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Thermodynamics of the binding of ligands by macromolecules

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Abstract

The thermodynamics of the binding of ligands by proteins and other biological macromolecules has been treated by Wyman and others on the basis of the binding polynomial and the binding potential. However, the thermodynamics of the binding of ligands by small molecules and the effects of ligands on the apparent equilibrium constants of biochemical reactions has been developed on the basis of Legendre transformed Gibbs energies of formation. This article brings these seemingly disparate approaches together by considering simple systems and the binding of oxygen by hemoglobin. When the ligand is H^+, examples involving small molecules show that the standard transformed Gibbs energy of formation of a reactant at a specified pH is equal to the negative of the binding potential plus a term related to the standard thermodynamic properties of the elements. The standard transformed Gibbs energies of formation of eight forms of deoxygenated and oxygenated hemoglobin are calculated here for a specific set of conditions. This is the most efficient way to store the information from the seven independent apparent equilibrium constants involved. In a second step, a Legendre transform is used to introduce the concentration of molecular oxygen as a natural variable and calculate the apparent equilibrium constant K'' for 2TotD = TotT at specified values of $[O_2]$, where TotD is the sum of the concentrations of the dimer and its oxygenated forms and TotT is the sum of the concentrations of the tetramer and its oxygenated forms.

Keywords: Transformed Gibbs energy; Thermodynamics of macromolecules; Hemoglobin; Binding potential; Binding polynomial; Subunit dissociation

1. Introduction

There are two areas of biochemical thermodynamics that seem to be unrelated, although they both deal with the binding of ligands. The thermodynamics of the binding of ligands by proteins and other biological macromolecules has been developed by Wyman [1] and others on the basis of binding polynomials P and the definition [2] of the binding potential Π:

$$\Pi = \mathrm{RT} \ln P \tag{1}$$

Wyman used the Russian L for the symbol of the binding potential (L for linkage), but Π has been used by Wyman and others when it is not convenient to use the Russian L. The binding potential P is the polynomial obtained by putting equilibrium expressions into the sum of the concentrations of all of the species containing the macromolecule M divided by the concentration of the unliganded macromolecule:

$$\mathrm{P} = \frac{[\mathrm{M}] + [\mathrm{ML}] + [\mathrm{ML}_2] + \ldots}{[\mathrm{M}]} \tag{2}$$

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The most striking example of this approach relates to the allosteric effects in hemoglobin. However, the thermodynamics of the binding of ligands by reactants in biochemical reactions is based on the apparent equilibrium constant K' of a biochemical reaction at specified T, P, pH, and concentrations of free metal ions that are bound. The prime is used to indicate that the apparent equilibrium constant depends on the free concentrations of H^+ and metal ions that are bound. The apparent equilibrium constant leads to the standard transformed Gibbs energy of a biochemical reaction $\Delta_r G'^\circ$ [3–5]:

$$\Delta_r G'^\circ = -RT \ln K' \tag{3}$$

$\Delta_r G'^\circ$ for a biochemical reaction can be expressed in terms of the standard transformed Gibbs energies of formation $\Delta_f G_i'^\circ$ of the reactants (sums of species) by use of

$$\Delta_r G'^\circ = \Sigma \nu_i' \Delta_f G_i'^\circ \tag{4}$$

The apparent stoichiometric number ν_i' for reactant (sum of species) i in a biochemical reaction is positive for a reactant and negative for a product. The prime is used to distinguish the stoichiometric numbers for reactants in a biochemical reaction from the stoichiometric numbers ν_i of species in the underlying chemical reactions.

Both the binding potential Π of a macromolecule and the standard transformed Gibbs energy of a reactant in a biochemical reaction depend on the standard thermodynamic properties of species that are in equilibrium with each other at specified concentrations of the ligand. The applications to biochemical reactions have been primarily to reactants of relatively low molar mass, but proteins and other macromolecules may also be reactants in biochemical reactions. This leads to the question as to what extent biochemical reactions involving proteins can be handled by Eqs. (3) and (4). In spite of superficial differences, these two areas of biochemical thermodynamics both involve Legendre transforms [6] to introduce the chemical potentials of ligands as natural variables for a thermodynamic potential. Natural variables are extremely important in thermodynamics, because when a thermodynamic potential like U, H, A, or G can be determined in terms of its natural variables, all of the thermodynamic properties of the system can be obtained by taking partial derivatives of the thermodynamic potential. Concentrations of ligands are not natural variables of U, H, A, and G, and so new thermodynamic potentials have to be defined in order to treat ligand binding. In a study of ligand binding by macromolecules, Wyman [7] showed how Legendre transforms can be used to introduce the chemical potentials of ligands as natural variables. Schellman [8] provided an early discussion of the binding potential using Legendre transforms. Wyman and Gill [9] discussed Legendre transforms and pointed out the identity $\Pi = -\tilde{G}_{\mathrm{W,X,Y}}$, where the tilde indicates division by the amount of macromolecule so that a molar property is obtained, and the W,X,Y subscript indicates Legendre transforms with respect to water and two ligands, respectively. They showed that the number of X molecules bound by a macromolecule is given by $\tilde{X} = (\partial \Pi / \partial \mu_{\mathrm{X}})_{\mathrm{T,P}}$.

The two approaches are compared here by considering two examples with small molecules and the oxygenation of hemoglobin from both points of view. The first example, inorganic phosphate in the neutral pH region in the presence of magnesium ions, is an example of linkage, because the binding of hydrogen ions affects the binding of magnesium ions and the binding of magnesium ions affects the binding of hydrogen ions. The second example, isomerization of an isomeric weak acid, illustrates an allosteric effect because the pH affects the proportions of the A and B forms.

2. Standard transformed Gibbs energies of formation of phosphate species

The fundamental equation of thermodynamics for the Gibbs energy G of an aqueous solution of inorganic phosphate in the neutral pH region in the presence of magnesium ion can be written as

$$\begin{aligned}(\mathrm{d}G)_{T,P} = {} & \mu(\mathrm{HPO_4^{2-}})\,\mathrm{d}n(\mathrm{HPO_4^{2-}}) + \mu(\mathrm{H_2PO_4^-})\,\mathrm{d}n(\mathrm{H_2PO_4^-}) + \mu(\mathrm{MgHPO_4})\,\mathrm{d}n(\mathrm{MgHPO_4}) \\ & + \mu(\mathrm{H^+})\,\mathrm{d}n(\mathrm{H^+}) + \mu(\mathrm{Mg^{2+}})\,\mathrm{d}n(\mathrm{Mg^{2+}})\end{aligned} \tag{5}$$

where μ is the chemical potential of the indicated species, and $\mathrm{d}n$ is the differential of the amount. The criterion for equilibrium at specified T and P is $(\mathrm{d}G)_{T,P} \leq 0$; i.e. at constant temperature and pressure, G can only decrease and $\mathrm{d}G$ is equal to zero at equilibrium, where G is at its minimum. In writing the fundamental equation in this way, we are using the Gibbs energy G and the chemical potentials μ_i at a specified ionic strength. As a simplification, we are going to assume that $\mathrm{pH} = -\log([\mathrm{H}^+]/c^\circ)$ and $\mathrm{pMg} = -\log([\mathrm{Mg}^{2+}]/c^\circ)$, where c° is the standard state concentration of 1 mol l^{-1}. Since we are considering inorganic phosphate to be analogous to a macromolecule, we want to consider the thermodynamic properties of the sum of species making up inorganic phosphate, represented by P_i, as a function of pH and pMg at specified T and P. When T, P, pH, and pMg are specified, the Gibbs energy does not provide the criterion for equilibrium, and it is necessary to use a Legendre transform to define a transformed Gibbs energy G' that does provide this criterion. This Legendre transform [3,4] is

$$G' = G - n'(\mathrm{H}^+)\mu(\mathrm{H}^+) - n'(\mathrm{Mg}^{2+})\mu(\mathrm{Mg}^{2+}) \tag{6}$$

where $n'(\mathrm{H}^+)$ is the total amount of hydrogen in the system, namely $n'(\mathrm{H}^+) = \Sigma N_\mathrm{H}(i)n_i$, and $n'(\mathrm{Mg}^{2+}) = \Sigma N_\mathrm{Mg}(i)n_i$. $N_\mathrm{H}(i)$ and $N_\mathrm{Mg}(i)$ are the numbers of H and Mg atoms in a molecule of species i. The transformed Gibbs energy G' provides the criterion of equilibrium because it can be shown that $(\mathrm{d}G')_{T,P,\mathrm{pH},\mathrm{pMg}} \leq 0$.

The Gibbs energy of the system can be obtained by integrating Eq. (5) at constant T and P, to obtain $G = \Sigma n_i \mu_i$. Substituting the expressions for G, $n'(\mathrm{H}^+)$, and $n'(\mathrm{Mg}^{2+})$ into Eq. (6) yields

$$\begin{aligned} G' &= \Sigma\, \mu_i n_i - \mu(\mathrm{H}^+)\Sigma\, N_\mathrm{H}(i)n_i - \mu(\mathrm{Mg}^{2+})\Sigma\, N_\mathrm{Mg}(i)n_i \\ &= \Sigma\left[\mu_i - N_\mathrm{H}(i)\mu(\mathrm{H}^+) - N_\mathrm{Mg}(i)\mu(\mathrm{Mg}^{2+})\right]n_i = \Sigma\, \mu'_i n_i \end{aligned} \tag{7}$$

where the transformed chemical potential μ'_i of species i is given by

$$\mu'_i = \mu_i - N_\mathrm{H}(i)\mu(\mathrm{H}^+) - N_\mathrm{Mg}(i)\mu(\mathrm{Mg}^{2+}) \tag{8}$$

Eq. (7) shows that the transformed Gibbs energy G' is additive in the transformed chemical potentials μ'_i of species, just like the Gibbs energy G is additive in the chemical potentials μ_i of species. Eq. (8) can be used to eliminate all of the chemical potentials μ_i from Eq. (5), except for the terms for H^+ and Mg^{2+}, since $\mu'_i = 0$ for these ions. When this is done we obtain

$$\begin{aligned} (\mathrm{d}G)_{T,P} &= \mu'(\mathrm{HPO_4^{2-}})\,\mathrm{d}n(\mathrm{HPO_4^{2-}}) + \mu'(\mathrm{H_2PO_4^-})\,\mathrm{d}n(\mathrm{H_2PO_4^-}) + \mu'(\mathrm{MgHPO_4})\,\mathrm{d}n(\mathrm{MgHPO_4}) \\ &\quad + \mu(\mathrm{H}^+)\,\mathrm{d}n'(\mathrm{H}^+) + \mu(\mathrm{Mg}^{2+})\,\mathrm{d}n'(\mathrm{Mg}^{2+}) \end{aligned} \tag{9}$$

where $n'(\mathrm{H}^+) = n(\mathrm{H}^+) + n(\mathrm{HPO_4^{2-}}) + 2n(\mathrm{H_2PO_4^-})$ is the total amount of hydrogen in the system (note that H in $\mathrm{H_2O}$ is omitted since it does not change) and the total amount of magnesium in the system is given by $n'(\mathrm{Mg}^{2+}) = n(\mathrm{Mg}^{2+}) + n(\mathrm{MgHPO_4})$. The prime indicates the amount of a component. Components are important in thermodynamics because their amounts are independent variables at chemical equilibrium, but amounts of species are not independent variables because they have to satisfy equilibrium conditions.

The Legendre transform (Eq. (6)) is used to make the intensive variables $\mu(\mathrm{H}^+)$ and $\mu(\mathrm{Mg}^{2+})$ natural variables, so that we can discuss the thermodynamics of inorganic phosphate at specified pH and pMg. The differential of the transformed Gibbs energy G', defined in Eq. (6), is

$$\mathrm{d}G' = \mathrm{d}G - n'(\mathrm{H}^+)\,\mathrm{d}\mu(\mathrm{H}^+) - \mu(\mathrm{H}^+)\,\mathrm{d}n'(\mathrm{H}^+) - n'(\mathrm{Mg}^{2+})\,\mathrm{d}\mu(\mathrm{Mg}^{2+}) - \mu(\mathrm{Mg}^{2+})\,\mathrm{d}n'(\mathrm{Mg}^{2+}) \tag{10}$$

Replacing dG in this equation with Eq. (9) yields

$$(\mathrm{d}G')_{T,P} = \mu'(\mathrm{HPO_4^{2-}})\,\mathrm{d}n(\mathrm{HPO_4^{2-}}) + \mu'(\mathrm{H_2PO_4^-})\,\mathrm{d}n(\mathrm{H_2PO_4^-}) + \mu'(\mathrm{MgHPO_4})\,\mathrm{d}n(\mathrm{MgHPO_4}) - n'(\mathrm{H^+})\,\mathrm{d}\mu(\mathrm{H^+}) - n'(\mathrm{Mg^{2+}})\,\mathrm{d}\mu(\mathrm{Mg^{2+}}) \qquad (11)$$

When the chemical potentials of H^+ and Mg^{2+} are held constant, the last two terms drop out, and this equation becomes

$$(\mathrm{d}G')_{T,P,\mathrm{pH},\mathrm{pMg}} = \mu'(\mathrm{HPO_4^{2-}})\,\mathrm{d}n(\mathrm{HPO_4^{2-}}) + \mu'(\mathrm{H_2PO_4^-})\,\mathrm{d}n(\mathrm{H_2PO_4^-}) + \mu'(\mathrm{MgHPO_4})\,\mathrm{d}n(\mathrm{MgHPO_4}) \qquad (12)$$

The details of some steps in shifting to pH and pMg from $\mu(\mathrm{H^+})$ and $\mu(\mathrm{Mg^{2+}})$ as natural variables have been omitted, but they are described elsewhere [3,4]. This equation looks like it has three terms on the right-hand side, but actually there is a single term because the transformed chemical potentials of HPO_4^{2-}, $H_2PO_4^-$, and $MgHPO_4$ are equal, and can be replaced with the transformed chemical potential of inorganic phosphate $\mu'(\mathrm{P}_i)$, where $\mu'(\mathrm{P}_i) = \mu'(\mathrm{HPO_4^{2-}}) = \mu'(\mathrm{H_2PO_4^-}) = \mu'(\mathrm{MgHPO_4})$. This equality is demonstrated in the Appendix. Therefore, Eq. (12) can be written as

$$(\mathrm{d}G')_{T,P,\mathrm{pH},\mathrm{pMg}} = \mu'(\mathrm{P}_i)\,\mathrm{d}n'(\mathrm{P}_i) \qquad (13)$$

where the total amount of inorganic phosphate in the system is given by $n'(\mathrm{P}_i) = n(\mathrm{HPO_4^{2-}}) + n(\mathrm{H_2PO_4^-}) + n(\mathrm{MgHPO_4})$. Thus, introducing the Legendre transform into Eq. (6) and specifying pH and pMg has had the effect of reducing the number of terms in the fundamental equation from five in Eq. (5) to one in Eq. (13). It is this type of simplification that is needed to treat a macromolecule with many liganded species as a single entity. This single term involves the transformed chemical potential μ'_i and the amount n'_i of the entity P_i at specified T, P, pH, and pMg.

The above equations are generally applicable, but as a simplification it is assumed that the chemical potential of species i is given by

$$\mu_i = \mu_i^\circ + RT\ln([i]/c^\circ) \qquad (14)$$

where c° is the standard state concentration of 1 M. When this is true, transformed chemical potentials of species or pseudoisomer groups are given by

$$\mu'_i = \mu_i'^\circ + RT\ln([i]/c^\circ) \qquad (15)$$

where i might be inorganic phosphate. Derivations are carried out in terms of chemical potentials, but thermodynamic calculations on systems are carried out with standard thermodynamic properties, such as those tabulated in the NBS Tables [10]. It is evident from Eq. (13) that $\mu'(\mathrm{P}_i)$ is the partial derivative of the transformed Gibbs energy with respect to the amount of inorganic phosphate, and so it can be replaced with the transformed Gibbs energy of formation of inorganic phosphate $\Delta_\mathrm{f}G'(\mathrm{P}_i)$ for the purpose of numerical calculations. Since we are assuming ideal solutions, $\Delta_\mathrm{f}G'(\mathrm{P}_i)$ at specified pH and pMg is given by

$$\Delta_\mathrm{f}G'(\mathrm{P}_i) = \Delta_\mathrm{f}G'^\circ(\mathrm{P}_i) + RT\ln([\mathrm{P}_i]/c^\circ) \qquad (16)$$

where c° is the standard state concentration (1 M). $\Delta_\mathrm{f}G'^\circ(\mathrm{P}_i)$ is the standard transformed Gibbs energy of formation of inorganic phosphate at specified T, P, pH, pMg, and ionic strength. Eq. (16) indicates that $\Delta_\mathrm{f}G'^\circ(\mathrm{P}_i)$ is the value of $\Delta_\mathrm{f}G'(\mathrm{P}_i)$ at $[\mathrm{P}_i] = 1$ M. This, of course, would not be an ideal solution, but Eq. (16) can be used to calculate $\Delta_\mathrm{f}G'(\mathrm{P}_i)$ at low concentrations of inorganic phosphate and a specified ionic strength. When this equation is used for $\Delta_\mathrm{f}G'^\circ$ for a protein, we do not imply that an actual solution of the protein at 1 M is possible or ideal, but it is assumed that Eq. (16) is obeyed at very low molar concentrations of the protein. We can use Eq. (16) to calculate $\Delta_f G'(\mathrm{P}_i)$ at concentration $[\mathrm{P}_i]$ and specified T, P, pH, and pMg, if we can calculate $\Delta_f G'^{\circ}(\mathrm{P}_i)$ from the standard Gibbs energies $\Delta_f G_i^{\circ}$ of formation of the species that make up P_i.

First, we need to consider the calculation of the transformed Gibbs energy of formation $\Delta_f G'^{\circ}(i)$ of a species. The chemical potentials in Eq. (8) are replaced by Gibbs energies of formation to obtain

$$\Delta_f G_i'^{\circ} = \Delta_f G_i^{\circ} - N_{\mathrm{H}}(i)\left[\Delta_f G^{\circ}(\mathrm{H}^+) - 2.303RT\mathrm{pH}\right] - N_{\mathrm{Mg}}(i)\left[\Delta_f G^{\circ}(\mathrm{Mg}^{2+}) - 2.303RT\mathrm{pMg}\right] \qquad (17)$$

where the terms $RT\ \ln([i]/c^{\circ})$ on both sides were cancelled. The standard Gibbs energy of formation $\Delta_f G^{\circ}(\mathrm{H}^+)$ of the hydrogen ion is zero at zero ionic strength by convention, but it is not zero at higher ionic strengths [11]. The corresponding equation for the standard transformed enthalpy of formation of species i is

$$\Delta_f H_i'^{\circ} = \Delta_f H_i^{\circ} - N_{\mathrm{H}}(i)\Delta_f H^{\circ}(\mathrm{H}^+) - N_{\mathrm{Mg}}(i)\Delta_f H^{\circ}(\mathrm{Mg}^{2+}) \qquad (18)$$

The standard transformed Gibbs energies of formation and the standard transformed enthalpies of the three species of inorganic phosphate at specified pH and pMg can be calculated, because their standard Gibbs energies of formation $\Delta_f G^{\circ}$ are given by the NBS Tables [10]. The next step is to combine the three values for the three species to obtain $\Delta_f G'^{\circ}(\mathrm{P}_i)$ and $\Delta_f H'^{\circ}(\mathrm{P}_i)$ for the entity inorganic phosphate at specified pH and pMg.

3. Calculation of the standard transformed Gibbs energies of formation of inorganic phosphate at specified *T*, *P*, pH, and pMg

The simplest way to understand the calculation of the standard transformed Gibbs energy of formation of inorganic phosphate $\Delta_f G'^{\circ}(\mathrm{P}_i)$ at specified T, P, pH, and pMg is to recognize that once pH and pMg are specified, $\mathrm{HPO_4^{2-}}$, $\mathrm{H_2PO_4^-}$, and $\mathrm{MgHPO_4}$ are pseudoisomers. Isomers have the same chemical potential at chemical equilibrium, and pseudoisomers have the same transformed chemical potential at chemical equilibrium at specified pH and pMg. This means that the equation used to calculate the standard Gibbs energy of formation of a group of isomers at chemical equilibrium can be used to calculate the standard transformed Gibbs energy of formation of a group of pseudoisomers at specified pH and pMg [12,13]. The standard transformed Gibbs energy of formation of inorganic phosphate under the conditions discussed here is given by

$$\begin{aligned}\Delta_f G'^{\circ}(\mathrm{P}_i) = {} & -RT\ \ln\left\{\exp\left[-\Delta_f G'^{\circ}(\mathrm{HPO_4^{2-}})/RT\right]\right. \\ & \left. +\exp\left[-\Delta_f G'^{\circ}(\mathrm{H_2PO_4^-})/RT\right] + \exp\left[-\Delta_f G'^{\circ}(\mathrm{MgHPO_4})/RT\right]\right\} \end{aligned} \qquad (19)$$

It is important to note that $\Delta_f G'^{\circ}(\mathrm{P}_i)$ is not a weighted average of the three standard transformed Gibbs energies of formation. It will always be more negative than any one of them; in other words, the reactant (pseudoisomer group) P_i is more stable than any of the pseudoisomers. The equilibrium mole fraction r_i of any one of the pseudoisomers i in the pseudoisomer group is given by

$$r_i = \exp\left\{\left[\Delta_f G'^{\circ}(\mathrm{P}_i) - \Delta_f G'^{\circ}(i)\right]/RT\right\} \qquad (20)$$

which is an analog of the Boltzmann distribution. The standard transformed enthalpy of formation of the pseudoisomer group is a mole fraction weighted average and is given by

$$\Delta_f H'^{\circ}(\mathrm{P}_i) = r(\mathrm{HPO_4^{2-}})\Delta_f H'^{\circ}(\mathrm{HPO_4^{2-}}) + r(\mathrm{H_2PO_4^-})\Delta_f H'^{\circ}(\mathrm{H_2PO_4^-}) + r(\mathrm{MgHPO_4})\Delta_f H'^{\circ}(\mathrm{MgHPO_4}) \qquad (21)$$

The standard transformed entropy of formation of the pseudoisomer group is given by

$$\Delta_f S'^{\circ}(\mathrm{P}_i) = \left[\Delta_f H'^{\circ}(\mathrm{P}_i) - \Delta_f G'^{\circ}(\mathrm{P}_i)\right]/T \qquad (22)$$

For inorganic phosphate, the standard formation properties of these three species are known and have been used to calculate $\Delta_f G'^{\circ}(P_i) = -1059.55$ kJ mol^{-1} and $\Delta_f H'^{\circ}(P_i) = -1299.13$ kJ mol^{-1} at 298.15 K, 1 bar, pH 7, pMg 3, and $I = 0.25$ M [4,14]. These values are with respect to the usual reference state of the elements in their standard states. This nomenclature has been approved by IUBMB and IUPAC [15].

The average number of hydrogen atoms bound by inorganic phosphate at specified T, P, pH, and pMg is given by

$$\bar{N}_H(P_i) = \frac{1}{2.303RT}\left(\frac{\partial \Delta_f G'^{\circ}}{\partial \mathrm{pH}}\right)_{T,P,\mathrm{pMg}} \tag{23}$$

The average number of magnesium atoms bound by inorganic phosphate at specified T, P, pH, and pMg is given by

$$\bar{N}_{Mg}(P_i) = \frac{1}{2.303RT}\left(\frac{\partial \Delta_f G'^{\circ}}{\partial \mathrm{pMg}}\right)_{T,P,\mathrm{pH}} \tag{24}$$

The relation between the binding of hydrogen and magnesium ions by inorganic phosphate is given by

$$\left[\frac{\partial \bar{N}_H(P_i)}{\partial \mathrm{pMg}}\right]_{T,P,\mathrm{pH}} = \left[\frac{\partial \bar{N}_H(P_i)}{\partial \mathrm{pH}}\right]_{T,P,\mathrm{pMg}} \tag{25}$$

This discussion of the binding of protons by inorganic phosphate has been from the point of view of the thermodynamics of biochemical reactions, but we now turn to the point of view of the binding of ligands by macromolecules and seek to rearrange Eq. (19) into the form of the binding potential Π, defined in Eq. (1). This is done by multiplying and dividing the sum of exponential terms in Eq. (19) by the first term, so that this term can be removed from the logarithmic expression. This yields

$$\Delta_f G'^{\circ}(P_i) = \Delta_f G'^{\circ}(\mathrm{HPO_4^{2-}}) - RT\ln\left\{1 + \frac{\exp[-\Delta_f G'^{\circ}(\mathrm{H_2PO_4^-})/RT]}{\exp[-\Delta_f G'^{\circ}(\mathrm{HPO_4^{2-}})/RT]} + \frac{\exp[-\Delta_f G'^{\circ}(\mathrm{MgHPO_4})/RT]}{\exp[-\Delta_f G'^{\circ}(\mathrm{HPO_4^{2-}})/RT]}\right\} \tag{26}$$

The expressions for $\Delta_f G'^{\circ}(\mathrm{HPO_4^{2-}})$, $\Delta_f G'^{\circ}(\mathrm{H_2PO_4^-})$, and $\Delta_f G'^{\circ}(\mathrm{MgHPO_4})$ can be obtained from Eq. (17) and substituted into Eq. (26). On rearranging the logarithmic expression, note that for the acid dissociation

$$\mathrm{H_2PO_4^{2-} \rightleftharpoons H^+ + HPO_4^{2-}} \tag{27}$$

the equilibrium constant is given by

$$K_a = \frac{[\mathrm{H^+}][\mathrm{HPO_4^{2-}}]}{[\mathrm{H_2PO_4^-}]c^{\circ}} = \exp\{-[\Delta_f G^{\circ}(\mathrm{H^+}) + \Delta_f G^{\circ}(\mathrm{HPO_4^{2-}}) - \Delta_f G^{\circ}(\mathrm{H_2PO_4^-})]/RT\} \tag{28}$$

There is a similar equation for the dissociation of the magnesium complex ion:

$$\mathrm{MgHPO_4 \rightleftharpoons Mg^{2+} + HPO_4^{2-}} \tag{29}$$

$$K_{Mg} = \frac{[\mathrm{Mg^{2+}}][\mathrm{HPO_4^{2-}}]}{[\mathrm{MgHPO_4}]c^{\circ}} = \exp\{-[\Delta_f G^{\circ}(\mathrm{Mg^{2+}}) + \Delta_f G^{\circ}(\mathrm{HPO_4^{2-}}) - \Delta_f G^{\circ}(\mathrm{MgHPO_4^-})]/\mathrm{RT}\} \tag{30}$$

Also note that $[\mathrm{H^+}] = \exp(\ln[\mathrm{H^+}])$. Using Eqs. (28) and (30) in Eq. (26) yields

$$\Delta_f G'^{\circ}(P_i) = \Delta_f G'^{\circ}(\mathrm{HPO_4^{2-}}) - RT\ln\left(1 + \frac{[\mathrm{H^+}]}{K_a} + \frac{[\mathrm{Mg^{2+}}]}{K_{Mg}}\right) = \Delta_f G'^{\circ}(\mathrm{HPO_4^{2-}}) - RT\ln P \tag{31}$$

Where P is the binding polynomial. Thus, the standard transformed Gibbs energy of formation of inorganic phosphate at specified pH and pMg is related to the binding potential Π by

$$\Delta_f G'^{\circ}(\mathrm{P}_i) = -\Pi + \Delta_f G'^{\circ}(\mathrm{HPO}_4^{2-}) \tag{32}$$

The standard transformed Gibbs energy of formation of inorganic phosphate at specified pH and pMg is equal to the negative of the binding potential plus a term $\Delta_f G'^{\circ}(\mathrm{HPO}_4^{2-})$ to connect with the standard Gibbs energies of the elements. Since $\Delta_f G'^{\circ}(\mathrm{HPO}_4^{2-})$ is given by

$$\Delta_f G'^{\circ}(\mathrm{HPO}_4^{2-}) = \Delta_f G^{\circ}(\mathrm{HPO}_4^{2-}) - \left[\Delta_f G^{\circ}(\mathrm{H}^+) + RT\ln([\mathrm{H}^+]/c^{\circ})\right] \tag{33}$$

(see Eq. (17)), its value is known with respect to the elements in their reference states. Eq. (32) shows that Π is a standard thermodynamic quantity and should have the superscript °.

This discussion has been concerned with deriving both $\Delta_f G'^{\circ}$ and Π for P_i at specified pH and pMg. It shows the connection between the binding potential and existing tables of chemical thermodynamics. We have seen that $\Delta_f G'^{\circ}$ and Π can be calculated when the thermodynamic properties of the species in a system are already available. In this case, the thermodynamic behavior of a reactant (sum of species) can be predicted and discussed as a function of T, P, pH, and pMg, for example. However, a common situation in the laboratory is that the thermodynamic properties of the species making up the reactant are not available and there is a need for the transformed thermodynamic properties of the reactant under a specified set of conditions. Consider ATP as an example. Acid titrations of ATP have been used to determine its acid dissociation constants, and acid titrations in the presence of Mg^{2+} have been used to determine the dissociation constants of complex ions containing Mg^{2+}. Since the equilibrium constants are not known for a complete series of reactions connecting some species of ATP with the elements involved, the standard thermodynamic properties of the various species cannot be related to the usual reference states of the elements. However, the thermodynamic properties of species in the ATP series can be calculated by assigning one of them $\Delta_f G_i^{\circ} = \Delta_f H_i^{\circ} = 0$. This was done a long time ago with hydrogen ions in water, because there was no way to determine the equilibrium constant for a reaction linking H^+ in water to molecular hydrogen. Thermodynamic tables constructed in this way work, provided the convention is always respected and the tables are not used to try to calculate the missing equilibrium constants. In the case of ATP species, Alberty and Goldberg [14] chose to assign $\Delta_f G_i^{\circ} = \Delta_f H_i^{\circ} = 0$ to uncharged adenosine, since the values of equilibrium constants linking adenosine with species of ATP are known. When at some future time the missing equilibrium constants and their temperature derivatives are determined, the values of $\Delta_f G_i^{\circ}$ and $\Delta_f H_i^{\circ}$ for the various species of ATP will be changed, but this will not alter the values of equilibrium constants and their temperature derivatives calculated from the current table. It is interesting to note that values of $\Delta_f S_i^{\circ}$ for species of ATP will not be changed, because $\Delta_f S_i^{\circ} = -(\partial \Delta_f G_i^{\circ}/\partial T)_{T,P}$ so the value of this derivative is not affected by adding a constant to $\Delta_f G_i^{\circ}$.

Although this simple example involves linkage, it does not illustrate allosteric effects. In order to do that, we consider an isomeric weak acid.

4. Binding of H^+ by an isomeric weak acid from the point of view of an enzyme-catalyzed reaction

Consider a monoprotic weak acid HA that isomerizes to a different form designated as HB. The acid dissociation constants are different for the two isomeric forms. The system is represented by

$$\begin{array}{ccc} \mathrm{A}^- + \mathrm{H}^+ & \overset{K}{=} & \mathrm{B}^- + \mathrm{H}^+ \\ \| \, K_{\mathrm{HA}} & & \| \, K_{\mathrm{HB}} \\ \mathrm{HA} & & \mathrm{HB} \end{array} \tag{34}$$

The dimensionless equilibrium constants are defined at a specified ionic strength by

$$K = [B^-]/[A^-] \tag{35}$$

$$K_{HA} = [H^+][A^-]/[HA]c^\circ \tag{36}$$

$$K_{HB} = [H^+][B^-]/[HB]c^\circ \tag{37}$$

The fundamental equation for the Gibbs energy of this system at specified temperature and pressure is

$$(dG)_{T,P} = \mu(A^-)\,dn(A^-) + \mu(HA)\,dn(HA) + \mu(B^-)\,dn(B^-) + \mu(HB)\,dn(HB) + \mu(H^+)\,dn(H^+) \tag{38}$$

To consider the system at a specified pH, a Legendre transform is used to define a transformed Gibbs energy G' for the system by

$$G' = G - n'(H^+)\mu(H^+) \tag{39}$$

where $n'(H^+) = \Sigma N_H(i) n_i$ is the total amount of hydrogen (the amount of the hydrogen component) in the system (excluding H_2O), and $N_H(i)$ is the number of hydrogen atoms in a molecule of species i. Substituting this expression and $G = \Sigma n_i \mu_i$ into Eq. (39) shows that the transformed Gibbs energy is given by

$$G' = \Sigma \mu_i' n_i \tag{40}$$

where

$$\mu_i' = \mu_i - N_H(i)\mu(H^+) \tag{41}$$

is the transformed chemical potential of species i. When this equation is used to eliminate the chemical potentials of four of the species from Eq. (38), we obtain

$$\begin{aligned}(dG)_{T,P} = {} & \mu'(A^-)\,dn(A^-) + \mu'(HA)\,dn(HA) + \mu'(B^-)\,dn(B^-) + \mu'(HB)\,dn(HB) \\ & + \mu(H^+)\,dn'(H^+)\end{aligned} \tag{42}$$

where $dn'(H^+) = dn(HA) + dn(HB) + dn(H^+)$.

The differential of the transformed Gibbs energy G' is

$$dG' = dG - n'(H^+)\,d\mu(H^+) - \mu(H^+)\,dn'(H^+) \tag{43}$$

Eliminating dG with Eq. (42) yields

$$\begin{aligned}(dG')_{T,P} = {} & \mu'(A^-)\,dn(A^-) + \mu'(HA)\,dn(HA) + \mu'(B^-)\,dn(B^-) + \mu'(HB)\,dn(HB) \\ & - n'(H^+)\,d\mu(H^+)\end{aligned} \tag{44}$$

At specified pH, $d\mu(H^+) = 0$, and

$$(dG')_{T,P,pH} = \mu'(A^-)\,dn(A^-) + \mu'(HA)\,dn(HA) + \mu'(B^-)\,dn(B^-) + \mu'(HB)\,dn(HB) \tag{45}$$

At equilibrium, this equation can be written in two ways depending on the measurements that can be made. For example, it may be possible to study A and B separately and determine the apparent equilibrium constant for their interconversion by use of an enzyme. Alternatively, A and B may rapidly interconvert without a catalyst. If A and B can be distinguished experimentally, we know that $\mu'(A^-) = \mu'(HA)$ and $\mu'(B^-) = \mu'(HB)$ at equilibrium at specified pH, so Eq. (45) can be written as

$$(dG')_{T,P,pH} = \mu'(A)\,dn'(A) + \mu'(B)\,dn'(B) \tag{46}$$

where $\mu'(A) = \mu'(A^-) = \mu'(HA)$, $n(A)' = n(A^-) + n(HA)$, and there are similar equations for B. Eq. (46) shows how the fundamental equation for the system can be expressed in terms of reactants A and B, rather than species. This is the point of view that is used in studying enzyme-catalyzed reactions. The biochemical reaction A = B, with $K' = [\mathrm{B}]/[\mathrm{A}]$, can be studied for a series of pH values, since A and B can be distinguished.

If A and B interconvert rapidly in the absence of a catalyst, we must consider the four species at equilibrium as an entity and discuss the thermodynamics of the A,B pseudoisomer group; this is the approach used in the study of binding by a macromolecule because it is very difficult to distinguish A and B experimentally. The first approach is possible with small reactants, but not with proteins having allosteric transitions. The first approach is discussed in the remainder of this section, and the second approach is discussed in the next section.

When we consider the reaction between A and B at a specified pH, the biochemical reaction equation can be written as

$$\mathrm{A} \rightleftharpoons \mathrm{B} \tag{47}$$

where A and B represent sums of species. The apparent equilibrium constant K' at a specified pH is defined by

$$K' = \frac{[\mathrm{B}]}{[\mathrm{A}]} = \frac{[\mathrm{B}^-] + [\mathrm{HB}]}{[\mathrm{A}^-] + [\mathrm{HA}]} \tag{48}$$

This equation can be derived from Eq. (46) by substituting $-\mathrm{d}n'(\mathrm{A}) = \mathrm{d}n'(\mathrm{B}) = \mathrm{d}\xi'$, where ξ' is the apparent extent of reaction (47). This leads to

$$\left(\frac{\partial G'}{\partial \xi'}\right)_{T,P,\mathrm{pH}} = \Delta_\mathrm{r} G' = -\mu'(\mathrm{A}) + \mu'(\mathrm{B}) \tag{49}$$

At chemical equilibrium the transformed Gibbs energy of the system is at a minimum. This derivative is equal to zero and yields the equilibrium condition, which is

$$\mu'(\mathrm{A}) = \mu'(\mathrm{B}) \tag{50}$$

Substituting

$$\mu'(\mathrm{A}) = \mu'(\mathrm{A})^\circ + RT \ln([\mathrm{A}]/c^\circ) \tag{51}$$

and a similar equation for B in Eq. (49), and shifting from transformed chemical potentials to the transformed reaction Gibbs energy yields

$$\Delta_\mathrm{r} G'^\circ = -RT \ln K' \tag{52}$$

where

$$\Delta_\mathrm{r} G'^\circ = \Delta_\mathrm{f} G'^\circ(\mathrm{B}) - \Delta_\mathrm{f} G'^\circ(\mathrm{A}) \tag{53}$$

As discussed in the case of inorganic phosphate, the values of the standard transformed Gibbs energies of formation of A and B can be calculated from tabulated values of standard Gibbs energies of formation of species, or be determined in the laboratory by acid titrations since A and B are stable in the absence of the isomerase. The standard transformed Gibbs energy of formation of A at specified pH and pMg is given by

$$\Delta_\mathrm{f} G'^\circ(\mathrm{A}) = -RT \ln\{\exp[-\Delta_\mathrm{f} G'^\circ(\mathrm{A}^-)/RT] + \exp[-\Delta_\mathrm{f} G'^\circ(\mathrm{HA})/RT]\} \tag{54}$$

where $\Delta_\mathrm{f} G'^\circ(\mathrm{A}^-) = \Delta_\mathrm{f} G^\circ(\mathrm{A}^-)$ and, according to Eq. (41),

$$\Delta_\mathrm{f} G'^\circ(\mathrm{HA}) = \Delta_\mathrm{f} G^\circ(\mathrm{HA}) - [\Delta_\mathrm{f} G^\circ(\mathrm{H}^+) - 2.303RT\mathrm{pH}] \tag{55}$$

Similar equations exist for B.

5. Binding of H^+ by an isomeric weak acid when the two forms cannot be distinguished experimentally

This system has been discussed by Wyman and Gill (see Sections 2.6 and 4.3 of Ref. [9]) since it is the simplest allosteric system, but the system will be treated here using the fundamental equation of thermodynamics and a Legendre transform. If A and B cannot be distinguished experimentally, we can write Eq. (45) in a different way at equilibrium, because A^-, HA, B^-, and HB are pseudoisomers at specified pH and $\mu'(A^-) = \mu'(HA) = \mu'(B^-) = \mu'(HB) = \mu'(A,B)$. Thus, Eq. (45) can be written in terms of the A,B pseudoisomer group:

$$(\mathrm{d}G')_{T,P,\mathrm{pH}} = \mu'(\mathrm{A,B})\,\mathrm{d}n'(\mathrm{A,B}) \tag{56}$$

where $\mu'(A,B)$ is the transformed chemical potential of the A,B pseudoisomer group at specified pH and $n'(A,B)$ is the total amount of weak acids and anions. The standard transformed Gibbs energy of formation of the A,B pseudoisomer group is given by

$$\Delta_f G'^\circ(\mathrm{A,B}) = -RT\ln\{\exp[-\Delta_f G'^\circ(\mathrm{A^-})/RT] + \exp[-\Delta_f G'^\circ(\mathrm{HA})/RT] + \exp[-\Delta_f G'^\circ(\mathrm{B^-})/RT] + \exp[-\Delta_f G'^\circ(\mathrm{HB})/RT]\} \tag{57}$$

In words, the standard transformed Gibbs energy of formation of the A,B pseudoisomer group at a specified pH can be calculated if the standard formation properties of the various species are known. Taking the first term out of the summation, as described above for inorganic phosphate, yields

$$\Delta_f G'^\circ(\mathrm{A,B}) = \Delta_f G'^\circ(\mathrm{A^-}) - RT\ln\left[1 + \frac{[\mathrm{H^+}]}{K_{\mathrm{HA}}} + K\left(1 + \frac{[\mathrm{H^+}]}{K_{\mathrm{HB}}}\right)\right] = \Delta_f G'^\circ(\mathrm{A^-}) - RT\ln P \tag{58}$$

where P is the binding polynomial. This equation can be written as

$$\Delta_f G'^\circ(\mathrm{A,B}) = -\Pi + \Delta_f G^\circ(\mathrm{A^-}) \tag{59}$$

since $\Delta_f G'^\circ(A^-) = \Delta_f G^\circ(A^-)$. The second term on the right-hand side of Eq. (59) provides the connection with the rest of thermodynamics. An acid titration of the rapidly interconverting system will yield a single pK, but if we have reasons to believe that there are two isomers with a certain relation between K_{HA} and K_{HB}, we can calculate the three parameters in the binding polynomial. As explained above, this can yield the standard thermodynamic properties of the four species. It may be necessary to assign $\Delta_f G^\circ(A^-) = 0$, so that $\Delta_f G'^\circ(A,B) = -RT\ln P$. $\Delta_f G^\circ(HA)$, $\Delta_f G^\circ(B^-)$, and $\Delta_f G^\circ(HB)$ can then be calculated.

6. The fundamental equation for the binding of oxygen by hemoglobin

The binding of oxygen by hemoglobin can be considered from the point of view of the fundamental equation of thermodynamics, as used above to consider the binding of H^+ and Mg^{2+} by inorganic phosphate and the binding of H^+ by the base form of an isomeric weak acid. However, in contrast with these simple systems, the binding of oxygen by hemoglobin cannot be treated directly by use of the binding potential P, because of the dissociation of the tetramer T into dimers D. The tetramer is made up of two alpha and two beta chains and can be represented by $(\alpha\beta)_2$. The dimer is made up of one alpha and one beta chain and can be represented by $\alpha\beta$. Although the oxygen binding of the dimer and tetramer can be represented separately by Eqs. (1) and (2), the treatment of the partially dissociated hemoglobin requires the inclusion of the equilibrium 2D = T. Ackers and Halvorson [16] derived an equation for calculating the fractional saturation Y of hemoglobin that is written in terms of seven equilibrium constants, four describing the binding of O_2 by T, two describing the binding of O_2 by D, and one for the association of dimers to tetramer. The fractional saturation of the tetramer can be represented by an Adair equation with four constants, and these equilibria can be expressed by a binding polynomial P. The fractional saturation of the dimer can be represented by an Adair equation with two constants, and these equilibria can be expressed by a smaller binding polynomial P. In the treatment of experimental data in the ranges of [heme] and $[O_2]$ that have been available so far, the presence of all eight forms of oxygenated and deoxygenated hemoglobin have to be taken into account [17,18].

In contrast with the preceding simple cases, the discussion of the fundamental equation for the binding of oxygen by hemoglobin starts with the transformed Gibbs energy G', because of the reactions of various forms of oxygenated and deoxygenated hemoglobin with H^+ and other species in the buffer. At specified T, P, pH, and buffer composition, the fundamental equation for the transformed Gibbs energy G' of the system hemoglobin plus oxygen is given by

$$\begin{aligned}(\mathrm{d}G')_{T,P,\mathrm{pH}} &= \mu'(\mathrm{D(O_2)_2})\,\mathrm{d}n'(\mathrm{D(O_2)_2}) + \mu'(\mathrm{D(O_2)})\,\mathrm{d}n'(\mathrm{D(O_2)}) + \mu'(\mathrm{D})\,\mathrm{d}n'(\mathrm{D}) + \mu'(\mathrm{T})\,\mathrm{d}n'(\mathrm{T}) \\ &\quad + \mu'(\mathrm{T(O_2)})\,\mathrm{d}n'(\mathrm{T(O_2)}) + \mu'(\mathrm{T(O_2)_2})\,\mathrm{d}n'(\mathrm{T(O_2)_2}) + \mu'(\mathrm{T(O_2)_3})\,\mathrm{d}n'(\mathrm{T(O_2)_3}) \\ &\quad + \mu'(\mathrm{T(O_2)_4})\,\mathrm{d}n'(\mathrm{T(O_2)_4}) + \mu'(\mathrm{O_2})\,\mathrm{d}n'(\mathrm{O_2}) \qquad (60)\end{aligned}$$

The transformed chemical potential of reactant i in the specified buffer is represented by μ'_i, and the sum of the amounts of species of reactant i is represented by n'_i. Only the pH is specifically referred to here as being held constant, but the same treatment applies to other bound species from the medium. The last term could be written as $\mu(O_2)\,\mathrm{d}n(O_2)$ without the primes because the Legendre transform that defines G' does not affect this term; however, the primes are included so that Eq. (60) can be represented by $\mathrm{d}G' = \sum \mu'_i\,\mathrm{d}n'_i$. Since $n'_i = n'_{i0} + \nu'_{ij}\xi'_j$, where n'_{i0} is the initial amount of reactant i in the system, ν'_{ij} is the apparent stoichiometric number of reactant i in reaction j at specified pH, and ξ'_j is the apparent extent of reaction j. Eq. (60) can be written in terms of the differential apparent extents of seven independent reactions between the nine reactants. The adjective "apparent" is used to distinguish these stoichiometric numbers from the stoichiometric numbers of the underlying chemical reactions. These rearrangements of Eq. (60), which are not written out here but are familiar in elementary thermodynamics [19], show that Eq. (60) yields seven independent equilibrium conditions of the form

$$\mu'(\mathrm{T}) + \mu'(\mathrm{O_2}) = \mu'(\mathrm{TO_2}) \qquad (61)$$

where these are transformed chemical potentials at equilibrium at specified T, P, pH, etc. Assuming ideal solutions in the sense of Eq. (15) yields the expressions for the seven reactions. Eq. (61) yields

$$K'_{41} = \frac{[\mathrm{TO_2}]c^\circ}{[\mathrm{T}][\mathrm{O_2}]} = \exp(-\Delta_\mathrm{r}G'^\circ_{41}/RT) \qquad (62)$$

The subscript follows the nomenclature of Mills et al. [20], and the use of the prime follows the IUBMB-IUPAC recommendation [15] to call such equilibrium constants apparent equilibrium constants and to indicate with a prime that T and TO_2 are sums of species at a specified pH.

The calculations here use values of the seven apparent equilibrium constants determined by Mills et al. [20]. The apparent equilibrium constants for an independent set of reactions of human hemoglobin at 21.5°C, 1 bar, pH 7.4, $[Cl^-] = 0.2$ M, and 0.2 M ionic strength are

$$\mathrm{D} + \mathrm{O_2} \rightleftharpoons \mathrm{D(O_2)} \quad K'_{21} = 3.253 \times 10^6 \qquad (63)$$

$$\mathrm{D(O_2)} + \mathrm{O_2} \rightleftharpoons \mathrm{D(O_2)_2} \quad K'_{22} = 8.155 \times 10^5 \qquad (64)$$

$$\mathrm{T} + \mathrm{O_2} \rightleftharpoons \mathrm{T(O_2)} \quad K'_{41} = 4.397 \times 10^4 \qquad (65)$$

$$\mathrm{T(O_2)} + \mathrm{O_2} \rightleftharpoons \mathrm{T(O_2)_2} \quad K'_{42} = 1.221 \times 10^4 \qquad (66)$$

$$\mathrm{T(O_2)_2} + \mathrm{O_2} \rightleftharpoons \mathrm{T(O_2)_3} \quad K'_{43} = 4.049 \times 10^5 \qquad (67)$$

$$\mathrm{T(O_2)_3} + \mathrm{O_2} \rightleftharpoons \mathrm{T(O_2)_4} \quad K'_{42} = 6.644 \times 10^5 \qquad (68)$$

$$2\mathrm{D} \rightleftharpoons \mathrm{T} \quad {}^\circ K'_2 = 4.633 \times 10^{10} \qquad (69)$$

where the apparent equilibrium constants are expressed using molar concentrations. These values are in good agreement with values obtained later by Chu et al. [21], who also give information on the effects of pH. When reporting values of association constants of hemoglobin, it is common practice in the literature to introduce statistical factors that are used to calculate intrinsic equilibrium constants when the binding sites are identical and independent. However, the binding sites in the tetramer are not identical and are not independent. It is important to remember that it is the apparent equilibrium constants without the statistical adjustments that determine the equilibrium composition. The set of reaction equations in Eqs. (63)–(69) is not unique, and a different set of seven independent reactions could be used. A set of reactions is independent if no reaction in the set can be obtained by adding and subtracting other reactions in the set. The primes indicate that the apparent equilibrium constants are functions of pH, $[Cl^-]$, etc. This treatment is meant to be general and to apply to hemoglobins with different properties. The values of the equilibrium constants for a particular hemoglobin under particular conditions are used later to illustrate how calculations are made.

These seven apparent equilibrium constants can be used to calculate $\Delta_f G'^\circ$ values for the eight reactants under the specified conditions. The standard Gibbs energy of formation of molecular oxygen in dilute aqueous solutions is known [10], and the standard apparent Gibbs energy of formation of the tetramer can be taken as zero, since its calculation is not possible from the elements involved. It is important to understand that the apparent equilibrium constants have the values they do, because the reactants each have standard transformed formation properties under the specified conditions which are independent of the way the reactions are written.

In order to calculate the standard transformed Gibbs energies of formation of the eight reactants, it is necessary to use the value of $\Delta_f G^\circ(O_2,aq)$ at 21.5°C. The NBS Tables [10] give $\Delta_f G^\circ(O_2,aq) = 16.4$ kJ mol^{-1} and $\Delta_f H^\circ(O_2,aq) = -11.7$ kJ mol^{-1} at 25°C. The use of the Gibbs–Helmholtz equation shows that at 21.5°C, $\Delta_f G^\circ(O_2,aq) = 16.1$ kJ mol^{-1}. The values of $\Delta_f G'^\circ$ for the eight reactants calculated using Eqs. (3) and (4) are given in Table 1. These standard formation properties can be calculated by starting with Eq. (65) and calculating one $\Delta_f G'^\circ$ from each reaction, or it can be done by solving seven simultaneous linear equations, as with LinearSolve in *Mathematica* [22]. More digits are retained in these calculations than are justified by the experimental data because calculations here are compared with those in the following article [23], which are made by an entirely different method. The corresponding $\Delta_f H'^\circ$ and $\Delta_f S'^\circ$ values for the eight reactants can be calculated using the corresponding $\Delta_r H'^\circ$.

Table 1

Standard transformed Gibbs energies of formation $\Delta_f G'^\circ$ of deoxygenated and oxygenated forms of hemoglobin at 21.5°C, 1 bar, pH 7.4, $[Cl^-] = 0.2$ M, and ionic strength 0.2 M, and standard further transformed Gibbs energies of formation $\Delta_f G''^\circ$ at specified concentrations of molecular oxygen

	$\Delta_f G'^\circ$ (kJ mol^{-1})	$\Delta_f G''^\circ$ (kJ mol^{-1})		
		$[O_2] = 5 \times 10^{-6}$ M	$[O_2] = 10^{-5}$ M	$[O_2] = 2 \times 10^{-5}$ M
$D(O_2)_2$	−7.799 31	19.807 30	16.411 00	13.014 80
$D(O_2)$	9.447 23	23.250 50	21.552 40	19.854 30
D	30.083 20	30.083 20	30.083 20	30.083 20
T	0	0	0	0
$T(O_2)$	−10.0922	3.711 09	2.012 97	0.314 85
$T(O_2)_2$	−17.0455	10.5611	7.164 83	3.768 59
$T(O_2)_3$	−32.5767	8.833 13	3.738 77	−1.355 60
$T(O_2)_4$	−49.3213	5.891 89	−0.900 59	−7.693 07
$\Delta_f G''^\circ$(TotT)	−49.3213 [a]	−0.736 51	−2.814 90	−8.069 06
$\Delta_f G''^\circ$(TotD)	−7.799 31 [a]	19.2404	16.1194	12.8668
$\Delta_r G''^\circ$(eq. (79))	−33.7226 [a]	−39.2174	−35.0538	−33.8027
K'' (eq. (80))	$9.508\,14 \times 10^5$ [a]	$8.957\,00 \times 10^6$	$1.637\,08 \times 10^6$	$9.824\,08 \times 10^5$

[a] These are the values that are reached at infinite $[O_2]$. The value of K'' agrees with that calculated using Eq. (82).

The values of $\Delta_f G'^\circ$ for the eight reactants provide the most efficient way to record the experimental information on equilibrium constants, because they can be used to calculate apparent equilibrium constants K' for any reaction that can be written between these eight reactants under the specified conditions. The $\Delta_f G'^\circ$ values can be used to calculate equilibrium concentrations starting with various total concentrations of hemoglobin and molecular oxygen, using a general equilibrium program like EQUCALCC (see the following article [23]). However, it is often more convenient to use $[O_2]$ as an independent variable, rather than as a dependent variable, and calculate the composition of the hemoglobin–oxygen system at a desired $[O_2]$. In order to do this it is necessary to define a further transformed Gibbs energy G'' by use of the Legendre transform [6]:

$$G'' = G' - n'(\mathrm{TotO_2})\,\mu(O_2) \tag{70}$$

where $\mu(O_2)$ is the specified chemical potential of molecular oxygen and $n'(\mathrm{TotO_2})$ is the total amount of molecular oxygen in the system, bound and unbound $(\Sigma N_{O_2(i)} n'_i)$. G'' is referred to as a further transformed Gibbs energy [24] because it is based on a second Legendre transform. The concept of a further transformed Gibbs energy has been used in considering the equilibrium that can be reached in a series of biochemical reactions when the concentrations of ATP, ADP, and P_i, for example, have steady state values as a result of other reactions that occur in a system. A consequence of Eq. (70) is the introduction of a further transformed chemical potential, as we have seen earlier in Eqs. (8) and (41):

$$\mu''_i = \mu'_i - N_{O_2}(i)\,\mu(O_2) \tag{71}$$

Note that $\mu''(O_2) = 0$. Eq. (71) can be used to eliminate μ'_i from Eq. (60):

$$\begin{aligned}(\mathrm{d}G')_{T,P,\mathrm{pH}} = {} & \mu''(\mathrm{D(O_2)_2})\,\mathrm{d}n'(\mathrm{D(O_2)_2}) + \mu''(\mathrm{D(O_2)})\,\mathrm{d}n'(\mathrm{D(O_2)}) + \mu''(\mathrm{D})\,\mathrm{d}n'(\mathrm{D}) + \mu''(T)\,\mathrm{d}n'(\mathrm{T}) \\ & + \mu''(\mathrm{T(O_2)})\,\mathrm{d}n'(\mathrm{T(O_2)}) + \mu''(\mathrm{T(O_2)_2})\,\mathrm{d}n'(\mathrm{T(O_2)_2}) + \mu''(\mathrm{T(O_2)_3})\,\mathrm{d}n'(\mathrm{T(O_2)_3}) \\ & + \mu''(\mathrm{T(O_2)_4})\,\mathrm{d}n'(\mathrm{T(O_2)_4}) + \mu'(O_2)\,\mathrm{d}n'(\mathrm{TotO_2})\end{aligned} \tag{72}$$

where the total amount of molecular oxygen in the system is represented by

$$\begin{aligned}n'(\mathrm{TotO_2}) = {} & 2n'(\mathrm{D(O_2)_2}) + n'(\mathrm{D(O_2)}) + n'(\mathrm{T(O_2)}) + 2n'(\mathrm{T(O_2)_2}) + 3n'(\mathrm{T(O_2)_3}) + 4n'(\mathrm{T(O_2)_4}) \\ & + n'(\mathrm{O_2})\end{aligned} \tag{73}$$

The differential of G'' (calculated from Eq. (70)) is

$$\mathrm{d}G'' = \mathrm{d}G' - n'(\mathrm{TotO_2})\,\mathrm{d}\mu(O_2) - \mu(O_2)\,\mathrm{d}n'(\mathrm{TotO_2}) \tag{74}$$

Substituting Eq. (72) and holding $\mu(O_2)$ constant yields

$$\begin{aligned}(\mathrm{d}G'')_{T,P,\mathrm{pH},[O_2]} = {} & \mu''(\mathrm{D(O_2)_2})\,\mathrm{d}n'(\mathrm{D(O_2)_2}) + \mu''(\mathrm{D(O_2)})\,\mathrm{d}n'(\mathrm{D(O_2)}) + \mu''(\mathrm{D})\,\mathrm{d}n'(D) \\ & + \mu''(\mathrm{T})\,\mathrm{d}n'(\mathrm{T}) + \mu''(\mathrm{T(O_2)})\,\mathrm{d}n'(\mathrm{T(O_2)}) + \mu''(\mathrm{T(O_2)_2})\,\mathrm{d}n'(\mathrm{T(O_2)_2}) \\ & + \mu''(\mathrm{T(O_2)_3})\,\mathrm{d}n'(\mathrm{T(O_2)_3}) + \mu''(\mathrm{T(O_2)_4})\,\mathrm{d}n'(\mathrm{T(O_2)_4})\end{aligned} \tag{75}$$

This equation has eight terms for eight reactants, one less term than Eq. (60) because the concentration of molecular oxygen is specified. The criterion for equilibrium for this system is $(\mathrm{d}G'')_{T,P,\mathrm{pH},[O_2]} \leq 0$. In other words, the equilibrium composition in terms of these eight reactants is the composition with the lowest possible further transformed Gibbs energy G'' consistent with the conservation equations. As discussed after Eq. (60), Eq. (75) can be written in terms of the apparent extents of reaction ξ''_j. Since $n'_i = n'_{i0} + \nu''_{ij}\xi''_j$, where n'_{i0} is the initial amount of reactant i in the system and ν''_{ij} is the apparent stoichiometric number of reactant i in reaction j, Eq. (75) can be written in terms of the differential extents of seven independent reactions between the eight reactants. These rearrangements show that Eq. (75) yields seven independent equilibrium conditions of the form

$$\mu''(\mathrm{T}) = \mu''(\mathrm{TO_2}) \tag{76}$$

where these are further transformed equilibrium chemical potentials at specified $[O_2]$. Assuming ideal solutions in the sense of Eq. (15) yields the expressions for the seven reactions. Eq. (76) yields

$$K''_{41} = \frac{[TO_2]}{[T]} = \exp(-\Delta_r G''^{\circ}_{41}/RT) \qquad (77)$$

Chemical potentials are used in derivations, but Gibbs energies of formation are used in actual calculations. Thus, Eq. (71) can be used in the form

$$\Delta_f G''^{\circ}_i = \Delta_f G'^{\circ}_i - N(O_2)[\Delta_f G^{\circ}(O_2) + RT\ln([O_2]/c^{\circ})] \qquad (78)$$

where $\Delta_f G^{\circ}(O_2)$ is the standard Gibbs energy of formation of O_2 in the aqueous phase. The standard further transformed Gibbs energies of formation $\Delta_f G''^{\circ}$ of the eight forms of hemoglobin under the specified conditions are given in Table 1 for three concentrations of molecular oxygen. Thus, $T(O_2)_4$, for example, has a certain $\Delta_f G''^{\circ}$ at pH 7 in the presence of 10^{-5} M oxygen, just as it has a certain $\Delta_f G'^{\circ}$ at pH 7, and a specified protonated form of $T(O_2)_4$ has a certain $\Delta_f G^{\circ}$ at any pH. The further transformed properties at $[O_2] = 6 \times 10^{-6}$, 10^{-5}, and 2×10^{-5} M can be used to calculate the corresponding apparent equilibrium constants K''. At specified $[O_2]$, the tetramer behaves like a single entity TotT, although it is made up of five forms. Similarly, the dimer behaves like a single entity TotD, although it is made up of three forms. At specified $[O_2]$, the reaction

$$2\text{TotD} = \text{TotT} \qquad (79)$$

can be discussed, and has an apparent equilibrium constant K'':

$$K'' = \frac{[\text{TotT}]}{[\text{TotD}]^2} \qquad (80)$$

Apparent equilibrium constants are dimensionless, but the c° that should be in the numerator to make K'' dimensionless is omitted as a simplification. At specified $[O_2]$, TotT has a standard further transformed Gibbs energy of formation $\Delta_f G''^{\circ}$ that can be calculated using equations like Eq. (19) and Eq. (57). In other words, the five forms of the tetramer are pseudoisomers, and the $\Delta_f G''^{\circ}$ of the pseudoisomer group can be calculated by summing the exponential terms. The values of $\Delta_f G''^{\circ}(\text{TotT})$ and $\Delta_f G''^{\circ}(\text{TotD})$ in Table 1 have been calculated in this way. These values can be used to calculate $\Delta_r G''^{\circ} = -RT \ln K''$ and K'' for reaction (79). This nomenclature has been used in discussing the standard transformed thermodynamic properties of dissolved carbon dioxide in aqueous solution at specified pH where $CO_2(aq)$, $H_2CO_3(aq)$, $HCO_3^-(aq)$, and $CO_3^{2-}(aq)$ are in equilibrium [25].

The dependence of the apparent equilibrium constant K'' on the concentration of oxygen at T, P, pH, $[Cl^-]$, etc., is given by

$$K'' = {}^{\circ}K'_2 \frac{1 + K'_{41}[O_2] + K'_{41}K'_{42}[O_2]^2 + K'_{41}K'_{42}K_{43}[O_2]^3 + K'_{41}K'_{42}K'_{43}K'_{44}[O_2]^4}{\{1 + K'_{21}[O_2] + K'_{21}K'_{22}[O_2]^2\}^2} \qquad (81)$$

This equation is given by Ackers and Halvorson [16], who refer to K'' as the overall macroscopic dimerization constant for subunit association. As indicated by this equation, the limiting value of K'' at high $[O_2]$ is given by

$$K''([O_2] = \infty) = \frac{{}^{\circ}K'_2 K'_{41} K'_{43} K'_{44}}{(K'_{21}K'_{22})^2} \qquad (82)$$

The limiting value at high $[O_2]$ is $9.508\,14 \times 10^5$, as indicated in Table 1, and the limiting value in the absence of molecular oxygen is 4.633×10^{10}. The values of K'' at $[O_2] = 5 \times 10^{-5}$, 10^{-5}, and 2×10^{-6} M are given in Table 1, and the same values are obtained in the following paper by a completely different method.

7. Application to biochemical reactions involving proteins

A number of biochemical reactions involve proteins, and so it is important to be able to determine $\Delta_f G_i'^{\circ}$ for proteins under specified conditions, and not just values of the binding potential Π. The values of $\Delta_f G_i'^{\circ}$ for protein are generally dependent on pH, and may be dependent on the concentrations of other species, as illustrated by the effect of molecular oxygen on hemoglobin.

There are many examples of biochemical reactions involving proteins, but there is not much information on the values of the apparent equilibrium constants K'. However, the values of a number of apparent equilibrium constants for reactions involving proteins have been listed by Goldberg and co-workers. Goldberg et al. [26] have summarized measurements on glucoside 3-dehydrogenase (EC1.1.99.13) [27] and cytochrome-c oxidase (EC 1.9.3.1) [28]. Goldberg and Tewari [29] have summarized measurements on [acyl-carrier-protein] *S*-acetyltransferase (EC 2.3.1.38) [30], [acyl-carrier-protein] *S*-malonyltransferase (EC 2.3.1.39) [31], protein kinase (EC 2.7.1.37) [32], and phosphomevalonate kinase (EC 2.7.4.2) [33]. These are all measurements at a single pH value, but it would be of interest to determine the dependence of K' on pH, pMg, and temperature. Reactions involving cytochromes, ferrodoxin, and flavoproteins are possibilities for quantitative studies. The standard thermodynamic properties of *Clostridium pasteurianum* ferrodoxin were calculated [34] from its standard reduction potential, since this value is independent of pH in the range 6.1-7.4. The value for the oxidized form was taken as equal to zero. The standard reduction potentials of proteins as functions of pH and pMg also provide the possibility of determining standard formation properties.

8. Discussion

The thermodynamic treatment of binding, linkage, and allosteric effects in proteins have their counterparts with small molecules. The interpretation of binding by small molecules is simplified by the possiblity of studying binding of particular "allosteric" forms. Since the thermodynamics of binding of small molecules is simpler, it is possible to treat these cases more precisely, and this clarifies the interpretation of the thermodynamic properties of macromolecules in the presence of ligands. These derivations show that the standard transformed Gibbs energy of formation $\Delta_f G'^{\circ}$ of a reactant is equal to the negative of the binding potential Π plus a term that makes a connection with the standard thermodynamic properties of the elements.

The binding of ligands by macromolecules has been considered here from the point of view of the fundamental equation of thermodynamics for the transformed Gibbs energy G' at a specified pH. This provides justification for the use of apparent equilibrium constants K' that are functions of pH. In order to use the concentration of molecular oxygen as an independent variable, a further transformed Gibbs energy G'' is defined by a Legendre transform, $G'' = G' - n'(\text{TotO}_2)\mu(O_2)$. This leads to apparent equilibrium constants K'' that are functions of $[O_2]$ as well as pH. The calculations here show how standard further transformed Gibbs energies of formation for TotT and TotD can be calculated at any desired $[O_2]$, and can be used to calculate the apparent association constant K'' for 2TotD = TotT. This reaction shows a positive cooperative effect, and the 4.9×10^5 decrease in this constant when human hemoglobin is fully oxygenated is striking.

None of this discussion has been concerned with any model because it is about the represention of experimental facts in a thermodynamic framework. All valid models must also represent these facts.

Apparent equilibrium constants for at least seven enzyme-catalyzed reactions involving proteins have been reported in the literature. These apparent equilibrium constants make it possible to calculate the standard transformed Gibbs energies of formation of the reacted form of the protein with respect to the unreacted form of the protein taken as a reference.

9. Nomenclature

c°	standard state concentration (1 M)
G	Gibbs energy (J)
G'	transformed Gibbs energy (J) (the intensive variable involved must be specified)
G''	further transformed Gibbs energy (J) (the intensive variables involved must be specified)
$\Delta_{\mathrm{f}}G_i$	Gibbs energy of formation of species i (J mol^{-1})
$\Delta_{\mathrm{f}}G_i^{\circ}$	standard Gibbs energy of formation of species i (J mol^{-1})
$\Delta_{\mathrm{f}}G_i'$	transformed Gibbs energy of formation of i at a specified concentration of i (K mol^{-1}) (i can be a species or a sum of pseudoisomers)
$\Delta_{\mathrm{f}}G_i'^{\circ}$	standard transformed Gibbs energy of formation of i (J mol^{-1}) (i can be a species or a sum of pseudoisomers)
$\Delta_{\mathrm{r}}G'^{\circ}$	standard transformed Gibbs energy of reaction (J mol^{-1})
I	ionic strength (M)
$\Delta_{\mathrm{f}}G_i''^{\circ}$	standard further transformed Gibbs energy of formation of i (J mol^{-1}) (i can be a species or a sum of pseudoisomers)
$\Delta_{\mathrm{r}}G_i''^{\circ}$	standard further transformed Gibbs energy of reaction (J mol^{-1})
$\Delta_{\mathrm{f}}H_i^{\circ}$	standard enthalpy of formation of i (J mol^{-1})
$\Delta_{\mathrm{f}}H_i'^{\circ}$	standard transformed enthalpy of formation of i (J mol^{-1})
K	equilibrium constant (dimensionless)
K_{a}	acid dissociation constant (dimensionless)
K_{Mg}	dissociation constant of magnesium complex ion (dimensionless)
K'	apparent equilibrium constant (dimensionless) [The concentrations of the species held constant must be specified)
K''	apparent equilibrium constant (dimensionless) (the concentrations of species and reactants held constant must be specified
n_i	amount of species i (mol)
n_i'	total amount of i in a system including the amounts in other species (mol)
n_i''	total amount of i in a system after a second Legendre transform (mol)
$N_{\mathrm{H}}(i)$	number of hydrogen atoms in species i (dimensionless)
$N_{\mathrm{Mg}}(i)$	number of magnesium atoms in species i (dimensionless)
$\mathrm{N}_{\mathrm{O}_2}(\mathrm{i})$	number of oxygen molecules in i (dimensionless)
$\bar{N}_{\mathrm{H}}(i)$	average number of moles of hydrogen in a mole of i under specified conditions (dimensionless)
$\bar{N}_{\mathrm{Mg}}(i)$	average number of moles of magnesium in a mole of i under specified conditions (dimensionless)
P	pressure on the system (bar)

P	binding polynomial (dimensionless)
pH	$-\log([H^+]/c^\circ)$ (dimensionless)
pMg	$-\log([Mg^{2+}]/c^\circ)$ (dimensionless)
r_i	mole fraction within an isomer group or a pseudoisomer group (dimensionless)
R	gas constant (8.314 51 J K^{-1} mol^{-1})
T	temperature (K)
$\Delta_f S_i^\circ$	standard entropy of formation of species i (J K^{-1} mol^{-1})
$\Delta_f S_i'^\circ$	standard transformed entropy of formation of species i (J K^{-1} mol^{-1})
Y	fractional saturation with oxygen (dimensionless)
μ_i	chemical potential of species i (J mol^{-1})
μ_i°	standard chemical potential of species i (J mol^{-1})
μ_i'	transformed chemical potential of species i or reactant i at a specified concentration of a ligand (J mol^{-1})
$\mu_i'^\circ$	standard transformed chemical potential of species i or reactant i at a specified concentration of a ligand (J mol^{-1})
μ_i''	further transformed chemical potential of i at specified pH and partial pressure of oxygen (J mol^{-1})
ν_i	stoichiometric number of species i in a chemical reaction (dimensionless) (positive for products and negative for reactants)
ν_{ij}'	apparent stoichiometric number of reactant i (sum of species) in biochemical reaction j (dimensionless) (positive for products and negative for reactants)
ν_{ij}''	apparent stoichiometric number of reactant i (sum of species) in biochemical reaction j when the concentration of a reactant is held constant (dimensionless) (positive for products and negative for reactants)
ξ_j'	apparent extent of reaction j (mol)
ξ_j''	apparent extent of reaction j when the concentration of a reactant is specified (mol)
Π	binding potential (J mol^{-1})

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Appendix A

The purpose here is to justify the statement just before of Eq. (13) that the transformed chemical potentials of the three phosphate species are equal at specified pH and pMg, so they can be considered to be pseudoisomers. The equilibrium conditions for the following reactions

$$H_2PO_4^- \rightleftharpoons H^+ + HPO_4^{2-} \tag{A1}$$

$$MgHPO_4 \rightleftharpoons Mg^{2+} + HPO_4^{2-} \tag{A2}$$

are

$$\mu(\mathrm{H_2PO_4^-}) = \mu(\mathrm{H^+}) + \mu(\mathrm{HPO_4^{2-}}) \tag{A3}$$

$$\mu(\mathrm{MgHPO_4}) \rightleftharpoons \mu(\mathrm{Mg^{2+}}) + \mu(\mathrm{HPO_4^{2-}}) \tag{A4}$$

The transformed chemical potentials of the three phosphate species calculated using Eq. (8) are

$$\mu'(\mathrm{HPO_4^{2-}}) \rightleftharpoons \mu(\mathrm{HPO_4^{2-}}) - \mu(\mathrm{H^+}) \tag{A5}$$

$$\mu'(\mathrm{H_2PO_4^-}) \rightleftharpoons \mu(\mathrm{H_2PO_4^-}) - 2\mu(\mathrm{H^+}) \tag{A6}$$

$$\mu'(\mathrm{MgHPO_4}) \rightleftharpoons \mu(\mathrm{MgHPO_4}) - \mu(\mathrm{H^+}) - \mu(\mathrm{Mg^{2+}}) \tag{A7}$$

Eliminating $\mu(\mathrm{H_2PO_4^-})$ and $\mu(\mathrm{MgHPO_4})$ from Eqs. (A6) and (A7) using Eqs. (A1) and (A2) yields

$$\mu'(\mathrm{H_2PO_4^-}) \rightleftharpoons \mu(\mathrm{HPO_4^{2-}}) - \mu(\mathrm{H^+}) \tag{A8}$$

$$\mu'(\mathrm{MgHPO_4}) \rightleftharpoons \mu(\mathrm{HPO_4^{2-}}) - \mu(\mathrm{H^+}) \tag{A9}$$

Eqs. (A5), (A8) and (A9) show that $\mu'(\mathrm{HPO_4^{2-}}) = \mu'(\mathrm{H_2PO_4^-}) = \mu'(\mathrm{MgHPO_4})$. Isomers have the same chemical potentials at equilibrium, and since these species have the same transformed chemical potentials at specified pH and pMg, they can be referred to as pseudoisomers.

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