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Thermodynamics of oxygen binding to arctic hemoglobins

The case of reindeer

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The most surprising characteristic of reindeer hemoglobin (Hb) concerns its response to changes in temperature. Thus, the shape of the oxygen-binding curve is strongly temperature dependent due to the difference in the enthalpy of oxygenation between the T and R state of the molecule. In fact, ΔH of oxygen binding to the T state is strongly exothermic whereas that of the R state is very close to zero or possibly positive after correction for the heat of oxygen solubilization. Moreover, the allosteric transition $T_o \leftrightarrow R_o$ has been found to display a negative ΔH and a contemporaneous decrease in entropy, a behavior which is precisely the opposite of what has been reported for other hemoglobins. As a whole, reindeer Hb represents a beautiful example of the significance that comparative studies may have in assessing the general validity of the main properties of the hemoglobin molecule.

1. Introduction

The basic function of the hemoglobins is to ensure an adequate supply of oxygen to all parts of the organisms in which they occur. In order to accomplish this task they have developed, in the course of evolution, a common molecular mechanism based on the principle of ligand-linked conformational change in a multisubunit structure [1–3]. Within the framework of this common mechanism, however, different hemoglobins have acquired special features to meet particular needs [4,5]. Very recently, this has also been illustrated by hemoglobin from arctic mammals [6–10] such as reindeer (*Rangifer tarandus tarandus*) and musk ox (*Ovibos muschatos*). In particular, the functional characterization of the hemoglobin from reindeer has provided new elements which appear to be of great significance for the understanding of the mechanisms which are at the basis of molecular adaptation to extreme environmental conditions. Thus, hemoglobin from reindeer is characterized by the fact that the effect of temperature is mainly on the shape of the binding curve, the overall oxygen affinity being very little affected by changes of this parameter. It has been suggested that this functional characteristic could be of great importance in ensuring an adequate oxygen supply at the level of those peripheral tissues (such as skin, legs etc.) which, under some external conditions, may well be at a temperature significantly lower than that of the lungs.

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From a thermodynamic point of view, the temperature dependence of the shape of the oxygen-binding curve is due to the fact that, as the reaction proceeds, the overall heat of oxygenation, ΔH, decreases. It should be recalled that in human Hb there is no such change, the shape of the binding curve being temperature invariant over most of its range [3,11].

The objective of this paper concerns the presentation of the oxygen equilibria of isolated reindeer Hb obtained at different temperatures from 10 to 30 °C. These equilibria are of interest not only because they illustrate the variations possible within the scope of an overall allosteric mechanism but also, even more, because this hemoglobin represents a typical case of the different strategies adopted during evolution to meet the physiological requirements of the particular species. On the whole the results of this investigation are such as to suggest its extension to other hemoglobins and also to the effects of a second ligand.

2. Materials and methods

The hemoglobin of reindeer (*R. tarandus tarandus*), collected as described previously [6], was stripped by passing the hemoglobin solution first through a Sephadex G-25 column, equilibrated with 0.01 M Tris buffer (pH 8.0), containing 0.1 M NaCl, and afterwards through a column of mixed-bed ion-exchange resin (Bio-Rad AG 501X8).

The oxygen equilibria were measured via a diffusion chamber technique [12] in which the stepwise increases in oxygen tension were generated by cascaded gas mixing pumps (Wosthoff) while absorbance changes between zero and full saturation were monitored on recorders with high-sensitivity units (Eppendorf, Radiometer). The application of small amounts of hemoglobin solution (layer thickness about 10 μm) minimized equilibration time and methemoglobin formation, which was always less than 1%.

Alternatively, oxygen dissociation curves were obtained spectrophotometrically by the tonometric method [13] at a protein concentration of 3–5 mg/ml.

The oxygen-binding data were fitted on the basis of the equation given for a simple MWC model [14]:

$$Y = \frac{XK_R(1+K_RX)^{n-1} + L_0XK_T(1+K_TX)^{n-1}}{(1+K_RX)^n + L(1+K_TX)^n}$$

where X denotes the ligand concentration, K_T and K_R the intrinsic association constants for the low- (T) and high-affinity (R) forms, respectively, and $L_0 = T_0/R_0$ the allosteric constant for the interconversion of the two quaternary states in the unliganded form.

The values for L_0, K_R and K_T at the various temperatures have been obtained by means of a nonlinear least-squares fitting method based upon a modified Marquardt algorithm [15].

Concentrated stock solutions of 2,3-diphosphoglyceric acid (2,3-DPG) were prepared by dissolving the sodium salt of 2,3-DPG (Sigma) in water or in buffer. The N-terminal sequence has been determined adopting the procedure reported below. The β-chain was purified by high-performance liquid chromatography (HPLC); the hemoglobin was applied in several aliquots on a reverse-phase column (Aquapore RP 300, 7 × 250 mm, Brownlee Labs), which was developed in 15 min with a gradient of acetonitrile in 0.2% trifluoroacetic acid (TFA) generated in a Beckman model 340 instrument at a flow rate of 3.0 ml/min.

The absorbance of the effluent was monitored at both 220 and 280 nm using a Beckman model 165 wavelength detector.

The β-chain (2 mg) was pyridylethylated essentially as described by Friedman et al. [16] and 2 nmol were loaded onto the gas-phase sequencer (Applied Biosystems model 470 A) for direct collection of sequence data.

3. Results and discussion

The oxygen-binding properties of reindeer Hb were investigated within the pH range 5.6–8.6 in both the absence and presence of saturating concentration of 2,3-DPG. Fig. 1 shows the effect of pH on the oxygen affinity of reindeer Hb and, for the sake of comparison, for human HbA under

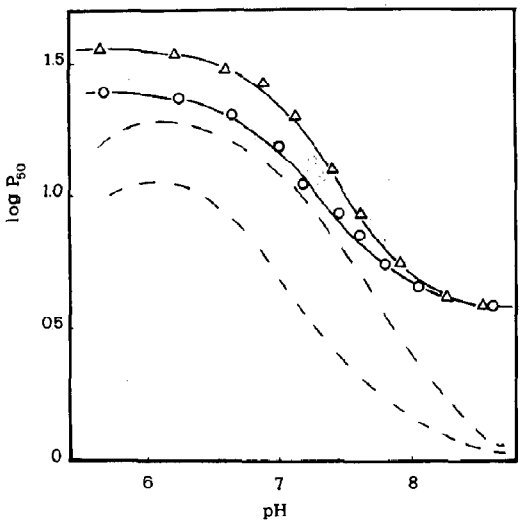

Fig. 1. Oxygen Bohr effect of reindeer Hb at 20°C in 0.1 M Bis-Tris buffer plus 0.1 M NaCl. (○) Stripped hemoglobins; (△) plus 3 mM 2,3-DPG. (— — —) Human hemoglobin A under the same experimental conditions.

both sets of conditions. As may be observed, addition of a saturating concentration of 2,3-DPG [8] results only in a slight decrease in oxygen affinity so that the extent of this effect appears to lack any physiological significance. The very minor effect of 2,3-DPG is very well correlated to the structural information available on the residues involved in the organic phosphates' interaction in human hemoglobin A [4,17,18]. This comparison is reported in table 1 where it may be seen that in β-chain of reindeer Hb the N-terminal residue Val (NA1) is lacking while His (NA2) is substituted by Met. These findings are in full agreement with what is known from other ruminant hemoglobins [4,5,17].

Table 1

Amino acid residues of β-chains responsible for the binding of 2,3-DPG in human HbA [11] and reindeer hemoglobin (this paper)

The helical designation refers to that used for HbA.

Residue	HbA	Reindeer Hb
NA1 (1)	Val	–
NA2 (2)	His	Met
EF6 (82)	Lys	Lys
H21 (143)	His	His

On the other hand, the oxygen affinity of reindeer Hb is very much affected by changes in chloride concentration, strongly indicating that, in this case, similarly to other related species [4,17], small anions may substitute for 2,3-DPG in modulating 'in vivo' the functional properties of hemoglobin.

As far as the Bohr effect is concerned, it should be outlined that its amplitude appears to be smaller than that of human hemoglobin in both the absence and presence of 2,3-DPG. Furthermore, the oxygen affinity tends towards a minimum value below pH 6.6 without showing any tendency to increase, thereby implying the absence of the so-called 'reverse Bohr effect' normally observed in hemoglobin from mammals.

On the whole, the most surprising characteristic of reindeer hemoglobin concerns its response to changes in temperature. In fact, the overall heat of oxygenation was found to be 2–3-times lower than that of human hemoglobin A under the same experimental conditions [6,8]. This finding could be of particular significance from an evolutionary point of view, since it should be considered in relation to the wide range of temperature changes, from −40 to 20°C, that reindeer encounter in the wild [19]. For this reason, a set of accurate oxygen-binding experiments have been carried out as a function of temperature within the range 10–30°C. The data, presented in the form of a Hill plot in fig. 2, extend over a saturation range broad enough to permit the evaluation of a number of thermodynamic parameters.

One feature, which shows up very clearly in fig. 2, is the strong temperature dependence of the shape of the binding curve. A very unusual characteristic is the temperature independence of the upper asymptote representative of the R state of the molecule; to our knowledge this is the very first time that the R state of a hemoglobin has been found to display such a small ΔH for ligand binding. In contrast, the lower asymptote, representative of the T state of the molecule, is very much affected by temperature, indicating a strong exothermic character of the oxygen binding.

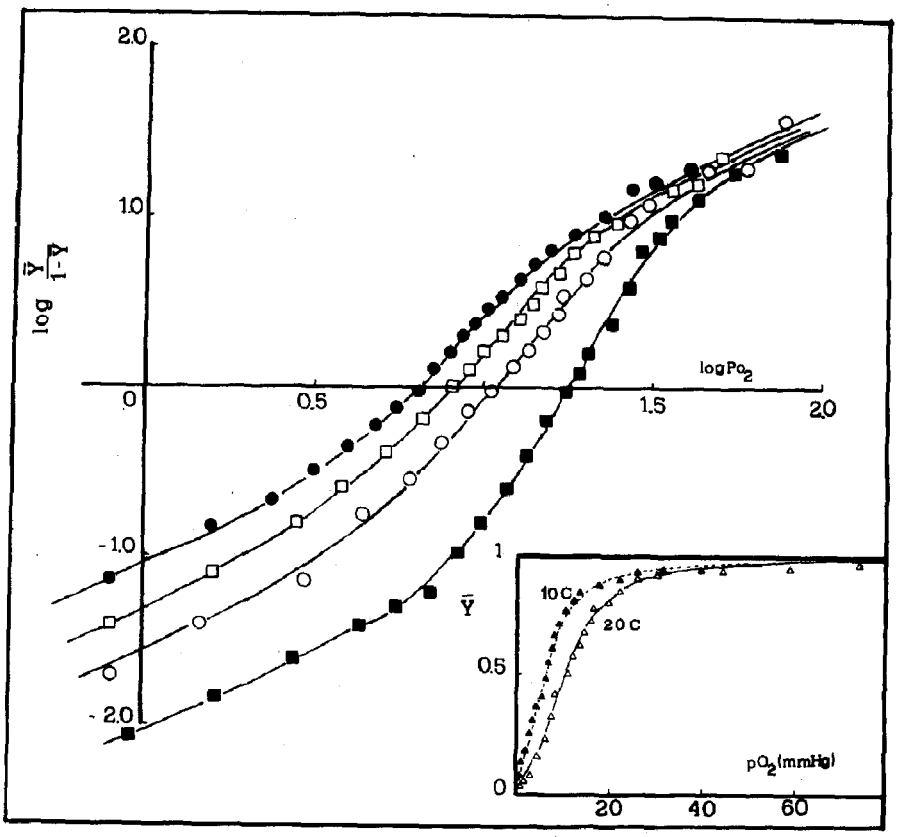

Fig. 2. Effect of temperature on oxygen equilibria of reindeer hemoglobin measured in 0.05 M Tris buffer plus 0.1 M NaCl at pH 7.4 in the presence of 4% carbon dioxide. (●) 10 °C; (□) 15 °C; (○) 20 °C; (■) 30 °C. (Inset) Comparison of the experimental points, obtained at 10 and 20 °C, with full curves calculated on the basis of the two states model with parameters given in table 2.

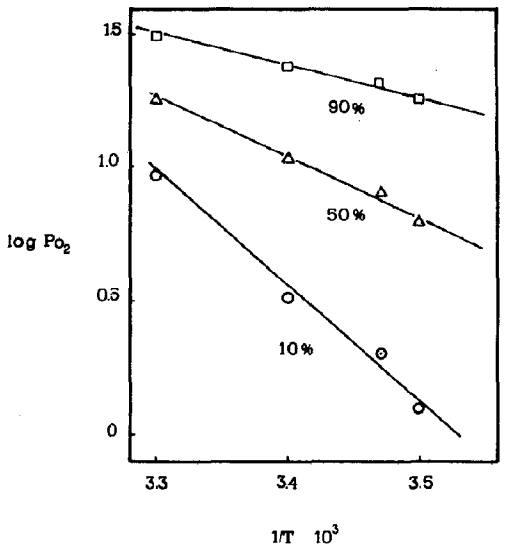

Fig. 3. Temperature dependence of the partial pressure of oxygen necessary to give 10, 50 and 90% oxygen fractional saturation. Corresponding overall ΔH values of oxygen binding: -17.0 ± 0.7, -9.0 ± 0.5 and -4.7 ± 0.5 kcal/mol of O_2.

concerning the $T_4 \leftrightarrow R_4$ allosteric transition were simply deduced according to the rule of microscopic reversibility.

Examination of the scheme shows that, in the absence of the ligand, the T_0 state is stabilized over R_0 (see also table 2) by a large entropic

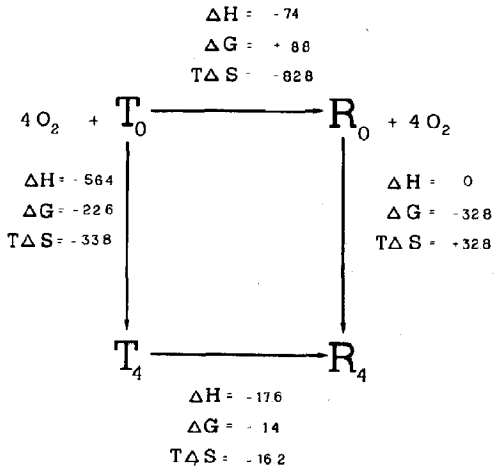

Fig. 4. Thermodynamic parameters (expressed in kcal/mol of tetramers) for oxygen binding to reindeer hemoglobin and for the associated allosteric transition at pH 7.4.

contribution which is not compensated by the negative enthalpic change related to the allosteric transition toward R_0. To the best of our knowledge this is the very first time among hemoglobins that the allosteric transition $T_0 \leftrightarrow R_0$ has been found to display negative values for ΔH and ΔS.

The same reasoning applies to the $T_4 \leftrightarrow R_4$ conformational transition which is characterized by a negative enthalpy change and by an almost equivalent negative entropic contribution; this results in a small value of the free energy change associated with the quaternary transition which suggests that the percentage of T state is not negligible even in the ligated species.

The large negative enthalpy change which characterizes the quaternary transition in the absence of the ligand ($T_0 \leftrightarrow R_0$) implies that the relative population of the conformational states is strongly temperature dependent, this being in line with the strong temperature dependence of the shape of the ligand-binding curve. In this respect, it should be pointed out as, differently from human hemoglobin A [3] and trout Hb [21,22], the exothermicity of the $T_0 \leftrightarrow R_0$ allosteric transition implies that, lowering the temperature, the deoxy-quaternary structure is progressively destabilized.

Moreover, for binding of oxygen to the T state, the negative free energy change turns out to be driven only by the negative value of the apparent

Table 2

Equilibrium constants for the reaction of reindeer hemoglobin with oxygen at various temperatures in 0.05 M Tris buffer plus 0.1 M NaCl at pH 7.4

The parameters refer to the intrinsic affinity constant for the T and R quaternary state. Standard deviations for both K_T and K_R are indicated. The equilibrium constant for the allosteric transition is also reported ($L_0 = R_0/T_0$). It should be emphasized that the fitting procedure has been performed by maintaining K_R at a fixed value. ΔG_i (RT ln K_T/K_R) represents the free energy of heme-heme interactions expressed in kcal/site.

T (°C)	K_R ($l\,mol^{-1}$)	K_T ($l\,mol^{-1}$)	L_0 (R_0/T_0)	ΔG_i (kcal/site)
10	$1.4\pm0.4\times10^6$	$4.0\pm0.1\times10^4$	2.2×10^{-5}	2.0
15	$1.4\pm0.4\times10^6$	$2.6\pm0.07\times10^4$	2.3×10^{-6}	2.3
20	$1.4\pm0.4\times10^6$	$1.7\pm0.05\times10^4$	2.4×10^{-7}	2.6
30	$1.4\pm0.4\times10^6$	$5.0\pm0.05\times10^3$	3.7×10^{-9}	3.4

enthalpy change, since the entropic contribution is greatly unfavorable. In contrast, binding of oxygen to the R state is peculiar insofar as the enthalpy change is either close to zero or even positive (once corrected for oxygen solubilization) and the reaction appears to be almost totally entropy driven. A structural interpretation of this behavior is still premature but it may be suggested that binding to the R state is likely to be associated with compensatory effects involving protein side chains in the proximity of the ligand-binding site.

In conclusion, the present results indicate a clear-cut flexibility of the hemoglobin molecule which seems to possess great potentialities of functional modulation. In this respect, reindeer Hb represents a beautiful example of the significance that comparative studies may have in assessing the main properties of the hemoglobin molecule, outlining the sophisticated mechanisms which may be at the basis of molecular adaptation to extreme environmental conditions.

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