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Fundamental equation of thermodynamics for protein–ligand binding

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Abstract

For the internal energy and every thermodynamic potential that can be defined by a Legendre transform, there is a fundamental equation that contains all the thermodynamic information about a system. For a system involving the binding of molecular oxygen and hydrogen ions by a protein, fundamental equations are given for the Gibbs energy G, the transformed Gibbs energy G' at specified pH, and the further transformed Gibbs energy G'' at specified pH and specified concentration of molecular oxygen. The Maxwell equations for these various Gibbs energies are important because they provide the connection with experimentally determined properties and increase our understanding of these properties. Measurements of the average number of oxygen molecules bound as a function of T, pH and concentration of molecular oxygen make it possible to calculate $\Delta_f G''^{\circ}$ of the reactant. Maxwell equations make it possible to calculate the average number of hydrogen ions bound, $\Delta_f S''^{\circ}$, $\Delta_f H''^{\circ}$ and their partial derivatives. These relations are illustrated with numerical calculations on a simple reaction system.

Keywords: Thermodynamics; Transformed Gibbs energy; Further transformed Gibbs energy; Allosteric protein; Linkage potential; Maxwell equations

1. Introduction

In studying biochemical reaction systems various choices can be made of independent variables to suit the situation in the laboratory or for the interpretation of the data. As Callen [1] commented in 1985, 'The choice of variables in terms of which a given problem is formulated, while a seemingly innocuous step, is often the most crucial step in the solution'. The criterion for spontaneous change and equilibrium for a one-phase system at specified T and P is provided by the Gibbs energy G; the number of independent variables is $N+2$ if there are no chemical reactions and N_c+2 if there are chemical reactions, where N is the number of different species and N_c is the number of components ($N_c=N-R$, where R is the number of independent reactions). Legendre transforms of the Gibbs energy can be used to define other thermodynamic potentials that provide the criteria for spontaneous change and equilibrium when, for example, concentrations of certain species are chosen as independent variables. For each thermody-

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namic potential defined in this way, there is a fundamental equation that expresses the differential of the particular thermodynamic potential in terms of the differentials of the independent variables for that potential. If a thermodynamic potential can be expressed as a function of its independent variables as a result of experimental work, all of the other thermodynamic properties of the system can be calculated by taking partial derivatives of the thermodynamic potential with respect to its independent variables. The fundamental equation for a system is also important because it leads to a number of Maxwell relations (second cross partial derivatives) that show how the various thermodynamic properties are related to each other and to experimental measurements. The Maxwell relations that connect thermodynamic properties with experimental measurements are of special importance because thermodynamic potentials like G cannot be determined directly.

The approach described here has been developed to treat the thermodynamics of metabolic reactions that are catalyzed by enzymes, but it also applies to protein–ligand reactions like the binding of molecular oxygen and hydrogen ions by hemoglobin. For this latter example of ligand binding, the fundamental equations has been given for the Gibbs energy G, the transformed Gibbs energy G' at specified pH, and the further transformed Gibbs energy G'' at specified pH and specified concentration of molecular oxygen [2]. However, since the emphasis in the 1996 article was on the dissociation of hemoglobin tetramers to dimers, other important aspects of the thermodynamics of ligand binding were omitted. In discussing the fundamental equation for the transformed Gibbs energy at specified T, P and pH, the terms involving dT, dP and dpH were omitted, and in discussing the further transformed Gibbs energy G'' at specified T, P, pH and concentration of molecular oxygen, the terms involving dT, dP, dpH and d$[O_2]$ were omitted. Because of these omissions, the various Maxwell equations were not discussed. Since the objective in 1996 was to calculate equilibrium compositions, the fact that hemoglobin is an allosteric protein that exists in more than one conformation was not considered. The objective here is to discuss these omissions. But since the full treatment of hemoglobin involves very long equations, the current discussion is limited to a macromolecule M that binds one oxygen molecule and has two acidic groups linked to the binding site. The research on the binding of oxygen molecules and hydrogen ions by hemoglobin and other proteins up to 1990 has been summarized by Wyman and Gill [3].

The simple reaction system considered here is

$$\begin{array}{l} \mathrm{M} \overset{pK_{1M}}{=} \mathrm{HM} \overset{pK_{2M}}{=} \mathrm{H_2M} \\ K\| \\ \mathrm{MO_2} \underset{pK_{1MO2}}{=} \mathrm{HMO_2} \underset{pK_{2MO2}}{=} \mathrm{H_2MO_2} \end{array} \tag{1}$$

where $K=[M][O_2]/[MO_2]$ and the hydrogen ions have been omitted in the acid dissociations. This simple reaction system was chosen because Antonini et al. [4] made a very thorough study of the equilibrium constants and enthalpies of reaction involved in human and horse hemoglobin. A system like this can be treated in three ways: (1) it can be discussed in terms of species by use of the Gibbs energy G. (2) It can be discussed at a specified pH by representing the system by $H_{av}MO_2=H_{av}M+O_2$, where the subscripts av indicate average numbers, by use of the transformed Gibbs energy G'. (3) It can be discussed at a specified pH and $[O_2]$ by representing the system by $H_{av}MO_{2av}$ and using the further transformed Gibbs energy G''. It is this third type of treatment that led Wyman [5] to introduce the binding potential, which he represented by the Russian L. The current article discusses this simple reaction system in all three ways. The transformed Gibbs energy was developed to treat metabolism at specified pH, and the further transformed Gibbs energy was developed to treat series and cycles of metabolic reactions at specified pH and specified concentrations of coenzymes. The thermodynamics of ligand binding and metabolism should not be considered to be separate subjects because proteins may be reactants in metabolic reactions. In fact thermodynamic properties of three proteins have already been included in the data base BasicBiochemData2 [6] for calculating apparent equilibrium constants of enzyme-catalyzed reactions.

These three proteins are cytochrome *c*, thioredoxin, and ferredoxin. The thermodynamic properties of the reactive sites of these proteins have been calculated from apparent equilibrium constants for enzyme-catalyzed reactions [7,8].

2. Fundamental equation for the Gibbs energy

The Gibbs energy G provides the criterion for spontaneous change and equilibrium at specified T and P in terms of species. The species involved are represented by M, HM, H_2M, MO_2, HMO_2, H_2MO_2, H^+ and O_2, where M represents the macromolecule. Although these symbols are used, we are really concerned with the binding site for molecular oxygen that is linked with two acid dissociations. As discussed earlier [9] this involves the assumption that the binding polynomial for hydrogen ions can be factored into a binding polynomial for the acid groups of the binding site and a binding polynomial for acid groups outside the site. The fundamental equation for the Gibbs energy is

$$\begin{aligned}\mathrm{d}G = {} & -S\mathrm{d}T + V\mathrm{d}P + \mu(\mathrm{M})\mathrm{d}n(\mathrm{M}) \\ & + \mu(\mathrm{HM})\mathrm{d}n(\mathrm{HM}) + \mu(\mathrm{H_2M})\mathrm{d}n(\mathrm{H_2M}) \\ & + \mu(\mathrm{MO_2})\mathrm{d}n(\mathrm{MO_2}) \\ & + \mu(\mathrm{HMO_2})\mathrm{d}n(\mathrm{HMO_2}) \\ & + \mu(\mathrm{H_2MO_2})\mathrm{d}n(\mathrm{H_2MO_2}) \\ & + \mu(\mathrm{H^+})\mathrm{d}n(\mathrm{H^+}) + \mu(\mathrm{O_2})\mathrm{d}n(\mathrm{O_2}) \qquad (2)\end{aligned}$$

This fundamental equation involves eight species, and there are five independent reactions between these species. The number C of components is given by $C = N - R = 8 - 5 = 3$ where N is the number of different species and R is the number of independent reactions. The components can be taken to be M, O_2 and H.

Since there are five equilibrium constant expressions and three conservation equations, the equilibrium concentrations of the eight species can be calculated if the equilibrium constants are known for five independent reactions at the desired T, P and ionic strength. However, in the absence of experimental methods for determining the equilibrium concentrations of all eight species, the five reactions cannot be used directly to determine the standard Gibbs energies of formation of the six species involving protein. The standard Gibbs energies of formation of O_2 and H^+ are known, and we will see that, in principle, the standard Gibbs energies of formation of all the other six species can be determined by studying the system at specified pHs and specified concentrations of molecular oxygen.

3. Fundamental equation for the transformed Gibbs energy at specified pH

When the pH is specified the Gibbs energy G does not provide the criterion for spontaneous change and equilibrium, and so it is necessary to use the Legendre transform [10–13]

$$G' = G - n_\mathrm{c}(\mathrm{H})\mu(\mathrm{H^+}) \qquad (3)$$

to define a transformed Gibbs energy G' that does. A Legendre transform always involves subtracting a product of conjugate variables from a thermodynamic potential. The conjugate property to the chemical potential of hydrogen ions is the amount $n_\mathrm{c}(\mathrm{H})$ of hydrogen atoms in the system. The amount of the hydrogen component is given by $n_\mathrm{c}(\mathrm{H}) = \Sigma N_\mathrm{H}(i) n_i$, where $N_\mathrm{H}(i)$ is the number of hydrogen atoms in species i. Since only differences in numbers of hydrogen atoms in products and reactants are actually involved in calculations, it is not necessary to count all of the hydrogen atoms in a protein molecule or a binding site. Substituting the expression for $n_\mathrm{c}(\mathrm{H})$ and $G = \Sigma\mu_i n_i$ in Eq. (3) yields the expression for the transformed chemical potential of species i.

$$\mu'_1 = \mu_i - N_\mathrm{H}(i)\mu(\mathrm{H^+}) \qquad (4)$$

This equation can be used to eliminate all μ_i from Eq. (2), except $\mu(\mathrm{H^+})$. This yields

$$\begin{aligned}\mathrm{d}G = {} & -S\mathrm{d}T + V\mathrm{d}P + \mu'(\mathrm{M})\mathrm{d}n(\mathrm{M}) \\ & + \mu'(\mathrm{HM})\mathrm{d}n(\mathrm{HM}) + \mu'(\mathrm{H_2M})\mathrm{d}n(\mathrm{H_2M}) \\ & + \mu'(\mathrm{MO_2})\mathrm{d}n(\mathrm{MO_2}) \\ & + \mu'(\mathrm{HMO_2})\mathrm{d}n(\mathrm{HMO_2}) \\ & + \mu'(\mathrm{H_2MO_2})\mathrm{d}n(\mathrm{H_2MO_2}) \\ & + \mu'(\mathrm{O_2})\mathrm{d}n(\mathrm{O_2}) + \mu(\mathrm{H^+})\mathrm{d}n_\mathrm{c}(\mathrm{H}) \qquad (5)\end{aligned}$$

At a specified pH, the species M, HM and H_2M are pseudoisomers; that is, the ratios of their equilibrium concentrations are independent of other reactions in the system. Since $\mu'(M) = \mu'(HM) = \mu'(H_2M)$ at equilibrium at specified pH, the three terms involving these transformed chemical potentials can be added, and their sum is represented by $\mu'(H_{av}M)dn'(H_{av}M)$, where $n'(H_{av}M)$ is the amount of this reactant. For the same reasons, the three terms for MO_2, HMO_2 and H_2MO_2 can be replaced with $\mu'(H_{av}MO_2)dn'(H_{av}MO_2)$.

The differential of the transformed Gibbs energy (Eq. (3)) is given by

$$dG' = dG - n_c(H)d\mu(H^+) - \mu(H^+)dn_c(H) \tag{6}$$

Substituting Eq. (5) into this equation leads to the fundamental equation for G'. However, the chemical potential of hydrogen ions is not a very useful independent variable because it is a function of both temperature and concentration [14]. The differential of the chemical potential of the hydrogen ion is given by

$$d\mu(H^+) = \left(\frac{\partial\mu(H^+)}{\partial T}\right)_{[H^+]} dT + \left(\frac{\partial\mu(H^+)}{\partial[H^+]}\right)_T d[H^+] \tag{7}$$

Taking the indicated derivatives of $\mu(H^+) = \mu^o(H^+) + RT\ln[H^+]$ yields

$$d\mu(H^+) = -\left(\bar{S}^o - R\ln[H^+]\right)dT - RT\ln(10)dpH \tag{8}$$

where $pH = -\log[H^+]$ and $\bar{S}^o(H^+)$ is the standard molar entropy of hydrogen ions. Substituting Eq. (8) into the combination of Eqs. (5) and (6) yields the fundamental equation for the transformed Gibbs energy:

$$\begin{aligned} dG' = &-S'dT + VdP + \mu'(H_{av}M)dn'(H_{av}M) \\ &+ \mu'(H_{av}MO_2)dn'(H_{av}MO_2) \\ &+ RT\ln(10)n_c(H)dpH + \mu'(O_2)dn'(O_2) \end{aligned} \tag{9}$$

where S' is the transformed entropy of the system; $S' = S - n_c(H)\bar{S}(H^+)$ and $\bar{S}(H^+)$ is the molar entropy of hydrogen ions. Note that at specified T, P and pH, Eq. (9) integrates to $G' = \Sigma\mu_j'n_j'$, so that the transformed Gibbs energy of a system is additive in the transformed chemical potentials of species.

The fundamental equation for G' involves three reactants, one reaction, and two components since hydrogen ions are no longer conserved in the system. The reaction at specified pH is

$$H_{av}MO_2 = H_{av}M + O_2 \tag{10}$$

Introducing the extent of reaction ξ into Eq. (9) yields

$$dG' = -S'dT + VdP + \Delta_rG'd\xi + RT\ln(10)n_c(H)dpH \tag{11}$$

where Δ_rG' is the transformed reaction Gibbs energy, $\Delta_fG'(H_{av}M) + \Delta_fG'(O_2) - \Delta_fG'(H_{av}MO_2)$. At equilibrium at specified T, P and pH, $dG'/d\xi = 0$. In the Gibbs–Duhem equation corresponding with Eq. (11), dG' is replaced with 0 and $\Delta_rG'd\xi$ is replaced with $-\xi d\Delta_rG'$. This makes it clear that Δ_rG' is a function of T, P and pH.

Fundamental Eq. (11) yields the expression for the apparent equilibrium constant K' for Eq. (10), and the usual routes can be used to calculate $\Delta_fG'^o$, $\Delta_fH'^o$ and $\Delta_fS'^o$ for $H_{av}M$ and $H_{av}MO_2$ as functions of T and pH. The apparent equilibrium constant is given by

$$\begin{aligned} K'_{O2} &= \frac{[H_{av}M][O_2]}{[H_{av}MO_2]} \\ &= K\frac{1 + 10^{pK_{1M} - pH} + 10^{pK_{1M} + pK_{2M} - 2pH}}{1 + 10^{pK_{1MO2} - pH} + 10^{pK_{1MO2} + pK_{2MO2} - 2pH}} \end{aligned} \tag{12}$$

where pK_1 and pK_2 are the pKs for the two oxygen-linked acid dissociations and K is equal to the apparent equilibrium constant at very high pH. The standard transformed Gibbs energy of reaction is given by

$$\begin{aligned} \Delta_rG'^o = -RT\ln K'_{O2} = &\Delta_fG'^o(H_{av}M) + \Delta_fG'^o(O_2) \\ &- \Delta_fG'^o(H_{av}MO_2) \end{aligned} \tag{13}$$

Ignoring the VdP term, fundamental Eq. (11) leads to three Maxwell relations:

$$\left(\frac{\partial \Delta_r G'}{\partial \mathrm{pH}}\right)_{T,P,\xi} = RT\ln(10)\left(\frac{\partial n_c(\mathrm{H})}{\partial \xi}\right)_{T,P,\mathrm{pH}} = RT\ln(10)\Delta_r N_H \tag{14}$$

The derivative of $n_c(\mathrm{H})$ with respect to the extent of reaction is the change in binding $\Delta_r N_H$ of hydrogen ions in the reaction. The second Maxwell relation is

$$\left(\frac{\partial S'}{\partial \xi}\right)_{T,P,\mathrm{pH}} = -\left(\frac{\partial \Delta_r G'}{\partial T}\right)_{\xi,P,\mathrm{pH}} = \Delta_r S' \tag{15}$$

The derivative of S' with respect to the extent of reaction is the change in transformed entropy $\Delta_r S'$ in the reaction. The third Maxwell relation is

$$\left(\frac{\partial S'}{\partial \mathrm{pH}}\right)_{T,P,\xi} = -R\ln(10)\left(\frac{\partial (Tn_c(\mathrm{H}))}{\partial T}\right)_{P,\xi,\mathrm{pH}} \tag{16}$$

This Maxwell relation does not introduce a new thermodynamic property, but it provides an unexpected relationship between properties.

The transformed enthalpy of the system does not appear in the fundamental equation, but $S' = H'/T - G'/T$ can be substituted in Eq. (15). This leads to the Gibbs–Helmholtz equation [15].

$$H' = -T^2(\partial(G'/T)/\partial T)_{P,\mathrm{pH},\xi} \tag{17}$$

Substituting $S' = H'/T - G'/T$ into Eq. (16) yields

$$\left(\frac{\partial H'}{\partial \mathrm{pH}}\right)_{T,P,\xi} = -RT^2\ln(10)\left(\frac{\partial n_c(\mathrm{H})}{\partial T}\right)_{P,\xi,\mathrm{pH}} \tag{18}$$

These three Maxwell relations and the two equations for the transformed enthalpy, which are derived using Maxwell relations, can be written in terms of standard transformed thermodynamic properties of reaction as follows:

$$\Delta_r N_H = \frac{1}{RT\ln(10)}\left(\frac{\partial \Delta_r G'^{\circ}}{\partial \mathrm{pH}}\right)_{T,P} \tag{19}$$

$$\Delta_r S'^{\circ} = -\left(\frac{\partial \Delta_r G'^{\circ}}{\partial T}\right)_{P,\mathrm{pH}} \tag{20}$$

$$\left(\frac{\partial \Delta_r S'^{\circ}}{\partial \mathrm{pH}}\right)_{T,P} = -R\ln(10)\left(\frac{\partial (T\Delta_r N_H)}{\partial T}\right)_{P,\mathrm{pH}} \tag{21}$$

$$\Delta_r H'^{\circ} = -T^2(\partial(\Delta_r G'^{\circ}/T)/\partial T)_{P,\mathrm{pH}} \tag{22}$$

$$\left(\frac{\partial \Delta_r H'^{\circ}}{\partial \mathrm{pH}}\right)_{T,P} = -RT^2\ln(10)\left(\frac{\partial \Delta_r N_H}{\partial T}\right)_{P,\mathrm{pH}} \tag{23}$$

Eq. (19) yields the change in binding of hydrogen atoms in the reaction, and Eq. (20) yields the standard transformed entropy of reaction. Eq. (21) shows that it is the change in $T\Delta_r N_H$ with temperature that determines the change in the transformed entropy of reaction with pH. The change in the binding of hydrogen ions $\Delta_r N_H$ in a reaction like this can be determined experimentally, and $\Delta_f G'^{\circ}$ plus an integration constant can be calculated by integrating Eq. (19). The integration constant can be calculated using the experimental fact that K'_{O_2} approaches K at high pH. It is of interest to note that German and Wyman [16] showed how it is possible to obtain relative values of the oxygen affinity of hemoglobin as a function of pH from titration curves for oxygenated and deoxygenated hemoglobin.

The properties in the preceding equations can be calculated if the standard Gibbs energies of formation are known for the species involved. Eq. (4) can be written in terms of the standard thermodynamic properties of a species as follows:

$$\Delta_f G'^{\circ}_i + RT\ln[i] = \Delta_f G^{\circ}_i + RT\ln[i] + N_H(i)\{\Delta_f G^{\circ}(\mathrm{H}^+) + RT\ln[\mathrm{H}^+]\} \tag{24}$$

where the $RT\ln[i]$ terms cancel. In treating metabolic reactions [10] the extended Debye–Huckel equation is used for activity coefficients, but in the calculations presented here, this adjustment is omitted as a simplification. Eq. (24) shows that the standard transformed Gibbs energy of a species containing $N_H(i)$ hydrogen atoms is given by

$$\Delta_f G'^{\circ}_i = \Delta_f G^{\circ}_i + N_H(i)RT\ln(10)\mathrm{pH} \tag{25}$$

When a reactant is made up of a sum of pseudoisomers, the standard transformed Gibbs energy of formation of the reactant (designated by j) is given by

$$\Delta_f G'^{o}_{j} = -RT\ln\sum\exp\left(-\Delta_f G'^{o}_{i}/RT\right) \tag{26}$$

which is the equation for calculating the standard transformed Gibbs energy $\Delta_f G'^{o}_{j}$ of a pseudoisomer group at a specified pH from the standard transformed Gibbs energies $\Delta_f G'^{o}_{i}$ of the various pseudoisomers. This equation can be written in terms of the binding polynomial for reactant j as follows [9]:

$$\Delta_f G'^{o}_{j} = \Delta_f G'^{o}_{1} - RT\ln(1 + 10^{-\mathrm{pH}+\mathrm{p}K_1} + 10^{-2\mathrm{pH}+\mathrm{p}K_1+\mathrm{p}K_2}) \tag{27}$$

where $\Delta_f G'^{o}_{1}$ is the standard transformed Gibbs energy of formation of species number 1, which is the species with the fewest hydrogen atoms. The equilibrium mole fractions r_i of species in the pseudoisomer group at a specified pH are given by

$$r_i = \exp\left\{\Delta_f G'^{o}_{j} - \Delta_f G'^{o}_{i}/RT\right\} \tag{28}$$

It is more difficult to calculate species properties from experimental values of standard transformed properties than to calculate reactant properties from species properties, and the inverse Legendre transform is useful in writing computer programs to do this [17]. Eq. (12) and $\Delta_r G'^{o} = -RT\ln K'_{O_2}$ make it possible to calculate $\Delta_r G'^{o}$ for the dissociation of oxygen from the macromolecule from laboratory data. When $\Delta_r G'^{o}$ is known as a function of pH and temperature, all the other thermodynamic properties of this reaction can be obtained as functions of pH and temperature by use of the Maxwell relations in Eqs. (19)–(23).

4. Fundamental equation for the further transformed Gibbs energy at specified pH and specified concentration of molecular oxygen

When the concentration of molecular oxygen is specified, the transformed Gibbs energy G' does not provide the criterion for spontaneous change and equilibrium, and so it is necessary to use the Legendre transform [2]

$$G'' = G' - n_c(O_2)\mu'(O_2) \tag{29}$$

to define a further transformed Gibbs energy G'' that does. The amount of the oxygen component is given by $n_c(O_2) = \Sigma N_{O_2}(j)n_j'$. Substituting this and $G' = \Sigma\mu_j' n_j'$ into Eq. (29) yields the expression for the further transformed chemical potential of reactant j:

$$\mu''_j = \mu'_j - N_{O_2}(j)\mu'(O_2) \tag{30}$$

where of course $\mu'(O_2) = \mu(O_2)$ since the species does not contain a hydrogen atom. The differential of G'' is

$$dG'' = dG' - n_c(O_2)d\mu'(O_2) - \mu'(O_2)dn_c(O_2) \tag{31}$$

Substituting Eq. (9) in Eq. (31) and using Eq. (30) yields

$$\begin{aligned} dG'' = {} & -S'dT + VdP + \mu''(H_{av}M)dn''(H_{av}M) \\ & + \mu''(H_{av}MO_2)dn''(H_{av}MO_2) \\ & + RT\ln(10)n_c(H)d\mathrm{pH} \\ & - n_c(O_2)d\mu'[O_2] \end{aligned} \tag{32}$$

However, the transformed chemical potential of molecular oxygen in aqueous solution $\mu'(O_2) = \mu'^{o}(O_2) + RT\ln[O_2]$ is not a very useful independent variable because it is a function of both temperature and concentration. The differential of the transformed chemical potential of molecular oxygen is given by

$$\begin{aligned} d\mu'(O_2) = {} & \left(\frac{\partial\mu'(O_2)}{\partial T}\right)_{[O_2]} dT \\ & + \left(\frac{\partial\mu'(O_2)}{\partial[O_2]}\right)_T d[O_2] \end{aligned} \tag{33}$$

Taking the indicated derivatives of $\mu'(O_2)$ yields

$$\begin{aligned} d\mu'(O_2) = {} & -\left(\bar{S}'^{o}(O_2) - R\ln[O_2]\right)dT \\ & + RTd\ln[O_2] \\ = {} & -\bar{S}'(O_2)dT + RTd\ln[O_2] \end{aligned} \tag{34}$$

where $\bar{S}'^{\mathrm{o}}(\mathrm{O_2})$ is the standard molar transformed entropy of molecular oxygen in aqueous solution. Substituting this into Eq. (32) yields

$$\begin{aligned}\mathrm{d}G'' = {} & -S''\mathrm{d}T + V\mathrm{d}P + \mu''(\mathrm{H_{av}M})\mathrm{d}n''(\mathrm{H_{av}M}) \\ & + \mu''(\mathrm{H_{av}MO_2})\mathrm{d}n''(\mathrm{H_{av}MO_2}) \\ & + RT\ln(10)n_{\mathrm{c}}(\mathrm{H})\mathrm{dpH} \\ & - RTn_{\mathrm{c}}(\mathrm{O_2})\mathrm{dln}[\mathrm{O_2}]\end{aligned} \tag{35}$$

where $S'' = S' - n_{\mathrm{c}}(\mathrm{O_2})\bar{S}'(\mathrm{O_2})$ and $\bar{S}'(\mathrm{O_2})$ is the transformed molar entropy of molecular oxygen, which of course is equal to the molar entropy $\bar{S}(\mathrm{O_2})$ of molecular oxygen in aqueous solution. At a specified concentration of molecular oxygen, $\mathrm{H_{av}M}$ and $\mathrm{H_{av}MO_{2av}}$ are pseudoisomers and have the same further transformed Gibbs energy of formation. Therefore these two terms in Eq. (35) can be replaced with a single term:

$$\begin{aligned}\mathrm{d}G'' = {} & -S''\mathrm{d}T + V\mathrm{d}P + \Delta_{\mathrm{f}}G''(\mathrm{H_{av}MO_{2av}}) \\ & \times \mathrm{d}n''(\mathrm{H_{av}MO_{2av}}) + RT\ln(10)n_{\mathrm{c}}(\mathrm{H})\mathrm{dpH} \\ & - \{RTn_{\mathrm{c}}(\mathrm{O_2})/[\mathrm{O_2}]\}\mathrm{d}[\mathrm{O_2}]\end{aligned} \tag{36}$$

where $\Delta_{\mathrm{f}}G''(\mathrm{H_{av}MO_{2av}}) = \mu''(\mathrm{H_{av}MO_{2av}})$. Note that at specified T, P, pH and $[\mathrm{O_2}]$, this equation integrates to $G'' = n''(\mathrm{H_{av}MO_{2av}})\Delta_{\mathrm{f}}G''(\mathrm{H_{av}MO_{2av}})$.

Eq. (36) indicates that when T, P, pH and $[\mathrm{O_2}]$ are held constant there is a single reactant, no reactions, and a single component. (Note that since there is a single component, no more Legendre transforms can be defined for this system.) Ignoring the $V\mathrm{d}P$ term, this fundamental equation has six Maxwell relations. The first Maxwell relation is

$$\begin{aligned}[\mathrm{O_2}]\left(\frac{\partial \Delta_{\mathrm{f}}G''}{\partial[\mathrm{O_2}]}\right)_{T,P,n'',\mathrm{pH}} &= -RT\left(\frac{\partial n_{\mathrm{c}}(\mathrm{O_2})}{\partial n''}\right)_{T,P,\mathrm{pH},[\mathrm{O_2}]} \\ &= -RT\bar{N}_{\mathrm{O_2}}\end{aligned} \tag{37}$$

This Maxwell relation provides the first direct connection in this section with experimental data because the average number of oxygen molecules bound $\bar{N}_{\mathrm{O_2}}$ by the macromolecule at specified $[\mathrm{O_2}]$ and pH can be determined spectrophotometrically.

The second Maxwell relation shows how the average binding of hydrogen ions by the binding site can be calculated from the pH dependence of $\Delta_{\mathrm{f}}G''(\mathrm{H_{av}MO_{2av}})$.

$$\begin{aligned}\left(\frac{\partial \Delta_{\mathrm{f}}G''}{\partial \mathrm{pH}}\right)_{T,P,n'',[\mathrm{O_2}]} &= RT\ln(10)\left(\frac{\partial n_{\mathrm{c}}(\mathrm{H})}{\partial n''}\right)_{T,P,\mathrm{pH},[\mathrm{O_2}]} \\ &= RT\ln(10)\bar{N}_{\mathrm{H}}\end{aligned} \tag{38}$$

This Maxwell relation provides the second direct connection with experimental data.

The next three Maxwell relations deal with the further transformed entropy of formation $\Delta_{\mathrm{f}}S''$ that can be calculated using

$$\begin{aligned}\left(\frac{\partial S''}{\partial n''}\right)_{T,P,\mathrm{pH},[\mathrm{O_2}]} &= -\left(\frac{\partial \Delta_{\mathrm{f}}G''}{\partial T}\right)_{n'',P,\mathrm{pH},[\mathrm{O_2}]} \\ &= \Delta_{\mathrm{f}}S''\end{aligned} \tag{39}$$

The following two Maxwell relations involving S'' do not introduce new properties, but they show that the dependence of S'' on pH is related to the change in the binding of hydrogen ions with temperature and the dependence of S'' on $[\mathrm{O_2}]$ is related to the change in the binding of molecular oxygen with temperature.

$$\left(\frac{\partial S''}{\partial \mathrm{pH}}\right)_{T,P,n'',[\mathrm{O_2}]} = -R\ln(10)\left(\frac{\partial(Tn_{\mathrm{c}}(\mathrm{H}))}{\partial T}\right)_{P,n'',\mathrm{pH},[\mathrm{O_2}]} \tag{40}$$

$$[\mathrm{O_2}]\left(\frac{\partial S''}{\partial[\mathrm{O_2}]}\right)_{T,P,n'',\mathrm{pH}} = -R\left(\frac{\partial(Tn_{\mathrm{c}}(\mathrm{O_2}))}{\partial T}\right)_{P,n'',\mathrm{pH},[\mathrm{O_2}]} \tag{41}$$

The sixth Maxwell relation shows the linkage between the binding of hydrogen ions and oxygen molecules.

$$\ln(10)[\mathrm{O_2}]\left(\frac{\partial n_{\mathrm{c}}(\mathrm{H})}{\partial[\mathrm{O_2}]}\right)_{T,P,\mathrm{pH},n''} = \left(\frac{\partial n_{\mathrm{c}}(\mathrm{O_2})}{\partial \mathrm{pH}}\right)_{T,P,[\mathrm{O_2}],n''} \tag{42}$$

The further transformed enthalpy is not directly involved in Eq. (36), but of course, $\Delta_f H''$ can be calculated under specified conditions using $\Delta_f G'' = \Delta_f H'' - T\Delta_f S''$. To obtain the function of T, pH and $[\mathrm{O_2}]$ for $\Delta_f H''$ we substitute $\Delta_f S'' = \Delta_f H''/T - \Delta_f G''/T$ in Eq. (39). This yields the Gibbs–Helmholtz equation:

$$\Delta_f H'' = -T^2\left(\partial(\Delta_f G''/T)/\partial T\right)_{P,\mathrm{pH},[\mathrm{O_2}]} \tag{43}$$

To obtain the derivative of $\Delta_f H''$ with respect to pH, $\Delta_f S'' = \Delta_f H''/T - \Delta_f G''/T$ is substituted in Eq. (40) to obtain

$$\left(\frac{\partial \Delta_f H''}{\partial \mathrm{pH}}\right)_{T,P,[\mathrm{O_2}]} = -RT^2\ln(10)\left(\frac{\partial \bar{N}_\mathrm{H}}{\partial T}\right)_{P,\mathrm{pH},[\mathrm{O_2}]} \tag{44}$$

Substituting $\Delta_f S'' = \Delta_f H''/T - \Delta_f G''/T$ in Eq. (41) yields

$$\left(\frac{\partial \Delta_f H''}{\partial [\mathrm{O_2}]}\right)_{T,P,\mathrm{pH}} = -\frac{R}{[\mathrm{O_2}]}\left(\frac{\partial\left(T^2\bar{N}_{\mathrm{O_2}}\right)}{\partial T}\right)_{P,\mathrm{pH},[\mathrm{O_2}]} \tag{45}$$

The six Maxwell relations and the three relations for the further transformed enthalpy that were calculated using Maxwell relations are especially useful when they are written in terms of standard molar properties.

$$[\mathrm{O_2}]\left(\frac{\partial \Delta_f G''^{\mathrm{o}}}{\partial [\mathrm{O_2}]}\right)_{T,P,\mathrm{pH}} = -RT\bar{N}_{\mathrm{O_2}} \tag{46}$$

$$\left(\frac{\partial \Delta_f G''^{\mathrm{o}}}{\partial \mathrm{pH}}\right)_{T,P,[\mathrm{O_2}]} = RT\ln(10)\bar{N}_\mathrm{H} \tag{47}$$

$$\left(\frac{\partial \Delta_f G''^{\mathrm{o}}}{\partial T}\right)_{P,\mathrm{pH},[\mathrm{O_2}]} = -\Delta_f S''^{\mathrm{o}} \tag{48}$$

$$\left(\frac{\partial \Delta_f S''^{\mathrm{o}}}{\partial \mathrm{pH}}\right)_{T,P,[\mathrm{O_2}]} = -R\ln(10)\left(\frac{\partial\left(T\bar{N}_\mathrm{H}\right)}{\partial T}\right)_{P,\mathrm{pH},[\mathrm{O_2}]} \tag{49}$$

$$[\mathrm{O_2}]\left(\frac{\partial \Delta_f S''^{\mathrm{o}}}{\partial [\mathrm{O_2}]}\right)_{T,P,\mathrm{pH}} = -R\left(\frac{\partial\left(T\bar{N}_{\mathrm{O_2}}\right)}{\partial T}\right)_{P,\mathrm{pH},[\mathrm{O_2}]} \tag{50}$$

$$\ln(10)[\mathrm{O_2}]\left(\frac{\partial \bar{N}_\mathrm{H}}{\partial [\mathrm{O_2}]}\right)_{T,P,\mathrm{pH}} = \left(\frac{\partial \bar{N}_{\mathrm{O_2}}}{\partial \mathrm{pH}}\right)_{T,P,[\mathrm{O_2}]} \tag{51}$$

$$\Delta_f H''^{\mathrm{o}} = -T^2\left(\partial(\Delta_f G''^{\mathrm{o}}/T)/\partial T\right)_{P,\mathrm{pH},[\mathrm{O_2}]} \tag{52}$$

$$\left(\frac{\partial \Delta_f H''^{\mathrm{o}}}{\partial \mathrm{pH}}\right)_{T,P,[\mathrm{O_2}]} = -RT^2\ln(10)\left(\frac{\partial \bar{N}_\mathrm{H}}{\partial T}\right)_{P,\mathrm{pH},[\mathrm{O_2}]} \tag{53}$$

$$\left(\frac{\partial \Delta_f H''^{\mathrm{o}}}{\partial [\mathrm{O_2}]}\right)_{T,P,\mathrm{pH}} = -\frac{R}{[\mathrm{O_2}]} \times\left(\frac{\partial\left(T^2\bar{N}_{\mathrm{O_2}}\right)}{\partial T}\right)_{P,\mathrm{pH},[\mathrm{O_2}]} \tag{54}$$

Since $\bar{N}_{\mathrm{O_2}}$ can be determined experimentally, the standard further transformed Gibbs energy of formation of $\mathrm{H_{av}MO_{2av}}$ plus an integration constant can be calculated by the following integration:

$$\Delta_f G''^{\mathrm{o}}(H_{\mathrm{av}}\mathrm{MO_{2av}}) + \mathrm{const} = -RT\int\left(\bar{N}_{\mathrm{O_2}}/[\mathrm{O_2}]\right)\mathrm{d}[\mathrm{O_2}] \tag{55}$$

We will see below that the integration constant is a function of T and pH.

Maxwell relations can be used if $\Delta_f G''^{\mathrm{o}}$ can be obtained as a function of T, pH and $[\mathrm{O_2}]$. For reaction system (Eq. (1)) the standard further transformed Gibbs energies of formation of $\mathrm{H_{av}M}$ and $\mathrm{H_{av}MO_2}$ can be calculated using Eq. (30).

$$\Delta_f G''^{\mathrm{o}}(\mathrm{H_{av}M}) = \Delta_f G'^{\mathrm{o}}(\mathrm{H_{av}M}) \tag{56}$$

$$\begin{aligned}\Delta_f G''^{\mathrm{o}}(\mathrm{H_{av}MO_2}) &= \Delta_f G'^{\mathrm{o}}(\mathrm{H_{av}MO_2}) - \Delta_f G(\mathrm{O_2}) \\ &= \Delta_f G'^{\mathrm{o}}(\mathrm{H_{av}MO_2}) - \left(\Delta_f G^{\mathrm{o}}(\mathrm{O_2,aq}) + RT\ln[\mathrm{O_2}]\right)\end{aligned} \tag{57}$$

where the standard Gibbs energy of formation of molecular oxygen in aqueous solution at 298.15 K can be calculated as a function of T using data in the NBS tables [18]. The standard transformed Gibbs energy of formation of the pseudoisomer group is given by

$$\begin{aligned}\Delta_f G''^{\circ}(H_{av}MO_{2av}) = &\\ -RT\ln\Big\{&\exp\big(-\Delta_f G''^{\circ}(H_{av}M)/RT\big)\\ &+\exp\big(-\Delta_f G''^{\circ}(H_{av}MO_2)/RT\big)\Big\}\end{aligned} \quad (58)$$

This equation can be written in terms of the binding polynomial for molecular oxygen as follows:

$$\begin{aligned}\Delta_f G''^{\circ}(H_{av}MO_{2av}) = \Delta_f G''^{\circ}(H_{av}M)&\\ -RT\ln\Big(1+[O_2]/K'_{O_2}\Big)&\end{aligned} \quad (59)$$

In this equation $\Delta_f G''^{\circ}(H_{av}M)$ is not a function of $[O_2]$ because $H_{av}M$ does not contain O_2. The expression for K'_{O_2} has already been given in Eq. (12). The relation between Eqs. (55) and (59) is discussed in Section 7. The equilibrium concentrations of $H_{av}M$ and $H_{av}MO_2$ are readily calculated since the equilibrium mole fractions r_j of reactants in the pseudoisomer group are given by

$$r_j = \exp\Big\{(\Delta_f G''^{\circ}_{k} - \Delta_f G''^{\circ}_{j})/RT\Big\} \quad (60)$$

5. Use of the transformed Gibbs energy to treat an allosteric protein

If protein M that binds one oxygen molecule at a site linked with two acid groups is in equilibrium with an isomer M*, then Eq. (36) will have one more term in it.

$$\begin{aligned}dG'' = &-S''dT + VdP + \mu''(H_{av}MO_{2av})\\ &\times dn''(H_{av}MO_{2av}) + \mu''(H_{av}M^{*}O_{2av})\\ &\times dn''(H_{av}M^{*}O_{2av}) + RT\ln(10)n_c(H)dpH\\ &-\Big\{RTn_c(O_2)/[O_2]\Big\}d[O_2]\end{aligned} \quad (61)$$

We can regard this as a system with two reactants, one reaction, and one component, but we can also regard it as having one reactant because $H_{av}MO_{2av}$ and $H_{av}M^{*}O_{2av}$ are pseudoisomers at specified pH and $[O_2]$. The expression for the further transformed Gibbs energy of formation of this pseudoisomer group contains all the information about the system. We expect that the functional dependence on pH and $[O_2]$ will have the same form as for a single protein. The Maxwell relations have the same form as those in Eqs. (46)–(54). Thus, even when there are a number of forms of $H_{av}MO_{2av}$, equilibrium experiments can be represented thermodynamically as if there is a single form. Of course the thermodynamic properties can then be interpreted in terms of isomerization of the reactant if there is other experimental data or a theory for how to do it.

6. Calculations of transformed thermodynamic properties for the reaction system

Given the pKs, K, and the corresponding standard enthalpies of reaction for the reaction system in Eq. (1), it is possible to calculate all the thermodynamic properties (1) at specified pH by using the reaction $H_{av}MO_2 = H_{av}M + O_2$ or (2) at specified pH and $[O_2]$ by representing the system with $H_{av}MO_{2av}$. The pKs used are those of Antonini et al. [4] because their very thorough investigation includes the effect of temperature on the equilibrium constants of the binding sites in human and horse hemoglobins. Their pKs are based on the assumption that the oxygen-linked groups are independent. However, pK_1 and pK_2 in Eq. (1) are the usual thermodynamic pKs independent of any assumptions. Actually some of the acid dissociations are cooperative, which indicates that they are not independent. The pKs used in the current calculations were obtained using

$$pK_1 = \log(10^{pK_\alpha} + 10^{pK_\beta}) \quad (62)$$

$$pK_2 = pK_\alpha + pK_\beta - pK_1 \quad (63)$$

where pK_α and pK_β are the pKs based on the assumption that the oxygen-linked acid groups are independent [9,19]. Eqs. (62) and (63) were used to calculate the pK_1 and pK_2 values given in Table 1 for the temperatures used by Antonini et al.

When the pKs for a dibasic acid are far apart, $pK_1 = pK_\alpha$ and $pK_2 = pK_\beta$, but pK_1 may be quite different form pK_α and pK_2 may be quite different from pK_β when pK_1 and pK_2 are close. If pK_1 and

Table 1
pKs of H_2M and H_2MO_2

T (K)	pK_1(M)	pK_1(MO_2)	pK_2(M)	pK_2(MO_2)
283.15	8.09	6.91	5.42	6.00
293.15	7.85	6.67	5.46	6.04
303.15	7.63	6.45	5.50	6.08
313.15	7.42	6.24	5.43	6.11

pK_2 are closer than 0.6, the acid dissociation is cooperative [9,19]. The effect of ionic strength is ignored here to simplify the calculations.

In order to be able to differentiate standard thermodynamic properties with respect to temperature, it is necessary to express pK_1 and pK_2 as functions of temperature. Antonini et al. [4] found that the standard enthalpy of acid dissociation is 37.7 kJ mol^{-1} for the pK_1 values and -6.3 kJ mol^{-1} for the pK_2 values. The pKs can be represented as functions of temperature by use of

$$\mathrm{p}K(T)=\mathrm{p}K(293.15)-\frac{\Delta H^{\mathrm{o}}}{R\ln(10)}\left(\frac{1}{293.15}-\frac{1}{T}\right) \tag{64}$$

Since this is a hypothetical reaction system, the value of K in Eqs. (1) and (12) is rather arbitrarily taken to be 10^{-5} at 293.15 K, with $\Delta_r H^{\mathrm{o}}=60.7$ kJ mol^{-1}, where the later value was used by Antonini et al. The equilibrium constant K for $MO_2=M+O_2$ can be represented as a function of temperature by

$$K(T)=10^{-5}\exp\left(\frac{\Delta H^{\mathrm{o}}}{R}\left(\frac{1}{293.15}-\frac{1}{T}\right)\right) \tag{65}$$

When the pH is specified, the function of T and pH that represents $\Delta_f G'^{\mathrm{o}}(H_{av}M)$ can be calculated using Eq. (27) in the form

$$\begin{aligned}\Delta_f G'^{\mathrm{o}}(H_{av}M)=\Delta_f G'^{\mathrm{o}}(M)\\ -RT\ln(1+10^{-\mathrm{pH}+\mathrm{p}K_{1M}}\\ +10^{-2\mathrm{pH}+\mathrm{p}K_{1M}+\mathrm{p}K_{2M}})\end{aligned} \tag{66}$$

where $\Delta_f G'^{\mathrm{o}}(M)=0$ because $\Delta_f G^{\mathrm{o}}(M)$ is set at zero at each temperature by convention and $N_H(M)=0$. When the functions of T for pK_{1M} and pK_{2M} are inserted in this equation, $\Delta_f G'^{\mathrm{o}}(H_{av}M)$ is obtained as a function of T and pH.

When the pH is specified, the function of T and pH that represents $\Delta_f G'^{\mathrm{o}}(H_{av}MO_2)$ can be calculated using Eq. (27) in the form

$$\begin{aligned}\Delta_f G'^{\mathrm{o}}(H_{av}MO_2)=\Delta_f G'^{\mathrm{o}}(MO_2)\\ -RT\ln(1+10^{-\mathrm{pH}+\mathrm{p}K_{1MO2}}\\ +10^{-2\mathrm{pH}+\mathrm{p}K_{1MO2}+\mathrm{p}K_{2MO2}})\end{aligned} \tag{67}$$

where $\Delta_f G'^{\mathrm{o}}(MO_2)=\Delta_f G^{\mathrm{o}}(MO_2)$ because $N_H(MO_2)=0$. The value of $\Delta_f G^{\mathrm{o}}(MO_2)$ can be calculated by using

$$\begin{aligned}-RT\ln K=\Delta_f G^{\mathrm{o}}(M)\\ +\Delta_f G^{\mathrm{o}}(O_2)-\Delta_f G^{\mathrm{o}}(MO_2)\end{aligned} \tag{68}$$

The standard Gibbs energy of formation of O_2 in aqueous solution can be calculated at temperature T by using

$$\begin{aligned}\Delta_f G^{\mathrm{o}}(O_2,\mathrm{aq})=(T/298.15)\Delta_f G^{\mathrm{o}}(298.15)\\ +(1-T/298.15)\Delta_f H^{\mathrm{o}}(298.15)\end{aligned} \tag{69}$$

that is based on the assumption that $\Delta_f H^{\mathrm{o}}(O_2)$ is independent of temperature in the range 283.15–313.15 K. At 298.15 K the NBS tables [18] give $\Delta_f G^{\mathrm{o}}(O_2,\ \mathrm{aq})=16.4$ kJ mol^{-1} and $\Delta_f H^{\mathrm{o}}(O_2,\ \mathrm{aq})=-11.7$ kJ mol^{-1}. Since K is given by Eq. (65), Eq. (68) makes it possible to obtain the function of temperature for $\Delta_f G^{\mathrm{o}}(MO_2)$. With K and the pKs as functions of T, Eq. (12) makes it possible to calculate K'_{O_2} as a function of T and pH. The standard transformed reaction Gibbs energy of $H_{av}MO_2=H_{av}M+O_2$ can be calculated using $\Delta_r G'^{\mathrm{o}}=-RT\ln K'_{O_2}$. This function of T and pH is very complicated, but it and its various derivatives can be calculated by use of *Mathematica*® [20].

Fig. 1 shows $\Delta_r G'^{\mathrm{o}}$ in joule per mole as a function of pH and T in the first plot.

The dissociation of molecular oxygen is unfavorable over the entire range, but the dissociation is greater at higher temperatures because the standard transformed enthalpy of reaction is positive, as shown by the third plot. The base ten logarithm of the apparent dissociation constant is shown in

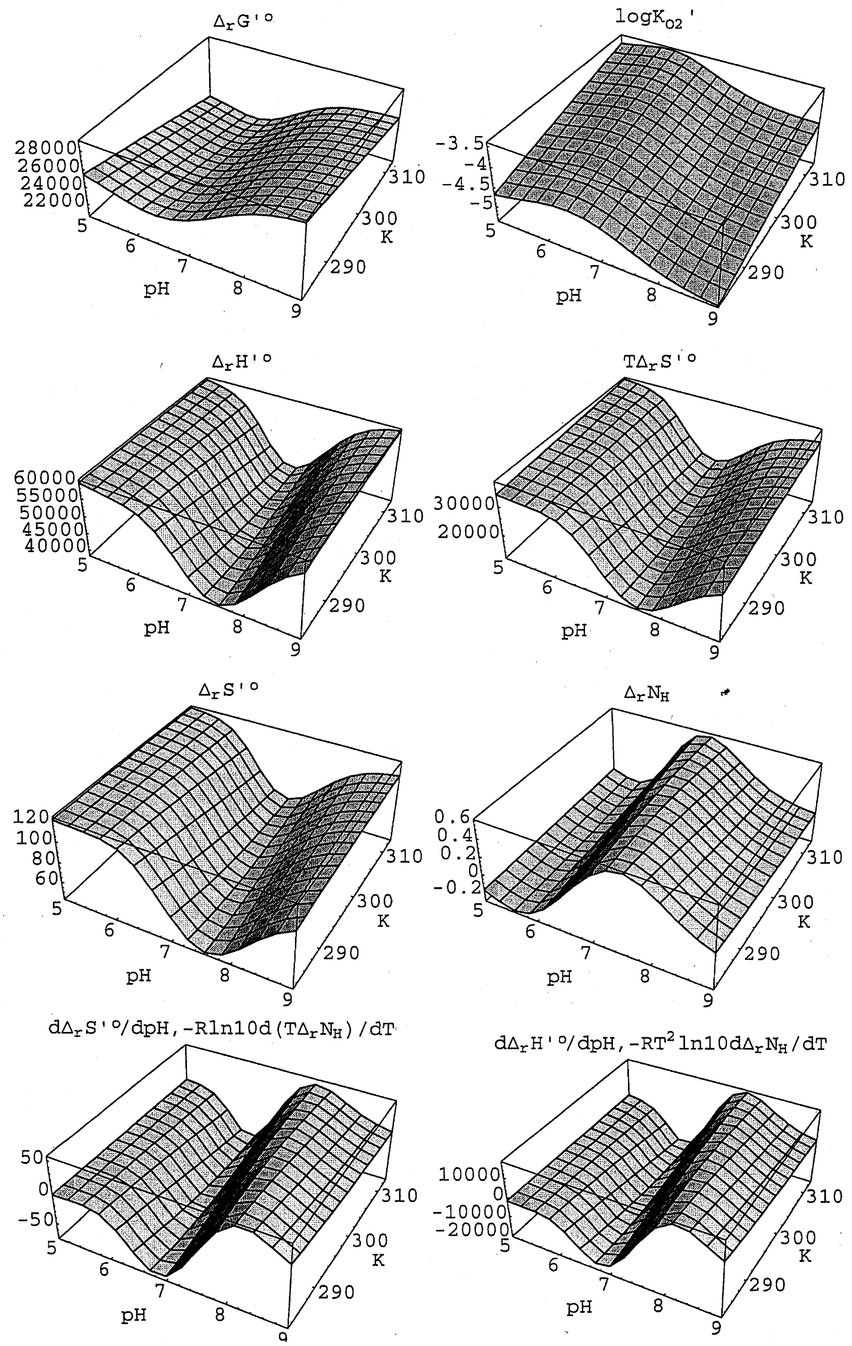

Fig. 1. Standard transformed thermodynamic properties for the reaction $H_{av}MO_2 = H_{av}M + O_2$ as functions of pH and *T*. Energies are given in joule per mole, and entropies are given in joule per kelvin per mole.

the second plot. The standard transformed entropy of reaction can be calculated by taking the derivative of $\Delta_r G'^{\circ}$ with respect to temperature, as shown by Maxwell relation (Eq. (20)). $\Delta_r S'^{\circ}$ is positive over the whole range, as shown by the fifth plot, and this favors dissociation, in opposition to the enthalpy change. The fourth plot gives $T\Delta_r S'^{\circ}$ in joule per mole, and when these values are subtracted from the plot for $\Delta_r H'^{\circ}$, the plot for $\Delta_r G'^{\circ}$ is obtained. Some increase in entropy upon the dissociation is expected because two molecules are produced out of one. It may be that $H_{av}M$ has a more random structure than $H_{av}MO_2$. Another factor to be considered in explaining the entropy change is that there is a change in the binding of hydrogen ions in the reaction, as shown by the sixth plot. This plot is obtained by use of Maxwell relation (Eq. (19)). At pH 7 and above there is an uptake of hydrogen ions when the dissociation of oxygen occurs. This decreases the entropy of dissociation of oxygen in that pH range. Maxwell relation (Eq. (21)) shows that the rate of change of the standard transformed entropy of reaction with respect to pH is proportional to the derivative of $T\Delta_r N_H$ with respect to temperature. This is an important new result. The seventh plot gives $d\Delta_r S'^{\circ}/dpH$, and $-R\ln(10)d(T\Delta_r N_H)/dT$ is given by the same plot. The eighth plot gives $d\Delta_r H'^{\circ}/$pH and $-RT^2\ln(10)dN_H/dT$ is given by the same plot. Plots 7 and 8 look like they have the same dependence on T and pH, but they are slightly different. These plots provide four checks on the consistency of these calculations because $\Delta_r G'^{\circ} = \Delta_r H'^{\circ} - T\Delta_r S'^{\circ}$, the third and fifth Maxwell relations are obeyed, and the plot for $d\Delta_r H'^{\circ}/dpH$ minus T times the seventh plot gives the plot for $\Delta_r N_H$ after division by $RT\ln(10)$.

When data on reaction system (Eq. (1)) are interpreted in terms of $H_{av}MO_2 = H_{av}M + O_2$, the experimental values of the apparent equilibrium constant K'_{O_2} can be used to calculate $\Delta_r G'^{\circ}$ as a function of T and pH. This function contains all the thermodynamic information on the system. The change in the binding of hydrogen ions $\Delta_r N_H$ in the reaction can be calculated using Maxwell relation (Eq. (19)). This property of the reaction can be obtained experimentally using a pHstat or can be calculated from acid titration curves of M and MO_2. At a specified temperature, $\Delta_r G'^{\circ}$ plus an integration constant can be calculated by integrating Eq. (19).

$$\Delta_r G'^{\circ} + \text{const} = RT\ln(10)\int \Delta_r N_H \, dpH \qquad (70)$$

This type of integration was used by Antonini et al. [4]. The value of the integration constant can be evaluated because $\Delta_r G'^{\circ} = -(8.3145)(0.29315)\ln 10^{-5}$ at 293.15 K in the limit of high pH.

7. Calculation of the further transformed thermodynamic properties for the reaction system

The standard further transformed Gibbs energies of formation of $H_{av}M$ and $H_{av}MO_{2av}$ are calculated by substituting Eqs. (66) and (67) in Eqs. (56) and (57). In Eq. (57), the value of $\Delta_f G^{\circ}(O_2, aq)$ is replaced with the function of T in Eq. (69). The functions for $\Delta_f G'^{\circ}(H_{av}M)$ and $\Delta_f G'^{\circ}(H_{av}MO_2)$ are substituted in Eq. (58) to obtain the function for the standard transformed Gibbs energy of formation of the pseudoisomer group. The function $\Delta_f G''^{\circ}(H_{av}MO_{2av})$ of T, pH and $[O_2]$ is very complicated, but it and its various derivatives can be calculated by use of *Mathematica*.

The first plot in Fig. 2 gives the standard further transformed Gibbs energy of formation of the system with respect to the standard Gibbs energy of formation of the macromolecule. The convention is that $\Delta_f G^{\circ}(M) = 0$ at each temperature, just like an element in familiar chemical thermodynamic tables. Adding oxygen or lowering the pH increases the stability of the system, that is $\Delta_f G''^{\circ}(H_{av}MO_{2av})$ decreases. This surface looks simple, but it contains a lot of information, as shown by the other plots that are all calculated from it. The second plot is obtained by use of Maxwell relation (Eq. (46)) to calculate the average number of oxygen molecules bound by the macromolecule. Oxygen is more tightly bound at pH 9. Since the average number of oxygen molecules bound can be determined spectrophotometrically, this plot provides the way to obtain $\Delta_f G''^{\circ}(H_{av}MO_{2av})$ by integration of $\bar{N}_{O_2}$ with respect to the oxygen concentration, as indicated

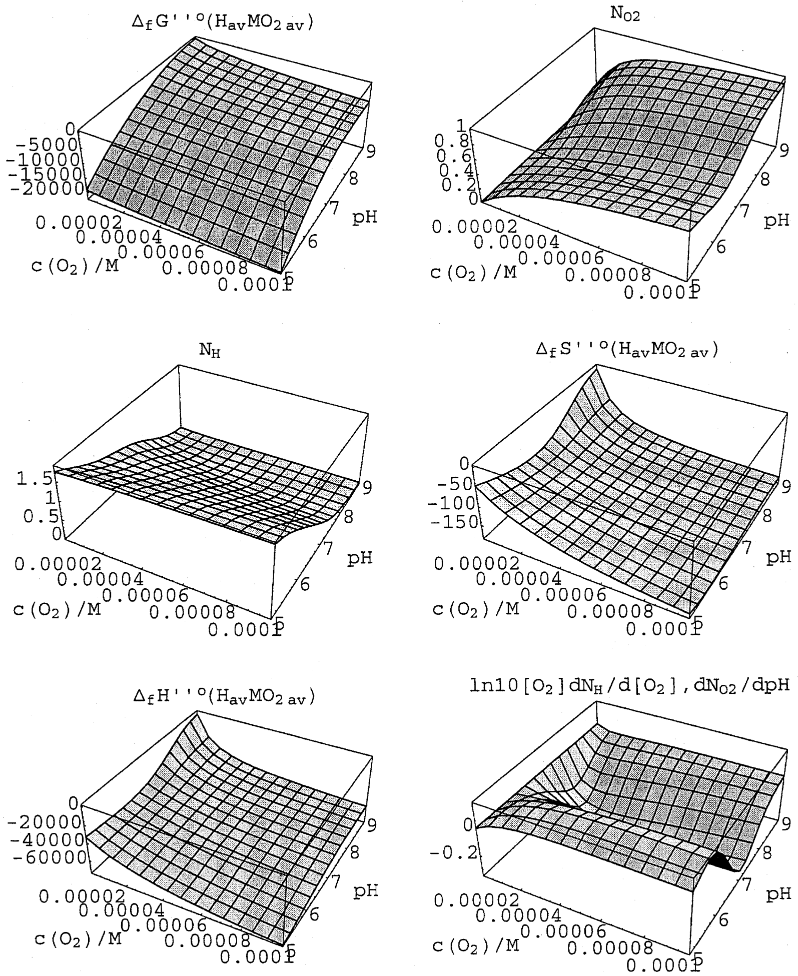

Fig. 2. Standard further transformed thermodynamic properties at 293.15 K for the reaction system. These properties can be readily calculated at other temperatures in approximately the range 273.15–323.15 K. Energies are given in joule per mole, and entropies are given in joule per kelvin per mole.

by Eq. (55). The third plot showing the average number of hydrogen ions bound by the macromolecule at each pH and concentration of molecular oxygen is obtained by use of Maxwell relation (Eq. (47)). At very high pHs no hydrogen ions are bound by the site, and at very low pHs two hydrogen ions are bound. At intermediate pHs the shape of this plot indicates the p*K*s of the acidic groups in M and MO_2. If the acid titration curves of M and MO_2 are determined in the laboratory at a particular temperature, the average binding of hydrogen ions can be calculated.

The standard further transformed entropy of the system given in the fourth plot is obtained by use of Maxwell relation (Eq. (48)). At high pH and in the absence of oxygen, the site is empty and the entropy of the binding site is equal to zero at each temperature. The standard further transformed entropy of this reaction system is always negative, and so it increases the standard further transformed Gibbs energy. The standard further transformed enthalpy of the system is obtained using the Gibbs–Helmholtz equation (Eq. (52)). Multiplying values in the entropy plot by the temperature

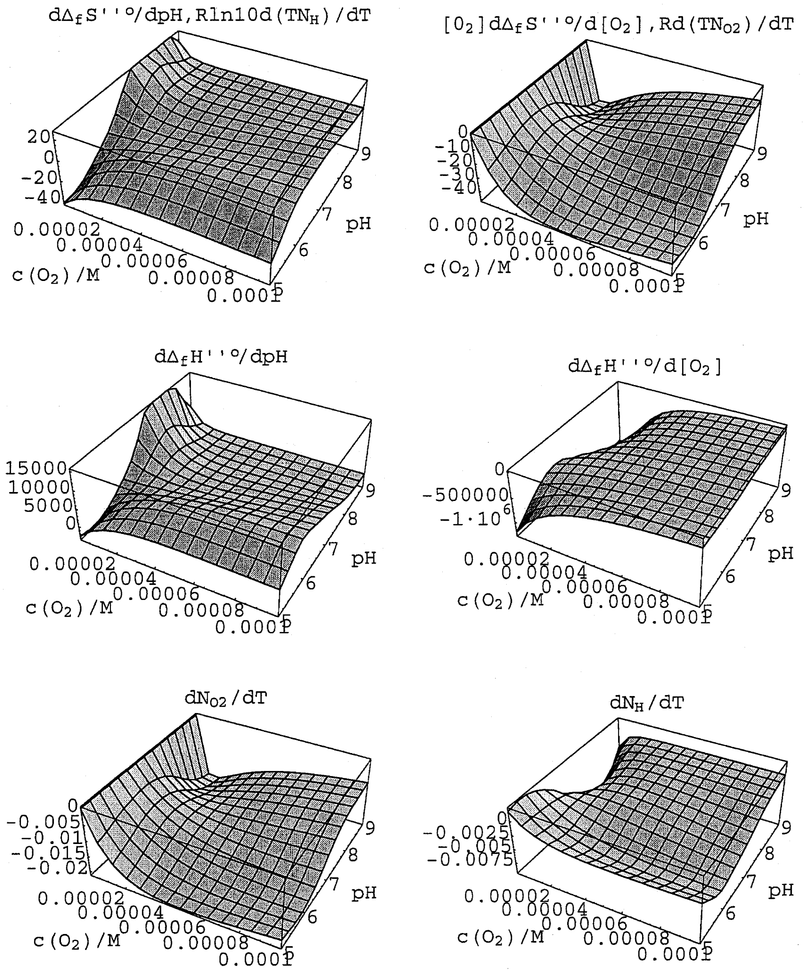

Fig. 3. Dependencies of $\Delta_f S''^{o}$ and $\Delta_f H''^{o}$ on pH and $[O_2]$ at 293.15 K for the reaction system. These properties can be readily calculated at other temperatures in approximately the range 273.15–323.15 K. Enthalpies are given in joule per mole, and entropies are given in joule per kelvin per mole.

and subtracting them from the values in the enthalpy plot yields the first plot, as expected from $\Delta_f G''^{o} = \Delta_f H''^{o} - T\Delta_f S''^{o}$. The sixth plot in Fig. 2 deals with Maxwell relation (Eq. (51)) that expresses the linkage between the binding of oxygen molecules and the binding of hydrogen ions. It shows how the average binding of hydrogen ions varies with the concentration of molecular oxygen and how the average binding of oxygen molecules varies with the pH.

The dependencies of $\Delta_f S''^{o}$ and $\Delta_f H''^{o}$ on pH and $[O_2]$ at 293.15 K are shown in Fig. 3. The derivative of $\Delta_f S''^{o}$ with respect to pH given in the first plot is obtained from Maxwell relation (Eq. (49)), which can also be labeled $-R\ln(10)\mathrm{d}(T\bar{N}_H)/\mathrm{d}T$. This provides an explanation of the dependence of the standard further transformed entropy of formation of $H_{av}MO_{2av}$ on pH because it shows that it is a result of the dependence of $T\bar{N}_H$ on temperature. The second plot of $[O_2]\mathrm{d}\Delta_f S''^{o}/\mathrm{d}[O_2]$ is obtained using Maxwell relation (Eq. (50)), and this plot can also be labeled $-R(\mathrm{d}(T\bar{N}_{O_2})/\mathrm{d}T)$. This shows that the dependence of the standard further transformed entropy of formation of $H_{av}MO_{2av}$ on the concentration of molecular oxygen is a result of the dependence of $T\bar{N}_{O_2}$ on temperature. These mathematical relationships have to be taken into account in interpreting the third plot in Fig. 2.

The third plot of $d\Delta_f H''^\circ/dpH$ in Fig. 3 is obtained using Maxwell relation (Eq. (53)). This plot can also be labeled $-RT^2\ln(10)(d\bar{N}_H/dT)$. The fourth plot of $d\Delta_f H''^\circ/d[O_2]$ is obtained from Maxwell relation (Eq. (54)). This plot can also be labeled $-(R/[O_2])(d(T^2\bar{N}_{O_2})/dT)$. The first four plots in Fig. 3 have shown implicitly how $d\bar{N}_{O_2}/dT$ and $d\bar{N}_H/dT$ vary with pH and $[O_2]$, and so they are shown explicitly in the fifth and sixth plots in Fig. 3.

This does not exhaust the partial derivatives that can be calculated. Di Cera et al. [21] have defined the binding capacity, which is analogous to the heat capacity, and have applied it to the binding of oxygen to hemoglobin. The binding capacity is of interest because it is a measure of cooperativity.

8. Discussion

These derivations and their application to a reaction system demonstrate the importance of fundamental equations and Maxwell relations in bringing together all the thermodynamic information on a complicated system. They also show how these equations can be extended to treat reaction systems with more reactants. $\Delta_f G''^\circ$ $(H_{ax}MO_{2av})$ is a complicated function of T, pH and $[O_2]$ that contains all the thermodynamic information about the reaction system in Eq. (1). This function is not directly accessible by experiment, but measurements of $\bar{N}_{O_2}$ make it possible to obtain it. The differentiation of $\Delta_f G''^\circ$ can be used to obtain $\Delta_f S''^\circ$, $\Delta_f H''^\circ$, and various derivatives of these and other thermodynamic properties of a reaction system.

In Section 2 on the fundamental equation for G, it was stated that 'in the absence of experimental methods for determining the equilibrium concentrations of all eight species, the five reactions cannot be used directly to determine the standard Gibbs energies of formation of the six species involving protein,' but determining K'_{O_2} or $\bar{N}_{O_2}$ makes it possible to obtain $\Delta_f G^\circ(MO_2)$ with respect to $\Delta_f G^\circ(M)$. The latter is set equal to zero by convention. The pKs determined from K'_{O_2} or $\bar{N}_H$ make it possible to calculate $\Delta_f G^\circ$ and $\Delta_f H^\circ$ for the other species.

These discussions have involved a different approach than that introduced by Wyman and Gill [3], but nevertheless these two approaches have a lot in common. The fundamental equations described here were developed to treat the enzyme-catalyzed reactions of metabolism, but proteins may be involved in these reactions, and so it should be possible to treat metabolism and ligand binding together.

Wyman [5] introduced the concept of the binding potential, which he represented by the Russian L for linkage. This is a molar thermodynamic property that is defined by use of a Legendre transform that introduces the chemical potential of the ligand as an independent intensive property. The binding potential of Wyman, which is represented here by L, is given by [3]

$$L = RT\ln\sum_{i=1}^{r}\nu_i e^{L_i/RT} \tag{71}$$

where L_i is the binding potential for the ith form, ν_i is the equilibrium mole fraction of the ith form, and r is the number of forms. The thermodynamic property used in the current paper that is comparable to L is the further transformed Gibbs energy of formation of $H_{av}MO_{2av}$ that is given by

$$\Delta_f G'' = -RT\ln\sum_{i=1}^{N''} e^{-\Delta_f G''^\circ_i/RT} + RT\ln[H_{av}MO_{2av}] \tag{72}$$

where $\Delta_f G''^\circ_i$ is the standard further transformed Gibbs energy of formation of ith form, N'' is the number of forms, and $[H_{av}MO_{2av}]$ is the total concentration of the macromolecule.

It may appear puzzling that the summation in Eq. (72) does not involve the equilibrium mole fractions that appear in Eq. (71). This is because the exponential terms in Eq. (72) involve the standard further transformed Gibbs energies of formation $\Delta_f G''^{o}$ of the various forms rather than the further transformed Gibbs energy of formation $\Delta_f G''$. The standard further transformed Gibbs energies of formation $\Delta_f G''^{o}_i$ of the various forms are given by

$$\begin{aligned}\Delta_f G''^{o}_i &= \Delta_f G''_i - RT\ln[M_i] \\ &= \Delta_f G''_i - RT\ln\left(r_i[H_{av}MO_{2av}]\right)\end{aligned} \qquad (73)$$

where r_i is the equilibrium mole fraction of the ith form of $H_{av}MO_{2av}$. Substituting Eq. (73) in Eq. (72) and setting $[H_{av}MO_{2av}] = 1$ M yields

$$\Delta_f G'' = -RT\ln\sum_{i=1}^{N''} r_i e^{-\Delta_f G''_i/RT} \qquad (74)$$

that is the same as Eq. (71) except for the sign differences. In the Wyman approach, a normalization is used to eliminate the term for the macromolecule in the fundamental equation, and so the concept of standard state for the macromolecule does not arise. This does not cause a problem in deriving Eq. (71), but the concentration of the macromolecule is needed in treating an enzyme-catalyzed reaction involving a macromolecule and to obtain all of the Maxwell relations discussed here.

9. Glossary

C	number of components
G	Gibbs energy of a system (J)
$\Delta_f G_i$	Gibbs energy of formation of species I ($J\ mol^{-1}$)
$\Delta_f G^{o}_i$	standard Gibbs energy of formation of species i ($J\ mol^{-1}$)
$\Delta_r G$	Gibbs energy of reaction ($J\ mol^{-1}$)
$\Delta_r G^{o}$	standard Gibbs energy of reaction ($J\ mol^{-1}$)
G'	transformed Gibbs energy of a system (J)
$\Delta_f G'$	transformed Gibbs energy of formation of species i or reactant j ($J\ mol^{-1}$)
$\Delta_f G'^{o}$	standard transformed Gibbs energy of formation of a species i or reactant j ($J\ mol^{-1}$)
$\Delta_r G'$	transformed Gibbs energy of reaction ($J\ mol^{-1}$)
$\Delta_r G'^{o}$	standard transformed Gibbs energy of reaction ($J\ mol^{-1}$)
G''	further transformed Gibbs energy of a system (J)
$\Delta_f G''$	further transformed Gibbs energy of formation of reactant j or k ($J\ mol^{-1}$)
$\Delta_f G''^{o}$	standard further transformed Gibbs energy of formation of reactant j or k ($J\ mol^{-1}$)
$\Delta_r G''$	further transformed Gibbs energy of reaction ($J\ mol^{-1}$)
$\Delta_r G''^{o}$	standard further transformed Gibbs energy of reaction ($J\ mol^{-1}$)
H	enthalpy of a system (J)
$\Delta_f H_i$	enthalpy of formation of species i ($J\ mol^{-1}$)
$\Delta_f H^{o}_i$	standard enthalpy of formation of species i ($J\ mol^{-1}$)
$\Delta_r H$	enthalpy of reaction ($J\ mol^{-1}$)
$\Delta_r H^{o}$	standard enthalpy of reaction ($J\ mol^{-1}$)
H'	transformed enthalpy of a system (J)
$\Delta_f H'$	transformed enthalpy of formation of species i or reactant j ($J\ mol^{-1}$)
$\Delta_f H'^{o}$	standard transformed enthalpy of formation of a species i or reactant j ($J\ mol^{-1}$)
$\Delta_r H'$	transformed enthalpy of reaction ($J\ mol^{-1}$)
$\Delta_r H'^{o}$	standard transformed enthalpy of reaction ($J\ mol^{-1}$)
H''	further transformed enthalpy of a system (J)
$\Delta_f H''$	further transformed enthalpy of formation of reactant j or k ($J\ mol^{-1}$)
$\Delta_f H''^{o}$	standard further transformed enthalpy of formation of reactant j or k ($J\ mol^{-1}$)
$\Delta_r H''$	further transformed enthalpy of reaction ($J\ mol^{-1}$)
$\Delta_r H''^{o}$	standard further transformed enthalpy of reaction ($J\ mol^{-1}$)
I	ionic strength (M)
K	equilibrium constant or acid dissociation constant
K'	apparent equilibrium constant
L	linkage potential ($J\ mol^{-1}$)
n_i	amount of species i (mol)
n_j'	amount of reactant j at specified pH (mol)
n_k''	amount of reactant k at specified pH and $[O_2]$ (mol)
$n_c(i)$	amount of component i (mol)
N	number of different species
N_c	number of components
$N_H(i)$	number of hydrogen atoms in species i
$N_{O_2}(i)$	number of oxygen molecules in species or reactant i
$\bar{N}_H$	average number of hydrogen atoms in a reactant
$\bar{N}_{O_2}$	average number of oxygen molecules in a reactant
$\Delta_r N_H$	change in number of hydrogen atoms bound in a reaction
$\Delta_r N_{O_2}$	change in number of oxygen molecules bound in a reaction
P	pressure (bars)
pH	$-\log[H^+]$
pK	$-\log K$
r_i	equilibrium mole fraction of i
R	gas constant (8.31451 $J\ K^{-1}\ mol^{-1}$)
R	number of independent reactions
S	entropy of a system ($J\ K^{-1}$)
$\bar{S}_i$	Molar entropy of a species ($J\ K^{-1}\ mol^{-1}$)

$\Delta_{\mathrm{f}}S_i$	entropy of formation of species *i* ($\mathrm{J\,K^{-1}\,mol^{-1}}$)
$\Delta_{\mathrm{f}}S_i^{\circ}$	standard entropy of formation of species *i* ($\mathrm{J\,K^{-1}\,mol^{-1}}$)
$\Delta_{\mathrm{r}}S$	entropy of reaction ($\mathrm{J\,K^{-1}\,mol^{-1}}$)
$\Delta_{\mathrm{r}}S^{\circ}$	standard entropy of reaction ($\mathrm{J\,K^{-1}\,mol^{-1}}$)
S'	transformed entropy of a system ($\mathrm{J\,K^{-1}}$)
$\Delta_{\mathrm{f}}S'$	transformed entropy of formation of species *i* or reactant *j* ($\mathrm{J\,K^{-1}\,mol^{-1}}$)
$\Delta_{\mathrm{f}}S'^{\circ}$	standard transformed entropy of formation of a species *i* or reactant *j* ($\mathrm{J\,K^{-1}\,mol^{-1}}$)
$\Delta_{\mathrm{r}}S'$	transformed entropy of reaction ($\mathrm{J\,K^{-1}\,mol^{-1}}$)
$\Delta_{\mathrm{r}}S'^{\circ}$	standard transformed entropy of reaction ($\mathrm{J\,K^{-1}\,mol^{-1}}$)
S''	further transformed entropy of a system ($\mathrm{J\,K^{-1}}$)
$\Delta_{\mathrm{f}}S''$	further transformed entropy of formation of reactant *j* or *k* ($\mathrm{J\,K^{-1}\,mol^{-1}}$)
$\Delta_{\mathrm{f}}S''^{\circ}$	standard further transformed entropy of formation of reactant *j* or *k* ($\mathrm{J\,K^{-1}\,mol^{-1}}$)
$\Delta_{\mathrm{r}}S''$	further transformed entropy of reaction ($\mathrm{J\,K^{-1}\,mol^{-1}}$)
$\Delta_{\mathrm{r}}S''^{\circ}$	standard further transformed entropy of reaction ($\mathrm{J\,K^{-1}\,mol^{-1}}$)
T	temperature (K)
ν_i	mole fraction in Eq. (71)
μ_i	chemical potential of species *i* ($\mathrm{J\,mol^{-1}}$)
μ_i°	standard chemical potential of species *i* ($\mathrm{J\,mol^{-1}}$)
μ'	transformed chemical potential of species *i* or reactant *j* ($\mathrm{J\,mol^{-1}}$)
μ'°	standard transformed chemical potential of species *j* or reactant *j* ($\mathrm{J\,mol^{-1}}$)
μ''	further transformed chemical potential of reactant *j* or *k* ($\mathrm{J\,mol^{-1}}$)
μ''°	standard further transformed chemical potential of reactant *j* or *k* ($\mathrm{J\,mol^{-1}}$)
ξ	extent of reaction (mol)

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