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Dithiothreitol Activation of the Insulin Receptor/Kinase Does Not Involve Subunit Dissociation of the Native $\alpha_2\beta_2$ Insulin Receptor Subunit Complex†

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ABSTRACT: The subunit composition of the dithiothreitol- (DTT) activated insulin receptor/kinase was examined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and gel filtration chromatography under denaturing (0.1% SDS) or nondenaturing (0.1% Triton X-100) conditions. Pretreatment of ^{32}P-labeled insulin receptors with 50 mM DTT followed by gel filtration chromatography in 0.1% SDS demonstrated the dissociation of the $\alpha_2\beta_2$ insulin receptor complex (M_r 400 000) into the monomeric 95 000 β subunit. In contrast, pretreatment of the insulin receptors with 1-50 mM DTT followed by gel filtration chromatography in 0.1% Triton X-100 resulted in no apparent alteration in mobility compared to the untreated insulin receptors. Resolution of this complex by nonreducing SDS-polyacrylamide gel electrophoresis and autoradiography demonstrated the existence of the $\alpha_2\beta_2$ heterotetrameric complex with essentially no $\alpha\beta$ heterodimeric or free monomeric β subunit species present. This suggests that the insulin receptor can reoxidize into the M_r 400 000 complex after the removal of DTT by gel filtration chromatography. Surprisingly, these apparently reoxidized insulin receptors were also observed to be functional with respect to insulin binding, albeit with a 50% decrease in affinity for insulin and insulin stimulation of the β subunit autophosphorylation. To prevent reoxidation, the insulin receptors were pretreated with 50 mM DTT followed by incubation with excess *N*-ethylmaleimide prior to gel filtration chromatography in 0.1% Triton X-100. Under these conditions the insulin receptors migrated as the M_r 400 000 $\alpha_2\beta_2$ complex. However, when this insulin receptor complex was subjected to nonreducing SDS-polyacrylamide gel electrophoresis, subsequent to gel filtration, only the M_r 95 000 β subunit was detected. These results demonstrate that treatment of the insulin receptors with high concentrations of DTT, followed by removal of DTT by gel filtration, results in reoxidation of the reduced $\alpha_2\beta_2$ insulin receptor complex. Further, these results document that although the DTT stimulation of the insulin receptor/kinase does involve reduction of the insulin receptor subunits, it does not result in dissociation of the native $\alpha_2\beta_2$ insulin receptor subunit complex.

The effects of insulin on cellular metabolism are initiated by the specific binding of insulin to its cell surface receptor on target cells (Czech, 1977; Kahn et al., 1981). The insulin receptor is an integral membrane glycoprotein composed of two M_r 130 000 (α) and two M_r 95 000 (β) subunits covalently linked by disulfide bonds to form the native $\alpha_2\beta_2$ heterotetrameric complex [for reviews see Jacobs and Cuatracasas (1983) and Pessin et al. (1985)]. The complete primary amino acid sequence of the human placental insulin receptor precursor has been deduced from two full-length cDNA clones (Ebina et al., 1985; Ullrich et al., 1985). These data suggest that the insulin binding α subunit is exclusively located on the extracellular face of the plasma membrane and is anchored to the cell surface by disulfide bonds with the transmembrane β subunit. This prediction has been recently confirmed by the release of ^{125}I-insulin cross-linked α subunits from placenta membranes treated with dithiothreitol (DTT)[1] and urea (Grunfeld et al., 1985). The β subunit encodes the tyrosine kinase activity of the insulin receptor (Avruch et al., 1982; Kasuga et al., 1982a,b, 1983a,b; Petruzzelli et al., 1982, 1984; Tamura et al., 1983; Zick et al., 1983), containing both an ATP binding site and tyrosine phosphorylation acceptor sites (Roth & Cassell, 1983; Shia & Pilch, 1983; Van Obberghen et al., 1983). Although extensive information has been accumulated regarding the structure of the insulin receptor, the mechanism by which insulin binding to the extracellular α subunit results in an intramolecular transmembrane signal that activates the intracellular β subunit kinase domain has not been elucidated.

Several reports have suggested that the oxidation state of the insulin receptor may play an important role in receptor structure and function. In particular, sulfhydryl reagents have been reported to inhibit insulin biological responsiveness and reducing agents have been observed to act as insulinomimetic

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[1] Abbreviations: DTT, dithiothreitol; SDS, sodium dodecyl sulfate; NEM, *N*-ethylmaleimide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ATP, adenosine 5'-triphosphate; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor.

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agents (Carter & Martin, 1969; Czech et al., 1974; Goko et al., 1981; Clark & Harrison, 1983; Crettaz et al., 1984). Further, the insulin receptor/kinase activity has been shown to be inhibited by the highly specific sulfhydryl alkylating reagent NEM (Shia et al., 1983; Zick et al., 1983; Pike et al., 1984) and to be activated by the reducing agent DTT (Shia et al., 1983; Petruzzelli et al., 1984; Fujita-Yamaguchi & Kathuria, 1985). The ability of DTT to activate the insulin receptor/kinase activity has been reported to correlate with the appearance of a heterodimeric $\alpha\beta$ half-receptor form (Fujita-Yamaguchi & Kathuria, 1985). In contrast, a previous study suggested that the $\alpha_2\beta_2$ heterotetrameric insulin receptor complex is the most active kinase structure but requires a reduced sulfhydryl group(s) for maximal enzymatic activity (Shia et al., 1983). However, both these studies utilized SDS–polyacrylamide gel electrophoresis (denaturing conditions) to examine the relative portions of insulin receptor complexes in the presence of DTT, whereas the insulin receptor/kinase activity was measured in the presence of 0.1% Triton X-100 (nondenaturing conditions). In order to more carefully define the insulin receptor structure after DTT treatment, we have employed gel filtration chromatography under nondenaturing conditions similar to those used to determine the insulin receptor/kinase activity. The data presented in this communication demonstrate that although the DTT stimulation of the insulin receptor/kinase does involve reduction of the insulin receptor subunits, it does not result in dissociation of the native $\alpha_2\beta_2$ insulin receptor subunit complex.

Experimental Procedures

Materials. Triton X-100, *n*-octyl β-D-glucopyranoside, DTT, NEM, and protease inhibitors were obtained from Sigma. [γ-^{32}P]ATP (3000 Ci/mmol) and NCS tissue solubilizer were purchased from New England Nuclear and Amersham, respectively. Bio-Gel A-1.5m and SDS were from Bio-Rad. XAR-5 film and Cronex Lightening Plus enhancing screens were purchased from Kodak and Du Pont, respectively. Polyacrylamide gel electrophoresis reagents were obtained from U.S. Biochemical Corp. Porcine insulin was a gift from Dr. R. Chance, Eli Lilly Co., and A_{14}-mono[^{125}I]iodoinsulin was kindly provided by the Diabetes Endocrinology Research Center, The University of Iowa.

Purification of Insulin Receptors. Insulin receptors were purified as previously described (Boyle et al., 1985; Sweet et al., 1985). Briefly, membranes (10 mg/mL) prepared from freshly obtained human placenta (Harrison & Itin, 1980) were solubilized for 1 h at 4 °C in 0.25 M sucrose, 10 mM Tris, pH 8.0, 2 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 25 mM benzamidine hydrochloride, 10 μM leupeptin, 50 trypsin-inhibiting units of aprotinin, 1 mM 1,10-phenanthroline, and 1 μM pepstatin A, with 2% Triton X-100. Following centrifugation at 104000*g* for 1 h at 4 °C, the soluble material was applied to a 5 × 100 cm Sephacryl S-400 gel filtration column equilibrated with 50 mM Tris, pH 8.0, 0.1% Triton X-100, and 0.02% NaN_3. The insulin binding activity eluted from the gel filtration column was directly applied to a 1.6 × 10 cm column of insulin coupled to Affi-Gel 10 (0.8 mg of insulin/mL of resin). Triton X-100 was exchanged with *n*-octyl β-D-glucopyranoside while the receptor was bound to the affinity resin by washing the column with 10–15 column volumes of 50 mM Hepes, pH 8.0, 1.0 M NaCl, and 0.6% *n*-octyl β-D-glucopyranoside. The insulin receptors were eluted from the resin with 50 mM sodium acetate, 1.0 M NaCl and 0.6% *n*-octyl β-D-glucopyranoside, pH 5.0, neutralized with 1.0 M Hepes, pH 8.0, and concentrated with Centricon miniconcentrators. The purified insulin receptors were then resuspended in 50 mM Hepes, pH 8.0, containing 10% glycerol and 0.6% *n*-octyl β-D-glucopyranoside.

Insulin Binding Activity. Purified insulin receptors were incubated with 0.25 nM ^{125}I-insulin in a final volume of 0.20 mL of Krebs–Ringer's–Hepes buffer (10 mM Hepes, pH 7.6, 5.1 mM KCl, 1.3 mM $MgSO_4$) with 0.1% BSA for 1 h at 23 °C. Free ^{125}I-insulin was separated from the bound hormone by serial additions of 0.1% bovine γ-globulin and 12.5% poly(ethylene glycol), followed by centrifugation at 12000*g* for 10 min in a Beckman Microfuge B. Nonspecific binding was determined in the presence of 10 μM unlabeled insulin. Binding data for Scatchard analysis (Scatchard, 1949) were generated by incubating the purified receptor with 0.01–0.25 nM ^{125}I-insulin and with 0.25 nM ^{125}I-insulin plus 0.75–250 nM unlabeled insulin. Bound and free hormone were separated as described above. Scatchard analysis was performed by using a modification of the computer program LIGAND kindly provided by the Diabetes Endocrinology Research Center, The University of Iowa.

Phosphorylation of Purified Insulin Receptors. Samples to be assayed for β subunit autophosphorylating activity were incubated for 30 min at 23 °C in the presence or absence of 200 nM porcine insulin or various concentrations of DTT in 50 mM Hepes, pH 7.6, 5 mM $MgCl_2$, and 5 mM $MnCl_2$. The phosphorylation reaction was initiated by the addition of [γ-^{32}P]ATP (1 mM, 3 μCi/nmol) to a final concentration of 100 μM ATP and terminated by the addition of Laemmli sample buffer containing 100 mM DTT. Following resolution by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970), the phosphorylated species were localized by autoradiography, excised, solubilized with NCS tissue solubilizer, and counted for ^{32}P in scintillation solution. The ^{32}P-labeled insulin receptors utilized for column chromatography studies were phosphorylated for 60 min at 23 °C as above except for the use of 8.0 μM [γ-^{32}P]ATP. The reaction was terminated by the addition of unlabeled ATP to a final concentration of 15.0 mM. Phosphorylated receptors were separated from free ATP by gel filtration chromatography using a 1.6 × 20 cm Sephadex G-50 column equilibrated with 50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Triton X-100, 0.1% BSA, and 0.02% NaN_3. The material voided from the Sephadex G-50 column was concentrated by using Centricon miniconcentrators, applied to a 1.6 × 50 cm Bio-Gel A-1.5m gel filtration column, and eluted with the same buffer. The phosphorylated receptor, which was partially included by the Bio-Gel A-1.5m column, was pooled and stored at 4 °C until use.

Bio-Gel A-1.5m Gel Filtration Chromatography. The purified and ^{32}P-labeled insulin receptors were resolved on Bio-Gel A-1.5m (1.6 × 46 cm) columns equilibrated in 50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% BSA, and 0.02% NaN_3 plus 0.1% Triton X-100 or 0.1% SDS at 23 °C. After the appropriate treatments, as described in the figure legends, the samples were applied to the Bio-Gel A-1.5m columns and 20.0 mL was voided before collecting 0.40-mL fractions at a flow rate of 15 mL/h. Aliquots were removed for scintillation counting (0.20 mL) and for separation by SDS–polyacrylamide gel electrophoresis (0.10 mL).

Results

The effects of DTT on the autophosphorylation of the insulin receptor β subunit were initially examined by incubating the purified human placental insulin receptor with various concentrations of DTT in the presence and absence of insulin. As shown in Figure 1, low concentrations of DTT (0–1.0 mM) stimulated the incorporation of ^{32}P into the β subunit in a

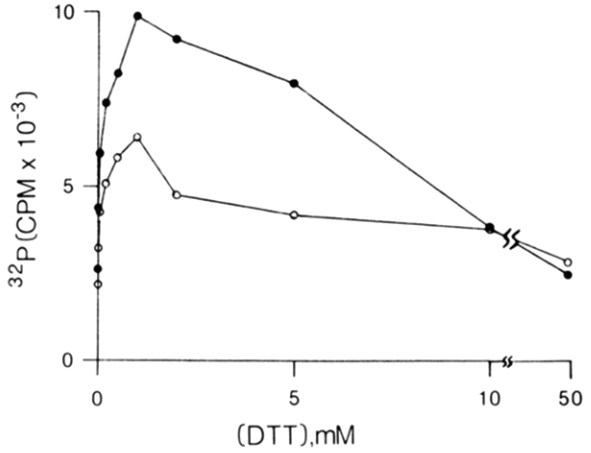

FIGURE 1: Dithiothreitol stimulation of β subunit autophosphorylation of purified human placental insulin receptors. Purified insulin receptors (1.0 μg) were incubated in the presence (●) or absence (○) of 200 nM insulin with various concentrations of DTT for 1 h at 23 °C. The phosphorylation reaction was initiated by the addition of 100 μM [γ-^{32}P]ATP (3 μCi/nmol) for 5 min and terminated by the addition of Laemmli sample buffer containing 100 mM DTT. The samples were resolved in a 7% SDS–polyacrylamide gel and the M_r 95 000 β subunit was excised and counted for ^{32}P as described under Experimental Procedures.

concentration-dependent manner, with a maximum stimulation of 4-fold being observed at 1.0 mM DTT. Over this range of concentrations, DTT increased the autophosphorylation both in the presence and absence of insulin and the combined stimulation by insulin plus DTT were observed to be approximately additive. Higher concentrations of DTT (2.0–50 mM) decreased the amount of autophosphorylation; however, even at 50 mM DTT the amount of β subunit autophosphorylation was greater compared to the ^{32}P incorporation in the absence of DTT. Additionally, the insulin stimulation of the autophosphorylating activity was lost in the presence of high concentrations of DTT (>10 mM). These results are similar to those previously reported for partially purified (Shia et al., 1983) and purified (Fujita-Yamaguchi & Kathuria, 1985) insulin receptors.

To examine the subunit structure of the DTT-stimulated insulin receptor/kinase, ^{32}P-autophosphorylated insulin receptors were treated with various concentrations of DTT and resolved by SDS–polyacrylamide gel electrophoresis under two different conditions (Figure 2). In Figure 2A, excess NEM was added to each sample before heating at 100 °C for 2 min in the presence of SDS. The sample was then subjected to SDS–polyacrylamide gel electrophoresis. NEM was added in order to inactivate any reducing equivalents present in the sample before heating and the addition of the denaturing agent SDS. This protocol is similar to that used by Shia et al. (1983). Figure 2B shows the effects of various DTT concentrations on the insulin receptor subunit structure when prepared for SDS–polyacrylamide gel electrophoresis in an inverse order to that used in Figure 2A. This protocol is similar to that used by Fujita-Yamaguchi and Kathuria (1985) in that SDS was added prior to heating and SDS–polyacrylamide gel electrophoresis. Under both conditions of NEM addition, the only insulin receptor species present in the absence of DTT was the $\alpha_2\beta_2$ complex. When NEM was added prior to SDS (Figure 2A), treatment with increasing concentrations of DTT (0.1–1.0 mM) initially resulted in the dissociation of the $\alpha_2\beta_2$ complex with the concomitant formation of $\alpha\beta$ heterodimers. Under these conditions, the presence of the monomeric M_r 95 000 β subunit could be detected only with treatment of the insulin receptors with concentrations of DTT greater than 1.0 mM. Complete reduction to the monomeric β subunit was observed at 50 mM DTT under these conditions. In contrast, when the insulin receptors were treated with SDS prior to the addition of NEM (Figure 2B), the DTT concentration-dependent dissociation of the $\alpha_2\beta_2$ complex was markedly altered from that observed when NEM was added prior to heating and the addition of SDS. The dissociation of the $\alpha_2\beta_2$ insulin receptor complex into $\alpha\beta$ heterodimers and to mostly monomeric β subunits was apparent even at 0.1 mM DTT, a concentration 10-fold less than that required to form monomeric β subunits when NEM was added prior to heating and the addition of SDS. The complete formation of monomeric β subunits from the $\alpha_2\beta_2$ insulin receptor complex under the condition of SDS addition prior to NEM treatment

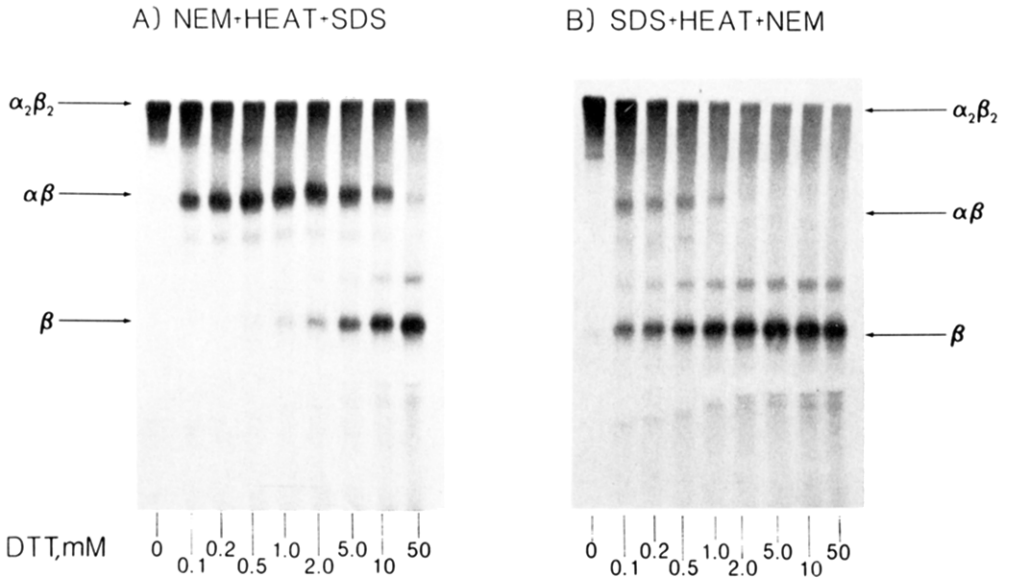

FIGURE 2: Effects of dithiothreitol on insulin receptor suubunit structure as determined by SDS–polyacrylamide gel electrophoresis. (A) ^{32}P-Autophosphorylated insulin receptors were treated with the indicated concentrations of DTT for 1 h at 23 °C, then with 150 mM NEM for 15 min, and followed by the addition of Laemmli sample buffer to achieve a final concentration of 1.4% SDS. The samples were heated at 100 °C for 2 min and resolved in a 3–10% linear gradient SDS–polyacrylamide gel under nonreducing conditions. The autoradiograph of the gel is presented. (B) ^{32}P-Autophosphorylated insulin receptors were treated as indicated for (A) except the DTT-treated insulin receptors were mixed with Laemmli sample buffer and heated prior to the addition of NEM.

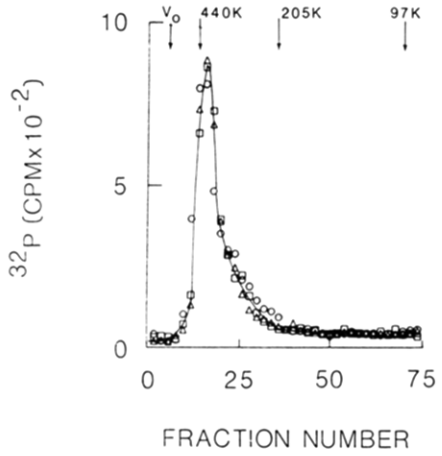

FIGURE 3: Effects of dithiothreitol on insulin receptor subunit structure determined by gel filtration chromatography. ^{32}P-Autophosphorylated insulin receptors were incubated for 1 h at 23 °C in the absence (○) and presence of 1.0 (□) or 5.0 mM (△) DTT in 0.1% Triton X-100. The samples were applied to a 1.6 × 46 cm Bio-Gel A-1.5m column equilibrated in 50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% BSA, 1.0 μM leupeptin, 0.02% NaN_3, and 0.1 Triton X-100. Fractions (0.40 mL) were collected after voiding 20.0 mL. Aliquots were removed and counted for ^{32}P. The arrows depict the elution positions of blue dextran (V_0), ferritin (440K), myosin (205K), and phosphorylase *b* (97K).

occurred at DTT concentrations greater than 1.0 mM. Thus, it is apparent that the conditions used to prepare the insulin receptors for SDS–polyacrylamide gel electrophoresis can result in different interpretations with regard to the subunit structure at various DTT concentrations (Figure 2).

Therefore, to define the insulin receptor subunit structure under conditions similar to those used to determine the DTT stimulation of the insulin receptor/kinase activity, we investigated the subunit structure of the untreated and DTT-treated insulin receptors by gel filtration chromatography under nondenaturing (Triton X-100) conditions (Figure 3). ^{32}P-Autophosphorylated insulin receptors were incubated without or with 1.0 mM and 5.0 mM DTT for 1 h at 23 °C prior to Bio-Gel A-1.5m gel filtration chromatography. In the absence of DTT pretreatment, the insulin receptors migrated as a single peak with an approximate M_r 400,000 and a K_{av} of approximately 0.19. Similarly, pretreatment of the ^{32}P-labeled insulin receptors with 1.0 or 5.0 mM DTT resulted in the identical gel filtration column profile as compared to the untreated insulin receptor preparation. This experiment suggested that these concentrations of DTT, at which the $\alpha_2\beta_2$ insulin receptor complex dissociates by SDS–polyacrylamide gel electrophoresis (Figure 2), do not result in dissociation of the insulin receptor under nondenaturing buffer conditions (Figure 3).

To further investigate this apparent inability of relatively low DTT concentrations to dissociate the insulin receptor, under conditions similar to those used to examine the tyrosine kinase activity (Figure 1; Shia et al., 1983; Fujita-Yamaguchi & Kathuria, 1985), the ^{32}P-autophosphorylated insulin receptors were treated with 50 mM DTT to maximize the reduction of the disulfide bonds in the nondenatured insulin receptor complex. As previously observed (Figure 3) in the absence of DTT pretreatment, the insulin receptors migrated as a single peak with an approximate M_r 400 000 in Bio-Gel A-1.5m gel filtration columns equilibrated with 0.1% Triton X-100 or 0.1% SDS (Figure 4A). SDS–polyacrylamide gel electrophoresis confirmed the species resolved by gel filtration chromatography in Triton X-100 (Figure 4B, lane 1) or SDS (Figure 4B, lane 2) as the M_r 400 000 $\alpha_2\beta_2$ heterotetrameric disulfide-linked complex. Pretreatment of the ^{32}P-auto-

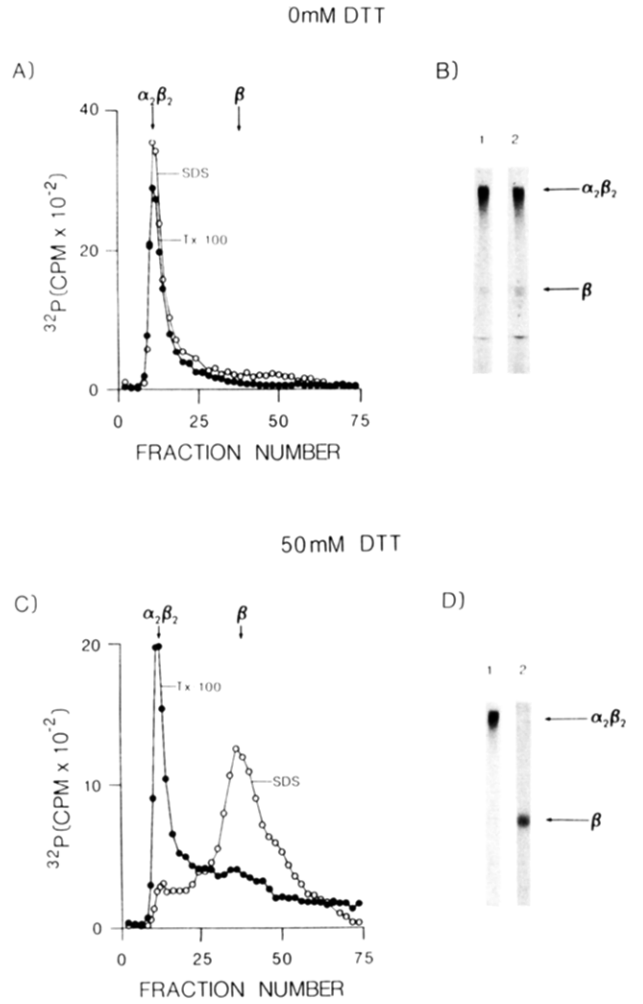

FIGURE 4: Dithiothreitol effects on insulin receptor subunit structure under denaturing and nondenaturing conditions. (A) ^{32}P-Autophosphorylated insulin receptors were incubated at 23 °C for 1 h in the presence of 0.1% Triton X-100 (●) or 0.1% SDS (○) and applied to 1.6 × 46 cm Bio-Gel A-1.5m columns equilibrated in 50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% BSA, 1.0 μM leupeptin, 0.02% NaN_3, and either 0.1% Triton X-100 or 0.1% SDS as described in Figure 3. (B) Autoradiograph of insulin receptor species recovered from the gel filtration columns. Pooled fractions 10–14 from the gel filtration columns equilibrated with 0.1% Triton X-100 (lane 1) or 0.1% SDS (lane 2) were resolved in a 3–10% SDS–polyacrylamide gel under nonreducing conditions. (C) ^{32}P-Autophosphorylated insulin receptors were incubated for 1 h at 23 °C in the presence of 50 mM DTT plus 0.1% Triton X-100 (●) or 50 mM DTT plus 0.1% SDS (○) and resolved by gel filtration chromatography as described above. (D) Autoradiograph of DTT-pretreated receptors recovered from the gel filtration columns. Fractions 10–14 (lane 1) from the gel filtration column equilibrated in 0.1% Triton X-100 and fractions 32–42 (lane 2) from the gel filtration column equilibrated in 0.1% SDS were resolved in a 3–10% linear gradient SDS–polyacrylamide gel under nonreducing conditions.

phosphorylated insulin receptors with 50 mM DTT, followed by gel filtration chromatography in the presence of 0.1% SDS, demonstrated the appearance of the M_r 95 000 species with the concomitant disappearance of the M_r 400 000 species (Figure 4C). Nonreducing SDS–polyacrylamide gel electrophoresis of the Bio-Gel A-1.5m fractions documented the formation of the M_r 95 000 β subunit in the presence of SDS (Figure 4D, lane 2). In contrast, insulin receptors pretreated with 50 mM DTT, followed by gel filtration chromatography in 0.1% Triton X-100, were resolved as a single sharp band centered at the same position as the untreated insulin receptors (Figure 4A), with no significant alteration in the elution profile (Figure 4C). Thus, in the presence of Triton X-100, insulin

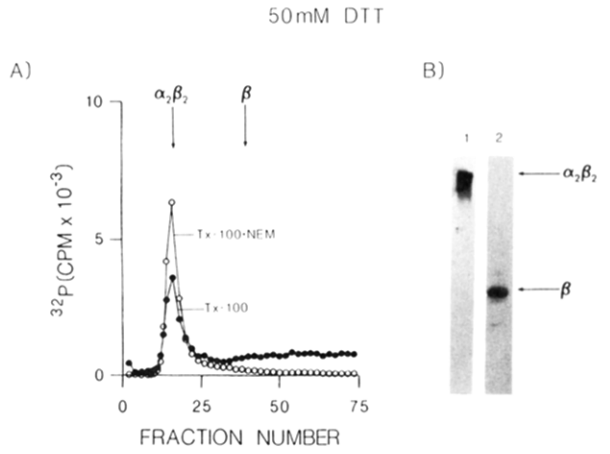

FIGURE 5: Gel filtration chromatography of fully reduced ^{32}P-labeled insulin receptors in the presence or absence of NEM. (A) ^{32}P-Autophosphorylated insulin receptors were treated with 50 mM DTT in 0.1% Triton X-100 for 1 h at 23 °C and then with (○) or without (●) 150 mM NEM prior to resolution on a 1.6 × 46 cm Bio-Gel A-1.5m gel filtration column in the presence of 0.1% Triton X-100 as described in Figure 3. (B) Autoradiograph of insulin receptor species resolved by SDS–polyacrylamide gel electrophoresis subsequent to gel filtration chromatography. The ^{32}P-labeled insulin receptor species that were recovered from the Bio-Gel A-1.5m columns (fractions 14–20) on which the insulin receptors treated with DTT only (lane 1) or DTT followed by NEM treatment (lane 2) were resolved in 3–10% linear gradient nonreducing SDS–polyacrylamide gels.

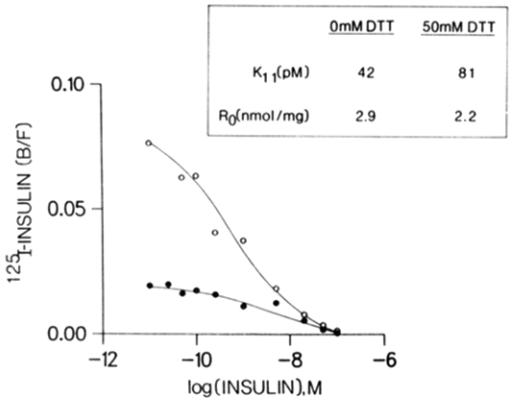

FIGURE 6: Competition–inhibition curves of insulin binding to untreated and DTT-treated insulin receptors. Unlabeled insulin receptors (15 μg) were treated with (●) or without 50 mM DTT (○) for 1 h at 4 °C and resolved by Bio-Gel A-1.5m columns equilibrated with 0.1% Triton X-100. The fractions demonstrating insulin binding activity were pooled and analyzed for insulin binding activity as described under Experimental Procedures. The high-affinity dissociation constant and total number of binding sites are indicated in the insert.

receptors treated with 50 mM DTT primarily maintained an apparent M_r 400 000 by gel filtration chromatography. Identical results were also obtained when the gel filtration columns were also preequilibrated with 50 mM DTT before the addition of the ^{32}P-labeled insulin receptors (data not shown). Further, SDS–polyacrylamide gel electrophoresis of this species, under nonreducing conditions, demonstrated the existence of the $\alpha_2\beta_2$ heterotetrameric complex with little or no significant formation of the $\alpha\beta$ heterodimeric complex or monomeric β subunits (Figure 4D, lane 1). These results suggest that the reduced insulin receptor can reoxidize to form a M_r 400 000 disulfide-linked complex when DTT is removed by gel filtration chromatography in the presence of 0.1% Triton X-100.

^{32}P-Labeled insulin receptors were then pretreated with 50 mM DTT, followed by incubation with or without excess NEM, prior to Bio-Gel A-1.5m gel filtration chromatography in 0.1% Triton X-100 to further examine the apparent reoxidation of the insulin receptors following DTT reduction and gel filtration chromatography (Figure 5). The inclusion of NEM in the sample did not alter the migration of the ^{32}P-autophosphorylated insulin receptors such that under both conditions the insulin receptors migrated as the M_r 400 000 $\alpha_2\beta_2$ complex (Figure 5A). As previously shown, when not treated with NEM prior to the gel filtration chromatography, the DTT-treated insulin receptor was resolved as the M_r 400 000 $\alpha_2\beta_2$ complex by SDS–polyacrylamide gel electrophoresis under nonreducing conditions (Figure 5B, lane 1). However, when NEM was added to the DTT-treated insulin receptors prior to the gel filtration chromatography, the resulting ^{32}P-labeled insulin receptor complex was resolved as the M_r 95 000 monomeric β subunit by nonreducing SDS–polyacrylamide gel electrophoresis (Figure 5B, lane 2). These results document that treatment of the insulin receptors with high concentrations of DTT reduces the interchain disulfide bonds but does not result in the insulin receptor subunit dissociation under nondenaturing buffer conditions. Further, removal of DTT by gel filtration chromatography results in the reoxidation of the reduced insulin receptor subunits back into an $\alpha_2\beta_2$ heterotetrameric disulfide-linked complex.

The functional consequences of the 50 mM DTT treatment and subsequent reoxidation of the insulin receptors were examined with respect to alterations in insulin binding activity (Figure 6). Treatment of the insulin receptors with 50 mM DTT followed by excess NEM treatment prior to gel filtration chromatography demonstrated a complete loss of insulin binding activity (data not shown). This suggests that at least some of the disulfide bonds critical to maintain the insulin binding activity of the insulin receptor have been reduced. Surprisingly, Scatchard analysis of insulin binding to the 50 mM DTT-treated and subsequently reoxidized insulin receptors after gel filtration chromatography indicated a 50% reduction in the high-affinity binding constant (42 vs. 81 pM for the untreated and DTT-treated insulin receptors, respectively) without significant change in the total number of binding sites when analyzed by a noncooperative two site model (Figure 6). This suggests that, after reduction of disulfide bonds necessary for insulin binding activity, the insulin receptor molecules can reoxidize back into a functional conformation. Further, the amount of insulin bound at saturation (R_0) is consistent with one insulin binding site per insulin receptor molecule, assuming a M_r 400 000 for the native $\alpha_2\beta_2$ insulin receptor complex.

The ability of insulin and DTT to stimulate the β subunit autophosphorylating activity of the 50 mM DTT-treated and reoxidized insulin receptors is shown in Figure 7. As previously observed for the recovery of insulin binding activity, the reoxidized insulin receptors were functional with regard to kinase activation by insulin as compared to the untreated insulin receptors. However, the maximal extent of insulin stimulation of the DTT-treated and reoxidized insulin receptors was found to be significantly reduced. Interestingly, the ability of DTT to stimulate the β subunit autophosphorylation of the insulin receptors was significantly impaired after the initial 50 mM DTT pretreatment and subsequent reoxidation whereas insulin was still capable of activating the β subunit autophosphorylation activity.

Discussion

The structural basis for the DTT activation of the insulin receptor/kinase has received much attention recently and may provide fundamental information for our understanding of the

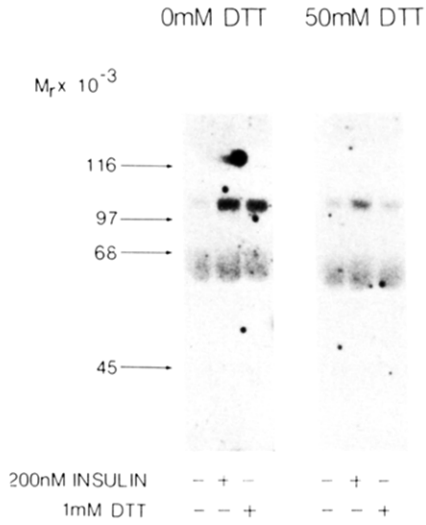

FIGURE 7: Autophosphorylation of untreated and DTT-pretreated insulin receptors after resolution by gel filtration chromatography. DTT-treated (50 mM) or untreated insulin receptors were resolved by Bio-Gel A-1.5m gel filtration chromatography, and the resulting peaks of insulin binding activity were pooled. These samples (20 ng) were then assayed for β subunit autophosphorylating activity in the presence and absence of 200 nM insulin or 1 mM DTT as shown in the figure. The phosphorylation reaction mixture was subjected to resolution in a reducing 7% SDS–polyacrylamide gel, and the autoradiograph is presented. The apparent decrease in mobility of the M_r 95 000 β subunit in this gel is due to the distortion of the band by the large amount of BSA present.

molecular mechanism involved in the intramolecular transmembrane single activation by insulin. It was originally proposed that relatively low concentrations of DTT can stimulate the insulin-dependent insulin receptor/kinase with little or no effect on basal kinase activity (Shia et al., 1983). At higher DTT concentrations, conversion of the $\alpha_2\beta_2$ heterotetrameric complex into $\alpha\beta$ insulin receptor heterodimers was observed concomitant with a reduction in the kinase activity. Thus, these authors concluded that the $\alpha_2\beta_2$ heterotetrameric disulfide-linked insulin receptor complex was the most kinase-active structure that required a reduced sulfhydryl group(s) to express maximal kinase activity. More recently, it was reported that the heterodimeric $\alpha\beta$ insulin receptor complex exhibits greater kinase activity than the heterotetrameric $\alpha_2\beta_2$ complex (Fujita-Yamaguchi & Kathuria, 1985). These studies were also based upon the examination of the DTT-stimulated insulin receptor/kinase activity in Triton X-100 (nondenaturing conditions) in comparison with SDS–polyacrylamide gel electrophoresis (denaturing conditions) to determine the degree of subunit dissociation. The discrepancy between the conclusions drawn by these two studies clearly results from the method of sample preparation used for the SDS–polyacrylamide gel electrophoresis (Figure 2). When the insulin receptors are heated in the presence of SDS and DTT (Figure 2B), at least a 10-fold increase in the apparent DTT sensitivity to subunit dissociation is observed by SDS–polyacrylamide gel electrophoresis. This is as compared to the insulin receptors that are first incubated with DTT, followed by excess NEM, to inactivate the reducing equivalents before heating and the addition of SDS (Figure 2A). It should be noted that, in some of the SDS–polyacrylamide gels run in the absence of reductants, the high molecular weight ^{32}P-autophosphorylated insulin receptors are not well resolved at the top of the gel. This is most likely due to the heat/SDS-dependent sulfhydryl rearrangement reported previously (Boyle et al., 1985). Nevertheless, these data document that the apparent altered sensitivity of the insulin receptor to DTT treatment previously reported by others (Shia et al., 1983; Fujita-Yamaguchi & Kathuria, 1985) results from the different conditions employed for SDS–polyacrylamide gel electrophoresis.

In order to directly compare the subunit structure of the insulin receptor with the DTT stimulation of the insulin receptor/kinase, we employed gel filtration chromatography under conditions similar to those used for the determination of the β subunit autophosphorylating activity. DTT concentrations (1–50 mM) that can completely dissociate the $\alpha_2\beta_2$ insulin receptor complex into monomeric α and β subunits in SDS (Figure 2) are unable to significantly dissociate the insulin receptor subunits in 0.1% Triton X-100 under these conditions (Figures 3 and 4). Further, after separation of the reduced $\alpha_2\beta_2$ insulin receptor complex from DTT,[2] the insulin receptor subunits spontaneously reoxidize into the M_r 400 000 disulfide-linked $\alpha_2\beta_2$ complex. To demonstrate that the reduced insulin receptor subunits did, in fact, remain associated under these conditions, NEM was added to alkylate all accessible sulfhydryl groups before the gel filtration chromatography (Figure 5). Recently, Pike et al. (1986) also observed that treatment of the insulin receptor with 2.0 mM DTT did not significantly alter its mobility by gel filtration chromatography under nondenaturing conditions. These results demonstrate that the reduced $\alpha_2\beta_2$ insulin receptor complex remains tightly associated in 0.1% Triton X-100 and only dissociates upon the addition of SDS. Thus, although the DTT stimulation of the insulin receptor/kinase does involve reduction of the insulin receptor subunits, it does not result in dissociation of the native $\alpha_2\beta_2$ insulin receptor subunit complex.

The fact that the insulin receptors, after reduction, are able to spontaneously reoxidize back into a functional $\alpha_2\beta_2$ complex (Figures 6 and 7) suggests that strong noncovalent interactions help maintain the insulin receptor subunits in their native conformational state. Surprisingly, the reduced and subsequently reoxidized insulin receptors are still capable of transmitting the insulin binding signal to the β subunit kinase domain. In contrast, these insulin receptors are unable to respond to the intramolecular signal produced by DTT. The reason for this variance is not yet apparent but may reflect fundamental differences in the molecular mechanisms of insulin and DTT activation of the insulin receptor/kinase.

Several laboratories have previously reported alterations of insulin receptor structure in response to insulin binding. These have included an increase in sensitivity of the α subunit to exogenously added proteases and changes in chromatographic gel profiles in detergent solutions (Krupp & Livingston, 1978; Pilch & Czech, 1980; Donner & Yonkers, 1983; Maturo et al., 1983). We have recently observed that insulin can potentiate the DTT-dependent reduction of the insulin receptor and suggested that this conformational change may be related to the mechanism of insulin receptor/kinase activation (Wilden et al., 1986). It should be pointed out that this insulin-potentiated DTT-dependent reduction of the insulin receptor is only apparent in SDS–polyacrylamide gels since the $\alpha_2\beta_2$ complex remains tightly associated in the absence of SDS. The results reported in this paper suggest that these previous observations do not reflect alterations in the association state of the insulin receptor subunits but probably represent changes in the conformational states of the native $\alpha_2\beta_2$ complex.

It has been recently reported that the EGF receptor/kinase may exist as a kinase-inactive noncovalently associated hom-

[2] The concentration of DTT in the various insulin receptors containing Bio-Gel A-1.5m fractions was determined to be less than 0.1 μM under the conditions of these experiments.

odimer (Biswas et al., 1985). The binding of EGF was suggested to convert the inactive homodimer to a kinase-active monomer state. On the basis of these results we would speculate that the $\alpha\beta$ heterodimeric form of the insulin receptor is a functional kinase unit equivalent to the monomeric EGF receptor. However, insulin-stimulation of the insulin receptor/kinase would then require the intact heterotetrameric $\alpha_2\beta_2$ complex. Currently we are developing methods to dissociate the native $\alpha_2\beta_2$ complex to form a functional $\alpha\beta$ heterodimeric insulin receptor structure.

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