PURIFICATION AND PROPERTIES OF INSULIN RECEPTORS FROM RAT LIVER MEMBRANES

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Received June 17,1977

Summary. Insulin receptors were solubilized from rat liver membranes with Triton X-100. The soluble receptors were purified on DEAE cellulose and then on an insulin-agarose affinity column. The purified receptor had a Stokes radius of 72Å on Sepharose 6B, an isoelectric point of 4.0, and could be adsorbed to Conanavalin A-agarose and specifically eluted with α-methylmannopyranoside. The eluate from the insulin-agarose column demonstrated a major band with an apparent molecular weight of 135,000 on SDS polyacrylamide gel electrophoresis. Because of the possibility of anomalous SDS binding, this molecular weight must be accepted with caution.

Insulin receptors are intrinsic membrane proteins. They have been solubilized with retention of binding activity (1), and some of their physical and chemical properties have been determined (2,3). Although techniques for insulin receptor purification have been available for several years (4,5,6), until now amounts of receptor sufficiently large for direct chemical characterization have not been obtained. In this report a procedure for large-scale purification of the insulin receptor and some of its properties are described. Some of the data presented here has appeared in a preliminary form previously (7).

Methods. [^{125}I]Insulin (175 μCi/μg) was prepared with chloramine T(8). Soluble insulin receptors were extracted from a crude rat liver microsomal fraction with 2% (v/v) Triton X-100 (6). Insulin binding to the soluble receptor was measured using the polyethylene glycol method (1). Because of the low concentration of protein during final stages of purification, and because Triton X-100 interferes with many methods of protein determination, florescamine (9) was used to measure protein with bovine serum albumin dissolved in an equal concentration of detergent as the standard.

Insulin-succinyl-diaminodipropylamino-agarose (0.6 mg/mg packed gel) was prepared and washed as previously described (4) except that epichlorhydrin-crosslinked agarose was used to provide better stability of the matrix backbone. Immediately prior to use the insulin-agarose was washed overnight with 4.5 M urea in 50 mM sodium acetate, pH 6.0. This washing procedure was also used to regenerate the column after use.

Concanavalin A-agarose was prepared as previously described (5), except that epichlorhydrin-agarose was used.

Preparative isoelectric focusing was performed at 4° in a sucrose gradient containing 0.2% Triton X-100 and 1% (w/v) ampholines with a 110 ml

ISSN 0006-291X

LKB column. Focusing was started at 800 V for two hours and continued at 1200 V for an additional 22 hours. Eluted fractions were titrated to pH 7.4 with 1N HCl or 1N NaOH before insulin binding activity was measured.

Gel filtration was performed with a Sepharose 6B column (1x30 cm) equilibrated with 0.5 M NaCl, 0.05 M $NaPO_4$, 0.2% Triton X-100, pH 7.4, and developed with this same buffer. The flow rate was 6 ml/hr. Insulin binding was measured in the eluted fractions.

Results. The Triton X-100 extract of the microsomal fraction was dialysed against 50 mM sodium acetate, 0.2% Triton X-100, pH 6.3. The fine precipitate which formed was removed by centrifugation at 100,000 x g for 30 minutes. The supernatant was chromatographed on DEAE cellulose as previously described (4). Seventy percent of the insulin binding activity obtained from 50 rat livers could be adsorbed and eluted from a 2 x 50 cm column. This step results in only 7-fold purification. However, it is a convenient means of decreasing the concentration of detergent below levels which will inhibit insulin-receptor binding (1), without excessive dilution or aggregation of proteins. For reasons which are not clear, this step increases the capacity of the subsequent insulin affinity column.

Insulin-Agarose Affinity Chromatography. Eluate fractions from the ion exchange column were pooled, dialysed against 0.1 M $NaPO_4$, 0.1% Triton X-100 pH7.4 and applied at room temperature to a 5 ml column of insulin- succinyl-diaminodipropylamino-agarose. Under these conditions more than 85% of the insulin binding activity obtained from 50 rat livers could be adsorbed to the column. The column was washed for 18 hours at 4° with 400 ml of 0.5 M NaCl, 0.05 M $NaPO_4$, 0.1% Triton X-100, pH 7.4, following which 10 to 25% of the applied insulin binding activity was eluted at room temperature with 4.5 M urea, 0.05 M sodium acetate, 0.1% Triton X-100, pH 6.0. Because of the time- dependent irreversible denaturation of the receptor by urea (3), the eluate was immediately diluted with an equal volume of buffer. The specific activity of the insulin binding activity present in the eluate from the insulin affinity column is 0.4 nmole/mg protein, which is 2,000-fold higher than in the crude membrane fraction.

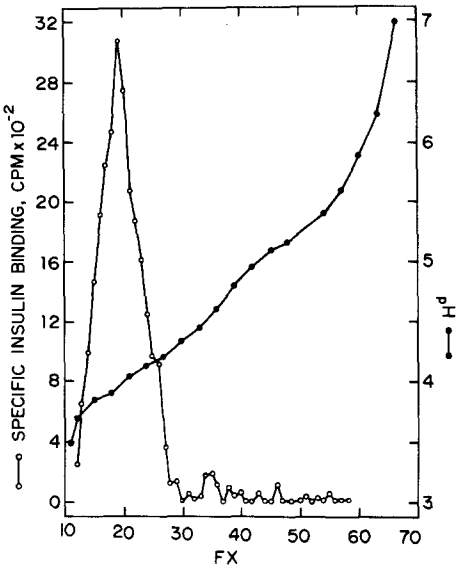
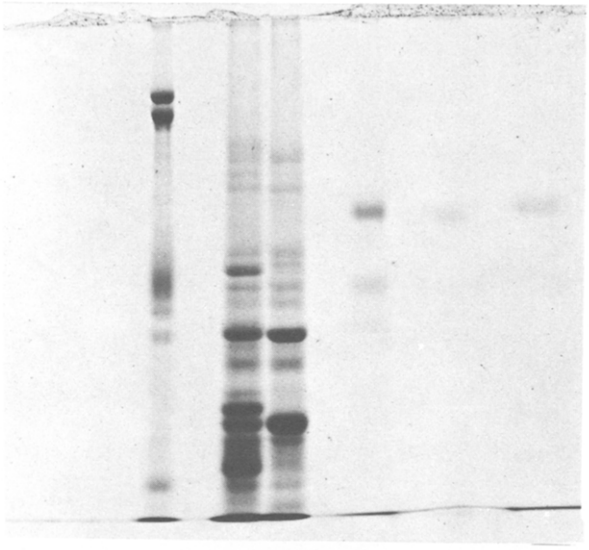


Figure 1. Preparative isoelectric focusing of the purified insulin receptor. The eluate from the insulin-agarose column was dialysed against 10 mM Tris-HCl, 0.1% Triton X-100, pH 7.4, and focused as described in Methods with pH 3.5-5.0 Ampholine®. The pH of eluant fractions was measured at 4°, and after neutralization binding activity was measured on 20 µl aliquots as described in Methods.

Gel Filtration - The eluate from the insulin affinity column was dialysed against 50 mM $NaPO_4$, 0.1% Triton X-100, pH 7.4, and concentrated 10- fold by further dialysis against solid polyethylene glycol. 100 µl of the concentrated material was applied to a Sepharose 6B column as described in Methods. The insulin binding activity eluted with a K_{av} of 0.32, corresponding to a Stokes radius of 72Å. Similar results have been obtained for the insulin receptor prior to purification from rat liver membranes (3), rat fat cell membranes (3), and turkey erythrocyte ghosts (10).

Isoelectric Focusing. The pI (Fig. 1) of the receptor measured by insulin binding activity was near 4.0 (range 3.9 to 4.2 in several runs.) The insulin receptor present in the eluate from DEAE cellulose prior to purification on insulin-Sepharose focuses at the same pI. However, when the DEAE eluate is used, a precipitate forms. If more than 10 mg of protein



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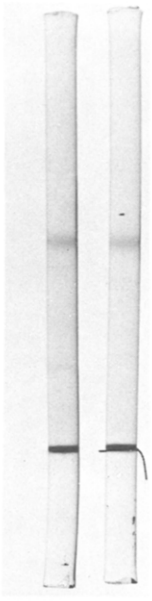

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Figure 2. SDS polyacrylamide gel electrophoresis of the insulin receptor at various stages of purification. Samples were boiled in 1% SDS, 20 mM dithiothreitol for 10 minutes and electrophoresed using the system described by Laemmli (12). The stacking gel contained 3% acrylamide, and the spacer gel 6.5% acrylamide. From left to right the samples are: (A) human erythrocyte ghosts used as molecular weight standards (13); (B) the Triton X-100 extract of liver membranes; (C) the eluate from DEAE cellulose; (D) the eluate from insulin-agarose; (E) the eluate from insulin-agarose after further purification on Sepharose 6B; (F) the eluate from insulin-agarose after further purification by isoelectric focusing and extensive dialysis to remove Ampholine. The gels were stained with Coomassie blue (13).

Figure 3. SDS polyacrylamide gel electrophoresis of the receptor purified on insulin-agarose before (left) and after (right) further purification on Concanavalin A-agarose. The procedure was the same as for figure 2 except that the spacer gel contailed 6% acrylamide. The wire marks the dye front.

are applied to the columm, the precipitate will disturb the gradient and interfere with elution.

Concanavalin A-Agarose Affinity Chromatography - It has been demonstrated previously that Concanavalin A and wheat germ agglutinin interact with the insulin receptor (5,11) and that these could be useful in its purification

(5). The eluate from the insulin affinity column was dialysed against 50 mM Tris-HCl, 0.1% Triton X-100, pH 7.4, adjusted to 2 mM $MgCl_2$, 2 mM $CaCl_2$ and applied to a 2.5 ml column of Concanavalin A-agarose. The column was washed with 50 ml of the same buffer and eluted with 0.5 M α-methylmanno-pyranoside in 50 mM Tris-HCl, 0.1% Triton X-100, pH 7.4. All of the insulin binding activity from 50 rat livers, which had been previously purified on insulin-agarose, could be adsorbed to the column; 40% to 60% could be eluted with the specific, simple sugar.

SDS-Polyacrylamide Gel Electrophoresis - The SDS-polyacrylamide gel electrophoretic patterns of the insulin receptor during different stages of purification are shown in Figures 2 and 3. The eluate from the insulin affinity column contains one major protein band with an apparent molecular weight of 135,000, and other minor bands of lower molecular weight. It is significant that the major band is not visible in the crude extract or in the eluate from the DEAE cellulose column, consistent with its being only a minor component of the total protein in these fractions. This same major protein band is present in eluate fractions containing insulin binding activity from the Sepharose 6B column (Fig. 2), the isoelectric focusing column (Fig. 2) and the Concanavalin A-agarose column (Fig. 3).

Displacement of [^{125}I]Insulin from the Purified Receptor- [^{125}I]Insulin can be displaced from the purified receptor by native insulin (Fig. 4). The concentration of native insulin required to displace 50% of the specifically bound, labeled insulin is 12 ng/ml. ACTH, glucagon and epidermal growth factor at concentrations of 1 μg/ml caused no significant displacement.

Stability of the Purified Receptor - The soluble receptor in the crude liver membrane extract is quite stable (3). However, following purifi-cation on insulin-agarose binding activity is rapidly destroyed by freezing and thawing. This can be partially prevented by freezing in 50% glycerol, 30% glucose or 20% albumin, or by storage at 4°.

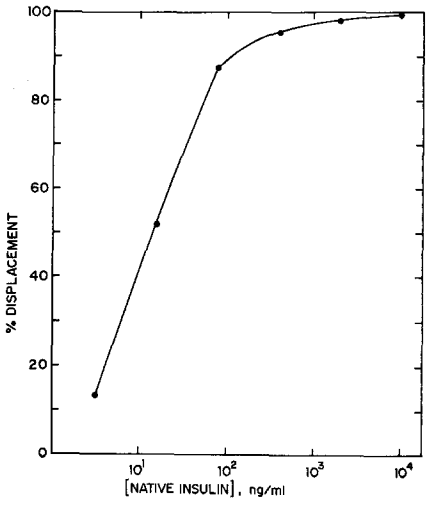

Figure 4. Displacement of [^{125}I]insulin from the purified receptor. The eluate from the insulin-agarose column was dialysed against 50 mM $NaPO_4$, 0.1% Triton X-100, pH 7.4. Aliquots of the dialysate containing 240 ng of protein were incubated with 55 pg of labeled insulin in 0.2 ml 100 mM $NaPO_4$, 0.1% albumin, pH 7.4, containing no native insulin or increasing concentrations of native insulin. The amount of labeled insulin bound to the receptor was determined by precipitating with polyethylene glycol (1). In the absence of native insulin, 16 pg of labeled insulin are bound.

Discussion. Using the purification scheme described in this report, 50 rat livers can easily be processed. Although the yield in terms of insulin binding activity is low, only about 10%, it is possible to obtain about 200 µg of purified protein.

The specific activity of the purified receptor, 0.4 pmol/µg, is about seven times less than predicted if it is assumed that the molecular weight of the receptor is 300,000 (3) and one mole of receptor binds one mole of insulin. There may be several reasons for this low specific activity. Measurement of insulin binding capacity is subject to large errors. The method used to determine protein, although reproducible, is non-specific and might be expected to overestimate the true protein concentration. Most importantly, the conditions used to elute the receptor from the insulin-agarose column are harsh and some irreversible denaturation of the receptor may be inevitable. However, the disparity between the experimentally determined

specific activity and the expected specific activity demands caution in accepting the major protein band present in the eluate from the insulin affinity column as the true receptor. To investigate this, the receptor purified on insulin-agarose was further purified by gel filtration chromatography, isoelectric focusing, and Concanavalin A affinity chromatography. Each of these procedures depends on very different physical or chemical properties for separation, yet in each case the same major protein band was present in the elution fractions possessing insulin binding activity. Furthermore, the minor disc gel bands varied between different batches of purified receptor and between different fractionation procedures, and were also visible in the crude Triton extract suggesting they were contaminating proteins not completely removed by insulin-agarose affinity chromatography.

Using the Stokes radius of the insulin receptor determined by gel filtration, and the reported (3) sedimentation constant, a molecular weight of 300,000 can be calculated if minimal detergent binding and a partial specific volume of 0.734 cm^3 per g are assumed (3). On SDS-polyacrylamide gel electrophoresis the reduced and denatured receptor has an apparent molecular weight of 135,000. Since glycoproteins and membrane proteins are notorious for anomalous SDS-binding, a considerably lower molecular weight is possible. This might suggest that the native receptor is composed of at least two and possibly more subunits, a result consistent with the finding that insulin causes the reversible dissociation of the receptor into subunits with a Stokes radius of 40 Å (10).

REFERENCES

1. Cuatrecasas, P. (1972) Proc. Nat. Acad. Sci. USA, 69, 318-322.
2. Cuatrecasas, P. (1971) J. Biol. Chem. 246, 7265-7274.
3. Cuatrecasas, P. (1972) J. Biol. Chem., 247, 1980-1991.
4. Cuatrecasas, P. (1972) Proc. Nat. Acad. Sci. USA, 69, 1277-1281.
5. Cuatrecasas, P. and Tell, G. P. E. (1973) Proc. Nat. Acad. Sci USA, 70, 485-489.
6. Cuatrecasas, P., and Parikh, I., Insulin receptors. In: Methods in Enzymol., Vol. XXXIV, Part B, ed. W. B. Jacoby and M. Wilchek, Academic Press, N. Y., pp. 670-688, 1974.
7. Jacobs, S., Parikh, I., Cuatrecasas, P., (1977) Fed. Proceed. 36, 710.

8. Cuatrecasas, P. (1971) Proc. Nat. Acad. Sci. USA 68, 1264-1268.
9. Böhlen, P., Stein, S., Dairman, W., and Udenfriend, S. (1973) Arch. Biochem. Biophys 155, 213-220.
10. Ginsberg, B. H., Kahn, C. R., Roth, J., De Meyts, P. (1976) Biochem. Biophys. Res. Commun. 73, 1068-1074.
11. Cuatrecasas, P. (1973) J. Biol. Chem 248, 3528-3534.
12. Laemmli, U. K. (1970) Nature (London) 227, 680-685.
13. Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971) Biochemistry 10, 2606-2617.