The interplay of temperature and protons in the modulation of oxygen binding by squid blood

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An extensive set of data relating to the binding of oxygen by haemocyanin from the squid *Todarodes sagittatus* has been collected under various experimental conditions. The results obtained show that, within the range of physiological pH, the concentration of protons affects mainly the high-affinity state of the molecule without significantly affecting the low-affinity state. As far as the effect of temperature is concerned, the data show a characteristic feature which is very similar to that previously described in the case of haemoglobins from Arctic mammals such as reindeer (*Rangifer tarandus*) and musk ox. (*Ovibos moschatus*). The shape of the oxygen equilibrium curve shows strong temperature-dependence, since the overall heat of the binding of oxygen to the low-affinity state of the molecule is strongly exothermic and that to the high-affinity state is very close to zero. The results provide an outline of the intramolecular compromise that, through the interplay of temperature and protons, optimizes the loading and unloading of oxygen under the various environmental conditions experienced by this species of squid.

INTRODUCTION

The temperature-sensitivity of haemocyanin–oxygen affinity and co-operativity has been recently investigated in many arthropod and molluscs (Bellelli *et al.*, 1988; Burnett *et al.*, 1988; Brix *et al.*, 1989a). In the light of previous results, it was possible to predict, on the basis of the temperature-sensitivity of the affinity for oxygen, the ecological distribution of various cephalopods. Moreover, it has been also demonstrated that the large Bohr shift characteristic of these animals cannot facilitate the unloading of oxygen at the level of tissues, since there would be no shift in pH between arterial and venous blood when the functional Haldane coefficient is about 1.0 (Brix *et al.*, 1981, 1989a). In this case, in fact, the Bohr shift would not come into operation via an arterial–venous difference in pH, but only via a contemporaneous and identical change in pH at the level of both the arterial and the venous blood. We can suggest three different physiological conditions under which these changes could occur: (i) anaerobiosis with formation of octopine or lactate; (ii) increased ventilation due to very active swimming; (iii) a change in temperature. Although anaerobiosis does occur in cephalopods, it is known that most of the metabolic end products are utilized at the level of muscles and, as a result, have very little influence, if any, on the pH of the blood; the second case has been discussed previously (Brix *et al.*, 1981); in the third case we may assume that the blood undergoes a significant change in pH in response to variations in temperature, in accord with the concept of constant relative alkalinity (Reeves, 1980). As far as this latter point is concerned, it should be noted that, for an animal whose respiratory pigment is characterized by a substantial Bohr shift, exothermic oxygenation and a blood pH that follows the Rosenthal curve, any change in temperature might have a serious impact on oxygen transport. In fact, if the temperature increases, then oxygen loading may be impaired as a result of the decrease in oxygen affinity brought about by both the direct effect of temperature and the Bohr shift. By contrast, if the temperature of the blood decreases, then oxygen unloading will soon become a problem.

The aim of the present investigation was to investigate the possible presence, in haemocyanin from a squid (*Todarodes sagittatus*), of molecular mechanisms that might compensate for the effects outlined above and optimize the transport of oxygen.

Such mechanisms, if present, would be of general interest for all respiratory pigments exposed to a similar situation, for example, the haemoglobins of arctic mammals (Brix *et al.*, 1989b, 1990; Giardina *et al.*, 1989, 1990).

MATERIALS AND METHODS

Squids (*Todarodes sagittatus*; five specimens) were obtained in the vicinity of Bergen, Norway. Blood was sampled from the vena cava cephalica, was centrifuged to remove cells and debris, and was stored frozen before experimentation (the samples were not pooled).

The oxygen equilibria were measured, at constant CO₂ partial pressure and at different temperatures, with a diffusion-chamber technique (Lykkeboe *et al.*, 1975), except that the stepwise increases in oxygen partial pressure 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). Alternatively oxygen dissociation curves were obtained spectrophotometrically by the tonometric method (Giardina & Amiconi, 1981) at an observation wavelength of 364 nm.

The pH was measured for oxygenated and deoxygenated blood, and the mean values were used as pH values for the oxygen-binding curves. The oxygen-binding data were fitted to the MWC (Monod–Wyman–Changeux) model (Monod *et al.*, 1965) as previously described (Brunori *et al.*, 1979; Brix *et al.*, 1989b).

Measurement of the haemocyanin oxygen affinities at different pH and temperature values makes possible the characterization of the temperature-sensitivity in terms of the total heat of oxygenation at fixed pH using the modified (Wyman, 1964) van't Hoff isochore:

$$\Delta H^{\text{app}} = R \Delta \ln P_{50} / \Delta (1/T)$$

The reported data were not corrected for the heat of solution of oxygen [−12.6 kJ (−3.0 kcal)/mol].

RESULTS AND DISCUSSION

A set of data relating to the binding of oxygen by *Todarodes sagittatus* haemocyanin has been collected under various experimental conditions.

Fig. 1. Oxygen-binding curves, expressed by means of Hill plots, for blood from *T. sagittatus*, as a function of temperature and pH

Δ , pH 7.7 and 6 °C, 20 °C (from left to right); $\circ$, pH 7.46 and 6 °C, 20 °C (from left to right); $\blacktriangledown$, pH 7.2 and 6 °C, 20 °C (from left to right). It should be mentioned that similar binding curves were obtained at 10 °C and used in the analysis of the data; we omitted these curves to avoid overcrowding. The fractional saturation with oxygen is indicated as $\bar{Y}$ and represents the ratio $\text{HcO}_2/(\text{Hc} + \text{HcO}_2)$, where Hc is haemocyanin. (Note: 1 mmHg $\equiv$ 133.3 Pa; $p\text{O}_2$ is the partial pressure of oxygen.)

Fig. 1 shows a series of Hill plots obtained at different values of pH and temperature. As the data in Fig. 1 show, the distribution of the Bohr protons all along the binding curve is strongly dependent on pH. In particular, going from pH 7.4 down to more acid values, the concentration of protons acts mainly on the upper asymptote, representative of the high-affinity state of the molecule, leaving unchanged the lower asymptote, which is representative of the low-affinity state of the molecule. This differential effect of pH on the high and the low asymptote is evident as a decrease in the free energy of interaction between the oxygen-binding sites and results in a lowering the overall cooperativity of ligand binding.

In addition to the effect of pH described above, Fig. 2 outlines the effect of temperature, as observed within the same range of pH values at a constant concentration of protons (see also Fig. 1). The data, presented in the form of a Hill plot, show a characteristic feature which is very similar to that previously described in the case of haemoglobins from Arctic mammals, such as reindeer (*Rangifer tarandus*), musk ox (*Ovibos moschatus*) and the lesser rorqual whale (*Balaenoptera acutorostrata*) (Brix *et al.*, 1989b, 1990; Giardina *et al.*, 1989, 1990). As in the case of those haemoglobins, the shape of the binding curve shows strong temperature-dependence, since an increase in temperature induces a great decrease in the association constant for the binding of oxygen to the low-affinity state of the molecule

Fig. 2. Differential effect of temperature and pH on low- and high-affinity states of haemocyanin from *T. sagittatus* blood

Δ , pH 7.18 and 20 °C; $\circ$, pH 7.46 and 6 °C; $\blacktriangle$, pH 7.2 and 6 °C. The fractional saturation with oxygen is indicated as $\bar{Y}$ and represents the ratio $\text{HcO}_2/(\text{Hc} + \text{HcO}_2)$, where Hc is haemocyanin. (Note: 1 mmHg $\equiv$ 133.3 Pa.)

Fig. 3. Overall heat of oxygenation (ΔH_{app}) for haemocyanin from *T. sagittatus* as a function of pH, at three different levels of saturation (as indicated)

The reported values are not corrected for the heat of solubilization of oxygen [-12.6 kJ (-3.0 kcal)/mol] and are expressed in kJ (kcal)/mol of oxygen.

without having any significant influence on that for binding to the high-affinity state. Thus, whereas the overall reaction of oxygen binding to the low-affinity state is strongly exothermic, the heat of reaction relative to the high-affinity state is very close to zero, and the reaction may even absorb heat if a correction is made for the heat of solubilization of oxygen. As indicated by the unchanged position of the upper asymptote and the contemporaneous marked shift of the lower one, cooperativity of

Fig. 4. Dependence on pH of the partial pressure of oxygen (pO_2) necessary to give 25, 50 and 95% saturation at 6 °C

oxygen binding increases substantially as the temperature is increased.

It should be emphasized that the different thermodynamics that characterize the low- and high-affinity states of haemocyanin from *T. sagittatus* appear to be strongly dependent on pH. This dependence is shown in Fig. 3, where the overall heat of oxygenation, measured at three different degrees of saturation, is reported as a function of pH within the range between pH 7.7 and pH 7.2. At alkaline pH values (about 7.7) the overall reaction of oxygen binding is strongly exothermic and very similar for the three degrees of saturation [from 54.4 to 62.8 kJ (13–15 kcal)/mol]. This observation implies that, at this pH, the binding curves are shifted, almost without any change in shape, with increasing temperature, as is often observed with other respiratory pigments (Brunori *et al.*, 1979, 1982). The same applies at pH 7.4, where, however, a smaller heat of binding is observed [from 29.3 to 37.6 kJ (–7 to –9 kcal)/mol]. Towards more acid pH values, whereas the enthalpy of oxygen binding at 25 and 50% saturation is seen to level off, that at 95% continues to decrease, tending to about –4.18 kJ (–1 kcal)/mol at pH 7.2. At this pH value, as outlined in Fig. 2, the dependence of the effect of temperature on the degree of saturation of the protein with oxygen is dramatic. These features are correlated with the particular differential effect of protons between the two conformational states of the protein. This aspect is outlined in Fig. 4 by the dependence on pH of the partial pressure of oxygen necessary to give 25, 50 and 95% saturation at 6 °C. It is immediately apparent that whereas between pH 7.7 and 7.4 the Bohr effect is almost equally distributed all along the saturation levels, at lower pH values protons act mainly on the high-affinity state of the protein.

The results reported here show that the functional behaviour of haemocyanin from *T. sagittatus* displays peculiar features which, like those previously observed with haemoglobins from reindeer and musk ox, make this protein a very interesting example of molecular adaptation to specific physiological requirements. These characteristic features result from the unusual interplay of protons and temperature (see Figs. 2 and 3). In fact, the strong dependence of the effect of temperature on the degree of oxygen saturation observed at pH 7.2 (see Fig. 3) can be explained in large part by the differential effect of protons between states with very high and very low saturation by the ligand.

Thus, whereas the Bohr effect in the case of the low-affinity

Fig. 5. Dependence on temperature of the Bohr coefficient, measured at three different levels of saturation (as indicated) and over a range of pH values from 7.2 to 7.46

state of the protein is almost absent at pH values lower than 7.4, that of the high-affinity state is operative over the entire range of pH values examined (see Fig. 4). Therefore, for pH values lower than 7.4, most of the Bohr protons are released with the binding of the last molecules of oxygen. In other words, below pH 7.4, the overall enthalpy of oxygen binding to the low-affinity state is no longer influenced by the endothermic release of the oxygen-linked protons and levels off at about –41.84 kJ (–10 kcal)/mol of oxygen; by contrast, the enthalpy of oxygenation of the high-affinity state is more and more diminished by the endothermic contribution of the Bohr effect that is still operative within this pH range. The net result of this interplay is the peculiar dependence on temperature of the shape of the binding curve, which is clearly discernable at pH 7.2, as shown in Fig. 2.

These functional properties seem to fit very well with the physiological requirements of *T. sagittatus*. This animal, like most cephalopods, is characterized by a high aerobic metabolism which has to be satisfied by a very efficient respiration. In this connection, as also outlined in the Introduction, it should be recalled that, for a functional Haldane coefficient of about 1.0 (Brix *et al.*, 1981, 1989a), the Bohr shift would not come into operation by means of an arterial–venous difference, but only by means of a change in pH brought about by temperature at the level of both arterial and venous blood. The particular physico-chemical properties of the blood of *T. sagittatus* seem, in this respect, very well designed to optimize the transport of oxygen when the temperature is allowed to change all over the animal's body. Thus the particular differential and opposite effects of protons and temperature will minimize the handicap in unloading of oxygen that is induced by a decrease in temperature and a subsequent increase in pH. In fact, as far as the concentration of protons is concerned, the position of the lower asymptote is fixed and is allowed to change only as a result of a change in temperature. This change will increase the oxygen affinity, represented by the lower asymptote of the binding curve, but not to the extent that would have been observed in the presence of a significant Bohr effect at these levels of saturation. As a consequence, and on the basis of the insensitivity of the high-affinity state to changes in temperature, a decreased co-operativity should have been observed; however, this effect is almost completely counterbalanced by the Bohr effect, which is operative only at high levels of saturation (below pH 7.4). Therefore, at the level of the high-affinity state, also, a minimization of the effect of contemporaneous changes in temperature and pH is observed. At this level, the increase in the oxygen affinity is solely determined by the change in pH and the effect of temperature is negligible. Moreover, the increased affinity, represented by the upper asymptote and brought about by pH, would increase the co-operativity and counteract the effect of temperature at the

level of the lower asymptote. The particular behaviour is also outlined in Fig. 5, where the Bohr coefficient at three different levels of saturation is shown as a function of temperature. Here, at 6 °C, where the temperature-induced change in pH becomes important, we are able to see the balance and that occurs between high and low levels of saturation. At high saturation (95 %) the Bohr coefficient is about -1.5 , whereas at low saturation it becomes about -0.6 . The results in Fig. 5 outline very clearly the intramolecular compromise that, through the interplay of temperature and protons, optimizes oxygen loading and unloading under the various environmental conditions experienced by these animals.

The example described here could be of general applicability and validity for all those animals in which temperature is allowed to change over the entire body.

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