

# Ab initio quantification of the oxygen-hemoglobin dissociation curve

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**ABSTRACT** Uptake of oxygen by hemoglobin (Hb), described by the oxygen-Hb dissociation curve, is obviously important for the existence of all vertebrates. Its sigmoidal curve shape indicates that oxygen binds more tightly if sites already are occupied, commonly referred to as the cooperative effect. The effect has been challenging to understand and quantify ever since its experimental demonstration in 1904. Here, we derive an ab initio analytical expression for the dissociation curve based on the fundamental principle of uniform oxygen chemical potential and absolute activity throughout the system at equilibrium using the grand partition function. The resulting analytical dissociation expression therefore only has four molecular oxygen-Hb binding energies as free variables, which are determined by fitting the analytical expression to measured data. The corresponding resulting negative reaction enthalpies identified in increasing magnitude are,  $\Delta H_1 = -41.6$ ,  $\Delta H_2 = -48.8$ ,  $\Delta H_3 = -51.2$ , and  $\Delta H_4 = -51.8$  kJ/mol, in the range observed experimentally. The difference between  $\Delta H_1$  and  $\Delta H_4$  is  $\sim 10$  kJ/mol, smaller than the maximum enthalpy difference measured experimentally,  $\sim 16.7$  kJ/mol. Hence, the cooperative effect can therefore be explained, from an energy point of view, as caused by the reaction enthalpy difference between  $\Delta H_1$  and the three subsequent enthalpy values. No impact of Hb's spatial and structural properties is assumed. The finding highlights the importance of identifying the ligand-receptor molecular binding energies, and thereby the reaction enthalpies, under different conditions as a way for calculating not only the oxygen-Hb but ligand-receptor dissociation curves in general under various conditions, a priori, since the procedure for determining these curves ab initio has been established.

**WHY IT MATTERS** The sigmoid shape and thereby the cooperative effect of the oxygen-hemoglobin dissociation curve has been derived ab initio using the grand partition function. No assumptions about hemoglobin's spatial and/or structural properties were assumed. The cooperative effect is hence explained from an energy point of view by the difference between the first and three subsequent binding enthalpies. The reason is that the binding enthalpy difference required to fit the dissociation curve is smaller than the enthalpy difference determined experimentally. The advance matters since the hemoglobin-oxygen system is regarded as the Hydrogen-atom of ligand-receptor systems. The method opens the possibility for determining not only the current but all ligand-receptor dissociation curves in general, a priori, when the relevant binding enthalpies are known.

## INTRODUCTION

In 1904, Christian Bohr, the father of the atom physicist, discovered that oxygen molecules bind nonlinearly to hemoglobin (Hb) as illustrated by the black bold curve in Fig. 1. The curve shows that binding of oxygen to Hb at low oxygen pressure occurs more tightly than expected due to its sigmoid shape (1,2).

A linear relationship for 1:1 binding of the smaller oxygen molecules (generally called ligands) to larger Hb molecules (generally called receptors) was expected since Hb at that time was assumed to be single stranded, well described by a conventional Langmuir isotherm (3), as indicated by the blue dotted curve.

Adair (4) later discovered, using accurate osmotic pressure measurements, that each Hb molecule in fact binds up to four oxygen molecules, typically written as  $\text{Hb}(\text{O}_2)_4$ , when fully occupied. It therefore seems like that subsequent binding of oxygen to Hb somehow occurs more tightly if oxygen already occupies some sites on the Hb molecule. This tendency

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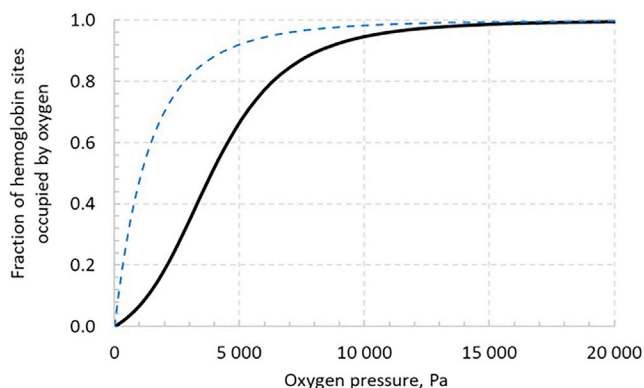


FIGURE 1 The oxygen-Hb dissociation curve at 37°C. The black bold curve shows that binding of oxygen appears to increase nonlinearly at low oxygen pressure due to its sigmoid shape if some of the four sites already are occupied by oxygen, as discovered experimentally by Bohr et al. (1). The blue dotted line illustrates expected curve without any cooperative effect present.

has therefore in general been referred to as positive cooperative binding ever since Bohr's discovery and it occurs frequently in biochemical systems in cases where the receptor, typically a large protein molecule, contains more than one binding site for the ligand molecules. The word positive indicates that the effect also could be opposite, which is indeed the case. A negative cooperative effect occurs when the ligand binding ability decreases for subsequent ligand molecules. Furthermore, cooperativity is called homotropic if the ligand only impacts binding of the same molecule or heterotropic if it also impacts binding of other ligand types (2). Quantification of the positive homotropic cooperative effect in the oxygen-Hb system is the focus of this work.

A vast amount of research has been undertaken related to the positive cooperative effect, particularly for the oxygen-Hb system due to its importance for the existence of all vertebrates, but also in general due to its frequent occurrence in many biochemical and physiological processes. The research has been focusing along two main lines, on the understanding of the effect's mechanism from a molecular biology/chemistry point of view (5) and on its quantitative description (6–11), the latter mostly advanced by physical-chemical scientists. None of the activities can be stated fully closed, particularly the latter, although large progress has been obtained over the years (5,7). Research related to quantification of the positive cooperative effect was initiated in 1910 when Hill published his famous equation describing general binding of ligands to receptors each having  $n$  available binding sites. It reads (2,12),

$$\theta_H = \frac{[L]^n}{K_D + [L]^n}, \quad (1)$$

where,  $\theta_H$  is the fractional receptor occupancy by ligands,  $K_D$  is an apparent dissociation constant, and  $[L]$  is the ligand concentration. The latter is typically replaced by the oxygen pressure,  $P_{O_2}$ , when considering the oxygen-Hb dissociation curve (Eq. 16).  $n$  is called the Hill exponent and its value was originally assumed to be equal to the number of binding sites on the receptor. It is smaller than unity for systems exhibiting negative cooperativity and vice versa for the case of positive cooperativity. The fractional receptor occupancy is defined as the number of binding sites occupied by ligands divided by the total number of sites available in the system. No bindings are present when  $\theta_H = 0$ , whereas all sites are occupied when  $\theta_H = 1$ . It was, however, realized early that Hill's equation was unable to capture all aspects of cooperativity. The exponent,  $n$ , is not four for the experimental oxygen-Hb curve but varies typically in the range from 1.7 to 3.2. It can therefore not represent the number of sites available on the receptor in general but is more interpreted as a cooperativity parameter or an interaction strength between ligand and receptor (13,14). In addition, the equation is semiempirical as the apparent dissociation constant,  $K_D$ , and the exponent,  $n$ , must be found by fitting the expression to experimental data. Many researchers have therefore proposed different expression for quantifying the dissociation curve more accurately based on different generic functions and partition functions, see, e.g., Adair et al. (6), Klotz (8), Pauling (9), Koshland et al. (10), and Monod et al. (11). These works have generalized Hill's equation by quantifying the impact of adding intermediate polynomial terms, originating from intermediate oxygen-Hb forms such as  $Hb(O_2)_{n=1,2 \text{ or } 3}$ , on the resulting dissociation curve. None have, however, to the knowledge of the author, used the grand partition function directly in a systematic way as the starting point for deriving the dissociation curve. Since varying particle number obviously is an intrinsic part of the challenge, using such an approach should be the most fundamental. It accounts for the varying occupation numbers by utilizing the fundamental principle of uniform oxygen chemical potential and hence absolute activity throughout the system at equilibrium regardless of whether oxygen resides in solution or is bonded to Hb. It is extensively used in thermodynamics, statistical mechanics, and solid-state physics, e.g., in semiconductor theory (3).

Hence, the aim of the work is to use the grand partition function to derive an ab initio analytical expression for the oxygen-Hb dissociation curve. The molecular binding site energies for each of the four oxygen molecules are then the only unknown variables left since no other dependencies are assumed, e.g.,

related to Hb's spatial and/or structural properties (5). The magnitude of the molecular binding energies will be identified by matching the analytical dissociation expression to measured curves and their magnitudes compared and discussed in relation to corresponding measured reaction enthalpies reported in the literature. Furthermore, the relationship of the current approach to Hill's equation is demonstrated and discussed. It should finally also be mentioned that, apart from temperature and the Bohr effect (impact of pH and CO<sub>2</sub> on the shape of the dissociation curve), other compounds have also been shown to affect the shape of the oxygen-Hb dissociation curve, e.g., protons per se, and 2,3-diphosphoglycerate (2,3-DPG) (15). A way to account for such additional effects when calculating dissociation curves a priori is also discussed.

## MATERIALS AND METHODS

### Derivation of an ab initio expression for the dissociation curve using the grand partition function

Consider systems characterized by particles formally of atomic or molecular size in general referred to as ligands called L, which typically are able to bind to a larger molecule called a receptor denoted R. The ligands will herein be oxygen and the receptor Hb, containing four binding sites ( $n = 4$ ) for ligands. Each of these ligands may have different molecular binding energies,  $\epsilon_1, \dots, \epsilon_4$ . These binding energies are relative to oxygen molecules at rest at infinite distance. If

Here,  $\lambda \equiv e^{\mu/\tau}$ , is the absolute activity where  $\mu$  is the internal chemical potential of oxygen of thermal origin and  $\tau$  is following the terminology by Kittel and Kroemer (3), the fundamental temperature given as  $\tau = k_B T$ . The sum in Eq. 3 is over all states for all number of particles, which gives,

$$\begin{aligned} Z_4 &= \lambda^0 e^0 + \binom{4}{1} \lambda e^{-\epsilon_1/\tau} + \binom{4}{2} \lambda^2 e^{-(\epsilon_1+\epsilon_2)/\tau} \\ &+ \binom{4}{3} \lambda^3 e^{-(\epsilon_1+\epsilon_2+\epsilon_3)/\tau} \\ &+ \lambda^4 e^{-(\epsilon_1+\epsilon_2+\epsilon_3+\epsilon_4)/\tau} \\ &= 1 + 4\lambda e^{-\epsilon_1/\tau} + 6\lambda^2 e^{-(\epsilon_1+\epsilon_2)/\tau} \\ &+ 4\lambda^3 e^{-(\epsilon_1+\epsilon_2+\epsilon_3)/\tau} \\ &+ \lambda^4 e^{-(\epsilon_1+\epsilon_2+\epsilon_3+\epsilon_4)/\tau}, \end{aligned} \quad (4)$$

where the unity term comes from the zero-occupancy case when  $n = \epsilon_0 = 0$  and  $\binom{A}{B} = \frac{A!}{B!(A-B)!}$  is the binomial coefficient. The fraction of sites occupied by oxygen on the Hb molecule versus the oxygen pressure, i.e., the oxygen dissociation curve, is then given as the probability of having one, two, three, or four oxygen molecules bonded to each Hb molecule normalized by Eq. 4. The fractional occupancy,  $\theta_4$ , is then given as,

$$\theta_4 = \frac{4\lambda e^{-\epsilon_1/\tau} + 6\lambda^2 e^{-(\epsilon_1+\epsilon_2)/\tau} + 4\lambda^3 e^{-(\epsilon_1+\epsilon_2+\epsilon_3)/\tau} + \lambda^4 e^{-(\epsilon_1+\epsilon_2+\epsilon_3+\epsilon_4)/\tau}}{1 + 4\lambda e^{-\epsilon_1/\tau} + 6\lambda^2 e^{-(\epsilon_1+\epsilon_2)/\tau} + 4\lambda^3 e^{-(\epsilon_1+\epsilon_2+\epsilon_3)/\tau} + \lambda^4 e^{-(\epsilon_1+\epsilon_2+\epsilon_3+\epsilon_4)/\tau}}. \quad (5)$$

energy must be supplied to remove oxygen from the Hb molecule, the binding energy will be negative, which is true for all cases herein. As free oxygen molecules mostly possess kinetic energy at ambient temperature in the order of  $k_B T$ , the same amount of kinetic energy must also be present in the bonded state when the system temperature is uniform.  $k_B$  is here Boltzmann's constant and  $T$  is the absolute temperature. The energies,  $\epsilon_1, \dots, \epsilon_4$ , therefore represent the net decrease in potential energy per molecule due to the exothermic chemical reaction taking place when oxygen is bonded to the actual Hb site (16). The aim is now to derive an ab initio expression for the dissociation curve,  $\theta_4$ , defined as,

$$\theta_4 = \frac{\text{Number of binding sites occupied by ligands}}{\text{Total number of binding sites available}}, \quad (2)$$

as a function of the oxygen pressure. It is believed that application of the grand partition function is the proper methodology for such derivations as the particle number is varying. Hence, for a receptor with four available sites, the grand partition function,  $Z_4$ , is given as (3),

$$Z_4 = \sum_{n=0,1,\dots,4} \sum_s \lambda^n e^{-\epsilon_s/\tau}. \quad (3)$$

At equilibrium, the chemical potential of oxygen is everywhere uniform, so the O<sub>2</sub> molecules bonded to Hb must have the same chemical potential as the O<sub>2</sub> molecules in solution. This implies that the absolute activity at constant temperature also must be the same for bonded and nonbonded oxygen molecules since  $\lambda = e^{\mu/\tau}$ . Hence,

$$\begin{aligned} \mu(\text{O}_2 \text{ solution}) &= \mu(\text{Hb}(\text{O}_2)_{1-4}) \rightarrow \\ \lambda(\text{O}_2 \text{ solution}) &= \lambda(\text{Hb}(\text{O}_2)_{1-4}). \end{aligned} \quad (6)$$

The chemical potential can be shown to be  $\mu = \tau \times \ln(\rho_{\#}/\rho_Q)$ , where  $\rho_{\#}$  is the number density of oxygen gas and  $\rho_Q$  is the quantum concentration (3). The latter is the concentration associated with one atom in a cube of side equal to the thermal average de Broglie wavelength and given as  $\rho_Q \equiv \left(\frac{m\tau}{2\pi\hbar^2}\right)^{3/2}$ . Here,  $m$  is the mass of the oxygen molecule and  $\hbar$  is Planck's constant divided by  $2\pi$ . Assuming that oxygen behaves like an ideal gas, that is  $P_{\text{O}_2} = \rho_{\#}\tau$ , implies that the absolute activity can be written,  $\lambda = e^{\mu/\tau} = \frac{\rho_{\#}}{\rho_Q} = \frac{P_{\text{O}_2}}{\tau\rho_Q}$ , which has the same value everywhere because of Eq. (6). Multiplying and dividing all terms in Eq. (5) with  $e^{(\epsilon_1+\epsilon_2+\epsilon_3+\epsilon_4)/\tau}$  and  $\lambda^4$ , respectively, gives,

$$\theta_4 = \frac{4 \left( \frac{\rho_Q \tau}{P_{O_2}} \right)^3 e^{(\varepsilon_2 + \varepsilon_3 + \varepsilon_4)/\tau} + 6 \left( \frac{\rho_Q \tau}{P_{O_2}} \right)^2 e^{(\varepsilon_3 + \varepsilon_4)/\tau} + 4 \left( \frac{\rho_Q \tau}{P_{O_2}} \right) e^{\varepsilon_4/\tau} + 1}{\left( \frac{\rho_Q \tau}{P_{O_2}} \right)^4 e^{(\varepsilon_1 + \varepsilon_2 + \varepsilon_3 + \varepsilon_4)/\tau} + 4 \left( \frac{\rho_Q \tau}{P_{O_2}} \right)^3 e^{(\varepsilon_2 + \varepsilon_3 + \varepsilon_4)/\tau} + 6 \left( \frac{\rho_Q \tau}{P_{O_2}} \right)^2 e^{(\varepsilon_3 + \varepsilon_4)/\tau} + 4 \left( \frac{\rho_Q \tau}{P_{O_2}} \right) e^{\varepsilon_4/\tau} + 1} \quad (7)$$

Multiplying all terms with  $P_{O_2}^4$  gives the final expression for the oxygen dissociation curve,

$$\theta_4 = \frac{4P_{2,3,4}^3 P_{O_2} + 6P_{3,4}^2 P_{O_2}^2 + 4P_4 P_{O_2}^3 + P_{O_2}^4}{P_{1,2,3,4}^4 + 4P_{2,3,4}^3 P_{O_2} + 6P_{3,4}^2 P_{O_2}^2 + 4P_4 P_{O_2}^3 + P_{O_2}^4}, \quad (8)$$

where the pressure coefficient terms are,

$$P_{1,2,3,4} = \left( \frac{m}{2\pi\hbar^2} \right)^{3/2} (k_B T)^{5/2} \times e^{(\varepsilon_1 + \varepsilon_2 + \varepsilon_3 + \varepsilon_4)/4k_B T}, \quad (9)$$

$$P_{2,3,4} = \left( \frac{m}{2\pi\hbar^2} \right)^{3/2} (k_B T)^{5/2} \times e^{(\varepsilon_2 + \varepsilon_3 + \varepsilon_4)/3k_B T}, \quad (10)$$

$$P_{3,4} = \left( \frac{m}{2\pi\hbar^2} \right)^{3/2} (k_B T)^{5/2} \times e^{(\varepsilon_3 + \varepsilon_4)/2k_B T}, \quad (11)$$

$$P_4 = \left( \frac{m}{2\pi\hbar^2} \right)^{3/2} (k_B T)^{5/2} \times e^{\varepsilon_4/k_B T}. \quad (12)$$

Equation 8 is an ab-initio-derived expression for the oxygen-Hb dissociation curve. The pressure coefficient terms in Eqs. 9–12 are dependent on the type of gas and the absolute temperature in addition to the molecular binding energies. The former are given by the system specifications leaving the magnitude of the molecular binding energies  $\varepsilon_1, \dots, \varepsilon_4$ , as the only free variables. They must be determined by matching the  $\theta_4$  curve shape versus oxygen pressure to measured data as demonstrated in the results.

## The relationship to Hill's equation

As shown by many (2,13,17), Hill's equation in Eq. 1 can be derived by considering the relative affinity of ligands, L, for being free or bonded to the receptor, R, characterized by association and dissociation constants called  $k_a$  and  $k_d$ , respectively. It can be observed that Eq. 1,  $\theta_H = \frac{[L]^n}{K_D + [L]^n}$ , also appears from the grand partition function (Eq. 3) for the case  $n = 4$  if all terms associated with binding of one, two, and three ligand molecules are neglected. This is in line with the assumption underlying Eq. 1, that no intermediate forms of the ligand-receptor forms are considered. Hence, the simplified version of the grand partition function in Eq. 4 then becomes,

$$Z_{L_4} = \sum_{n=0 \text{ and } 4} \sum_s \lambda^n e^{-\varepsilon_s/\tau} = 1 + \lambda^4 e^{-4\varepsilon/\tau}. \quad (13)$$

In analogy to Eq. 5, the dissociation curve, identical to Hill's equation, then becomes,

$$\theta_H = \frac{\lambda^4 e^{-4\varepsilon/\tau}}{1 + \lambda^4 e^{-4\varepsilon/\tau}} = \frac{\rho_{\#}^4}{K_D + \rho_{\#}^4} = \frac{[L]^4}{K_D + [L]^4}, \quad (14)$$

where,  $\lambda = \frac{\rho_{\#}}{\rho_Q}$  has been used, [L] is the molar concentration of ligands in solution, and  $K_D = \bar{K}_D^4 = \left( \frac{\rho_Q}{N_A} \right)^4 e^{4\varepsilon/\tau}$ .  $N_A$  is Avogadro's number and the quantum concentration,  $\rho_Q$ , must here be given in the unit,  $\#/\text{dm}^3$ . If the version of the absolute activity expression containing the oxygen pressure is applied, the dissociation curve following from Eq. 13 instead becomes,

$$\theta_H = \frac{P_{O_2}^4}{K_{D,P_{O_2}} + P_{O_2}^4}, \quad (15)$$

with  $K_{D,P_{O_2}} = (\rho_Q \tau)^4 e^{4\varepsilon/\tau}$ . For the purpose of characterizing the extent of cooperativity, Hill's equation is conventionally linearized, and expressed as,

$$\log\left(\frac{\theta_H}{1 - \theta_H}\right) = n_H \times \log[P_{O_2}] - \log K_{D,P_{O_2}}, \quad (16)$$

where the prefactor,  $n_H$ , is now called the Hill coefficient and its value is normally in the range from 1.7 to 3.2 (14). Log is here  $\text{Log}_{10}$ . Equations 15 and 16 will be used for discussion and comparison purposes herein, the latter in a form where the Hill fractional occupancy,  $\theta_H$ , is replaced by a general fractional occupancy,  $\theta$ .

## RESULTS

### Quantification of the oxygen-Hb dissociation curve

Identifying the grand partition function characterizing the oxygen-Hb system, is considered key for determining the proper dissociation curve as shown in the materials and methods. The fraction of sites occupied by oxygen ( $\theta_4$ ) versus oxygen pressure ( $P_{O_2}$ ), i.e., the oxygen-Hb dissociation curve, is derived there and given as Eq. 8,

$$\theta_4 = \frac{4P_{2,3,4}^3 P_{O_2} + 6P_{3,4}^2 P_{O_2}^2 + 4P_4 P_{O_2}^3 + P_{O_2}^4}{P_{1,2,3,4}^4 + 4P_{2,3,4}^3 P_{O_2} + 6P_{3,4}^2 P_{O_2}^2 + 4P_4 P_{O_2}^3 + P_{O_2}^4}, \quad (17)$$

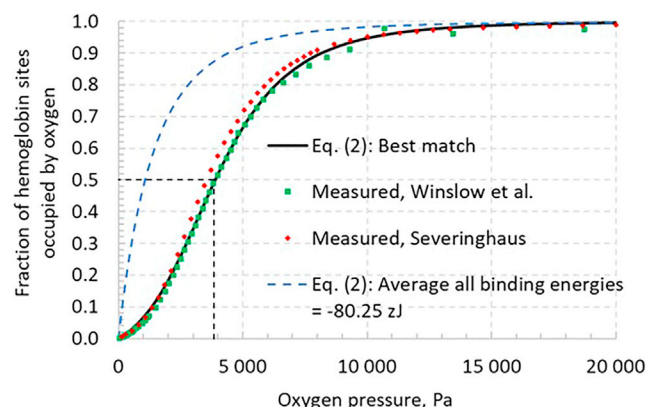
where the pressure coefficient terms,  $P_{1,2,3,4}$ ,  $P_{2,3,4}$ ,  $P_{3,4}$ , and  $P_4$ , are defined in Eqs. 9–12. It should be noted that these terms are derived ab initio and therefore contain the absolute activity of oxygen. The latter is inversely proportional to the fundamental quantity referred to as the quantum concentration whose value is determined by solving the single-atom Schrödinger equation for a cube with sides equal to the thermal average of the de Broglie wavelength (3). Hence, the only free variables left in Eq. 17 are the four molecular oxygen-Hb binding energies,  $\varepsilon_1, \dots, \varepsilon_4$ , stemming from

**TABLE 1 Numerical molecular binding energy values used to determine the best match to experimental data in Fig. 2 together with corresponding reaction enthalpies,  $\Delta H$**

Planck's constant divided by $2\pi, \hbar$	$1.055 \times 10^{-34}$ Js
Boltzmann's constant, $k_B$	$1.38 \times 10^{-23}$ J/K
Mass oxygen $O_2, m$	$5.31 \times 10^{-26}$ kg
Molecular binding energy $\epsilon_1$ ; reaction enthalpy $\Delta H_1 = \epsilon_1 N_A$	- 69 zJ; - 41.6 kJ/mol
Molecular binding energy $\epsilon_2$ ; reaction enthalpy $\Delta H_2 = \epsilon_2 N_A$	- 81 zJ; - 48.8 kJ/mol
Molecular binding energy $\epsilon_3$ ; reaction enthalpy $\Delta H_3 = \epsilon_3 N_A$	- 85 zJ; - 51.2 kJ/mol
Molecular binding energy $\epsilon_4$ ; reaction enthalpy $\Delta H_4 = \epsilon_4 N_A$	- 86 zJ; - 51.8 kJ/mol

the grand partition function expression. They must be determined by fitting Eq. 17 to measured data since no known method exists for calculating the change in molecular binding energies for these reactions a priori. By adjusting the molecular binding energies to the values given in Table 1, the best match  $\theta_4$  curve to the experimental data reported by Winslow et al. (18) and Severinghaus (19) was obtained as shown in Fig. 2.

The calculated curve matches the measured data closely and was identified using the following molecular binding energy values,  $\epsilon_1 = -69$ ,  $\epsilon_2 = -81$ ,  $\epsilon_3 = -85$ , and  $\epsilon_4 = -86$  zJ. Corresponding measurable reaction enthalpy values all in kJ/mol are,  $\Delta H_1 = -41.6$ ,  $\Delta H_2 = -48.8$ ,  $\Delta H_3 = -51.2$ , and  $\Delta H_4 = -51.8$ . The fit was ob-



**FIGURE 2 Best match dissociation curve (Eq. 17) fit to measured data at 37°C. The match was obtained by adjusting the four molecular binding energies to the following values:  $\epsilon_1 = -69$ ,  $\epsilon_2 = -81$ ,  $\epsilon_3 = -85$ , and  $\epsilon_4 = -86$  zJ. The numerical value of  $k_B T = 4.28$  zJ at 37°C. These values completely determine the pressure coefficient terms (Eqs. 9–12 in the materials and methods) to the following values:  $P_{1,2,3,4} = 5646$ ,  $P_{2,3,4} = 2350$ ,  $P_{3,4} = 1655$ , and  $P_4 = 1472$  Pa. The pressure at 50% occupation is, 3,850 Pa (29 mmHg). Experimental data from Winslow et al. (18): temperature 37°C and pH 7.42,  $P_{CO_2} = 5333$  Pa, 2,3-DPG = 0.89  $\mu\text{mol}$  per  $\mu\text{mol}$  of Hb tetramer. Severinghaus (19): temperature 37°C and pH 7.4.**

tained by adjusting the values manually followed by visual inspection. Since measurements of heat release normally take place under constant pressure experimentally, such values are conventionally reported as changes in molar reaction enthalpy,  $\Delta H$ . The relationship between  $\epsilon$  and the corresponding molar reaction enthalpy value is therefore that the latter equals  $\epsilon$  multiplied with Avogadro's number, i.e.,  $\Delta H_1 = \epsilon_1 N_A$ ,  $\Delta H_2 = \epsilon_2 N_A$ , etc. The enthalpy values above are in the range typically measured for the oxygen-Hb system (5,6,20–22). The most important feature is, however, that the enthalpy difference between the smallest and largest value is only  $10.2 \frac{\text{kJ}}{\text{mol}}$ , well within the highest enthalpy difference measured experimentally according to Eaton et al. (5), equal to  $4 \frac{\text{kcal}}{\text{mol}}$  or  $16.7 \frac{\text{kJ}}{\text{mol}}$ . It should, furthermore, be noticed that the energies required to occupy the three last sites are quite similar and therefore significantly more negative than for the first site. Varying the value of  $\epsilon_1$  relative to the other three energies by keeping them equal and more negative than  $\epsilon_1$  shows that the gap in fact is responsible for capturing the positive cooperative effect from an energy point of view. The requirement is that the gap energy must exceed approximately  $\sim 10$  zJ. Varying the  $\epsilon_2$ ,  $\epsilon_3$ , and  $\epsilon_4$  values individually impacts the shape of the dissociation curves from lower to higher fractional values on the y axis. Although each individual value can vary slightly, their magnitudes and sequence can be considered quite unique due to their coupled appearance in the pressure coefficient terms. The dissociation curve shape is, e.g., very sensitive to variation in these binding energy values inducing changes in the fraction of Hb sites occupied in the order of up to  $\pm 0.6$  by varying each of the individual binding energies only  $\pm 2\%$  from their best match values. It can also be shown that the cooperative effect disappears completely, blue dotted curve, by putting all binding site energies equal using, e.g., the average of the four values defining the best match,  $\epsilon_{\text{Avg}} = -80.25$  zJ. The magnitude of all these binding energies is finally believed to possess physical/chemical significance and not only act as fit parameters, since the correct dissociation expression has been identified.

It can therefore be concluded that the proposed dissociation expression (Eq. 17), together with the molecular binding energies identified, is able to explain the positive cooperative effect in the oxygen-Hb system, from an energy point of view, since the enthalpy difference required to match measured dissociation curves is well within the differences measured experimentally. The difference in enthalpy values between

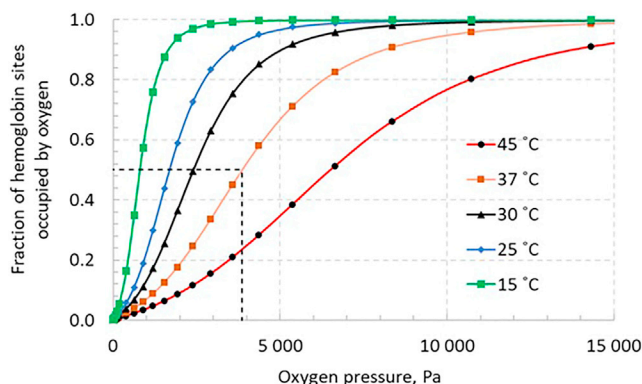


FIGURE 3 Calculated dissociation curves for varying temperatures. The curves show how the best match dissociation curve at 37°C varies with temperature. It is assumed that all the temperature dependency is due to the explicit temperature terms in the pressure coefficients, [materials and methods](#), Eqs. 9–12.

the first and the second reaction induces the positive cooperative effect. It should be noted that no impacts of Hb's spatial and structural properties have been assumed (5,11,23–25). Such effects may, however, contribute to the explanation for the binding energy variation observed but considered outside the main scope of this work. One potential proposal related to the issue will, however, be briefly discussed below. The main advancement of the current approach is therefore believed to be identification of the correct mathematical expression for the dissociation curve since it is based on an ab initio derivation using the principle of uniform chemical potential as previously mentioned. Hence, the somewhat complex relation-

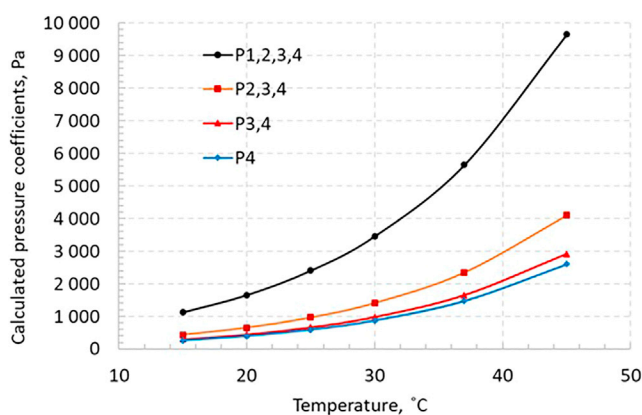


FIGURE 4 Calculated temperature dependency of the pressure coefficient terms,  $P_{1,2,3,4}$ ,  $P_{2,3,4}$ ,  $P_{3,4}$ , and  $P_4$ . All temperature dependencies are assumed to be accounted for by the  $T^{5/2}$  term plus the Boltzmann factors, [materials and methods](#), Eqs. 9–12. Since the  $T^{5/2}$  term is common for all terms, the Boltzmann factors are responsible for the differences seen. All the molecular binding energies are assumed independent of temperature and constant equal to the values in [Table 1](#) over the temperature range considered.

ship between the different molecular binding energies (Eq. 8) therefore follows and is a direct consequence of the grand partition function originally established by Gibbs, one of the most tested and well-supported relationships occurring in statistical physical chemistry. The appearance of the molecular binding energy terms in the dissociation curve expression can hence therefore not be justified by any deeper theory/principle, simply because none are presently known.

A proposal for further research will finally be briefly discussed. According to Dunne et al. (26) considering adsorption of gases on solid surfaces: *An increase in heat of adsorption with gas loading is characteristic of nonheterogeneous adsorbents (e.g., graphitized carbon) with constant gas-solid energies of interaction. The increase is due to cooperative interactions between adsorbed molecules.* Assume each oxygen site on Hb initially is identical from a reaction point of view so no special preference for any of the four sites exists. The close proximity of an occupied site to the remaining sites could hence induce a change in subsequent reaction energies in a similar way as described for the gas adsorption process. A lot of work has already been performed addressing the issue as previously discussed. There is, however, to the knowledge of the author, not much work published using, e.g., molecular simulations. Such an approach could be useful due to practical experimental challenges related to tracking of the origin of the different energies involved. It is therefore proposed to investigate the issue in more detail using molecular simulations with the aim of clarifying the relative contributions from the different underlying causes quantitatively responsible for the net cooperative effect observed.

Calculated dissociation curves using best-match molecular binding energies at 37°C as the base are depicted in [Fig. 3](#) for varying temperatures. The difference between the curves is in qualitative line with measured data (27). The temperature dependency of the pressure coefficient terms is assumed fully accounted for by the  $T^{5/2}$  term plus the Boltzmann factor (Eqs. 9–12 in the [materials and methods](#)) since they are derived ab initio and any minor variation in molecular binding energy versus temperature is neglected.

[Fig. 4](#) shows the temperature dependency for each of the pressure coefficient terms. The variations are caused solely by the Boltzmann factors since all the terms have the  $T^{5/2}$  term in common. They are therefore as expected according to the variation in molecular binding energies,  $\epsilon_1$ , being significantly smaller than the others in absolute value. Hence, the terms  $P_{2,3,4}$ ,  $P_{3,4}$ , and  $P_4$  behave quite similar, whereas the term,  $P_{1,2,3,4}$ , which contains  $\epsilon_1$ , shows a significant stronger temperature dependency. Since the effect of temperature is in qualitative line with measured

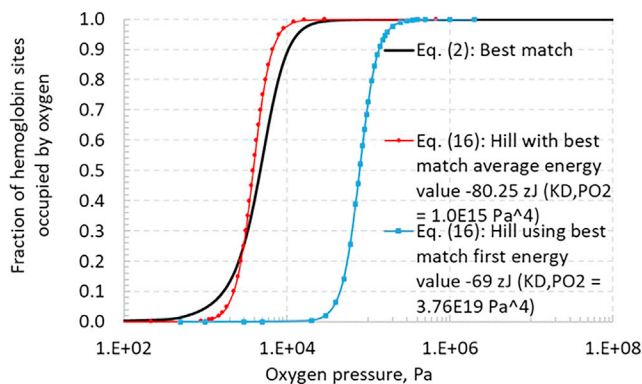


FIGURE 5 Oxygen dissociation curves for the best match compared with two cases of Hill's equation (Eq. 15). The black bold curve is the best match. The red curve is Hill's equation with  $K_{D,P_{O_2}} = 1.0 \times 10^{15} \text{ Pa}^4$  calculated from the average molecular binding energy value ( $\epsilon_{Ave} = -80.25 \text{ zJ}$ ) from the best match. The blue curve is Hill's equation with  $K_{D,P_{O_2}} = 3.76 \times 10^{19} \text{ Pa}^4$ , calculated using the first molecular binding energy value ( $\epsilon_1 = -69 \text{ zJ}$ ) in the best match.

data and captured well by the pressure coefficient terms identified, the results in Figs. 3 and 4 add additional empirical support to the methodology used, i.e., applying the grand partition function for deriving the dissociation curve.

### Relationship to the Hill equation and its linearized form

As shown in the materials and methods, Hill's equation can be derived from the grand partition function, if ne-

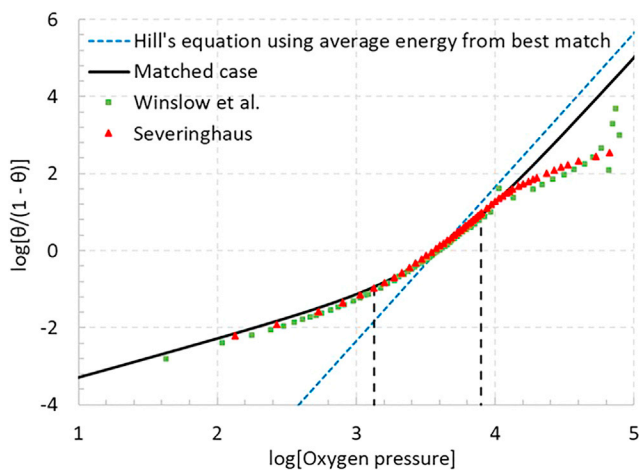


FIGURE 6 Hill plots (materials and methods, Eq. 16) for calculated and experimentally measured dissociation curves. The black bold and blue dotted curves show the best match and Hill's equation using the average binding energy for the best match, respectively. The square green and triangle red dots show experimental data according to Winslow et al. (18) and Severinghaus (19), respectively. The vertical dotted lines indicate pressure values corresponding to fractional occupancies of 0.1 and 0.9, respectively.

glecting the influence of all intermediate forms of the  $\text{Hb}(\text{O}_2)_n$  complex, i.e., for the  $n = 1, 2,$  and  $3$  cases. Two cases using Hill's equation, materials and methods, Eq. 15, are compared in Fig. 5 to the best match indicated as the black bold curve. The red curve is based on the average molecular binding energy ( $\epsilon_{Ave} = -80.25 \text{ zJ}$ ) for the best match giving a  $K_{D,P_{O_2}} = 1.0 \times 10^{15} \text{ Pa}^4$ . The curve shape confirms the inability for Hill's equation to fit the best match curve and thereby experimental data at low and high fractional occupancies. It is interesting to note that Severinghaus (19) was able to improve the fit to measured data considerably by including only one additional term proportional to the oxygen pressure in the Hill expression in Eq. 1, ad hoc. The blue curve is based on the first molecular binding energy identified for the best match,  $\epsilon_1 = -69 \text{ zJ}$ , which gives a significantly larger value for  $K_{D,P_{O_2}} = 3.76 \times 10^{19} \text{ Pa}^4$ . The curve location confirms the general sensitivity of the dissociation expressions to the magnitude of the molecular binding energy. Shifting the value from,  $-80.25$  to  $-69 \text{ zJ}$ , shifts the dissociation curve more than an order of magnitude to the right. Fig. 5 therefore shows that the presence of the intermediate terms in the dissociation expression are significant. Their presence supports the hypothesis proposed by Adair et al. (6) almost 100 years ago, advanced by subsequent researchers, stating that the Hb molecule is built up and broken down in stages such that the system at all times contains all forms of the complex  $\text{Hb}(\text{O}_2)_{1,\dots,4}$ .

The linearized form of Hill's equation (called a Hill plot) is given in Eq. 16 in the materials and methods. Applied to the proposed dissociation curve expression (materials and methods, Eq. 8), it becomes,  $\log\left(\frac{\theta_4}{1-\theta_4}\right) = \log\left[\frac{4P_{2,3,4}^3 P_{O_2} + 6P_{3,4}^2 P_{O_2}^2 + 4P_4 P_{O_2}^3 + P_{O_2}^4}{P_{1,2,3,4}^4}\right]$  versus  $\log[P_{O_2}]$ . Hill plots for the experimental data, the Hill equation using average energy from the best match together with the best match, are shown in Fig. 6 where the general  $\log\left(\frac{\theta}{1-\theta}\right)$  term is plotted versus  $\log[P_{O_2}]$  for all cases. The dotted vertical lines mark pressure values corresponding to fractional occupancies of 0.1 and 0.9, respectively. The Hill equation, shown as the dotted blue line, gives a straight line with slope 4. The value therefore confirms that the Hill exponent overestimates the magnitude of the Hill coefficient,  $n_H$ , since the experimental curves have slopes of approximately, 2.4 and 3, over the middle range. The proposed dissociation curve is, however, able to closely fit the experimental data over the whole range up to  $\log[P_{O_2}]$ , approximately equal to 4.1. This corresponds to a  $\log\left(\frac{\theta}{1-\theta}\right)$  value of approximately 1.5, which again corresponds to a fractional occupancy of approximately

0.97. The deviation between the calculated linearized dissociation curve and the experimental data in the upper region above this value is believed to be caused by two main effects. The experimental uncertainty is higher in this region than elsewhere (18,19,28) and the calculated theoretically dissociation curve is very sensitive and in principle unbounded here. Hence, from a practical experimental point of view, uncertainty in the measured fractional occupancy is therefore likely to limit the maximum reliable value of the term  $\log\left(\frac{\theta}{1-\theta}\right)$  to less than two. A value of two corresponds for example to a fractional occupancy of  $\theta = 0.99$ .

## DISCUSSIONS

The results reported show that oxygen-Hb dissociation curves possibly also can be calculated under different conditions, e.g., under the influence of the Bohr effect (1,2) (variations with pH and CO<sub>2</sub>) or varying 2,3-DPG concentration a priori, if the impact of these compounds is quantified from a reaction enthalpy point of view. Imai and Yonetani (21) report, e.g., a change in reaction enthalpy for increased content of 2,3-DPG and inositol hexaphosphate. Wyman (22) reports a change in reaction enthalpy of approximately  $-13 \frac{\text{kJ}}{\text{mol}}$  from  $-42$  to  $-54 \frac{\text{kJ}}{\text{mol}}$  to occur when pH increases from 6.8 to 9.5 in qualitative line with the dissociation curve expression proposed. The results presented therefore highlight the importance of increased focus on the quality of such reaction enthalpy measurements as a promising way for calculating reliable oxygen-Hb dissociation curves under various conditions a priori.

As previously mentioned, much work has been performed related to quantification of the cooperative effect in the oxygen-Hb system. Adair (4) proposed a dissociation curve of the form,  $\theta_A = \frac{0.25(Kx)+0.25(Kx)^2+0.25(Kx)^3+(Kx)^4}{1+Kx+0.5(Kx)^2+0.333(Kx)^3+(Kx)^4}$ , where  $x$  is oxygen pressure and  $K$  is related to kinetic theory, already in 1925. Subsequent fundamental works by Pauling (9), Klotz (8), and Koshland et al. (10), all end up with similar expressions although the rationales behind and the coefficients turn out differently (2,5). All the resulting expressions are generally in a form similar to the dissociation curve proposed herein but contain in general several additional parameters, which makes it challenging to derive reliable consequences. Procedures for fitting all parameters involved to measured data is therefore commonly applied (29). The proposed ab-initio-derived dissociation curve could therefore also appear advantageous from a scientific falsification point of view, as it has fewer free variables, i.e., only the four binding energies, which addi-

tionally are relatively accessible from an experimental point of view.

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## DECLARATION OF INTERESTS

The author declares no conflicting interests related to the paper.

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