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PHOTOREACTIVE INSULIN ANALOGUES USED TO CHARACTERISE THE INSULIN RECEPTOR

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Summary Three photoreactive insulin analogues ("photoprobes") have been prepared in which an aryl azide group was substituted at either the A1, B1 or B29 positions of the insulin molecule. When incubated with rat liver plasma membranes and irradiated all three photoprobes covalently labelled specific insulin binding sites within the membrane. SDS-polyacrylamide gel electrophoresis of plasma membranes covalently tagged with either of the three ^{125}I-photoprobes resolved one major specifically labelled polypeptide with an apparent molecular weight of 130,000. The labelled polypeptide migrated anomalously in SDS-polyacrylamide gels and a molecular weight of 90,000 for the polypeptide was determined from a 'Ferguson' plot using the combined results from gels of different acrylamide concentrations. Column chromatography of detergent solubilised photoprobe-labelled membranes indicated that the labelled polypeptide may be a subunit of a larger protein complex.

The use of hormone analogues containing photoreactive groups allowing covalent linkage to the receptor provides a powerful new approach to the study of mechanisms of hormone action. Recently a number of photoreactive derivatives of hormones and circulating proteins have been prepared (1,2,3). Following UV irradiation these photoreactive analogues covalently label specific binding sites on cells or in isolated subcellular fractions. We report here a characterisation of the rat liver plasma membrane insulin receptor using three photoreactive insulin analogues (insulin photoprobes). Although the kinetics of insulin binding to cells and isolated plasma membranes have been studied in some detail (for review see Ref 4) and some progress has been made in isolating and purifying the insulin receptor (5,6,7), these studies have been hampered by the small amounts of receptor present in the plasma membrane. The advantage of using photoreactive insulins is that the receptor can be labelled specifically and covalently with radio-iodinated photoprobe thus allowing the identification of the receptor after solubilisation of the membranes and during subsequent purification procedures. Our characterisation of the covalently labelled proteins

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in rat liver plasma membranes was carried out using insulin photoprobes in which an aryl azide group was specifically substituted at the B1, B29 or A1 amino acids (8,9). These were as follows: [Phe (4-azido)^{B1}] insulin, B-29-(4-azidophenylacetyl)-insulin and [γ-(4-azidophenylacetyl)-D-α,γ-diaminobutyrl ^{A1}]-insulin. Previous studies using chemically modified insulins (10) showed that the B29 Lys and A1 Gly are close to the receptor binding region of insulin whereas the B1 Phe lies away from this site at the end of a flexible amino acid chain.

Methods Lipogenesis was measured (11) using isolated rat adipocytes (12). Rat liver plasma membranes derived predominantly from the blood sinusoidal surface of the hepatocyte were prepared as described previously (13). These plasma membranes contained very little insulin degrading activity (14). Insulin photoprobes were iodinated with ^{125}I using chloramine T (15). Plasma membranes (0.8-1.2 mg) were incubated in the dark at 30°C for 30 minutes with 11-13 nM (690-800 Ci/mmol) of ^{125}I-photoprobe in Krebs-Ringer phosphate buffer - 1% bovine serum albumin pH 7.8 (final volume 250 µl). Non-specific labelling was determined in the presence of 17.4 µM beef insulin. The photoreactive insulins were then activated by irradiation for 20 minutes at 4°C with a high pressure mercury lamp (Phillips HPK 125 W/L UV lamp, 8 cm from sample). Non-covalently bound photoprobe was disscoiated off the membranes by diluting the sample to 18 ml in Krebs-Ringer phosphate buffer pH 7.8 and incubating at 30°C for 15 minutes. The plasma membranes were pelleted by high-speed centrifugation (95,000 g_{av} for 30 minutes) and then washed once more in the buffer. The final plasma membrane pellet was dissolved in 6M urea - 10% SDS-1% mercaptoethanol and heated at 90°C for 5 minutes. Polyacrylamide gel electrophoresis was carried out in SDS-Tris-glycine buffers (16) using a Raven Scientific (Haverhill, Suffolk, UK) slab gel electrophoresis apparatus. The gels were discontinuous, with a 3.6% (w/v) acrylamide spacer gel, pH 6.7 and a pH 8.9 resolving gel (ratio of acrylamide: bis-acrylamide was 100 : 1). After electrophoresis at 30mA for 16 h the gels were stained for protein with Coomassie Blue (16). Radioactivity was measured in 2 mm slices of the gel cut with an automatic gel slicer (Bio-Rad Laboratories, Richmond, Ca, U.S.A.). Molecular weights were determined from the position of the following marker proteins: soybean trypsin inhibitor, 21,500; RNA polymerase subunits, α 39,000, β 155,000, β' 165,000 ovalbumin monomer and dimer, 45,000 and 90,000; monomer, dimer and trimer of bovine serum albumin, 68,000, 136,000 and 204,000; and cross-linked hemocyanin; 70,000, 140,000, 210,000 and 280,000.

The biological potency of the B1 and B29 photoprobes in an adipocyte lipogenesis bioassay, was 80 - 100% compared to beef insulin whereas the A1 photoprobe had a lower biological potency of 67% compared to beef insulin. The photoprobes were iodinated (15) with ^{125}I and it was shown that photoreactive arylazide did not incorporate ^{125}I and was stable to the conditions of iodination (17). All three ^{125}I-insulin photoprobes were shown to bind specifically to liver plasma membranes, Following irradiation and subsequent dilution up to

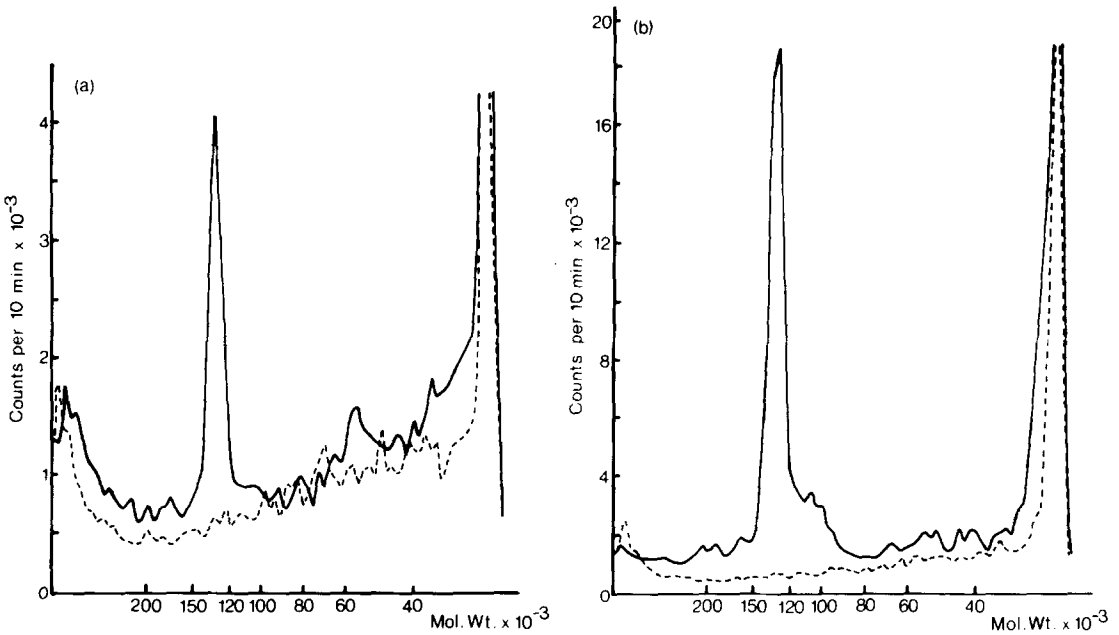

Figure 1 Sodium dodecyl sulphate - polyacrylamide gel electrophoresis of plasma membranes covalently labelled with ^{125}I-A1 photoprobe (a) or ^{125}I-B29 photoprobe (b).

Plasma membranes were covalently labelled with ^{125}I-photoprobes as described in the methods. SDS-polyacrylamide gel electrophoresis was carried out using a 7% (w/v) acrylamide resolving gel. The solid line represents total counts bound and the broken line non-specific labelling.

50% of the initial counts bound were still bound to liver plasma membranes after 19 h at 30°C. Under the same conditions ^{125}I-insulin was fully dissociated from its receptor (18). This result was particularly significant for the ^{125}I-B1 photoprobe since it was thought that this region of the molecule did not participate in receptor binding (10). However, we expected that sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis might demonstrate some differences in the membrane polypeptides labelled with the B1 photoprobe compared to those labelled with the A1 and B29 photoprobes.

Figure 1a shows that SDS-polyacrylamide gel electrophoresis in reducing conditions resolved only one major polypeptide of apparent molecular weight 130,000 which was specifically and consistently labelled by ^{125}I-A1 photoprobe. Other polypeptides were non-specifically labelled within the plasma membranes. As shown in Figure 1b ^{125}I-B29 photoprobe specifically labelled the same

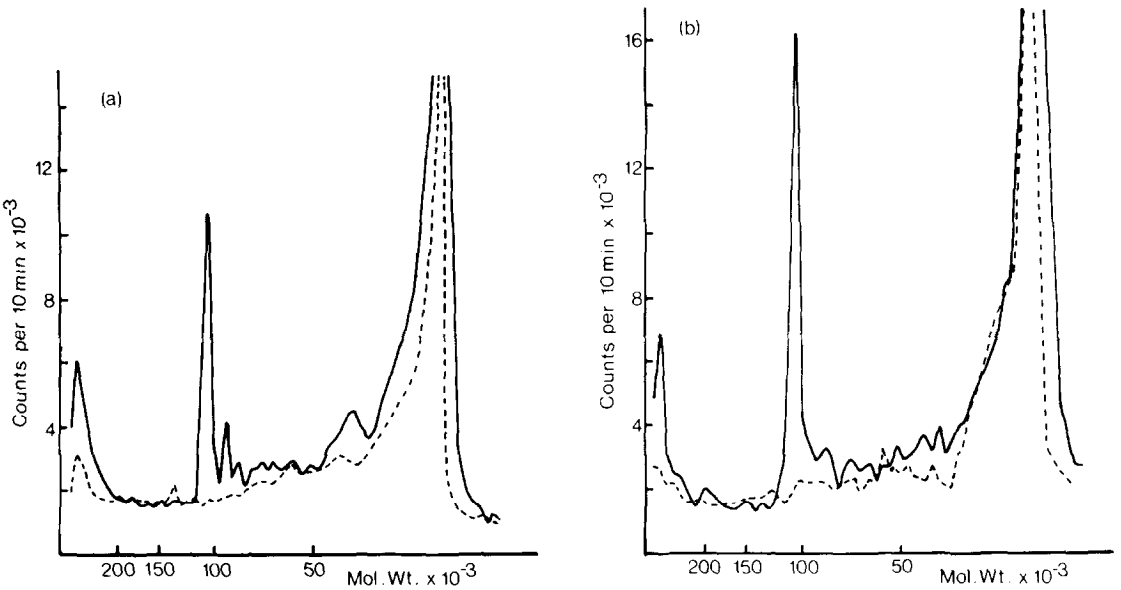

Figure 2 SDS-polyacrylamide-gel electrophoresis of rat liver plasma membranes covalently labelled with ^{125}I-A1 photoprobe (a) and ^{125}I-B1 photoprobes (b). SDS-polyacrylamide gel electrophoresis was also carried out using an 8% (w/v) acrylamide resolving gel. The solid line represents total counts bound and the broken line non-specific labelling in the presence of excess insulin.

molecular weight polypeptide as did the ^{125}I-A1 photoprobe. In many gels, but not all, there was a specifically labelled shoulder to the major peak which had an apparent molecular weight (in 7% acrylamide) of 100 - 110,000. This may be another specifically labelled polypeptide within the plasma membrane or may be the result of limited non-specific proteolysis or specific processing of the major photoprobe-binding polypeptide. The number of counts bound was lower with the A1 compared to the B29 photoprobe and this may be a reflection of the probable lower binding affinity of the A1 photoprobe, suggested by its reduced biological potency.

It was evident from the results shown in Figure 2 that the same molecular weight polypeptide was labelled by the ^{125}I-B1 photoprobe as was labelled by ^{125}I-A1 material and hence as the B29 photprobe. The apparent molecular weight of this polypeptide in 8% acrylamide gels was 110,000 and this difference will be explained below. However, the important observation that the labelling pattern observed with the B1 photoprobe was the same as that observed with the

A1 and B29 analogues indicates that although the B1 region of insulin may not participate in the initial hormone-receptor binding interaction it eventually lies close enough to the membrane for covalent linkage of a B_1 labelled photoprobe to occur.

The apparent molecular weight of 130,000 in 7% acrylamide gels for the specifically labelled polypeptide compares with an apparent molecular weight of 135,000 (in 6.5% acrylamide gel) for an insulin binding polypeptide purified from Triton X100 solubilised rat liver membranes by insulin-agarose affinity chromatography (16). Disuccinimidyl suberate has been used to cross-link ^{125}I-insulin to an adipocyte plasma membrane component of 140,000 (19) and Yip et al, (20) using an uncharacterised preparation of photoreactive insulin have shown the specific labelling of a polypeptide in rat adipocyte plasma membranes with an apparent molecular weight of 130,000 (10% gel).

The difference in apparent molecular weight of our photoprobe-labelled polypeptide with change in acrylamide concentration may be due to anomalous SDS binding observed with many plasma membrane proteins. Lectins have been shown to inhibit the binding of insulin to its receptor on rat adipocytes and mimic some of the effects of insulin (21) suggesting that the insulin receptor may be a glycoprotein. An indication of the true molecular weight of the photoprobe-labelled polypeptide was obtained from the 'Ferguson' plot (22) of the relative mobility (Rf) of the polypeptide in gels of different percentage acrylamide concentration (Figure 3). Theoretical and experimental analysis of the behaviour of uncharged proteins in SDS-polyacrylamide gel electrophoresis has shown that the slope of the line in the Ferguson plot (K'r) is proportional to the molecular weight of these proteins and that they have a similar mobility (Rfo) at zero acrylamide concentration (23,24,25). Figure 3 shows that the Rfo of the photoprobe-labelled polypeptide was significantly different from the mobilities of the uncharged marker proteins indicating that this polypeptide binds SDS anomalously. From a plot of K'r against molecular weight of the marker proteins, a molecular weight of 90,000 may be ascribed to the photoprobe-labelled polypeptide.

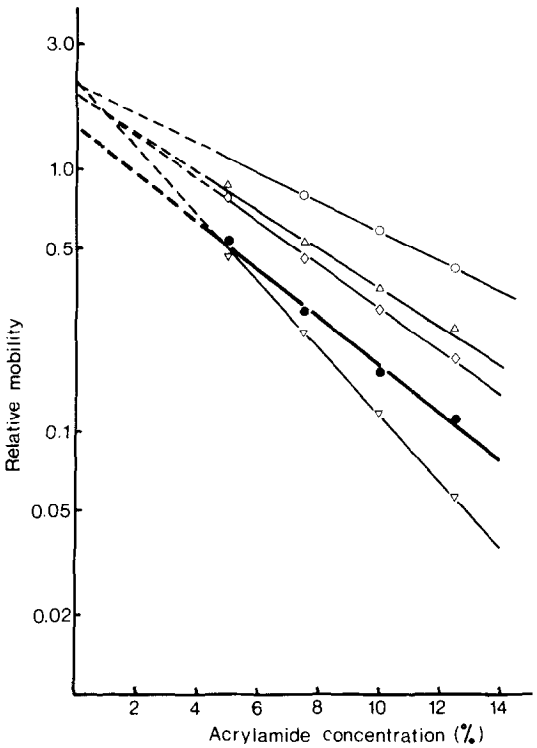

Figure 3 Determination of the molecular weight of the ^{125}I-B29 photoprobe labelled plasma membrane polypeptide by means of a 'Ferguson' plot from the combined results of SDS-polyacrylamide gel electrophoresis. Photoprobe-labelled membranes were resolved by SDS-polyacrylamide gels with different resolving gel acrylamide concentrations as described in the Methods. The logarithm of the mobilities of the specifically labelled photoprobe binding polypeptide and the molecular weight markers relative to the tracking dye (bromophenol blue) was plotted against the percentage (w/v) acrylamide concentration.

For the sake of clarity only four markers were plotted: (○)ovalbumin, 45,000; (△) bovine serum albumin, 68,000; (◇) a dimer of ovalbumin, 90,000; (▽) and a dimer of hemocyanin 140,000, (●) the plasma membrane polypeptide specifically labelled by ^{125}1-B29 photoprobe.

Studies with detergent solubilised membranes have indicated that the specific insulin receptor may be a protein of approximately 300,000 molecular weight (5). Preliminary studies with photoprobe labelled liver plasma membranes solubilised in Triton X100 and separated by Sepharose 6B column chromatography also resolved a specifically labelled peak of molecular weight 300,000. We suggest that the photoprobe labelled polypeptide is probably glycosylated and is the specific binding component of the insulin receptor but is associated in the plasma membrane with other polypeptides to form a larger protein complex. These results further illustrate the potential usefulness of highly purified

photoreactive hormone analogues in the purification and characterisation of hormone receptors.

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References

1 Das, M., Miyakawa, T., Fox, C.F., Pruss, R.M., Aharonov, A. and Herschman, H. R. (1977) Proc. Nat. Acad. Sci USA 74, 2790-2794.
2 Bregman, M.D., Cheng, S. and Levy, D. (1978) Biochim. Biophys. Acta 539, 489-495.
3 Glenn, K.C. and Cunningham, D.D. (1979) Nature 278, 711-714.
4 Ginsberg, B.H. (1977) in Biochemical Actions of Hormones (Litwack, G. ed) Academic Press NY.
5 Cuatrecasas, P. (1972) J. Biol. Chem. 247, 1980-1991.
6 Jacobs, S., Schechter, Y., Bissell, K. and Cuatrecasas, P. (1977) Biochem. Biophys. Res. Commun. 77, 981-988.
7 Ginsberg, B.H., Cohen, R.M., Kahn, C.R. and Roth, J. (1978) Biochim. Biophys. Acta. 542, 88-100.
8 Thamm, P. and Brandenburg, D. (1978) in Proceedings of 15th Europ. Peptide Symposium. In press.
9 Saunders, D.J. and Brandenburg, D. in preparation.
10 Pullen, R.A., Lindsay, D.G., Wood, S.P., Tickle, I.J., Blundell, T.L., Wollmer, A., Krail, G., Brandenburg, D., Zahn, H., Gliemann, J. and Gammeltoft, S. (1976) Nature 259, 369-373.
11 Moody, A.J., Stan, M.A., Stan, M and Gliemann, J. (1974) J. Horm. Metab. Res. 6, 12-16.
12 Gliemann, J. (1967) Diabetologia 3, 382-388.
13 Wisher, M.H. and Evans, W.H. (1975) Biochem J. 146, 375-388.
14 Wisher, M.H., Dron. D.I., Thomas, J.H. and Sönksen, P.H. (1977) Biochem. Soc. Trans. 5, 313-316.
15 Greenwood, F.C., Hunter, W.H. and Glober, J.S. (1963) Biochem. J. 89 114-123.
16 Maizel, J.V. (1971), Methods in Virology (Maramorosch, K. and Koprowski, H. eds) vol 5, pp 180-246. Academic Press N.Y.
17 Saunders, D.J., Thamm, P. and Brandenburg, D. (1979) in Proceedings of the 2nd International Insulin Symposium, Aachen. De Gruyter and Co. Berlin. In press.
18 Wisher, M.H., Thamm, P., Saunders, D.J., Sönksen, P.H. and Brandenburg, D. (1979) in Proceedings of the 2nd International Insulin Symposium, Aachen. De Gruyter and Co. Berlin. In press.
19 Pilch, P.F. and Czech, M.P. (1979) J. Biol. Chem. 254, 3375-3381.
20 Yip, C.C., Yeung, C.W.T. and Moule, M.L. (1978), J.Biol.Chem. 253, 1743-1745.
21 Cuatrecasas, P. and Tell, G.P.E. (1973). Proc. Nat. Acad. Sci. USA 70, 485-489.
22 Ferguson, K.A. (1964)Metabolism 13, 985-1002.
23 Rodbard, D. and Chrambach, A. (1971) Anal. Biochem. 40, 95-134.
24 Neville, D.M (1971) J. Biol. Chem. 246, 6328-6334.
25 Hayashi, K., Matsutera, E. and Ohba, Y. (1974) Biochem. Biophys. Acta 342, 185-194.