Structure of covalent insulin–receptor complexes (I–S–S–R) in isolated rat adipocytes and human placental membranes

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The structure of naturally-formed covalent disulphide-linked complexes between insulin and its receptor was examined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. To prevent destabilization of disulphide bonds at alkaline pH the standard discontinuous electrophoresis conditions were changed to a continuous buffer system at pH 7.0. ^{125}I -insulin was first bound to either rat adipocytes or human placental membranes for 10 min at 37°C. After washing, non-dissociable radioactivity was extracted from cells or membranes in Triton X-100 and immunoprecipitated with an antiserum (B-2) to the insulin receptor. Electrophoresis of the immune precipitate revealed the two smaller of the three reported species of native insulin receptor (M_r values approx. 350 000, 290 000 and 260 000); in addition, a species of M_r 200 000 was also frequently observed in adipocytes. When non-dissociable ^{125}I -insulin was chemically crosslinked to adipocytes or placental membranes, prior to solubilization and immunoprecipitation, all three species of the native receptor were labelled; after reduction, only a single species of M_r 130 000 was observed. These findings indicate that disulphide exchange of insulin occurs with the M_r 130 000 (α) binding subunit within partially reduced species of the native, oligomeric receptor. The degree of disulphide binding of insulin could therefore depend on the relative abundance of partially reduced receptor species and on the redox state of the cell membrane.

We have previously demonstrated that a small fraction of the insulin specifically bound to rat adipocytes becomes covalently attached to its receptor via a thiol–disulphide interchange reaction (Clark & Harrison, 1982), and have provided evidence that the formation of these covalent complexes (I–S–S–R) is not an artefact from ^{125}I -labelling of insulin and indeed may be a necessary post-binding step in insulin action (Clark & Harrison, 1983). Although we have shown by gel filtration that the M_r of these covalent complexes is similar to that of ^{125}I -insulin–receptor complexes that have been chemically crosslinked (Clark & Harrison, 1982), their structure has not been studied in detail.

Disulphide exchange appears to require at least one receptor thiol group, since the reaction can be inhibited by thiol group blocking agents (Clark & Harrison, 1982). This reactive group could pre-

exist in the receptor or be exposed after the initial binding event and should be more abundant in a reduced or partially reduced receptor species. Evidence for multiple partially reduced forms of the native receptor has been reported (Yip & Moule, 1983). When identified by affinity cross-linking with ^{125}I -insulin the native insulin receptor on intact cells has been reported to exist as a single, high- M_r species in the absence (Massague *et al.*, 1981) or in the presence of thiol group blocking agents (Heidenreich *et al.*, 1983; Yip & Moule, 1983). However, in the absence of thiol group blocking agents, three receptor species are usually observed with intact cells (Heidenreich *et al.*, 1983; Yip & Moule, 1983), isolated membrane preparations (Massague *et al.*, 1980, 1981) or highly purified insulin receptors (Fujita-Yamaguchi *et al.*, 1983). Although there is some discrepancy between the molecular sizes reported for these three species, with M_r values ranging from 450 000 to 330 000, 350 000 to 300 000 and 290 000 to 230 000, possibly due to the different electrophore-

Abbreviations used: SDS, sodium dodecyl sulphate; DSS, disuccinimidyl suberate; NEM, *N*-ethylmaleimide.

sis conditions used, it is interesting to note that their ratio is identical in membranes and highly purified receptors (Massague *et al.*, 1983; Fujita-Yamaguchi *et al.*, 1983) and in intact adipocytes (Heidenreich *et al.*, 1983; Yip & Moule, 1983). Partial reduction of the receptor can give rise to species of intermediate size, namely M_r 210000 and M_r 160000 (Massague *et al.*, 1980), although an M_r 210000 form has been postulated to be a glycosylated but non-cleaved precursor of the insulin receptor subunits (Hedo *et al.*, 1983). On complete reduction insulin receptor subunits of M_r 130000 (α -subunit) and M_r 90000 (β -subunit) are revealed by a variety of techniques (Massague *et al.*, 1980; Van Obberghen *et al.*, 1981; Kasuga *et al.*, 1982; Yip & Moule, 1983; Fujita-Yamaguchi *et al.*, 1983; Heidenreich *et al.*, 1983).

It seems reasonable to propose that the multiple forms of the native receptor may provide a basis for thiol redox reactions such as disulphide exchange with insulin. The present studies were therefore performed to determine the structure of I-S-S-R and its relationship to the multiple redox forms of the native receptor.

Experimental

Materials

All electrophoresis reagents were obtained from Bio-Rad, except for M_r standards which were obtained from Pharmacia. Anti-receptor serum, obtained from a patient (B-2) with the syndrome of severe insulin resistance and acanthosis nigricans (Flier *et al.*, 1975), contained IgG antibodies specific for the binding subunits of the receptor (Kasuga *et al.*, 1981). Staphylococcal protein A from *S. aureus* Cowan I, which had been formalin-fixed and heat-killed, was obtained as a 10% suspension from the Commonwealth Serum Laboratories, Melbourne, Australia. DSS was obtained from the Pierce Chemical Co., Rockford, IL, U.S.A. All other reagents were as previously described (Clark & Harrison, 1982).

Preparation of isolated adipocytes

Male Sprague-Dawley rats fed *ad libitum* and weighing 150–200 g were killed by cervical dislocation. Isolated adipocytes were prepared by the method of Rodbell (1964) in Dulbecco buffer, pH 7.4 (Dulbecco & Vogt, 1954) containing collagenase (3 mg/ml) and albumin (40 mg/ml).

Preparation of placental membranes

Particulate placental membranes were prepared by differential centrifugation of an homogenate of human placenta as described previously (Harrison *et al.*, 1978) except that the buffer was 0.1 M-sodium phosphate, pH 7.4.

^{125}I -insulin binding

Pig ^{125}I -insulin (100–150 $\mu\text{Ci}/\mu\text{g}$) was prepared by a modification of the chloramine-T method (Roth, 1975). Adipocytes [(2–4) $\times 10^6$ cells/ml] or placental membranes (approx. 5 mg of protein/ml) were incubated with ^{125}I -insulin (25 ng/ml) in Dulbecco buffer, pH 7.4, containing albumin (10 mg/ml) for 10 min at 37°C. In some cases excess unlabelled insulin (10 $\mu\text{g}/\text{ml}$) was added to determine non-specific binding (always less than 15% of the total binding). Cells or membranes were washed three times for 10 min at 37°C and non-dissociable radioactivity was extracted in 1% Triton X-100 as previously described (Clark & Harrison, 1982). Most of the receptor-bound ^{125}I -insulin in this preparation is resistant to SDS (Clark & Harrison, 1982). This non-dissociable radioactivity was either chemically crosslinked, as described below, or immunoprecipitated prior to SDS/polyacrylamide-gel electrophoresis.

Immunoprecipitation of insulin-receptor complexes (Harrison *et al.*, 1979)

A 200 μl sample of solubilized insulin-receptor complexes [(10–50) $\times 10^3$ c.p.m.] was incubated with 10 μl of either control serum or antireceptor serum B-2 (final dilution 1:400) for 16 h at 4°C and then with protein A (50 μl of a 10% suspension) for 30 min at 4°C. Immune precipitates obtained by centrifugation at 4000g for 10 min were counted for radioactivity in an Auto-Gamma spectrometer and solubilized in electrophoresis sample buffer.

Chemical crosslinking (Massague *et al.*, 1981)

After ^{125}I -insulin binding at 37°C, placental membranes were pelleted by centrifugation and washed twice in ice-cold 0.1 M-sodium phosphate, pH 7.4. The membranes were resuspended in this buffer and incubated with DSS (1 mM) for 15 min at 4°C. The reaction was stopped and the membranes washed with 20 mM-Tris/HCl/1 mM-EDTA before being solubilized in 1% Triton X-100, immunoprecipitated and electrophoresed using the discontinuous system as described below. After ^{125}I -insulin binding to adipocytes at 37°C the cells were washed twice in Dulbecco buffer, pH 7.4, containing 1% bovine albumin at 13–15°C. Cells were resuspended in this buffer, incubated with DSS (1 mM) for 15 min at 13–15°C and then treated as for the placental membranes. In some experiments only bound ^{125}I -insulin resistant to acid was used for crosslinking. In these experiments, after ^{125}I -insulin binding, membranes or cells were washed twice in 1 M-acetic acid before being resuspended in either sodium phosphate buffer pH 7.4 (placental membranes) or Dulbecco buffer, pH 7.4, containing 1% albumin (fat cells) and crosslinked with DSS as described above.

Gel chromatography

Two aliquots of solubilized, non-dissociable radioactivity were chromatographed simultaneously on Sepharose CL-6B columns (1.5 cm $\times$ 30 cm) equilibrated and eluted with 0.5 M-Tris/HCl containing 0.1% SDS at either pH 7.0 or pH 8.8. The columns were calibrated with dextran blue, DSS-crosslinked ^{125}I -insulin-receptor complexes, ^{125}I -insulin and Na^{125}I .

SDS/polyacrylamide-gel electrophoresis

Continuous buffer system (modified from Maizel, 1971). Slab gels (1.0 mm thick) were prepared with an acrylamide gradient of 3.5 to 7.5% [acrylamide/bisacrylamide (100:1) in 0.1 M-sodium phosphate, pH 7.0, with 0.1% SDS]. A stacking gel was not used, sample wells being formed directly in the top of the gradient gel. Immune pellets dissolved in 20 μl of sample buffer (0.01 M-sodium phosphate, pH 7.0, containing 1% SDS, 1% glycerol and 0.001% Bromophenol Blue), were heated at 60°C for 10 min. Electrophoresis was carried out in 0.1 M-sodium phosphate/0.1% SDS, pH 7.0, at 60 mA for approx. 5 h.

Discontinuous buffer system (Laemmli, 1970). Gels of two acrylamide concentrations were used: 7.5% (acrylamide/bisacrylamide 100:3) and 5% (acrylamide/bisacrylamide 100:1), with a 4% stacking gel in both cases. Electrophoresis was performed at 20 mA for about 3 h. Both the

continuous and discontinuous gels were stained, dried and autoradiographed according to Laemmli (1970). Autoradiograms were scanned using a LKB 2202 Ultrosan laser densitometer.

Protein standards were thyroglobulin (M_r 330 000), ferritin (M_r 200 000 and 18 500), myosin (M_r 200 000), β -galactosidase (M_r 116 500), phosphorylase *b* (M_r 94 000), bovine serum albumin (M_r 67 000) and ovalbumin (M_r 43 000).

Results

Native insulin-receptor complexes (I-S-S-R): un-reduced

Initial attempts to demonstrate an I-S-S-R structure electrophoretically using the standard Laemmli discontinuous buffer system proved unsuccessful, even though we had previously shown that I-S-S-R was resistant to SDS (Clark & Harrison, 1982). In the Laemmli system the pH of the resolving gel buffer is 8.8 and of the running gel

Fig. 1. Gel filtration at pH 7.0 and 8.8 of non-dissociable ^{125}I -insulin radioactivity solubilized from adipocytes. Adipocytes $[(2-4) \times 10^6/\text{ml}]$ were incubated with ^{125}I -insulin (25 ng/ml) for 10 min at 37°C. After extensive washing of the cells non-dissociable radioactivity was extracted in Triton X-100 as described in the Experimental section. Samples of the extract (0.5 ml) were applied to Sepharose CL-6B columns (1.5 cm $\times$ 30 cm) and eluted with 0.5 M-Tris/HCl containing 0.1% SDS at either pH 7.0 (—) or pH 8.8 (---). The columns were calibrated with Dextran Blue (V_0), ^{125}I -insulin chemically crosslinked to its receptor, ^{125}I -insulin and Na^{125}I (V_t).

Fig. 2. Electrophoretic analysis of native covalent ^{125}I -insulin-receptor complexes from adipocytes. Adipocytes $[(2-4) \times 10^6/\text{ml}]$ were incubated with ^{125}I -insulin (25 ng/ml) for 10 min at 37°C, followed by extensive washing of the cells and extraction of non-dissociable radioactivity into Triton X-100. The extracted radioactivity was immunoprecipitated with either control serum or anti-receptor serum B-2. The immune pellets were dissolved in sample buffer, electrophoresed in a continuous buffer system, pH 7.0, in 3.5%–7.5% gradient gels (acrylamide/bisacrylamide 100:1) and the gels were stained, dried and scanned by densitometry, as described in the Experimental section.

Fig. 3. Electrophoretic analysis of native covalent ^{125}I -insulin-receptor complexes from human placental membranes

Particulate placental membranes (approx. 5 mg of protein/ml) were incubated with ^{125}I -insulin for 10 min at 37°C in the presence or absence of excess unlabelled insulin ($20\text{ }\mu\text{g/ml}$). After extensive wash-

buffer 8.3, and it is known that alkaline pH favours destabilization and re-shuffling of disulphide bonds (Liu, 1977; Konishi & Scheraga, 1980). To test whether alkaline pH was responsible for the loss of radioactivity from I-S-S-R complexes, gel chromatography of non-dissociable radioactivity was performed at 24°C in 0.5 M -Tris/HCl with 0.1% SDS at either pH 7.0 or pH 8.8. At pH 8.8 the recovery of I-S-S-R complexes was only 50% of that at pH 7.0 (Fig. 1). An electrophoresis system was therefore employed using a continuous buffer of pH 7.0.

When the non-dissociable radioactivity solubilized after ^{125}I -insulin binding to adipocytes was precipitated with anti-receptor serum and analysed by SDS/polyacrylamide-gel electrophoresis at pH 7.0 bands of M_r 290 000, 235 000 and 200 000 were seen (Fig. 2). No bands were visible when control serum was used (results not shown). When particulate placental membranes were used as a source of covalent complexes only two high- M_r bands of 290 000 and 260 000 were specifically immunoprecipitated (Fig. 3, top). These species were not seen when binding of ^{125}I -insulin was carried out in the presence of excess unlabelled insulin (results not shown). If the immunoprecipitation was performed in the presence of 5 mM -NEM the total radioactivity precipitated was unchanged, with mainly a single more heavily labelled band of M_r 290 000 present (Fig. 3, bottom). Again this band was absent when binding was performed in the presence of excess unlabelled insulin (results not shown).

When non-dissociable ^{125}I -insulin was chemically crosslinked, immunoprecipitated and electrophoresed in a discontinuous buffer in the absence of reducing agents several high- M_r bands were observed: for adipocytes bands of M_r 360 000, 290 000 and 210 000, with very low labelling of a band at M_r 260 000 (Fig. 4, lane c) and for placenta bands of M_r 360 000, 290 000, 260 000 and 210 000 (Fig. 4, lane g). These bands were identical with those seen when total bound ^{125}I -insulin (dissociable and non-dissociable) was crosslinked (Fig. 4, lanes a and e).

Subunit structure of I-S-S-R complexes

I-S-S-R complexes by their very nature are susceptible to reduction and their structure cannot

ing of the membranes and extraction of non-dissociable radioactivity into Triton X-100, complexes were immunoprecipitated with either control serum or anti-receptor serum B-2 in the presence (bottom panel) or absence (top panel) of 5 mM -NEM. The immune precipitates were analysed as described in the legend to Fig. 2.

Fig. 4. Electrophoretic analysis of non-dissociable ^{125}I -insulin radioactivity chemically crosslinked to either rat adipocytes or human placental membranes: non-reduced

Adipocytes $[(2-4) \times 10^6/\text{ml}]$ (lanes *a-d*) or placental membranes (approx. 5 mg of protein/ml) (lanes *e-h*) were incubated with ^{125}I -insulin for 10 min at 37°C . After removal of free ^{125}I -insulin the samples were treated with DSS, either before (lanes *a, b, e, f*) or after (lanes *c, d, g, h*) extensive washing at 37°C . After solubilization the radioactivity was immunoprecipitated with either anti-receptor serum B-2 (lanes *a, c, e, g*) or control serum (lanes *b, d, f, h*). Immune pellets were dissolved in sample buffer and electrophoresed in a 5% gel (acrylamide/bisacrylamide 100:1) using a discontinuous buffer system, as described in the Experimental section.

therefore be ascertained, unmodified, in reduced gels. To circumvent this problem and thereby define which of the receptor subunits (themselves disulphide-linked) is involved in the disulphide exchange with ^{125}I -insulin, non-dissociable ^{125}I -insulin was first exposed to concentrations of DSS known to crosslink insulin chemically to the M_r 130000 receptor binding subunit (Massague *et al.*, 1980). Radioactivity solubilized from either intact adipocytes or placental membranes after this procedure was precipitated with anti-receptor serum B-2. Analysis of the immune precipitates in discontinuous SDS/polyacrylamide-gel electrophoresis under reducing conditions revealed a single band of M_r 130000 (Fig. 5, lanes *d* and *h*), identical with the subunit obtained when total ^{125}I -insulin (dissociable and non-dissociable) was chemically crosslinked and immunoprecipitated (Fig. 5, lanes *b* and *f*). Not all non-dissociable radioactivity is necessarily covalently bound (Clark & Harrison, 1982); however, identical results were obtained if placental membranes or adipocytes were treated with 1 M-acetic acid before chemical crosslinking and electrophoresis (results not shown).

Discussion

The present findings provide further evidence for covalent binding of insulin to its receptor and reveal the molecular size of covalent complexes in both the native and reduced state of the receptor. Our ability to detect covalent complexes in gels only after changing the standard alkaline electrophoresis conditions to a neutral, continuous phosphate-buffered system most likely reflects the alkaline lability of disulphides (for review see Liu, 1977). Others (Creighton, 1979; Konishi & Scheraga, 1980) have also found that in order to stabilize and prevent re-shuffling of disulphides the pH of the medium must be decreased.

Native I-S-S-R appears to exist as no more than two high- M_r species of M_r 290000 and 235000 in adipocytes (Fig. 2) and M_r 290000 and 260000 in placental membranes (Fig. 3, top). These M_r values are similar to the two smaller of the three unreduced species of oligomeric receptor observed by others (Massague *et al.*, 1981; Fujita-Yamaguchi *et al.*, 1983; Heidenreich *et al.*, 1983; Yip & Moule, 1983). The highest M_r species reported (M_r 350000–380000) was never observed for I-S-S-R

Fig. 5. Electrophoretic analysis of non-dissociable ^{125}I -insulin radioactivity chemically crosslinked to either rat adipocytes or human placental membranes: reduced

Adipocytes (lanes *a*–*d*) and placental membranes (lanes *e*–*h*) were incubated with ^{125}I -insulin and treated with DSS as described in the legend to Fig. 4. Solubilized radioactivity was immunoprecipitated with either control serum (lanes *a*, *c*, *e*, *g*) or anti-receptor serum B-2 (lanes *b*, *d*, *f*, *h*). Immune pellets were dissolved in sample buffer containing 100 mM-dithiothreitol and electrophoresed in a 7.5% gel (acrylamide/bisacrylamide 100:3) with a discontinuous buffer system.

under these conditions. The work of Yip & Moule (1983) suggests that the three receptor species are in a redox equilibrium, being interconvertible from the most oxidized (M_r 380000) to the most reduced (M_r 230000). Disulphide exchange probably occurs to a receptor containing free thiol groups since the reaction can be inhibited by thiol group blocking agents (Clark & Harrison, 1982). On the basis of our present findings we would postulate that disulphide exchange occurs only with the partially-reduced lower- M_r receptor species. Presumably, the relative abundance of these partially reduced species, and thus the degree of covalent binding, would be a reflection of the redox state of the cell membrane.

Massague *et al.* (1981) using intact adipocytes found only one receptor species in the absence of reduction whereas we (Fig. 4) and others (Heidenreich *et al.*, 1983; Yip & Moule, 1983) consistently find three. Massague *et al.* (1981) proposed that the three high- M_r receptor species are generated by limited proteolysis of the largest form (M_r 350000). However, it has been shown that the presence of a range of proteinase inhibitors cannot prevent the appearance of these three receptor species (Heidenreich *et al.*, 1983; Yip & Moule, 1983).

Consistent with reports on crosslinking of photoreactive ^{125}I -insulins (Heidenreich *et al.*,

1983; Yip & Moule, 1983) we find that NEM prevents the appearance of two receptor species in placental membranes, leaving a single denser species of M_r 290000 (Fig. 3). Since we did not add the NEM until the immunoprecipitation step, this finding indicates that there must be a continuous process of thiol-dependent receptor conversion. This process could be non-enzymic or effected by an oxidoreductase system coupled to the receptor; alternatively, the lower- M_r receptor species could be generated by the action of a thiol-dependent proteinase which is inhibited by NEM. NEM did not cause the appearance of an M_r 350000 receptor species as reported for covalently linked photoreactive ^{125}I -insulin (Yip & Moule, 1983), perhaps because disulphide binding of insulin to the partially reduced species blocks conversion of the receptor to its fully oxidized form.

Chemical crosslinking of both non-dissociable and acid-resistant radioactivity reveals three high- M_r species in the absence of reduction, including an M_r 360000 species (Fig. 4). This result is identical with that obtained for total bound insulin (dissociable and non-dissociable) (Fig. 4) and implies that although covalent binding is not to an M_r 360000 receptor this species is still present in the membrane. Under these conditions DSS may crosslink non-covalently-bound ^{125}I -insulin to the M_r 360000 species or may be able to crosslink

receptor components that are not actually involved in disulphide exchange, leading to the appearance of all three M_r species.

In several studies using both adipocytes and placental membranes we observed a protein band of M_r 200000–210000 (Figs. 2 and 4). A protein of this M_r has been observed by others and is purported to be a $(\alpha\beta)$ ‘half’ of an intact $(\alpha\beta)_2$ receptor (Massague *et al.*, 1981). Its generation did not affect insulin binding or transmembrane signalling (Massague & Czech, 1982). Although we have no evidence that our protein band represents such a receptor species, it may be that at the higher temperature of our binding assay (37°C) a membrane reducing system is active and leads to the appearance of this intermediate form. It is unlikely that this species represents the fully glycosylated form of the receptor subunit precursor which has escaped cleavage and been inserted in the membrane, as reported by Hedo *et al.* (1983), because in our chemical crosslinking experiments the M_r 210000 band was reducible (Figs. 4 and 5) whereas the receptor precursor is not.

Complete reduction of DSS-crosslinked non-dissociable complexes yields an M_r = 130000 band (Fig. 5) indicating, as might be expected, that covalent binding occurs to the α -binding subunit of the insulin receptor. We saw no evidence of labelling of the M_r 90000 β -subunit and therefore can probably assume that disulphide exchange is not a mechanism for crosslinking α and β subunits via an ‘insulin bridge’ (Clark & Harrison, 1982). The β -subunit is only rarely observed by methods that utilize insulin as the radioactive affinity probe (Massague *et al.*, 1981; Heidenreich *et al.*, 1983) and is proposed to have only a minor role, if any, in binding insulin.

In conclusion, these data indicate that disulphide exchange of insulin occurs with partially-reduced lower- M_r forms of the native insulin receptor and that the insulin in the I-S-S-R complex is bound to the M_r 130000 (α) binding subunit of the receptor. They support the idea that the redox state of the receptor may be an important regulator of insulin action.

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