Linked Functions in Allosteric Proteins: An Exact Theory for the Effect of Organic Phosphates on Oxygen Affinity of Hemoglobin†

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ABSTRACT: A new thermodynamic analysis has been developed for the linkage between binding of two ligands (e.g., oxygen and organic phosphate) to a nondissociating macromolecule (e.g., hemoglobin tetramer). The theory relates the median ($\bar{X}$) of an oxygenation curve, measured in the presence of organic phosphate, to any three of the following equilibrium constants: K_4 (for binding four oxygens onto the tetramer in the absence of organic phosphate), K_{D4} (for binding four oxygens onto the tetramer under saturating amounts of organic phosphate), ${}^0K_D$ (for binding organic phosphate onto unliganded tetramers), and ${}^4K_D$ (for binding organic phosphate onto fully oxygenated tetramers). In contrast with previous linkage theories [cf. Wyman, J. (1964) *Adv. Protein Chem. 19*, 223–286], the present development relates observable properties of binding isotherms for one ligand to the *total* concentrations of the second ligand, and of the macromolecule. In most experimental situations it is the total concentration of the second ligand which is known, whereas the concentration of its free (i.e., unbound) form is not accessible. In the case of human hemoglobin, the new theory opens up the possibility of carrying out interpretable experiments in previously inaccessible regions of the experimental variables where the concentrations of hemoglobin–phosphate complexes and unbound phosphate are of comparable magnitude. Some illustrative applications are presented, and the ranges of validity of the earlier approximate theories have been evaluated. The new theory is found especially pertinent to allosteric systems where binding of an effector molecule occurs with high affinity such as with inositol hexaphosphate binding to human hemoglobin. The theoretical approach employed in this work is general and can be applied to linked binding systems other than hemoglobin. Some examples are mentioned. The relationship of this work to the corresponding theory for subunit polymerization [Ackers, G. K., & Halvorson, H. R. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4312–4316; Johnson, M. L., Halvorson, H. R., & Ackers, G. K. (1976) *Biochemistry 15*, 5363–5371] is discussed, and it is shown how the approach can also be extended to cases of ligand binding linked with conformational equilibria.

The oxygen binding properties of vertebrate hemoglobins are regulated under physiological conditions by the reversible binding of organic phosphates (Benesch & Benesch, 1967, 1974; Chanutin & Curnish, 1967), notably 2,3-diphosphoglycerate (DPG), adenosine triphosphate (ATP), and inositol hexaphosphate (IHP). The general mechanism for this regulation has been shown to lie in the preferential binding of the organic phosphate molecule (usually a single molecule) to the deoxygenated form of the hemoglobin tetramer [cf. Benesch & Benesch (1974) for a general review]. X-ray structural studies have provided a stereochemical rationale for this preferential binding (Arnone & Perutz, 1974). In addition to their physiological role, much interest in these molecules stems from their use as tools (particularly IHP) for probing and manipulating the quaternary structural transitions in normal, mutant, and chemically modified hemoglobins. Although these effects have been studied for several years and approximate formulas used to describe the experimental observations (Benesch et al., 1971; Berger et al., 1973; Bare et al., 1974; Baldwin, 1975; Szabo & Karplus, 1976), there has not been an exact theory for the dependence of hemoglobin oxygen affinity upon the experimental concentrations of total organic phosphate and hemoglobin and upon the fundamental binding constants of the system. In this paper such a relationship is derived and some illustrative applications to the case of human hemoglobin are shown. The new theory opens up the possibility of carrying out interpretable experiments in previously inaccessible regions of the experimental variables where the concentrations of hemoglobin–phosphate complexes and unbound phosphate are of comparable magnitude. Additionally it provides a means to evaluate conditions under which the approximate theories are valid. The new theory is general so that it can be applied to other systems of experimental interest.

The most commonly observed manifestation of the linkage between oxygen binding and organic phosphate binding is a progressive shift of oxygen binding curves toward decreasing affinities with increasing concentrations of organic phosphate, as predicted by the linkage theory of Wyman (1964) and demonstrated originally by Benesch et al. (Benesch & Benesch, 1967; Benesch et al., 1971). At any fixed total concentration of organic phosphate, (D_t), a general measure of the overall hemoglobin affinity for oxygen is provided by the median oxygen activity, ($\bar{X}$) (or corresponding partial pressure) as defined in eq 1

$$\int_0^{(\bar{X})} \bar{Y}\, d \ln (X) = \int_{(\bar{X})}^{\infty} (1 - \bar{Y})\, d \ln (X) \qquad (1)$$

where $\bar{Y}$ is the fractional saturation corresponding to the molar concentration (X) of dissolved oxygen (assuming ideal behavior for the oxygen solubility so that concentrations and activities are equal). The concentration (X) is equal to the product of partial pressure, P, and the Henry's law constant, K_H, i.e., $(X) = K_H P$. The median oxygen concentration, ($\bar{X}$), or the corresponding median pressure, P_m, provides a measure of the free energy, ΔG, required to fully oxygenate the macromolecule (hemoglobin tetramer). This energy is given by $\Delta G = -RT \ln K$, where $K = (\bar{X})^{-4} = (K_H P_m)^{-4}$. The energy ΔG determined in this way is independent of the actual shape of the oxygenation curve.[1] A determination of the median values

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for a series of oxygen binding curves, each in the presence of a different concentration of organic phosphate, provides a rigorous determination of the variation in total binding free energy induced by the phosphate effector (Wyman, 1964). In order to resolve the overall free energy into groups of constituent energies pertaining to the various binding processes, two relationships have been utilized as represented by formulas 2 and 3.

$$\log P_m = \log P_m^0 + \frac{1}{4}\log\frac{1 + {}^0K_D(\bar{D})}{1 + {}^4K_D(\bar{D})} \quad (2)$$

$$\log P_m = \log P_m^0 + \frac{1}{n}\log\frac{1 + {}^0K_D(\bar{D})}{1 + K'(\bar{D})} \quad (3)$$

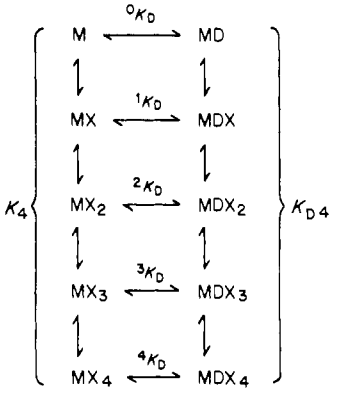
In both formulas, P_m is the median pressure of an oxygen binding curve in the presence of phosphate, $(\bar{D})$ is the concentration of free (i.e., unbound) phosphate in equilibrium with the hemoglobin *at the median point* P_m of the oxygen binding curve, ${}^0K_D$ is the binding constant of organic phosphate to the unliganded tetramer, and P_m^0 is the median pressure in the absence of organic phosphate. In eq 2, which is thermodynamically rigorous and model independent (Szabo & Karplus, 1976), ${}^4K_D$ is the binding constant of organic phosphate to fully oxygenated hemoglobin. Equation 3 is an empirical formula in which n is the Hill coefficient and K' represents binding of organic phosphate to species representing intermediate stages of oxygenation (Benesch et al., 1971, 1976, 1977; Edalji et al., 1976).

With additional assumptions, both eq 2 and 3 have been shown capable of providing accurate descriptions of experimental data over certain ranges (Benesch et al., 1971, 1976, 1977; Berger et al., 1973; Bare et al., 1974; Baldwin, 1975; Szabo & Karplus, 1976; Edalji et al., 1976). A difficulty in applying either of these formulas lies in the fact that the appropriate concentration, $(\bar{D})$, of free phosphate is always unknown. In fact, the concentration (D) of unbound phosphate will change throughout the oxygenation curve whenever ${}^0K_D$ and ${}^4K_D$ have different values. In practice, the total concentration, (D_t), has generally been used instead of $(\bar{D})$ on the grounds that $(D_t) \approx (\bar{D})$ if the ratio of (D_t) to hemoglobin concentration, (M_t), is sufficiently large. Szabo & Karplus (1976) have assumed $(D_t)/(M_t) \geq 10$ to be a safe ratio for DPG binding, whereas Benesch et al. (1977) and Edalji et al. (1976) have chosen a factor of five in the case of IHP. To date there has existed no exact theory which would permit evaluation of the circumstances where such assumptions can be used with impunity, nor permit a calculation of errors involved in the use of the thermodynamically rigorous eq 2 under the assumption $(\bar{D}) = (D_t)$ for particular values of ${}^0K_D$, ${}^4K_D$, and P_m^0.[2] In some cases, it is highly desirable, or necessary, to conduct studies under conditions in which the concentrations of free D, free hemoglobin, M, and MD complexes are all of comparable magnitude (Edalji et al., 1976). The ability to do so is of particular importance in the case of IHP, as will be shown. In addition, the ability to interpret data from the lower concentrations of organic phosphates may in certain instances eliminate complications due to effects of solution nonideality and "secondary" binding. The use of high concentrations of hemoglobin is also desirable to eliminate effects arising from the presence of dissociated dimers (White, 1976). Such high concentrations may lead to significantly nonideal solutions for phosphate concentrations approaching saturating levels, thus necessitating use of data from the lower ratios of phosphate to hemoglobin.

Theory

In this section, an exact theory is derived for the dependence upon total concentrations of organic phosphate, (D_t), and hemoglobin, (M_t), of the median oxygen concentration, $(\bar{X})$. The theory relates these experimentally determinable quantities to the binding constants ${}^0K_D$ and ${}^4K_D$, and the median oxygen concentration of hemoglobin in the absence of organic phosphate. A method for exact calculation of the concentration $(\bar{D})$ at the median point also follows from this theory.

(*a*) *Definitions and Assumptions.* We consider a macromolecule M (e.g., hemoglobin tetramer) which binds four molecules of a ligand X (e.g., oxygen) and one molecule of a second ligand D (e.g., organic phosphate). The simultaneous equilibria depicted in the linkage scheme of eq 4 are then of interest.



(4)

We assume that the macromolecule M does not dissociate or polymerize. The system is described thermodynamically by nine independent constants from the 13 equilibria shown. The Adair constants for oxygen binding reactions depicted on the right and left sides of the scheme are defined by eq 5.

$$K_i = \frac{(MX_i)}{(M)(X)^i} \quad K_{Di} = \frac{(MDX_i)}{(MD)(X)^i} \quad i = 1, 2, 3, 4 \quad (5)$$

For organic phosphate binding, the association equilibrium constants are

$${}^iK_D = \frac{(MDX_i)}{(MX_i)(D)} \quad i = 0, 1, 2, 3, 4 \quad (6)$$

There are several ways of choosing the nine independent constants required to define the linkage scheme. An experimentally convenient choice might include all the constants of eq 5 plus either ${}^0K_D$ or ${}^4K_D$. We define the binding polynomials

$$Z_4 = \sum_{i=0}^{4} K_i(X)^i \quad Z_{D4} = \sum_{i=0}^{4} K_{Di}(X)^i \quad K_0 \equiv 1, K_{D0} \equiv 1 \quad (7)$$

and note that $Z_4 = [1/(M)]\sum(MX_i)$ and $Z_{D4} = [1/(MD)]\sum(MDX_i) = {}^0K_D(M)(D)Z_{D4}$. Using these quantities, we can write the binding isotherms for oxygen in compact form for the two sides of the linkage scheme (eq 4).

$$\bar{Y}_4 = \frac{1}{4}\frac{d \ln Z_4}{d \ln (X)} \quad \bar{Y}_{D4} = \frac{1}{4}\frac{d \ln Z_{D4}}{d \ln (X)} \quad (8)$$

These isotherms for stripped and phosphate-saturated hemoglobin are illustrated diagrammatically in Figure 1, along with an intermediate binding curve corresponding to an arbitrary

[1] If the curves are symmetric in shape, the partial pressure at half-saturation, P_{50}, provides an accurate approximation to the median pressure, P_m. Oxygenation curves measured in the presence of organic phosphates are frequently not symmetric.

[2] Equation 2, although thermodynamically exact, will be referred to in this paper as an "approximate formula" solely to indicate its use with the assumption $(\bar{D}) = (D_t)$, corresponding to the usual experimental situation where only (D_t) is known.

total concentration, (D_t), of effector. As noted earlier, the medians of the two extreme curves $\bar{Y}_4$ and $\bar{Y}_{D4}$ are directly related to the oxygen binding constants K_4 and K_{D4} through the relations $(\bar{X})_4^{-4} = K_4$ and $(\bar{X})_{D4}^{-4} = K_{D4}$, whereas the exact shape of these binding curves is dependent in each case upon all four Adair constants. For the intermediate curve of Figure 1, the Adair constants are given by ${}^0K_D(1 + K_{Di})/(1 + K_i)$ (cf. Szabo & Karplus, 1976) so that all nine independent constants of the linkage scheme would be required to define the exact shape. However, since the median value for each of these curves is independent of the exact shape, the use of Wyman's ingenious relationship, $\Delta G = 4RT \ln (\bar{X})$ (Wyman, 1964), makes it possible to estimate accurately the total binding energy from data which does not exhibit the ultimate precision necessary for resolution of the stepwise binding constants. It is the object of the present derivation to provide an interpretation of the median $(\bar{X})$ for the general binding isotherm (the intermediate curve of Figure 1) in terms of any *three* of the constants K_4, K_{D4}, ${}^0K_D$, and ${}^4K_D$, corresponding to energies of the outer sides of the linkage scheme (eq 4).

(*b*) *Phosphate Binding Constant at Fractional Oxygen Saturation.* For analyzing the effects of interest, an especially useful linkage function is the quantity ${}^XK_D$ which we define to represent binding of the ligand D to the macromolecule as a function of the concentration (X) of unbound oxygen. At any particular value of (X), this function is defined as in eq 9, where the sums are again taken from $i = 0$ to $i = 4$.

$$ {}^XK_D = \frac{\text{(concn of tetramers with D bound)}}{\text{(D)(concn of tetramers without D)}} = \frac{\sum(MDX_i)}{(D)\sum(MX_i)} = {}^0K_D\frac{Z_{D4}}{Z_4} \quad (9)$$

It is of interest to note that ${}^XK_D$ is independent of (D) and is a function only of the concentration of unbound ligand (X) and the nine fundamental binding constants of the linkage scheme contained in the quantities Z_4, Z_{D4}, and ${}^0K_D$. The binding isotherm for D at any value of (X) is given by eq 10

$$ f_D = \frac{{}^XK_D(D)}{1 + {}^XK_D(D)} \quad (10)$$

where f_D is the fractional saturation with phosphate, equal to $\sum(MDX_i)/[\sum(MX_i) + \sum(MDX_i)]$. In the limit of oxygen saturation $[(X) \rightarrow \infty]$, the function ${}^XK_D$ is equal to ${}^4K_D$, whereas for unliganded hemoglobin it equals ${}^0K_D$. As a function of oxygen concentration, it varies over the range of values between ${}^0K_D$ and ${}^4K_D$ in a manner which depends upon the nine microscopic constants. In principle, an experimental determination of ${}^XK_D$ as a function of (X) could be used to resolve all nine microscopic constants of the linkage scheme by least-squares analysis of the data in relation to eq 9. In practice it would be desirable to combine such data with information obtainable from oxygen binding curves.

The function ${}^XK_D$ is analogous to the linkage function ${}^XK_2$ for the dimer-tetramer equilibrium as a function of (X) which has been discussed previously (Ackers & Halvorson, 1974; Johnson et al., 1976) and for which experimental determinations have recently been made (Valdes et al., 1978). The variation of ${}^XK_D$ with (X) provides a measure of the difference between the two binding isotherms for (X) corresponding to the extreme concentrations of D:

$$ \frac{d \ln {}^XK_D}{d \ln (X)} = 4(\bar{Y}_{D4} - \bar{Y}_4) \quad (11)$$

When the hemoglobin is in equilibrium with an arbitrary concentration of D, the binding isotherm $\bar{Y}$ will be, at any value (X) of the free oxygen concentration, a weight average of the isotherms $\bar{Y}_4$ and $\bar{Y}_{D4}$

$$ \bar{Y} = \bar{Y}_{D4} + f_0(\bar{Y}_4 - \bar{Y}_{D4}) \quad (12)$$

where f_0 is the fraction of hemoglobin molecules with no D bound ($f_0 = 1 - f_D$). Substituting into eq 12 from eq 8 and 11 for Y_{D4} and $(\bar{Y}_4 - \bar{Y}_{D4})$, respectively, and then substituting the resulting $\bar{Y}$ into eq 1, one obtains eq 13, after evaluation of integrals.

$$ \ln (\bar{X})^4 + \ln K_{D4} = \int_{(X)=0}^{(X)=\infty} f_0 \, d \ln {}^XK_D \quad (13)$$

Equation 13 provides a very general formula which can be applied to a variety of cases. In order to evaluate the right side of eq 13, an expression for f_0 as a function of ${}^XK_D$ is needed. The relevant form of f_0 for a particular experimental situation will depend upon the other variables desired, as will be shown in the next two sections.

(*c*) *General Formula.* Since we are interested in relating (X) to the total concentrations (D_t) and (M_t), we first evaluate f_0 in terms of these variables prior to integration. When (M_t) is expressed on a molar heme basis, $(D) = (D_t) - (1/4)(1 - f_0)(M_t)$. Substituting into eq 10 (with $f_0 = 1 - f_D$) and solving the resulting quadratic, we have

$$ f_0 = \frac{-(1 + \phi {}^XK_D + \sqrt{(1 + \phi {}^XK_D) + (M_t){}^XK_D}}{\frac{1}{2}(M_t){}^XK_D} \quad (14)$$

where $\phi = (D_t) - (1/4)(M_t)$. Substituting this expression for f_0 into eq 13 and evaluating the resulting integral between limits of $[(X) \rightarrow 0; {}^XK_D \rightarrow {}^0K_D]$ and $[(X) \rightarrow \infty; {}^XK_D \rightarrow {}^4K_D]$, we find

$$ \log P_m = \log P_m{}^0 + \frac{1}{4}\log\frac{{}^0K_D}{{}^4K_D} + \frac{1}{2(M_t)}\left[\frac{1}{{}^4K_D} - \frac{1}{{}^0K_D}\right] + \frac{1}{2(M_t)}\phi \ln\frac{{}^0K_D}{{}^4K_D} + \frac{A_1}{2(M_t){}^0K_D} - \frac{A_2}{2(M_t){}^4K_D} + \left[\frac{1}{4} + \frac{\phi}{2(M_t)}\right]\ln\frac{\frac{A_1 + 1}{{}^0K_D} + \frac{2\phi + (M_t)}{2}}{\frac{A_2 + 1}{{}^4K_D} + \frac{2\phi + (M_t)}{2}} + \frac{\phi}{2(M_t)}\ln\frac{A_2 + \phi {}^4K_D + 1 + \frac{(M_t)}{2\phi}}{A_1 + \phi {}^0K_D + 1 + \frac{(M_t)}{2\phi}} \quad (15)$$

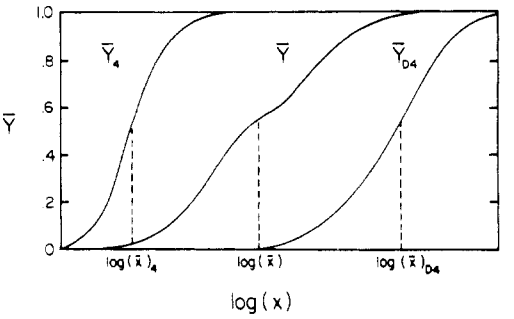


FIGURE 1: Effect of organic phosphate on oxygen binding curves (schematic). $\bar{Y}_4$ is the binding curve in the absence of effector, and Y_{D4} is the isotherm for saturating levels of organic phosphate. $\bar{Y}$ is the binding curve in the presence of an arbitrary total concentration (D_t) of organic phosphate. The median values for each curve (dashed lines) are defined by eq 1.

where

$$A_1 = \sqrt{(1 + \phi^0K_D)^2 + (M_t)^0K_D}$$

$$A_2 = \sqrt{(1 + \phi^4K_D)^2 + (M_t)^4K_D}$$

Equation 15 provides the desired exact relationship between the overall affinity for oxygen (P_m) of a solution at hemoglobin concentration, (M_t), the *total* organic phosphate concentration, (D_t), and the parameters ${}^0K_D$, ${}^4K_D$, and $P_m{}^0$ representing three sides of the linkage scheme (eq 4). This function is completely general and defines the relationship between these variables over their entire ranges of physically permissible values. The function may be used to estimate one or more of the three constants ${}^0K_D$, ${}^4K_D$, K_4 (or alternatively K_{D4} since $K_{D4} = {}^4K_DK_4/{}^0K_D$) from experimentally determined values of the other parameters or by least-squares analysis of experimental data relating the median pressure P_m (or concentration ($\bar{X}$)) to (D_t). With sufficient accuracy, such data alone could be used to estimate all three constants. Equation 15 can also be used to evaluate the accuracy and valid ranges of the approximate formulas. A numerical exploration of these possibilities is described in a later section. It may be noted at this point that the theory developed here could be applied to systems other than hemoglobin. The present formulation, applicable to any system which conforms to the linkage scheme (eq 4), can be extended to schemes with other stoichiometries.[3]

(*d*) *Simpler Cases.* While eq 15 pertains to all ratios of $(D_t)/(M_t)$, a somewhat simpler formula arises in the special case where equimolar concentrations of hemoglobin tetramers and ligand D are present. In this *equimolar case* ($\phi = 0$), we have

$$f_0 = \frac{-1 + \sqrt{1 + 4(D_t)^XK_D}}{2(D_t)^XK_D}$$

and the evaluation of eq 13 leads to

$$\log P_m = \log P_m{}^0 + \frac{1}{4}\log\frac{{}^0K_D}{{}^4K_D} + \frac{1}{8(D_t)}\left[\frac{1}{{}^4K_D} - \frac{1}{{}^0K_D}\right] + \frac{\sqrt{1 + 4(D_t)^0K_D}}{8(D_t)^0K_D} - \frac{\sqrt{1 + 4(D_t)^4K_D}}{8(D_t)^4K_D} + \frac{1}{4}\ln\frac{(\sqrt{1 + 4(D_t)^4K_D} - 1)(\sqrt{1 + 4(D_t)^0K_D} + 1)}{(\sqrt{1 + 4(D_t)^4K_D} + 1)(\sqrt{1 + 4(D_t)^0K_D} - 1)} \quad (16)$$

A second case of interest is that corresponding to either eq 2 or 3, where we may be interested in the variation of P_m with free phosphate concentration. In this case the relevant expression for evaluation of $f_0 = (1 - f_D)$ in terms of ${}^XK_D$ is given by eq 10. Substitution, then, of $f_0 = (1 + {}^XK_D)^{-1}$ into eq 13 and integration leads (with $K_4 = K_1K_{D4}/{}^0K_D$) directly to eq 2. Equation 2 is thus found by using the approach of eq 13 to be the thermodynamically correct expression in terms of the concentration of unbound phosphate. This derivation is different from those previously employed (Baldwin, 1975; Szabo & Karplus, 1976) and provides an independent verification of the theoretical basis of eq 2.

(*e*) *Estimation of* ($\bar{D}$). By using both eq 15 and 2, it is possible to make an accurate estimation of the concentration ($\bar{D}$) of unbound organic phosphate and, consequently, the fraction bound at the median point of any oxygenation curve. In principle, the values of ${}^0K_D$, ${}^4K_D$, and $P_m{}^0$ could be determined (e.g., by least-squares analysis) from determinations of ($\bar{X}$) or P_m, corresponding to experimentally predetermined values of (D_t) and (M_t). Subsequently the value ($\bar{D}$) of unbound phosphate corresponding to the median oxygen pressure may be explicitly calculated by rearrangement of eq 2:

$$(\bar{D}) = \frac{1 - \left(\frac{P_m}{P_m{}^0}\right)^4}{{}^4K_D\left(\frac{P_m}{P_m{}^0}\right)^4 - {}^0K_D} \quad (17)$$

Values of f_0 and ${}^XK_D$ corresponding to the median point may subsequently be estimated since at the median $f_0 = (\bar{D})/(D_t)$ and then, from eq (10), ${}^XK_D = [(D_t) - (\bar{D})]/(\bar{D})^2$.

Application to Experimental Cases

In this section we summarize some explorations of the theoretical results in terms of their applicability to existing knowledge on organic phosphate binding by human hemoglobin. In addition to simulations of the behavior of the functions, a nonlinear least-squares fitting program was employed to investigate the resolution of parameters and some aspects of the relationships between eq 2, 3, and 15. The general fitting program has been described previously (Johnson et al., 1976). It employs a modified Gauss–Newton algorithm and estimates the confidence limits for fitted parameters according to the procedure of Box (1960). In all calculations reported here, the confidence limits at 65% probability were used. The program also evaluates correlation coefficients between the fitted parameters. It was of particular interest to compare the correlation between parameters of eq 2 and

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[3] The type of behavior shown in Figure 2 is also characteristic of the more general case where more than one molecule of the second ligand binds, except that the upper limit value of P_m becomes larger. Extension of the theoretical treatment to this more complex case can be carried out along similar lines to the derivation of eq 13, with the result

$$\ln(\bar{X})^4 + \ln K_{D4} = \int_{(X)=0}^{(X)=\infty} f_0 \, d \ln {}^XK_D - \int_{(X)=0}^{(X)=\infty} f_2 \, d \ln {}^XK_{D2}$$

where f_2 is the fraction of tetramers with two molecules of bound D and ${}^XK_{D2}$ is the binding constant for the second molecule of D at partial saturation (X). All other symbols have the same significance as in eq 13. Evaluation of the right side again depends upon conditions imposed. To derive the expression in terms of (D) corresponding to eq 2, we use

$$f_0 = \frac{1}{1 + {}^XK_D(D) + {}^XK_D{}^XK_{D2}(D)^2}$$

$$f_2 = \frac{{}^XK_D{}^XK_{D2}(D)^2}{1 + K_D(D) + {}^XK_D{}^XK_{D2}(D)^2}$$

which leads to

$$\log P_m = \log P_m{}^0 + \frac{1}{4}\log\frac{1 + {}^0K_D(D) + {}^0K_D{}^0K_{D2}(D)^2}{1 + {}^4K_D(D) + {}^4K_D{}^4K_{D2}(D^2)}$$

where ${}^0K_{D2}$ and ${}^4K_{D2}$ are respectively the stepwise binding constants for the second molecule of D onto tetramers which have no bound X and which are fully saturated with X. This is identical with an equation derived by Szabo & Karplus (1976) by using a different approach. In order to evaluate f_0 and f_2 in terms of (D_t), so that analysis corresponding to eq 15 can be carried out, we can write the formulas corresponding to eq 14 which turn out to be cubic equations. The solution of these for positive real roots and subsequent evaluation of the integrals is analytically cumbersome, but readily accomplished by numerical methods. A detailed development of the various cases will be presented elsewhere.

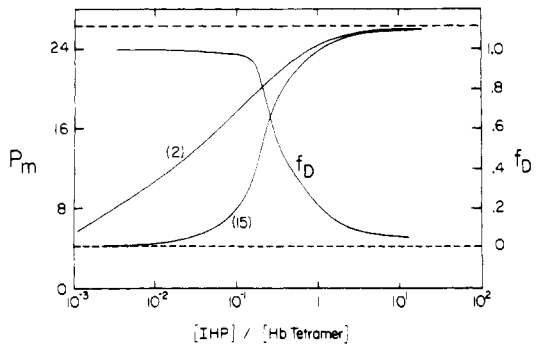

FIGURE 2: Variation in median pressure of oxygenation curves for human hemoglobin as predicted by eq 2 and 15 in the presence of inositol hexaphosphate (IHP). Both formulas use the independently determined values of ${}^0K_D = 1.67 \times 10^7$ M^{-1} and ${}^4K_D = 1.11 \times 10^4$ M^{-1} (Edalji et al., 1976) and assume a value of 4.22 mmHg for the median pressure. The hemoglobin concentration used in eq 15 was 2.4×10^{-4} M (heme). f_D, the fraction of IHP bound as a function of IHP concentration for the same conditions.

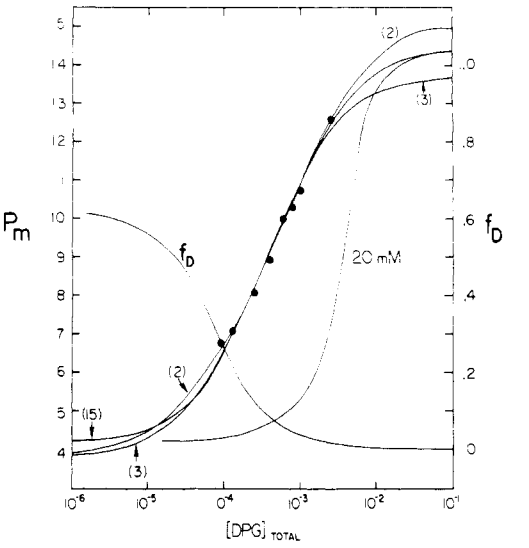

FIGURE 3: Effect of DPG on the oxygen binding curves of human hemoglobin. Circles are the experimental data of Benesch et al. (1971), at a hemoglobin concentration of 2.0×10^{-4} M (heme). The solid curves 2, 3, and 15 are generated from the parameters found from least-squares fitting of the data points to each of the respective formulas. Values of the fitted parameters are given in Table I. f_D is the corresponding fraction of DPG bound. The solid curve to the right was calculated by eq 15 for an approximately physiological concentration of hemoglobin (20 mM heme) by using the same best fit parameters as for curve 15.

3 where these formulas differ in physical interpretation. Calculations were performed on a Hewlett-Packard 1000 system. Results of the simulations and analyses have been grouped into several categories representing the issues of interest.

(*a*) *Where the New Theory Is Required: Interactions with IHP.* Under any conditions where the concentrations of bound and free phosphate are of comparable magnitude, eq 2 and 3 will fail to describe correctly the variation in P_m with (D_t). With certain values of the constants ${}^0K_D$, ${}^4K_D$, and K_4, this situation will obtain where also a significant fraction of the total variation in P_m with (D_t) occurs. An important example of such a case is the binding of IHP by human hemoglobin. Here the value of ${}^0K_D$ is almost immeasurably high (10^7 to 10^8), whereas ${}^4K_D$ is in a range of commonly measurable values (e.g., 10^4 to 10^6). Figure 2 shows the variation in P_m with (D_t) for a protein concentration of 240 μM (heme) and by using the values of ${}^0K_D$ and ${}^4K_D$ estimated by independent measurements (Edalji et al., 1976; Benesch et al., 1977). For comparison, the curve predicted from eq 2 is also shown. The dramatic failure of eq 2 under the assumption $(D_t) = (\bar{D})$ is seen over most of the range of variation of the experimental variable, P_m. A second striking feature of Figure 2 is the low ratio of IHP to hemoglobin concentrations required to effect the shift in P_m. The major change is seen to occur between a ratio of 0.1 and 0.5. At values of this ratio higher than unity, the effect of ignoring the difference between (D_t) and (D) is negligible since the two curves nearly coincide. Thus, the assumption made by Edalji et al. (1976) and Benesch et al. (1977) of a "safe" factor of five is certainly valid. It should be noted, however, that, in the "safe region" where values of (IHP/Hb) exceed unity, there is very little range left for measuring the variation of P_m with IHP concentration. In the experimental results reported to date on the effect of IHP, this problem of inaccuracy due to limited and least-sensitive range may be compounded by additional problems. (a) Oxygen binding curves in the presence of small amounts of IHP are asymmetric (even biphasic) so that the use of P_{50} values rather than true medians P_m can lead to substantial error. (b) With low hemoglobin concentrations employed (e.g., 5 μM tetramers), the effects of IHP binding by dissociated dimers (White, 1976) may lead to significant deviations from the behavior predicted by eq 15. It is possible also that hemoglobin tetramers might bind a second molecule of IHP and this is suggested by the fact that experimental values of P_{50} exceed that predicted by eq 15 by using the literature values for ${}^0K_D$, ${}^4K_D$, and $P_m^{0.3}$. However, the calorimetric titrations of Gaud (1977) exhibit sharp end points at an (IHP)/(hemoglobin tetramer) ratio of unity. Thus, factors a and b would seem more likely as a source of this discrepancy. An extensive set of oxygen binding curves as a function of IHP concentration would be desirable over the range of IHP/Hb ratios shown in Figure 2, and at sufficiently high concentrations of hemoglobin to eliminate possible effects of dissociation. Least-squares fitting of eq 15 (or a more complex analogue of this formula[3]) to the median values from such curves would permit an accurate estimate to be made for the IHP binding constants since values of K_4 and possibly K_{4D} may be independently estimated. Such a set of data does not presently exist in the literature for interaction of IHP with any hemoglobin system. In addition to its role in physiological regulation, much interest in this molecule stems from its use as a powerful effector for quaternary structure transitions in normal, mutant, and chemically modified hemoglobins. Phenomenological theories such as that developed here provide a guide to useful types of experiments and their optimization.

(*b*) *Range of Validity for Use of Equations 2 and 3: Analysis of DPG Interaction.* With binding constants similar to the IHP case described above, the curves approach each other only at the ends of the range of variation in P_m so that only eq 15 correctly describes the process in a sensitive region for experimental determinations (Figure 2). However, with equilibrium constants corresponding to the binding of DPG by human hemoglobin (${}^0K_D \approx 10^4$ to 10^5 and ${}^4K_D \approx 10^2$ to 10^3), the approximation afforded by eq 2 or 3 is good over most of the range for variation of P_m. This conclusion is derived from numerical analyses of several sets of data for the effects of DPG upon oxygenation curves including those of Benesch et al. (1971), Tyuma et al. (1973), and Bunn et al. (1971). For purposes of illustration, only the data of Benesch et al. will be discussed here, consisting of oxygenation curves obtained on human hemoglobin at a concentration of 60 μM in tetramer ($(M_t) = 2.4 \times 10^{-4}$ M heme) and total DPG concentrations ranging between 9×10^{-5} M and 2.5×10^{-3} M.

Table I: Least-Squares Analysis of DPG Binding Data by Equations 2, 3, and 15

parameter	est value	confidence limits[b]	variance of fit
A. Best Fits with ${}^0K_D$ Constrained to 6.67×10^4 M^{-1} [a]			
fit to eq 2			
${}^4K_D$	3.36×10^2 M^{-1}	$(2.12 \times 10^2, 4.82 \times 10^2)$	2.6×10^{-2}
$P_m{}^0$	4.06 mmHg	(3.96, 4.18)	
fit to eq 3 with $n = 2.68$, $P_m{}^0 = 3.8$ mmHg			
${}^4K_D$	3.18×10^3 M^{-1}	$(2.36 \times 10^3, 4.28 \times 10^3)$	4.5×10^{-1}
fit to eq 15			
${}^4K_D$	4.85×10^2 M^{-1}	$(2.76 \times 10^2, 8.54 \times 10^2)$	5.31×10^{-2}
$P_m{}^0$	4.22 mmHg	(4.03, 4.42)	
B. Best Fits without Constraints on ${}^0K_D$			
fit to eq 2			
${}^0K_D$	8.54×10^4 M^{-1}	$(6.24 \times 10^4, 1.12 \times 10^5)$	
${}^4K_D$	3.48×10^2 M^{-1}	$(2.13 \times 10^2, 4.75 \times 10^2)$	2.26×10^{-2}
$P_m{}^0$	3.83 mmHg	(3.57, 4.05)	
fit to eq 3 with $n = 2.68$			
${}^0K_D$	3.49×10^4 M^{-1}	$(2.76 \times 10^4, 4.55 \times 10^4)$	
K'	1.15×10^3 M^{-1}	$(3.05 \times 10^2, 1.82 \times 10^3)$	8.48×10^{-2}
$P_m{}^0$	3.83 mmHg	(fixed)	
fit to eq 15, $(M_t) = 2.4 \times 10^4$ M heme			
${}^0K_D$	1.03×10^5 M^{-1}	$(1.02 \times 10^5, 1.07 \times 10^5)$	
${}^4K_D$	4.93×10^2 M^{-1}	$(3.05 \times 10^2, 5.00 \times 10^2)$	8.02×10^{-3}
$P_m{}^0$	3.80 mmHg	(3.51, 4.02)	

[a] The value of ${}^0K_D$ is from equilibrium dialysis experiments of Benesch et al. (1971). [b] Upper and lower bounds correspond to one standard deviation. Exact definition and interpretations of these limits are discussed in Johnson et al. (1976).

The binding curves of Benesch et al. (1971) are fairly symmetric and values of P_{50} were found nearly identical with the medians. These values are shown as the plotted points in Figure 3. Previous least-squares analyses of the five highest points have been reported by Szabo & Karplus (1976), yielding values of ${}^0K_D = 8.5 \times 10^4$ M^{-1}, ${}^4K_D = 3.3 \times 10^2$ M^{-1}. A best fit over the entire data set according to eq 3 has been reported by Benesch et al. (1971). Using their independently estimated value of 6.67×10^4 M^{-1} for ${}^0K_D$, they obtained a best value of $K' = 3.3 \times 10^3$ M^{-1}. By using the least-squares fitting program described earlier, which also evaluates the confidence limits on estimated parameters, a best value for K' of 3.18×10^3 M^{-1} was obtained from fits of all the data to eq 3 with $n = 2.68$ and constraining ${}^0K_D$ to its independently determined value. These and other results of the fitting are summarized in Table I. It can be seen that the confidence limits (corresponding to one standard deviation) on the value of K' include the value of 3.3×10^3 obtained by Benesch et al. (1971). Likewise a least-squares analysis according to eq 2 of the five highest data points, but without any constraints, yielded values for ${}^0K_D$ and ${}^4K_D$ in close agreement with those reported by Szabo & Karplus (1976). Only slightly different values are obtained when all the data points are included in the fit as shown in Table I, and the fits according to the exact eq 15 yield values (Table I) which are also in generally good agreement with those of eq 2. The goodness of fit to these data was found generally comparable for all three equations, as judged by values of the variance obtained and the observed randomness in the distribution of residuals to the best fits (not shown).

Figure 3 shows solid curves generated according to the three equations 2, 3, and 15 by using in each case the best fit values of the parameters as shown in Table I, part A. It can be seen that the three curves coincide very closely over their central region which includes nearly all the data points and encompasses the major fraction of the range for variation of P_m. The curves do differ near their low and high points, indicating that data in these regions would be required in order to distinguish between the abilities of the three formulas to describe the experimental results. It should be noted that in this data set the lowest data point corresponds to the nearly equimolar condition. At this point, the degree of saturation f_D is equal to 0.27. For all the other data points, it is considerably lower.

The effects of protein concentration on the DPG case is illustrated by comparison of the data points of Figure 3 with the solid curve on the right which was generated by eq 15 by using the same constants (Table I, part A) but for a hemoglobin concentration of 20 mM heme, corresponding approximately to physiological concentrations of hemoglobin within the red cell.

It can be seen from these results that the new theory does not supersede the earlier formulas for DPG interaction in terms of providing a more accurate description in the ranges of presently available data. For calculations within this "safe" middle region, formula 2 has the distinct advantage of simplicity. The new formula does, however, provide a unique means of verifying that eq 2 can be safely used in this range.

(*c*) *Why Formulas 2* and *3 Work Equally Well.* As noted above, formulas 2 and 3 are both capable of describing the experimental results over most of the possible range for DPG binding with essentially equal accuracy. The reason for this lies in the extremely high correlation between the parameters wherein the two formulas differ. A numerical exploration of the general formula

$$\log P_m = \log P_m{}^0 + \frac{1}{Z_1} \log \frac{1 + {}^0K_D(\bar{D})}{1 + Z_2(\bar{D})} \qquad (18)$$

reveals that many pairs of values for Z_1 and Z_2 may be used to define curves of log P_m vs. $(\bar{D})$ which have nearly identical form. Thus a decrease in Z_1 can be accommodated by an increase in Z_2 without appreciable change in the shape of the curves over the regions of experimental data. This correlation property is illustrated by the curves in Figure 3 and also in Table II, where values are presented of the analysis by each of the equations to "synthetic data" generated by the other equation. By using each equation and the corresponding constants listed in Table I, part B, a series of 19 pairs of values $(P_m, (D_t))$ was generated. The synthetic data points in each

Table II: Reciprocal Analyses of Equations 2 and 3[a]

parameter	value	confidence limits	variance
	A. Analysis by Eq 2 of Synthetic Data Generated with Eq 3		
^0K_D	1.22×10^5 M^{-1}	(8.89×10^4, 1.61×10^5)	4.1×10^{-2}
^4K_D	5.31×10^2 M^{-1}	(4.51×10^2, 6.18×10^2)	
$P_m{}^0$	3.57 mmHg	(3.33, 3.76)	
	B. Analysis by Eq 3 of Synthetic Data Generated with Eq 2		
^0K_D	2.35×10^4 M^{-1}	(1.81×10^4, 2.98×10^4)	5.0×10^{-2}
K'	7.71×10^2 M^{-1}	(6.76×10^2, 8.71×10^2)	
$P_m{}^0$	4.16 mmHg	(3.91, 4.37)	

[a] Synthetic data generated by each equation by using the constants of Table I, part B, were subjected to least-squares analysis according to the other equation (see text for details).

case covered a range of organic phosphate concentration between 10^{-6} M and 5.5×10^{-2} M corresponding to >95% of the range for P_m, and had a precision of approximately six decimal places. The set of values resulting from each of the equations was then subjected to least-squares analysis to obtain the best fit to the contrasting equation. Results of these fits are presented in Table II. Several points are of interest. First, it may be noted that the variance of fit is nearly the same in both cases A and B. These variances which arise solely from the difference in functional form of the two equations are also of comparable magnitude to those of the fits to experimental data (Table I, part B) corresponding to a slightly narrower range of the variables. A second point illustrated by these calculations is the compensation of variables Z_1 and Z_2. In Table II, part A, for example, the best fitted value of $^4K_D = 5.31 \times 10^2$ in contrast to $K' = 1.15 \times 10^3$ from Table I, part B. Reciprocally, of course, Z_1 has changed from 2.68 to 4.0. Similarly, the best fitted value for K' in Table II, part B, is 7.7×10^2, whereas the "data" were generated from a value of 3.48×10^2 for ^4K_D (Table I, part B). Additionally, it is found that some compensation is also exhibited in the altered values of the other parameters ^0K_D and $P_m{}^0$ since these synthetic data cover most of the end regions of the curves shown in Figure 3. When such synthetic data are restricted to the middle region corresponding to most of the experimental points, the functions are made almost totally isomorphic by reciprocal variations in Z_1 and Z_2 alone.

Since eq 2 is theoretically exact when Z_2 is defined to be the equilibrium constant ^4K_D for phosphate binding to fully oxygenated hemoglobin, it is of interest to ask whether the physical significance of the fitting parameter K' can be ascertained. It should be noted in this regard that both eq 2 and 15 assume a definite stoichiometric binding of organic phosphate by fully oxygenated hemoglobin. If the assumption is incorrect, then a different constant ^jK_D will replace ^4K_D in eq 2

$$^jK_D = \frac{(MDX_j)(X)^{4-j}}{(MX_4)(D)} \qquad (19)$$

where j is the highest oxygen ligation state for which a molecule of D is bound. But in this case Z_1 will still equal 4. This factor arises merely from the number of sites available for binding by oxygen. A proof of these results is given in the Appendix. Thus, when Z_1 is set equal to the value of Hill's constant n as in eq 3, then K' does not pertain to the binding by any single species of the linkage scheme.

(d) Pitfalls of Single-Point Estimates of ^4K_D. In many experimental circumstances the values of ^0K_D and $P_m{}^0$ can be determined independently so that the main interest in using eq 2 is to estimate ^4K_D which represents binding so weak as

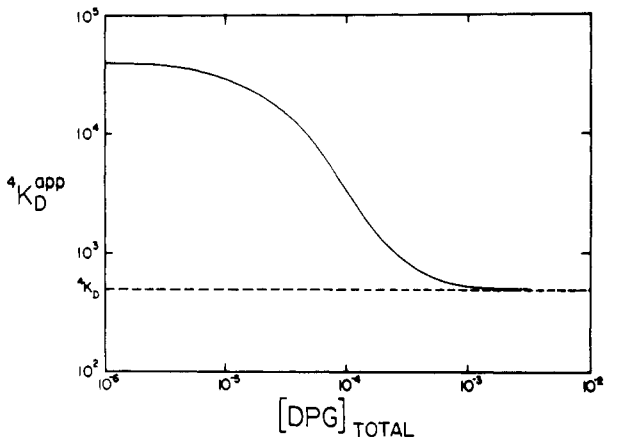

FIGURE 4: Apparent values of the binding constant ^4K_D for DPG to oxyhemoglobin as a function of DPG concentration. Values were calculated according to eq 20 with the assumption of accurately known parameters ^0K_D, P_m, $P_m{}^0$ having the values listed in Table I, part A. The horizontal line is the "correct" value for $^4K_D = 4.85 \times 10^2$ M^{-1}. Deviations from this value are solely a consequence of inaccuracies in the assumption $(D_t) = (\bar{D})$.

to prohibit its independent evaluation. By rearrangement of eq 2, we have

$$^4K_D = \frac{1 + {}^0K_D(\bar{D}) - \left(\frac{P_m}{P_m{}^0}\right)^4}{\left(\frac{P_m}{P_m{}^0}\right)^4 (\bar{D})} \qquad (20)$$

By assuming that $P_m{}^0$, ^0K_D, P_m, and (D_t) are known to great accuracy, but with the assumption $(D_t) = (\bar{D})$, an important question is how sensitive the right side of eq 20 may be to errors in the value of $(\bar{D})$. An investigation of this question reveals that even relatively small errors in $(\bar{D})$ of a few percent as encountered in the "safe" regions for eq 2 can lead to enormous errors in the calculated value of ^4K_D under experimental conditions commonly employed. The errors of such single-point estimates are shown in Figure 4 for values of the parameters given in Table II. It can be seen that enormous errors can arise in the estimated value, $^4K_D^{app}$, even when the other terms of the equation are accurate. These errors arise from the fact that the sum of terms in the numerator of eq 20 are nearly equal so that relatively small errors in $\bar{D}$) are amplified greatly in their effect upon the calculated ^0K_D. This problem is avoided in a least-squares analysis of a series of data points according to eq 2.

Discussion

This work comprises an extension to the theory of linkage (Wyman, 1964) for the binding of two ligands by a macro-

molecule. It provides an exact thermodynamic basis for the analysis of results under the usual experimental conditions where the total concentration of organic phosphate is known, but not the concentration of unbound effector. The new theory does not provide a molecular mechanism for the interaction of organic phosphates with hemoglobin. Like all thermodynamic theories, it defines constraints which must be met by all mechanisms proposed to explain the behavior of such systems.

The further development and use of organic phosphates as structural probes may be of value in assessing the validity of mechanistic models. For example, the two-state MWC model (Monod et al., 1965) can be used to explain oxygen binding by stripped normal tetrameric human hemoglobin with allosteric parameters requiring the deoxy molecule to be essentially all in the "T state". The values of ${}^0K_D$ for various organic phosphates should therefore represent the respective binding energies of the "T-state" form. Upon complete oxygenation, the phosphate-free tetramers are converted almost entirely into the "R state", whereas in the presence of saturating levels of IHP complete conversion into the "T state" is widely presumed to occur. Thus, the constant ${}^4K_D$ of IHP should reflect the sum of energies for conversion from R $\rightarrow$ T and for binding to the "T state", i.e., ${}^4K_D/{}^0K_D = Lc^4$, where L is the allosteric constant for the equilibrium between R and T forms and c is the ratio of intrinsic binding constants for the two forms of the molecule. Typical values of L and c for hemoglobin A are 10^5 and 10^{-3}, respectively, so that Lc^4 is on the order of 10^{-7}. Comparison with predictions from ${}^0K_D = 1.67 \times 10^7\ M^{-1}$ and ${}^4K_D = 1.11 \times 10^4$ (Edalji et al., 1976) shows a large discrepancy since ${}^4K_D/{}^0K_D = 4 \times 10^{-4}$. It is clear from our earlier discussion that the source of this discrepancy could be incorrect values of ${}^0K_D$ or ${}^4K_D$. The calculation method, however, serves to illustrate how the various relationships between constants of the linkage scheme (eq 4) might be used with reliable constants to impose constraints upon theories of hemoglobin structure and function.

The special feature of the approach used in this work lies in the definition and use of the linkage function ${}^0K_D$ which provides an especially convenient route to the general formula, eq 15. The approach is analogous to that used previously in analyzing the linkage between polymerization and oxygen binding in human hemoglobin (Ackers & Halvorson, 1974; Johnson et al., 1976). In addition to its usefulness in the derivation of eq 15, the linkage function ${}^XK_D$ is of considerable interest in its own right. The definition and characteristics of this function point to the desirability of its experimental determination. Methods are beginning to be developed which should make this possible (cf. G. Greaney & D. Powers, 1979, unpublished results). It can be seen from eq 11 that knowledge of ${}^XK_D$ as a function of (X) combined with one of the isotherms $\bar{Y}_4$ or $\bar{Y}_{D4}$ could be used to compute the other isotherm, which might be experimentally inaccessible. ${}^XK_D$ as a function of (X) could, in principle, be used to estimate all nine of the independent constants of the linkage scheme. No determination of these constants has yet been made for any hemoglobin system. The oxygen binding data of Tyuma et al. (1973) represent the only presently available data base from which such a determination could be made. However, their results, all obtained at low hemoglobin concentration, have been found by Szabo & Karplus (1976) to be inconsistent with the linkage scheme (eq 4). It seems likely that the use of experimental values for ${}^XK_D$ in combination with oxygen saturation curves would provide a sound basis for determination of all the microscopic constants of the linkage scheme. The situation is analogous to that previously studied involving the linkage between oxygen binding and dimer–tetramer association (Ackers & Halvorson, 1974; Johnson et al., 1976; Valdes et al., 1978) where both kinds of data were required (Mills et al., 1976; Mills & Ackers, 1979; Ip & Ackers, 1977). As in that linkage system, the ability to determine the microscopic constants over a range of conditions could be used to provide information regarding the intermediate states of the hemoglobin molecule during oxygenation.

It should be noted that the theory developed here is general so that it could be applied to systems other than hemoglobin. One possible application would be the determination of binding constants for a small regulatory species by an enzyme molecule from steady-state kinetics of the catalyzed reaction measured as a function of substrate concentration. For such a system, the theory would relate median values of substrate concentration for the kinetic curves in the presence of varying concentrations (D_t) of the regulatory species. The two determinable constants, corresponding to ${}^0K_D$ and ${}^4K_D$, would pertain to binding of the regulatory molecule in the absence of substrate and at saturating substrate levels.

Finally it is of interest to discuss briefly the third area for application of the common approach to linkage functions which has been employed both in this study and in those of the work from this laboratory on ligand-linked subunit polymerization (Ackers & Halvorson, 1974; Johnson et al., 1976; Valdes et al., 1978). The approach is based upon the use of a general constant of polymerization (${}^XK_2$) or of ligand binding (${}^XK_D$) which varies continuously with the concentration of a second interacting molecule of interest (X). Relationships are then established which permit interpretation of the binding isotherm for the ligand (X) corresponding to an experimentally realizable constraint (e.g., fixed total concentration of protein or of organic phosphate). This approach is readily extended to the third general class of macromolecular transformations, that of conformational change. If we consider an equilibrium between two conformational states of a macromolecule M and M′, each capable of binding a ligand X, and define an equilibrium constant for the conformational transition at partial saturation with X

$$ {}^XK_I = \frac{\sum(M'X_i)}{\sum(MX_i)} = {}^0K_I\frac{Z'}{Z} \tag{21} $$

so that

$$ \frac{d \ln {}^XK_I}{d \ln (X)} = 4(\bar{Y}' - \bar{Y}) \tag{22} $$

where Z' and Z are the binding polynomials of species M′ and M and $\bar{Y}'$ and $\bar{Y}$ are the corresponding isotherms. In this linked system, the position of the equilibrium between M and M′ is shifted continually during the course of ligation as a result of the differential binding affinities of ligand X for the two conformations. Derivation entirely analogous to that of eq 13 and subsequent evaluation of the integral leads to

$$ (\bar{X}) = \frac{1 + {}^0K_I}{1 + {}^4K_I}\frac{1}{K_4} \tag{23} $$

where $(\bar{X})$ is the median concentration as usual, ${}^0K_I$ and ${}^4K_I$ are isomerization constants for unliganded and fully liganded macromolecule, respectively, and K_4 is the macroscopic binding constant for fully ligating macromolecule M.

Equations 21–23 provide a general treatment of this system. With a few simplifying assumptions, this treatment reduces to the allosteric model of Monod et al. (1965). We identify

${}^0K_I$ with the allosteric constant L of their model, and specify that all intrinsic binding constants to state M are identical (equal to $1/K_R$). Then $K_4 = K_R^{-4}$ and ${}^4K_I = Lc^4$ where $c = K_R/K_T$. Further noting that $(\bar{X})^{-4} = k_{44}$, the fourth tetramer Adair constant, we can rearrange eq 23 to

$$k_{44} = \frac{(1 + Lc^4)}{(1 + L)} \frac{1}{K_R^4} \quad (24)$$

This expression has been derived from the MWC model in a completely independent manner (Imai, 1973). Development of the binding isotherm for this special case of the linked isomerizing system, analogously to that of eq 9–12, leads directly to the binding isotherm expression of Monod et al. (1965).

These considerations, taken together with those of Ackers & Halvorson (1976) and Johnson et al. (1976) and of the theoretical section of this paper, provide a complete picture of the simplest ways in which this general approach may be applied to the three classes of macromolecular interaction when these processes are linked to the binding of a ligand species (X).

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Appendix

Here we summarize the derivation of eq 19 for the case where MX_j is the highest complex of M with X capable of binding D. In this situation all constants K_{Di} are zero for $i = j + 1$ up through 4. We define ${}^jK_D$ according to eq 19 and note that ${}^jK_D K_4 = {}^0K_D K_{Dj}$. The binding polynomial for the right side of the scheme (eq 4) becomes $Z_{Dj} = 1 + K_{D1}(X) + \ldots + K_{Dj}(X)^j$ and the binding isotherm for X at saturating concentrations of D is given by

$$\bar{Y}_{Dj} = \frac{1}{4} \frac{d \ln Z_{Dj}}{d \ln (X)} \quad (A-1)$$

For the binding constant ${}^XK_D$, we have

$${}^XK_D = {}^0K_D \frac{Z_{Dj}}{Z_4} \quad \frac{d \ln {}^XK_D}{d \ln (X)} = 4(\bar{Y}_{Dj} - \bar{Y}_4) \quad (A-2)$$

and the overall binding isotherm is

$$\bar{Y} = \bar{Y}_{Dj} - f_0(\bar{Y}_{Dj} - \bar{Y}_4) \quad (A-3)$$

Combining these relations with those unaffected by the change in stoichiometry and using the same derivation for eq 13, we have, with $f_0 = 1/[1 + {}^XK_D(D)]$:

$$\ln (\bar{X})^4 + \ln K_{Dj} = \int_{(X)=0}^{(X)=\infty} \frac{d \ln {}^XK_D}{1 + {}^XK_D(D)} \quad (A-4)$$

Since $\lim_{(X)\to\infty} {}^XK_D = {}^jK_D$ and $\lim_{(X)\to 0} {}^XK_D = {}^0K_D$, we have as the final result

$$\log P_m = \log P_m{}^0 + \frac{1}{4} \log \frac{[1 + {}^0K_D(D)]}{[1 + {}^jK_D(D)]} \quad (A-5)$$

Thus on model-independent thermodynamic grounds, the value of Z_1 in eq 18 is always equal to 4 regardless of the value of j, whereas Z_2 will be equal to ${}^jK_D$ as defined by eq 19.

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