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Effect of temperature on oxygen affinity and anion binding of bovine hemoglobin

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Measurements of oxygen binding to bovine hemoglobin have been carried out over the temperature range 15–37°C at pH 7.33. The standard enthalpy of oxygenation after correction for the heat of oxygen solution and of the Bohr protons is found to be −7.1 or −7.2 kcal/mol in the presence of 0.1 M chloride or bromide, respectively. This value is well below the −14.4 kcal/mol determined for human hemolglobin under identical experimental conditions. As reported by Fronticelli et al. (C. Fronticelli, E. Bucci and A. Razynska, J. Mol. Biol. 202 (1988) 343), the preferential binding of anions by bovine hemoglobin recognizes the various halides. Measurements at various temperatures reveal that this is true only above 25°C. The halide recognition and the less exothermic enthalpy of oxygenation of bovine hemoglobin are probable due to oxygen-linked hydrophobic effects that are larger in bovine than in human hemoglobin.

1. Introduction

Heterotropic modulation of oxygen affinity in hemoglobin involves, besides the classic Bohr effect, the linkage between oxygen and anion binding which is accomplished in vivo in a variety of ways. In human hemoglobin in vivo anion regulation is produced by 2,3-DPG, in bovine hemoglobins anion modulation in vivo is achieved by chloride anions, as the red blood cells lack 2,3-DPG.

Heterotropic modulation conforms to a common mechanism, i.e., a preferential binding of the effector molecule to the deoxy form of hemoglobin whereby the oxygen affinity is lowered as a consequence of basic linkage principles [2]. Bovine hemoglobin appears to have developed a finer tuning of oxygen affinity through additional binding sites for monovalent anions that are not available to organic phosphates [1]. Its higher sensitivity to anions provides a rationale for the modulation of oxygen affinity by chloride in the physiological concentration range near 0.1 M. Bovine hemoglobin can also discriminate among halides on the basis of their charge density, unlike the case of human hemoglobin [1]. These peculiar features motivated the further investigation of the system in terms of heat effects. The results show

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Abbreviations: Hb, hemoglobin; 2,3-DPG, 2,3-diphosphoglyceric acid.

that the discrimination among halides is temperature dependent and disappears at temperatures below 25 °C. They also show that the enthalpy of oxygenation of bovine hemoglobin is distinctly less exothermic than that of human hemoglobin.

2. Materials and methods

Purified bovine Hb was purchased from Biopure (Boston, MA) and extensively dialyzed against 0.1 M NaCl and water. The sample was then recycled through a mixed-bed resin to remove organic and inorganic anions and cations. The sample concentration was measured spectrophotometrically using an extinction coefficient of $E = 0.868$ cm^2/mg for the CO derivative at 540 nm.

Chromatographically pure human hemoglobin was obtained following a previously described procedure [3].

Oxygen equilibria were measured using the thin-layer optical technique developed by Dolman and Gill [4]. The protein concentration was kept between 3 and 5 mM heme to avoid the effect of dimer formation. Changes in optical absorbance upon stepwise changes in the oxygen partial pressure were followed at 442 nm using a Cary 14 spectrophotometer. The underlying relation is

$$\Delta A_i = \Delta A_T \Delta \Theta_i \qquad (1)$$

where ΔA_i denotes the absorbance change at each dilution step, $\Delta \Theta_i$ the corresponding change in fractional saturation of hemoglobin with oxygen and ΔA_T the total absorbance change obtained in going from deoxy- to oxyhemoglobin [5]. Analysis of the isotherms gave the median ligand activity P_m [5]. The absorption spectra of the samples were practically identical at the beginning and end of the experiments, otherwise the measurements were discarded.

The effect of chloride and bromide on the oxygen binding of bovine Hb was studied by measuring the oxygen binding curve over a wide range of chloride and bromide concentrations (from 0.013 to 1.5 M). The experiments were performed in 0.013 M Tris buffer at pH 7.33 at 15, 25 and 37 °C. At each temperature the pH was precisely adjusted by titration of the buffer with either HCl or HBr using a Radiometer M26 pH meter. The linkage between oxygen and chloride or bromide binding was analyzed in terms of the change in oxygen affinity with anion concentration. Assuming one anion-binding site per heme, the affinity constants for deoxy-(L_d) and oxy-(L_o) hemoglobin were estimated using the linkage equation

$$\ln P_m = \ln P_0 + \ln\left[(1 + L_d y)/(1 + L_o y)\right] \qquad (2)$$

where P_0 represents the value of P_m in the absence of anions, whose activity is y. The free energy of heterotropic linkage per mol oxygen bound was calculated from

$$\Delta G_L = RT \ln(L_d/L_0). \qquad (3)$$

The number of anions released upon oxygen binding was estimated from

$$\Delta A = \mathrm{d} \ln P_m / \mathrm{d} \ln a \qquad (4)$$

where ΔA denotes the difference in the number of anions bound per heme between deoxy- and oxyhemoglobin, and a the concentration of free anions.

The enthalpy change associated with the oxygen-binding reaction to bovine Hb was determined by measurements made over the same temperature range, in 0.01 M phosphate buffer (pH 7.33) and in the presence of 0.1 M chloride or bromide. The standard enthalpy change, $\Delta H°$, observed per mol of oxygen bound was derived from the Gibbs-Helmholtz equation

$$\mathrm{d} \ln P_m / \mathrm{d}(1/T) = \Delta H°/R \qquad (5)$$

where R is the gas constant and T the absolute temperature.

3. Results

3.1. Effect of chloride and bromide

Fig. 1 shows the effect of bromide and chloride on the oxygen affinity of bovine hemoglobin at different temperatures. The interpolated lines were

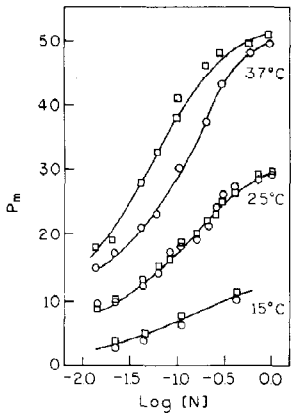

Fig. 1. Effect of chloride (□) and bromide (○) on the oxygen affinity of bovine hemoglobin at 15, 25 and 37 °C. The continuous lines were drawn using eq. 2 and the parameters listed in table 1.

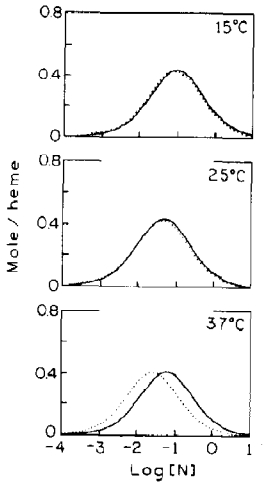

Fig. 2. Number of chloride (———) and bromide (·····) ions released per mol oxygen bound by bovine hemoglobin as function of anion concentration at 15, 25 and 37 °C, respectively. The lines were drawn according to eq. 4 with the parameters listed in table 1.

drawn according to eq. 2, which assumes one anion-binding site per heme. At 25 and 15 °C, the simulations for Cl^- and Br^- were so similar that only the simulation data for Cl^- are shown as continuous lines. Eq. 2 was the simplest model which gave an adequate simulation of the data. The values of the affinity constants L are listed in table 1 for chloride and bromide at three different temperatures.

At 37 °C bromide has a higher affinity than chloride for both the deoxy and oxy forms of bovine Hb. The difference vanishes at and below 25 °C. The free energy of linkage is positive for both chloride and bromide, because anions bind preferentially to deoxyhemoglobin. This free energy does not depend on temperature, which is consistent with the observation that the same binding sites are available to chloride and bromide [1]. It also indicates that the enthalpy change upon anion binding is similar in both the oxy and deoxy forms of hemoglobin.

From eq. 4, one can derive the number of anions released per mol oxygen bound as a function of anion concentration. The relative linkage graphs are shown in fig. 2. The maximum number of anion molecules linked to oxygenation of bovine Hb is similar for both anions at all temperatures. At 37 °C, the maximum value for bromide is shifted to the left as expected from affinity constants reported in table 1.

Table 1

Affinity constants (L) and free energy of etherotrophic linkage (ΔG_L) of chloride and bromide for bovine hemoglobin at various temperatures in 0.01 M phosphate buffer at pH 7.33

Values were obtained by nonlinear least-squares analyses of the data presented in fig. 1, using eqs 2 and 3, respectively.

Anion	T (C°)	L_d (M^{-1})	L_0 (M^{-1})	ΔG_L (kcal/mol)
Cl^-	15	25±6	5±1	1.05±0.12
	25	53±3	8±1	1.07±0.12
	37	40±2	7±1	1.05±0.11
Br^-	15	28±4	7±1	1.15±0.09
	25	56±2	9±1	1.08±0.08
	37	96±9	17±2	1.03±0.25

3.2. *Effect of temperature*

The effect of temperature on the oxygen affinity of bovine 3 at constant chloride or bromide concentrations is shown in fig. 3 as Van't Hoff plots, and the related enthalpy values are listed in table 2. For comparison fig. 3 and table 2 include data obtained from human hemoglobin under

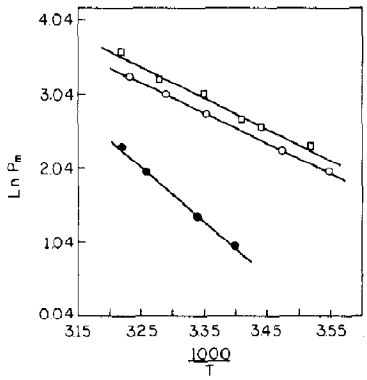

Fig. 3. Van't Hoff plot for the temperature dependence of the oxygen affinity of bovine (open symbols) and human hemoglobin (closed symbols) in the presence of 0.1 M chlorides (□) and bromides (○). The lines were drawn according to eq. 5 using the parameters listed in table 2.

identical solution conditions. The enthalpy of oxygenation obtained in the presence of 0.1 M chloride or bromide is significantly different from that observed from human hemoglobin. For bovine hemoglobin, correction for the heat of solution of oxygen (−3 kcal/mol) yields an enthalpy value of -4.9 ± 0.1 kcal/heme in the presence of chloride and -5.2 ± 0.4 kcal/heme in the presence of bromide. For human hemoglobin the enthalpy value is -11.2 ± 0.3 kcal/heme. In order to obtain an estimate of the intrinsic heat of oxygenation, further corrections are necessary for taking into account the contribution of Bohr protons. Anion binding per se does not yield heat effects other than those due to Bohr protons [6]. The number of protons linked to oxygenation at 25°C and pH 7.33 in human hemoglobin is 0.55/heme [7]. The number of Bohr protons for bovine hemoglobin at 25°C at pH 7.33, under the same solution conditions used for measuring the enthalpy change was computed in this laboratory from measurements of oxygen affinity. It was found to be 0.4 protons/heme. The heat of proton release in the pH region of 7.0–8.5 is due to histidyl residues with an enthalpy of 5.8 kcal/mol [7]. Therefore, the estimated value of the intrinsic enthalpy of oxygenation of bovine hemoglobin is -7.2 ± 0.1 kcal/heme from the chloride studies and -7.5 ± 0.3 kcal/mol from the bromide studies. For human hemoglobin the corrected value is -14.4 ± 0.3 kcal/heme, in very good agreement with data reported by others [7,8]. There is a difference of about 7.0 kcal/heme in the enthalpies of human and bovine hemoglobin. This difference is too large to be attributable to errors in the corrections for the heat of the Bohr protons.

Table 2

Van't Hoff heat of oxygenation of human and bovine hemoglobins in 0.01 M phosphate buffer at pH 7.33, in the presence of Cl^- and Br^-

The data of ΔH_{obs} were corrected for the heat of oxygen solution. The value of $\Delta H°$ was obtained after correction for the heat of the Bohr protons as discussed in the text.

Protein	Anion (0.1 M)	Bohr protons (equiv./heme)	ΔH_{obs} (kcal/mol)	$\Delta H°$ (kcal/mol)
Hb A	Cl^-	0.55	-11.2 ± 0.3	-14.4 ± 0.3
Hb bovine	Cl^-	0.40	-4.9 ± 0.1	-7.2 ± 0.1
Hb bovine	Br^-	0.40	-5.2 ± 0.4	-7.5 ± 0.4

4. Discussion

The effect of chloride and bromide on the oxygen affinity of bovine hemoglobin has already been addressed in a previous study [1], but the analysis presented here allows for more quantitative conclusions. Both anions lower the oxygen affinity by binding preferentially to the deoxy form of bovine hemoglobin. At 37°C both the deoxy and oxy forms of bovine hemoglobin have higher affinity for bromides than for chlorides. It has been proposed that these differences result from a hydrophobic modulation of the interaction of bovine hemoglobin with halides [1]. It is known that hydrophobic interactions tend to vanish at low temperature. This may explain why the different affinities for halides disappear below 25°C.

The effect of temperature on the oxygen affinity of bovine hemoglobin deserves particular attention. The intrinsic heat of oxygenation per heme is less exothermic than that found for human hemoglobin with a difference of about 7.0 kcal/mol. Besides the heat of oxygen binding to the heme, this intrinsic heat still includes the heat of salt-bridge formation or breakage, and the heat of R-to-T conformational transition. Any of these

events may be responsible for all or part of the difference observed with respect to human hemoglobin. The similarity between the heme pockets of bovine and human hemoglobin suggests that only a small, if any, difference should arise from binding to the heme site. Formation of extra oxygen-linked salt-bridges, or else a salt-bridge more buried in bovine than human hemoglobin could be responsible for a less exothermic heat of oxygenation.

Finally, it should be stressed that the different enthalpy of oxygenation of human and bovine hemoglobins does not match the difference of their free energy of oxygen binding which is below 1 kcal/heme [1,9]. This implies a compensation produced by a larger oxygen linked entropy change in bovine hemoglobin, which again is consistent with increased hydrophobic interactions.

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