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Characterization of solubilized insulin receptors from rat liver microsomes

Existence of two receptor species with different binding properties

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Insulin receptors were solubilized from rat liver microsomes by the nonionic detergent Triton X-100. After gel filtration of the extract on Sepharose CL-6B, two insulin-binding species (peak I and peak II) were obtained. The structure and binding properties of both peaks were characterized. Gel filtration yielded Stokes radii of 9.2 nm (peak I) and 8.0 nm (peak II). Both peaks were glycoproteins. At 4°C peak I showed optimal insulin binding at pH 8.0 and high ionic strength. In contrast, peak II had its binding optimum at pH 7.0 and low ionic strength, where peak I binding was minimal. For peak I the change in insulin binding under different conditions of pH and ionic strength was due to a change in receptor affinity only. For peak II an additional change in receptor number was found. Both peaks yielded non-linear Scatchard plots under most of the buffer conditions examined. At their binding optima at 4°C the high affinity dissociation constants were 0.50 nM (peak I) and 0.55 nM (peak II).

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of peak I revealed five receptor bands with M_r 400000, 365000, 320000, 290000, and 245000 under non-reducing conditions. For peak II two major receptor bands with M_r 210000 and 115000 were found. The peak II receptor bands were also obtained after mild reduction of peak I. After complete reduction both peaks showed one major receptor band with M_r 130000. The reductive generation of the peak II receptor together with molecular mass estimations suggest that the peak I receptor is the disulfide-linked dimer of the peak II receptor. Thus, Triton extracts from rat liver microsomes contain two receptor species, which are related, but differ considerably in their size and insulin-binding properties.

Insulin receptors have been found in almost all mammalian tissues investigated so far. Their properties have been examined thoroughly during the last decade. To investigate their structure, native or labeled insulin receptors have been subjected to gel filtration [1 – 9], electrophoresis [10 – 14], various centrifugation techniques [4 – 8] and radiation inactivation [15]. Recently the primary structure of the human insulin receptor has been determined [16]. Receptor binding data have been analyzed by displacement curves, Scatchard plots and non-linear curve-fitting programs. However, in spite of these efforts the results obtained to date do not offer a clear picture of the insulin receptor. While some authors have observed only one receptor species with a Stokes radius of 7.2 nm and an M_r of about 350000 [1, 3, 4] others have found two species, the smaller of which could be generated from the larger one [2, 5, 8 – 10]. Furthermore, under the denaturing conditions of SDS-PAGE, multiple receptor forms have been detected [11 – 14]. Resolving the problem of receptor homogeneity or heterogeneity is important for the interpretation of binding data. With a few exceptions [6, 17, 18], insulin receptor binding data yield non-linear Scatchard plots. Usually this non-linearity is attributed either to the existence of heterogeneous binding sites [19, 20] or to the negative cooperative behavior of a single receptor class [21]. However, this subject is still controversial and other factors may be involved as well.

The aim of this study was to characterize the insulin receptors obtained from Triton X-100 extracts of rat liver microsomes. Following gel filtration of these extracts we found two receptor species whose structure and binding properties were investigated. Part of these results have previously been presented in a preliminary form [22].

MATERIALS AND METHODS

Bovine insulin was obtained from Hoechst (Frankfurt/Main); $Na^{125}I$ was from Amersham-Buchler (Braunschweig); ^{125}I-insulin was prepared by a modification of the chloramine-T method with a specific activity of 210 μCi/μg [23]; Triton X-100 (spec. purified) (Triton), poly(ethyleneglycol) 6000, dithioerythritol, *N*-ethylmaleimide, bovine serum albumin, bovine immunoglobulin G, concanavalin A, sodium dodecyl sulfate (SDS), acrylamide, *N,N'*-methylenebisacrylamide and *N,N,N',N'*-tetramethylethylenediamine were obtained from Serva (Heidelberg); methyl-α-D-mannopyranoside (99%), blue dextran, myosin, β-galactosidase,

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Abbreviations. MABI, NεB29-mono(4-azidobenzoyl)-insulin; SDS-PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis.

Enzymes. β-Galactosidase (EC 3.2.1.23); phosphorylase *b* (EC 2.4.1.1); aldolase (EC 4.1.2.13); catalase (EC 1.11.1.6); RNA polymerase (EC 2.7.7.6).

phosphorylase *b*, bovine serum albumin, Trizma base, and Trizma pre-set 8.7 were from Sigma (St Louis); Sepharose CL-6B, Sephadex G-50 fine, SP-Sephadex C-25, thyroglobulin and ferritin (from the high-molecular-weight gel filtration calibration kit) were obtained from Pharmacia (Uppsala); aldolase, catalase, and RNA-polymerase were from Boehringer (Mannheim); spectrins I and II were prepared from bovine erythrocytes; all other chemicals used were of reagent grade.

Preparation of rat liver microsomal extract

Liver microsomes were prepared from male or female Sprague-Dawley rats (130–150 g) by differential centrifugation as previously described [23]. The insulin receptor was extracted from the microsomes (20–25 mg protein/ml) with 1.5% (w/v) Triton X-100 at room temperature for 50 min. The suspension was diluted to a Triton concentration of 0.5% (w/v) by addition of cold 0.01 M phosphate buffer pH 7.4. Insoluble material was removed by centrifugation at 100000 × *g* (45 min) at 4°C. The protein content of the extract was about 6 mg/ml. The extract was stored at −30°C without loss of binding activity.

Determination of protein was performed according to the method of Lowry et al. [24] with 0.1% bovine serum albumin as standard.

^{125}I-Insulin-binding assay

All binding assays were done in triplicate. 200 µl solubilized receptor preparation (0.05–0.15 mg protein/ml) was incubated with ^{125}I-insulin (0.04 pmol) at 4°C in a total volume of 300 µl phosphate buffer and in the presence of 0.06% Triton until a steady state was reached (40 h). The molarity and the pH of the phosphate buffer are indicated in the legends of the figures. In displacement studies the tubes contained unlabeled insulin at concentrations between 0.08 nM and 5000 nM. After incubation the ^{125}I-insulin-receptor complex was separated from unbound ^{125}I-insulin by precipitation with poly(ethyleneglycol) [23] and the pellets were counted for radioactivity. Specific binding was determined by subtraction of non-specific binding in the presence of unlabeled insulin (5 µM) from total binding and is indicated as percentage specifically bound = 100 × cpm (specifically bound)/cpm (total).

Binding data were analyzed by Scatchard plots and by a computerized method of non-linear curve-fitting corresponding to the binding model applied by Munson and Rodbard [25]. Each experiment was fitted assuming the existence of either one class of identical binding sites (one-site model), or of two classes of different binding sites (two-site model).

Degradation of ^{125}I-insulin was estimated by precipitation with 5% trichloroacetic acid [23]. Degradation is indicated as percentage degraded = 100 × cpm (trichloroacetic-acid-soluble)/cpm (total).

Gel filtration of microsomal extracts

5 ml microsomal extract containing about 6 mg protein/ml and 0.5% Triton were applied to a Sepharose CL-6B column (2.7 × 120 cm) and eluted with the indicated buffer containing 0.1% Triton. Fractions of 6–8 ml each were collected. For molecular size determinations the following standard proteins were used: thyroglobulin (8.5 nm), ferritin (6.1 nm), catalase (5.2 nm) and aldolase (4.8 nm).

Photoaffinity labeling of the receptors

NεB29-mono(4-azidobenzoyl)insulin (MABI) was prepared according to Yip et al. [26] with the following modifications: 40 mg insulin was suspended in 3.2 ml dimethylformamide and 15 µl triethylamine. 0.5 ml freshly prepared solution of (4-azidobenzoyl)-*N*-hydroxysuccinimide in dimethylformamide (9.5 mg/ml) was added. After allowing the reaction to proceed for 5 h at room temperature, 6 ml chilled 1 M acetic acid was added and the pH adjusted to 5.4 with 1 M NH_3. The reaction mixture was kept at 4°C for 1 h and the precipitate recovered by centrifugation. MABI was separated from the precipitate by ion-exchange chromatography on SP-Sephadex C-25 with a gradient of 0.02–0.4 M NaCl in 1.5 M acetic acid in the presence of 6 M urea. The fractions containing MABI were pooled and dialyzed first against 0.02 M ammonium acetate buffer pH 8.0 and then against H_2O. After lyophilisation, MABI was desalted by gel filtration on a Sephadex G-50 fine column with 1 M acetic acid as elution buffer, lyophilized and stored at −20°C in the dark.

Iodination of MABI was performed in the dark as described for insulin [23]. In the radioreceptor assay ^{125}I-MABI was about 45% as potent as ^{125}I-insulin. More than 95% of the radioactivity of ^{125}I-MABI was precipitable with anti-insulin antibodies. The solubilized insulin receptors were incubated with ^{125}I-MABI at 4°C for 20 h in the dark in 0.1 M phosphate buffer pH 8.0 containing 0.06% Triton. After incubation, the tubes were placed on ice and ultraviolet-irradiated for 10 min with a Philips HPK 125-W lamp at a distance of about 15 cm. The samples were protected from the short-wave ultraviolet light by a glass plate. After photolysis, 30–50% of the specifically bound ^{125}I-MABI was covalently linked.

SDS-PAGE of the photoaffinity labeled receptors

SDS/polyacrylamide gel electrophoresis (PAGE) was carried out in slab gels according to Laemmli [27] using stacking gels of 3.5% acrylamide and separating gels with a linear pore gradient of 4–10% acrylamide. The total gel length was 230 mm. Electrophoresis was performed at 15°C with a constant current of 36 mA for 5 h. Samples were prepared in sample buffer [27] containing 5% SDS and 0.5 mM EDTA in the absence or presence of the indicated concentrations of dithioerythritol and boiled for 3 min. After electrophoresis, gels were stained with Coomassie blue [28], destained, dried and autoradiographed at −80°C on Kodak X-OMAT-AR films with Dupont Hi Plus intensifying screens. For molecular mass determinations the following standard proteins were used: spectrins I (240000) and II (220000), myosin (205000), RNA-polymerase β′ subunit (165000) and β subunit (155000), β-galactosidase (116000), phosphorylase *b* (94000), and serum albumin (68000).

RESULTS

Preparation of peaks I and II

Microsomal Triton extracts were subjected to gel filtration on Sepharose CL-6B. Depending on the buffer used for the determination of insulin binding in the eluate, two binding peaks (I and II) were obtained (Fig. 1). Buffers of high molarity and basic pH yielded both peak I and peak II, whereas buffers of low molarity and neutral pH yielded peak II only. Buffers of intermediate molarity or pH produced

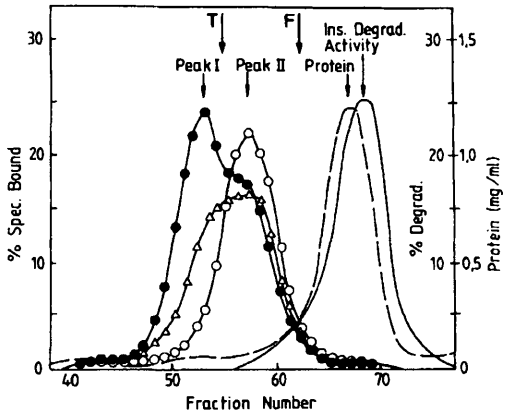

Fig. 1. *Preparation of peaks I and II.* 5 ml microsomal Triton extract (6 mg of protein/ml) was subjected to gel filtration on Sepharose CL-6B as described in Materials and Methods. Elution was performed with 0.01 M phosphate buffer (PB) pH 7.4 containing 0.1% Triton. 200-µl aliquots of the fractions were assayed for specific insulin binding with 0.1 M PB pH 8.0 (●), 0.1 M PB pH 7.4 (△), and 0.01 M PB pH 7.0 (○) as final assay buffers (see Materials and Methods). Insulin degradation (——) and protein content (– – – –) were measured as described in Materials and Methods. T, thyroglobulin; F, ferritin

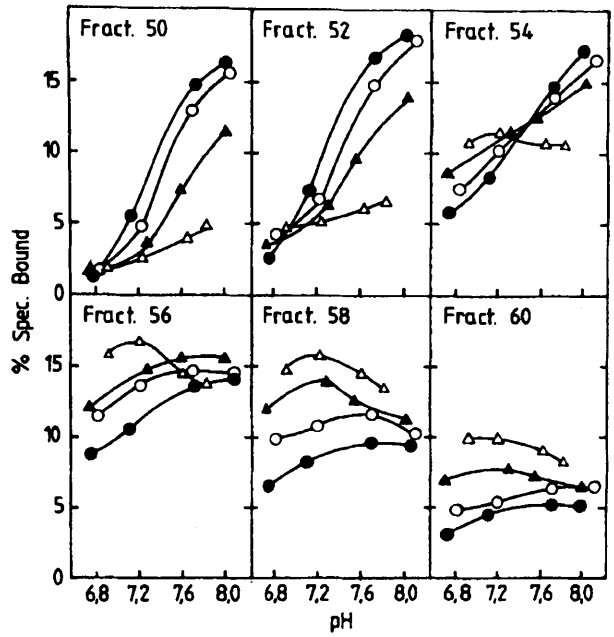

Fig. 2. *Effect of the concentration and the pH of phosphate buffer on the insulin binding of fractions from peaks I and II.* 200-µl aliquots of fractions 50, 52, 54, 56, 58, and 60 (see Fig. 1) were assayed for specific insulin binding in 0.02 M (△), 0.06 M (▲), 0.11 M (○) and 0.15 M (●) phosphate buffer at the indicated pH values (see Materials and Methods)

one mixed peak in an intermediate position. Peak I and peak II could not be separated completely from each other, but were separated from the bulk of the protein and the insulin-degrading activity present in microsomal Triton extracts. Both peaks could be rechromatographed symmetrically and without a shift of their elution volumes. Thus, during gel filtration no reversible or irreversible conversion of the peaks occurred. The same elution profiles were also obtained using 0.01 M Tris/HCl or phosphate buffers of high molarity as elution buffers. Both binding peaks could be stored without loss of activity in the lyophilized or frozen state at −30 °C.

If microsomal extracts were treated with concanavalin A (1 mg/mg of extract protein, pH 7.4), 70–80% of the insulin receptors were precipitated, whereas the bulk of the extract protein containing all of the insulin degrading activity was not precipitated and thus separated [23]. The precipitate was solubilized by buffers containing methyl-α-D-mannopyranoside. When this solution was subjected to gel filtration, peak I and peak II were found in the same quantitative proportion and with the same elution volumes shown in Fig. 1. Therefore, both peaks are glycoproteins.

Determination of the molecular sizes of peaks I and II by gel filtration on Sepharose CL-6B yielded a Stokes radius of 9.2 ± 0.2 nm for peak I and 8.0 ± 0.2 nm for peak II (mean ± SD, $n = 4$). Thus, both peaks represent very large molecular complexes. The same molecular sizes were obtained either if Sepharose CL-4B was used, or if the elution buffer contained no Triton or had a lower molarity. However, if peaks I and II were photoaffinity labeled before gel filtration, smaller Stokes radii of 8.7 ± 0.2 nm (peak I*) and 7.6 ± 0.2 nm (peak II*) were obtained. At present it is unknown whether peaks I* and II* lack a fragment or have undergone a conformational change after binding of ^{125}I-MABI and photolysis.

Dependence of the insulin binding of peaks I and II on ionic strength and pH

It is obvious from Fig. 1 that peaks I and II showed completely different insulin binding in different incubation

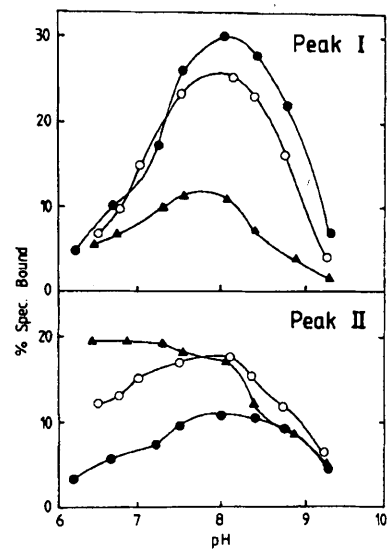

Fig. 3. *Effect of the concentration and the pH of Tris/HCl buffer on the insulin binding of peaks I and II.* Fractions from peak I and peak II (corresponding to fractions 48–53 and 57–62 in Fig. 1) were pooled separately. 200-µl aliquots of pooled peak I (above) and peak II (below) were assayed for specific insulin binding in 0.03 M Tris/HCl (▲), 0.12 M Tris/HCl (○), and 0.34 M Tris/HCl (●) at the indicated pH values (see Materials and Methods)

buffers. Therefore, fractions of peak I and peak II were examined for the dependence of insulin binding on buffer concentration and pH (Fig. 2). Fractions 50 and 52, which belong to peak I, showed minimal binding in buffers of low molarity and neutral pH, but maximal binding in high-molarity and basic pH buffers. In contrast fractions 58 and 60, which belong to peak II, exhibited optimal insulin binding at low molarity

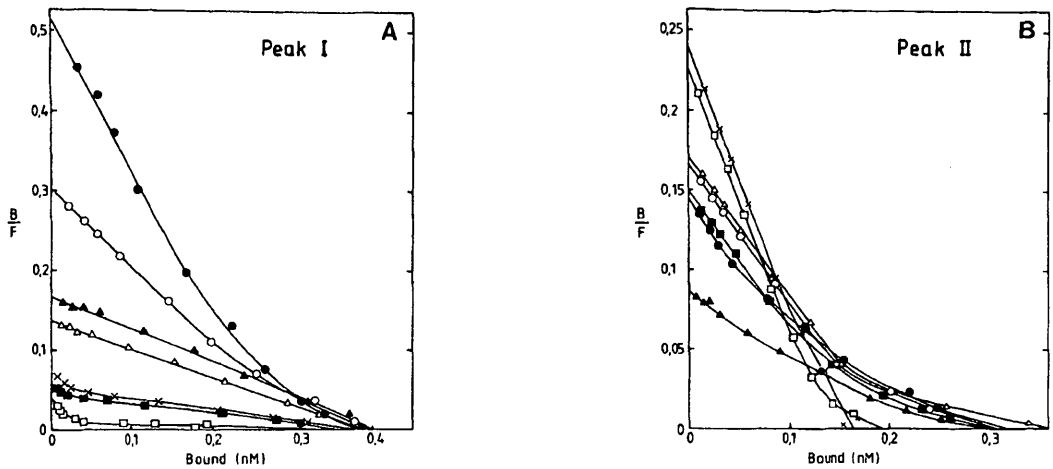

Fig. 4. *Scatchard plots of peaks I and II under different buffer conditions.* Peaks I and II were pooled as described in Fig. 3. 200-µl aliquots of pooled peak I (A) and peak II (B) were assayed for insulin binding in the following buffers in the presence of increasing concentrations of unlabeled insulin (see Materials and Methods). Binding data were plotted according to Scatchard. Buffer 1, 0.06 M phosphate buffer (PB) pH 8.0/0.2 M Na_2SO_4 (●); buffer 2, 0.1 M PB pH 8.0 (○); buffer 3, 0.06 M PB pH 7.4/0.2 M Na_2SO_4 (▲); buffer 4, 0.1 M PB pH 7.4 (△); buffer 5, 0.03 M PB pH 7.4 (×); buffer 6, 0.1 M PB pH 7.0 (■); buffer 7, 0.01 M PB pH 7.0 (□)

and neutral pH values. For these fractions, increasing the buffer molarity resulted in a decrease in insulin binding. Simultaneously the optimum pH shifted to the same basic pH values at high buffer molarity observed for peak I. Fractions 54 and 56, which contain both peaks I and II, exhibited intermediate binding characteristics.

The optimum pH was also examined in the Tris/HCl buffer system (Fig. 3). With this buffer the same dependence of insulin binding on buffer concentration and pH was observed. Rechromatography of peaks I and II also did not change this binding behavior.

The former experiments suggested that the influence of buffer concentration on insulin binding was an ionic strength effect. To verify this assumption, insulin binding was determined in a buffer system which was composed of a phosphate buffer of low concentration and increasing amounts of sodium sulfate. As expected, the increase in salt concentration had the same effect on insulin binding as the increase in buffer concentration in Figs 2 and 3 (data not shown).

Table 1. *Binding data of peaks I and II*
Peaks I and II were assayed for insulin binding in buffers 1 – 7 as described in Fig. 4. The binding data were analysed using the non-linear curve-fitting program (see Materials and Methods). K_{d1} values are indicated as means ± SD (n = 6). N_{tot} values are taken from a representative binding experiment (n = 4)

Buffer	Peak I		Peak II	
	K_{d1}	N_{tot}	K_{d1}	N_{tot}
	nM			
1	0.50 ± 0.06	0.35	1.11 ± 0.24	0.23
2	1.04 ± 0.06	0.39	1.02 ± 0.19	0.21
3	2.25 ± 0.21	0.42	2.46 ± 0.30	0.25
4	2.90 ± 0.39	0.40	1.18 ± 0.17	0.26
5	4.09 ± 0.90	0.36	0.61 ± 0.05	0.18
6	6.74 ± 0.80	0.37	1.27 ± 0.15	0.23
7	–	–	0.55 ± 0.10	0.14

Analysis of the insulin binding of peaks I and II at different conditions of ionic strength and pH

Fig. 4 shows the Scatchard plots obtained from binding studies of peaks I and II investigated under seven different buffer conditions. Most of the plots, especially those of peak II, exhibit more or less pronounced non-linearity, which varied partially for different experiments. The steep initial parts of the curves of peak I for buffers 5 – 7 were due to a small contamination by peak II. Fig. 4 shows that the different buffers led to a change in the slope of the curve, i.e. the dissociation constant, K_d. On the other hand, a significant change of the total number of binding sites, N_{tot}, was only observed for peak II with the two low-ionic-strength buffers 5 and 7.

The analysis of insulin binding by the non-linear curve-fitting program yielded data as shown in Table 1. Only the values of the dissociation constants for the high-affinity binding sites, K_{d1}, are shown, as these were highly reproducible. In contrast, the values of the dissociation constants for the low-affinity binding sites, K_{d2}, are not indicated since they could not be precisely determined. Usually K_{d2} was in the range of 5 – 15 nM, but values of 2 nM or 200 nM were also obtained.

Table 1 demonstrates that the K_{d1} values of both peak I and peak II were about 0.5 nM at their optimal buffer conditions. With buffers 2 and 3 both peaks had virtually the same binding affinity. With buffer 1, peak I bound with higher affinity than peak II, with buffers 4 – 6 the opposite was true. Under the conditions of low ionic strength and neutral pH (buffer 7) peak II showed its highest binding affinity, whereas peak I bound too poorly to be determined. Thus, the dependence of the dissociation constants of peaks I and II on ionic strength and pH was completely different. To a lesser extent this was also true for the total number of binding sites, N_{tot} (Table 1). For peak I, N_{tot} was independent of the buffer conditions used. For peak II, however, N_{tot} was diminished for buffer 5 and especially for buffer 7.

For all receptor preparations investigated so far, specific insulin binding decreases with increasing temperature. This was also true for peak I and peak II (data not shown). Further

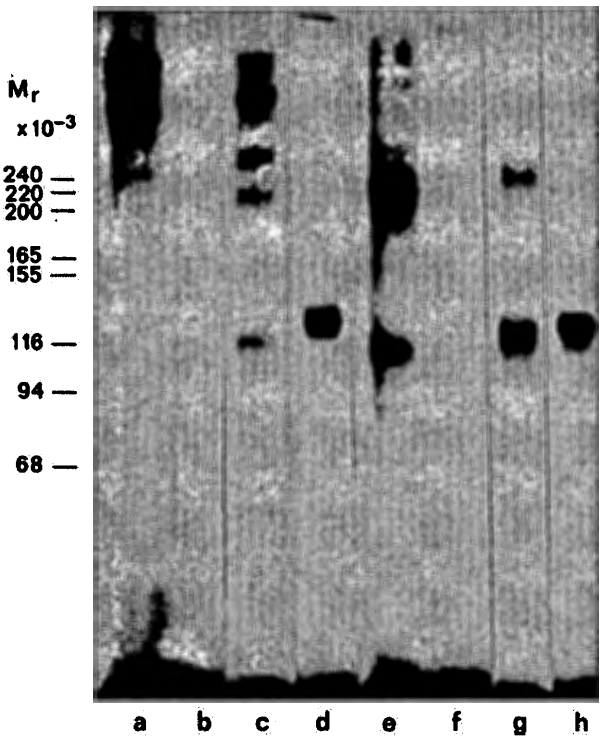

Fig. 5. *SDS-PAGE of peaks I and II.* Peaks I and II were pooled as described in Fig. 3. 200-µl aliquots of pooled peak I (lanes a–d) and peak II (lanes e–h) were photoaffinity labeled as described in Materials and Methods. Non-specific labeling was performed in the presence of native insulin (5 µM) (lanes b, f). The labeled samples were then prepared for electrophoresis in the absence (lanes a, b, e, f) or in the presence of 0.5 mM (lanes c, g) or 10 mM (lanes d, h) dithioerythritol. After SDS-PAGE, gels were stained, destained, dried and autoradiographed (see Materials and Methods)

examination of this effect revealed that for peak I the decrease in insulin binding with increasing temperature was due to a change in its binding constants, whereas for peak II, receptor denaturation seemed to play an important role.

SDS-PAGE of peaks I and II

When the photoaffinity-labeled peaks were subjected to SDS-PAGE under non-reducing conditions, peak I showed five receptor bands with M_r values of 400000, 365000, 320000, 290000 and 245000, whereas for peak II two major receptor bands of M_r 210000 and 115000 were found (Fig. 5). In addition, peak II occasionally revealed two minor bands of M_r 190000 and 160000. All of these bands were labeled specifically. The peak II receptor bands were also produced after mild reduction of peak I. After complete reduction, both peak I and peak II revealed one major receptor band of M_r 130000. In addition, peak II showed a minor band of M_r 120000, which was found to be a deglycosylation product of the M_r 130000 subunit. The M_r 130000 receptor band can be demonstrated to be identical with the M_r 115000 receptor band of the non-reduced peak II, from which it differs only by the extent of internal disulfide reduction.

Lane c in Fig. 5 shows that peak II is generated from peak I by reduction. However, this reaction takes place not only under the denaturing conditions of SDS-PAGE, but also under non-denaturing conditions. When non-labeled Triton extracts were treated for 1 h at 4 °C with dithioerythritol (2–20 mg/ml) and subjected to gel filtration, binding determination of the eluates revealed a marked increase of peak II and a corresponding decrease of peak I (data not shown). This result was confirmed by another experiment. If the sulfhydryl-blocking agent *N*-ethylmaleimide (5 mM) was present during the preparation of liver microsomes and Triton extracts to prevent reduction, the amount of peak II found after gel filtration of the extracts was considerably diminished compared to peak I.

DISCUSSION

Preparation and molecular sizes of peaks I and II

When insulin receptors are solubilized from rat liver microsomes by Triton X-100, gel filtration of the extracts reveales two insulin-binding peaks (I and II) with Stokes radii of 9.2 ± 0.2 nm and 8.0 ± 0.2 nm. Since Triton extracts from purified plasma membranes and nuclear envelopes of rat liver yielded the same two peaks [29], the possibility that the two receptor species are derived from different cell compartments can be excluded. It must be emphasized that both peaks were only obtained if the binding activity in the eluate was examined with appropriate buffers. In contrast, other groups obtained only one receptor species and a Stokes radius of about 7.2 nm for the native insulin receptor on Sepharose 6B or 4B [1–5, 9]. From the Stokes radius of 7.2 nm this receptor could be assumed to correspond to peak II. However, the receptor bands found in SDS-PAGE for this 7.2-nm receptor [3, 12, 13] and for the peaks I and II (Fig. 5) indicate that the 7.2-nm receptor is identical with peak I or with a mixture of peak I and peak II.

In contrast to the authors cited above, several other groups have reported receptor sizes which are comparable with those described here [6–8, 15]. Among these, the data of Aiyer [8] correspond quite well with our results. Investigating the insulin receptors from turkey erythrocytes, this author found two receptor species after sucrose density gradient centrifugation with Stokes radii of 8.9 ± 0.3 nm (species I) and 7.6 ± 0.3 nm (species II) as determined by Sepharose 4B chromatography. Since his experiments were done using affinity-labeled erythrocyte membranes, the data agree very well with the Stokes radii of peak I* (8.7 ± 0.2 nm) and peak II* (7.6 ± 0.2 nm) we observed.

Using SDS-PAGE, the M_r values obtained for the receptor species from peak I and peak II were 400000 and 210000 respectively (Fig. 5). These values correspond well to those of the $(\alpha\beta)_2$ and $(\alpha\beta)$ receptor forms found by others [11] and also correspond to the M_r values which were determined for the native receptor species I and II by Aiyer [8] from gel filtration and velocity sedimentation experiments.

Effect of pH and ionic strength on the binding behavior of peaks I and II

Peak I showed optimal binding at pH 8.0 and at high ionic strength, whereas peak II bound optimally at pH 7.0 and at low ionic strength. At the optimal conditions peak I and peak II bound insulin with a K_{d1} of 0.50 nM and 0.55 nM respectively (Table 1).

The Scatchard plots obtained using the seven different buffer conditions yielded predominantly non-linear curves for peak I and peak II (Fig. 4). In contrast to the Triton extracts, where non-linear Scatchard plots can be interpreted by the presence of the two distinct receptor species, peak I and

peak II, the occurrence of non-linear curves for the separated species of peak I and peak II is much more difficult to explain.

One possibility is a heterogeneity of binding sites [19, 20] since we observed different receptor forms after SDS-PAGE of peaks I and II under non-reducing conditions (Fig. 5). Furthermore, heterogeneity of the ligand must be considered [30]. This problem applies not only to the different affinities of ^{125}I-insulin and unlabeled insulin, but also to the heterogeneity of ^{125}I-insulin itself, which was iodinated by the chloramine-T method. However, if this heterogeneous ^{125}I-insulin was replaced by A14-mono-^{125}I-insulin, prepared according to Jørgensen et al. [31], non-linearity of the Scatchard plots was still evident (data not shown). Similarly the degradation of ^{125}I-insulin during the incubation [17] should play no role since peak I and peak II were free of insulin-degrading activity.

The mathematical analysis of insulin binding of peaks I and II, under the different buffer conditions described, was performed using non-linear curve fitting. Binding experiments which yielded non-linear Scatchard plots were best fitted using the two-site model, whereas for linear Scatchard plots this model provided no improvement compared to the one-site model. The dissociation constants of the high-affinity binding sites, K_{d1}, and the total number of binding sites, N_{tot}, could be determined with good accuracy and were used for the comparison of the insulin binding of peaks I and II under the seven buffer conditions examined (Table 1). For peak I, it is shown that the change of the insulin binding was caused only by a change of the affinity of the binding sites. In contrast, for peak II a change both of affinity and of the number of binding sites (for buffers 5 and 7) was responsible for the change of insulin binding.

At present it is not clear whether the change in affinity under the different conditions of pH and ionic strength is the result of a direct effect on the interactions between insulin and its receptor-binding site, or is accomplished indirectly by a conformational change of the receptor. In particular, it is unknown by which mechanism the complicated binding behavior of peak II is produced, which is in marked contrast to the rather simple dependence of peak I binding on ionic strength and pH.

Generation of peak II from peak I

Treatment of peak I with non-denaturing or denaturing detergents, or with different conditions of ionic strength or pH did not result in the generation of peak II. In addition, in contrast to the findings of others [2, 9, 10] we found no evidence that peak I was converted to peak II or another smaller receptor subunit in the presence of insulin (10 – 30 ng/ml).

The peak II receptor species could be produced from the peak I receptor only by reduction. This reaction proceeded not only under the denaturing conditions of SDS-PAGE (Fig. 5), but also under non-denaturing conditions when non-labeled Triton extracts were reduced with dithioerythritol. It could also be achieved by mild treatment of plasma membranes with reductants [32] (and A. Deger, unpublished observation). However, when the membranes were prepared in the presence of *N*-ethylmaleimide, the generation of peak II was largely inhibited. These results suggest that the reductive conversion of peak I to peak II may be both a physiological process within the intact cell membrane and an artificial process occurring during membrane and extract preparation. In addition, the formation of peak II within the membrane could be responsible for the controversial effects on insulin binding observed after treatment of membranes and cells with dithiothreitol [15, 32 – 35].

A comparison of peak I and peak II with the receptor species I and II found by Aiyer [8] shows that in both cases the smaller receptor species can be generated from the larger one (the native insulin receptor) by reduction. In addition, the receptor bands observed in SDS-PAGE for peaks I and II (Fig. 5) are largely identical with those of the receptor species I and II [14]. Together with the identity of the molecular sizes, these results indicate that the two receptor species found by our group and by Aiyer represent the same receptor species.

The appearance of smaller subunits after gel filtration of the partially reduced insulin receptor has also been reported by others [5, 9]. These subunits were able to bind insulin and exhibited Stokes radii of 3.8 nm [9] and 3.6 nm [5] respectively. Maturo et al. [9] speculated that the 3.8-nm species is identical with the ($\alpha\beta$) subunit of the insulin receptor, but this is in marked contrast to the Stokes radius of 8.0 nm found for peak II. On the other hand, Baron et al. [5] postulated that the 3.6-nm species represents the α-subunit of the receptor, but these authors obtained the same sedimentation constant (s = 6.5) as that observed for receptor species II [8]. These inconsistencies remain to be clarified.

For the discussion of the structural relationship between the peak I and peak II receptor species the following observations from SDS-PAGE (Fig. 5) are relevant. (a) The peak II receptor was generated from the peak I receptor by reduction; (b) All of the receptor bands produced by partial reduction of the peak I receptor were characteristic for the peak II receptor. After complete reduction, both peak I and peak II yielded the same band of M_r 130000, which represents the insulin-binding α-subunit of the receptor; (c) In agreement with others [8, 11] the M_r of the solubilized peak I receptor was approximately twice that of the peak II receptor. From these results we conclude that the peak I receptor is the disulfide linked dimer of the peak II receptor. This is consistent with the conclusion drawn by Aiyer [8] from his studies on turkey erythrocytes and also corresponds to the relationship between the $(\alpha\beta)_2$ and ($\alpha\beta$) receptor forms found by Czech et al. [11]. The physiological significance, however, of the conversion of the peak I receptor to the peak II receptor and the possible role of the peak II receptor in insulin action or receptor metabolism remains to be elucidated.

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