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Identification of the $\alpha\beta$ monomer of the adipocyte insulin receptor by insulin binding and autophosphorylation

(hydrodynamic studies/native oligomeric structure)

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ABSTRACT **The insulin receptor consists of an insulin-binding subunit (α) of 135,000 daltons. More recently, it has been documented that the receptor undergoes insulin-stimulated autophosphorylation that predominantly labels a 95,000-dalton (β) subunit. We solubilized rat adipocyte insulin receptors in Triton X-100 and partially purified the protein on a wheat germ agglutinin-Sepharose affinity column. Subsequently, we labeled the two subunits of the receptor independently by using ^{125}I-labeled insulin for the 135,000-dalton α-subunit and ^{32}P for the 95,000-dalton β-subunit. Sucrose density gradient sedimentation and NaDodSO$_4$/PAGE were used to characterize the native, oligomeric structure of the receptor. In 0.1% Triton X-100, the receptor sedimented as a single peak of $s_{20,w}$ = 10.2 S as detected by both ^{125}I and ^{32}P. NaDodSO$_4$/PAGE under nonreducing conditions revealed a large species that appeared to be $\alpha_2\beta_2$ and, to a lesser extent, $\alpha\beta$. Treatment of the solubilized, partially purified receptor with 10 mM dithiothreitol led to the partial conversion of the 10.2S species to a smaller one sedimenting at 6.6 S. The composition of this species was determined to be $\alpha\beta$ by nonreducing NaDodSO$_4$/PAGE. Our results suggest that detergent-solubilized insulin receptors can exist as dimers and monomers. The oligomeric structure of receptors functional in the cell membrane cannot be immediately deduced from these results due to the possibility of artifacts arising from membrane disruption and extraction procedures. However, the ability to label the two subunits of the receptor separately should facilitate a detailed study of its oligomeric structure both in solution and in the membrane.**

In earlier reports from this laboratory, R. Aiyer (1, 2) characterized the hydrodynamic properties and subunit composition of insulin receptors from turkey erythrocyte plasma membranes solubilized in nondenaturing detergents. The receptor species were detected by covalently attaching ^{125}I-labeled insulin (^{125}I-insulin) using disuccinimidyl suberate (DSS). In Triton X-100, two species were observed: a monomer [$s_{20,w}$ = 6.6 S, M_p ($M_{protein}$) = 180,000 ± 45,000] and its disulfide-linked dimer ($s_{20,w}$ = 10.2 S, M_p = 350,000 ± 65,000). Their minimum subunit compositions were inferred from NaDodSO$_4$/PAGE analyses to be $\alpha\beta$ and $\alpha_2\beta_2$, respectively, where α is the insulin-binding subunit of M_{app} ($M_{apparent}$) = 135,000. The presence of an additional β-subunit (M_{app} = 80,000–90,000) was deduced from the observation that the 135,000-dalton polypeptide aggregated to species of higher molecular weight during NaDodSO$_4$/PAGE under nonreducing conditions that were not integral multiples of the mass of the α-subunit. In all of these studies, the smaller β-subunit could not be visualized directly because it did not label with ^{125}I-insulin. Similar results were also obtained for insulin receptors solubilized from rat adipocyte membranes, with the difference that the native dimer ($s_{20,w}$ = 10.2 S) was the only receptor species observed.

The presence of the β-subunit was definitively established when Kasuga and co-workers (3, 4) showed that insulin specifically stimulated the phosphorylation of the 95,000-dalton β-subunit of its own receptor in cultured human IM-9 lymphocytes and rat hepatoma cells. Subsequently, several other investigators demonstrated phosphorylation of the β-subunit of the insulin receptor in other intact cell systems including adipocytes (5) and hepatocytes (6) as well as cell-free systems such as Triton X-100 extracts of membranes from rat adipocyte (5), rat liver (4, 6–8), and human placenta (9–11). In the latter instances, phosphorylation appears to occur predominantly on tyrosine residues in the protein.

We have used the ability to selectively label the 95,000-dalton subunit as a probe into the subunit structure of the native receptor oligomer in detergent-solubilized rat adipocyte membranes. We labeled the two subunits independently by using ^{125}I-insulin for the α-subunit and ^{32}P for the β-subunit. The sedimentation behavior of receptor preparations labeled in the two separate ways was identical, indicating that the ^{32}P-labeled polypeptide is, indeed, part of the receptor oligomer and that autophosphorylation can be used as a structural probe. In addition, by using the disulfide reducing agent dithiothreitol, the dimer ($\alpha_2\beta_2$) of the adipocyte receptor could be dissociated into the monomeric ($\alpha\beta$) structure.

EXPERIMENTAL PROCEDURES

Materials. Male Charles River CD rats were used as the source of adipocytes. Porcine insulin was from Eli Lilly, CNBr-activated Sepharose was from Pharmacia, and ^{125}I-insulin was from New England Nuclear.

Solubilization of Insulin Receptors from Rat Adipocytes. Rat adipocyte membranes were prepared following the method of McKeel and Jarrett (12). The membranes were stored at −20°C in buffer A (50 mM Hepes, pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride. Just prior to phosphorylation experiments, the membrane suspension was thawed, and membrane proteins were extracted by first incubating the membrane suspension in 1% (vol/vol) Triton X-100 for 20 min at room temperature; this was followed by centrifugation at 100,000 × g for 60 min. This procedure usually resulted in solubilization of 50% of the total membrane protein. The supernatant at this stage will be referred to as "total extract."

Partial Purification of the Solubilized Receptor by Wheat Germ Agglutinin (WGA)-Sepharose. WGA was coupled to CNBr-activated Sepharose following the procedure suggest-

Abbreviations: DSS, disuccinimidyl suberate; WGA, wheat germ agglutinin.

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ed by the manufacturer (Pharmacia). Typically, 5 mg of WGA was coupled to 1 ml of Sepharose. Two milliliters of total extract (1 mg/ml of protein) was incubated with 1 ml of WGA-Sepharose for 16 hr at 4°C with gentle rocking. Subsequently, the WGA-Sepharose beads were washed extensively with 100 ml of buffer B (50 mM Hepes, pH 7.4/0.1% Triton X-100) containing 150 mM NaCl to remove proteins that may be nonspecifically associated with the beads. Specific elution was achieved by incubating the washed beads with 0.3 M *N*-acetylglucosamine in buffer B for 2 hr at room temperature with gentle rocking. Aprotinin (100 μl/ml; 1000 Kallikrein units/ml) was added at each stage to inhibit neutral proteases.

Phosphorylation Assays. Total and WGA-Sepharose-purified extracts were incubated with and without 1 μM insulin in buffer B, in a total assay volume of 100 μl. After 1 hr of incubation at room temperature, $MgCl_2$, $MnCl_2$, and [γ-^{32}P]ATP were added to final concentrations of 10 mM, 2 mM, and 5 μM, respectively. The incubation was allowed to continue for an additional 15 min at room temperature, at which time either (*a*) an equal volume of twice-concentrated Laemmli sample buffer (13) was added, the mixture was boiled for 3 min, and the samples were analyzed by $NaDodSO_4$/PAGE and autoradiography or (*b*) the reactions were stopped by the addition of NaF, $Na_2P_2O_7 \cdot 10\ H_2O$, EDTA, and ATP to final concentrations of 50, 10, 5, and 5 mM, respectively, and the samples were velocity sedimented through linear sucrose density gradients.

Preparation of Solubilized Receptors Covalently Labeled with ^{125}I-insulin. Adipocyte plasma membranes (2 mg/ml) were incubated with 5–10 nM ^{125}I-insulin with or without 2–5 μM porcine insulin (control for nonspecific insulin binding) in a total volume of 1 ml in buffer A. The incubation was carried out for 2 hr at room temperature, at the end of which the reaction mixtures were centrifuged in a Beckman Microfuge for 2 min. The supernatants were discarded and the insulin-bound membranes were allowed to react with 1 mM DSS for 1 min at room temperature as described elsewhere (1). The reaction was terminated with 20 mM NH_4Cl and the membranes were resuspended and washed twice with ice-cold buffer A and extracted with 3% (wt/vol) Triton X-100.

Velocity Sedimentation. Velocity sedimentation was carried out by sucrose density gradient ultracentrifugation as described elsewhere (1). The values of $s_{20,w}$ were estimated by using the following marker enzymes: catalase, 11.3 S; fumarase, 9.0 S; lactate dehydrogenase, 7.3 S; and malate dehydrogenase, 4.3 S.

$NaDodSO_4$/PAGE and Autoradiography. $NaDodSO_4$/PAGE analysis was carried out by using the Laemmli system (13): the separating gel was made with 7.5% acrylamide for reducing conditions and 5% acrylamide for nonreducing conditions with a stacking gel containing 4% acrylamide in both cases. The standard proteins used to estimate apparent molecular weights were: myosin, 200,000; β-galactosidase, 116,000; phosphorylase b, 94,000; transferrin, 77,000; bovine serum albumin, 68,000; and catalase, 58,000. Kodak XR-50 film together with Cronex intensifying screens were used to obtain autoradiograms of the dried gels. The relative intensities of the radioactive bands were estimated by using a scanning densitometer (model Quick Scan from Helena Laboratories).

RESULTS AND DISCUSSION

Detergent-solubilized rat adipocyte insulin receptors were covalently labeled with ^{125}I-insulin and sedimented through 5–20% linear sucrose density gradients. As seen in Fig. 1, a single peak of specific insulin binding activity was observed sedimenting with a $s_{20,w}$ of 10.2 S. The radioactivity in this peak was displaced when labeling with ^{125}I-insulin was car-

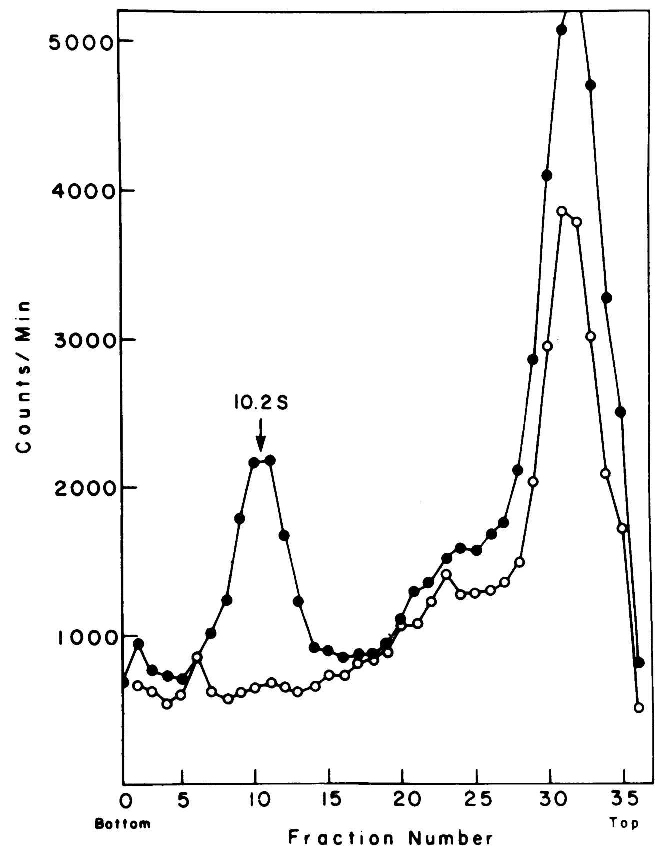

FIG. 1. Velocity sedimention of Triton X-100-solubilized rat adipocyte insulin receptors. Rat Adipocyte plasma membranes were bound and cross-linked with ^{125}I-insulin and extracted in 3% Triton X-100. Two hundred microliters of this extract was layered onto 4.8 ml of 5–20% (wt/vol) linear sucrose density gradients in buffer B. They were sedimented in a Beckman SW 50.1 rotor at 48,000 rpm, 3°C, for 9 hr. The gradients were fractionated and assayed for radioactivity in a Beckman γ-counter. ○, Binding was carried out in the presence of 2 μM unlabeled insulin (control); ●, binding was carried out in the absence of excess unlabeled ligand.

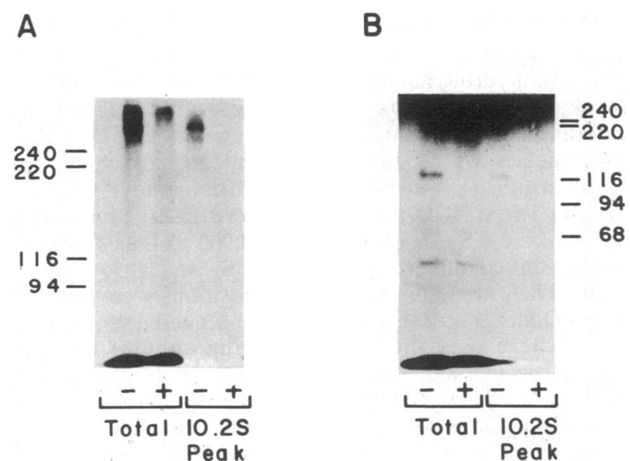

FIG. 2. $NaDodSO_4$/PAGE of ^{125}I-insulin-labeled adipocyte receptors under reducing and nonreducing conditions. Rat adipocyte receptors were labeled with ^{125}I-insulin in the absence (−) and presence (+) of excess unlabeled insulin and extracted in Triton X-100. The labeled samples were analyzed by $NaDodSO_4$/PAGE by using the Laemmli system (13) under reducing conditions (10% 2-mercaptoethanol) with a separating gel of 7.5% acrylamide (*B*) and nonreducing conditions with a separating gel of 5% acrylamide (*A*). Molecular masses are shown in kilodaltons.

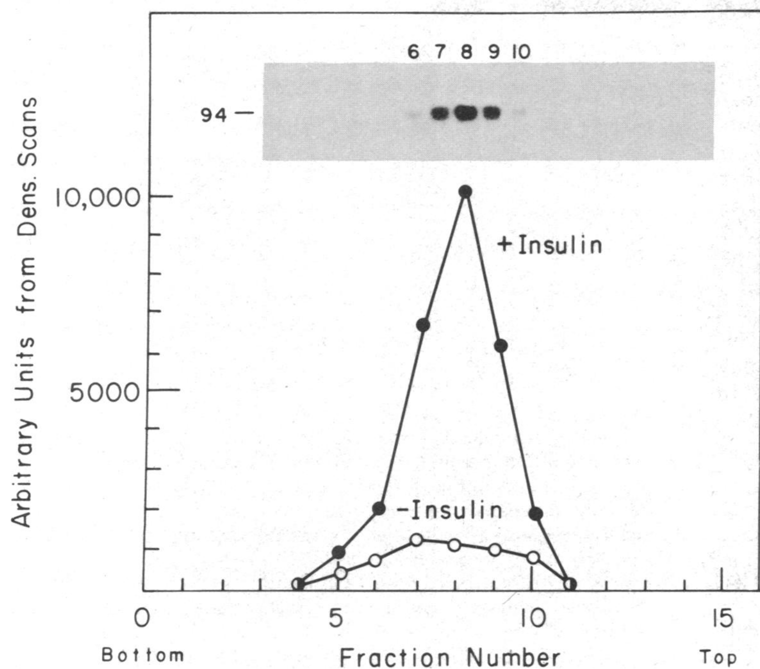

FIG. 3. Velocity sedimentation of WGA-purified and phosphorylated receptor. WGA-purified receptor preparation was incubated for 1 hr at room temperature in the presence and absence of 1 μM insulin. Two hundred microliters of each sample was layered on 4.8 ml of 5–20% linear sucrose density gradients and sedimented. The fractions of each gradient were analyzed by NaDodSO$_4$/PAGE under reducing conditions on a Laemmli system (13) using a separating gel of 7.5% acrylamide. The autoradiograms were scanned in a densitometer and the areas of density peaks were estimated by weighing. The graph shows the density profiles obtained for the ^{32}P-labeled 95,000-dalton band with and without preincubation with insulin. (*Inset*) Autoradiogram of the 95,000-dalton band for the insulin-stimulated case (molecular mass is shown in kilodaltons). The autoradiogram for the non-insulin-stimulated autophosphorylation is not shown because ^{32}P incorporation in this case is very faint, as can be judged from the ratios of the areas in the graph.

ried out in the presence of 2 μM unlabeled insulin. Partial purification of solubilized receptor on a WGA-Sepharose affinity column did not alter its sedimentation behavior. As shown in Fig. 2, NaDodSO$_4$/PAGE analysis of the 10.2S species under reducing conditions revealed that the 135,000-dalton α-subunit (Fig. 2*B*), is the predominant insulin-labeled component. Under nonreducing conditions (Fig. 2*A*), the β-subunit aggregated to larger-sized bands, which were not well resolved.

It has been shown that the α-subunit is associated with a smaller subunit of 95,000 daltons that can be labeled by [γ-^{32}P]ATP (3, 4). When we analyzed the WGA-purified, ^{32}P-labeled preparation by velocity sedimentation through 5–20% linear sucrose density gradients (Fig. 3), we obtained a single peak of ^{32}P-labeled material whose extent of labeling was stimulated by insulin preincubation. The peak had a $s_{20,w}$ value of 10.2 S. Conversely, when unlabeled receptors were first sedimented and then phosphorylated, with and without preincubation with insulin, an insulin-stimulatable kinase activity sedimented at the same position as the insulin labeled and phosphorylated polypeptide (data not shown). These results are consistent with evidence from several other laboratories (14-17) that the kinase activity is part of the native, oligomeric receptor molecule.

The identical sedimentation behavior of the insulin-binding and phosphorylated polypeptides was more clearly demonstrated by means of a double-label experiment (Fig. 4). We first labeled membrane-bound insulin receptors covalently with ^{125}I-insulin and, following extraction in Triton X-100 and partial purification by WGA-Sepharose affinity chromatography, we phosphorylated the receptors with [γ-^{32}P]ATP. The two labels were used to identify the two subunits independently of each other: ^{125}I-insulin for the α-subunit and ^{32}P for the β-subunit. As shown in Fig. 4, they comigrate following velocity sedimentation, as judged by the intensity of the respective radiolabeled bands in NaDodSO$_4$/PAGE. This result clearly demonstrates that 135,000- and 95,000-dalton polypeptides are part of the same native oligomer.

In hydrodynamic studies of native insulin receptors from turkey erythrocyte and liver membranes (1, 2), it was determined that the protein can exist as a mixture of $\alpha_2\beta_2$ dimers ($s_{20,w}$ = 10.2 S) and $\alpha\beta$ monomers ($s_{20,w}$ = 6.6 S) in detergent extracts. There was also evidence that the monomers were held together in the dimeric structure by means of disulfide bonds. In solubilized extracts of adipocyte plasma membranes, however, dimers were the only receptor species observed after velocity sedimentation (Fig. 1).

To determine whether the dimeric structure of the adipocyte receptor was also held together by disulfides, we studied the sedimentation behavior of ^{32}P-labeled adipocyte re-

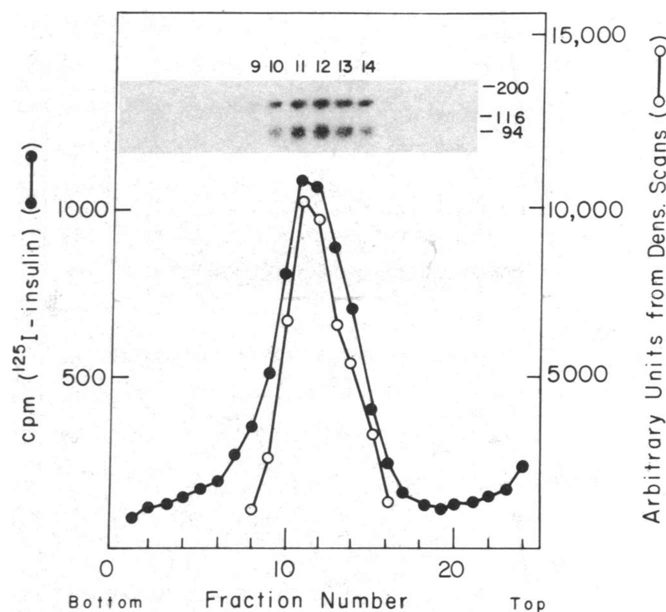

FIG. 4. Velocity sedimentation of ^{125}I-insulin- and ^{32}P-labeled receptor. Rat adipocyte insulin receptors were first covalently labeled with ^{125}I-insulin, then extracted with 2% Triton X-100, and subsequently purified on WGA-Sepharose. Next, they were incubated for 1 hr at room temperature in the absence and presence of 1 μM insulin before labeling with [γ-^{32}P]ATP. The double-labeled samples were analyzed by velocity sedimentation and NaDodSO$_4$/PAGE as described for Fig. 3. ●, cpm of ^{125}I-insulin specifically bound as detected by a Beckman 4000 γ-counter; ○, relative intensity of the 95,000-dalton band in the fractions as determined from densitometry scan of the autoradiogram. (*Inset*) Actual autoradiogram used for densitometry (molecular masses are shown in kilodaltons).

ceptors that were treated with 10 mM dithiothreitol. The results are shown in Fig. 5.

Treatment with the reducing agent produced one significant change: the appearance of a 6.6S peak, which is most likely the monomer form ($\alpha\beta$) observed earlier in detergent-solubilized extracts of turkey erythrocyte and rat liver membranes (1, 2). In addition, a shift in the position of the dimer peak from 10.2 S to 11.3 S was consistently observed. The amount of ^{32}P label incorporated into the 10.2 S receptor species (Fig. 5*A*) was divided quantitatively between the 11.3S and 6.6S receptor species following dithiothreitol treatment (Fig. 5*B*).

The material under all three peaks was analyzed by NaDodSO$_4$/PAGE. Under reducing conditions (Fig. 5), only the ^{32}P-labeled β-subunit was observed under each peak. In another experiment with ^{125}I-insulin-labeled receptors, the same procedure produced the ^{125}I-insulin-labeled 135,000-dalton α-subunit (data not shown).

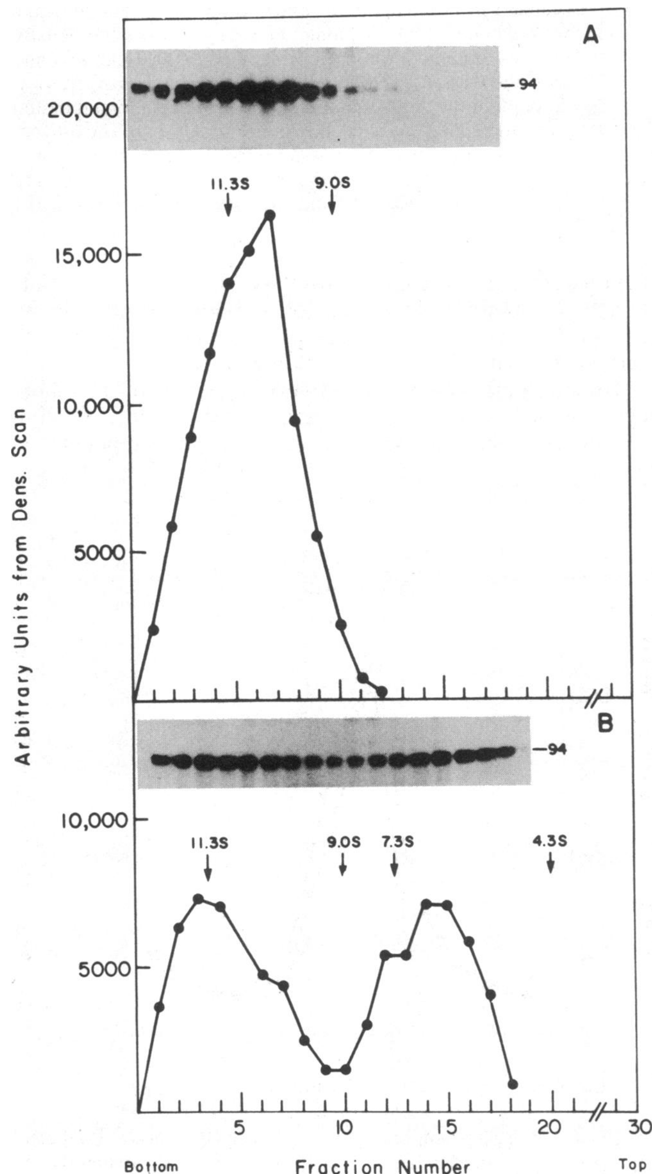

FIG. 5. Effect of dithiothreitol on the sedimentation behavior of phosphorylated insulin receptors. Insulin receptors were treated with mM dithiothreitol for 15 min at room temperature following WGA-Sepharose purification and phosphorylation and then subjected to velocity sedimentation. (*A*) Extract that was not treated with dithiothreitol; (*B*) extract that was allowed to react with 10 mM dithiothreitol. The corresponding autoradiograms are shown in the *Inset* for each graph (molecular mass is shown in kilodaltons). The values for $s_{20,w}$ were determined by using the appropriate marker enzymes.

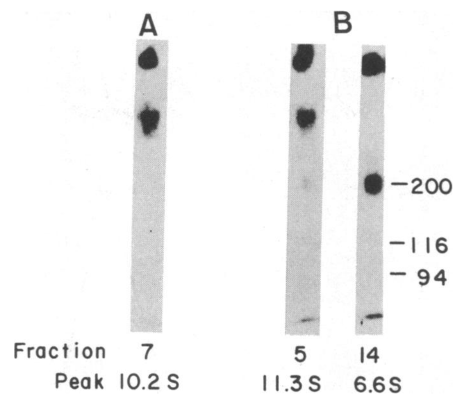

FIG. 6. NaDodSO$_4$/PAGE of 10.2S and 6.6S insulin receptor species under nonreducing conditions. The peak fractions from the gradients shown in Fig. 5 were analyzed by NaDodSO$_4$/PAGE under nonreducing conditions by using the Laemmli system (13) and a separating gel of 5% acrylamide. (*A*) Peak fraction from the sample that was not treated with dithiothreitol; (*B*) peak fractions from the sample that was treated with 10 mM dithiothreitol prior to sucrose density gradient sedimentation (molecular masses are shown in kilodaltons).

Under nonreducing conditions (Fig. 6), the 6.6S peak revealed a distinct ^{32}P-labeled species of 210,000 daltons, which clearly is the disulfide-linked $\alpha\beta$-monomer of the receptor. The larger peaks of 10.2 S and 11.3 S produced aggregates greater than 210,000, quite possibly $\alpha_2\beta_2$ dimers. It should be pointed out that the 210,000-dalton species is virtually absent in both of these peaks.

Massague and Czech (18) have reported that reduction of rat adipocyte receptors with 10 mM dithiothreitol and subsequent analysis under nonreducing conditions revealed a 210,000-dalton ^{125}I-insulin-labeled species, which they designated $\alpha\beta$. Aiyer (2) has also observed that the monomeric receptor species of turkey erythrocytes, which sedimented at 6.6 S in sucrose density gradients, yielded a 200,000-dalton $\alpha\beta$ species on nonreduced gels.

CONCLUSIONS

Velocity sedimentation of insulin receptors ^{32}P-labeled by autophosphorylation yields a peak with a sedimentation coefficient of 10.2 S, exactly as observed with receptors that were labeled with ^{125}I-insulin. Our sedimentation and NaDodSO$_4$/PAGE results taken together confirm that the basic monomer of the receptor is composed of a 135,000-dalton α-subunit that binds insulin and a 95,000-dalton β-subunit that is phosphorylated and may itself be the kinase.

The adipocyte insulin receptors were obtained only as $\alpha_2\beta_2$ dimers sedimenting at 10.2 S in nondenaturing detergent solution in contrast to detergent extracts of the turkey erythrocyte and liver membranes, where both monomers and dimers were observed (1, 2). However, we have shown that $\alpha_2\beta_2$ dimers can be partially dissociated into monomers by treatment with dithiothreitol. This was evidenced by the appearance of a peak of sedimentation at 6.6 S corresponding to monomers after reduction.

These results present hydrodynamic evidence for the presence of $\alpha\beta$ monomers in the native structure of the adipocyte insulin receptor in detergent solution and raise questions as to the functional role of $\alpha\beta$ monomers in insulin action. In particular, it should be possible to determine whether the monomer can undergo autophosphorylation and/or act as a kinase on another substrate. Furthermore, the ability to specifically label both subunits of the receptor should prove to

be an effective way to probe the details of its structure both in solution and in the membrane. The regulation of the oligomeric state of the protein and the role of insulin, if any, in its regulation are questions that can be addressed through this approach.

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1. Aiyer, R. A. (1983a) *J. Biol. Chem.* **258,** 14992–14999.
2. Aiyer, R. A. (1983b) *J. Biol. Chem.* **258,** 15000–15003.
3. Kasuga, M., Karlsson, F. Anders & Kahn, C. R. (1982) *Science* **215,** 185–187.
4. Kasuga, M., Zick, Y., Blithe, D. L., Crettaz, M. & Kahn, C. R. (1982) *Nature (London)* **298,** 667–669.
5. Haring, H. U., Kasuga, M. & Kahn, C. R. (1982) *Biochem. Biophys. Res. Commun.* **108,** 1538–1545.
6. Van Obberghen, E. & Kowalski, A. (1982) *FEBS Lett.* **143,** 179–182.
7. Zick, Y., Kasuga, M., Kahn, C. R. & Roth, J. (1983) *J. Biol. Chem.* **256,** 75–80.
8. Zick, Y., Whittaker, J. & Roth, J. (1983) *J. Biol. Chem.* **258,** 3431–3434.
9. Machicao, F., Urumow, T. & Wieland, O. H. (1982) *FEBS Lett.* **149,** 96–100.
10. Petruzelli, L. M., Ganguly, S., Smith, C. J., Cobb, M. H., Rubin, C. S. & Rosen, O. M. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 6792–6796.
11. Avruch, J., Nemenoff, R. A., Blackshear, P. J., Pierce, M. W. & Osathanondh, R. (1982) *J. Biol. Chem.* **257,** 15162–15166.
12. McKeel, D. W. & Jarrett, L. (1970) *J. Cell. Biol.* **44,** 417–432.
13. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
14. Roth, J. & Cassell, D. J. (1983) *Science* **219,** 299–301.
15. Van Oberghen, E., Rossi, B., Kowalski, A., Gazzano, H. & Ponzio, G. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 945–949.
16. Rosen, O. M., Herrera, R., Olowe, Y., Petruzzelli, L. M. & Cobb, M. H. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 3237–3240.
17. Stadtmauer, L. A. & Rosen, O. M. (1983) *J. Biol. Chem.* **258,** 6682–6685.
18. Massague, J. & Czech, M. P. (1982) *J. Biol. Chem.* **257,** 6729–6738.