Insulin Binding to Rat Liver Membranes Predicts a Homogeneous Class of Binding Sites in Different Affinity States That May Be Related to a Regulator of Insulin Binding†

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ABSTRACT: Insulin binding to pericanicular, liver plasma membranes was measured at equilibrium as a function of temperature from 4 °C to 37 °C. Scatchard plots of the binding data obtained at temperatures from 4 °C to 15 °C were linear and the Hill plots were characterized by Hill coefficients equal to unity. Thus, insulin binding under these conditions was consistent with the presence of a single class of homogeneous, noninteracting binding sites. However, the Scatchard plots of binding data obtained above 15 °C were curvilinear, and the Hill coefficients derived from these data were about 0.75. This apparent change in the complexity of the binding with increasing temperature was not due to gross ligand or receptor degradation and care was taken to ensure that all assumptions inherent in interpreting the equilibrium binding data were valid. Changes in membrane fluidity or the presence of a cryptic population of receptors which surface with increasing temperature also could not account for this apparent increase in the complexity of the binding above 15 °C because identical observations were made using nonionic and ionic detergent-solubilized liver plasma membranes. Thus, we were able to rule out heterogeneity of binding sites as a model to explain the increased complexities of the binding above 15 °C. We conclude that the temperature dependence of insulin binding in impure but intact receptor preparations is consistent with a two-state model of the insulin receptor. Using this model, we predict that one conformational state of the insulin receptor exists below 15 °C but that two affinity states of the receptor exist at higher and physiological temperature. Our results are consistent with two states of the receptor resulting from the temperature-dependent interaction of the receptor with an affinity regulator of insulin binding.

The first event in insulin action is the binding of insulin to its specific plasma membrane receptor. How this insulin receptor transduces insulin action into a cell is still unclear, although recent studies of the structure and function of the insulin receptor are beginning to shed some light on this area. We now know that the insulin receptor is a specific transmembrane glycoprotein of about 450 kDa (Helmerhorst et al., 1986). It is minimally composed of two 130-kDa α subunits and two 90-kDa β subunits in a disulfide-linked complex. The complete amino acid sequences of the α and β subunits of the human insulin receptor have recently been deduced from a human placental cDNA clone (Ullrich et al., 1985).

It is now well established that insulin binds to the α subunit (Yip et al., 1978) and that this interaction activates the β subunit which is an insulin-responsive tyrosine kinase (Avruch et al., 1982). Domains implicated in insulin binding variably have been located within the cysteine-rich region and at both the N-terminal and C-terminal regions of the insulin receptor (Demeyts et al., 1990; Gustafson & Rutter, 1990; Waugh et al., 1989; Wedekind et al., 1989; Zhang & Roth, 1991). The tyrosine kinase activity of the insulin receptor is believed to be important for the transmission of the signal of insulin into the cell. The mechanism of signal transduction from the occupied insulin binding subunit to the tyrosine kinase subunit is unknown but probably involves conformational change (Chiacchia, 1988; Gammeltoft et al., 1978; Ginsberg et al., 1978; Pilch & Czech, 1980; Wilden et al., 1986). The nature of these conformational changes awaits the elucidation of the three-dimensional structure of the insulin receptor and a better understanding of the complex kinetics of insulin binding.

The kinetics of insulin binding to its receptor are described in a huge volume of literature published over the last 20 years. However, no consensus has been reached over a model that describes the kinetics of insulin binding to its receptor at equilibrium. Some investigators have concluded that the insulin receptor population is characterized by a single class of homogeneous, noninteracting binding sites, whereas others have concluded that there are multiple classes of binding sites. On the other hand, several investigators have concluded that there is negative cooperativity between insulin receptors [reviewed in Gammeltoft (1984)].

Several technical difficulties have impeded our understanding of the kinetics of insulin binding to its receptor. Until recently, one obstacle has been the nonavailability of a radioactive insulin analog with a biological potency indistinguishable from that of native insulin. This requirement is critical because the mathematical models normally used to interpret equilibrium binding data assume that the labeled and unlabeled ligand behave identically. Yet, the majority of insulin binding studies have been performed using heterogeneous ^{125}I-labeled insulin preparations with biological potencies significantly different from that of native insulin (Gammeltoft, 1984). Consequently, most of the published insulin binding data cannot be analyzed quantitatively. Several other problems also add to the complexity of interpreting the insulin binding data from different studies. For example, many

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studies do not account for the binding of insulin degradation products. There is evidence to suggest that this may lead to a curvilinear bias of the Scatchard plots (Davidson & Venkatesan, 1982; Donner, 1980a), leading to an incorrect interpretation of heterogeneity of receptors or the presence of cooperative interactions. In addition, receptor degradation is infrequently monitored in insulin binding studies. Another common problem encountered in many studies is the inappropriate correction of binding data for the nonspecific binding of insulin (Mendel et al., 1985). Furthermore, the binding parameters are often incorrectly derived from the Scatchard plots of the data (Mendel et al., 1985). Moreover, insulin binding studies have been performed in a large number of different tissues and under widely varying conditions of pH, temperature, and ionic strength.

In this study, we have measured insulin binding to particulate and detergent-solubilized liver plasma membranes as a function of temperature. We discuss our findings considering the different models describing the kinetics of insulin binding.

EXPERIMENTAL PROCEDURES

Materials. Bovine serum albumin, bacitracin, *N*-ethylmaleimide, human γ-globulin, and poly(ethylene glycol) 6000 were purchased from Sigma Chemical Co. (St. Louis, MO). [[^{125}I]Tyr^{A14}]Iodoinsulin was purchased from Amersham. Radioiodinated $N^{\epsilon B29}$-(monoazidobenzoyl)insulin was prepared as previously described (Yip et al., 1980). All other chemicals were of reagent grade.

Preparation of Liver Plasma Membranes. Plasma membranes were prepared from the livers of 175–200-g male Wistar rats according to the method of Ray (1970). The 5′-nucleotidase activity of these membranes was enhanced about 30-fold in comparison to the crude liver homogenates. Electron microscopy of the plasma membrane preparations demonstrated the presence of large sheets of membranes connected by various junctional complexes. The membranes were free from any recognizable organelles.

Solubilization of Liver Plasma Membranes. Plasma membranes (10 mg of protein/mL) were incubated for 30 min at 25 °C in 50 mM Tris-HCl, pH 7.5, containing 0.1% bovine serum albumin, 100 units/mL bacitracin and either 1% Triton X-100, 1% Tergitol NP40, or 0.6% CHAPS. These preparations were then centrifuged at 178000*g* for 1 h at 4 °C. The supernatants were removed and diluted 10-fold with 50 mM Tris-HCl containing 0.1% bovine serum albumin, pH 7.5, and 100 units/mL bacitracin. These solubilized membrane preparations were stored at 4 °C and used within 16 h of preparation.

Insulin Binding to Plasma Membranes. Plasma membranes (12.5–50 μg of protein) were incubated in 0.2 mL of 50 mM Tris-HCl, pH 7.5, containing 0.1% bovine serum albumin, 100 units/mL bacitracin, 8 fmol of [[^{125}I]Tyr^{A14}]insulin and a millimolar to picomolar range of unlabeled insulin. Since the p*K* of the Tris-HCl buffer changes as a function of temperature (from a p*K* of 8.66 at 5 °C to a p*K* of 7.81 at 35 °C), a specified amount of sodium chloride (0, 3, 6, 7, 8, 10, 13, and 18 mM at 4, 10, 15, 17, 20, 25, 30, and 37 °C, respectively) was added to the incubation mixture to maintain a constant ionic strenth as a function of temperature at pH 7.5. When detergent-solubilized membranes were used, the final concentration of detergent in the incubation was 0.05%.

Separation of the Bound and Free Insulin. Insulin bound to the plasma membranes was separated from the unbound insulin by one of three methods. (1) Filtration: 175 μL of a membrane suspension (see preceding paragraph) was collected on a Whatmann GF/C glass fiber filter (2-cm diameter) under vacuum. The filter was washed with 2 mL of ice-cold 50 mM Tris-HCl, pH 7.5, containing 0.1% bovine serum albumin immediately before and immediately after collection of the plasma membranes. The filter then was removed and its radioactivity was determined. (2) Centrifugation: plasma membranes were pelleted by centrifugation at 15000*g*, 4 °C, in microfuge tubes. The supernatant was carefully aspirated and the tip of the microfuge tube containing the plasma membrane pellet was cut off and its radioactivity was determined. (3) Poly(ethylene glycol) precipitation: 50 μL of 0.4% bovine γ-globulin and 250 μL of 20% poly(ethylene glycol) in 50 mM Tris-HCl, pH 7.5, were added to the 0.2-mL incubation mixture (see preceding paragraph) and vigorously vortex-mixed. The suspension was kept on ice for 15 min and then centrifuged at 8500*g*, 4 °C, for 15 min. The supernatant was aspirated, and the tip of the microfuge tube containing the membrane–protein pellet was cut off and its radioactivity was determined.

Photoaffinity Labeling of Insulin Receptors. Plasma membranes (50 μg of protein) were incubated for 2 h at 4 °C in the dark in 0.2 mL of 50 mM Tris-HCl, pH 7.5, containing 0.1% bovine serum albumin, 100 units/mL bacitracin, and 50 nM radioiodinated $N^{\epsilon B29}$-(monoazidobenzoyl)insulin in the presence or absence of 2.5 μM native insulin. Following this incubation the solution was photolysed for 30 s with a focused light source from a 100-W high-pressure mercury lamp. The photolabeled plasma membranes were pelleted by centrifugation at 10000*g*, 4 °C for 15 min. The pellet was washed with 50 mM Tris-HCl, pH 7.5, containing 0.1% bovine serum albumin and 100 units/mL bacitracin. The pellet was then resuspended in 0.2 mL of this buffer and incubated for an additional 16 h at 4 °C, 2 h at 25 °C, or 45 min at 37 °C. Following this incubation, the pellet was resuspended and then boiled for 5 min in 62.5 mM Tris-HCl, pH 6.8, containing 3% sodium dodecyl sulfate, 10% glycerol, and either 100 mM dithiothreitol (reducing conditions) or 10 mM *N*-ethylmaleimide (nonreducing conditions). Electrophoresis of the solubilized proteins was performed in 3–10% gradient polyacrylamide gels.

Analysis of Binding Data. Binding data were analyzed by the method of Scatchard (1949) using the Ligand program (Munson & Rodbard, 1980). The data were fitted with either a one-site model or a two-site model. The goodness of fit of a model was analyzed with the Ligand program using the Runs test (Bennett & Franklin, 1954). This test predicts whether the scatter of points about a fit is likely due to chance, and therefore, whether a given model provides a significant fit to the data. Whether or not a two-site model provided a statistically better fit over a one-site model was tested with the Ligand program using an F-test criterion on the residual variances of the two models. Statistical comparisons between curves within a given model were also tested using the Ligand program. Nonspecific binding was handled as a computer-fitted parameter, and all points within an analysis were weighted equally. The binding data also were analyzed using Hill plots (Hill, 1910).

RESULTS

Time Course of Insulin Binding. Insulin binding to purified liver plasma membranes was monitored as a function of time at different temperatures. The results of some typical time courses of insulin binding are illustrated in Figure 1. Insulin binding increased until equilibrium was reached. The equilibrium reached was stable at all temperatures from 4 °C to 37 °C when bacitracin was included to inhibit insulin degradation. Similar time courses of binding were obtained

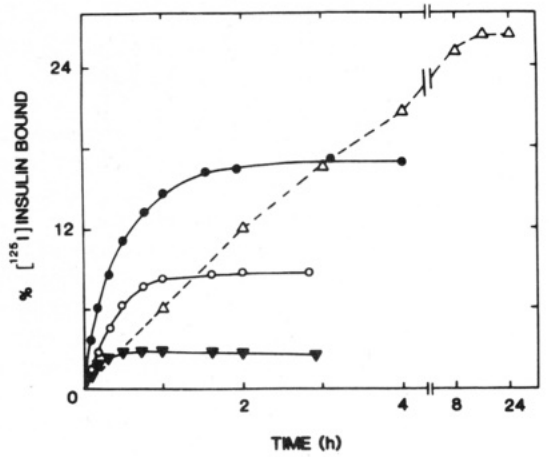


FIGURE 1: Time courses of specific insulin binding. Insulin binding to liver plasma membranes was determined as function of time at 4 (△), 15 (●), 25 (○), and 37 °C (▼) according to the procedures detailed in Experimental Procedures. Specific binding was calculated from the difference between total and nonspecific binding. Total and nonspecific binding were determined from the binding of 8 fmol of [[^{125}I]Tyr^{A14}]insulin in the absence and presence of 1 μM native insulin, respectively.

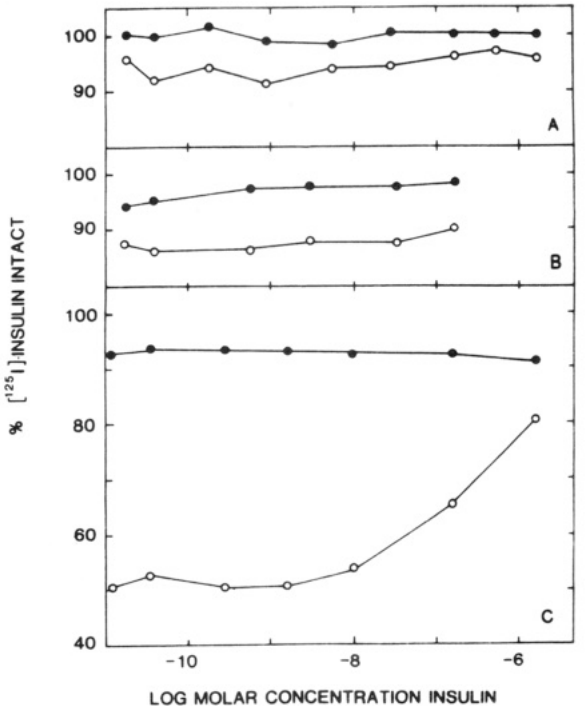


FIGURE 2: Integrity of insulin during binding to liver plasma membranes: Insulin degradation was estimated following a 45-min incubation at 37 °C by measuring the precipitability of 8 fmol of [[^{125}I]Tyr^{A14}]insulin in 5% trichloroacetic acid. The integity of receptor bound (●) or unbound (○) [[^{125}I]Tyr^{A14}]insulin was determined as a function of total insulin concentration in the presence of bacitracin (A) or *N*-ethylmaleimide (B) or in the absence (C) of an inhibitor of insulin degradation.

over a wide range of membrane concentration. In addition, the time taken to reach equilibrium was not dependent on the insulin concentration. Furthermore, the equilibrium was reached in a path-independent manner. Thus, if equilibrium was reached at 37 °C and then the temperature of the incubation was decreased to 4 °C, then the new equilibrium reached was identical to the 4 °C incubation alone. In subsequent binding studies, equilibrium was taken to be reached following 16 h at 4 °C, 6 h at 10 °C, 4 h at 15 °C, 3 h at 17 °C, 2.5 h at 20 °C, 2 h at 25 °C, 1 h at 30 °C, and 45 min at 37 °C.

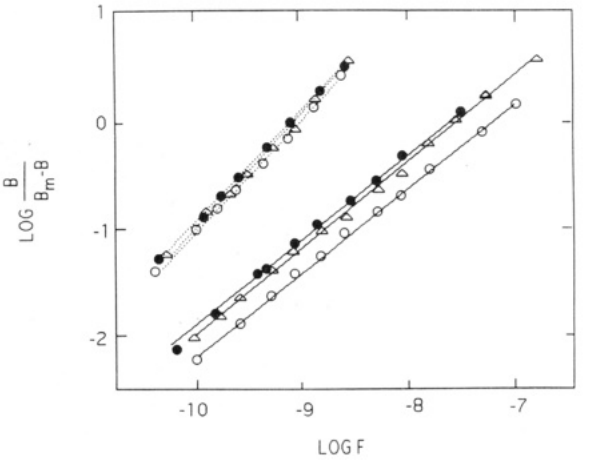


FIGURE 3: Evaluation of the effect of bacitracin on the equilibrium binding of insulin to receptors using the Hill plot. Equilibrium [[^{125}I]-Tyr^{A14}]insulin binding to liver plasma membranes was measured as a function of the total insulin concentration at 4 °C (dotted lines) or 37 °C (solid lines) in the presence (●) or absence (○) of bacitracin. Data collected in the absence of bacitracin also were plotted following correction for trichloroacetic acid precipitable insulin degradation products (△).

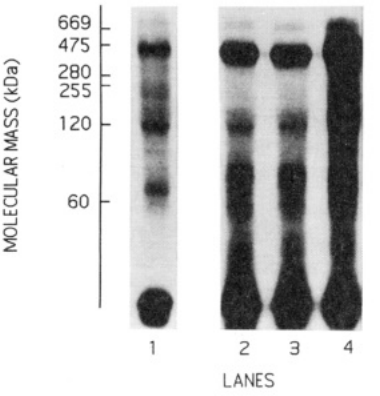


FIGURE 4: Integrity of insulin receptors in liver plasma membranes. Liver membranes were photoaffinity labeled with radioiodinated $N^{\alpha B29}$-(monoazidobenzoyl)insulin in the presence (lane 1) or absence (lanes 2–4) of an excess of native insulin. The membranes then were incubated for 16 h at 4 °C (lane 2), 2 h at 25 °C (lane 3), or 45 min at 37 °C (lane 4) before the labeled proteins were resolved on a 3–10% gradient polyacrylamide gel.

Degradation of Insulin. Insulin degradation was estimated following a 45-min incubation at 37 °C by measuring the precipitability of ^{125}I-insulin in 5% trichloroacetic acid. The receptor-bound insulin was greater than 90% precipitable at insulin concentrations in the range of 1 mM to 10 pM (Figure 2). By contrast, the unbound insulin was less than 60% precipitable below an insulin concentration of 10 mM. The inclusion of either bacitracin or *N*-ethylmaleimide in the binding buffer markedly decreased the degradation of unbound insulin (Figure 2). Bacitracin was most effective with 100% of bound insulin and more than 95% of the unbound insulin being precipitable at all insulin concentrations. The effect of bacitracin on the interpretation of the insulin binding data derived at either 4 °C or 37 °C is illustrated by Hill plot analyses in Figure 3. At 4 °C there was little apparent difference between the Hill coefficients (estimated from the slopes of the lines) or the averaged association constants (estimated from F when log $[B/Bm - B] = 0$) in the presence or absence of bacitracin (Figure 3) or *N*-ethylmaleimide (data not shown). At 37 °C there was little difference between the Hill coefficients but the average association constant decreased in the presence of bacitracin or *N*-ethylmaleimide. However, the average association constant of binding data which was

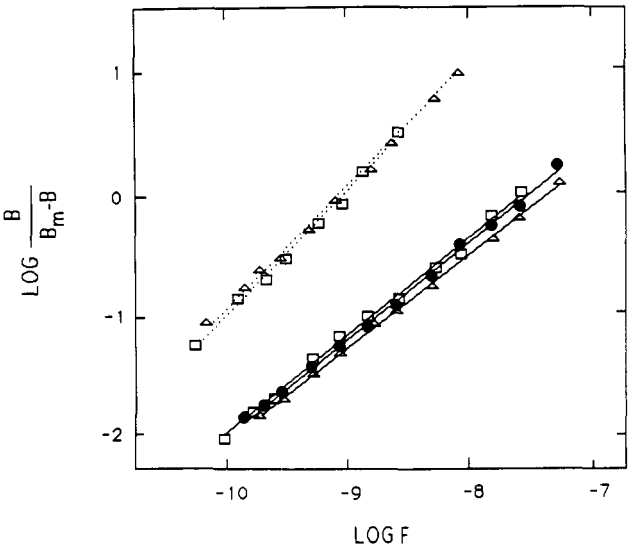


FIGURE 5: Evaluation of different methods for separating bound and unbound ligand on the equilibrium binding of insulin to receptors using the Hill plot. Equilibrium [[^{125}I]Tyr^{A14}]insulin binding to liver plasma membranes was measured as a function of the total insulin concentration at 4 °C (dotted lines) or 37 °C (solid lines) using poly(ethylene glycol) precipitation (□), centrifugation (●), or filtration methods (△) to separate plasma membrane bound and unbound ligand.

derived in the absence of bacitracin or *N*-ethylmaleimide but corrected for insulin degradation (see Experimental Procedures) approached the data derived in the presence of bacitracin or *N*-ethylmaleimide. Moreover, the inclusion of either bacitracin or *N*-ethylmaleimide in the binding buffer did not alter the obvious temperature dependent difference in the Hill coefficients. In light of these results, we selected bacitracin as the choice inhibitor of insulin degradation and subsequently included it in all binding studies.

Degradation of the Insulin Receptor. The effect of the incubation conditions on the degradation of insulin receptors was estimated following the procedure outlined by Kahn et al. (1974). Liver plasma membranes were preincubated for 16 h at 4 °C or for 45 min at 37 °C in binding buffer containing a range of 1 mM to 10 pM insulin concentrations. ^{125}I-Insulin was then added to each tube, and the incubations were continued for an additional 16 h at 4 °C or for 45 min at 37 °C when equilibrium was achieved. A comparison between insulin bound by these membranes and membranes that had not undergone the preincubation demonstrated that less than 2% and 5% of the insulin receptors were degraded during the preincubation at 4 °C and 37 °C, respectively (data not shown).

The integrity of the insulin receptors also was monitored by examining the structure of the insulin receptors in particulate and solubilized (data not shown) liver membranes that were photoaffinity labeled with radioiodinated $N^{\epsilon B29}$-(monoazidobenzoyl)insulin and then incubated for various times at a range of temperatures (Figure 4). A 450-kDa protein was specifically labeled (it was the predominant band displaced by a 50-fold excess of native insulin), and the appearance of this band was largely independent of the time and temperature of incubation. A slight increase in degradation of the 450-kDa band was observed as the temperature was raised from 25 °C to 37 °C. Under reducing conditions each preparation migrated on polyacrylamide gel as an intensely labeled band at 130 kDa and a weakly labeled band of 90 kDa, and the appearance of these two specifically-labeled bands was also independent of the time and temperature of the incubation (data not shown).

Separation of Bound and Free Insulin. Hill plot analyses of the binding data derived at 4 °C or 37 °C using either

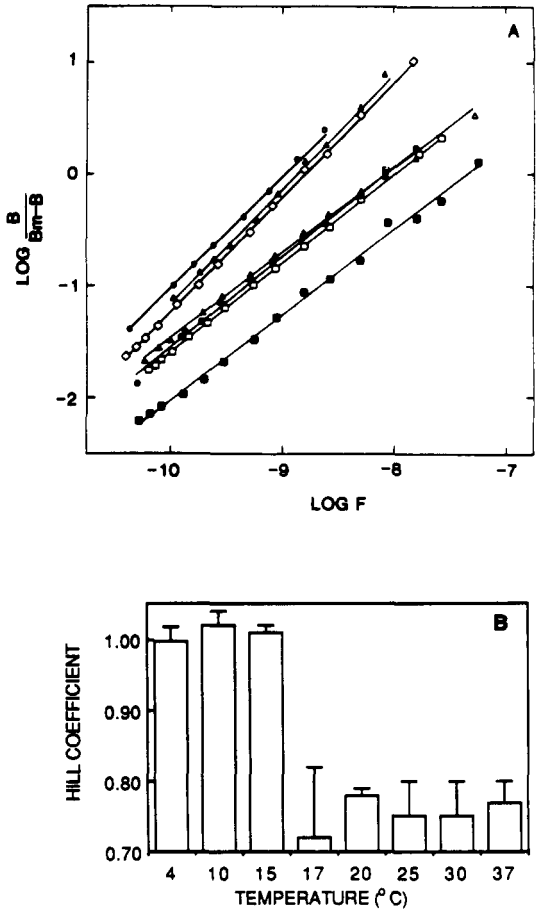


FIGURE 6: Hill plot analysis of the equilibrium binding of insulin to receptors as a function of temperature. (A) Equilibrium [[^{125}I]Tyr^{A14}]-insulin binding to liver plasma membranes was measured as a function of the total insulin concentration and temperature of binding. Typical Hill plots derived at 4 (○), 10 (▲), 15 (◇), 20 (●), 25 (△), 30 (□), and 37 °C (■) are shown. (B) The average Hill coefficients ± the standard deviations from three experiments are plotted as a function of temperature.

centrifugation, poly(ethylene glycol) precipitation, or filtration methods to separate the membrane bound and unbound insulin (see Experimental Procedures for details) are illustrated in Figure 5. The coordinates of the Hill plots at either 4 °C or 37 °C were virtually identical irrespective of the separation method used to generate the binding data. Moreover, a temperature-dependent difference in the Hill coefficients was observed irrespective of the method used for separating the bound and free insulin.

Receptor Concentration and Insulin Binding. Insulin binding was monitored at 4 °C and 20 °C with increasing concentrations of plasma membranes (62.5 μg of protein/mL–300 μg of protein/mL; data not shown). The insulin receptor concentrations which were determined from Scatchard plots of the binding data increased proportionately with increasing plasma membrane concentrations. The Hill coefficients and average affinity constants determined from Hill plots of the binding data were independent of plasma membrane concentration.

Insulin Binding at Different Temperatures. Hill plots and Scatchard plots of insulin binding data which were derived at temperatures from 4 °C to 37 °C are illustrated in Figure 6 and Figure 7, respectively. Hill plot analyses of the binding data demonstrated a marked temperature-dependent change between 15 °C and 17 °C (Figure 6). Indeed, the Hill coefficient was not significantly different from unity between 4 °C and 15 °C ($P < 0.001$) but was decreased to about 0.75 from 17 °C to 37 °C. The Scatchard plots of the data were

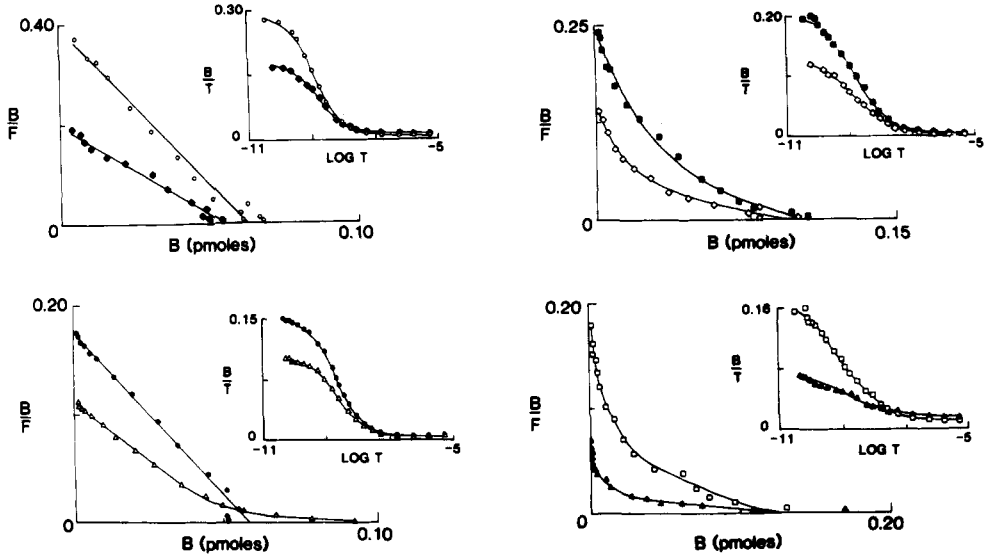


FIGURE 7: Scatchard analysis of the equilibrium binding of insulin to receptors as a function of temperature. Equilibrium [[^{125}I]Tyr^{A14}]insulin binding to liver plasma membranes was measured as a function of the total insulin concentration and temperature of binding. Typical Scatchard plots derived at 4 (○), 10 (◆), 15 (●), 17 (△), 20 (■), 25 (◇), 30 (□), and 37 °C (△) are shown. The insets illustrate the associated competitive dispacement data.

fitted assuming the presence of either single or multiple classes of binding sites. The Scatchard plots were linear from 4 °C to 15 °C in accord with a model assuming a single class of binding sites. However, above 15 °C a model assuming a single class of binding sites did not provide an adequate fit to the data. These data were well fitted by a model assuming two classes of independent binding sites.

Insulin Binding to Solubilized Insulin Receptors at Different Temperatures. Liver plasma membranes were solubilized in Triton X-100, CHAPS, or Tergitol NP40. Insulin binding to the detergent-solubilized membranes at 4 °C and 25 °C were analyzed with Hill plots (Figure 8) and Scatchard plots (not shown). The Hill plots illustrate a marked temperature dependence of the insulin binding in each detergent-solubilized membrane preparation. In particular, the average affinity constants and the Hill coefficients were decreased at 25 °C. A one-site model provided a good fit to the Scatchard plots of the binding data derived from the detergent-solubilized membranes at 4 °C. However, a two-site model was required to provide a significant fit to the Scatchard plots of the data derived from the detergent-solubilized receptors at 25 °C.

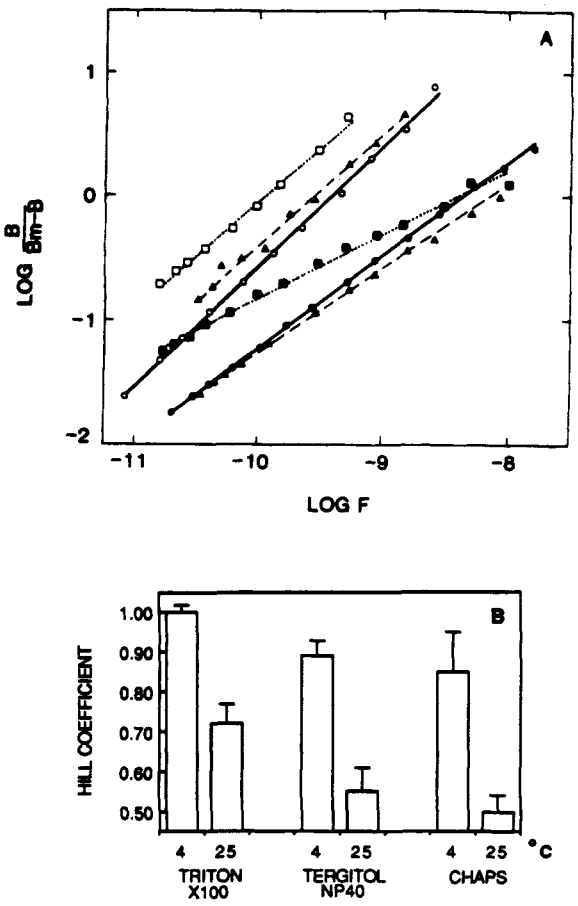


FIGURE 8: Evaluating the equilibrium binding of insulin to detergent-solubilized receptors using the Hill plot. (A) Equilibrium [[^{125}I]-Tyr^{A14}]insulin binding to liver plasma membranes solubilized in 1% Triton X-100 (○, ●), 1% Tergitol NP40 (△, ▲) or 0.6% CHAPS (□, ■) was measured as a function of the total insulin concentration at 4 °C (open symbols) or 25 °C (closed symbols). (B) The average Hill coefficients ● the standard deviations from three experiments are plotted as a function of temperature.

DISCUSSION

Several assumptions are implicit in the analysis of equilibrium binding data. One of the principal assumptions is that the binding is measured at a true thermodynamic equilibrium. In this study, a steady-state equilibrium was achieved that was stable for several hours at each temperature from 4 °C to 37 °C (Figure 1). The equilibria were not perturbed by insulin degradation (Figure 2) when either bacitracin or *N*-ethylmaleimide were included in the binding buffer. The inclusion of these inhibitors of insulin degradation did not bias the trends observed in this study (Figure 3). Furthermore, the receptor preparation remained structurally (Figure 4) and functionally [assessed using the rebinding studies described by Kahn et al. (1974)] intact irrespective of the time and temperature of the incubation. The binding equilibrium was not perturbed during the separation of the bound and free insulin since the results obtained using three different methods for separating the bound and free insulin were indistinguishable (Figure 5). Furthermore, one of us has previously demonstrated that the insulin–receptor interaction is fully reversible under the conditions of this study (Helmerhorst, 1987). Thus, we are confident that the steady-state equilibrium achieved at each temperature in this study was indeed representative of true thermodynamic equilibria. Another important assumption is that the labeled and

unlabeled ligands behave identically. Insulin monodiodinated exclusively on its A14-tyrosine residue was used in this study since this derivative has been shown to have binding properties and biological potencies indistinguishable from native insulin (Hansen et al., 1980).

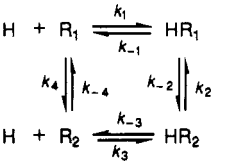
Insulin binding to liver plasma membranes was markedly dependent on temperature (Figures 6 and 7). Without exception, the Scatchard plots of insulin binding at temperatures below 17 °C were linear, and the Hill coefficients derived from the Hill plots of the binding data were not significantly different from unity. These observations demonstrate that insulin was interacting with a homogeneous class of receptors at temperatures below 17 °C. In contrast, however, the Scatchard plots of the binding data at temperatures above 15 °C were always curvilinear and the Hill coefficients derived from the Hill plots of this data were significantly ($P < 0.01$) less than unity. These observations demonstrate that above 15 °C the interaction between insulin and its receptor was more complex than observed at lower temperature.

Consistent with the findings in this study, Waelbroeck et al. (1979) found that the steady-state binding of insulin to its receptor on human lymphocytes of the IM-9 cell line was markedly dependent on temperature. Moreover, they also noted a change in the trend of their data at about 20 °C. They attributed this change to the nonclassical thermodynamic behavior of the insulin–receptor interaction and suggested that this probably resulted from the loss of nonpolar surface residues in the formation of the insulin–receptor complex. In contrast to our findings with rat liver plasma membranes, these investigators qualitatively noted that the Scatchard plots of their binding data were curvilinear at all temperatures. This may reflect the different insulin receptor soruces being used. Alternatively, some artifactual curvilinearity may have been introduced with the use of a heterogeneous ^{125}I-labeled insulin preparation in their study. That critical changes in the nature of insulin binding to its receptor occur at about 20 °C is also supported by the studies of Dwenger and Zick (1984). They found that insulin binding to human erythrocytes exhibits a regular thermodynamic behavior between 0 °C and 22 °C but an irregular thermodynamic behavior between 22 °C and 37 °C. This irregular thermodynamic behavior above 22 °C could only be partially explained by insulin degradation and the binding of insulin degradation products.

The apparent increase in the complexity of the binding which we observed above 15 °C cannot be ascribed to changes in membrane fluidity or to the presence of an independent population of cryptic insulin receptors which surface with increasing temperature because identical observations were made using particulate plasma membranes and nonionic and zwitterionic detergent-solubilized plasma membranes (Figure 8). Thus, we cannot reconcile the binding data in this study by reference to the presence of a heterogeneous population of insulin receptors. Rather, our data demonstrate that insulin interacts with a single class of homogeneous binding sites in liver plasma membranes.

Several investigators have suggested that the insulin–insulin receptor interaction can be best described by a model assuming negative cooperative interactions (De Meyts, 1976; De Meyts et al., 1973, 1976). Experiments have been performed to support this model which demonstrate that the dissociation rate of receptor bound ^{125}I-labeled insulin is enhanced when the dissociation is induced by dilution in the presence of free native insulin. However, one of us has recently demonstrated that it is invalid to infer negative-cooperative interactions among insulin receptors on the basis of these ligand dissociation experiments (Helmerhorst, 1987). Several other investigators also have challenged the interpretation of the ligand dissociation experiments when used to infer cooperativity among receptors (Gammeltoft, 1984; Pollet et al., 1977; Sandvig et al., 1976; Verrier et al., 1974). It is also interesting to note that accelerated dissociation of ^{125}I-labeled insulin in the presence of an excess of unlabeled insulin has been demonstrated for biologically inert substances such as talc (Cuatrecasas & Hollenburg, 1975). Levitzki (1984) suggests that a possible explanation of the accelerated dissociation of ^{125}I-labeled insulin in the presence of an excess of unlabeled insulin is that "two ligand molecules can occupy, however briefly, the same binding site, especially if it is an extended one"—as he suggests is the case with insulin which has many attachment sites to the receptor.

Corin and Donner (1982) proposed a two-state model for the insulin receptor to explain the biphasic kinetics observed when insulin dissociates from its receptor. Our data can be explained using this model illustrated below. We propose



that the insulin receptor exists in predominantly one state ($R_1 > R_2$) below 17 °C. This would be the case if $k_4 < k_{-4}$. We propose that as the equilibrium binding temperature is raised, k_4 and k_{-4} become similar and therefore the two receptor states (R_1 and R_2) are observed. Curvilinear Scatchard plots characteristic of a heterogeneous population of receptors then would be expected provided k_2/k_{-2} are not fast events relative to k_1/k_{-1}, k_3/k_{-3}, and k_4/k_{-4}. In other words, it may be equally or more rapid for HR_1 to dissociate to H + R_1, followed by the conversion of R_1 to the R_2 form which can then associate with H to form HR_2. Lipkin et al. (1986) could find no evidence that insulin (H) could directly dissociate from HR_2 or associate with R_2. However, their studies were performed with fat cells at 15 °C. As proposed above, this temperature probably did not favor the formation of R_2 from R_1.

There is strong support for the above two-state model and the findings in this study. Krupp and Livingston (1979, 1980) demonstrated that insulin binding in rat adipocytes and liver plasma membranes could be chromatographically separated into two interconvertible affinity states. Apparently, this interconversion between affinity states of the receptor could by mediated by insulin. In addition, Mortensen et al. (1992) noted that mild reduction of adipocyte plasma membranes with dithiothreitol, before insulin binding, increases the fraction of hormone binding with high affinity without significantly changing the total number of receptor-binding sites. In the presence of guanosine triphosphate (GTP), the amount of receptor with high affinity for insulin in the reduced membranes decreases to that present in the absence of reduction; the effect of the nucleotide is concentration- and temperature-dependent (Mortensen et al., 1992). The effect of temperature on the inhibition of insulin binding by GTP is marked. At 4 °C there is no effect, but above 20 °C the effect is dramatic. These data imply that G-proteins modulate insulin binding and are consistent with our observations on the temperature dependence of insulin binding.

The association of an affinity regulator of insulin binding with the insulin receptor has been implicated in many studies. Harmon et al. (1980, 1981, 1983) used the technique of radiation inactivation to demonstrate that the insulin receptor in rat liver membranes is composed of at least two functional

components: an insulin binding component and a component which acts as an affinity regulator. Kohanski and Lane (1985) concluded that a peripheral membrane glycoprotein which modulates the affinity of the adipose insulin receptor is removed during receptor purification on insulin agarose, and we have made similar observations using human placental membranes (data not shown). Other studies also support the presence of an affinity regulator of insulin binding (Baldini et al., 1991; Ciaraldi & Maisel, 1989; Davies & McDonald, 1990). Furthermore, interconvertible affinity states have been reported for many other receptor systems (Donner, 1980b; Donner et al., 1978; Lad et al., 1977; Landreth et al., 1980; Ross et al., 1977; Williams et al., 1977).

We conclude that the insulin–receptor interaction is complex but that there is a single class of homogeneous, noninteracting insulin receptors in liver plasma membranes. These insulin receptors undergo temperature-dependent change with only one receptor state predominating below 17 °C but with two receptor states existing at physiological temperature. The two states of the receptor may reflect conformational transitions resulting from the interaction of the receptor with an affinity regulator of insulin binding.

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