EFFECT OF HAEMOGLOBIN CONCENTRATION ON THE OXYGEN AFFINITY OF INTACT LAMPREY ERYTHROCYTES

S. AIRAKSINEN AND M. NIKINMAA

Department of Biosciences, Division of Animal Physiology, Box 17, FIN-00014 University of Helsinki, Finland

Summary

We investigated whether the oxygen affinity of lamprey haemoglobin decreases with increasing oxygen concentration at the high (10–$25\,mmol\,l^{-1}$ monomeric) haemoglobin concentrations prevailing within the erythrocytes. The intracellular concentration of haemoglobin was experimentally adjusted by shrinking the cells osmotically: the osmolality of the equilibration medium was increased from approximately $250\,mosmol\,kg^{-1}$ by $90\,mosmol\,kg^{-1}$ to $340\,mosmol\,kg^{-1}$ or by $180\,mosmol\,kg^{-1}$ to $430\,mosmol\,kg^{-1}$ by adding sucrose in the medium. This increased the mean cellular haemoglobin concentration from $16.9\pm0.23\,mmol\,l^{-1}$ (monomeric haemoglobin) to $20.0\pm0.20\,mmol\,l^{-1}$ (monomeric haemoglobin) and to $23.0\pm0.36\,mmol\,l^{-1}$ (monomeric haemoglobin), respectively (means ± S.E.M., N=35–40; all the samples from 7–8 different pools of blood at each osmolality combined). The oxygen equilibrium curves at each osmolality were determined by Tucker's method. An increase in haemoglobin concentration shifted the oxygen equilibrium curve to the right as indicated by the P_{50} values, which were $4.26\pm0.07\,kPa$ at the lowest, $4.64\pm0.13\,kPa$ at the intermediate and $5.64\pm0.40\,kPa$ (means ± S.E.M., N=7–8) at the highest haemoglobin concentrations. The decrease in haemoglobin oxygen-affinity was attributed to the volume changes, since the intracellular pH did not decrease with increasing mean cellular haemoglobin concentration. Thus, the variations in red blood cell volume commonly observed during hypoxia may play a role in the regulation of haemoglobin function.

Key words: Agnatha, oxygen transport, red blood cell, lamprey, haemoglobin, *Lampetra fluviatilis*.

Introduction

The current model for the behaviour of lamprey haemoglobin in solution implies that all cooperative phenomena, and the presence of Bohr and Haldane effects, are the result of association/dissociation reactions between high-affinity monomers and low-affinity oligomers (Perutz, 1990). Since haemoglobin within lamprey erythrocytes shows distinct cooperativity and large Bohr and Haldane effects (Nikinmaa and Mattsoff, 1992; Ferguson *et al.* 1992; Nikinmaa, 1993), it is likely that association/dissociation reactions also occur in intact erythrocytes. This being the case, the equilibrium between oligomers and monomers within intact erythrocytes should depend on haemoglobin concentration, as is observed in dilute solutions; an increase in concentration should increase the proportion of the low-affinity oligomers and a decrease in concentration should increase the proportion of high-affinity monomers. Thus, changes in haemoglobin concentration should affect the haemoglobin oxygen-affinity in intact erythrocytes.

Up to the present, it has been difficult to separate the effect of concentration and the effect of pH on the oxygen affinity of lamprey haemoglobin (Nikinmaa, 1992). However, Virkki and Nikinmaa (1994) recently observed that when lamprey erythrocytes were shrunk osmotically, they did not exhibit a regulatory volume increase and, furthermore, their intracellular pH was the same as or higher than that of erythrocytes in isotonic conditions. Osmotic shrinkage therefore provided a means of varying the mean cellular haemoglobin concentration independently from the intraerythrocytic pH and thereby of investigating the role of haemoglobin concentration as such on the haemoglobin oxygen-affinity within intact lamprey erythrocytes.

Materials and methods

River lampreys, *Lampetra fluviatilis* (N=40; 30–90 g), were caught during their spawning run from the rivers Kymijoki and Lestijoki, Finland, and were acclimated to laboratory conditions [dechlorinated Helsinki tapwater, 10–15 °C, oxygen tension (P_{O_2}) >15 kPa, carbon dioxide tension (P_{CO_2}) <0.1 kPa, pH 7.2–7.6] for at least 2 weeks before experiments. Blood was taken from the caudal vessels of anaesthetized (MS-222, $1\,g\,l^{-1}$) lampreys into heparinized syringes. Blood from about five animals was pooled to ensure a sufficient sample size. Red cells were separated from plasma by centrifugation ($20\,000\,g$)

and washed three times for 10 min with the saline used in the experiments (composition: $110 \, \text{mmol} \, l^{-1}$ NaCl, $6 \, \text{mmol} \, l^{-1}$ NaHCO$_3$, $5 \, \text{mmol} \, l^{-1}$ KCl, $1 \, \text{mmol} \, l^{-1}$ MgCl$_2$, $1 \, \text{g} \, l^{-1}$ glucose), which closely resembles lamprey plasma. ^{14}C-labelled 5,5-dimethyl-2,4-oxazolidinedione (DMO; $10 \pm 1 \, \mu l \, ml^{-1}$ erythrocyte suspension) was added to the incubation medium for intracellular pH measurements.

Each pooled sample was divided into three portions. The first portion was incubated in isotonic saline (approximately $250 \, \text{mosmol} \, kg^{-1}$) so that the normal cell volume was maintained. The other two portions were placed in salines with the same composition as the isotonic saline except that they also contained either 90 or $180 \, \text{mmol} \, l^{-1}$ sucrose to increase the osmolality of the medium by $90 \, \text{mosmol} \, kg^{-1}$ (to $340 \, \text{mosmol} \, kg^{-1}$) or by $180 \, \text{mosmol} \, kg^{-1}$ (to $430 \, \text{mosmol} \, kg^{-1}$). Using these salines, it was possible to shrink the cells to two different degrees. As known from earlier studies (Virkki and Nikinmaa, 1994), lamprey red blood cells shrink and remain at the reduced volume when exposed to hypertonic medium.

The oxygen equilibrium curves were determined at $18 \, ^{\circ}$C and $1 \, \text{kPa} \, P_{CO_2}$ using Tucker's (1967) method. Five points on the curve were determined using P_{O_2} values of 1, 2, 4, 8 and $20 \, \text{kPa}$ starting alternately at the highest or the lowest P_{O_2}; the cells were tonometered for 30 min at each oxygen tension, except for the first measurement, when they were tonometered for 1 h. In each case, the maximal oxygen capacity was also determined by tonometering the cells at $20 \, \text{kPa} \, P_{O_2}$ in the absence of carbon dioxide to enable calculation of haemoglobin oxygen-saturation. Humidified gases were used, and the gas tensions were obtained using cascaded Wösthoff (Bochum, Germany) gas-mixing pumps. The equilibration flasks were fitted with rubber stoppers through which two hypodermic needles were inserted, enabling the withdrawal of samples into syringes without contact with air.

In addition to the oxygen saturation, the haemoglobin concentration, haematocrit and extracellular and intracellular pH were determined at each oxygen tension. The water content of the cells was determined at the start and at the end of each experiment.

Haemoglobin concentration was determined using the cyanmethaemoglobin method and the haematocrit value by centrifugation (3 min at $13\,000\,g$). The mean cellular haemoglobin concentration (MCHC) was calculated by dividing the haemoglobin concentration by the haematocrit. The extracellular pH was measured immediately after sampling using a Radiometer BMS3 Mk2 and PHM 72 apparatus. The remaining sample was divided into two parts: one portion was used for determinations of intracellular pH and the other, which was placed in pre-dried and weighed Eppendorf tubes, for determinations of water content. In both samples, the red cells and the medium were separated by centrifugation (2 min at $20\,000\,g$). $100 \, \mu l$ of the supernatant was saved and the rest was carefully removed together with the uppermost layer of cells. The water content of the pelletted cells was determined by weighing, drying (80 °C, 48 h) and

reweighing (Nikinmaa and Huestis, 1984). The red cell pH was determined as described by Nikinmaa and Huestis (1984) using the DMO method. Briefly, both the weighed red cell pellet and $100 \, \mu l$ of supernatant were deproteinized in $300 \, \mu l$ of $0.6 \, \text{mol} \, l^{-1}$ perchloric acid and centrifuged for 5 min at $20\,000\,g$. Thereafter, $150 \, \mu l$ of the supernatant was placed in scintillation vials, scintillation liquid (Wallac, OptiPhase 'Hi Safe' 2) was added, and radioactivity was counted using a Wallac 1211 Minibeta scintillation counter. The intracellular pH was then calculated from the extracellular pH and intra- and extracellular concentration (in $\text{disints} \, \text{min}^{-1} \, l^{-1}$) of radioactive DMO using the formula:

$$pHi = pK_{DMO} + \log\{[DMO]_i/[DMO]_e \times (10pHe - pK_{DMO} + 1) - 1\},$$

in which the pK_{DMO} was taken to be 6.25, pHi and pHe are intracellular and extracellular pH, respectively, and e and i refer to extra- and intracellular compartments, respectively.

Statistical analyses of the data were carried out by the Wilcoxon matched-pairs signed-ranks test (when the data were not normally distributed) or the least-squares difference (LSD) test with paired design of the ANOVA/MANOVA module of Statistica Software (StatSoft Inc, Tulsa, USA).

Results and discussion

The water content of packed lamprey red cells in isotonic medium was $72.1 \pm 0.52 \%$ (N=12). When the osmolality of the medium was increased by $90 \, \text{mosmol} \, kg^{-1}$, the water content decreased significantly to $69.3 \pm 0.7 \%$ (N=12), and when the osmolality was increased by $180 \, \text{mosmol} \, kg^{-1}$, the water content was further significantly reduced to $67.2 \pm 1.27 \%$ (N=10). At every osmolality, the values at the start and at the end of the experiment were combined, since the mean values at these points were not significantly different. The change in
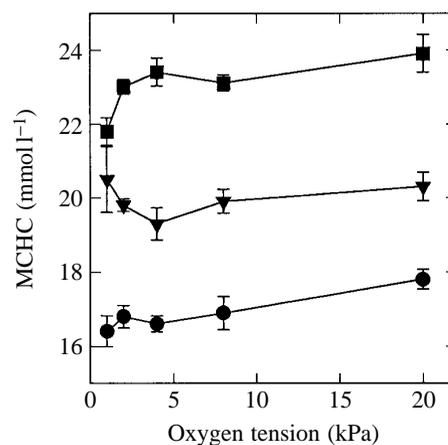

Fig. 1. The mean cellular haemoglobin concentration (MCHC, in $\text{mmol} \, l^{-1}$ monomeric haemoglobin) of lamprey red blood cells as a function of the oxygen tension (in kPa) at approximately $250 \, \text{mosmol} \, kg^{-1}$ (circles), $340 \, \text{mosmol} \, kg^{-1}$ (triangles) and $430 \, \text{mosmol} \, kg^{-1}$ (squares). Means ± S.E.M. are given, N=7–8. There was a significant (P<0.05) difference between the means at the different osmolalities at every oxygen tension (ANOVA).

Table 1. *The* P_{50} *value and red cell pH at* P_{50} *value at different mean cellular haemoglobin concentrations in intact lamprey erythrocytes*

MCHC ($mmol\,l^{-1}$)	P_{50} (kPa)	pHi at P_{50} value
16.9±0.23 (*N*=40)	4.26±0.07 (*N*=8)	7.279±0.026 (*N*=6)
20.0±0.20 (*N*=40)	4.64±0.13 (*N*=8)	7.327±0.021 (*N*=6)
23.0±0.36 (*N*=35)	5.64±0.40 (*N*=7)	7.331±0.018 (*N*=5)

MCHC, mean cellular haemoglobin concentration.

Means ± S.E.M. are given. For MCHC, *N* indicates the total number of samples from 7–8 pools of blood.

P_{50} values were estimated from individual oxygen equilibrium curves, and pHi values at P_{50} values were estimated from individual pHi *versus* oxygen saturation relationships.

The P_{50} values at the different mean cellular haemoglobin concentrations differed significantly from each other (*P*<0.05; Wilcoxon matched-pairs signed-ranks test), whereas a significant difference in the intracellular pH values was observed only between the lowest and the highest MCHC values.

water content was reflected in the mean cellular haemoglobin concentrations (Fig. 1), which increased significantly with increasing osmolality of the medium. The oxygen tension of the incubation medium did not have a significant effect on the mean cellular haemoglobin concentration at a given osmolality – the only statistically significant difference (*P*<0.05) was observed at the highest osmolality between the value at 1 kPa and the values at other oxygen tensions. Thus, we were able to change the mean cellular haemoglobin concentration in a predictable fashion by osmotically shrinking the cells. Importantly, an increase in mean cellular haemoglobin concentration was not associated with a decrease in intracellular pH at comparable oxygen saturations (Table 1). Rather, there was a tendency for an increase in pH with increasing mean cellular haemoglobin concentration. Since lamprey haemoglobin oxygen-affinity increases markedly with increasing pH (Nikinmaa, 1993), a decrease in haemoglobin oxygen-affinity occurring with an increase in haemoglobin concentration cannot be due to intracellular pH changes. Furthermore, although cellular shrinking undoubtedly increases intracellular solute concentrations, the oxygen affinity of lamprey haemoglobin is independent of levels of both organic phosphates and inorganic ions (at least within the concentration range 100–480 $mmol\,l^{-1}$ for NaCl; Nikinmaa and Weber, 1993). In conclusion, we were able to produce conditions in which the effect of haemoglobin concentration on the haemoglobin oxygen-affinity within intact lamprey erythrocytes could be studied.

The P_{50} values at the different mean cellular haemoglobin concentrations are given in Table 1, and Hill plots are given in Fig. 2. It is clear from the data that an increase in mean cellular haemoglobin concentration decreases the haemoglobin oxygen-affinity. Thus, the present results show that haemoglobin concentration affects the oxygen affinity of intact lamprey erythrocytes, as it does in dilute haemoglobin
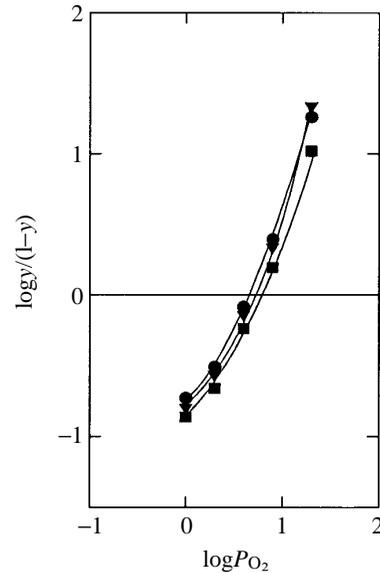

Fig. 2. Hill plots for lamprey haemoglobin within intact erythrocytes at 16.9 $mmol\,l^{-1}$ (circles), 20.0 $mmol\,l^{-1}$ (triangles) and 23.0 $mmol\,l^{-1}$ (squares) mean cellular haemoglobin concentrations. Values are means of 7–8 oxygen saturation determinations at the different oxygen tensions. The approximate Hill coefficients at P_{50} value were 1.60 at the lowest, 1.59 at the intermediate and 1.44 at the highest mean cellular haemoglobin concentration. These values do not differ significantly from each other.

solutions (e.g. Dohi *et al.* 1973). The present results are thus further (albeit circumstantial) evidence for the occurrence of association/dissociation reactions of haemoglobin molecules within intact erythrocytes (see Nikinmaa and Weber, 1993; Nikinmaa, 1993).

It is notable that in intact lamprey erythrocytes the effect of pH and the effect of haemoglobin concentration can be separated. At present, however, it is not known whether the concentration effect as such is utilized in the physiological regulation of haemoglobin function. It is known that when lampreys are exposed to low oxygen tensions, their mean cellular haemoglobin concentration decreases (Nikinmaa and Weber, 1984). On the basis of the present results, the reduced mean cellular haemoglobin concentration could increase haemoglobin oxygen-affinity. The reason for the hypoxia-associated increase in the red cell volume of lamprey (Nikinmaa and Weber, 1984) is not known. One possibility is that the Na^+/H^+ exchange of lamprey erythrocytes is oxygenation-sensitive, as is the adrenergically activated Na^+/H^+ exchange in fish erythrocytes (Motais *et al.* 1987). Notably, Ferguson *et al.* (1992) observed that the intracellular Na^+ concentration of deoxygenated *Petromyzon marinus* erythrocytes was higher than that of oxygenated ones. A pronounced activation of Na^+/H^+ exchange, as occurs during acidification of lamprey erythrocytes (Virkki and Nikinmaa, 1994), would primarily increase intracellular pH and, if it also caused a marked increase in the red cell volume, could secondarily cause the increase of haemoglobin oxygen-affinity *via* the concentration effect.

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