Insulin binding to its cellular receptors is markedly dependent on the temperature. The thermodynamic parameters for the reaction of insulin with the high affinity state of its receptor have been evaluated from equilibrium studies at multiple temperatures between 5°C and 37°C. The thermodynamics of the insulin-receptor interaction is not classical. The van't Hoff plot is not linear. Both the enthalpy and entropy changes, due to the formation of the hormone-receptor complex, decrease markedly with temperature, corresponding to a large heat capacity change of $-766 \text{ cal/(mol deg)}$ at 25°C. The reaction is endothermic and entropically driven at low temperature and exothermic and enthalpically driven at higher temperature. This thermodynamic behavior is suggestive of a hydrophobic reaction and supports Blundell's concept that the loss of nonpolar surface residues in the formation of the hormone-receptor complex is an important driving force for the reaction. Alternatively, this nonclassical behavior may indicate that the reaction of insulin with its receptor involves more than one step.

An increasing body of information has been gathered over the last few years on the nature of the reaction of insulin with its receptors in the membrane of target cells (1-5). In particular, parallel studies of x-ray diffraction, circular dichroism, biological potency, and receptor binding of a variety of native and chemically modified insulins have allowed the mapping of the presumed receptor-binding region on the surface of the insulin monomer (6-12) that is responsible for inducing the biological activity of the hormone ("bioactive site"), as well as a discrete surface region responsible for inducing the negatively cooperative interactions among the receptor sites (13, 14). Blundell and coworkers have suggested (12, 15) that the so-called "hydrophobic reaction" (16) plays an important role in the reaction with its receptor of insulin and other polypeptide hormones like glucagon.

Indeed, the insulin monomer appears to be folded around an inside core of buried hydrophobic residues, but there are also two patches of clustered hydrophobic residues on the surface of the monomer. The first hydrophobic domain is part of the putative receptor-binding region, constitutes most of the "cooperative site" (14), and is involved in the monomer-monomer interactions in the insulin dimer whereas the second hydrophobic domain is involved in dimer-dimer interactions in the insulin hexamer.

If hydrophobic effects play an important role in the formation of the insulin-receptor complex, it should be possible to demonstrate it by a thermodynamic analysis of the reaction. Edelhoch and Osborne et al. have indeed shown that most, if not all, protein reactions in which nonpolar surface groups lose their contact with water, including protein renaturation, ligand binding, and protein-protein associations, show a marked dependence of enthalpy on temperature and a negative heat capacity change (17-19).

In the present work, we have attempted such a thermodynamic analysis for the reaction of insulin with its receptor on cultured human lymphocytes. Such a detailed analysis has not previously been performed for the reaction of a polypeptide with its receptor.

**MATERIALS AND METHODS**

**Insulin**—Crystalline porcine insulin was purchased from Elanco. Carrier-free Na$^{131}$I was bought from Amersham. Insulin was iodinated with $^{131}$I at a specific activity of 200 to 220 $\mu$Ci/$\mu$g by a modification of the chloramine-T method as previously described (20, 21).

**Cells**—Cultured human lymphocytes of the IM-9 line (21) were cultured at 37°C in RPMI medium supplemented with 10% fetal calf serum. Stock cultures were transferred every 2 or 3 days in 2 volumes of fresh media. Cells were harvested by centrifugation at 600 $\times$ g for 10 min and washed twice in assay buffer prior to use.

**Assay Buffer**—The composition of assay buffer was Tris(hydroxymethyl)aminomethane, 100 mM; NaCl, 120 mM; KCl, 5 mM; MgSO$_4$, 1.2 mM; sodium acetate, 15 mM; glucose, 10 mM; EDTA, 1 mM; bovine serum albumin (Fraction V from Armour), 10 mg/ml. The pH was measured at the temperature of the experiment.

**Establishment of the Equilibrium Condition**—A thermodynamic analysis of binding data requires that the system studied is truly at equilibrium. It is usually assumed in receptor studies that when the association of the hormone with the receptor reaches a plateau at time, this steady state reflects a thermodynamic equilibrium, and binding data are analyzed by methods derived for true equilibrium (Scatchard plots, Hill plots...). However, if the binding reaction is complicated by other processes like hormone or receptor degradation, irreversibility of the interaction, or internalization of the hormone or receptor, or both, the plateau reflects a steady state and not an equilibrium and cannot be analyzed by methods based on simple thermodynamics.

In the case of insulin binding to lymphocyte receptors, degradation of hormone and receptor were negligible at all temperatures in our experimental conditions (21). There is no internalization of labeled ligand (22).

The closeness to a true equilibrium was also demonstrated by the two following original experiments. First, we demonstrated that the same level of binding is reached whether a given concentration of unlabeled insulin is added simultaneously with a tracer of labeled insulin, or if the addition of unlabeled insulin is delayed until after the label has reached a steady state (Figs. 1 and 2). In the latter case, the tracer dissociates until the fraction of tracer bound is identical with the fraction bound when unlabeled insulin is present from time...
thermodynamics of the insulin receptor

measure the kinetics of association. At 90 min, 40 μl of buffer containing unlabeled insulin (10 ng/ml = 1.67 × 10⁻⁹ M) was added to the tube containing no unlabeled insulin and centrifugation of aliquots from both tubes pursued at the times indicated. This was true for all the concentrations of unlabeled insulin used in the experiments.

Second, we studied the dissociation kinetics of the insulin-receptor complex under two conditions: (a) in an "infinite dilution" (10₀-fold) of the complex in buffer, a condition in which the tracer dissociates slowly, reflecting the kinetics of the high affinity state of the receptor, and (b) in a 10₀-fold dilution of the complex in buffer containing an excess of unlabeled insulin, a condition in which the tracer dissociates much faster, reflecting the kinetics of the insulin-induced low affinity state of the receptor. We demonstrated that the addition of unlabeled insulin to the dissociating buffer could be delayed up to 8 h after the initiation of dissociation without loss of the fast reversal induced by insulin (Fig. 3).

In these conditions, we feel entitled to apply thermodynamic analysis to this ligand-receptor interaction with a reasonable chance of being close to a thermodynamic equilibrium.

Equilibrium Analysis of Binding Data—[¹²⁵I]-Insulin (5 × 10⁻¹¹ M) and increasing concentrations of native insulin (0 to 1.7 × 10⁻⁹ M) were added simultaneously to freshly harvested cells resuspended in assay buffer at a final concentration of 2 × 10⁶ cells/ml at various temperatures between 5 and 37°C. After a time sufficient to reach equilibrium (see legend of Fig. 4), bound and free insulin are measured by transferring duplicate 20₀-μl samples of the incubation to microfuge tubes containing 200 μl of chilled buffer and centrifuged for 1 min in a Beckman microfuge. In these conditions, degradation of [¹²⁵I]-insulin was less than 5% at all temperatures.

The plot of bound/free radioactivity versus bound hormone or Scatchard plot (21) was curvilinear at all temperatures, due to the presence of negative cooperativity in the binding reaction (2-5, 21) which complicates the analysis. The receptor concentration determined by computer curve fitting of the data to a negatively cooperative model as previously described (23, 24) did not vary with temperature, so changes in binding could be ascribed to changes in affinity.

zero. This was true for all the concentrations of unlabeled insulin used to define the "standard curve" submitted to equilibrium analysis. Second, we studied the dissociation kinetics of the insulin-receptor complex under two conditions: (a) in an "infinite dilution" (10₀-fold) of the complex in buffer, a condition in which the tracer dissociates slowly, reflecting the kinetics of the high affinity state of the receptor, and (b) in a 10₀-fold dilution of the complex in buffer containing an excess of unlabeled insulin, a condition in which the tracer dissociates much faster, reflecting the kinetics of the insulin-induced low affinity state of the receptor.

In the eight other series of tubes, the addition of unlabeled insulin was delayed, respectively, by 1 to 8 h from the time of initiation of dissociation. At intervals, a tube of each set was centrifuged, and the radioactivity in the cell pellet was counted. The radioactivity on the cells, expressed as a percentage of the radioactivity present at time zero, is plotted as a function of the time elapsed after the dilution of the system.

van't Hoff plot of insulin binding to receptors in cultured human lymphocytes. Cultured human lymphocytes (2 × 10⁶ cells) were incubated at various temperatures in assay buffer with a tracer amount (1 × 10⁻¹¹ M) of [¹²⁵I]-insulin during the minimal time necessary to reach equilibrium at the temperature considered (5°C, 4 h; 10°C, 3 h; 15°C, 2 h; 20°C, 1½ h; 25°C, 1 h; 30°C, 30 min; 37°C, 15 min). In these conditions, degradation of [¹²⁵I]-insulin was less than 5% in each case. The affinity constant (R₁) for the high affinity state of the receptor was determined at 15°C from Scatchard plots and average affinity profiles as explained in Ref. 20 and plotted as a function of the reciprocal of the absolute temperature (bottom curve "measured" values). These values were corrected according to Equation 1 to yield the top curve ("corrected" values).
The equilibrium constant for the high affinity state of the receptor \( (K_\text{h}) \) can be easily determined even in the absence of a suitable model or computer curve-plotting program from Scatchard plots or average affinity profiles as previously described (25). \( K_\text{h} \) is in fact simply \( (B/F)/(1+R) \) when the insulin concentration approaches zero. Since the constant for the low affinity state is less precisely determined, we will report here on the effect of temperature on the high affinity state of the receptor \( (K_\text{h}) \), that is on the state of the receptor in the absence of site-site interactions.

Correction for pH Effects Varying With the Temperature—The binding of insulin depends markedly on the pH, with a sharp optimum. Thus, to interpret the effects of the temperature on binding in terms of hydrophobic contributions, we should take into account the effect of temperature on the pH dependence of the binding, since the groups involved may also have significant enthalpies of ionization. This factor has often been neglected in thermodynamic analyses. We found indeed that the pH optimum of insulin binding varies markedly with the temperature. We thus measured the binding at the pH optimum corresponding to each temperature (pH 8.2 at 5°C, 8.1 at 10°C, 8.0 at 15°C, 7.9 at 20°C, 7.8 at 25°C, 7.7 at 30°C, 7.6 at 37°C) and corrected the data to take into account the ionization constants. The method used for this correction is detailed in another paper1 describing a theoretical analysis of the pH dependence of the insulin receptor and only briefly summarized here.

We assumed, adapting the generalized pH theory of Reiner (26) to receptor binding, that binding is optimal when certain groups of the insulin and the receptor are protonated, whereas other groups must be deprotonated. If one assumes that only the moieties in the correct ionization state can bind, we can derive an equation giving the "true" affinity constant \( (K_\text{true}) \) of the insulin-receptor complex as a function of the pH, the number, and pKs of the groups whose state of ionization matters. By computer curve-fitting, one can derive the number of groups involved, their pK values, and the enthalpies of ionization of the groups involved. In the case of insulin, only one group was found to be involved on each side of the pH optimum, and the equation simplifies to (Equation 1):

\[
\log (K_\text{true}) = \log (K_\text{measured}) - \log (1 + 10^{(pH-pK)}) - \log (1 + 10^{(pH-pK)})
\]

where \( K_\text{measured} \) is the apparent affinity of the insulin-receptor complex when both the insulin and the receptor are in the correct ionic state, \( pK \) is the pK (\( = -\log K_\text{uncharged} \)) of the group which must be deprotonated for binding, and \( pK_\text{true} \) is the pK of the group which must be protonated for binding.

It must be stressed, however, that the conclusions drawn from the corrected values were identical with the conclusions drawn from the uncorrected ones, the correction changing only moderately the values of the thermodynamic parameters obtained.

RESULTS

Equilibrium Constant and Free Energy Change—The equilibrium constant for the association reaction in the high affinity state, \( K_\text{h} \), varies markedly with temperature. A van't Hoff plot of the data (Fig. 4) is curvilinear with a maximum around 20°C for the corrected data. The standard free energy change is \( \Delta G^0 = -RT \ln K_\text{h} \) (Equation 2) where \( R \) is the gas constant and \( T \) the absolute temperature. A plot of \( \Delta G^0 \) against the temperature (Fig. 5) also shows a marked curvilinearity with a maximum at 37°C. The enthalpy change, \( \Delta H^0 \), is usually determined from the slope of the van't Hoff plot at various temperatures, but this procedure would give inexact values in this case because of the curvature of the plot. Therefore, we used the same procedure as Osborne et al. (18) and performed a regression analysis of the free energy change by using Equation 3:

\[
\Delta G^0 = -RT \ln K_\text{h} - A + BT + CT^2
\]  

The best fit analysis of the experimental data is represented by the solid line in Fig. 5 and the coefficients shown in Equation 4:

\[
\Delta G^0 = 110,630 - 797.8 T + 1.286 T^2
\]  

Therefore, one obtains

\[
\Delta H^0 = \delta(\Delta G^0/\delta(1/T)) = 110,630 - 1.286 T
\]  

\[
\Delta S^0 = \delta(\Delta G^0/\delta T) = + 797.8 - 2.57 T
\]

where \( \Delta S^0 \) is the entropy change of the reaction. \( \Delta C_p^0 \), the heat capacity change, can also be derived

\[
\Delta C_p^0 = \delta(\Delta H^0/\delta T) = -2.57 T
\]

1 M. Waelbroeck, manuscript in preparation.
As shown in Fig. 6, the changes in both enthalpy and entropy for the insulin-receptor association decrease continuously and within the limits studied vary quasi linearly with temperature. The thermodynamic stability of the complex ($\delta \Delta G^0 / \delta T = -\Delta S^0 = 0$) is maximum at 37°C. The change in heat capacity, that is the slope of the $\Delta H^0$ curve, is negative, indicating that the heat capacity for the separated insulin and receptor molecules is greater than in the hormone-receptor complex. Although the plot of $\Delta H^0$ against temperature appears linear within the experimental errors and the range of temperatures studied, it is represented by a second degree equation (Equation 5), and its first derivative, $\Delta C P^0$, also varies with temperature (Fig. 3). The absolute value of the variation is, however, a second derivative of the initial data plot and thus relatively imprecise.

The value of $\Delta C P^0$ is traditionally expressed at 25°C and is in this case $-766 \text{ cal/(mol deg)}$.

Fig. 7 presents a complete analysis of the driving forces in the formation of the insulin-receptor complex in the case where the effect of temperature on ionization is “eliminated” by appropriate correction.

**DISCUSSION**

The characteristics of the thermodynamic parameters of the association of insulin to its receptor are strikingly similar to those of other types of interactions determined by so-called hydrophobic effects (17-19). Our results are remarkably comparable to those obtained by Osborne et al. in a thermodynamic study of the self association of the reduced and carboxymethylated form of apo-A-II protein from the human high density lipoprotein complex (18). That reaction showed a similar shape in the temperature dependence of the free energy change, with a maximum near 38°C (37°C in the case of insulin), the same inverse relationship between $\Delta H^0$ or $\Delta S^0$ and the temperature, and a quite comparable change in heat capacity between the unassociated and associated molecules $-1250 \text{ cal/(mol deg)}$ versus $-766$ in our case.

Similarly, analysis of the free energy change of association of glutamic dehydrogenase between 10 and 40°C showed a linear dependence of the enthalpy on temperature with positive values below 28°C and negative values above 28°C, and a heat capacity change of $-600 \text{ cal/(mol deg)}$. Many similar findings are listed in Ref. 17.

This type of behavior is typical of the interactions with water on the nonpolar moieties of a variety of compounds such as acids, bases, alcohols, amino acids, and surfactants and contrasts with the interactions of polar moieties with water (17). This “hydrophobic effect” and its consequences on the thermodynamics of various model systems has been lucidly analyzed by Edelhoch et al. (17) and understanding its basis may prove crucial in many hormone-receptor interactions.

The most useful thermodynamic parameter that expresses the interaction of various groups with water is the heat capacity change (17-19). From the large negative heat capacity change observed in our study, it appears that the insulin-receptor interaction is critically dependent on the properties of water. Since ionic and polar groups interact strongly with water, a large part of the driving force in the insulin-receptor interaction is probably contributed by the removal of water from the less polar moieties (probably surface residues of both proteins) and the resultant changes in the cooperative organization of the hydrogen binding between water molecules.

Using Tanford's estimates for the heat capacity associated with the exposure of various nonpolar groups (27), we have calculated the theoretical contribution of the nonpolar residues in the putative receptor-binding regions of insulin (12), assuming that they change from totally exposed in the medium to completely buried in the hormone-receptor complex (A16 Tyr, B20 Phe, B20 Phe, B20 Tyr, B15 Val, B16 Tyr).

It amounts to $-460 \text{ cal/(mol deg)}$. Thus, insulin may contribute more than half of the heat capacity change of the reaction as determined in our study, $(-766 \text{ cal/(mol deg)})$, and burying hydrophobic residues from the receptor or membrane site must contribute the other half. However, more precise calculations must be done, taking in account the accessibility of each group and the hydrophobic contribution from polar groups. This quantification will need further studies.

These data strongly support Blundell's concept that hydrophobic forces play an important role in the interaction of polypeptide hormones with their receptors. They are also in line with the general principles of protein-protein recognition proposed by Chothia and Janin (28), whereby hydrophobicity is the major factor which stabilizes protein-protein association while van der Waals and polar interactions determine which proteins may recognize each other since the proper formation of hydrogen bonds and of van der Waals contacts requires complementarity of the surfaces involved.

Similar data were recently reported in the interaction of steroids with their cytoplasmic receptors (29). This interpretation of our data must, however, be taken with caution since it is based on the critical assumption that the reaction of insulin with the initial state of its receptor is a simple, one-step reaction, $H + Re \rightleftharpoons HRe$, with a simple high affinity equilibrium constant $= K_c$.

An alternative explanation of our data would be that the reaction involves more than one step, for example $H + Re \rightleftharpoons HRe \rightleftharpoons HRe_c$, or that there is another coupled reaction. Even if the thermodynamics of each step was classical (i.e. linear van’t Hoff plot), the resulting steady state may yield curvilinear van’t Hoff plots even in the absence of hydrophobic effects. The fact that the kinetics of dissociation of many receptors is not first order could certainly suggest that such an explanation is plausible. This restriction is not unique to our case but could be valid also in other systems, like protein denaturation.
where thermodynamic analysis has been applied. Extensive work is clearly required to refine the modeling of the insulin-receptor interaction.

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