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A SIMPLIFIED METHOD FOR DETERMINATION OF OXYGEN AFFINITY OF HEMOPROTEINS-ESTIMATION OF THERMODYNAMIC CONSTANTS

Oxygen affinity of respiratory proteins is characterized by two parameters: p_{50} and "n". They are derived from so called oxygen dissociation curves determined usually by the spectrophotometric method of Asakura et al. This paper presents a considerably simplified modification of this procedure. The use of a pipette with mercury for measurement of the introduced air is eliminate and the equation for calculation of partial oxygen pressure in the tonometer is simplified. This convenient procedure cannot be applied, however, to proteins of low oxygen affinity (e.g. hemocyanin). From the oxygen dissociation curves the oxygenation equilibrium constant K can be also derived which enables calculation of thermodynamic constants of the reaction.

Introduction

From the biological standpoint the primary feature of respiratory proteins is their capacity for reversible oxygen binding. This reaction does not involve metal oxidation but is accompanied by changes of molecular shape ("pulsating molecules") and alterations of spectral and magnetochemical properties of respiratory proteins. Oxygen affinity of these proteins is characterized by two parameters: p_{50} ($p_{1/2}$, $\log p_{50}$, $\lg p_{1/2}$) i.e. partial oxygen pressure at which the protein is half - saturated with oxygen, and n - exponent of the Hill equation.

The relation between hemoglobin oxygenation and partial oxygen pressure (pO_2) was given by Hill in the form of the equation:

$$\frac{y}{1-y} = K \cdot p^n$$

Logarithming of this equation yields:

$$\lg \frac{y}{1-y} = n \lg p + \lg K$$

where: y - % protein saturated with oxygen,
K - equilibrium constant.

A plot of the latter equation gives a straight line (y = ax + b) enabling an easy estimation of the interaction coefficient n (regression coefficient a = n); lgK(b) and p_{50} (lg 50/50). At half - saturation of the protein with the ligand: $-\lg K = n \lg p_{50}$.

Therefore, estimation of the relationship between partial oxygen pressure (pO_2) and per cent oxygenation, e.g. HbO_2, permits calculation of the three parameters of oxygen affinity of a protein: K, p_{50}, and n (Fig. 1).

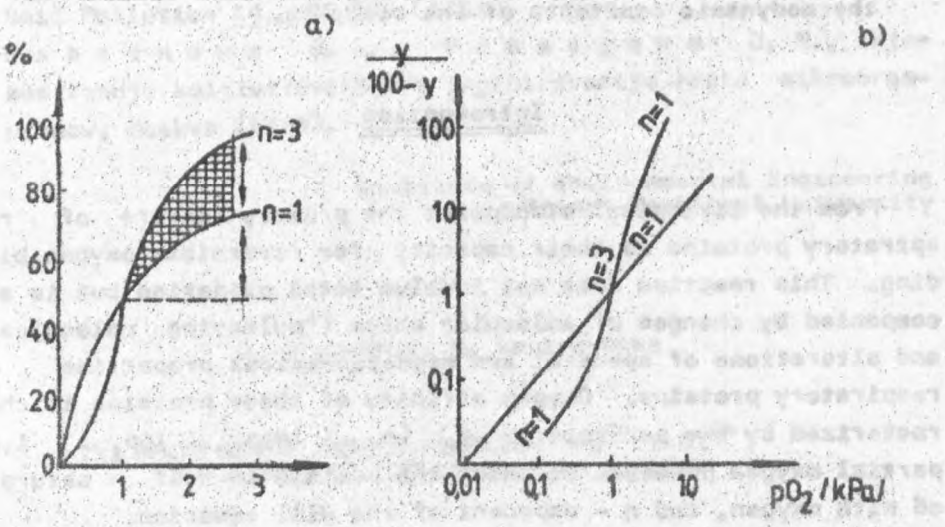

Fig. 1. Hypothetical oxygen dissociation curves and their logarithmic transformate. The curves were calculated for the same p_{50} and various n values

Hipotetyczne krzywe dysocjacji tlenowej i ich transformacje logarytmiczna. Krzywe wykreślono dla białek o tej samej wartości p_{50} i różnym n

The capacity of respiratory proteins for oxygen binding is conditioned by a lot of physico-chemical factors, including pH, CO_2 (the Bohr and Root effects), temperature, concentration of some salts (DPG, ATP, Cl^-, Mg^{2+}, Ca^{2+} and PO_4^{2-})[1, 3]. In most vertebrates, especially mammals, a strict dependence is observed between the amount of liberated protons and the O_2 binding: 0.7 mole of H^+ was found to be released per each mol of oxygen bound. The protons released within tenth and hundredth fractions of a second are called Bohr protons. It is known that the reaction of hemoglobin with oxygen depends on the pH of the medium and on the kind of animal. For example, oxygen affinity curves of fish hemoglobin differ significantly from those of other animals (the Root effect).

One way of determination of the Bohr effect is a determination of oxygen dissociation at various pH, and making use of the relation: $\phi = \Delta \lg p_m / \Delta pH$.

In the above equation $\phi = \Delta H^+$ denotes the difference in the number of protons between the oxy and the deoxy form of a protein. When the oxygen dissociation curve is symmetrical, $p_m = p_{50}$, and therefore $\phi = \Delta \lg p_{50} / \Delta pH$. The ϕ value denotes the amount of H^+ released by mole of a protein upon binding of a mole of O_2. In an equivalent form, $\phi = x / \Delta pH$, where $x = \Delta n\, O_2 / \Delta pO_2$. When $\phi = 0$, the oxygen dissociation curve is independent of pH in the physiological range. Many vertebrate hemoglobins and chlorocruorins and cephalopod hemocyanins have normal or negative Bohr effect, indicating a decrease in the oxygen affinity with decreasing pH. A value of $\phi = -1$ was found for numerous respiratory pigments studied. On the contrary, reverse or positive Bohr effect was revealed in gastropod hemocyanins [4].

Determination of thermodynamic parameters (ΔG, ΔS and ΔH) is a fundamental method of estimation of effects of physico-chemical factors on the special structure of macromolecules. In order to determine the free energy, enthalpy (ΔH) and entropy (ΔS), the value of equilibrium constant K must be known. It can be found from oxygen dissociation curves obtained at different temperatures.

The heat of reaction of oxygen binding ($-\Delta H$) can be determined from the relationship:

$$\Delta H = \Delta RT\, \Delta \lg K$$

where: R - gas constant;
T - absolute temperature;
K - equilibrium constant.

In the above equation, K may be substituted for p_{50} leading to:

$$\Delta H = -\frac{\Delta RT}{\Delta \lg p_{50}} = \frac{\Delta \lg p_{50}}{2{,}3\Delta RT}$$

or $\lg p_{50} = \Delta H\ 2{,}3\ RT$.

The last equation can be presented graphically as a plot of $\lg p_{50} = f(1/T)$ enabling calculation of H from the relation: $\Delta H = \operatorname{tg} \alpha \cdot 2{,}3\ R$ (Fig. 2).

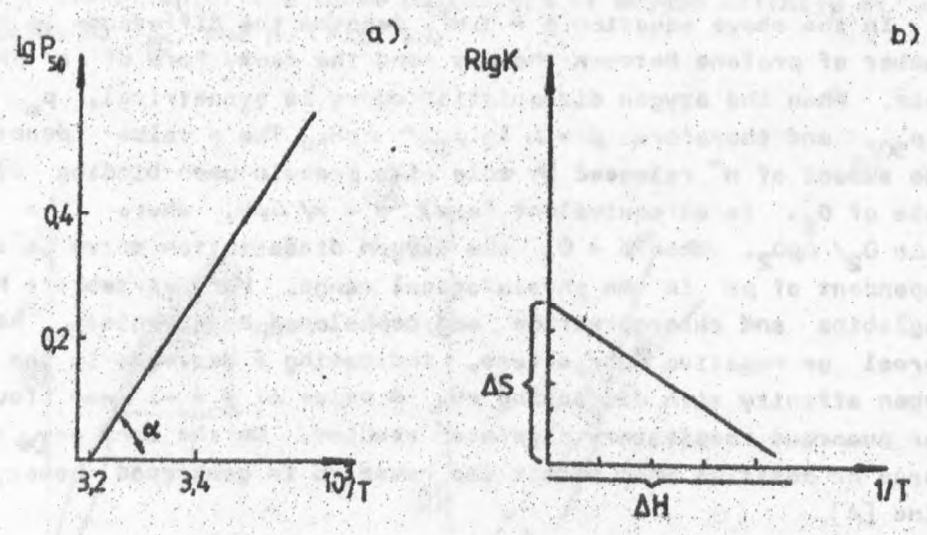

Fig. 2. Temperature dependence of oxygenation and equilibrium constant

Zależność utlenowenie oraz stałej równowagi K od temperatury

The temperature dependence of interaction of respiratory proteins with oxygen is characterized by so called temperature coefficient

$$K_T = \Delta \lg p_{50} / \Delta T$$

It is known that the oxygen affinity decreases with increasing temperature, according to the to the equation:

$$\Delta H = 0{,}0192 \left(\frac{T^1 T^2}{T^2 - T^1} \right) \lg \left(p^1_{50} / p^2_{50} \right) \text{kJ mol}^{-1}$$

with: ΔH = heat of oxygenation:

T^1, T^2 - lower and higher temperatures, respectively (in K);

p^1_{50}, p^2_{50} referring to respective temperatures (T^1 and T^2).

For majority of respiratory proteins, ΔH ranges from -40 to -65 kJ mol^{-1}. Lower values of ΔH indicate a more stable molecular configuration upon transition from the deoxy to the oxy form. The oxygen dissociation curves obtained at different temperatures have the same shape indicating that the heat of oxygenation is the same for all hemes in the molecule. Moreover, heme groups of different hemoglobines have practically, the same values of the heat of oxygenation. Those data are important for understanding of the phenomenon of cooperativity. Proteins which do not exhibit the interaction phenomenon have a value of $n = 1$ and a hyperbolic oxygen dissociation curve. Sigmoidal curves are characteristic for $n > 1$, up to a maximal value of 6.

The phenomenon of cooperativity is constant under physiological conditions and is typical only for those proteins which are aggregates of at least 4 subunits. It is noteworthy that interaction not necessarily occure in huge protein aggregates (hemocyanine).

The value of entropy gives the due for explanation of these diverse behaviors of respiratory proteins. At constant temperature,

$$\Delta S = \frac{\Delta H - \Delta G}{T}$$

Introduction of K into this equation yields:

$$\Delta S = \frac{\Delta H}{T} + R \ln K$$

This formula permits plotting a linear relationship:

$$R \lg K = f\left(\frac{1}{T}\right)$$

from which ΔS and ΔH can be calculated, since the slope of the line gives ΔH, and y - intercept of the line is equal to ΔS (Fig. 1b).

It was found that oxygenation is accompanied by a considerable decrease in entropy, indicating that oxygen binding leads to a considerable decrease in the degree of freedom of protein molecules.

It results from the presented data that determination of oxygen dissociation curves is indispensable for physico-chemical characterization of respiratory proteins.

Oxygen dissociation curves are usually derived by the spectrophotometric method. Recently, volumetric or polarographic methods are also employed [1].

A convenient spectrophotometric method of determination of oxygen dissociation curves was proposed by A s a k u r a et al. [2]. Introduction of slight modifications of this procedure, especially avoiding the pipette with mercury for measurement of the air added, as well as neglecting one term of the equation led renders this method more simple and efficient.

Material and methods

Principle

Spectrum of hemoglobin devoid of oxygen (deoxyhemoglobin, Hb^{2+}) with one absorption peak at 560 nm differs from that of oxyhemoglobin (HbO_2), with two absorption maxima at 540 and 578 nm, and a minimum at 560 nm.

In a tight vessel (modified Thunberg tube) in the presence of a reducing agent ($NaBH_4$, or $Na_2S_2O_3$) the solution of HbO_2 is devoid of oxygen and converted into Hb with accompanying characteristic change in spectrum. The decrease of the absorption maxima at 540 nm and 578 nm, and appearance of the maximum at 560 nm indicates a complete conversion. Readdition of air

leads to a gradual oxygenation of Hb to HbO_2 accompanied by reappearance of the maxima at 540 and 578 nm, and disappearance of the peak at 560 nm. The amount of oxyhemoglobin formed (% HbO_2) is calculated on the basis of increase in absorbance at 578 nm, assuming the absorbance corresponding to complete Hb oxygenation (open tonometer) as 100%.

Procedure

Hb solution of concentration of about 4×10^{-5} M (A = 0.5-0.7) in appropriate buffer (e.g. 0.2 M phosphate, pH 7.0) is placed in a tonometer with spectrophotometric cuvette. A pinch (1-2 mg) of $NaBH_4$ is added and the solution is deaerated under a vacuum aspirator until bubbling disappears (3-5 min). During deaeration the tonometer is well shaken. Then the tonometer is closed by turning the side arm and absorbance of the solution is measured at 540, 560 and 578 nm (absorption maxima) and at 565 and 585 nm (isobestic point). Appropiate values of absorbance evidence a complete conversion of HbO_2 into Hb. Changes in absorbance at isobestic point indicate protein denaturation.

Air (oxygen) present in the side arm of the tonometer is introduced into the tonometer. This is accomplished by a tight closing of the inlet with a finger covered with a rubber thimble and a turn of the side arm. The hemoglobin solution is gently but thoroughly shaken and its absorbance is measured at the abovementioned wavelengths. This manipulation is repeated 5-8 times. The amounts of air introduced are dependant on the volume of the inlet tube and Hb concentration. The following Tab. 1 is useful for calculation and plotting of the dissociation curve according to the method of least squares.

Before the measurament, the volume of the inlet tube must be determined as it corresponds to the volume of air added at each step ($V_0 = V_1 = V_2$). This volume is determined by weighing the tonometer with the inlet tube filled with water and with empty inlet tube. The weight of water divided by its density (ρ) at the temperature of measurement yields the volume of air introduced (V_0).

Table 1

Table of measurements

No	ml of air added V_o	A_{578}	ΔA_s	% HbO_2 (y)	pO_2 kPa (x)	lg pO_2 (x′)	$\frac{y}{1-y}$	$\lg \frac{y}{1-y}$ (y′)	$(x')^2$	$(y')^2$	$(x'\ y')$
1	0	A_o	0	0%							
2	V_1	A_1	$A_1 = (A_1 - A_o)$								
3	V_2	A_2	$A_2 = (A_2 - A_o)$								
4	V	A	$A = (A - A_o)$	100%							
						$\Sigma x'$		$\Sigma y'$	$\Sigma(x')^2$	$\Sigma(y')^2$	$\Sigma x'\ y'$

After the measurement the tonometer with the HbO_2 solution is weighed (m_1). The solution is poured off and the empty tonometer is weighed (m_2). Then the tonometer is filled with water and weighed again (m_3). Total volume of the tonometer is $V = (m_3 - m_2)\rho$. The difference $(m_3 - m_1)\rho$ corresponds to V_c (volume of the tonometer above the Hb solution). Volume of the Hb solution equals to $V - V_c$. Concentration of Hb in the solution is estimated according to one of the known methods [1]. The value of y is calculated from the relation

$$y = \frac{\Delta A_m}{\Delta A}$$

where: A_m - increase in absorbance of the solution at 578 nm after addition of a portion of air, referred always to A_o i.e. $(A_m - A_o)$;

ΔA - increase in absorbance of the solution at 578 nm after complete oxygenation $(A = A - A_o)$;

A_o - absorbance at 578 nm after complete deoxygenation.

All the above steps are necessary to calculate partial oxygen pressure according to the formula:

$$pO_2 = \frac{\frac{1}{V_c}}{p_a V_o} - 760\,\frac{1.36\ xy}{100}$$

with: pO_2 - partial oxygen pressure in the tonometer;

p_a - oxygen pressure in the air (kPa at 20°C);

V_o - volume of air introduced (sum);

V_c - volume of the gas phase above the Hb solution (in ml);

y - % HbO_2;

x - amount of Hb (in g).

In this equation the factor $760 \cdot 1.36\ xy/100 = 1.003\ xy$ is negligible with respect to other factors and may be neglected in the calculation which makes the determination of the oxygen dissociation curve much simpler.

From the obtained data (Tab. 1) the plot of $\lg\ y/1-y = f\ \lg\ pO_2$ is constructed, and the line is positioned by the method of least squares. The slope of the line ($tg\ \alpha$) corresponds to n. The value of n may be also calculated from the relation

$$a = \frac{\bar{y}' - b}{\bar{x}'}$$

The value lg K is found as the y - intercept of the line, or calculated as the value of b of the line. The ratio y/1-y is equal [1] at 50% oxygen saturation, i.e. its logarithm equals to 0. The value of lg p corresponding to this point represents lg p_{50} (x - intercept of the line).

Therefore all values describing oxygen affinity of a protein may be read from the plot.

The above procedure can be used without reservations for determination of oxygen affinities of almost all hemoproteins. In some cases the appropriate values of characteristic absorption maxima of oxy- and deoxy- forms must be taken into account [4]. The applicability of this procedure is limited in cases of proteins of low oxygen affinity. This concerns, among others, hemocyanins for which oxygen affinity is determined in large tonometers (about 300 ml) containing more solution 10-20 ml portions of air are added. In this case, changes in absorbance are monitored at 340 nm (absorption maximum for HcO_2). Moreover, the buffer in which copper proteins are dissolved must contain Ca^{2+}, as its absence results in dissociation of the large aggregates into much smaller components (5 S).

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UPROSZCZONY SPOSÓB WYZNACZANIA POWINOWACTWA TLENOWEGO HEMOPROTEIN. OKREŚLENIE STAŁYCH TERMODYNAMICZNYCH

W poszechnie stosowanej metodzie wyznaczania krzywych powinowactwa tlenowego hemoprotein metodą spektrofotometryczną wg A s a k u r y i wsp. wprowadzono dwie zmiany. Zaniechano uciążliwego odmierzania powietrza przy użyciu pipety z rtęcią. Wprowadzono natomiast dodawanie stałej ilości powietrza równej objętości rurki wlotowej zmodyfikowanego komercyjnego tonometru Thunberga.

Ponadto pominięto część końcową równania na obliczanie w układzie wartości ciśnienia parcjalnego tlenu. Wprowadzone dwie niewielkie zmiany upraszczają niesłychanie wykonanie krzywych dysocjacji, a tym samym wyznaczenie z nich parametrów powinowactwa tlenowego, tj. p_{50} n i K. Obliczenie wartości K pozwala ponadto określić stałe termodynamiczne danej reakcji (ΔH, ΔG, ΔS).