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High Molecular Weight Forms of the Insulin Receptor†

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ABSTRACT: The insulin receptor of liver, adipose, and placental plasma membranes was photoaffinity labeled with radioiodinated $N^{\epsilon B29}$-(monoazidobenzoyl)insulin. Three specifically labeled bands of 450, 360, and 260 kilodaltons (kDa) were identified in each tissue by polyacrylamide gel electrophoresis of the membranes solubilized in sodium dodecyl sulfate (SDS). The 360- and 260-kDa bands corresponded to partially reduced forms of the 450-kDa band. The distribution of radioactivity between the three insulin receptor bands was dependent on the tissue, the purity of the receptor preparation, and the conditions of solubilization in SDS. The 360- and 260-kDa bands became more prominent in each tissue with an increasing time of solubilization in SDS. However, with a short solubilization time in SDS, the 450-, 360-, and 260-kDa bands of the receptor were distributed approximately in a ratio of 85:15:0 in all three tissues. Inclusion of sulfhydryl alkylating reagents during solubilization in SDS altered this ratio to about 95:5:0. We conclude that the 450-kDa band represents the predominant form of the photolabeled insulin receptor and that the 260-kDa and probably the 360-kDa form as well were generated during the experimental manipulations preceding identification of the receptor. However, the appearance of the 360- and 260-kDa bands was not due to reductant present in SDS or buffer solutions and could not be accounted for by proteolytic degradation of the receptor. Furthermore, purification of the receptor over 2000-fold did not prevent the appearance of the 360- and 260-kDa bands. The effect of sulfhydryl alkylating reagents [*p*-(chloromercuri)benzenesulfonate, iodoacetamide, and *N*-ethylmaleimide] on the appearance of the receptor suggests that a sulfhydryl component of the membranes, which copurifies with the insulin receptor, can reduce specific disulfide bonds of the insulin receptor during solubilization in SDS.

The insulin receptor is minimally composed of a 130-kilodalton (kDa)[1] (α) insulin binding subunit and a 90-kDa (β) protein kinase subunit. cDNA cloning of the human insulin receptor has recently demonstrated that α and β subunits of the receptor are the products of a single gene (Ullrich et al., 1985). However, a number of high molecular weight forms of the plasma membrane receptor are observed on nonreducing SDS–polyacrylamide gels. To account for these high molec-

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[1] Abbreviations: SDS, sodium dodecyl sulfate; kDa, kilodalton(s); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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ular weight forms of the insulin receptor, several investigators have proposed that the insulin receptor is assembled as a disulfide-linked heterodimer, $(\alpha\beta)_2$ [reviewed by Ullrich et al. (1985)]. However, additional putative receptor subunits of 40 (Goren et al., 1983; Hofmann et al., 1981; Yip & Moule, 1983), 65 (Baron & Sonksen, 1983), and 85 kDa (Wang et al., 1982; Yip & Moule, 1983) have also been postulated to associate with the insulin receptor at the cell surface. Yip and Moule (1983) postulated that the plasma membrane insulin receptor is composed of one α subunit and one β subunit as well as one 85-kDa subunit and two 40-kDa subunits. The significance of these other putative receptor subunits is unknown. Interestingly, however, some recent investigations suggest that a 44-kDa class I major histocompatability complex antigen may be associated with the plasma membrane insulin receptor (Brossette et al., 1984).

Three high molecular weight forms of the insulin receptor have been previously identified in this laboratory (Yip & Moule, 1983).[2] Similarly, multiple high molecular weight forms of the insulin receptor have been observed by other investigators using cross-linking and immunochemical techniques (Massague & Czech, 1980; Massague et al., 1980; Berhanu et al., 1982; Heidenreich et al., 1983; Crettaz et al., 1984; Endo & Elsas, 1984). It is generally agreed that the smaller molecular weight forms of the receptor correspond to partially reduced species (Yip & Moule, 1983; Massague & Czech, 1980; Crettaz et al., 1984; Endo & Elsas, 1984). It is unclear, however, whether these different high molecular weight forms of the insulin receptor are native species of the receptor (Massague & Czech, 1980; Crettaz et al., 1984) which subserve different functions or are artifactually generated (Endo & Elsas, 1984). Furthermore, it has been observed in this laboratory (Yip & Moule, 1983) and subsequently in others (Heidenreich et al., 1983; Endo & Elsas, 1984) that the largest receptor form predominates when the insulin receptor is solubilized in SDS containing sulfhydryl alkylating reagents but it is not known how these reagents produce their effect.

We set out to determine whether the multiple high molecular weight forms of the insulin receptor are all native forms of the receptor or whether they are fully or in part artifactual. To this end, the photoaffinity-labeled insulin receptors of adipose, hepatic, and placental preparations were compared, and the experimental manipulations preceding their identification were examined. The possibility that the multiple high molecular weight forms of the receptor arise as a consequence of proteolysis (Massague et al., 1980, 1981; Berhanu et al., 1982) or alkaline lysis (Endo & Elsas, 1984) of disulfide bonds during solubilization in SDS was also assessed. Finally, we compared the effects of different sulfhydryl alkylating reagents in an attempt to understand how they may influence the structure of the insulin receptor.

Experimental Procedures

Radioiodinated $N^{\epsilon B29}$-(monoazidobenzoyl)insulin was prepared as previously described (Yip et al., 1980). Bovine insulin was a gift from Connaught Laboratories (Canada). Chemicals for polyacrylamide gel electrophoresis and Affigel-10 were obtained from Bio-Rad Laboratories (Richmond, CA). Bovine serum albumin, bacitracin, phenylmethanesulfonyl fluoride, iodoacetamide, *N*-ethylmaleimide, and *p*-(chloromercuri)-benzenesulfonate were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade.

Rat adipose and liver plasma membranes were prepared following the methods of Jarett (1974) and Ray (1970), respectively. Human placental plasma membranes were prepared following the method of Harrison and Itin (1980). The insulin receptors of placental or liver plasma membranes were purified more than 2000-fold on an insulin–Affigel-10 column according to the method of Fujita-Yamaguchi et al. (1983). Photolabeling of plasma membranes was carried out as follows. Plasma membranes (50–200 μg/mL) were incubated for 1 h at 18 °C in 50 mM Tris-HCl, pH 7.5, containing 0.1% bovine albumin, 1 mM bacitracin, and 10 nM radioiodinated $N^{\epsilon B29}$-(monoazidobenzoyl)insulin. Nonspecific binding of the photoprobe was determined in the presence of 5 μM bovine insulin. The incubation mixture was photolyzed for 30 s with a focused light source of a 100-W high-pressure mercury lamp. The membranes were then separated from the incubation mixture by centrifugation (8000*g*, 15 min, 4 °C) and washed twice with 50 mM Tris-HCl, pH 7.5, containing 0.5% bovine serum albumin. The membranes were then solubilized in 62.5 mM Tris-HCl, pH 6.8, containing 3% SDS and 10% glycerol (SDS solubilization buffer) unless otherwise stated. Electrophoresis was performed on 3–10% gradient slab polyacrylamide gels, and autoradiography of the gels was performed as previously described (Yip et al., 1983). Molecular weight markers used for gels were thyroglobulin (669 kDa), thyroglobulin intermediates (475, 280, and 255 kDa), catalase dimer (120 kDa), and catalase monomer (60 kDa). Protein was electroeluted from gel slices by using the method described by Bhown et al. (1980).

Results

When the photoaffinity-labeled plasma membrane preparations of adipose, liver, and placental tissue were solubilized in SDS and resolved by electrophoresis on polyacrylamide gels without reduction, three major radioactive bands were detected (peaks 1–3, Figure 1A–C). Insulin specifically inhibited the labeling of these bands. The apparent molecular weights of these bands were determined to be approximately 450 000, 360 000 and 260 000. A 40-kDa band was also detected in the photolabeled liver preparation (peak 7, Figure 1C), and insulin also specifically inhibited the labeling of this band. The inclusion of a wide range of proteolytic inhibitors including soybean trypsin inhibitor, bacitracin, benzamidine, and phenylmethanesulfonyl fluoride did not affect the labeling of the 450-, 360-, 260-, or 40-kDa bands. However, the 65-kDa band observed in the photolabeled adipose plasma membrane preparation (peak 9, Figure 1A) was not inhibited when labeling was performed in the presence of an excess of insulin (This band probably represented the nonspecific labeling of bovine serum albumin.) The 360-, 260-, and 40-kDa bands of the receptor corresponded to partially reduced forms of the receptor since the 450-kDa band, after electroelution from appropriate gel slices and partial reduction with 0.1 mM dithiothreitol, could also be resolved into bands of 450, 360, 260, and 40 kDa (figure 1D). Additional bands of 210, 125, 90, and 25 kDa (peaks 4, 5, 6, and 8, respectively) were also observed. Complete reduction of the electroeluted 450-kDa band resulted in most of the radioactivity appearing in the 125-kDa insulin binding subunit.

The distribution of radioactivity obtained in the 450-, 360-, and 260-kDa bands was dependent on the time of solubilization of each tissue in SDS (Figure 2). As an illustration, an autoradiogram of the photoaffinity-labeled insulin receptor of liver plasma membranes is shown in Figure 2A. Figure 2B

[2] The 450-, 360-, and 260-kDa bands of the insulin receptor were previously published by this laboratory as 380-, 300-, and 230-kDa bands, respectively.

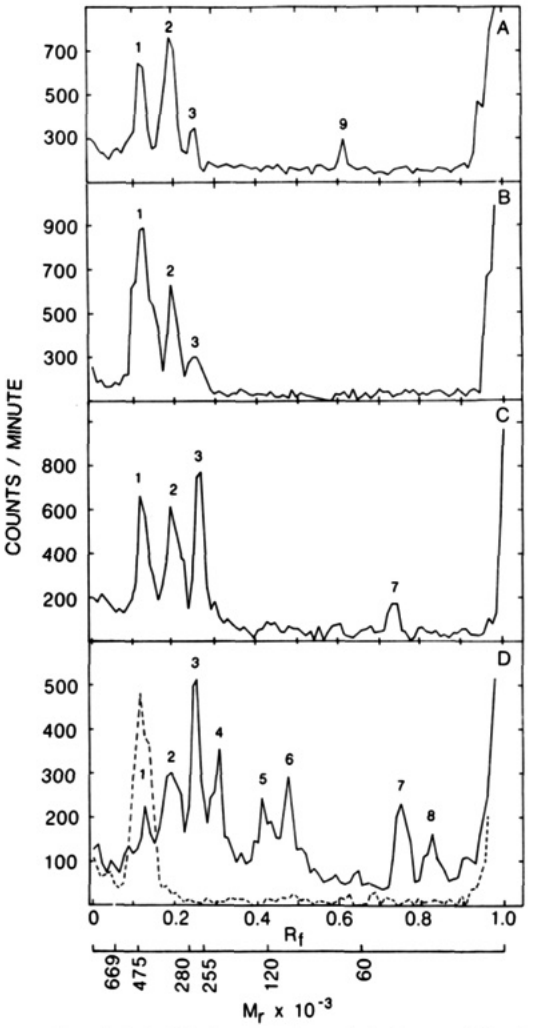

FIGURE 1: Photolabeled high molecular weight forms of the insulin receptor in adipocyte (A), placental (B), and liver (C) plasma membranes and the generation of partially reduced forms of the insulin receptor by dithiothreitol (D). Photolabeled plasma membranes were solubilized by boiling for 15 min in 3% SDS solubilization buffer and then electrophoresed on 3–10% gradient SDS–polyacrylamide slab gels as described under Experimental Procedures. Tracks were excised from the gels, cut into 1-mm slices, and counted. The photolabeled receptor was electroeluted from gel slices corresponding to peak 1 (450-kDa band) obtained from liver plasma membranes. The electroeluted material was lyophilized, boiled for 10 min in 3% SDS solubilization buffer (---) containing 0.1 mM dithiothreitol (—), and then electrophoresed on a 3–10% gradient SDS–polyacrylamide slab gel. The appropriate tracks were excised, cut into 1-mm slices, and counted (D). Peaks 1–9 correspond to molecular masses of 450, 360, 260, 210, 125, 90, 40, 25, and 65 kDa, respectively.

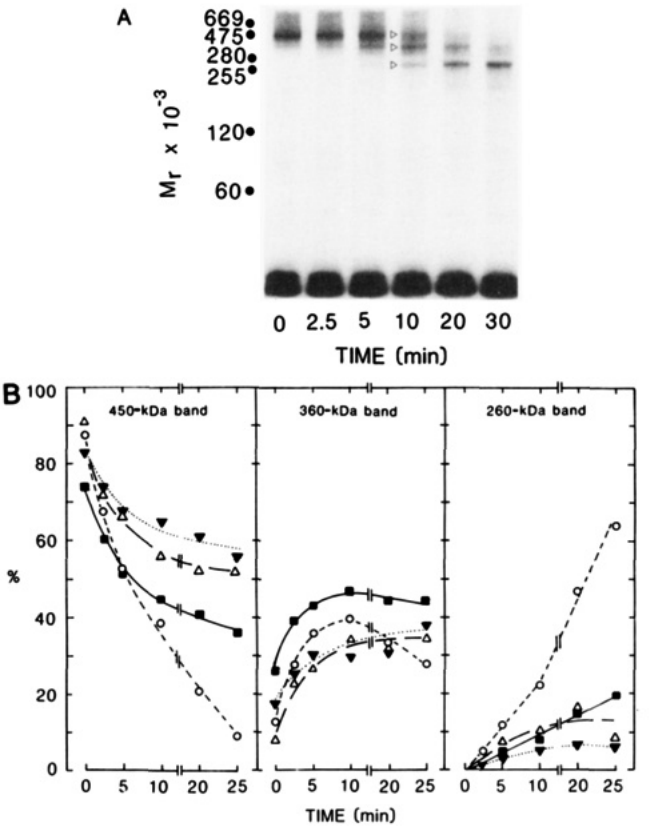

FIGURE 2: Effect of solubilization time on the high molecular weight forms of the photolabeled insulin receptor. Plasma membranes were solubilized for different times by boiling in 3% SDS solubilization buffer and then electrophoresed on 3–10% gradient SDS–polyacrylamide slab gels. An autoradiogram of photolabeled liver plasma membranes solubilized for different times and the positions of the 450-, 360-, and 260-kDa bands are shown in (A). In (B), the 450-, 360-, and 260-kDa bands were excised from the slab gels obtained from 2000-fold-purified placental insulin receptor (▼) as well as adipose (■), liver (○), and placental plasma membranes (△), and the radioactivity in each band was expressed as a percent of the total radioactivity in the three bands. Zero boiling time represents samples solubilized on ice for 5 min.

shows that in the three tissues studied, a predominant 450-kDa band was obtained with a short solubilization time in SDS. When the tissues were solubilized for 5 min on ice (zero boiling time), the 450-kDa band accounted for 80–90% of the total radioactivity associated with the three high molecular weight bands, and almost all of the remaining radioactivity was present in the 360-kDa band. In each tissue, the percent of radioactivity declined in the 450-kDa band and increased in the 360- and 260-kDa bands with increasing times of solubilization. However, the 260-kDa band of photolabeled liver plasma membranes became prominent only after long solubilization times in SDS (at least 10 min) (Figure 2B), and this was accompanied by a dramatic loss of the 450-kDa band. The time-dependent appearance of the 260- and 360-kDa bands during solubilization in SDS was unaffected by a 2000-fold purification of the insulin receptor from placental plasma membranes by affinity chromatography. Similarly, affinity purification of the insulin receptor from liver plasma membranes did not prevent the appearance of the 360- and 260-kDa bands during solubilization in SDS.

The pH of the solubilization solution had a marked influence on the distribution of radioactivity in the three receptor bands (Figure 3). In particular, a large increase in the radioactivity appearing in the 260-kDa band occurred, apparently at the expense of the 450-kDa band, when the pH was raised from 8.5 to 9.5. Increasing the pH further from 9.5 to 10.5 had little additional effect. However, when the pH was raised above 10.5, the 450-kDa band was almost entirely replaced by the 360- and 260-kDa bands. Additional bands of 115–130 and 95 kDa were also observed at pHs above 10.5.

Photoaffinity-labeled liver plasma membranes were treated with sulfhydryl alkylating reagents before or during a 20-min, 100 °C solubilization in SDS. In the absence of these reagents, the insulin receptor appeared predominantly as 360- and 260-kDa bands (Figure 4, lanes 1 and 6). Iodoacetamide, *N*-ethylmaleimide, and *p*-(chloromercuri)benzenesulfonate, when included during the solubilization in SDS, maintained the receptor predominantly as the 450-kDa band (Figure 4, lanes 7, 8 and 9, respectively). Under these conditions, the 360-kDa band appeared as a minor band, and the 260-kDa band was not obtained. However, when the photolabeled membranes treated with these reagents were thoroughly

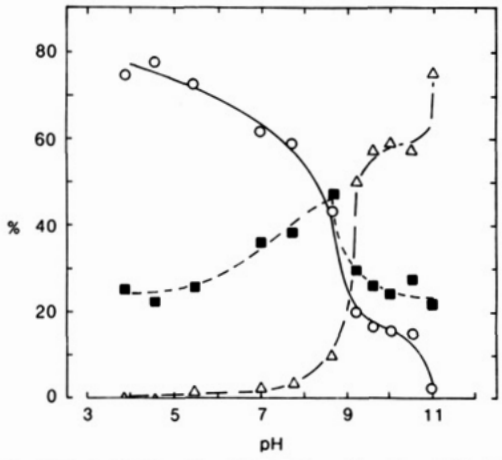

FIGURE 3: Effect of pH on the distribution of radioactivity between the high molecular weight forms of the insulin receptor. Photolabeled liver plasma membranes were solubilized by boiling for 1 min in 3% SDS and 10% glycerol buffered at different pHs by a solution 10 mM in citrate, Tris, and glycine. The solubilized membranes were then electrophoresed on 3–10% gradient SDS–polyacrylamide slab gels. The 450- (○), 360- (■), and 260-kDa (△) bands were then excised from the slab gels, and the radioactivity in each band was expressed as a percent of the total radioactivity in the three bands.

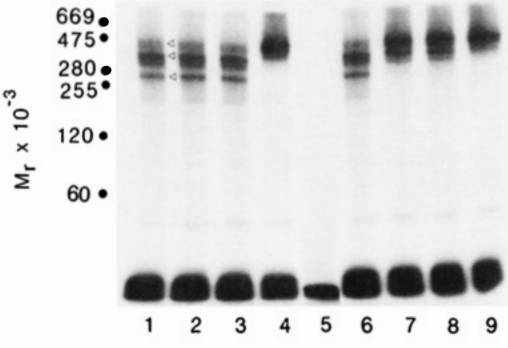

FIGURE 4: Autoradiograms showing the effect of sulfhydryl alkylating reagents on the high molecular weight forms of the insulin receptor. Photolabeled liver plasma membranes were incubated for 1 h at 18 °C in 50 mM Tris-HCl, pH 7.4 (lane 1), containing 25 mM iodoacetamide (lane 2), 10 mM *N*-ethylmaleimide (lane 3), or 1 mM *p*-(chloromercuri)benzenesulfonate (lane 4). The membranes were washed 3 times by resuspending the pellet obtained by centrifugation (20000*g*, 20 min) in 50 mM Tris-HCl, pH 7.4, containing 1% bovine serum albumin using a syringe fitted with a 22-gauge needle. The washed membranes were then solubilized by boiling for 20 min and electrophoresed on 3–10% gradient polyacrylamide slab gels. Photolabeled liver plasma membranes were solubilized by boiling for 20 min in solubilization buffer (lane 6) containing 25 mM iodoacetamide (lane 7), 10 mM *N*-ethylmaleimide (lane 8), or 1 mM *p*-(chloromercuri)benzenesulfonate (lane 9). Lane 5 was obtained when liver plasma membranes were photolabeled in the presence of 5 μM insulin. The positions of the 450-, 360-, and 260-kDa bands are shown.

washed before solubilization in SDS, the effect of iodoacetamide or *N*-ethylmaleimide was not observed (Figure 4, lanes 2 and 3, respectively). In contrast, the effect of *p*-(chloromercuri)benzenesulfonate persisted in spite of thorough washing (Figure 4, lane 4). In order to determine if this effect of *p*-(chloromercuri)benzenesulfonate was due to a carry-over of the reagent into the solubilization step, we treated unlabeled membranes with the reagent, which were then thoroughly washed. We then solubilized them together with untreated photolabeled membranes by boiling for 20 min in SDS. The photolabeled receptor appeared predominantly as 360- and 260-kDa bands, indistinguishable from those observed with photolabeled membranes not treated with the reagent (Figure 4, lanes 1 and 6). The persistent effect of *p*-(chloromercuri)benzenesulfonate despite thorough washing was therefore not due to the carry-over of the reagent.

Discussion

Several investigators using either affinity labeling or immunoprecipitation techniques (Massague & Czech, 1980; Massague et al., 1980; Berhanu et al., 1982; Heidenreich et al., 1983; Crettaz et al., 1984; Endo & Elsas, 1984) have observed that the insulin receptor can be resolved as multiple high molecular weight forms corresponding to partially reduced receptor. This study, in confirming these observations, also demonstrates that the resolution of the adipose, hepatic, or placental insulin receptor into these multiple high molecular weight forms is dependent on the conditions of solubilization in SDS before gel electrophoresis. Since the 360- and 260-kDa forms of the receptor were prominent only after a long time of solubilization in SDS and since their appearance during solubilziation was inhibited by sulfhydryl alkylating reagents, we conclude that the 450-kDa band of the insulin receptor represents the predominant form of the receptor in these tissues. While the 360-kDa form of the receptor was always detected and could not be totally abolished by sulfhydryl alkylating reagents, the appearance of the 260-kDa form required extended periods of solubilization in SDS. The significance of the 360-kDa form of the receptor remains to be determined. However, it is interesting to note that we have recently demonstrated a doubling of receptor affinity for insulin following enzymatic conversion of the receptor to predominantly a 360-kDa form.[3]

Conversion of the insulin receptor from the 450- to the 360- and 260-kDa forms during solubilization in SDS is presumably the result of the dissociation of receptor subunits. It is clear from Figure 2 that the subunits which are presumed to dissociate from the receptor during solubilization in SDS are not photolabeled. That all the subunits of the insulin receptor are not detected by photoaffinity labeling techniques has been observed previously (Yip & Moule, 1983). This observation is expected since only those subunits accessible to the photoreactive group of the ligand will be photolabeled. It has previously been shown, and confirmed in this study (data not shown), that the photoprobe we used labels both α and β subunits in the 450-, 360-, and 260-kDa forms of the insulin receptor (Yip & Moule, 1983). Therefore, if one adopts the $(\alpha\beta)_2$ heterodimer model for the insulin receptor, then one must conclude that one α subunit and one β subunit are not photolabeled but are dissociated from the receptor complex during solubilization in SDS. This would be correct if only one molecule of insulin was bound per receptor. Indeed, Ullrich et al. (1985) have speculated that insulin may interact with a single binding domain bounded by the two α subunits. An alternative model proposed by Yip and Moule (1983) suggests that the plasma membrane insulin receptor is composed of single α and β subunits as well as other subunits of 40 and 85 kDa. Other investigators have also identified putative receptor subunits of 40 (Goren et al., 1983; Hofmann et al., 1981), 65 (Baron & Sonksen, 1983), and 85 kDa (Wang et al., 1982). Thus, the subunits dissociated from the receptor during solubilization in SDS may conceivably be subunits other than the α or β subunits of the insulin receptor.

Alkaline lysis of disulfide bonds (Endo & Elsas, 1984) and proteolysis (Massague et al., 1980, 1981; Berhanu et al., 1982) of the insulin receptor have been proposed as a mechanism to account for the appearance of the multiple forms of the insulin receptor. However, alkaline lysis of disulfide bonds by either an α- or a β-elimination mechanism normally occurs

[3] F. Haynes, E. Helmerhorst, and C. C. Yip (unpublished results).

only at a pH above 10 (Cecil & McPhee, 1959; Danehy, 1966). Furthermore, since the time-dependent dissociation of subunits from the insulin receptor also occurred under acidic conditions (pH 4–7), alkaline lysis of disulfide bonds is evidently not the mechanism of subunit dissociation from the receptor during solubilization in SDS under these conditions. The effect of increasing the pH from 7 to 10 on increased dissociation of subunits from the receptor (Figure 3) can be attributed to the ionization of a residue of pK about 9. Nonetheless, the additional dissociation observed above pH 10 (Figure 3) may be due to some alkaline lysis of disulfide bonds.

Proteolysis of the receptor could result in the appearance of multiple receptor forms. However, this is unlikely for the following reasons. First, we and others (Heidenreich et al., 1983; Endo & Elsas, 1984) have shown that a wide range of protease inhibitors does not affect the appearance of the multiple high molecular weight forms of the receptor. Second, a denatured protein substrate should generally be more susceptible to proteolytic activity than the native substrate (Weber et al., 1972). However, no change was observed in the electrophoretic mobility of the 450- or 360-kDa bands after they had been electroeluted off SDS–polyacrylamide gels and mixed with fresh membranes for solubilization in SDS (data not shown). Third, treatment with 8 M urea before solubilization in SDS did not prevent the appearance of the multiple high molecular weight forms of the receptor (data not shown). Fourth, dissociation of subunits from the receptor by treatment in SDS at 100 °C was observed over an extended period of time (30 min), indicating that the effect is probably nonenzymatic (Weber et al., 1972). Finally, when liver plasma membranes were treated with N-ethylmaleimide or iodoacetamide and then thoroughly washed free of these alkylating reagents before solubilization in SDS, dissociation of the receptor was not prevented. Thus, it cannot be argued that the effect of alkylating reagents is through the inactivation of sulfhydryl proteases since these enzymes should be readily inactivated by such treatment (Weber et al., 1972).

The appearance of the multiple high molecular weight forms of the insulin receptor could not be due to reducing agents present in SDS or Tris buffer solutions. Quadrupling the concentration of Tris did not alter the distribution of radioactivity between the receptor bands. In addition, the appearance of the multiple high molecular weight forms of the insulin receptor could be prevented by treatment with p-(chloromercuri)benzenesulfonate before solubilization in SDS. Similarly, the action of contaminating reducing equivalents (such as reduced glutathione) during solubilization in SDS could not be responsible in our studies for the following reasons. First, a 2000-fold purification of the liver or placental insulin receptor did not prevent the appearance of the multiple high molecular weight forms of the receptor during solubilization in SDS. Second, the reducing component displayed different reactivities to sulfhydryl alkylating reagents, suggesting the involvement of tertiary structure. In this context, it is relevant to note that, as already described above, no change was observed in the electrophoretic mobility of the 450- or 360-kDa bands after they were electroeluted and resolubilized in SDS together with fresh, untreated membranes.

It is apparent that a sulfhydryl component is involved in the generation of the multiple high molecular weight forms of the insulin receptor since the receptor was maintained predominantly as a 450-kDa band when membranes were treated with sulfhydryl alkylating reagents. Furthermore, a residue(s) of pK about 9 which dramatically influence(s) the distribution of high molecular forms of the insulin receptor during solubilization in SDS (Figure 3) is (are) also consistent with the involvement of a sulfhydryl residue(s). The sulfhydryl residue(s) implicated is (are) most probably "buried" since only p-(chloromercuri)benzenesulfonate was an effective modifier of this (these) residue(s) without the denaturation of the membrane protein in SDS (Figure 4). Iodoacetamide and N-ethylmaleimide were effective modifiers of this (these) residue(s) only if added to the solubilization buffer. This may explain why some investigators (Massague & Czech, 1980; Crettaz et al., 1984) did not observe an effect of N-ethylmaleimide on the appearance of the insulin receptor when resolved by SDS–polyacrylamide gels. Partial reduction of the insulin receptor during solubilization in SDS by a sulfhydryl component of the receptor, or some sulfhydryl component which copurifies with the receptor, could lead to the generation of the multiple high molecular weight forms of the insulin receptor. Our data are consistent with this mechanism.

Several investigators have concluded that oxidation–reduction of disulfide bonds within the insulin receptor may be important in the mediation of insulin action. Whether a sulfhydryl component, such as that which we have described in this study, might be correlated with in vivo receptor function cannot be deduced from these studies. Nevertheless, it is interesting that this sulfhydryl component and the insulin receptor appear to require some minimal tertiary structure for apparent reduction to occur during solubilization in SDS and that, in addition, the activity of this sulfhydryl component is directed toward specific disulfide bonds within the insulin receptor. Specific disulfide bonds are implicated because no free subunits of the insulin receptor (which are disulfide linked) were observed irrespective of the length of the solubilization time at pH 6.8 in SDS. Indeed, other investigators (Endo & Elsas, 1984; Massague et al., 1981) have observed the differential reactivity of disulfide bonds linking subunits of the insulin receptor.

We conclude from the results of this study that the 450-kDa band of the photolabeled insulin receptor represents the predominant form of the receptor.[4] Partially reduced forms of the receptor are generated during experimental manipulations. However, we cannot exclude the possibility that partially reduced forms of the receptor may play a role in receptor function. We suggest that a sulfhydryl component of the plasma membrane reduces specific disulfide bonds within the receptor during solubilization in SDS. The significance of this interaction remains to be established.

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[4] After submission of the manuscript, we noted that Boyle et al. (1985) reached conclusions which corroborate those we arrived at in this study.

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Active Nonaromatic Intermediates in the Conversion of Steroidal Estrogens into Catechol Estrogens†

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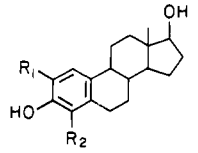
ABSTRACT: A mechanism is proposed for mixed-function oxidase-catalyzed formation of the catechol estrogens 2-hydroxy- and 4-hydroxyestradiol from estradiol. This mechanism involves nonaromatic epoxyenones as intermediates. The isomeric 1α,2α-epoxy-17β-hydroxyestr-4-en-3-one and 1β,2β-epoxy-17β-hydroxyestr-4-en-3-one (the latter as its 17-acetate) were synthesized from 17β-hydroxy-5α-estran-3-one. The isomeric 4α,5α-epoxy-17β-hydroxyestr-1-en-3-one and 4β,5β-epoxy-17β-hydroxyestr-1-en-3-one were prepared from 19-nortestosterone. From incubations of [6,7-^{3}H]estradiol with microsomes from MCF-7 human breast cancer cells, which principally catalyze the formation of 2-hydroxyestradiol from estradiol, we were able to isolate a ^{3}H-labeled product with the chromatographic properties of 1β,2β-epoxy-17β-hydroxyestr-4-en-3-one (as its 17-acetate). The soluble protein fraction of homogenates of rat liver, which is devoid of estrogen 2-/4-hydroxylase activity, has been shown to catalyze the formation of 2- and 4-hydroxyestradiol from the 1α,2α-epoxide and from the 4α,5α- and 4β,5β-epoxides, respectively. We suggest that these results taken together strongly support a role for epoxyenones as intermediates in the formation of catechol estrogens.

The formation of catechol estrogens from natural and synthetic steroidal estrogens is the major pathway of metabolism of these hormones (Ball & Knuppen, 1980; MacLusky et al., 1981; Fishman, 1983). In vivo, the endogenous female sex hormone estradiol (**1**) is metabolized in large part to 2-hydroxyestradiol (**2**) and in a much smaller amount to 4-hydroxyestradiol (**3**). The formation of catechol estrogens has been demonstrated to occur in a wide variety of normal and neoplastic tissues in experimental animals and humans (Poth et al., 1983). It is now apparent that catechol estrogens cannot be regarded merely as weak estrogens, since they bind to estrogen receptors and have biological effects as agonists or antagonists of the parent hormone (MacLusky et al., 1983; Schneider et al., 1984).



1 $R_1 = R_2 = H$
2 $R_1 = OH$, $R_2 = H$
3 $R_1 = H$, $R_2 = OH$

The formation of catechol estrogens is catalyzed by P-450 mixed-function oxidase (estrogen 2-/4-hydroxylases) found in numerous tissues, with highest activity in the mammalian liver (Poth et al., 1983). Estrogen 2-hydroxylase activity from mouse liver represents the contribution of at least four, and from rat liver of five, separable forms of mixed-function oxidases (Lang & Nebert, 1981; Ryan et al., 1982). However,

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