Titration Calorimetry of Anesthetic-Protein Interaction: Negative Enthalpy of Binding and Anesthetic Potency

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ABSTRACT Anesthetic potency increases at lower temperatures. In contrast, the transfer enthalpy of volatile anesthetics from water to macromolecules is usually positive. The transfer decreases at lower temperature. It was proposed that a few selective proteins bind volatile anesthetics with negative ΔH, and these proteins are involved in signal transduction. There has been no report on direct estimation of binding ΔH of anesthetics to proteins. This study used isothermal titration calorimetry to analyze chloroform binding to bovine serum albumin. The calorimetrically measured ΔH_{cal} was -10.37 kJ · mol^{-1}. Thus the negative ΔH of anesthetic binding is not limited to signal transduction proteins. The binding was saturable following Fermi-Dirac statistics and is characterized by the Langmuir adsorption isotherms, which is interfacial. The high-affinity association constant, K, was 2150 ± 132 M^{-1} (K_D = 0.47 mM) with the maximum binding number, B_{max} = 3.7 ± 0.2. The low-affinity K was 189 ± 3.8 M^{-1} (K_D = 5.29 mM), with a B_{max} of 13.2 ± 0.3. Anesthetic potency is a function of the activity of anesthetic molecules, not the concentration. Because the sign of ΔH determines the temperature dependence of distribution of anesthetic molecules, it is irrelevant to the temperature dependence of anesthetic potency.

INTRODUCTION

The hydrophobic interaction of anesthetics is well known by the Meyer-Overton rule, where the potency of anesthetics correlates linearly with their olive oil/water partition coefficient. Because olive oil is a mixture of several components, octanol is now preferred to represent the organic phase. Octanol/water partition coefficient is now established as a standard hydrophobicity parameter in the quantitative structure-activity relationship (QSAR) in analyzing the binding of a ligand to host molecules (Hansch, 1971). Oil/water partition coefficients of volatile anesthetics decrease when the temperature is lowered (positive temperature dependence), whereas anesthetic potencies increase (negative temperature dependence).

To deal with this inconsistency, Dickinson et al. (1993) expressed EC_{50} (the concentration that anesthetizes 50% of the population) by

$$EC_{50} = \Delta H/RT + \text{constant}$$

and proposed that the negative ΔH of anesthetic binding is imperative to satisfy the negative temperature dependence of anesthetic potencies. They reported that the enthalpy of anesthetic binding to firefly luciferase was negative, and proposed that only a few selected proteins are involved in anesthesia mechanisms. The enthalpy was estimated from the temperature dependence of anesthetic action on the light intensity of firefly luciferase.

To our knowledge, there has been no direct binding study to estimate the ΔH of anesthetic-protein complex formation. This study used isothermal differential titration calorimetry (DTC) to measure the ΔH_{cal} of chloroform binding to lipid-free bovine serum albumin (BSA). Studies of the binding mode of anesthetics have difficulty in maintaining constant concentrations of highly volatile agents during the procedure. DTC has the advantage of estimating the association constant by avoiding the separation procedure, thereby leaving the equilibrium state of binding undisturbed. From the DTC data, the free and bound ligand concentrations were obtained, and the association constant was estimated.

MATERIALS AND METHODS

BSA (essentially fatty acid free) was obtained from Sigma (St. Louis, MO), and chloroform was from EM Science (Cherry Hill, NJ). Water was purified by distillation followed by two-stage mixed-bed ion-exchanger columns, an activated charcoal column, and an ultrafilter.

BSA was dissolved in 0.1 M phosphate buffer (pH 6.0) at various concentrations. The solvent pH value of 6.0 was selected to avoid the pH-induced conformational changes of BSA: N⇌F transition below pH 4.5 and N⇌B transition above pH 7.0. Chloroform was dissolved in the same buffer. To cover an extended ligand/host concentration ratio, three BSA concentrations were used: ~1.0, 0.1, and 0.01 mM. The chloroform concentration was set at about 50 mM. The chloroform concentration appears to be excessive when compared to BSA concentration, but chloroform is diluted to about 1/65 when injected into the BSA solution. The theory for the interpretation of the calorimetry data of this weakly binding ligand is described in the Appendix.

The solvent density was measured by a pycnometer with an internal volume ~12 cm^3. The density was 1.007 g · cm^{-3} at 25.0°C. This value was used to calculate the molarity of the solutes in the sample solutions.

An OMEGA differential titration calorimeter (Microcal, Northampton, MA) was used. The titration cell (1.2955 cm^3) was filled with the BSA solution and titrated with chloroform solution in the injection microsyringe at 25°C. The chloroform solution was injected into the BSA solution in fractional doses under automatic control from the system computer. The volume of each injection was 20 μl, and the intervals between injections

Received for publication 22 November 1996 and in final form 23 January 1997.

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0006-3495/97/04/1812/06 $2.00

were 10 min. To correct for the dilution effect by the injection of chloroform solution, two controls were obtained: titrating the BSA solution by the buffer for BSA dilution, and titrating the buffer solution by chloroform solution for dilution of chloroform. The overflow of the reaction mixture by injection of ligand solution was corrected by the computer.

RESULTS

Fig. 1 shows the DTC data of BSA titration by chloroform. The left figure is the highest BSA concentration, where 0.998 mM BSA was titrated with 53.9 mM chloroform. The center figure is the intermediate, where the BSA/chloroform concentration ratio was 0.101/56.3 mM, and the right figure is the lowest ratio, 0.0105/51.4 mM.

Fig. 1 *A* shows the raw titration data. The upper tracings (marked 1) show the computer-controlled injection of 20 μl chloroform solution into the BSA solution in the titration chamber. The area under the kth downward spike represents the amount of heat, $\Delta Q(k)$, released at the kth injection. The lower tracings (marked 2) are the control, created by injecting the same volume of buffer solution without chloroform into the BSA solution. The area under the kth upward spike represents the amount of heat, $\Delta Q^0(k)$, absorbed by the dilution of the BSA concentration at the kth injection. Contrary to the expectation that the dilution heat of chloroform is large, the injection of chloroform solution into the buffer solution showed negligibly small upward peaks (<0.1 μcal/s) and was ignored.

The difference between the two, $\Delta Q(k) - \Delta Q^0(k)$, represents the total amount of heat released during the injection. It consists of the partial molar enthalpy change of the anesthetic-protein complex formation and the partial molar enthalpy change of the free ligand in the solution.

Fig. 1 *B* is the integrated titration heats per mole of chloroform during injection. The lower tracing (marked 1) is the titration with chloroform, and the upper tracing (marked 2) is the control without chloroform. Fig. 1 *C* represents the molar enthalpy change between the absence and presence of chloroform after correcting the BSA dilution factor ($h - h^0$). It is plotted as a function of mole ratio of chloroform to BSA.

From the initial slope of the curve at high BSA concentration (0.998 mM) and from the final slope of the curve at low BSA concentration (0.01 mM), the averaged enthalpy changes of the chloroform-BSA complex formation and the numbers of bound and free chloroform concentrations at each injections were obtained (see Appendix). The averaged

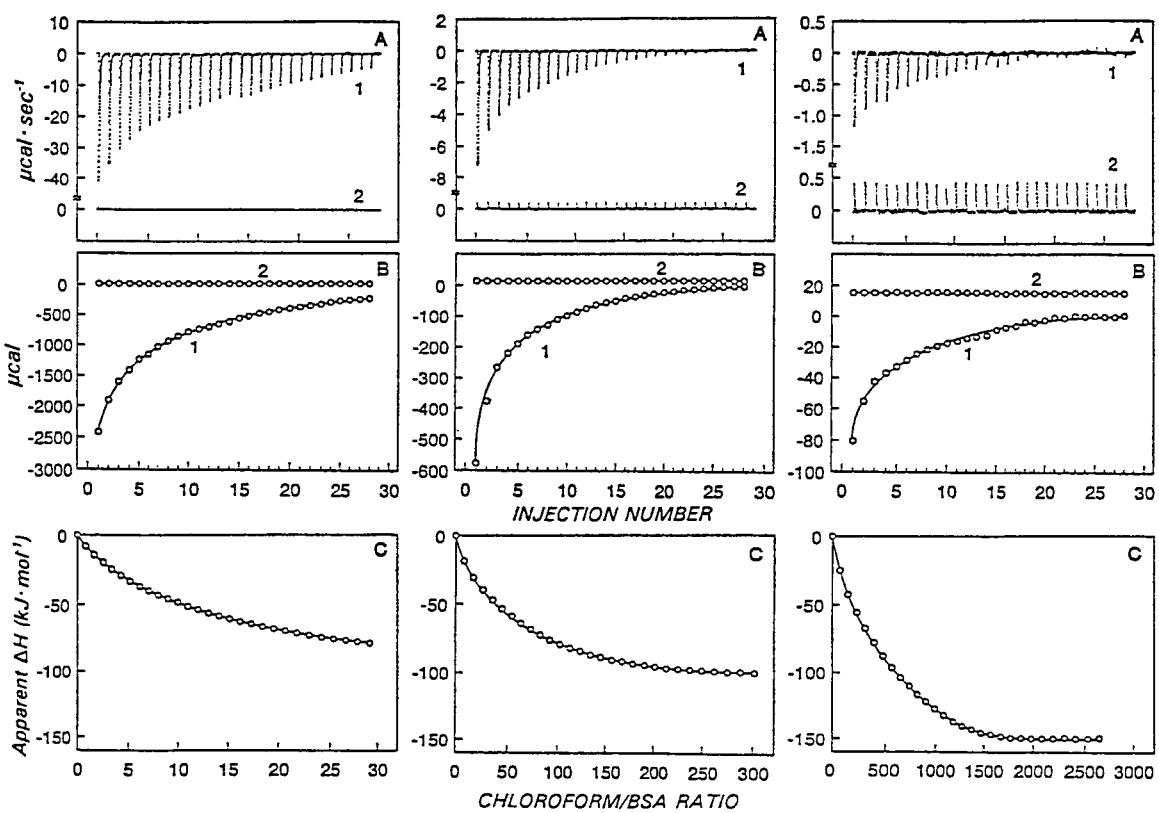

FIGURE 1 Isothermal titration calorimetry profiles at 25°C. Figures are from the left. BSA/chloroform ratio 0.998/53.9 mM, 0.101/56.3 mM, and 0.0105/51.4 mM, respectively. (*A*) Raw titration tracing. Ordinate is the heat flow (μcal · s^{-1}). Abscissa is the time elapsed. Downstroke is the heat evolved and upstroke is the heat absorbed. 1: Titration with chloroform solution; 2: titration with buffer alone. (*B*) Integrated titration heat for each injection. Ordinate is the area under each peak (μcal). Abscissa is the injection number. 1: Titration with chloroform solution; 2: titration with buffer alone. (*C*) Difference in the molar enthalpy of the BSA solution between the absence and presence of chloroform at each injection. Abscissa is the chloroform/BSA mole ratio.

enthalpy change of chloroform-BSA complex formation is assumed to be the standard molar enthalpy change, ΔH, of chloroform. The values of the three BSA concentrations were similar, and the averaged ΔH_{cal} value was -10.37 ± 0.29 kJ · mol^{-1}. The binding isotherms are constructed in Fig. 2 from the free and bound chloroform concentrations.

By fitting free and bound anesthetic molecules to the Scatchard plot (Fig. 3), the affinity constants and the binding numbers of chloroform to BSA were estimated. These plots were nonlinear. It indicates multiple classes of binding sites. Because of the difficulty of identifying many intermediate classes of binding, the high- and low-affinity binding classes were analyzed. The high-affinity interaction was estimated at the lowest chloroform/BSA concentration ratio, where almost all added chloroform molecules are bound to BSA. This corresponds to the initial part of the titration in the high BSA concentration. The low-affinity interaction was estimated at the highest chloroform/BSA concentration ratio, where almost all chloroform-binding sites are occupied. This corresponds to the final part of the titration in the low BSA concentration. The results showed that the high- and low-affinity association constants, K, were 2150 ± 132 (K_D = 0.47 mM) and 189 ± 3.8 M^{-1} (K_D = 5.29 mM), respectively, and the maximum binding numbers for the high- and low-affinity binding were 3.7 ± 0.2 and 13.2 ± 0.3, respectively.

The thermodynamic parameters were estimated from the affinity constant, K, by the equation

$$-RT \ln K = \Delta G = \Delta H - T\Delta S,$$

where R is the gas constant, T is the absolute temperature, G is the Gibbs free energy, and S is the entropy. The thermodynamic parameters for the high- and low-affinity interactions were $\Delta G = -19.0$ kJ · mol^{-1} and $\Delta S = 28.1$ J · K^{-1} · mol^{-1} for high-affinity binding and $\Delta G = -12.9$ kJ · mol^{-1} and $\Delta S = 8.8$ J · K^{-1} · mol^{-1} for low-affinity binding.

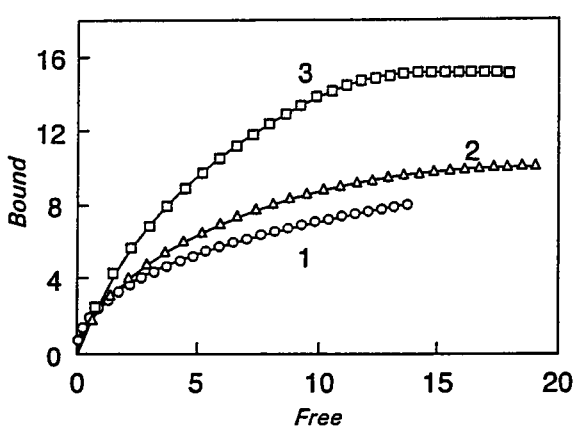

FIGURE 2 Binding isotherms of chloroform to BSA at 25°C. Ordinate: bound chloroform concentration per mole BSA. Abscissa: free chloroform concentration (mM) in water. BSA concentrations: ○, 0.998 mM; △, 0.101 mM; □, 0.0105 mM.

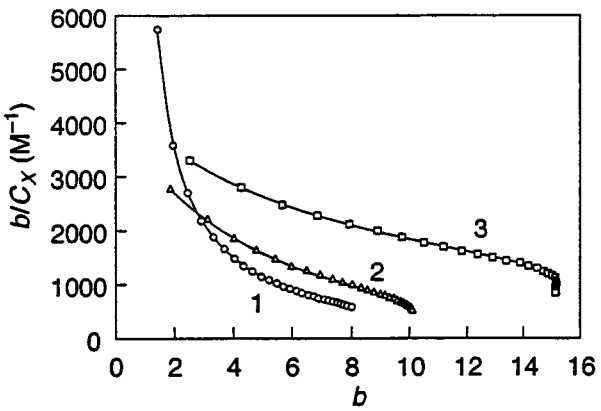

FIGURE 3 The Scatchard plot. Ordinate: the ratio between bound and free chloroform. Abscissa: bound chloroform molecule. C_X is the molarity of free ligand molecules, and b is the averaged number of ligand molecules bound to a BSA molecule. The symbols are the same as Fig. 2.

DISCUSSION

Anesthetic-protein binding mode

Reports on the binding of volatile anesthetics to proteins are few. Dubois and Evers (1992) used ^{19}F-NMR and gas chromatography for isoflurane binding to BSA and reported that the dissociation constant, K_D, was 1.4 mM, with a maximum binding number of 3–4. Dubois et al. (1993) reported K_D values of three other anesthetics on BSA, also using ^{19}F-NMR: halothane, 1.3 mM; methoxyflurane, 2.6 mM; and sevoflurane, 4.5 mM. The binding of anesthetics was inhibited by oleic acid, and they concluded that anesthetics bind to BSA at or near the oleic acid-binding site. However, by a photoaffinity labeling study, Eckenhoff (1996) determined the [^{14}C]halothane binding site on BSA. He demonstrated that the halothane-binding domain is different from the oleic acid-binding domain. Eckenhoff and Shuman (1993) used photoaffinity labeling with [^{14}C]halothane to estimate binding to several lipid-free proteins and polypeptides. They reported that the K_D of halothane binding to BSA was 0.3–0.5 mM. Johanson et al. (1995) used intrinsic fluorescence and obtained a K_D of 1.8 mM for halothane binding to BSA.

The present study showed that the binding was saturable, following Fermi-Dirac statistics (Eyring et al., 1982). The data conformed the Langmuir adsorption isotherms, which is interfacial.

Because the Scatchard plot was nonlinear, the association constant, K, is not a single value and consists of at least two binding classes. The values for the high- and low-affinity bindings were, respectively, 2150 M^{-1} (K_D 0.47 mM) and 189 M^{-1} (K_D 5.3 mM) for the affinity constants, and 3.7 and 13.2 for the maximum binding numbers.

These differences between high- and low-affinity bindings, plus the differences in experimental methods and the choice of plotting equations, probably explain the wide variation in the reports on the dissociation constants of anesthetics with proteins.

Hydrophobic interaction has long been considered an entropy-driven process with positive ΔH values. Recently, however, the hydration effect on nonpolar solute in water and the resulting change in the heat capacity of the solutes led to scrutinization of the concept of hydrophobic effect (Baldwin, 1986; Murphy et al., 1990; Dill, 1990). Seelig and Ganz (1991) reported that several hydrophobic ligands interacted with lipid membranes with negative ΔH. They denoted the binding with negative ΔH as a "nonclassical" hydrophobic effect, as opposed to the "classical" hydrophobic effect, where ΔH is positive.

Katz and Diamond (1974) reported that when the water solubility of ligand decreases, the affinity constant increases and the enthalpy change of the ligand transfer from water (w) to lipid (l), $\Delta H_{w\to l}$, becomes less positive, and the sign would eventually shift from positive to negative. They predicted that in the alcohol series, elongation of the hydrocarbon chain increases the lipid/water partition coefficients and ultimately reverses the sign of $\Delta H_{w\to l}$ to a negative value. Rowe et al. (1995) reported that the binding of octanol to a variety of phospholipid bilayer membranes is accompanied by negative $\Delta H_{w\to l}$ in the range of -1 to -2 kcal/mol (-4 to -8 kJ $\cdot$ mol^{-1}).

Together with the present negative $\Delta H_{w\to p}$ (where subscript p signifies protein) of chloroform interaction with BSA, negative ΔH of transfer of anesthetics from water to macromolecules is not limited to the selected signal transduction proteins.

Temperature dependence of anesthetic potency

It is important to distinguish between concentrations and activities of anesthetics when dealing with potencies. Ferguson (1939) stressed the importance of anesthetic activities at the action site, which is difficult to obtain. He used the chemical potential of anesthetics, which is equal in all phases in equilibrium, and defined the "thermodynamic activity" of anesthetics. Thermodynamic activity is expressed by the ratio of anesthetic partial pressure in the gas phase that anesthetizes half the population, and the vapor pressure of the anesthetic at the same temperature. It is analogous to relative humidity. The humidity one feels is not the amount of water molecules contained in the air (absolute humidity). It is the ratio [water content in the air]/[saturating water content in the air at the same temperature] (relative humidity). When the temperature decreases, the amount of saturating water vapor in the gas phase decreases. One feels more humid at lower temperatures, even though the water content in the air remains constant.

When the anesthetic potency is expressed by MAC (minimum alveolar concentration of anesthetics that induces anesthesia in 50% of the population), the thermodynamic activity of anesthetics is the ratio between MAC (expressed by partial pressure) and the vapor pressure of the neat anesthetics (reference) at the same temperature. The reference vapor pressure is taken in a one-component, two-phase system. Because the degree of freedom is 1, the vapor pressure is fixed by the temperature. Anesthetic potency expressed by partial pressure always increases (MAC decreases) at low temperatures because the vapor pressure of neat anesthetic must decrease at lower temperatures, as dictated by the phase rule. It is the thermodynamic activity of anesthetics that determines the anesthetic potency, not the number of anesthetic molecules present at the action site. Of course, an increase in the number of anesthetic molecules at the action site increases the potency when the anesthetic vapor pressure remains constant. The potency of anesthetics is not absolute to their concentrations, but is relative to their standard state.

The statement of Dickinson et al. (1993) that anesthetic potency always increases at lower temperature fails when the anesthetic concentration is expressed by aqueous concentrations. Anesthetic potencies vary, depending upon where the concentration is measured. When the concentration is expressed in the gas phase, the rank order of potency is methoxyflurane > chloroform > halothane > isoflurane > enflurane > diethylether. When the concentration is expressed in the aqueous phase, the order is halothane > isoflurane > methoxyflurane > enflurane > chloroform > diethylether. The variation is caused by the difference in the solvation of anesthetics in water.

Cherkin and Catchpool (1964) showed that low temperature decreased the potency of diethylether. The EC_{50} of diethylether in goldfish was 1.85 g/liter water at 30°C and increased to 2.56 g/liter water at 5°C. In contrast, the EC_{50} of halothane was 0.0935 g/liter water at 30°C and decreased to 0.0467 g/liter water at 5°C. When the aqueous concentrations are used, lower temperature does not necessarily increase the anesthetic potency.

These discrepancies occur because the anesthetic potency is expressed by the concentration in an arbitrary phase. Anesthetic potency is a function of activity, not concentration. The sign of ΔH measures the temperature-dependent balance of anesthetic concentrations in two phases. Hence the sign of $\Delta H_{w\to p}$ has little importance for the temperature dependence of anesthetic potency.

Living animals are in a highly complex dynamic state of the network of numerous systems. A change in the temperature induces a cascade of a series of stress reactions, involving hormone levels and other signal transduction pathways. The problem of how these changes affect consciousness remains.

APPENDIX

Consider a system composed of a solution of n_M^T moles of protein M and n_X^T moles of ligand X dissolved in n_W moles of solvent W. Provided that the protein molecule has q sites, each capable of combining with the ligand molecule, then q species of protein-ligand complex MXi ($i = 1, 2, \ldots, q$)

are formed in the solution. The enthalpy of such a system is written in the form

$$H = n_W h_W + n_X h_X + \sum_{i=0}^{q} n_i h_i, \quad (1)$$

where n_W and h_W are the number of moles and the partial molar enthalpy of the solvent, respectively; n_X and h_X are those of the free ligand; and n_i and h_i are those of the free protein ($i = 0$) and of the complex MXi ($i = 1, 2, \ldots, q$). The numbers of moles of total ligand and protein molecules are given by

$$n_X^T = n_X + \sum_{i=0}^{q} i n_i \quad (2)$$

and

$$n_M^T = \sum_{i=0}^{q} n_i, \quad (3)$$

respectively. Eq. 1 can be written in the form

$$H = n_W h_w + n_M^T h_0 + n_X^T h_X + \sum_{i=0}^{q} n_i (h_i - h_0 - i h_X). \quad (4)$$

The fourth term of the right side of this equation is related to the formation of the protein-ligand complexes. If we define the averaged partial molar enthalpy change of the complex formation per ligand molecule Δh by

$$\Delta h = \frac{\sum_{i=0}^{q} n_i (h_i - h_0 - i h_X)}{\sum_{i=0}^{q} i n_i} \quad (5)$$

and the averaged number of ligand molecules bound to a protein molecule b by

$$b = \frac{\sum_{i=0}^{q} i n_i}{\sum_{i=0}^{q} n_i} = \frac{n_X^T - n_X}{n_M^T}, \quad (6)$$

we obtain

$$H = n_W h_W + n_M^T h_0 + n_X^T h_0 + n_X^T h_X + n_M^T b \Delta h. \quad (7)$$

Because the enthalpy of the system is a function of temperature T, pressure p, n_W, n_X, and n_i ($i = 0, 1, \ldots, q$), its total differential at constant temperature and pressure is written in the form

$$dH = h_W dh_W + h_X dn_X + \sum_{i=0}^{q} h_i dn_i. \quad (8)$$

Similarly, from Eqs. 1–7 we can derive

$$dH = h_W dn_W + h_0 dn_M^T + h_X dn_X^T + \Delta h d(n_M^T b). \quad (9)$$

For a solution composed of the same amount of solvent and protein as above, but without ligand, the enthalpy of this solution and its total differential at constant temperature and pressure are given by

$$H^0 = n_W h_W^0 + n_M^T h_0^0 \quad (10)$$

and

$$dH^0 = h_W^0 dn_W + h_0^0 dn_M^T, \quad (11)$$

respectively, where superscript 0 means the solution without the ligand. If we assume that the partial molar enthalpies of solvent and free protein in the solution with ligand are equal to the corresponding partial molar enthalpies in the solution without ligand, the difference in the enthalpy between the solutions with or without ligand H_{dif} (the net change in the enthalpy by the addition of ligand to the solution) gives the third and fourth terms of the right side of Eq. 7:

$$H^{dif} \equiv H - H^0 = n_X^T h_X + n_M^T b \Delta h. \quad (12)$$

Furthermore, using Eqs. 9 and 11, we obtain the total differential of H^{dif} in the form

$$dH^{dif} = h_X dn_X^T + \Delta h d(n_M^T b). \quad (13)$$

In the practical analysis of experimental data, thermodynamic quantities per mole of protein are often used. Dividing Eq. 12 by n_M^T, we obtain the enthalpy difference per mole of protein:

$$h^{dif} = \frac{H^{dif}}{n_M^T} = \frac{n_X^T}{n_M^T} h_X + b \Delta h = \alpha_X^T h_X + b \Delta h. \quad (14)$$

Using Eqs. 12 and 13, the total differential of h^{dif} is derived as follows:

$$dh^{dif} = d\left(\frac{H^{dif}}{n_M^T}\right) = h_X d\left(\frac{n_X^T}{n_M^T}\right) + \Delta h db = h_X d\alpha_X^T + \Delta h db. \quad (15)$$

In Eqs. 14 and 15, we have introduced α_X^T ($= n_X^T/n_M^T$) to denote the molar ratio of total ligand molecules to total protein molecules. Applying Eqs. 14 and 15 to the experimental data of h^{dif} as a function of α_X^T, we obtain information about the protein-ligand binding. From Eq. 15 we have

$$\left(\frac{\partial h^{dif}}{\partial \alpha_X^T}\right)_{T,p} = h_X + \Delta h \left(\frac{\partial b}{\partial \alpha_X^T}\right)_{T,p}. \quad (16)$$

Because most of the binding sites on protein molecules are empty in the very low range of α_X^T, almost all of the ligand molecules added in the system bind to the protein molecules, i.e.,

$$\left(\frac{\partial b}{\partial \alpha_X^T}\right) \simeq 1, \quad (17)$$

so that we have

$$\lim_{\alpha_X^T \to 0} \left(\frac{\partial h^{dif}}{\partial \alpha_X^T}\right)_{T,p} = h_X + \Delta h. \quad (18)$$

On the other hand, in the very high range of α_X^T, the binding sites are almost saturated by the ligand molecules, i.e.,

$$\left(\frac{\partial b}{\partial \alpha_X^T}\right)_{T,p} \simeq 0, \quad (19)$$

and we have

$$\lim_{\alpha_X^T \to \infty} \left(\frac{\partial h^{dif}}{\partial \alpha_X^T}\right)_{T,p} = h_X. \quad (20)$$

Thus we can obtain the partial molar enthalpy of ligand h_X by using Eq. 20, and the averaged partial molar enthalpy change of the complex formation per ligand molecule Δh by using

$$\Delta h = \lim_{\alpha_X^T \to 0} \left(\frac{\partial h^{dif}}{\partial \alpha_X^T}\right)_{T,p} - \lim_{\alpha_X^T \to \infty} \left(\frac{\partial h^{dif}}{\partial \alpha_X^T}\right)_{T,p}. \quad (21)$$

Furthermore, because Eq. 14 is rewritten as

$$b = \frac{h^{\mathrm{dif}} - \alpha_X^T h_X}{\Delta h}, \qquad (22)$$

the averaged number of ligand molecules, b, bound to a protein molecule can be estimated.

This study was supported by the DVA Medical Research Funds.

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