Subunit Structure of Insulin Receptor of Rat Adipocytes As Demonstrated by Photoaffinity Labeling†

C. C. Yip,* M. L. Moule, and C. W. T. Yeung

ABSTRACT: Isolated rat adipocytes were incubated in the dark with either one of two radioiodinated photoreactive insulin derivatives, $N^{\epsilon B29}$-(azidobenzoyl)insulin (B29-MABI) and $N^{\alpha B1}$-(azidobenzoyl)insulin (B1-MABI), and were then exposed to light. Sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis and radioautography of the crude plasma membrane fraction after reduction showed that B29-MABI labeled specifically three proteins of M_r 130 000, 90 000, and 40 000, whereas B1-MABI labeled specifically two proteins of M_r 130 000 and 40 000. B1-MABI also variably labeled some bands of intermediate M_r between 130 000 and 90 000. In contrast, the labeling of the 40-kilodalton protein was not observed in our previous studies in which photolabeling was carried out on isolated plasma membrane preparations [Yip, C. C., Yeung, C. W. T., & Moule, M. L. (1980) *Biochemistry 19*, 70–76; Yeung, C. W. T., Moule, M. L., & Yip, C. C. (1980) *Biochemistry 19*, 2196–2203]. Without reduction, an M_r 300 000 band and a larger band which barely entered a 5–15% gradient gel were specifically labeled by both photoreactive insulins. Reduction of these two high molecular weight bands gave rise to the 130-, 90-, and 40-kilodalton bands. The labeling of these proteins was affected neither by the time or temperature of incubation nor by the addition of methylamine, chloroquine, bacitracin, phenylmethanesulfonyl flouride, *p*-(chloromercuri)benzenesulfonic acid, Trasylol, *N*-ethylmaleimide, or benzamidine. The labeling of these proteins by the photoreactive insulin derivatives was inhibited by first incubating the adipocytes with a human autoimmune serum to insulin receptor. We therefore conclude that these proteins are subunits of the insulin receptor in intact adipocytes.

We have previously demonstrated that two proteins of M_r 130 000 and 90 000, respectively, were specifically photolabeled when rat liver or adipocyte plasma membrane preparations were incubated with the photoreactive azidobenzoyl derivatives of insulin and photolyzed (Yip et al., 1978, 1980a; Yeung et al., 1980). Photolabeling of a protein similar to the 130-kilodalton protein was observed also by others using different photoreactive insulin derivatives (Jacobs et al., 1979; Wisher et al., 1980). A protein of M_r 125 000 was specifically cross-linked to insulin when adipocyte plasma membranes were incubated with insulin and a bifunctional cross-linking reagent (Pilch & Czech, 1980). We have since shown that the photolabeling of the 130- and 90-kilodalton proteins by (azidobenzoyl)insulin was inhibited by an autoimmune antiserum to insulin receptor and that the same antiserum reacted with these two labeled proteins (Yip et al., 1980b). These observations strongly support our conclusion that the insulin receptor on the isolated plasma membranes is composed of at least these two nonidentical subunits. In order to establish that these subunits detected by using isolated plasma membranes are indeed those present on the surface of intact cells, we have incubated isolated intact rat adipocytes with both of our photoreactive insulin derivatives. Our results show that an additional protein of M_r 40 000, likely another subunit of the insulin–receptor complex, was specifically labeled.

Experimental Procedures

Materials. $N^{\epsilon B29}$-(Azidobenzoyl)insulin (B29-MABI) and $N^{\alpha B1}$-(azidobenzoyl)insulin (B1-MABI) were prepared from bovine insulin (a gift from Connaught Laboratories Ltd., Canada), purified, fully characterized with respect to their chemical and biological properties, and radioiodinated as we have described previously (Yip et al., 1980a; Yeung et al., 1980). Chemicals for polyacrylamide gel electrophoresis were obtained from Bio-Rad Laboratories. Collagenase (EC 3.4.24.3) used for the isolation of adipocytes was purchased from Worthington Biochemical Corp., Freehold, NJ. Neuraminidase (EC 3.2.1.18) (type IX), methylamine, phenylmethanesulfonyl fluoride (PMSF), *p*-(chloromercuri)benzenesulfonic acid (PCMPS), bacitracin, *N*-ethylmaleimide (NEM), and chloroquine were obtained from Sigma Chemical Co., St. Louis, MO. Trasylol was obtained from FBA Pharmaceuticals, Montreal. Benzamidine was from Aldrich Chemical Co., Inc., Milwaukee, WI. All other chemicals used were of reagent grade. Adipocytes were isolated from the epididymal fat pads of 180–200-g Wistar rats by digestion with collagenase according to the method described by Rodbell (1964) and were fully responsive to insulin. Autoimmune human antiserum to insulin receptor was a gift from Dr. R. Kahn, National Institutes of Health.

Incubation and Photolabeling. Isolated adipocytes, approximately 1.4×10^7 cells, were incubated in the dark in 8 mL of Krebs–Ringer bicarbonate buffer containing 2% human serum albumin (KRB–HSA), 0.1 mg/mL glucose, and approximately 10 nM radioiodinated (azidobenzoyl)insulin. Native bovine insulin, 62.5 μg/mL, was added where appropriate to determine nonspecific binding. The temperature of incubation was 18 °C unless specified otherwise. The incubation was carried out in a 50-mL polycarbonate round-bottom centrifuge tube. The cells were kept in suspension by gentle stirring using a small magnetic stirring bar. After incubation, the cell suspension was exposed for 30 s to a vertically focused beam of light from a 100-W high-pressure mercury lamp (PRA Photochemical Research Associates Inc., London, Ontario). The light beam, cooled by being passed through a quartz reservoir of circulating cold tap water, was aimed directly down the polycarbonate tube at the cell suspension. Stirring was maintained during this brief period of photolysis.

† From the Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontario M5G 1L6, Canada. *Received October 30, 1981.* This work was supported by research grants from the Medical Research Council (Canada), the C. H. Best Foundation, and the Juvenile Diabetes Foundation. A preliminary account of this work was presented at a minisymposium at the 71st Annual Meeting of the American Society of Biological Chemists, New Orleans, LA, June 1–5, 1980 (Yip et al., 1980c).

0006-2960/82/0421-2940$01.25/0

In experiments where the effect of neuraminidase or methylamine was studied, the adipocytes were first incubated at 37 °C with the enzyme (45 milliunits/mL) for 30 min or with the amine (10 mM) for 20 min. The cells treated were than used immediately for photolabeling. Photolabeling was also carried out in the presence of bacitracin (0.8 mg/mL), benzamidine (1 mM), chloroquine (0.25 mM), *N*-ethylmaleimide (1 mM), PMSF (10 μg/mL), PCMPS (0.1 mM), or Trasylol [1000 kallikrein inactivator units (KIU)/mL].

The effects of these treatments on glucose oxidation by the adipocytes were examined by the bioassay method of Moody et al. (1974). Under the conditions described, insulin stimulation of glucose oxidation was reduced 50%, 40%, 35%, and 35% by neuraminidase, PCMPS, methylamine, and chloroquine, respectively. At the concentration used, bacitracin, PMSF, and benzamidine did not affect the stimulatory response of the adipocytes to insulin. However, NEM abolished the response to insulin completely.

Membrane Preparation and Electrophoresis. We followed the method of McKeel & Jarett (1970) to prepare crude plasma membrane fraction from the cell suspension. The cell suspension was washed twice with KRB–HSA buffer and then twice with sucrose–Tris buffer (0.25 M sucrose in 5 mM Tris, pH 7.5) before homogenization. In some experiments, we added also benzamidine, NEM, PMSF, PCMPS, or Trasylol to the homogenization medium. The crude membrane fraction was obtained after centrifugation for 20 min at 16000*g*. Solubilization and reduction of the membrane fraction, sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis of the solubilized membranes, and radioautography were carried out as described previously (Yip et al., 1980a; Yeung et al., 1980). Gradient gel electrophoresis was performed on a slab gel prepared by mixing an equal volume of a 5% and a 15% acrylamide solution in a gradient maker. A stock solution of 30% acrylamide and 0.8% bis(acrylamide) in water was diluted in buffer to contain 5% or 15% acrylamide, $NaDodSO_4$ (0.1%), Tris-HCl (375 mM, pH 8.8), ammonium persulfate (1.25 mg/10 mL), and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (10 μL/30 mL). The 5% and 15% acrylamide solutions contained also 6.25% and 16.25% sucrose, respectively. A stacking gel layer of 3% acrylamide–0.05% sucrose, in Tris-HCl (125 mM, pH 6.8), was used. Electrophoresis was carried out at a constant voltage of 200 V. Protein molecular weight standards used for calibration were the following: myosin, 200 000; β-galactosidase, 116 500; phosphorylase *b*, 94 000; bovine serum albumin, 68 000; ovalbumin, 43 000. Radioactive bands excised from dried but unstained gels were digested by using the method of Cleaveland et al. (1977) for in-gel digestion of proteins.

Results

Photolabeling of isolated intact adipocytes with B29-MABI resulted in the specific covalent labeling of three major bands in the plasma membrane fraction after solubilization and reduction (Figure 1A). The approximate molecular weights of these bands were 130 000, 90 000, and 40 000. The radioactive A chain which contained about 80% of the radioiodine of the radioactive analogues and the radioactive analogues that were bound but not covalently linked to the membranes account for the high radioactivity detected at the gel front (Yip et al., 1980a). In contrast, labeling with B1-MABI yielded specific covalent labeling of two major bands of M_r 130 000 and 40 000 together with several bands of intermediate molecular weights (Figure 1B). Figure 1 shows also that the pattern of specific labeling was not changed by increasing the period of binding.

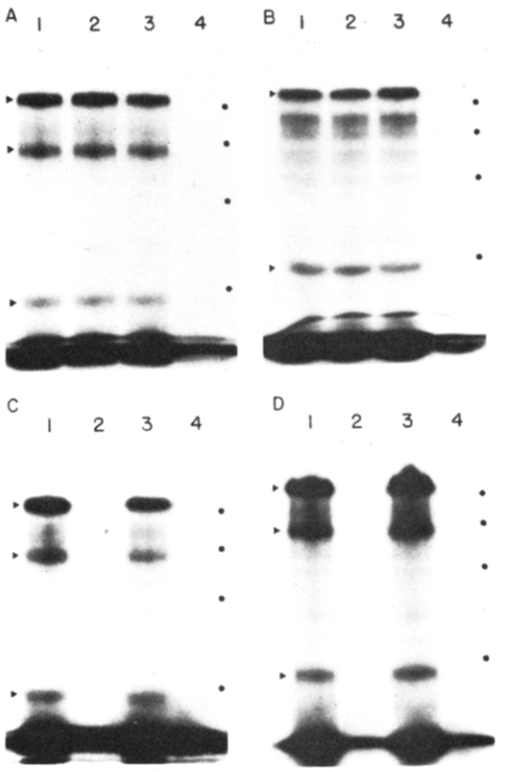

FIGURE 1: (A) Effect of incubation time on the specific photolabeling by [^{125}I]B29-MABI. Before photolysis, the adipocytes were incubated with photoreactive insulin at 17 °C for (1) 15, (2) 30, (3) 60, and (4) 60 min in the presence of excess insulin. From top to bottom, the dots mark the molecular weight standards of β-galactosidase (116 500), phosphorylase *b* (94 000), bovine serum albumin (68 000), and ovalbumin (43 000). The triangles mark the 130-, 90-, and 40-kilodalton labeled bands. (B) Same as (A), except that the cells were incubated with [^{125}I]B1-MABI. The triangles mark the 130- and 40-kilodalton bands. (C) Effect of temperature shift on the specific photolabeling by [^{125}I]B29-MABI. The adipocytes were incubated for 30 min at 17 °C with the photoreactive insulin and then photolyzed. An ultraviolet transmission filter (BG-3, Leitz) was used during photolysis. The cell suspension was then washed and incubated for 30 min at 17 °C (lanes 1 and 2) or at 37 °C (lanes 3 and 4). Lanes 2 and 4 were obtained from incubation in the presence of excess insulin. (D) Effect of benzamidine on the specific photolabeling by [^{125}I]-B29-MABI. The adipocytes were incubated with the photoreactive insulin in the presence (lanes 1 and 2) or absence (lanes 3 and 4) of 1 mM benzamidine. Lanes 2 and 4 were from incubation containing excess insulin. The triangles mark the 130-, 90-, and 40-kilodalton bands. A similar lack of effect was obtained with other inhibitors.

In our previous study, we did not observe the specific labeling of an M_r 40 000 band when we carried out photolabeling on an isolated plasma membrane preparation of adipocytes (Yip et al., 1980a; Yeung et al., 1980). In order to determine if the appearance of the 40-kilodalton band could be the result of proteolytic degradation when intact adipocytes were used, we have therefore examined the effects of several proteolytic inhibitors, namely, bacitracin (Carpentier et al., 1979), benzamidine (Ensinck et al., 1972), Trasylol, NEM (Duckworth et al., 1979), and PMSF (Jacobs & Cuatrecasas, 1980). None of these compounds affected the appearance of the 130-, 90-, and 40-kilodalton bands whether these compounds were added to the incubation or to the homogenization medium. Since the appearance of the 40-kilodalton band could have resulted from the internalization of the insulin–receptor complex, we have studied also the effects of methylamine which inhibits clustering and internalization of ligand–receptor complexes in fibroblasts (Maxfield et al., 1979), and chloroquine, a lysosomotrophic agent, which inhibits intracellular proteolysis (Wibo & Poole, 1974). Neither of these agents affected the

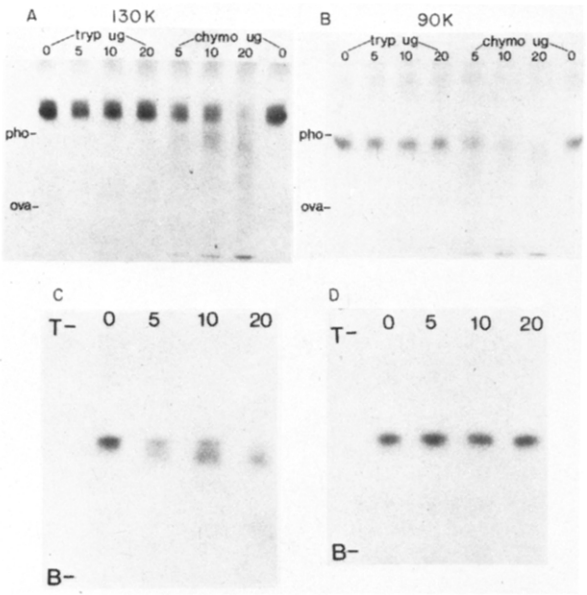

FIGURE 2: (A and B) In-gel tryptic (tryp) and chymotryptic (chymo) digestion of the 130- and 90-kilodalton bands obtained from photolabeling of adipocytes with [^{125}I]B29-MABI. These bands located by radioautography were excised from unstained slab gel. Positions of two molecular weight markers, phosphorylase *b* (pho), 94000, and ovalbumin (ova), 43000, are indicated. Separating gel was 10%. (C and D) In-gel digestion of the 40-kilodalton band obtained from photolabeling with [^{125}I]B1-MABI and [^{125}I]B29-MABI, respectively. The number above each lane indicates the microgram amount of trypsin used. Top (T) and bottom (B) of the separating gel are indicated. Separating gel was 15%.

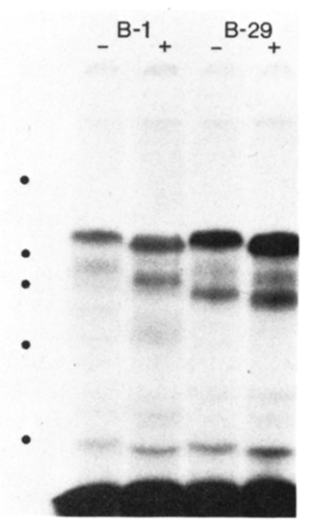

FIGURE 3: Effect of neuraminidase digestion of adipocytes on the photolabeling of insulin receptor proteins by [^{125}I]B1-MABI (B-1) and [^{125}I]B29-MABI (B-29). Cells were incubated with (+) or without (−) neuraminidase before photolabeling. The dots mark the positions of protein molecular weight standards, from top to bottom: myosin, β-galactosidase, phosphorylase *b*, albumin, and ovalbumin.

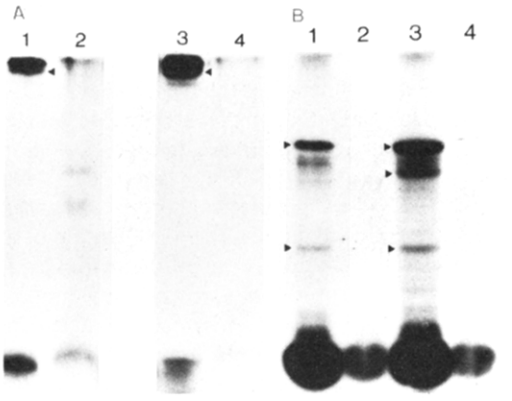

FIGURE 4: (A) Adipocytes were photolabeled with [^{125}I]B1-MABI (lanes 1 and 2) or with [^{125}I]B29-MABI (lanes 3 and 4). Radioautograms were obtained from gradient $NaDodSO_4$–polyacrylamide gel electrophoresis analysis of the membranes without reduction. Lanes 2 and 4 were obtained in the presence of excess insulin. The triangle marks the position of the 300-kilodalton band. (B) Same as in (A), except that the membranes were reduced with dithiothreitol. Lanes 2 and 4 were obtained in the presence of excess insulin.

appearance of the 40-kilodalton band. Figure 1D shows the pattern of specific labeling by B29-MABI in the presence or absence of benzamidine, illustrating the lack of any effect of these compounds. Incubation temperature, which should affect the proteolytic degradation and has been shown to influence the internalization of ligand–receptor complexes, was without effect on the pattern of specific labeling by the photoreactive insulins. Thus, labeling at 18 or 37 °C produced the same patterns of radioactive bands (not shown); changing the temperature from 18 to 37 °C after binding and photolysis produced also no detectable effect (Figure 1C).

If the photolabeled 40-kilodalton band is a proteolytic product of the 130- or 90-kilodalton protein, it might be possible to generate such a band by limited digestion with proteases, such as trypsin and chymotrypsin. The in-gel digestion technique of Cleaveland et al. (1977) was used to digest the 130- and 90-kilodalton bands with several concentrations of trypsin or chymotrypsin. Figure 2A,B shows the results of such an experiment using B29-MABI as the photolabel. However, no band of the size of 40 kilodaltons was obtained, although the original radioautogram showed some degree of proteolysis at 20 μg of trypsin. Similar results were obtained with the 130-kilodalton band labeled with B1-MABI. By use of the same technique, digestion of the 40-kilodalton band with trypsin showed that the reactivity of the band labeled with B29-MABI toward the enzyme differed from that of the band labeled with B1-MABI. Thus, the 40-kilodalton band labeled with B1-MABI was partially digested while that labeled with B29-MABI remained intact (Figure 2C,D).

We had demonstrated previously that the electrophoretic mobilities of the 130- and 90-kilodalton bands labeled with B29-MABI were increased when isolated plasma membranes were first treated with the enzyme neuraminidase before labeling (Yip et al., 1980b). We concluded that these bands were glycoproteins. In the present study, the electrophoretic mobility of the 40-kilodalton band and those of the 130- and 90-kilodalton bands were all increased when the intact adipocytes were first treated with the enzyme before labeling with the photoreactive insulins (Figure 3). These findings are consistent with our previous conclusion and are supported by the recent report of Hedo et al. (1981) on the incorporation of amino sugars into insulin receptor proteins of IM-9 lymphocytes.

The detection of these labeled bands required the reduction of the plasma membranes with dithiothreitol. Radioautography of the gradient $NaDodSO_4$–polyacrylamide gel electrophoresis of the plasma membranes obtained from the adipocytes labeled with either B29-MABI or B1-MABI showed that most of the radioactivity appeared near the top of the gel if the membranes were not reduced (Figure 4A, tracks 1 and 3). Two highly labeled bands were visible: one barely entering the gel and the other having an M_r of about 300000, based on the extrapolation of the standard calibration curve obtained in the same gel. The labeling of these two bands was inhibited by the addition of excess insulin to the

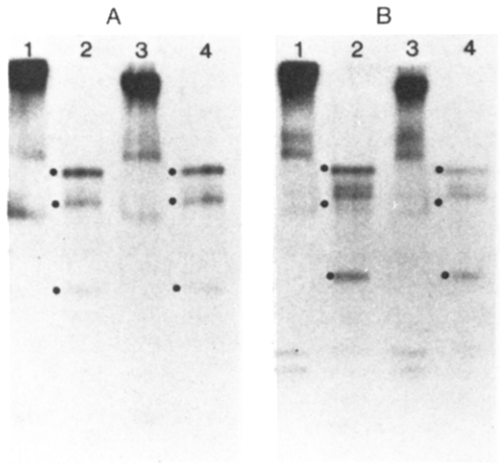

FIGURE 5: Direct reduction of high molecular weight bands photolabeled by (A) [^{125}I]B1-MABI and (B) [^{125}I]B29-MABI. The radioactive band barely entering the gel and the 300-kilodalton bands obtained as shown in Figure 4A were separately excised from unstained gels. They were then boiled in $NaDodSO_4$–sample buffer with or without dithiothreitol for 10 min. The gel pieces were then inserted into the sample slots of another 5–15% gradient gel for electrophoresis. In both (A) and (B), the lanes represented the following: (1) unreduced band barely entering the original 5–15% gradient gel; (2) same band after reduction; (3) 300-kilodalton band without reduction; (4) 300-kilodalton band after reduction. The dots mark the positions of the 130-, 90-, and 40-kilodalton bands.

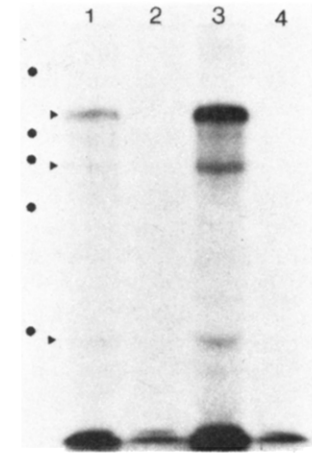

FIGURE 6: Effect of human autoimmune anti-receptor serum on the photolabeling of adipocytes by [^{125}I]B29-MABI. Before being labeled, the cells were incubated at 37 °C for 30 min with the antiserum (lanes 1 and 2) or with normal human serum (lanes 3 and 4). Both sera were diluted 1:80 for use. Lanes 2 and 4 were obtained when labeling was carried out in the presence of excess insulin. The dots mark the molecular weight standards. The triangles indicate the 130-, 90-, and 40-kilodalton bands.

incubation medium (Figure 4A, tracks 2 and 4). Reduction of the plasma membranes yielded the 130-, 90-, and 40-kilodalton bands in the case of labeling with B29-MABI, and the 130- and 40-kilodalton bands with B1-MABI (Figure 4B, tracks 1 and 3). It thus seemed likely that the appearance of these bands was the result of the reduction of the 300-kilodalton band and the band that barely entered the gel. In order to demonstrate directly this relationship, we excised the two radioactive bands from the gel, reduced each one separately, and analyzed each one by gradient $NaDodSO_4$–polyacrylamide gel electrophoresis again. Figure 5 shows that the direct reduction of both bands generated the appropriate radioactive banding pattern. The radioactive A chain, containing about 80% of the radioiodine in the radioactive photolabels (Yip et al., 1980a), was split off these bands and lost from the gel slices to the medium during reduction, thus accounting for the apparent loss of radioactivity and the absence of radioactivity at the gel front. Without reduction, the two bands retained their original electrophoretic mobilities, and there was no obvious interconversion between them. These experiments clearly show that the photolabeled bands of 130, 90, and 40 kilodaltons are linked by disulfide bonds and that they are components of larger protein units.

Incubation of the adipocytes with the human autoimmune anti-receptor serum before photolabeling with the insulin derivatives inhibited the specific binding of the photoprobe by about 75%. Incubation with normal human serum was without effect. Analysis of the labeled plasma membranes showed that the labeling of the 130-, 90-, and 40-kilodalton bands by B29-MABI was greatly reduced by the antiserum (Figure 6). The labeling of the 130- and 40-kilodalton bands by B1-MABI was similarly inhibited (data not shown).

Discussion

In this study using intact adipocytes, we have confirmed our previous findings that two fully characterized photoreactive insulins, B29-MABI and B1-MABI, specifically photolabeled two protein bands of 130 and 90 kilodaltons and one protein band of 130 kilodaltons, respectively, in isolated plasma membranes (Yip et al., 1980a; Yeung et al., 1980). However, an additional protein band of 40 kilodaltons was now also specifically labeled by these two insulin derivatives when intact adipocytes were used. In order to explain this difference we considered the possibility that the 40-kilodalton band might be a product of either the extracellular degradation or the intracellular proteolysis of the insulin–receptor complex subsequent to receptor internalization by the intact cells. It is evident from the data presented that this is unlikely, since the labeling of this 40-kilodalton protein band was not affected by the time or temperature of incubation and was not inhibited by bacitracin, benzamidine, *N*-ethylmaleimide, PMSF, PCMPS, Trasylol, methylamine, or chloroquine. The addition of protease inhibitors to the homogenization medium failed also to alter the labeling of these bands. Nor were we able to generate a labeled band of similar molecular weight by the digestion of either the 130- or the 90-kilodalton band with trypsin or chymotrypsin, although we cannot rule out the possibility that a specific enzyme, but not trypsin or chymotrypsin, might be involved in the degradation of the labeled proteins by the adipocytes and that its activity might not be affected by the inhibitors tested. On the other hand, the labeled 40-kilodalton band could not be a degradative product of the 90-kilodalton protein, since the photoreactive B1-MABI did not label a 90-kilodalton protein, and yet a labeled 40-kilodalton band was detected when this derivative was used. If the 40-kilodalton protein band was derived from the degradation of the labeled 130-kilodalton protein, one would expect the 40-kilodalton proteins labeled respectively by the two insulin derivatives to be very similar to each other. It appears, however, that they may be different from each other as evident from their differing sensitivity to trypsin. The possibility remains, nonetheless, that this difference might be the result of the labeling by the two different derivatives. The labeling of the 40-kilodalton protein by B1-MABI might have rendered it insensitive to trypsin. A full characterization of this (these) 40-kilodalton protein(s) would require the isolation and purification of the insulin receptor. The effects of neuraminidase treatment on the electrophoretic mobilities of the 130-, 90-, and 40-kilodalton proteins show that these proteins are glycoproteins and that, like the 130- and 90-kilodalton proteins, the 40-kilodalton protein labeled by either insulin derivatives

is at least partially exposed to the exterior of the adipocyte. It is also evident from the results of the experiments using dithiothreitol that these proteins are most likely disulfide-linked subunits of a larger structure. Based on these considerations and data obtained, we conclude that the 40-kilodalton protein band is another subunit of the insulin receptor. This conclusion is consistent also with the observation that the autoimmune antiserum to insulin receptor inhibited not only the specific labeling of the 130- and 90-kilodalton proteins but also the 40-kilodalton protein, although the same antiserum did not precipitate a biosynthetically labeled 40-kilodalton protein from cultured IM-9 lymphocytes (Van Obberghen et al., 1981; Hedo et al., 1981). This discrepancy may be due to possible tissue difference.

Our data show that, in addition to the 130- and 40-kilodalton proteins, several radioactive bands of intermediate molecular weights between 130 and 90 kilodaltons were obtained with B1-MABI but not with B29-MABI. The intensity of labeling of these bands varied considerably between experiments. We are not certain as to the nature of these bands. It is apparent that they were also produced from the direct reduction of the 300-kilodalton band and the one other higher molecular weight band. They could have been generated from the 130-kilodalton band during the process of reduction, although their appearance was not affected by the concentration of reductant used (from 1 to 100 mM) or by the period of reduction (from 5 to 90 min). The appearance of these bands was not inhibited by the various inhibitors used. The origin and significance of these labeled bands remain to be investigated.

There is a general agreement in the literature on the subunit nature of the insulin receptor, but there are considerable variations in the reported number and size of the subunits. Based on the immunoprecipitation of radioiodinated solubilized and purified insulin receptor, Jacobs et al. (1980) concluded that in liver tissue the native insulin receptor of 310 kilodaltons is composed of two 135- and two 45-kilodalton subunits in disulfide linkages. On the other hand, Massague et al. (1980) using bifunctional cross-linking reagents concluded that in adipocytes the native insulin receptor of 350 kilodaltons is composed of two 125- and two 90-kilodalton subunits, although their data did not clearly show the presence of the 90-kilodalton band. They further concluded that proteolysis of the 90-kilodalton subunits gave rise to 49-kilodalton products and that this accounted for their detection of the insulin receptor in three major structural forms of 350, 320, and 290 kilodaltons. Van Obberghen et al. (1981) and Hedo et al. (1981) using a human autoimmune serum to insulin receptor to precipitate biosynthetically labeled receptor proteins of cultured human IM-9 lymphocytes concluded that the 130- and 90-kilodalton proteins were subunits of the insulin receptor. Harrison & Itin (1980) detected three proteins of 126, 90–94, and 42 kilodaltons, respectively, in solubilized and purified insulin receptor from human placenta and suggested that the 90–94-kilodalton species might be a dimer of the 42-kilodalton species. Recently Siegel et al. (1981) also reported the presence of a 45-kilodalton protein in the insulin receptor purified from human placenta. We (Yip et al., 1980a; Yeung et al., 1980) have previously concluded from photoaffinity labeling of plasma membranes of adipose and liver tissues that the 130- and 90-kilodalton proteins were subunits of the insulin receptor. It is therefore evident from the published data and from the results presented in this study that three subunit species of 125–135, 90–94, and 40–49 kilodaltons have been detected by different methods and in different tissues as components of the insulin receptor and that they are linked by disulfide bonds to form a larger structure of 300–350 kilodaltons. Furthermore, it has been generally observed that the 125–135-kilodalton species is the major labeled band by either chemical cross-linking, photoaffinity labeling, or radioiodination of the insulin receptor. This species is possibly the primary binding subunit of the insulin receptor. On the other hand, the 90-kilodalton species has been clearly detected by us in this and previous studies (Yip et al., 1980a–c; Yeung et al., 1980) and by others (Van Obberghen et al., 1981; Hedo et al., 1981; Harrison & Itin, 1980). The uncertainty in the subunit structure of the receptor is the origin of the 40–49-kilodalton species as to whether they are derived from the 90-kilodalton species through proteolysis as suggested by Massague et al. (1980) or they are monomers forming the 90–94-kilodalton species as suggested by Harrison & Itin (1980). We have already discussed the experimental evidence which supports our conclusion that the 40-kilodalton band is not derived from the 90-kilodalton protein and is itself a subunit of the insulin receptor. The evidence includes the lack of an effect on the labeling of the 40-kilodalton band when various inhibitors were used and when the temperature of incubation was altered, the failure to generate a band of similar size to the 40-kilodalton species from the 90-kilodalton band, and the detection of the 40-kilodalton species with B1-MABI while no 90-kilodalton species was observed. Further, the difference in sensitivity to trypsin between the 40-kilodalton species labeled by the two photoreactive insulins suggests that there may be two separate 40-kilodalton subunits. Our inability to detect the 40-kilodalton band in our studies by using isolated plasma membrane preparations (Yip et al., 1980a,b; Yeung et al., 1980) emphasizes the importance of using intact viable cells in such labeling experiments. The process of membrane preparation might have altered the spatial relationship of the receptor subunits such that the 40-kilodalton species is not within the reactive range of the nitrene and thus not labeled by the photoreactive insulins. The alteration might have resulted from a change of ionic environment or from the loss of certain membrane components.

We therefore propose that the insulin receptor on the surface of the intact cell is composed of four disulfide-linked glycoprotein subunits consisting of one 130-, one 90-, and two 40-kilodalton subunits. Although the sum of the estimated molecular weights of these four subunits in this proposed model is in good agreement with the estimated size of the unreduced receptor being 300–350 kilodaltons (Pilch & Czech, 1980; Jacobs et al., 1980a), these values should be considered only as estimates because of possible inherent errors in the molecular weight determination of large glycoproteins by Na-DodSO_4–polyacrylamide gel electrophoresis. The intensity of photolabeling of each band reflects the proximity of the subunit to the reactive nitrene on the photoreactive insulin for covalent bond formation and cannot be used as a quantitative stoichiometric measurement of the amount of each subunit in the intact receptor. The proposed subunit structure of the insulin receptor is consistent with the following experimental observations: (a) a 130-kilodalton band was labeled by either B1-MABI or B29-MABI; (b) the 90-kilodalton band was labeled by B29-MABI only; (c) the two photoreactive insulins labeled apparently two different 40-kilodalton proteins; (d) without reduction, a 300-kilodalton band was specifically labeled; and (e) direct reduction of this 300-kilodalton band produced the 130-, 90-, and 40-kilodalton bands. It is worth noting that, without reduction, another band of molecular weight more than 300 000 was also specifically labeled and

that this band, when reduced directly, also generated the 130-, 90-, and 40-kilodalton species. There was, however, no apparent interconversion between this and the 300-kilodalton species. The 130-kilodalton subunit is postulated here as the major binding or recognition subunit of the receptor. The other subunits may function by interacting with another insulin–receptor complex or with other membrane components. Such interactions could produce the required signals or messages for the appropriate biological responses.

Acknowledgments

We thank Helga Hsu for her excellent technical assistance.

References

Carpentier, J.-L., Gorden, P., Freychet, P., Le Cam, A., & Orci, L. (1979) *J. Clin. Invest. 63*, 1249–1261.
Cleaveland, D. W., Fischer, S. G., Kirshner, M. W., & Laemmli, U. K. (1977) *J. Biol. Chem. 252*, 1102–1106.
Duckworth, W. C., Stentz, F. B., Heinemann, M., & Kitabchi, A. E. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 635–639.
Ensinck, J. W., Shephard, C., Dudl, R. J., & Williams, R. H. (1972) *J. Clin. Endocrinol. Metab. 35*, 463–467.
Harrison, L. C., & Itin, A. (1980) *J. Biol. Chem. 255*, 12066–12072.
Hedo, J. A., Kasuga, M., Van Obberghen, E., Roth, J., & Kahn, R. C. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4791–4795.
Jacobs, S., & Cuatrecasas, P. (1980) *J. Clin. Invest. 66*, 1424–1427.
Jacobs, S., Hazum, E., Schechter, Y., & Cuatrecasas, P. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4918–4921.
Jacobs, S., Hazum, E., & Cuatrecasas, P. (1980) *J. Biol. Chem. 255*, 6937–6940.
Massague, J., Pilch, P. F., & Czech, M. P. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7137–7141.
Maxfield, F. R., Willingham, M. C., Davies, P. J. A., & Pastan, I. (1979) *Nature (London) 277*, 661–663.
McKeel, D. W., & Jarett, L. (1970) *J. Cell Biol. 44*, 417–432.
Moody, A. J., Stan, M. A., Stan, M., & Gliemann, J. (1974) *Horm. Metab. Res. 6*. 12–16.
Pilch, P. F., & Czech, M. P. (1980) *J. Biol. Chem. 255*, 1722–1723.
Rodbell, M. (1964) *J. Biol. Chem. 239*, 375–380.
Siegel, T. W., Ganguly, S., Jacobs, S., Rosen, O. M., & Rubin, C. S. (1981) *J. Biol. Chem. 256*, 9266–9273.
Van Obberghen, E., Kasuga, M., Le Cam, A., Hedo, J. A., Itin, A., & Harrison, L. C. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1052–1056.
Wibo, M., & Poole, B. (1974) *J. Cell Biol. 63*, 430–440.
Wisher, M. H., Brown, M. D., Jones, R. H., Sonksen, P. H., Saunder, D. J., Thamm, P., & Brandenburg, D. (1980) *Biochem. Biophys. Res. Commun. 92*, 492–498.
Yeung, C. W. T., Moule, M. L., & Yip, C. C. (1980) *Biochemistry 19*, 2196–2203.
Yip, C. C., Yeung, C. W. T., & Moule, M. L. (1978) *J. Biol. Chem. 253*, 1743–1745.
Yip, C. C., Yeung, C. W. T., & Moule, M. L. (1980a) *Biochemistry 19*, 70–76.
Yip, C. C., Moule, M. L., & Yeung, C. W. T. (1980b) *Biochem. Biophys. Res. Commun. 96*, 1671–1678.
Yip, C. C., Yeung, C. W. T., & Moule, M. L. (1980c) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 1664.

Interaction of Photosynthetic Electron Transport Inhibitors and the Rieske Iron–Sulfur Center in Chloroplasts and the Cytochrome b_6–f Complex†

Richard Malkin

ABSTRACT: The interaction of electron transport inhibitors with the Rieske iron–sulfur center in chloroplast membranes and in a purified chloroplast cytochrome complex (the b_6–f complex) has been studied by using electron paramagnetic resonance (EPR) spectroscopy. Several quinone inhibitors, all of which contain a halogen substituent and a bulky alkyl side chain, cause a shift in the EPR signal of the reduced Rieske iron–sulfur center from $g = 1.90$ to $g = 1.94$. This g-value shift occurs in untreated membranes as well as in the cytochrome complex, which contains the Rieske center and no other iron–sulfur centers. Other compounds known to inhibit electron transport in the region of the iron–sulfur center cause a smaller alteration in the EPR signal of the Rieske center and are able to interact with the quinone-binding site as evidenced by their displacement of quinones from the Rieske center. One substrate, plastoquinone 9, was also able to displace quinones from the Rieske center while others, such as plastoquinone 1 and duroquinone, did not show this effect. These results are considered in relation to the mode of interaction of quinones with the Rieske center in the photosynthetic membrane.

Electron transport between the two light reactions of chloroplast photosynthesis involves a series of carriers which include plastoquinone, the Rieske iron–sulfur center, cytochrome f, and plastocyanin (Trebst, 1974; Bendall, 1977; Crofts & Wood, 1978; Velthuys, 1980). Recent studies with the inhibitory plastoquinone analogue, DBMIB[1] (Trebst et al., 1970), have provided evidence for the interaction of quinones with the Rieske iron–sulfur center and have indicated that the iron–sulfur center can function to stabilize a semiquinone

† From the Department of Plant and Soil Biology, University of California, Berkeley, California 94720. *Received September 3, 1981.* This work was supported in part by a grant from the National Institutes of Health.

[1] Abbreviations: DBMIB, 2,5-dibromo-3-methyl-5-isopropylbenzoquinone; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole; DNP-INT, 2,4-dinitrophenyl ether of iodonitrothymol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; BBB, 2-bromo-5-*tert*-butylbenzoquinone; DBBB, 2,3-dibromo-5-*tert*-butylbenzoquinone; DIBB, 2,3-diiodo-5-*tert*-butylbenzoquinone; DBDIB, 3,5-dibromo-2,6-diisopropylbenzoquinone; $NaDodSO_4$, sodium dodecyl sulfate.

0006-2960/82/0421-2945$01.25/0