membrane may be. Observation of dissociation using an independent approach will be necessary to establish conclusively whether the receptor halves dissociate upon reduction.

Somewhat clearer is the mechanism of the effect of insulin on the reoxidation of the class I sulfhydryls. The reaction can be described by

$$2R \underset{k_d}{\overset{k_a}{\rightleftharpoons}} RR \xrightarrow{k_0} R_2$$

where R_2 is tetramer, RR is the noncovalently bound dimer of heterodimers, R is the heterodimer, k_0 is the rate constant for reoxidation of the class I disulfides, k_d is the rate constant for dissociation of the reduced heterodimers, and k_a is their association rate constant.

The presence and absence of insulin are not likely to affect the value of k_a. In the absence of insulin, the value of k_d must be greater than k_0 in order to account for the absence of any reoxidation. There are two limiting explanations for the observation that the presence of insulin results in the appearance of tetramer stabilized by disulfide bonds (R_2). Insulin may decrease the value of k_d, the dissociation rate constant, leading to an increase in the amount of tetramer compared to the situation in the absence of insulin. On the other hand, the value of k_0, the rate constant for the reoxidation, may be increased in the presence of insulin. As shown in Figure 12, if loss of cross-linkage between the α subunits indicates dissociation of the receptor halves, it is complete long before the completion of the reoxidation. Assuming this interpretation, rather than affecting the association between receptor halves, insulin would appear to act by increasng k_0, the reoxidation constant of the receptor halves which have already attained the proper conformation or dimerization state. Of interest is the fact that insulin binding has been shown to increase the lability of the class I disulfides to DTT (Wilden et al., 1986): this result and those described here are readily explained if the effect of insulin binding is to expose the class I disulfides to the solvent to a greater extent than in the unbound receptor. Thus, the disulfides would be more accessible to DTT reduction in the native receptor, and the reduced sulfhydryl would be more exposed in the reduced receptor. Clearly, the dimerization state of the reduced receptor halves is a crucial question: interpretation of many of the above results requires knowledge of whether the reduced heterodimers retain noncovalent interactions. Independent methods for investigating this question must be pursued.

Acknowledgments

Thanks are due to Guido Guidotti, in whose laboratory these experiments were performed, for invaluable advice and guid-

ance. Thanks are also due to William Brennan, Lloyd Cantley, Frank Gorga, and Eric Mortenson for many helpful discussions and for reading the manuscript (E.M.).

Registry No. ATP, 56-65-5; Mg, 7439-95-4; insulin, 9004-10-8.

REFERENCES

Anfinsen, C. B. (1973) *Science (Washington, D.C.) 181*, 223–230.

Blundell, T., Dodson, G., Hodgkin, D., & Marcola, D. (1972) *Adv. Protein Chem. 26*, 279–402.

Boni-Schnetzler, M., Rubin, J. B., & Pilch, P. F. (1986) *J. Biol. Chem. 261*, 15281–15287.

Boni-Schnetzler, M., Scott, W., Waugh, S. M., DiBella, E., & Pilch, P. F. (1987) *J. Biol. Chem. 262*, 8395–8401.

Boyle, T. R., Campana, J., Sweet, L. J., & Pessin, J. E. (1985) *J. Biol. Chem. 260*, 8593–8600.

Chance, R. E., Ellis, R. M., & Bromer, W. W. (1968) *Science (Washington, D.C.) 161*, 165–167.

Czech, M. P., & Fain, J. N. (1972) *J. Biol. Chem. 247*, 6218–6223.

Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graf, L., Clauser, E., Ou, J. H., Masiarz, F., Kan, Y. W., Goldfine, I. D., Roth, R. A., & Cutter, W. H. (1985) *Cell (Cambridge, Mass.) 40*, 747–758.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70–77.

Forsayeth, J. R., Caro, J. F., Sinha, M. K., Maddux, B. A., & Goldfine, I. D. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 3448–3451.

Fujita-Yamaguchi, Y., & Kathuria, S. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6095–6099.

Givol, D., De Lorenzo, F., Goldberger, R. F., & Anfinsen, C. B. (1965) *Proc. Natl. Acad. Sci. U.S. A. 53*, 676–684.

Grunfeld, C., Shinegaga, J. K., & Ramachandran, J. (1985) *Biochem. Biophys. Res. Commun. 133*, 389–396.

Kosower, N. S., Kosower, E. M., Newton, G. L., & Ranney, H. M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3382–3386.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Massague, J., & Czech, M. P. (1982) *J. Biol. Chem. 257*, 6729–6738.

Massague, J., Pilch, P. F., & Czech, M. P. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7137–7141.

Massague, J., Pilch, P. F., & Czech, M. P. (1981) *J. Biol. Chem. 256*, 3182–3190.

May, J. F. (1985) *J. Biol. Chem. 260*, 462–467.

Monken, C. E., & Gill, G. N. (1980) *J. Biol. Chem. 255*, 7067–7070.

Pang, D. T., Lewis, S. D., Sharma, B. R., & Shafer, J. A. (1984) *Arch. Biochem. Biophys. 234*, 629–638.

Pike, L. J., Eakes, A. T., & Krebs, E. G. (1986) *J. Biol. Chem. 261*, 3782–3789.

Ratnam, M., Nguyen, D. L., Rivier, J., Sargent, P. B., & Lindstrom, J. (1986) *Biochemistry 25*, 2633–2643.

Resh, M. D. (1982) *J. Biol. Chem. 257*, 11946–11952.

Resh, M. D., Nemenoff, R. A., & Guidotti, G. (1980) *J. Biol. Chem. 255*, 10938–10945.

Shia, M. A., Rubin, J. B., & Pilch, P. F. (1983) *J. Biol. Chem. 258*, 14450–14455.

Steiner, D. F. (1967) *Trans. N.Y. Acad. Sci. 30*, 60–68.

Sumikawa, K., Houghton, M., Smith, J. C., Bell, L., Richards, B. M., & Barnard, E. A. (1982) *Nucleic Acids Res. 10*, 5809–5822.

Sweet, L. J., Wilden, P. A., & Pessin, J. E. (1986) *Biochemistry 25*, 7068–7074.

Tamura, S., Brown, T. A., Whipple, J. H., Fujita-Yamaguchi, Y., Dubler, R. E., Cheng, K., & Larner, J. (1984) *J. Biol. Chem. 259*, 6650–6658.

Ullrich, A., Bell, J. R., Chen, E. Y., Herrera, R., Petruzzeli, L. M., Dull, T. J., Gray, A., Coussens, L., Liao, Y.-C., Tsubokawa, M., Mason, A., Seeburg, P. H., Grunfeld, C., Rosen, O. M., & Ramachandran, J. (1985) *Nature (London) 313*, 756–761.

Velicelebi, G., & Aiyer, R. A. (1984) *J. Biol. Chem. 81*, 7693–7697.

Wilden, P. A., Boyle, T. R., Swanson, M. L., Sweet, L. J., & Pessin, J. E. (1986) *Biochemistry 25*, 4381–4388.

Young, E. F., Ralston, E., Blake, J., Ramachandran, J., Hall, Z. W., & Stroud, R. M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 626–631.

Zick, S. K., & Taylor, S. S. (1982) *J. Biol. Chem. 257*, 2287–2293.