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KINETIC AND THERMODYNAMIC PARAMETERS FOR OXYGEN BINDING TO THE ALLOSTERIC STATES OF *PANULIRUS INTERRUPTUS* HEMOCYANIN

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The temperature dependence of the oxygen binding equilibria and kinetics of *Panulirus interruptus* hemocyanin has been analyzed within the context of the two-state allosteric model. Oxygenation of the T-state is characterized by a more negative value of ΔH than that of the R-state; therefore, cooperative effects in oxygen binding to *P. interruptus* hemocyanin are thermodynamically governed by favorable entropy changes. The allosteric transition in the unliganded derivative shows an enthalpy-entropy compensation effect. The activation enthalpies for oxygenation and deoxygenation of the T-state are larger than those for the R-state, while the activation entropies are favorable for the T-state and unfavorable for the R-state. Thus, the activation free energies for oxygen binding to the T- and R-states are similar, while for the deoxygenation reaction $\Delta G^{\ddagger}$ is smaller for the T-state. The analysis reported confirms the applicability of the Monod-Wyman-Changeux two-state allosteric model to *P. interruptus* hemocyanin and yields a complete thermodynamic characterization of oxygen binding under both equilibrium and dynamic regimes.

1. Introduction

Hemoglobins and hemocyanins constitute two classes of O_2-transport proteins widely distributed in nature. In spite of their large chemical differences (e.g., hemoglobin has iron-porphyrin and hemocyanin copper), their functional properties display common general features. These aspects have been recently discussed [1].

A more complete comparison of the structural and functional properties of these two classes of respiratory proteins demands quantitative studies of the thermodynamics and kinetics of O_2 binding. However, in the case of hemocyanins this type of investigation is still very limited, and in fact partially contradictory. Two papers, one on *Helix pomatia* β-hemocyanin [2], and the other on *Levantina hierosolima* hemocyanin [3], have shown that the driving force for the quaternary structural change may be different for different proteins.

If knowledge on the equilibrium thermodynamics is limited, essentially no information is available for the activation parameters of the oxygen reaction with hemocyanins. Therefore, any attempt to compare quantitatively hemocyanins with hemoglobins, is hindered by lack of experimental information, especially on the kinetics.

Previous studies performed at pH 9.6, in the presence or absence of calcium, indicated [4] that *Panulirus interruptus* hemocyanin may exist either as a cooperative O_2-binding hexamer or as a monomer, which binds O_2 noncooperatively and may be taken to represent the low-affinity state of the protein.

In this paper we report a characterization of the equilibria and kinetics of O_2 binding to this hemocyanin, in the framework of a simple two-state model: within the limitations of this approach the

data show (i) that cooperativity is mainly, but not exclusively, entropy driven; (ii) that the activation enthalpy for the oxygen reactions are different for the two allosteric states; (iii) that a favorable entropy of activation is observed only for the deoxygenation of the liganded T-state. These results are briefly discussed with reference to other respiratory proteins.

2. Materials and methods

Hemocyanin was isolated as described by Kuiper et al. [5] and stored lyophilized.

Dissociated *P. interruptus* hemocyanin was separated into its components by column chromatography and monomeric fraction I was used throughout. The hemocyanin concentration, expressed taking 75 000 as the molecular weight of the oxygen-binding site, was determined from the absorbance at 280 nm ($E^{1\%}_{1\,cm} = 1.38$).

Ethanolamine buffer (0.1 M) at pH 9.6 was used throughout.

Oxygen-binding experiments were carried out as described by Rossi Fanelli and Antonini [6].

Stopped-flow measurements at different temperatures were carried out with a Gibson-Durrum apparatus, having a dead-time of approx. 4 ms. The oxygen dissociation time course was followed by mixing a solution of oxygenated hemocyanin with degassed buffer containing excess sodium dithionite [7]. The reaction was monitored at 400 and 570 nm, corresponding to absorption bands of oxygenated hemocyanin.

Temperature-jump relaxation measurements [8,9] were carried out with an instrument built by Messanlägen Gesellschaft, Göttingen. A temperature-jump cell with a 1 cm light path and a volume of 7.8 ml was used throughout; a temperature increase of 4.5°C was obtained by discharging the condenser at 30 kV. Changes in transmission were followed around 360 nm. The concentration of free oxygen-binding sites was calculated from the total protein concentration and the oxygen-binding isotherm. A protein solution equilibrated with air at the temperature of the experiment was properly diluted with deoxygenated buffer in order to cover a range of oxygen saturations. Oxygen saturations were determined directly in the T-jump cell by measuring the absorption spectra in a Cary-14 recording spectrophotometer.

2.1. Analysis of temperature dependencies

Enthalpy changes for O_2 binding to hexameric and monomeric *P. interruptus* hemocyanin were calculated from the temperature dependence of the O_2 affinities using a Van't Hoff plot on the assumption of linearity over the temperature range explored. The heat of oxygen solvation ($\Delta H_s = -3.0$ kcal mol^{-1}) was taken into account when necessary.

Oxygen affinities of the T- and R-states at different temperatures were obtained from the low and high asymptotes of a Hill plot [10].

Fitting of the oxygen-binding isotherms was obtained by computer analysis according to Colosimo et al. [15] taking the number of interacting sites as being equal to 6 at every temperature. Computations were carried out using a Hewlett Packard 9830 A minicomputer.

Activation energies were obtained from the Arrhenius equation:

$$\frac{d \ln k}{d1/T} = -\frac{E_a^{\ddagger}}{R} \tag{1}$$

Corresponding activation entropies were calculated according to:

$$k = \frac{\underline{k}RT}{N\hbar} e^{-\Delta G^{\ddagger}/RT} = \frac{\underline{k}RT}{N\hbar} e^{-\Delta H^{\ddagger}/RT} e^{\Delta S^{\ddagger}/R} \tag{2}$$

where: $\Delta H^{\ddagger} = E_a^{\ddagger} - RT$, $\underline{k}$ is taken to be unity [11] and $\hbar$ is Planck's constant.

2.2. Simulation procedure

Simulation of the O_2-dissociation kinetics was carried out on a desk-top computer model HP87 equipped with an HP 7040 digital plotter using the analytical solution for a chain of n monomolecular and irreversible processes provided by Bateman [12].

The optical contribution of each intermediate species to the overall signal was calculated assuming identical extinctions for the R- and T-states and taking into proper account the different levels of saturation.

Switching between the two quaternary states is defined by the so-called switch-over point, i.e., the number obtained from the allosteric parameters on the basis of the formula $i_s = -\log L/\log c$ [13] which represents the average number of occupied sites on the macromolecule when the transition occurs.

3. Results

3.1. Oxygen binding of monomeric and hexameric hemocyanin

At pH 9.6 *P. interruptus* hemocyanin is hexameric in the presence of 10 mM $CaCl_2$ and monomeric in its absence. Oxygen-binding data of hexameric hemocyanin at three temperatures are shown in fig. 1, together with the results for monomeric fraction I.

At every temperature the monomer binds oxygen noncooperatively, while the undissociated hemocyanin exhibits cooperative oxygen-binding behavior. Furthermore, the position of the oxygen-binding curve of the monomer is consistent, at each temperature, with the lower asymptote of the oxygen isotherm of the hexamer. Thus, the O_2-binding behavior of monomeric fraction I mimics very well the low O_2-affinity state of the hexameric protein, as previously proposed [4].

Increase in temperature is associated with a decrease in oxygen affinity and in the case of the hexamer also with a change in shape of the binding curve (with an increase in the free energy of interaction per site).

The equilibrium parameters of the hexamer and the monomer are summarized in table 1; the same table also reports the equilibrium constants for the two allosteric states (K_R and K_T) as obtained from the asymptotic values of the binding isotherms, as well as the corresponding enthalpies (ΔH_R and ΔH_T).

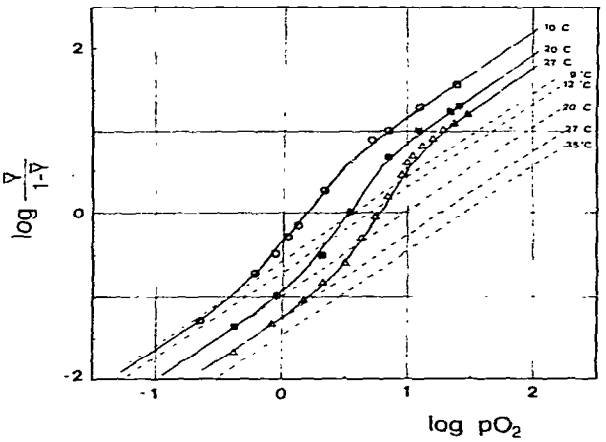

Fig. 1. Hill plots of oxygen equilibrium curves of the monomeric fraction I (dashed line) and hexameric (continuous line) *P. interruptus* hemocyanin at different temperatures. Conditions: 0.1 M ethanolamine (pH 9.6), ionic strength 0.1, in the absence or presence of 10 mM $CaCl_2$. $\bar{Y}$, fractional saturation with oxygen: p_{O_2}, oxygen pressure (mmHg). Continuous lines are computed isotherms for a two-state model with $r = 6$ [15]. Values of the characteristic parameters of the model are reported in table 1.

3.2. Temperature jump of monomer (fraction I)

At different initial temperatures and oxygen-saturation levels, temperature-jump experiments show one resolvable relaxation effect which corresponds to a decrease in the copper-oxygen absorption band, in agreement with the exothermic character of binding (see above). Relaxation spectra are always monophasic, indicating simple kinetics, and the dependence of the reciprocal relaxation time ($1/\tau$) on the concentration of free reactants (hemocyanin and oxygen) follows simple bimolecular behavior. The oxygen association and dissociation rate constants at different temperatures are given in table 2. It may be seen that the effect of temperature is much larger on the values of the O_2-dissociation rate constants.

3.3. Temperature jump of hexamer at high saturation levels

Temperature-jump experiments were carried out at $Y > 0.90$, where the hexamer is in the R-state, as inferred from the Hill plots presented in fig. 1. The relaxation spectra are homogeneous, indicating simple kinetics of O_2 binding to the R-state; the oxygen combination rate constants reported in table 2 indicate a very small temperature depen-

Table 1
Effect of temperature on the oxygen-binding properties of monomer and hexamer of *P. interruptus* hemocyanin

The data for the hexamers were fitted with a number of oxygen-binding sites, $r = 6$. Conditions: 0.1 M ethanolamine (pH 9.6), ionic strength 0.1 M in the absence or presence of 10 mM $CaCl_2$. The values of $p_{1/2}$ are given in mmHg to allow an easy comparison with data on other oxygen-binding proteins, which are usually expressed in partial pressure of oxygen. The corresponding binding constants may be calculated from the $p_{1/2}$ taking into account the solubility of oxygen at each temperature and knowing that at $T = 20°C$, $p_{O_2} = 1$ mmHg corresponds to a concentration of 1.7 μM and that $\Delta H_{sol} = -3$ kcal/mol. $\Delta F_i = [RT/\bar{Y}(1-\bar{Y})](1-1/n)$, see ref. 10.

Hexamer									Monomer			
T (°C)	$p_{1/2}$ (mmHg)	n	ΔF_i (cal mol^{-1})	L_0 ($= R_0/T_0$)	K_T (mmHg)	K_R (mmHg)	ΔH_T (kcal mol^{-1})	ΔH_R (kcal mol^{-1})	T (°C)	$p_{1/2}$ (mmHg)	n	ΔH (kcal mol^{-1})
10	1.5	2.0	1020	4×10^{-3}	4.2	0.67			9	3.7	1	
20	3.5	2.25	1150	1.6×10^{-3}	9.6	1.25	−11	−6	12	4.7	1	
27	6.0	2.4	1410	6.5×10^{-4}	17.4	1.90			20	9.5	1	−11
									27	17.4	1	
									35	26.6	1	

dence, and therefore the uncertainty in the corresponding activation energy is large.

3.4. Oxygen dissociation of hexameric hemocyanin determined by stopped-flow

Over the temperature range explored, $Y \geq 0.985$ in air and thus the dithionite method allows the estimation of the O_2 dissociation from the liganded R-state.

Table 2
Temperature effect on kinetic parameters of oxygen binding to monomeric (Fraction I) and hexameric *P. interruptus* hemocyanin

(a) Obtained by temperature jump. (b) Calculated from k_{on} (given in this table) and K_{eq} (given in table 1). (c) Obtained by stopped flow.

	T (°C)	k_{on} (10^{-6} M^{-1}s^{-1}) (a)	T (°C)	k_{off} (s^{-1})	
				a	b
Monomer	12.0	20	12.0	280	200
(T-state)	16.5	22	16.5	400	300
	24.5	37	24.5	1250	900
	31.5	44	31.5	2750	1700
				c	b
Hexamer	18.5	30	7.5	13	–
(R-state)	24.5	34	15	25	47
	31.5	44	21.4	36	71
			28.8	80	126

At every temperature the time course of the decrease in absorbance is autocatalytic and independent of dithionic concentration. At the higher temperatures the increase in reaction rate results in a progressive loss of optical density in the dead-time of the apparatus in all cases, however, the dissociation rate constant characteristic of the high

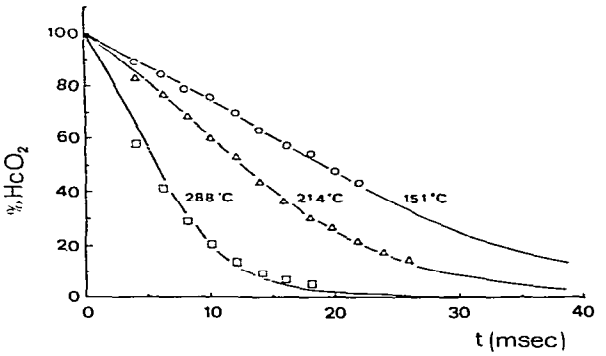

Fig. 2. Time course of the deoxygenation of hexameric *P. interruptus* hemocyanin at different temperatures. The solid lines have been calculated at each temperature on the basis of the model illustrated in section 2, using as values for the intrinsic dissociation rate constants of the T- and R-states those listed in table 2 and a switch-over point of 3, as demanded by the equilibrium data (given in fig. 1). Conditions: 0.1 M ethanolamine, ionic strength 0.1, in the presence of 10 mM $CaCl_2$.

oxygen-affinity state of the protein ($^{R}k_{off}$) can be estimated by extrapolation of the apparent time course towards time zero [13].

The values obtained at different temperatures (see table 2) can be checked for consistency with the independent information provided by equilibrium experiments. The solid lines shown in fig. 2, in fact, represent the time courses expected for a system of six sites undergoing a quaternary conformational transition from a high- to a low-affinity state during ligand dissociation (see section 2). The conformational transition never rate limits the overall kinetics, and this is the only 'a priori' assumption used throughout the calculation. Another basic feature of the model, which is, however, demanded by the symmetric shape of the equilibrium isotherms of fig. 1 * is that the transition occurs around 50% saturation. The good agreement between theoretical curves and experimental points reinforces our confidence in the correctness of both features.

4. Discussion

Analysis of the temperature dependence of ligand-binding equilibria and kinetics of hemocyanins within the context of the two-state allosteric Monod-Wyman-Changeux model [14] leads to the estimate of the thermodynamic parameters for O_2 binding by the two states (R and T) and for the ligand-linked conformational change.

As shown in fig. 1, an increase in temperature (from 10 to 27°C) results in a moderate increase in cooperativity ($n_H = 2$ to 2.4) and a concomitant increase in the free energy of interaction per site (see also table 1). The observed temperature dependence of the binding curve of the hexamer indicates that the heats of oxygenation of the T- and R-states are both exothermic and quantitatively different.

The binding isotherms of the hexamer were fitted to a two-state model, taking six as the number of interacting sites ($r = 6$) [15] which corresponds to the total number of sites per molecule. The solid lines in fig. 1 represent the fit of the results with only one disposable parameter at each temperature, namely L_0.

In agreement with previous conclusions [4] the monomer appears to mimic very well the low-oxygen affinity (T-) -state of the hexamer at the same pH and temperature, therefore the binding parameters of the subunit(s) are applied to the T-state of the hexamer. This is by itself a remarkable fact, since generally for hemoglobins and other hemocyanins the isolated subunits are similar to the high-affinity state of the corresponding protein, and their functional parameters have been taken to represent the R-state [7,16,17].

The extensive kinetic data reported above allow, for the first time, a complete characterization of the dynamics of O_2 binding to a hemocyanin.

As shown in table 2, the oxygen-association rate constants of the T- and R-state are fairly similar over the whole temperature range; on the other hand, the temperature dependences of the oxygen-dissociation rate constants of the two states are quite different. These new results confirm that the kinetic basis of cooperative oxygen binding resides in a large dependence of the oxygen-dissociation rate constant on the quaternary state of the molecule [4]. This conclusion seems very general, and it is based on the results obtained for several hemocyanins of both molluscs and arthropods [18]. The stopped-flow experiments carried out with hexameric *P. interruptus* hemocyanin indicate that the autocatalytic nature of the dissociation time course is fully consistent with a progressive population of the T-state of the protein during deoxygenation, given the fact that $^{T}k_{off} \gg {}^{R}k_{off}$ (see table 2 and section 3).

The activation parameters for the reaction with O_2 are given in table 3. In the case of O_2 combination the free energy of activation is similar (≈ 7 kcal mol^{-1}) for the two states; however, the activation entropy of the T-state is very close to zero (0.84 e.u.), while that for the R-state (in spite of the greater uncertainty) is clearly unfavorable (-14 e.u.). In the case of deoxygenation the relatively high activation enthalpy of the T-state is largely compensated by a favorable activation en-

* The values obtained for the switch-over point (see section 2) on the basis of the parameters in table 1 range, in the temperature interval from 10 to 27°C, between 3.01 and 3.13.

Table 3
Activation parameters for the oxygenation reactions of *P. interruptus* hemocyanin in the T- and R-state

Values are calculated from the equations reported in section 2. The activation free energy is given at 20°C.

	Oxygenation			Deoxygenation		
	$\Delta G^{\ddagger}$ (kcal mol^{-1})	$\Delta H^{\ddagger}$ (kcal mol^{-1})	$\Delta S^{\ddagger}$ (cal deg^{-1}mol^{-1})	$\Delta G^{\ddagger}$ (kcal mol^{-1})	$\Delta H^{\ddagger}$ (kcal mol^{-1})	$\Delta S^{\ddagger}$ (cal deg^{-1}mol^{-1})
T-state	7.1	7.4	0.84	13.2	18.1	16.6
R-state	7.1	3(a)	−14	14.8	14	−3.1

[a] This parameter is known with considerable uncertainty, ranging from 1 to 5 kcal mol^{-1}.

tropy (+16.6 e.u.), which accounts for the much faster oxygen-dissociation rate constant.

In spite of the great differences in the structure of the active site, the kinetic features of the oxygen reaction in hemoglobin(s) are similar to those reported in this paper for *P. interruptus* hemocyanin. Thus, for example, the low-O_2 affinity of the T-state of hemoglobin is determined by the higher O_2-dissociation rate constant [7,19] which is controlled by favorable entropy of activation [20]. It is tempting to propose that, in view of the very large differences in the mode of binding of O_2 in the two classes of respiratory proteins, the kinetic control of the reaction is achieved by the protein, independently of the chemistry of the site.

The activation parameters of the oxygen and deoxygenation reactions of the T- and R-state of *P. interruptus* hemocyanin may be used to obtain the corresponding thermodynamic parameters from elementary relationships. The comparison shown in table 4 is, in most cases, more than satisfactory and increases considerably the limits of confidence in the estimates obtained by the equilibrium analysis.

The enthalpy change for oxygen binding to both allosteric states of *P. interruptus* hemocyanin is negative, although the reaction of the T-state is more exothermic. In the case of *H. pomatia* β-hemocyanin [2], the intrinsic heat of oxygenation is approximately the same for both states ($\Delta H = -11$ kcal mol^{-1}), while a completely different result was reported for *L. hierosolima* hemocyanin [3] ($\Delta H_T = +3$ kcal mol^{-1} and $\Delta H_R = -11$ kcal mol^{-1}). These differences in thermodynamic parameters are not understood; however, they may possibly be ascribed to contributions from the

Table 4
Thermodynamic parameters for the binding of oxygen to *P. interruptus* hemocyanin (equilibrium and kinetic data)

Values in the table are expressed as kcal mol^{-1} (calculated at 20°C).

	$\Delta G_0'$ [c]	$(\Delta G_{on}^{\ddagger} - \Delta G_{off}^{\ddagger})$	$\Delta H_0'$	$(\Delta H_{on}^{\ddagger} - \Delta H_{off}^{\ddagger})$	$T\Delta S_0'$	$T(\Delta S_{on}^{\ddagger} - \Delta S_{off}^{\ddagger})$
Binding to T-state [a]	−6.3	−6.1	−11.0	−10.7	−4.7	−4.6
Binding to R-state [a]	−7.6	−7.8	−6.0	−11.0	+1.0	−3.4
Quaternary transition [b] ($T_0 \rightarrow R_0$)	+3.7		−18		−21.7	

[a] Values given per mol site.
[b] Values given per mol hexamer.
[c] The figure for $\Delta G_0'$ has been calculated from the corresponding equilibrium constants (given in table 1) on the basis of the formula $\Delta G_0' = -RT \ln K$.

binding of oxygen-linked solvent components (such as H^+ or Ca^{2+}), which may be difficult to account for. As an example, in the case of *H. pomatia* β-hemocyanin [2] the observed heat of oxygenation of the T-state was -17.5 kcal mol^{-1}, and the ionization heat of the Bohr protons within the T-state contributed $+5.4$ kcal mol^{-1}. For *P. interruptus* hemocyanin, however, the observed heat may be considered intrinsic values, since at pH 9.6 the Bohr effect is no longer operative and no effect of Ca^2 on the heat of oxygenation of the T-state was found. In fact, the oxygen-binding enthalpy of monomers is similar to that obtained from the lower asymptote of the cooperative hexamer. Thus, cooperative oxygen binding to *P. interruptus* hemocyanin is produced by a favorable difference in binding entropy, which outweighs the unfavorable enthalpy change ($+4.4$ kcal mol^{-1}), as calculated from $\Delta H_I = \Delta H_R - \Delta H_T$ [20].

As given in table 4, the allosteric transition ($T_0 \rightarrow R_0$) is an exothermic process, with a $\Delta H_L = -18$ kcal mol^{-1} (hexamer), consistent with the observation that increase in temperature is associated with an increase in homotropic interactions (as shown in fig. 1). This result is most significant and, in some ways, peculiar. It may be recalled that in hemoglobins the allosteric transition is associated with a positive enthalpy change, with values of approximately $\Delta H_{L_0} = +16.7$ kcal mol^{-1} (tetramer) for human Hb and approximately $\Delta H_{L_0} = +28$ kcal mol^{-1} (tetramer) for trout HbI, two of the more extensively investigated cases [21,22]. As a result, the entropy change associated with the allosteric transition is favorable in the case of hemoglobins, while is unfavorable in the case of *P. interruptus* hemocyanin ($T\Delta S = -21.7$ kcal mol^{-1} of hexamer). This important finding seems fully consistent with the fact that: (a) the isolated *Panulirus* subunits are characterized by low-O_2 affinity, contrary to the isolated α- and β-subunits of HbA [7] or to the isolated subunits of *Limulus polyphemus* hemocyanin [23]; and (b) the deoxygenated derivative of *P. interruptus* hemocyanin (i.e., the T-state) dissociates into subunits more rapidly than the oxygenated one (i.e., the R-state) [5], which is also opposite to the behavior characteristic of human hemoglobin [7]. It seems, therefore, most remarkable that the assembly of monomers into a liganded hexamer leads, in the case of *P. interruptus* hemocyanin, to a more 'compact' structure with a lower entropy content. This provides, among other things, the possibility to study the properties of a monomer in the T-state, to be compared with monomers of other species which are stabilized in the R-state.

In summary, the thermodynamics of oxygen binding by *P. interruptus* hemocyanin may be described with the allosteric two-state model, and thus the intrinsic thermodynamic properties of the two functionally relevant states may be determined. The transition $T_0 \rightarrow R_0$ in this hemocyanin is favored by a negative enthalpy term, which, however, is outweighed by an even larger unfavorable entropy change. In hemoglobins the enthalpy change for the allosteric transition is positive and thus opposite to that reported above for *P. interruptus* hemocyanin. The thermodynamic behavior of various respiratory proteins may reveal different features of molecular control of cooperative ligand binding, although additional effects of oxygen-linked solvent components may come into play and their role needs to be elucidated.

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