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*Report*

# The Use of Microcalorimetry to Measure Thermodynamic Parameters of the Binding of Ligands to Insulin

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Flow microcalorimetry was used to measure the free energies, enthalpies, and entropies of interactions between the hormone insulin and small ligand molecules or ions. Measurable amounts of heat were obtained for binding of four phenolic preservative molecules—phenol, meta-cresol, resorcinol, and methylparaben—to both two-zinc and zinc-free insulin and for binding of zinc ions to zinc-free insulin. All of the reactions were spontaneous, but the phenolic binding was driven by enthalpy, while that of zinc was entropy-driven. A combination of van der Waals interactions, hydrophobic effects, and protein conformational changes appeared to be involved in binding of the phenolic ligands. Zinc ions displayed two types of binding to insulin, both involving ion–dipole interactions.

KEY WORDS: calorimetry; phenol; zinc; hexamer.

## INTRODUCTION

Commercially available insulin preparations have phenol, meta-cresol, or methylparaben included for their bacterial preservative activity (1), although a significant binding interaction between these compounds and insulin occurs (2). Phenol is known to be an insulin denaturant (3) and has been implicated in the crystallization of a "six-zinc insulin hexamer" (4). Clearly, the extent of the interaction between these compounds and insulin must be determined before the actual activity of insulin and of preservative in the preparations can be established. In addition, phenolic molecules have recently been shown to stabilize the two-zinc insulin hexamer in such preparations (5), and the molecules are structurally similar to ligands designed to bind to and stabilize two-zinc insulin (6).

Zinc ions play a vital role in the biosynthesis, storage, and ultimate utilization of insulin in the body. The remarkable insolubility of the zinc insulin hexamer promotes the conversion of proinsulin to insulin, protects the newly formed insulin from degradative enzymes, and facilitates its release into the bloodstream from storage granules (7). In the commercial manufacture of insulin preparations, extraction of insulin in the presence of zinc yields insulin crystals that are substantially free of such impurities as proinsulin, preproinsulin, and glucagon (1).

In the present work, microcalorimetry was used to determine the thermodynamic parameters of the insulin–ligand interaction. For the phenolic compounds, which display only one type of binding site, complete thermodynamic information ($\Delta G$, $\Delta H$, and $\Delta S$) was obtained from a single binding curve. In addition, a good estimate of the stoichiometry of the reaction could be established. In the case of zinc ions, which exhibit two types of binding, enthalpy values were measured for each binding type, and the experimentally determined enthalpies were combined with association constants from the literature. The free energies and entropies of binding could then be calculated.

## MATERIALS AND METHODS

Crystalline porcine zinc insulin, porcine zinc-free insulin, and recombinant human zinc insulin, generously provided by Eli Lilly and Company, were used without further purification. All ligands were at least reagent grade. Phenol, resorcinol, and zinc nitrate hexahydrate were purchased from Fisher, meta-cresol was purchased from Sigma, and methylparaben was supplied by Lilly. Chelex 100 (100–200 mesh) was purchased from Bio-Rad Laboratories, Richmond, California. All solutions were prepared using double-distilled water.

Crystals of zinc-free insulin were prepared from the zinc insulin by modification of a published procedure (8). Zinc insulin solutions (5 mg/ml; pH 8.0 in 0.05 *M* Tris–HCl buffer) were passed over a Chelex 100 column to remove all traces of zinc ions. The zinc-free insulin was precipitated (pH 5.3) and filtered, then redissolved in water, precipitated, and filtered again to remove all traces of Tris–HCl from the crystals. The residue was redissolved in water, and the solvent was removed by lyophilization. Zinc-free insulin cakes prepared in this manner (yield, approximately 80%) were stored frozen until needed in the study. It has been previously shown (9,10) that zinc-free insulin prepared on a Chelex 100 column contained a zinc/insulin ratio of less than 0.002. In the present study, the zinc-free insulin from the Chelex 100

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column was found to give calorimetric heats equivalent to those of the zinc-free insulin supplied by Lilly.

Separate aqueous solutions of insulin and ligand were prepared for this work. The insulin solutions were prepared so that after mixing with ligand the final insulin concentration would be held constant at approximately 3.6 mg/ml (0.1 m*M* hexamer). The concentrations of the ligand solutions were varied. All solutions were adjusted to pH 7.4. Since no buffer was used in the solutions, the final pH after mixing was checked periodically; it was not found to deviate beyond ±0.1 pH unit, except at those zinc concentrations where precipitation occurred.

An LKB Flow Microcalorimeter (LKB Produkter AB, Bromma, Sweden) was used to measure the heat of mixing the insulin and phenolic ligand solutions. The entire calorimeter was submerged in a water bath maintained at exactly 25°C by a Tronac PTC-40 temperature controller (Tronac Inc., Orem, Utah). This modification has been reported to increase the stability and sensitivity of the instrument approximately 10-fold over the commercial product, which uses an air bath (11). The two solutions were pumped into the calorimeter by means of an LKB Microperpex Peristaltic Pump, Model 2132 (LKB Produkter AB, Bromma, Sweden). The flow rate was usually set to 30 ml/hr, so that the residence time in the mixing cell was approximately 1 min. Reactions were performed at faster and slower speeds to ascertain that complete reaction did occur in the allowed residence time. The electrical heat of mixing was amplified by a Keithley 150B Microvolt Ammeter (Keithley Instruments Inc., Cleveland, Ohio) and recorded on a chart recorder.

An instrument more sensitive than the LKB Flow Microcalorimeter was needed to measure the interaction between zinc and zinc-free insulin, because the heat of mixing was very small for the concentrations stated above. An LKB thermal activity monitor (TAM) (Thermometric AB, Jarfalla, Sweden) was found to be sufficiently sensitive to measure zinc binding to insulin at 25°C. The insulin and zinc solutions were pumped into the TAM by two Altex HPLC pumps operating at 0.5 ml/min (Altex Scientific Inc., Berkeley, California), which gave a more stable baseline than the LKB peristaltic pump. The two solutions were mixed in the flow-mixing cell of the calorimeter, then pumped through the flow-through cell before being discarded. The TAM output is actually a comparison of heats measured in the two cells. By passing solution through both of the cells, extraneous heats due to friction with the tubing or turbulence in the flow were canceled. The electrical heat of mixing was internally amplified and recorded on a chart recorder.

On both calorimeters, the pen response to the mixing was compared with the response to an electrically introduced calibration heat. This procedure was performed after each run on the LKB Flow, and the calibration heater was periodically checked by measuring the heat of mixing HCl and NaOH solutions. Calibration of the TAM was performed periodically throughout the study, and the calibration heater of this instrument was checked by measuring heat of dilution of an NaCl solution.

The measured heat of mixing in either calorimeter is actually the sum of three heats: the heat of reaction between ligand and insulin, the heat of dilution of ligand, and the heat of dilution of insulin. The respective heats of dilution were obtained in separate experiments (by mixing ligand or insulin with water) and subtracted from the total heat to obtain the heat of reaction. In all cases, the heats of dilution were small, usually less than 25%, in comparison to the heat of reaction.

### Theory

For one or more identical and independent ligand binding sites on a macromolecule such as insulin, the following equation has been derived (12,13):

$$\frac{1}{Q} = \frac{1}{Q_{max}} + \frac{1}{Q_{max}K_B[A]} \tag{1}$$

where $Q$ is the experimental heat of reaction per mole of insulin, $Q_{max}$ is the heat of reaction per mole of insulin at complete saturation of all binding sites, $K_B$ is the intrinsic binding constant per site, and $[A]$ is the concentration of unbound ligand. Equation (1) can be plotted in "double reciprocal" form, i.e., $1/Q$ vs $1/[A]$. The concentration of unbound ligand can be determined from the following equation:

$$[A] = [A]_T - n\frac{Q}{Q_{max}}[I]_T \tag{2}$$

where $[A]_T$ is the formal concentration of ligand, $[I]_T$ is the formal insulin concentration, and $n$ is the number of binding sites per insulin molecule.

In the present study, the double-reciprocal plot and calculations were performed on a computer by means of an iterative least-squares treatment of the data for assumed values of $n$. The value of $n$ giving the best linear fit was interpreted to be the actual number of binding sites per molecule of insulin.

As shown in Eq. (1), $\Delta H^\circ_B$ and $K_B$ for the binding reaction can be obtained from the slope and intercept of the double reciprocal plot. Once these values are known, $\Delta G^\circ_B$ and $\Delta S^\circ_B$ may be calculated from the following expressions:

$$\Delta G^\circ_B = -RT \ln (nK_B) \tag{3}$$

$$\Delta S^\circ_B = \frac{\Delta H^\circ_B - \Delta G^\circ_B}{T}. \tag{4}$$

Hence, apparent values of all thermodynamic parameters may be obtained from a single plot.

## RESULTS

### Phenolic Ligands

Typical results of studies on the binding of four different ligands—phenol, meta-cresol, resorcinol, and methylparaben—to both zinc insulin and zinc-free insulin (3.6 mg/ml final insulin concentration), are shown in Fig. 1; the experimental heat of reaction is plotted against free ligand concentration in a double reciprocal format according to Eq. (1). Calculated thermodynamic parameters are summarized in Table I. Approximate experimental errors in this table are of the order of ±0.2 kcal/mol for $\Delta G^\circ$, ±0.5 kcal/mol for $\Delta H^\circ$,

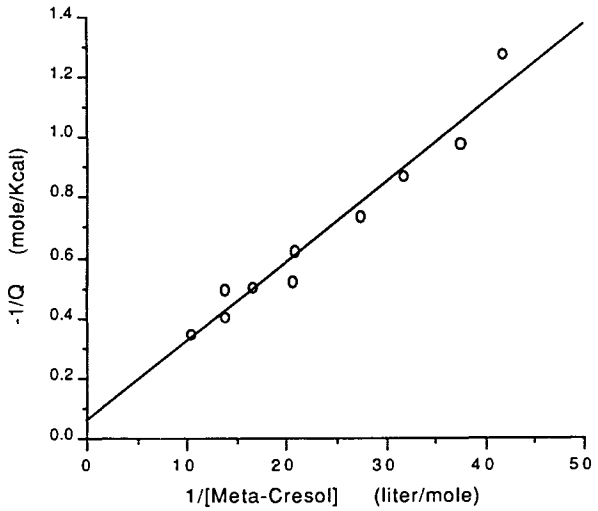



Fig. 1. Double reciprocal plot for meta-cresol binding to zinc insulin.

±2 entropy units for $\Delta S°$, and ±30 $M^{-1}$ for $K_B$. The results indicate that the binding reaction is a spontaneous process. In all cases the reaction is exothermic, but the entropy of the system either decreases substantially or remains relatively constant. Thus, the interaction is driven by enthalpy.

In all cases the data provided a good linear fit to the proposed model for $n = 1$ (one binding site per insulin monomer), over the concentration range studied. For example, assuming $n = 1$ for the interaction between zinc insulin and meta-cresol gave a sum of squared residuals value ($r^2$) of 0.962. Although this is evidence that only one phenolic binding site per monomer exists, it cannot be unequivocally stated that such is the case. The value of $n$ plays only a small role in the mathematics of the equations used to make the double reciprocal plots, Eqs. (1) and (2). Since these equations may be plotted and replotted many times during the iterative search for the value of $Q_{max}$, and since the experimental data will contain some errors in the beginning, the assumed value of $n$ has little effect on the eventual outcome. For the insulin–cresol data, assumptions of $n = 2$ or $n = 3$ also gave reasonably good linear fits ($r^2 = 0.961$ and 0.957, respectively), although significant deviations from linearity were observed at larger $n$ values (for $n = 10$, $r^2 = 0.905$, for $n = 12$, $r^2 = 0.881$). The existence of one phenol binding site per monomer of insulin is consistent with literature data on the two-zinc hexamer (5).

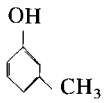
Bolen and co-workers (13) pointed out that a verification of the value of $n$ could be made if experiments were performed such that the ligand concentration was held constant while that of the protein varied. Unfortunately, the suggested technique is not very suitable in the present case where the complex formed is weak and where the insulin aggregation state changes with concentration. Bjurulf and Wadso (14) encountered the same problems in their study of lysozyme-inhibitor binding.

### Zinc Ion Binding to Zinc-Free Insulin

Experimental heats of reaction per mole of monomer from the present study are plotted against the formal zinc:hexamer ratio in Fig. 2. The most striking thing about this graph is the bend that occurs slightly below a zinc:hexamer ratio of two. The bend clearly indicates that two types of binding are occurring in this system: a high-affinity binding at low zinc concentrations, as evidenced by the steep slope in that region of the graph; and a lower-affinity binding at higher zinc concentrations, as evidenced by the shallow slope in that region. The bend in the graph occurs just below two zincs per hexamer, indicating that the high-affinity sites are capable of binding two zinc ions per hexamer. These experimental results are consistent with the reported data for zinc binding to insulin (15–18).

If the zinc:hexamer ratio could be increased further than

Table I. Thermodynamic Parameters for Binding of Phenolic Ligands to Insulin

| Ligand | Insulin | $K_B$ | $\Delta H°$ (kcal/mol) | $\Delta G°$ (kcal/mol) | $\Delta S°$ (cal/mol K) |
|---|---|---|---|---|---|
|  Phenol | Zinc | 74 | −2.3 | −2.5 | 0.93 |
| | Zinc free | 1.7 | −9.8 | −0.31 | −32 |
|  Meta-Cresol | Zinc | 2.2 | −17 | −0.46 | −57 |
| | Zinc free | 6.1 | −5.9 | −1.1 | −16 |
|  Resorcinol | Zinc | 37 | −9.1 | −2.1 | −23 |
| | Zinc free | 0.98 | −19 | 0.012 | −64 |
|  Methylparaben | Zinc | 220 | −3.2 | −3.2 | −0.12 |
| | Zinc free | 380 | −2.7 | −3.5 | 2.9 |

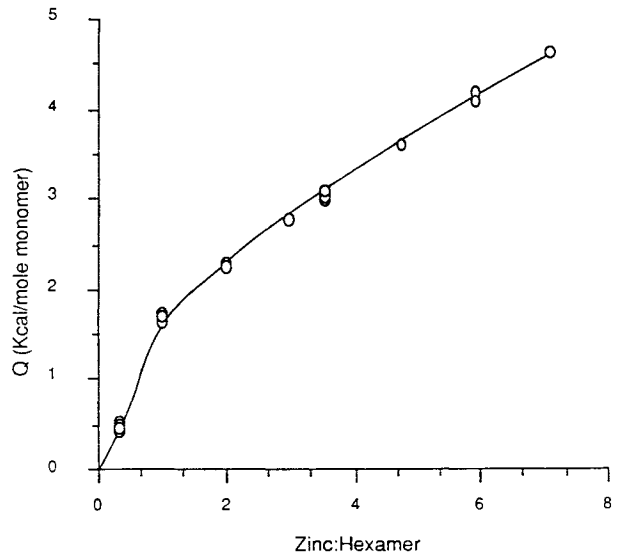



Fig. 2. Heats obtained from zinc binding to zinc-free insulin.

in the present study, the heat of binding should eventually stop increasing at saturation of the low-affinity sites, and a graph such as Fig. 2 should reach a plateau at high zinc:hexamer values. The plateau would be expected to occur at approximately eight zinc ions per hexamer. Unfortunately, data could not be obtained to show this phenomenon because of zinc insulin precipitation at the high zinc concentrations. From a visual inspection of the mixed solutions, it became evident that a zinc:hexamer ratio of six to one was the highest zinc concentration that could be used and still obtain meaningful binding data at pH 7.4. A similar problem was encountered by Bolen and Rajender (19) in their calorimetric study of zinc binding to rhodanese.

Since the insulin hexamer contains two different types of zinc binding sites, Eqs. (1) and (2) do not apply unless certain assumptions are made. The association constants for the two binding types differ by some two orders of magnitude. It may therefore be assumed that heats obtained at low zinc concentrations are entirely due to high-affinity binding and that heats at high zinc concentrations are due to low-affinity binding. Thus, data obtained at low zinc:hexamer ratios may be plotted according to Eqs. (1) and (2), to obtain thermodynamic parameters of high-affinity zinc binding.

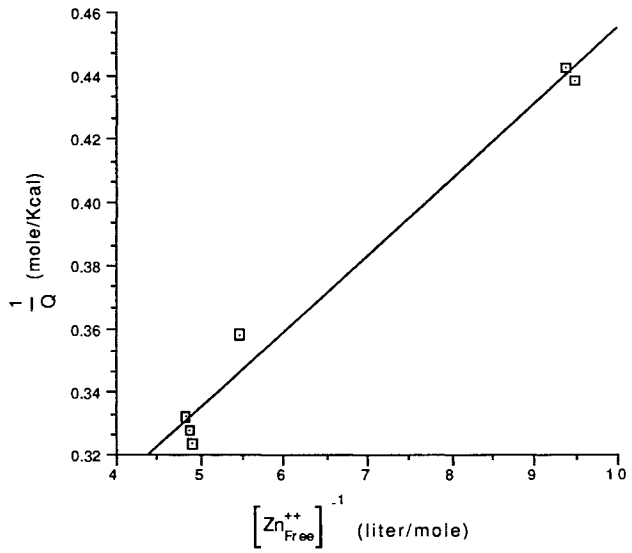



Fig. 3. Double reciprocal plot of zinc binding data.

The double reciprocal plot for high-affinity zinc binding is shown in Fig. 3. A satisfactory linear relationship was obtained for data at and slightly beyond the break in the Fig. 2 graph. Data at lower zinc concentrations could not be used because the free zinc concentration was very small in such cases. A $\Delta H^\circ$ of 4.66 kcal/mol was calculated for binding to the high-affinity sites, and $\Delta H^\circ \approx 4$ kcal/mol was estimated from Fig. 2 for binding to the low-affinity sites. It must be stressed that the enthalpy value for each type of binding was calculated assuming that interference from the other type did not occur and that precipitation of the zinc–insulin complex was negligible or slow in comparison to the flow rate. Significant contributions from such interfering processes would invalidate the method.

## DISCUSSION

### Phenolic Ligands

As shown in Table I, negative $\Delta S^\circ$ values were calculated for most of the insulin–ligand systems. Entropy changes such as these have been noted by other groups studying other ligand-macromolecule interactions; the values have usually been attributed to conformational changes in the macromolecule (20,21). In the present study, it is likely that the binding of these ligands causes a change in the conformation of the hexamer, to produce a more compact, less flexible structure. It has recently been shown that six phenol or meta-cresol molecules will bind to two-zinc insulin and induce a conformational change in residues B1–B8 of each of the six insulin monomers (5). Protein conformational changes may also make a negative contribution to the enthalpy of reaction (20,21). The negative $\Delta H^\circ$ values measured in the present work may well contain such a contribution.

From the structures of the ligand molecules, a combination of van der Waals effects, hydrogen bonding, and hydrophobic interactions is probably responsible for binding to the protein. Hydrogen bonds and van der Waals contacts have been shown to exist when phenol is bound to the two-zinc hexamer (5). Such a combination of interactions might be expected to make an overall positive contribution to $\Delta S^\circ$, due to the decrease in solvent structuring which accompanies hydrophobic interactions (22), and an overall negative contribution to $\Delta H^\circ$, due to van der Waals effects (23). The experimental results in Table I do show negative $\Delta H^\circ$ values, but the observed entropies are also negative, probably because of the larger negative contributions from protein conformational changes.

### Zinc Ion Binding

Several groups have measured the stoichiometry and association constants of the zinc–insulin interaction using methods such as equilibrium dialysis, sedimentation equilibrium, and X-ray crystallography (15–17). Their results are summarized in Table II. As the table indicates, there are two

Table II. Binding Sites and Association Constants from the Literature for Zinc Binding to Insulin

| $n$ | $K_a$ ($M^{-1}$) | pH | Ref. No. |
|---|---|---|---|
| | High-affinity binding | | |
| 2 | $4.7 \times 10^6$ | 8.0 | 15 |
| 2 | $1.9 \times 10^6$ | 7.0 | 16 |
| | Low-affinity binding | | |
| 6 | $3.5 \times 10^4$ | 8.0 | 15 |
| 6 | $22. \times 10^4$ | 8.0 | 17 |

levels of zinc binding to insulin. The higher-affinity type of binding can accommodate two zinc ions per hexamer and has an intrinsic association constant of about $10^6$ $M^{-1}$ per site (15,16). This binding most likely refers to the associations of zinc with B10 histidine residues at the ends of the central cavity of the insulin hexamer, as observed in X-ray crystallographic studies of the two-zinc hexamer (and hence the name, "two-zinc hexamer") (18). The weaker type of binding can incorporate up to six zinc ions per hexamer and is characterized by an association constant some two orders of magnitude lower than that of the high-affinity binding (15,17). Three of these sites are probably located near the B13 glutamate residues in the central cavity (7), with the other three on the exterior surface of the hexamer.

Enthalpy values obtained in the present study are combined with association constants from the literature in Table III. Free energies and entropies for the reactions have also been calculated. The results indicate that both types of binding are spontaneous processes. The entropies of the systems increase substantially, but the reactions are endothermic. Thus, the interactions are driven by entropy.

The large, positive $\Delta S°$ values are characteristic of ion–dipole interactions between an ionic species and polar functional groups on the protein structure. Entropy is positive because of the loss in solvent structuring which accompanies dehydration of the ligand in such interactions and is the driving force for binding (24). The experimental results are consistent with reported structural data showing that the high-affinity sites have zinc ions in octahedral coordination with three embedded water molecules and three B10 histidine imidazole groups (18).

In contrast to entropy, $\Delta H°$ values are unfavorable for both types of zinc binding. The disruption of ion–solvent interactions upon binding is probably responsible for the observed endothermic heats. Positive enthalpy changes have also been noted for other zinc–protein interactions; in fact, it is rare for a zinc–protein interaction to result in anything other than a positive enthalpy change (19,25).

It does not appear from the data that significant protein conformational changes occur when zinc binds to insulin under the experimental conditions employed in this study. Such changes would be expected to make negative contributions to both $\Delta H°$ and $\Delta S°$, as discussed earlier. Since the hexamer form of two-zinc insulin predominates under the experimental conditions of the present study (26), it can be concluded that a substantial amount of insulin still exists as the hexameric species at this concentration, even in the absence of zinc. The data of Pekar and Frank (27) on zinc-free insulin support this conclusion.

Table III. Summary of Thermodynamic Parameters for Zinc binding to Insulin

| $n$ | $K_a$ ($M^{-1}$) | $\Delta H°_B$ (kcal/mol) | $\Delta G°_B$ (kcal/mol) | $\Delta S°_B$ (eu) | Source |
|---|---|---|---|---|---|
| | | High-affinity binding | | | |
| 2 | $3.3 \times 10^6$ | | −9.30 | | Literature |
| 2 | | 4.66 | | 47 | This work |
| | | Low-affinity binding | | | |
| 6 | $13 \times 10^4$ | | −8.03 | | Literature |
| ~6 | | ~4 | | 40 | This work |

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