Prestegard, J. H., Koerner, T. A. W., Jr., Demou, P. C., & Yu, R. K. (1982) *J. Am. Chem. Soc. 104*, 4993–4995.
Richards, J. C., Perry, M. B., & Kniskern, P. J. (1985) *Can. J. Biochem. Cell Biol. 63*, 953–968.
Richarz, R., & Wuthrich, K. (1978) *J. Magn. Reson. 30*, 147–150.
Robbins, J. B., Lee, C.-J., Rastogi, P. H., Schiffman, G., Austrian, R., Henrichsen, J., Magela, P. H., & Broome, C. V. (1983) *J. Infect. Dis. 148*, 1136–1149.
Shaka, A. J., Keeler, J., Frenkiel, T., & Freman, R. (1983) *J. Magn. Reson. 52*, 335–338.
Smith, A. R. W., Zamze, S. E., Munro, S. M., Carter, K. J., & Hignett, R. C. (1985) *Eur. J. Biochem. 149*, 73–78.
Wagner, G. (1983) *J. Magn. Reson. 55*, 151–156.
Waugh, T. G., Dorin, A. G., McNeil, M., & Albersheim, P. (1983) *Carbohydr Res. 123*, 281–304.
Williams, J. M. (1975) *Adv. Carbohydr. Chem. Biochem. 31*, 9–79.

Quantitative Analysis of Linkage in Macromolecules When One Ligand Is Present in Limited Total Quantity†

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ABSTRACT: We present a general framework for analysis of two closely related problems in biochemical studies: (1) The first is analysis of binding data obtained under conditions in which a second, linked ligand is present in limited total quantity. In such conditions the free activity of the second ligand varies throughout the primary ligand binding curve, and the resultant behavior can be quite complex. Analysis of such curves enables one to quantitatively extract detailed information regarding the linkage of the two ligands at intermediate stages of ligation. The treatment is applied in an accompanying paper to oxygen binding in human hemoglobin in the presence of organic phosphates [Robert, C. H., Fall, L., & Gill, S. J. (1988) *Biochemistry* (following paper in this issue)]. (2) The second treatment we outline regards the analogous problem of analyzing differential scanning calorimetry (DSC) data obtained for a macromolecule binding a ligand present in limited quantity. A simple model is presented that accounts for dual transitions like those already seen in DSC data for human serum albumin in the presence of nonsaturating amounts of fatty acids [Ross, P., & Shrake, A. (1987) *Abstracts of the 42nd Calorimetry Conference*, University of Colorado, Boulder, CO].

The cellular environment can influence the function of a heterotropic linkage system (Wyman, 1964) in two major ways. In one case it may provide a buffering capacity for one ligand, as is often seen in the case of hydrogen ions, maintaining that ligand at a particular activity throughout the binding process of another, primary ligand. However, another common situation is where the total amount of a particular ligand in the cell is limited. The free concentration of this ligand, whose binding affinity for the macromolecule is either increased (positive linkage) or decreased (negative linkage) upon the binding of the primary ligand, can then undergo large variations during the primary binding process. The resultant binding curve of the primary ligand can then be far more complex than that seen under conditions where the secondary ligand is buffered at a particular value. A related complication can appear in thermal transition curves when the macromolecule is subjected to limited quantities of ligand. The thermodynamic analysis of the effects of limiting the total amount of a ligand is the subject of the present paper.

In the red blood cell a classic example of variation in a linked ligand's activity is that occurring with the effector compound 2,3-diphosphoglycerate (DPG) during oxygen binding to hemoglobin (Benesch & Benesch, 1968). The affinity of hemoglobin for DPG decreases upon oxygen saturation of the molecule. Since the DPG molecule is not free to pass through the cell membrane, its activity increases with increasing oxygenation, and the result can be a complex, biphasic effect on the oxygen binding curve under low chloride conditions (Imai & Tyuma, 1973; Herzfeld & Stanley, 1974). In 1979 Ackers formulated the dependence of the median of the oxygen binding curve under such situations (Ackers, 1979) for a second ligand binding with one-to-one stoichiometry, later applied to the analysis of overall linkage of inositol hexasulfate to oxygen binding in hemoglobin (Ackers et al., 1982). Imai and Tyuma (1973) described the effects one would see on the Adair oxygen binding curve for one-to-one stoichiometry of binding of the second ligand, and Herzfeld and Stanley (1974) used a detailed allosteric model to show similar effects. However, a general phenomenological treatment of the binding curve and median under limited total effector conditions thus far has been unavailable.

The increased complexity of the binding curve often seen in the case of the fixed, nonsaturating amount of second ligand makes it apparent that experiments undertaken with such constraints can furnish more information than can experiments conducted under buffered or excess ligand conditions. In this paper we outline a thermodynamic description of the various equilibria present in systems of a macromolecule binding two types of ligand, especially when one of the ligands, which we

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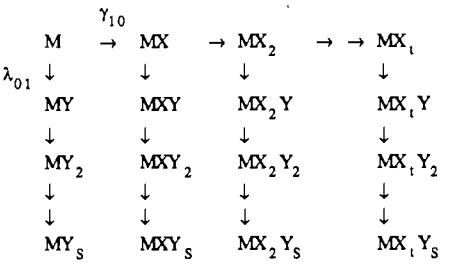


FIGURE 1: Stoichiometric species of a macromolecule in equilibrium with two ligands, X and Y, binding up to t X's and s Y's. Overall binding equilibrium constants are shown for the first reaction of each ligand with the unligated macromolecule.

shall refer to as the second ligand, is present in fixed total amount. Included are the effects both on the detailed shape of the primary ligand binding curve and on its overall position (the median), a measure of the free energy of ligation of the macromolecule. The approach of the present paper applies to any stoichiometry of either primary or secondary ligand. The theory presented here constitutes a complete framework for analysis of the effects of fixed, nonsaturating amounts of organic phosphates on the oxygen binding reactions of human hemoglobin and is thus immediately applied in the accompanying paper (Robert et al., 1988). We also show that the approach developed here applies equally well to the analysis of heat capacity curves from temperature-scanning experiments in the presence of a fixed, nonsaturating amount of ligand. Heat capacity data for such systems have been reported for human albumin transitions in the presence of fatty acids (Shrake & Ross, 1987).

The Partition Function for a Macromolecule with Two Ligands. The binding potential and the binding polynomial have been examined in detail by various authors (Wyman, 1965; Schellman, 1975; Gill et al., 1985; Hill, 1985), so we shall give here only the results for ease in defining the binding curve and its median in later sections.

A system of a nondissociating (and nonaggregating) macromolecule with its ligands can be described by the binding potential Π (the original designation was a Russian L), as shown by Wyman (1965). The extent of binding $\bar{X}$ (the binding curve) is given by $\bar{X} = (\partial\Pi/\partial\mu_X)$, where the partial derivative is evaluated at constant T, p, and μ_Y. (The μ's are the chemical potentials of the ligands.) For mass-law binding of the ligands one can write the statistical mechanical interpretation of Π in terms of P, the binding partition function or the binding polynomial (Wyman, 1965, 1967)

$$\Pi = RT \ln P(x,y) \tag{1}$$

The binding partition function describes the populations of the free energy levels of the macromolecular system relative to a ground-state level, usually the unligated macromolecule. Equivalently, the terms in P are the various macromolecular species concentrations relative to that of the unligated macromolecule.

For the macromolecule in equilibrium with two ligands, the relevant macromolecular species appear schematically in Figure 1. The concentrations of all the species in this array relative to that of the completely unligated macromolecule (in the upper left corner) can be described by a double summation over the x and y bound species, or

$$P = \sum\sum\beta_{ij}x^iy^j \tag{2}$$

where x and y are the respective ligand activities and where the overall equilibrium constant β_{ij} for the reaction $M + iX + jY \rightarrow MX_iY_j$ can be broken up into an X binding part and Y binding part. As shown in Figure 1 we use

$$\beta_{ij} = \lambda_{0j}\gamma_{ij} \tag{3}$$

where the λ_{0j} apply to the unligated molecule binding j of the Y ligands in the absence of X and the γ_{ij} refer to the X binding reactions of the macromolecule while it is ligated with j of the Y ligands. A given species is thus indicated relative to the unligated species by a path moving down the left side of the rectangular array and then across to the appropriate XY ligated species. The binding polynomial 2 is then written

$$P = \sum_j y^j\lambda_{0j}\sum_i\gamma_{ij}x^i \tag{4}$$

where $0 \le i \le t$ and $0 \le j \le s$ and γ_{00} and λ_{00} are both defined equal to unity. It is convenient to define "subbinding polynomials" for X, which are each a summation over the X-ligated species in one particular row j of Figure 1 with the appropriate factor λ_{0j} relating that row to the unligated macromolecule; i.e.

$$P_j(x) \equiv \lambda_{0j}\sum_i\gamma_{ij}x^i \tag{5}$$

and to note that they are not functions of y.[1]

The Binding Curve. As indicated above, the binding curve of the primary ligand X is given by

$$\bar{X} = \left(\frac{\partial \ln P}{\partial \ln x}\right)_y = \frac{x}{P}\frac{\partial P}{\partial x} = \frac{\sum_j y^j x\dfrac{\partial P_j(x)}{\partial x}}{P} \tag{6}$$

and that for the second ligand Y by

$$\bar{Y} = \left(\frac{\partial \ln P}{\partial \ln y}\right)_x = \frac{\sum_j jy^jP_j(x)}{P} \tag{7}$$

These equations provide the binding curves for each ligand provided the value of the activity of the other ligand is specified. The simplest specification is a constant value of the second ligand's activity. Taking X as our primary ligand and Y as the secondary ligand, this is equivalent to assuming that the macromolecular system is effectively buffered by a reservoir of Y at activity y. Imposing a new activity of the second ligand then results in a smooth shift of the first ligand's binding curve, intermediate between that described by the first row of Figure 1 when the activity of Y is zero and that described by the bottom row when the Y activity is "infinite" (saturating). This effect is demonstrated in a simulation shown in Figure 2 by the three parallel X binding curves for a macromolecule binding four X ligands ($t = 4$) and one Y ligand ($s = 1$) at three different but constant values of the activity of Y. At each constant value of the chemical potential of the second ligand, the curves are smooth and parallel. In contrast, the biphasic curve in this figure shows the complications that arise when the second ligand is not buffered and the total amount of the second ligand Y is limited. We shall now incorporate the imposition of such a constraint into the general expression for the binding curve.

When Y is present in limited quantity, one incorporates conservation of mass by noting that the total Y (y_T) that is present must be either bound or free. Identifying the concentration of free ligand Y with its activity[2] y, we then write

$$y_T = y + \bar{Y}\cdot m_T \tag{8}$$

[1] Of course, an alternative formulation enables description of the species in Figure 1 in terms of the columns instead of the rows. This choice leads to $P = \sum_i\gamma_{i0}x^i\sum_j\lambda_{ij}y^j$. The Y subbinding polynomials are thus defined analogously as $P_i(y) \equiv \gamma_{i0}\sum_j\lambda_{ij}y^j$, where each are functions of y but not x. Expressing the binding polynomial in terms of the $P_i(y)$ facilitates writing an expression for the median activity of ligand X.

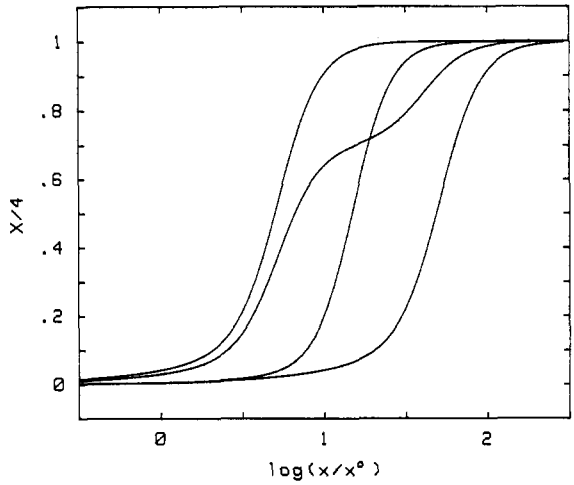


FIGURE 2: Examples of binding of the primary ligand X to a macromolecule in the presence of a second ligand Y for two cases: sigmoidal curves, second ligand buffered at constant activity, zero concentration of Y ligand (left curve), [Y] = 1×10^{-5} M (middle curve), and [Y] = 1×10^{-2} M (saturating, right curve); biphasic curve, limited total amount of second ligand = 0.3 mM (total macromolecule concentration m_T = 1 mM).

where m_T is the total concentration of the macromolecule. We must solve eq 8 for y: Substituting for $\bar{Y}$ from eq 7, we see that the denominator of $\bar{Y}$, that is, the binding polynomial P, is an s degree polynomial in y (where it may be recalled that s is the number of Y binding sites on the macromolecule). Thus, solution of eq 8 involves an equation in y whose degree is one greater than the stoichiometry of Y binding; single-site binding of Y yields a quadratic, two-site binding yields a cubic, and so on. A computer root-finding algorithm serves to find solutions when higher stoichiometry than 2:1 Y binding is required.

Thus, solution of eq 8 with eq 7 at a particular primary ligand activity x provides the equilibrium activity of Y, and both values are then substituted into eq 6 or 7 for the desired value of either binding curve.

Solution of Free Ligand Activity for 1:1 Binding Stoichiometry of Y. As an example of the preceding general formulation, we show the result for one-to-one binding stoichiometry of the second ligand, where there are only two rows of species in Figure 1 and the degree of Y ligation $\bar{Y}$ given by eq 7 is thus

$$\bar{Y} = yP_1(x)/[P_0(x) + yP_1(x)] \qquad (9)$$

where $P_0(x)$ and $P_1(x)$ are the x subbinding polynomials for zero Y saturation and full Y saturation (1 molecule), respectively, as given by 5. The corresponding solution of the quadratic resulting from substitution of eq 9 into 8 is

$$y = \left\{-\left(m_T - y_T + \frac{P_0(x)}{P_1(x)}\right) + \left[\left[m_T - y_T + \frac{P_0(x)}{P_1(x)}\right]^2 + 4y_T\frac{P_0(x)}{P_1(x)}\right]^{1/2}\right\} \Big/ 2 \qquad (10)$$

This equation allows calculation of the free second ligand concentration at any given activity of the primary ligand (x).

Substituting the value of the free activity of Y into the extent-of-binding eq 6 for X, we arrive at the complete formulation of the X binding curve under limiting conditions of Y for this example for one-to-one stoichiometry of Y binding. This is also the result obtained by Imai and Tyuma (1973). In Figure 3 we show a three-dimensional representation of binding curves for X calculated for a series of limiting total amounts of Y. The example shown in Figure 3 is that of human hemoglobin binding oxygen under conditions of different limited total amounts of inositol hexaphosphate (IHP) as described in the following paper (Robert et al., 1988). The binding curve when Y is in limited quantity can be seen to be biphasic in the region of subsaturating total Y.

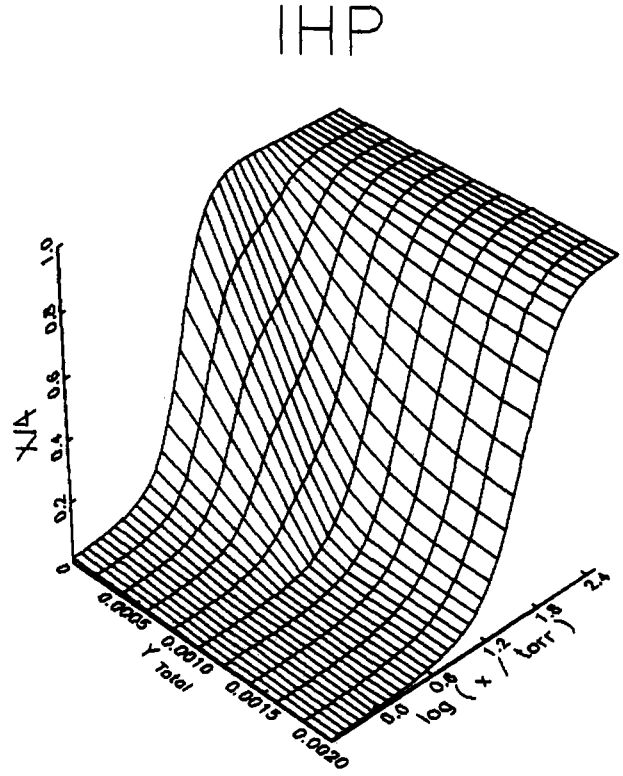


FIGURE 3: Three-dimensional representation of the binding curves ($\bar{X}$) for oxygen to human hemoglobin (t = 4), in the presence of the 1:1 stoichiometry (thus s = 1) second ligand inositol hexaphosphate (IHP) (25 °C, 0.1 M HEPES, 0.1 M Cl^-, pH 7.5, [Hb] = 0.001 M), with overall binding constants as determined by Robert et al. (1988): for oxygen $\gamma_{10} = 0.18$ Torr^{-1}, $\gamma_{20} = 0.011$ Torr^{-2}, $\gamma_{30} = 0$ Torr, and $\gamma_{40} = 0.0022$ Torr^{-4}; for IHP binding to each oxygenated form $\lambda_{01} = 4.1 \times 10^6$ M^{-1}, $\lambda_{11} = 6 \times 10^5$ M^{-1}, $\lambda_{21} = 3 \times 10^5$ M^{-1}, and $\lambda_{41} = 1.6 \times 10^3$ M^{-1}.

The Median of the Binding Curve. It is useful to define the overall position of the binding curve by its median. This property is related to the overall work of ligation per mole of macromolecule (Wyman, 1964):

$$W_X = RT \ln x_m^t \qquad (11)$$

for a macromolecule with t sites for binding ligand X. The work of ligation is a function of the particular constraints held on the linked Y component and is thus dependent on whether or not the activity of Y is constant or the total amount of Y is limited.

The median of the binding curve is that chemical potential of X, μ_{Xm}, at which the area below the binding curve to the left of that potential and the area above the binding curve, bounded by $\bar{X} = t$, to the right of that potential are equal. Defining μ_{Xm} for the integration just described is then

$$\int_{\mu_X=-\infty}^{\mu_{Xm}} \bar{X}\, d\mu_X = \int_{\mu_{Xm}}^{\mu_X=\infty} (t - \bar{X})\, d\mu_X \qquad (12)$$

In order to evaluate the effect of the ligand Y upon the median

[2] Such an approximation holds for solutions dilute in Y. At higher concentrations, particularly with charged ligands, conditions can often be chosen (e.g., high ionic strength from buffers etc.) such that the activity coefficient is effectively constant, and thus it can formally be absorbed into the equilibrium constants for the various reactions.

chemical potential μ_{Xm}, one must express each of the quantities in eq 12 in terms of the chemical potential of Y.

Since $\bar{X}$ is the partial differential of $\Pi(\mu_X,\mu_Y)$ with respect to μ_X, the total differential (under conditions of constant p and T) allows the substitution into eq 12 of

$$\bar{X}\,d\mu_X = d\Pi - \bar{Y}\,d\mu_Y \tag{13}$$

and the integrals in eq 12 can be combined to yield

$$\partial\Pi\int_{-\infty}^{\infty} = t\mu_X\int_{\mu_{Xm}}^{\infty} + \int_{\mu_X=-\infty}^{\mu_X=\infty}\bar{Y}\,d\mu_Y \tag{14}$$

The binding potential is next expressed in terms of the logarithm of the binding polynomial P (eq 2), and it may be noted that the overall binding polynomial evaluated at the extremes $x = 0$ ($\mu_X = -\infty$) and $x = \infty$ ($\mu_X = \infty$) is just the two extreme Y binding polynomials $P_0(y)$ and $P_t(y)$ (left and right columns in Figure 1 as shown in footnote 1) multiplied by the X activity x raised to the 0th and the tth power, respectively. Expression of the differential of the chemical potential at constant T, i.e., $d\mu_X = RT\,d\ln x$, and cancellation of RT factors then leaves

$$\ln\frac{x_\infty^t P_t(y_{x=\infty})}{P_0(y_{x=0})} = t\ln\frac{x_\infty}{x_m} + \int_{x=0}^{x=\infty}\frac{\bar{Y}}{y}\,dy \tag{15}$$

which can be further reduced and rearranged to

$$t\ln x_m = \ln\frac{P_0(y_{x=0})}{P_t(y_{x=\infty})} + \int_{x=0}^{x=\infty}\frac{\bar{Y}}{y}\,dy \tag{16}$$

When the activity of Y is constant, as in buffered conditions, the integral remaining in eq 16 is zero, and the median of the X binding curve is modulated solely by the ratio of the two extreme Y binding polynomials. The behavior of the median under such conditions for the binding of organic phosphates was described by Szabo and Karplus (1976). However, in the case of limited total amount of Y, the Y activity changes so that the integral in eq 15 is not zero. Rearranging the conservation eq 8 gives

$$\frac{\bar{Y}}{y} = \frac{1}{y}\frac{y_T}{m_T} - \frac{1}{m_T} \tag{17}$$

Incorporation into eq 16 and integration then yields after rearrangement

$$\ln x_m = \frac{1}{t}\left[\ln\frac{P_0(y_{x=0})}{P_t(y_{x=\infty})} + \frac{y_T}{m_T}\ln\frac{y_{x=\infty}}{y_{x=0}} - \frac{1}{m_T}(y_{x=\infty} - y_{x=0})\right] \tag{18}$$

Again we must solve eq 8 for the free activity of Y, but here we must solve it at the two extremes $x = 0$ and $x = \infty$. For this task it is easiest to evaluate $\bar{Y}_{x=0}$ and $\bar{Y}_{x=\infty}$. These quantities can be evaluated directly from eq 7 by eliminating respectively higher or lower order terms in x. However, the general result is more easily seen when eq 7 is rewritten in a form analogous to eq 6 for X binding; that is, the species array shown in Figure 1 can be described in terms of the columns (Y subbinding polynomials) rather than the rows (X subbinding polynomials) as shown in footnote 1. The quantities $\bar{Y}_{x=0}$ and $\bar{Y}_{x=\infty}$ describing the extent of Y saturation at these limiting X activities can then be written compactly:

$$\bar{Y}_{x=0} = \partial\ln P_0(y)/\partial\ln y \tag{19}$$

and

$$\bar{Y}_{x=\infty} = \partial\ln P_t(y)/\partial\ln y \tag{20}$$

where the partial derivative here refers to constancy of p and T. Equations 19 and 20, when substituted in turn into eq 8, provide the limiting values of the free Y activity for use in eq 18. Equation 18 thus provides the dependence of the overall position of the X binding curve in the presence of a fixed quantity of ligand Y.[3]

Median Dependence for 1:1 Stoichiometry of Y Binding. Again we show an example of how the general eq 18 applies to the case of one-to-one binding of the second ligand Y: In this situation the solutions of eq 8 combined with eq 19 or 20 to give $y_{x=0}$ and $y_{x=\infty}$, respectively, are

$$y_{x=0} = \frac{-\left(m_T - y_T + \frac{1}{\lambda_{01}}\right) + \left[\left(m_T - y_T + \frac{1}{\lambda_{01}}\right)^2 + 4y_T\frac{1}{\lambda_{01}}\right]^{1/2}}{2} \tag{21}$$

and

$$y_{x=\infty} = \frac{-\left(m_T - y_T + \frac{1}{\lambda_{t1}}\right) + \left[\left(m_T - y_T + \frac{1}{\lambda_{t1}}\right)^2 + 4y_T\frac{1}{\lambda_{t1}}\right]^{1/2}}{2} \tag{22}$$

For a single Y binding site $P_0(y) = 1 + \lambda_{01}y$ and $P_t(y) = \gamma_{t0}(1 + \lambda_{t1}y)$. Thus, for the case of 1:1 stoichiometry of Y binding to the macromolecule these equations, when combined with eq 18, allow one to describe the median dependence on the concentration of Y in the system and the total concentration of macromolecule. Although of a different form and arrived at in a different way, this relation for 1:1 stoichiometry of Y binding shows the same dependence as that obtained by Ackers (1979).

Linkage Relations. A standard linkage relation for evaluating the number of Y ligands linked to the binding of X is

$$(\partial\bar{Y}/\partial\bar{X})_{\mu_Y} = -(\partial\mu_X/\partial\mu_Y)_{\bar{X}} \tag{23}$$

(Wyman, 1964). One can thus measure the change of the chemical potential of X required to achieve a given X saturation versus the change in chemical potential of Y and, with eq 23, interpret this quantity as the differential amount of release of the Y ligand with X binding at that saturation. Particular applications have been the reactions of hemoglobin with oxygen as the primary ligand, where either the release of the second ligand was buffered by a chemical buffer, as in the case of protons (Antonini & Brunori, 1971; Imai, 1982) or the second ligand was a dissolved gas in equilibrium with a large gas phase, as in the case of CO_2 (Doyle et al., 1987; Imaizumi et al., 1982). In other cases the second ligand was present in large excess, as occurs in the case of chloride ion (Imaizumi et al., 1979).

However, when the second ligand Y is neither buffered nor present in large excess, the relation (23) is not as easily obtained since the chemical potential of the second ligand varies throughout the binding curve. In this situation it is convenient to consider another of the group of potentials derivable by successive Legendre transformations of the normalized Gibbs energy (Wyman, 1984). It is helpful to write these in terms of the total amounts of X and Y in the system normalized to the amount of macromolecule (ν_X and ν_Y) and the two chemical potentials μ_X and μ_Y. In this case, a single transformation

[3] We initially obtained these equations in the course of preparing a book manuscript (J. Wyman and S. J. Gill, in preparation); they appeared in a slightly modified form in a recent paper (Kister et al., 1987) in which the source was inadvertently omitted (Kister et al., 1988).

with respect to the ν_X, μ_X parameter couple applies, leaving the ν_Y, μ_Y couple unaffected. This potential, termed $\bar{G}^{II}$ (Wyman, 1984), is relevant to the situation at hand, with natural variables μ_X and ν_Y: the chemical potential of X and the total amount of Y.

If the system were identified as the macromolecule alone, the normalized quantities ν_X and ν_Y would be simply $\bar{X}$ and $\bar{Y}$. But here we identify the system as the macromolecule together with the available surrounding solution, so that the quantities ν_X and ν_Y refer to the total amounts of each ligand relative to the total amount of the macromolecule component, and thus include both bound and free ligand contributions, or

$$\nu_X = \bar{X} + x/m_T \tag{24}$$

and the analogous equation holds for Y. The system in this way can be effectively considered as two phases, one being the macromolecular phase and the other the intermacromolecular solution. The components X and Y are free to exchange from one phase to the other. For a fixed quantity of the two phases the state of such a system at equilibrium can be completely defined by the chemical potential of X and the total amount of Y. A general approach to macromolecular multiphase systems has appeared (Wyman & Gill, 1975).

$d\bar{G}^{II}$ is an exact differential, yielding the linkage relation relevant to the present situation of fixed total Y

$$(\partial\nu_X/\partial\nu_Y)_{\mu_X} = -(\partial\mu_Y/\partial\mu_X)_{\nu_Y} \tag{25}$$

where again T and p are understood to be held constant. The measurement implied in this relation is better seen when it is noted by eq 24 that at constant μ_X the differential $d\nu_X$ is given by $d\bar{X}$, resulting in

$$(\partial\bar{X}/\partial\nu_Y)_{\mu_X} = -(\partial\mu_Y/\partial\mu_X)_{\nu_Y} \tag{26}$$

The left side of eq 26 can thus be evaluated graphically from Figure 2 or 3 by observation of the change in $\bar{X}$ that one sees following along a vertical line at a given μ_X resulting from a change in the total amount of Y in the system. The increments in total Y in Figure 3 are spaced equally so that it is evident that for a given X binding curve the magnitude of this function increases to an intermediate point in the binding curve and then decreases. Equation 26 then shows that this behavior can be taken as the differential change in the chemical potential of Y at each point in the binding curve.

In order to illustrate the linkage under conditions of limited quantities of one ligand, we have evaluated analytically the derivative on the left-hand side of eq 26 for the example of oxygen and inositol hexaphosphate binding to hemoglobin as determined in the accompanying paper. This function is plotted in Figure 4 versus the logarithm of x activity for a series of total amounts of the second ligand. Equation 26 shows that this behavior is equivalent to the negative of the change in the chemical potential of Y that results from a given change in the chemical potential of X at fixed total amount of Y. In other words, this linkage equation enables one to evaluate the efficiency of the signal transduction, the conversion of a chemical signal X into another chemical signal Y.

Heat Capacity Effects. The binding partition function for a macromolecule is a function of all the physical variables, usually T and p, and the chemical variables μ_X, μ_Y, etc. (Gill et al., 1985). The effect of limiting a ligand's total amount has been so far discussed in relation to binding another ligand. However, the same idea carries over into other thermodynamic properties such as the enthalpy or volume. An example of this is a temperature-scanning experiment in which the heat capacity, the temperature derivative of the enthalpy, of a macromolecule is observed as the temperature is scanned. The

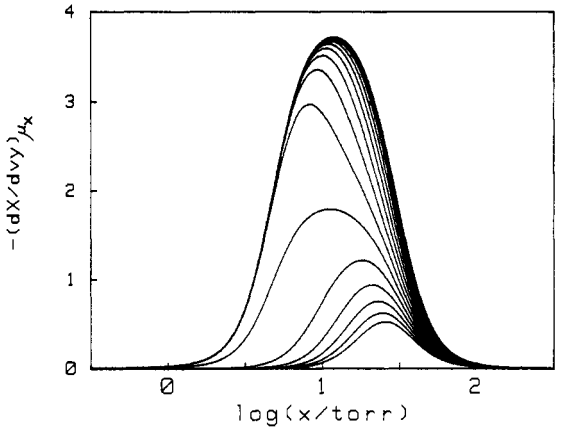
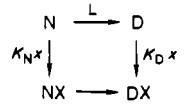


FIGURE 4: Family of curves depicting the efficiency of transduction of a change in chemical potential of X into a change of chemical potential of Y, where the total amount of Y is limited in the system. Total concentration of macromolecule m_T = 0.001 M. The concentrations of Y ($\nu_Y \cdot m_T$) used for the curves are as follows (from top): 0.0001, 0.0002, ..., 0.0015 M. The functions described are calculated for the Hb/O_2/IHP example given in Figure 3.

presence of fixed, nonsaturating amounts of a single ligand can result in complex behavior of the heat capacity curve. Such an effect has been reported by Shrake and Ross (1987) for denaturation curves of human albumin in the presence of limiting amounts of long-chain fatty acids. We shall show here briefly how such temperature effects can be handled using essentially the same formalism just outlined.

A simple system to illustrate this point is a macromolecule that undergoes a two-state thermal denaturation and is subject to ligand binding at a single site on both native and denatured forms, or



A linkage of the ligand binding to the temperature will be seen if the equilibrium constants or enthalpy changes (or both) for the ligand binding to the native (N) and the denatured (D) forms are different. Thus, for convenience we shall assume that there is no binding of the ligand to the denatured form of the macromolecule (i.e., $\kappa_D = 0$). The partition function for the resulting system is

$$P = (1 + \kappa_N x) + L \tag{27}$$

where x is as usual the activity of ligand X. In practice P will be normalized to the species or set of species desired as the reference state for expression of a given thermodynamic quantity. For denaturational heat capacity changes the normalization factor P_0 is the binding polynomial for the native macromolecule, including all its native states, or $P_0 = 1 + \kappa_N x$. As can be verified by the van't Hoff equation, the enthalpy of a nondissociating macromolecular system with one ligand X is obtained from the normalized binding partition function by a logarithmic partial derivative with respect to the inverse temperature (Gill et al., 1985):

$$\bar{H} - \bar{H}_0 = -R[\partial \ln (P/P_0)/\partial(1/T)]_{p,\mu_X} \tag{28}$$

where $\bar{H}_0$ thus refers to the enthalpy of the system in the native state. For an open system with ligand X held at constant chemical potential, the heat capacity of the system ΔC_p is the partial derivative of $\bar{H} - \bar{H}_0$ with respect to T at constant pressure and μ_X:

$$\Delta C_{p,\mu_X} = [\partial(\bar{H} - \bar{H}_0)/\partial T]_{p,\mu_X} \tag{29}$$

where the Δ in front of C_p indicates the change from the reference state heat capacity (native macromolecule).

The effect of limiting the total amount of the ligand is incorporated just as it was in the previous case: The conservation of mass equation is written

$$x_{tot} = x + \bar{X}m_{tot} \quad (30)$$

and the $\bar{X}$ quantity must be evaluated. For ligation properties such as $\bar{X}$ the normalization factor P_0 is the partition function for the unligated state, or 1 + L for this case. We write $\bar{X}$ by the derivative

$$\bar{X} = [\partial \ln (P/P_0)/\partial \ln x]_{T,p} \quad (31)$$

where it is seen that the normalizing factor will cancel since it is not a function of x. The solution of eq 30 after substitution of eq 31 provides the free activity of the ligand X at any point in the scanning experiment. Again, for single-site binding of the ligand, eq 30 with 31 yield a quadratic in the ligand activity x, two binding sites yield a cubic, and so on.

The system is closed with respect to ligand, whose activity will vary according to its linkage to temperature through the state of the macromolecule. Thus, it is important to recognize that in obtaining the heat capacity derivative of the enthalpy for the case of limited total ligand, one must incorporate differential changes in the ligand activity as well. The proper derivative can easily be calculated numerically, or else eq 29 can be used: at constant pressure $\bar{H} - \bar{H}_0 = f(T,\mu_X)$ so that

$$\Delta C_p = \left[\frac{\partial(\bar{H} - \bar{H}_0)}{\partial T}\right]_{\mu_X} + \left[\frac{\partial(\bar{H} - \bar{H}_0)}{\partial \mu_X}\right]_T \cdot \frac{d\mu_X}{dT} \quad (32)$$

where constant pressure is assumed in all terms and the two quantities in the right-hand term must be calculated from eq 28 and 30.[4]

To illustrate, we have simulated the heat capacity behavior of lysozyme in the presence of less than saturating amounts of substrate, the compound tri-*N*-acetylglucosamine (NAG_3). The quantities related to the thermal denaturation in the presence of ligand or substrate were taken from the data of Velicelebi and Sturtevant (1979), whose values agree with measurements by Privalov and Khechinashvili (1974). Binding constants and heats of binding for the substrate were given by Pace and McGrath (1980); again these values agree with earlier determinations by Banerjee and Rupley (1973). The actual lysozyme system in the absence of substrate exhibits a single, smooth transition in scanning calorimetric experiments (Privalov, 1974; Sturtevant, 1979). As can be seen from Figure 5, plotting a series of simulated curves under increasing limiting amounts of substrate shows the predicted behavior of this system under conditions of limiting ligand to be quite complex: a dual-transition effect is observed, resulting from stabilization provided by the substrate to the limited fraction of macromolecules to which it is bound.

The enthalpy versus temperature curve for the system, the derivative of which is the heat capacity curve, is shown in Figure 6. Unlike the ligand binding quantity $\bar{X}$ of Figure 3, $\bar{H}$ here shows an asymptotic behavior characterized by a constant slope, reflecting the constant heat capacity change of the denaturation. The double hump in the derivative curve of Figure 5 better reveals the slightly biphasic behavior seen of the enthalpy versus T curve, in analogy to the biphasic behavior of the primary ligand binding curve versus μ_X for the two-ligand case.

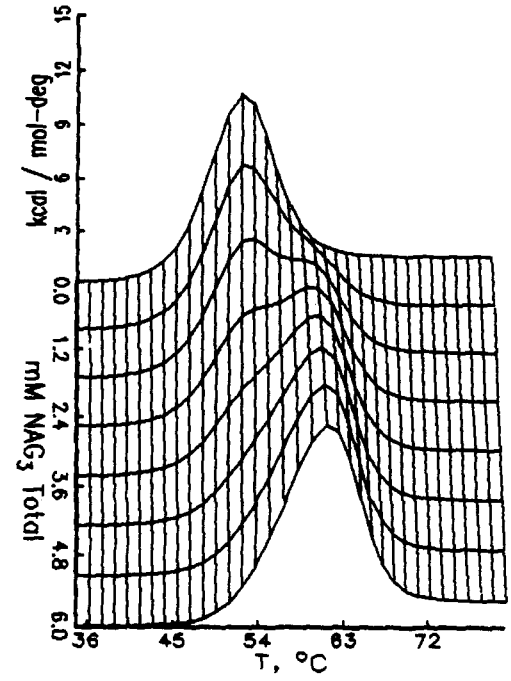


FIGURE 5: Simulation of heat capacity of lysozyme under subsaturating substrate conditions. Heat capacity surface shown is calculated at constant pressure relative to that of the native state for a simulated differential scanning calorimetry of the denaturation of lysozyme with a single binding site for the substrate ligand tri-*N*-acetylglucosamine (NAG_3) as described in the text. Constants used in the simulation: $L(57\ °C) = 1$, $\Delta H_L(57\ °C) = 91$ kcal/mol, and $\Delta C_p = 1.6$ kcal/mol (Velicelebi & Sturtevant, 1979); $K_N(53\ °C) = 10^3\ M^{-1}$ and $\Delta H_K = -8.2$ kcal/mol, constant over this temperature range (Pace & McGrath, 1980); $m_T = 0.005$ M.

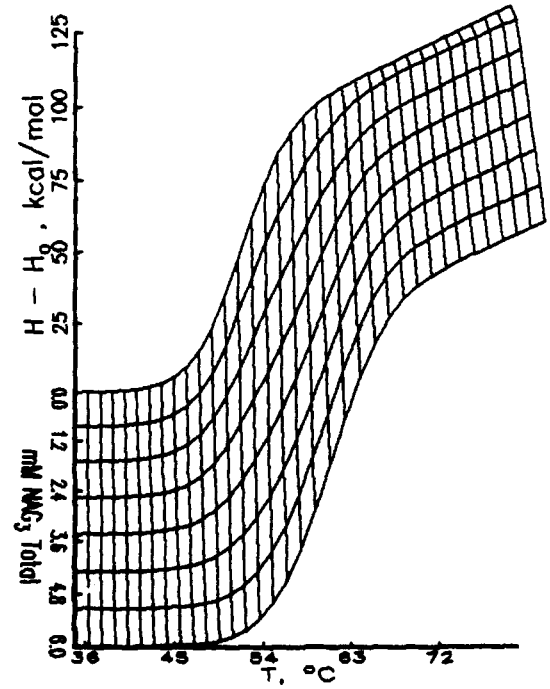


FIGURE 6: Three-dimensional view of the average enthalpy per mole of lysozyme simulated as in Figure 5. Derivatives of this surface with respect to temperature at each total ligand concentration are the heat capacity curves shown in Figure 5.

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[4] When species other than the macromolecule interact with the ligand, as does a buffer, the term $d\mu_X/dT$ will include the effect of temperature on the buffer binding reaction as well as the macromolecular linkage (eq 30).

of their scanning calorimetric experiments with human albumin. We express thanks to Enrico Di Cera regarding evaluation of the heat capacity under limiting ligand conditions and to Alfredo Colosimo, who provided assistance in the simulation of lysozyme denaturation.

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REFERENCES

Ackers, G. K. (1979) *Biochemistry 18*, 3372-3380.
Ackers, G. K., Benesch, R. E., & Edalji, R. (1982) *Biochemistry 21*, 875-879.
Antonini, E., & Brunori, M. (1971) *Haemoglobin and Myoglobin in Their Reactions with Ligands*, North-Holland, Amsterdam.
Bannerjee, S. K., & Rupley, J. A. (1973) *J. Biol. Chem. 248*, 2117-2124.
Benesch, R., Benesch, R. E., & Yu, C. I. (1968) *Proc. Natl. Acad. Sci. U.S.A. 59*, 526-532.
Doyle, M. L., Di Cera, E., Robert, C. H., & Gill, S. J. (1987) *J. Mol. Biol. 196*, 927-934.
Gill, S. J., Richey, B., Bishop, G. A., & Wyman, J. (1985) *Biophys. Chem. 21*, 1-14.
Herzfeld, J., & Stanley, H. E. (1974) *J. Mol. Biol. 82*, 231-265.
Hill, T. (1986) *Cooperativity Theory in Biological Systems*, Springer-Verlag, Heidelberg.
Imai, K. (1982) *Allosteric Effects in Human Haemoglobin*, Cambridge University Press, Cambridge.
Imai, K., & Tyuma, I. (1973) *Biochim. Biophys. Acta 293*, 290-294.
Imaizumi, K., Imai, K., & Tyuma, I. (1979) *J. Biochem. (Tokyo) 86*, 1829-1840.
Imaizumi, K., Imai, K., & Tyuma, I. (1982) *J. Mol. Biol. 159*, 703-719.
Kister, J., Poyart, C., & Edelstein, S. J. (1987) *Biophys. J. 52*, 527-535.
Kister, J., Poyart, C., & Edelstein, S. J. (1988) *Biophys. J.* (Erratum) (in press).
Pace, C. N., & McGrath, T. (1980) *J. Biol. Chem. 255*, 3862-3865.
Privalov, P. L., & Khechinashvili, N. N. (1974) *J. Mol. Biol. 86*, 665-685.
Robert, C. H., Fall, L., & Gill, S. J. (1988) *Biochemistry* (following paper in this issue).
Schellman, J. A. (1975) *Biopolymers 14*, 999-1018.
Shrake, A., & Ross, P. (1987) *Abstracts of the Calorimetry Conference*, University of Colorado, Boulder, CO.
Szabo, A., & Karplus, M. (1976) *Biochemistry 15*, 2869-2877.
Velicelebi, G., & Sturtevant, J. M. (1979) *Biochemistry 18*, 1180-1186.
Wyman, J. (1964) *Adv. Protein Chem. 19*, 223-286.
Wyman, J. (1965) *J. Mol. Biol. 11*, 631-644.
Wyman, J. (1967) *J. Am. Chem. Soc. 89*, 2202-2218.
Wyman, J. (1984) *Q. Rev. Biophys. 17*, 453-488.
Wyman, J., & Gill, S. J. (1975) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5239-5242.

Linkage of Organic Phosphates to Oxygen Binding in Human Hemoglobin at High Concentrations†

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ABSTRACT: We have performed high-precision oxygen binding studies on human hemoglobin tetramers in the presence of a series of limited, subsaturating amounts of the effector compounds 2,3-diphosphoglycerate (DPG) and inositol hexaphosphate (IHP). The use of thin-layer optical methods enabled the use of high hemoglobin concentrations, preventing complications arising from the dissociation of the tetramer into dimers. Model-independent, simultaneous analysis of all data for each effector demonstrated that the intrinsic oxygen binding characteristics of the molecule are in agreement with those determined in earlier high-precision studies [e.g., Gill, S. J., Di Cera, E., Doyle, M. L., Bishop, G. A., & Robert, C. H. (1987) *Biochemistry 26*, 3995-4002] and that the affinity of the tetramer for the tightly binding effector IHP changes most markedly between the second and fourth oxygen binding steps, perhaps indicating a large conformational change. The data were then analyzed by using the truncated allosteric model [Di Cera, E., Robert, C. H., & Gill, S. J. (1987) *Biochemistry 26*, 4003-4008], which is based on the hypothesis that a quaternary conformational change occurs in the hemoglobin tetramer before the third and fourth oxygen molecules bind.

Organic phosphates are well-known regulators of the oxygen binding affinity of human hemoglobin. Of the physiological effectors, 2,3-diphosphoglycerate (DPG) is of primary importance (Benesch et al., 1968, 1971), while of the non-endogenous compounds, inositol hexaphosphate (IHP) has proven valuable because of its strong linkage. These compounds bind with a stoichiometry of one molecule per hemoglobin tetramer (Benesch et al., 1968, 1971; Janig et al., 1971; Edalji et al., 1976) and are thought to bind at the same site. It is likely that the mechanisms of their linkage to oxygen binding are similar as well. Their study can be seen to be especially germane when one considers that the ligand binding site, situated between the β-chain termini of the $\alpha_2\beta_2$ tetramer (Arnone, 1972; Arnone & Perutz, 1974; Perella et al., 1975;

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