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Water regulates oxygen binding in hagfish (*Myxine glutinosa*) hemoglobin

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Summary

Hagfish hemoglobin (Hb) is considered to represent a transition stage between invertebrate and vertebrate hemoglobins. The Hb system of *Myxine glutinosa* consists of three monomeric hemoglobins, which upon deoxygenation associate to form primarily heterodimers and heterotetramers. *Myxine glutinosa* is an osmoconformer, whose red blood cells show the exceptional ability to swell and remain swollen under hyposmotic conditions. In order to determine whether water activity regulates hemoglobin function, the effect of changes in osmolality on hemoglobin-O_2 affinity was investigated by applying the osmotic stress method to purified hemoglobins as well as intact red blood cells. Oxygen affinity decreases when water activity increases, indicating that water molecules stabilize the low-affinity, oligomeric state of the hemoglobin. This effect is opposite to that observed in tetrameric vertebrate hemoglobins, but resembles that seen in the dimeric hemoglobin of the marine clam *Scapharca inaequivalvis*. Our data show that water may act as an allosteric effector for hemoglobin within intact red cells and even in animals that do not experience large variations in blood osmolality.

Key words: water effect, allostery, hagfish, *Myxine glutinosa*, hemoglobin, bicarbonate, osmolality, oxygen affinity, cooperativity, linkage plot.

Introduction

A central role for water in determining structure and regulating function of proteins is becoming increasingly evident. Many examples are known where water acts as an allosteric effector, by preferentially binding to a specific protein conformation, and is loaded or unloaded during biological reactions that involve conformational transitions (see Parsegian et al., 2000). Functionally significant changes in protein hydration are conveniently studied by the osmotic stress method, where water activity of the solution is altered by changing the concentrations of solutes (typically polyols, sugars and amino acids) that do not interact with the protein surface. The method is, however, of limited usefulness in the study of water–protein interactions within intact cells, due to cell damage and membrane rupture following osmotic challenge. It is now widely accepted that water acts as allosteric effector in purified proteins *in vitro*, but whether these effects have a real physiological role is still controversial.

Osmotic stress studies have shown opposite effects of water activity on the O_2 affinity of tetrameric vertebrate hemoglobins and dimeric hemoglobin of the clam *Scapharca inaequivalvis*. In human hemoglobin, approximately 60 water molecules are bound per tetramer in the transition from the low-affinity T to the high-affinity R state, which is in good agreement with the increase in solvent-accessible area that follows the T–R allosteric transition (Colombo et al., 1992; Colombo and Bonilla-Rodriguez, 1996; Colombo and Seixas, 1999; Arosio et al., 2002). By contrast, upon O_2 binding the clam hemoglobin releases 6–8 water molecules that stabilize the hemoglobin in the deoxy state by forming highly structured water bridges at the dimer interface (Royer et al., 1996).

Hagfishes, together with lampreys, belong to the Cyclostomes, and occupy a crucial phylogenetic position as the most ancient craniates (Martini, 1998). Their hemoglobin system is thus considered to represent the transition state between invertebrate and vertebrate hemoglobins (Goodman, 1981). The hemoglobin system of *Myxine glutinosa* consists of three major hemoglobin fractions: HbI, HbII and HbIII (Paléus et al., 1971), occuring in a ratio of approximately 15:50:35 (Fago et al., 2001), and are monomeric in the oxygenated state, but reversibly associate into dimers and tetramers when deoxygenated. This monomer–dimer–tetramer equilibrium between high-O_2-affinity monomers and low-affinity oligomers replaces the T–R equilibrium of vertebrate tetrameric hemoglobins and is basic to the allosteric properties of hagfish hemoglobins, such as cooperative O_2 binding and the effects of pH and of bicarbonate, the latter being a major physiological allosteric effector in *M. glutinosa* (Fago and Weber, 1995, 1998; Fago et al., 1999). With the exception of HbII, which self-aggregates at acidic pH, interactions between monomers under physiological conditions occur mainly between HbI and HbII and between HbII and HbIII in the presence of bicarbonate, whereas HbI and HbIII do not show functional interaction (Fago et al., 2001).

The red blood cells of *M. glutinosa* are unable to recover

their original volume after osmotic swelling or shrinking (Brill et al., 1992; Nikinmaa et al., 1993; Dohn and Malte, 1998). This unusual feature is attributed to the lack of membrane proteins essential to volume regulatory responses, such as those involved in the K^+/Cl^- cotransport and in the taurine efflux pathways (Nikinmaa et al., 1993). Moreover, absence of the anion exchanger band III membrane protein (Ellory et al., 1987; Peters et al., 2000) has important consequences for CO_2 and O_2 transport, whereby bicarbonate, a major regulator of O_2 affinity in *M. glutinosa* hemoglobin (Fago et al., 1999), is transported within the red blood cells rather than in plasma. Hagfishes are probably the only marine organisms to have evolved without entering freshwater (see Nikinmaa et al., 1993), and have one of the highest blood electrolyte concentrations, with an osmolality close to that of seawater, which is almost entirely due to high levels of inorganic ions (Robertson, 1963). They are osmoconformers and unable to regulate the osmotic concentration in their blood in response to that of the ambient seawater. While sodium and chloride concentrations in the blood plasma are the same as in seawater, the concentrations of the divalent cations calcium and magnesium are regulated (Robertson, 1963; Fänge, 1998).

These unusual characteristics provide a unique opportunity to study the effects of water activity on hemoglobin function at both the molecular and the cellular (erythrocytic) levels and to gain insight into the evolutionary origin of water effects in hemoglobins. We report here the effect of changes in osmolality on O_2 binding in isolated hemoglobins and in intact red blood cells of the hagfish *M. glutinosa*, using the osmotic stress approach.

Materials and methods

Hemolysate preparation and hemoglobin purification

Specimens of *Myxine glutinosa* L. were trapped at the Kristineberg Marine Biology Station, Sweden, and transported to the Department of Zoophysiology, University of Aarhus, where they were kept in running seawater at 11°C in the dark for at least 2 weeks before blood sampling. Blood was drawn using heparinized syringes from the caudal sinuses of unanaestethized animals after suspending them at the anterior end. Each sampling typically took 1–2 min. Blood samples were centrifuged for plasma removal and the red blood cells were washed three times with 3.4% NaCl. For complete hemolysis the red blood cells were frozen twice in liquid N_2 and thawed. Three volumes of 10 mmol l^{-1} Hepes buffer, pH 7.7, 0.5 mmol l^{-1} EDTA, were then added per unit volume of thawed cells before centrifugation at 200 ***g*** for 30 min to remove cellular debris.

Because of the high degree of polymorphism and hemoglobin multiplicity (Paléus et al., 1971; Fago and Weber, 1995; Fago et al., 2001), individual hemolysates from 91 animals were analysed on a 0.2 mm-thick polyacrylamide isoelectric focusing gel containing a 1:2 ratio of 3.5–10 and 7–9 Ampholines using the Multiphor II System (Amersham Pharmacia Biotech, Uppsala, Sweden). Samples showing identical multiplicity and having three major bands only were selected and pooled (see Fago et al., 2001). Hemoglobin was 'stripped' by gel filtration on a Sephadex G-25 Fine (23×350 mm) column equilibrated with 50 mmol l^{-1} Tris buffer, pH 7.8, 0.1 mol l^{-1} NaCl. The hemoglobin solution was then dialysed against CO-equilibrated 10 mmol l^{-1} Tris buffer, pH 7.9 and the three individual components were separated by anion exchange chromatography on a DEAE-Sephacel column (23×140 mm), equilibrated with 10 mmol l^{-1} Tris buffer, pH 7.9, and eluted in a 0–0.25 mol l^{-1} NaCl linear gradient as described (Fago et al., 2001). The isolated hemoglobins and the stripped hemolysate were concentrated under CO pressure in an Amicon Ultrafiltration Cell or in Ultrafree-4 Centrifugal Filter Units (Millipore, Billerica, MA, USA) (5000 MW cut-off) and dialysed against three changes of CO-equilibrated 10 mmol l^{-1} Hepes buffer, pH 7.7, 0.5 mmol l^{-1} EDTA. The samples were then divided into small portions that were thawed immediately before O_2 equilibrium measurements according to standardized procedures (Weber, 1992; Weber et al., 2000). All preparative steps were performed at 0–4°C. Recordings of the visible spectrum (450–700 nm) during hemoglobin preparation showed no evidence of oxidation, as judged by absorbance at 630 nm.

O_2 equilibria of hemoglobin solutions at various osmolalities; the osmotic stress method

O_2 equilibria of the hemolysate and of solutions containing HbI and HbII or HbII and HbIII at 1:1 molar ratios were measured at different osmolalities at 10°C, 0.5 mmol l^{-1} heme concentration, in 0.1 mol l^{-1} Hepes buffer at pH 7.3, close to the physiological pH in hagfish red blood cells (Tufts and Boutilier, 1990). A modified gas diffusion chamber connected to cascaded Wösthoff gas pumps, mixing pure N_2 (>99.998%), O_2 and air, was used to obtain stepwise increases in O_2 saturation, while changes in absorption at 436 nm were continuously recorded (Weber, 1981). Bicarbonate was added to hemoglobin solutions by introducing 4% CO_2 in the gas mixture and adding 1 μmol l^{-1} carbonic anhydrase to catalyse the rapid conversion of CO_2 into bicarbonate and hydrogen ions. Oxidation during O_2 equilibrium measurements was typically less than 5%, as judged from changes in absorbance between 0% and 100% oxygenation. Hill plots, $\log[S/(1–S)]$ *versus* $\log P_{O_2}$ (where S is the fractional saturation), were used to interpolate O_2 affinity (P_{50}, half-saturation O_2 tension) and cooperativity (expressed as Hill coefficient n_{50}, the slope of the Hill plot at half-saturation).

A BMS 2 MK 2 (Radiometer, Copenhagen, Denmark) thermostatted microelectrode was used to measure pH. In O_2 equilibrium experiments made in the presence of CO_2, pH measurements were carried out in hemoglobin subsamples placed in microtonometers equilibrated near P_{50} and in the presence of 4% CO_2.

Different water activities (a_w) in the samples were obtained by adding glucose or glycine to final concentrations ranging between 1 and 1000 mmol l^{-1}. These solutes were chosen for their opposite effects on the dielectric constant of the solution,

in order to eliminate factors related to electrostatic effects that may affect O_2 affinity (Colombo and Bonilla-Rodriguez, 1996). Osmolalities (Osm) were measured on a Semi-Micro Osmometer (Knauer, Kiel, Germany) in 50 μl samples and water activities (a_w) were calculated (Colombo and Bonilla-Rodriguez, 1996) as:

$$\log_e a_w = -\mathrm{Osm}/M_w\,, \quad (1)$$

where Osm is the solution osmolality (osmol kg^{-1}) and M_w is the molality of pure water (55.56 mol kg^{-1}). The number of water molecules bound upon heme oxygenation was derived from the Wyman linkage equation (Wyman, 1964):

$$\partial\log_e(P_{50})/\partial\log_e(a_w) = -(n_w{}^{oxy} - n_w{}^{deoxy}) = -\Delta n_w\,, \quad (2)$$

where P_{50} is the O_2 affinity obtained in the presence of glycine or glucose, and $n_w{}^{oxy}$ and $n_w{}^{deoxy}$ are the number of water molecules bound in the oxy and deoxy form of hemoglobin, respectively. It should be recalled that the Wyman linkage equation can quantify binding of an allosteric effector (in this case, water) only when other effectors are present at constant activity. Since solute concentration also changes in osmotic stress experiments, it is essential that solute molecules do not undergo O_2-linked binding to the protein molecule. Accordingly, the effect of direct solute binding was examined by:

$$\partial\log_e(P_{50})/\partial\log_e\mathrm{Osm} = -(n_s{}^{oxy} - n_s{}^{deoxy}) = -\Delta n_s\,, \quad (3)$$

where $n_s{}^{oxy}$ and $n_s{}^{deoxy}$ indicate the number of glycine or glucose molecules bound in the oxy and deoxy form of hemoglobin, respectively. Δn_s values close to zero indicate that the solute is indeed inert and suitable for osmotic stress experiments.

O_2 equilibria of red blood cells suspensions at various osmolalities

Individual blood samples were centrifuged at 40 ***g*** for plasma removal, and the red blood cells were washed in ice-cold 1000 mosmol kg^{-1} Ringer consisting of (mmol l^{-1}): 504 NaCl, 8 KCl, 5 $CaCl_2$, 3 $MgSO_4$, 9 $MgCl_2$, 5 glucose and 13.4 $NaHCO_3$. The cells were then resuspended by adding the same volume of Ringer as the volume of plasma removed and stored on ice overnight. The cells were then washed twice in several volumes of either 250, 500, 1000, 2000, 3000 or 4000 mosmol kg^{-1} saline Ringer, centrifuged and resuspended by adding solutions of the different molalities to obtain the original blood volumes. The Ringer solution was made hypo-osmotic by adding distilled water containing 13.4 mmol l^{-1} $NaHCO_3$ and hyperosmotic by adding 1 mol l^{-1} NaCl. The ensuing shrinkage/swelling of the red blood cells was confirmed by measurement of the hematocrit and followed the linear relationship $Hct/Hct_{1000}=0.71(\text{mosmol kg}^{-1}/1000)+0.29$, where Hct_{1000} is the hematocrit at 1000 mosmol kg^{-1} (Dohn and Malte, 1998).

Oxygen equilibrium curves were measured by the diffusion chamber method described above. Spectra in the range 410–600 nm (2 nm wavelength interval and a measuring time of 0.4 s at each wavelength) were recorded by a Cary 50 Probe spectrophotometer (Palo Alto, CA, USA) connected to a computer employing the Cary WinUV program for spectra analysis. After baseline recording with the microscope slide in the chamber, a 1 μl sample of red blood cell suspension was spread on the measurement area of the slide and left in the chamber for stabilization for at least 20 min. Spectra were measured after equilibration to 1, 2, 4, 8, 16, 32 and 55% air, as well as in pure N_2 (deoxy) and 100% air (oxy). The concentration of CO_2 in the gas mixtures entering the chamber was held constant at 0.5% (approx. 3.5 mmHg, 0.47 kPa). All measurements were carried out at 12°C. A spread-sheet programme (developed by H. Malte and S. Frische, Aarhus University) was used to calculate the O_2 saturations at each O_2 tension by linear least-square fitting of the oxy and deoxy reference spectra to the observed spectra. P_{50} and n_{50} values were interpolated from Hill plots as described above.

The number of O_2-linked water and solute molecules was calculated from Equations (2) and (3), respectively. The osmolality values employed for the calculations were those of the Ringer solution.

Results

The linkage plots for hemoglobin solutions (Fig. 1) show the relative changes in O_2 affinity [$\log_e(P_{50}/P_{50}^0)$], where P_{50}^0 values are those obtained in the absence of glycine or glucose)] as a function of water activity ($\log_e a_w$). The hemoglobins known to show cooperative functional interaction at pH 7.3, namely the equimolar mixtures HbI+II and HbII+III, were investigated in the presence and absence of bicarbonate (from CO_2). As evident from Fig. 1, the effects of glycine and glucose on O_2 binding are equivalent despite their dissimilar chemical properties, showing that the observed increases in O_2 affinity upon their addition are related to changes in water activity and not to the concentrations of the solutes, in agreement with previous data on human hemoglobin (Colombo and Bonilla-Rodriguez, 1996). There is a clear inverse relationship between O_2 affinity and water activity in all cases investigated, with the exception of HbII+HbIII (Fig. 1E,F), where the P_{50} remains practically unchanged for the range of osmolalities investigated. The decreased O_2 affinity with increasing water activity (decreasing osmolality) indicates that water molecules preferentially bind to the low-affinity, aggregated state of the hemoglobin, and are liberated upon O_2 binding. The number of O_2-linked water molecules per heme that are involved in the stabilization of the oligomeric, aggregated state can be calculated from the slopes of the linear regressions of the linkage plots and are shown in Table 1. The complex formed by HbI+II shows the highest number of water molecules involved in its stabilization, namely approx. 35 per heme in the presence of bicarbonate, whereas the hemolysate shows a lower dependence on water activity (approx. 10 water molecules per heme stabilize the oligomeric state). CO_2 increases the slopes of the linkage plots and the number of water molecules bound by deoxy-hemoglobin.

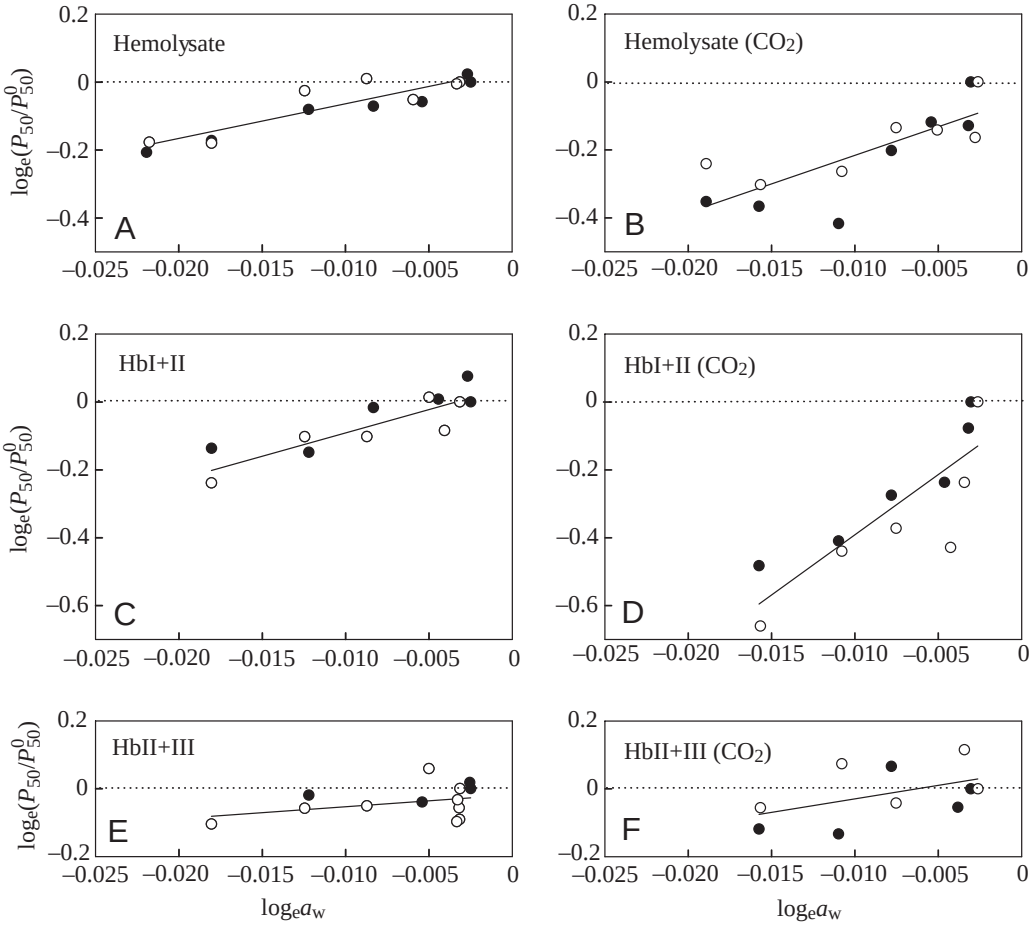

Fig. 1. Relative change in P_{50} ($\log_e P_{50}/P_{50}^0$) as a function of water activity $\log_e a_w$ in stripped hemolysate (A,B) and in equimolar hemoglobin mixtures of HbI and HbII (HbI+II; C,D) and HbII and HbIII (HbII+III; E,F) in the absence (A,C,E) and presence (B,D,F) of CO_2, and the presence of glycine (filled circles) or glucose (open circles). For regression coefficients, see Table 1.

In order to evaluate direct binding of solutes (glycine and glucose) to hemoglobin, the relative P_{50} shifts given in Fig. 1 were plotted against the logarithm of solution osmolality, which is directly proportional to solute activity (Fig. 2). The calculated slopes are either close to zero or slightly negative (Table 1), and according to Wyman's linkage equation (Wyman, 1964), this indicates that these solutes do not interact with the protein or, that if they do, they do not perturb the allosteric equilibrium, thus meeting the requirement for their use in osmotic stress experiments. The Hill coefficient, n_{50}, remained approximately constant within each series of experiments, regardless of the nature of solute used (not shown), justifying the use of P_{50} instead of the median O_2 tension, P_m, in the linkage equations (Wyman and Gill, 1990). The hemolysate and hemoglobin equimolar mixtures show low cooperativity, with Hill coefficients in the range 1.0–1.3, in agreement with previous data (Bauer et al., 1975; Fago and Weber, 1995; Fago et al., 2001).

Fig. 3 shows O_2 equilibria of red blood cell suspensions at different osmolalities. An increase in osmolality of the medium from 1000 to 4000 mosmol kg^{-1} causes shrinkage of the red blood cells and increased O_2 affinity (the O_2 equilibrium curve shifts to the left), whereas O_2 affinity decreases upon swelling (the curve shifts to the right), while cooperativity remains close to unity for all osmolalities, which agrees with data for *M. glutinosa* blood (Perry et al., 1993). Hemoglobin concentrations in intact red blood cells (before equilibration at different osmolalities) ranged from 9.3 to 11.7 mmol l^{-1} heme. Following equilibration at different osmolalities, protein concentration linearly increased from 3.3±0.4 mmol l^{-1} heme (250 mosmol kg^{-1}) to 22.3±2.5 mmol l^{-1} heme (4000 mosmol kg^{-1}). Fig. 4 shows the relative shifts in P_{50} values as a function of water activity for both intact red blood

Table 1. *Number of water ($-\Delta n_w$) and solute molecules ($-\Delta n_s$) released during transition from deoxy- to oxy-hemoglobin samples*

	$-\Delta n_w$		$-\Delta n_s$	
	No CO_2	+CO_2	No CO_2	+CO_2
HbI+II	13.55±2.46	35.51±7.22	–0.10±0.02	–0.27±0.48
HbII+III	3.54±2.70	7.96±5.04	–0.02±0.02	–0.05±0.04
Hemolysate	10.33±0.03	16.81±3.76	–0.09±0.01	–0.15±0.03
RBC	–	7.31±1.07	–	–0.11±0.01

Values are means ± S.E.M. calculated from the slopes of the plots in Figs 1, 2 and 4 according to Equations 2 and 3 in Materials and methods.

HbI+II, HbII+III and hemolysate were studied in presence and absence of 4% CO_2, and red blood cells (RBC) in the presence of 0.5% CO_2.

Values for RBC refer to the osmolality range 0.25–2.0 osmol kg^{-1}.

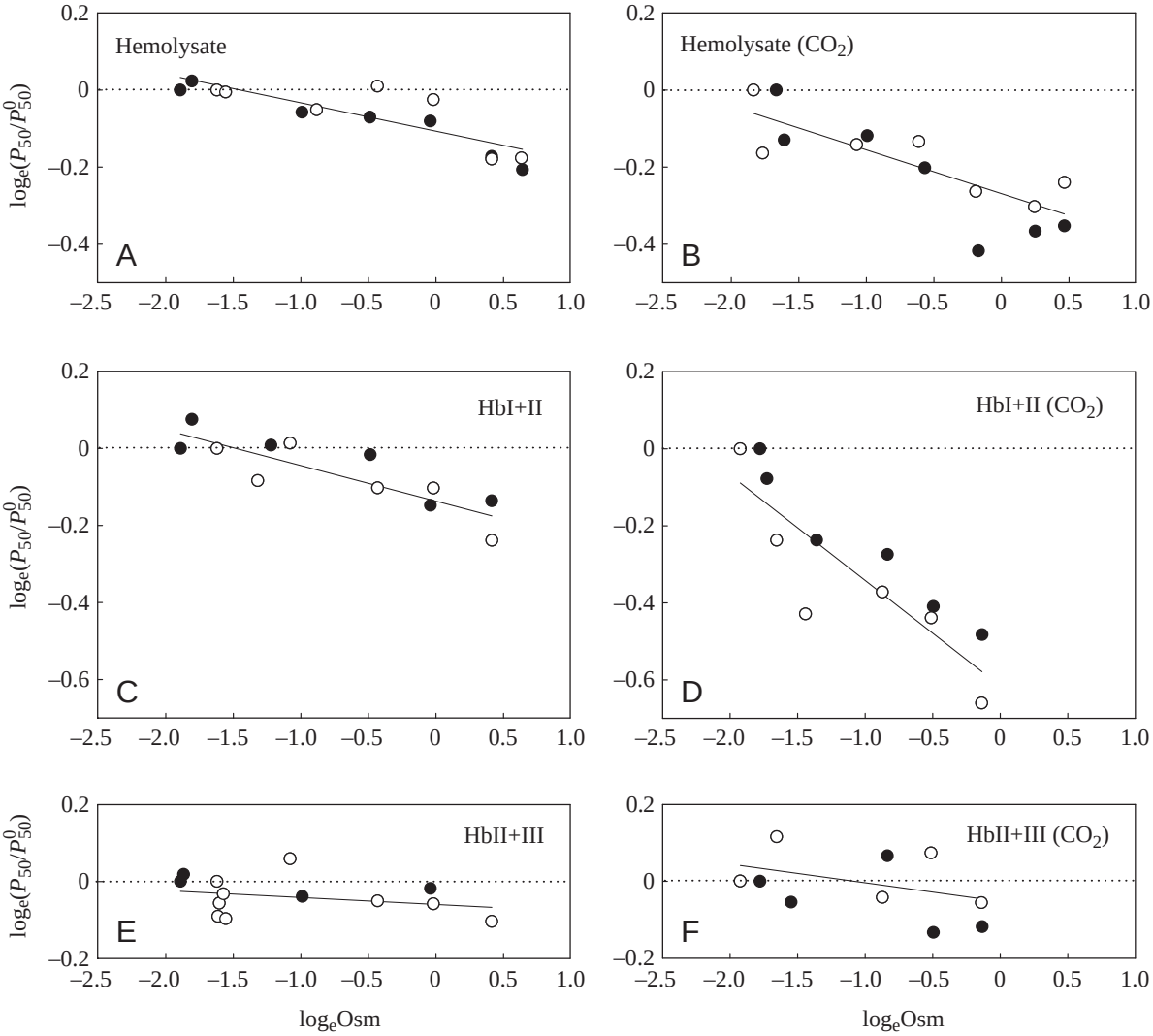

Fig. 2. Relative change in P_{50} ($\log_e P_{50}/P_{50}^0$) as a function of solution osmolality ($\log_e$Osm) in stripped hemolysate (A,B) and in equimolar hemoglobin mixtures of HbI and HbII (HbI+II; C,D) and HbII and HbIII (HbII+III; E,F) in the absence (A,C,E) and presence (B,D,F) of CO_2, and the presence of glycine (filled circles) or glucose (open circles). For regression coefficients, see Table 1.

cells and the hemolysate in the absence and presence of CO_2. As shown, the slope of the regression line for red blood cells is remarkably similar to that of the hemolysate in the range 0.25–2.0 osmol kg^{-1}, but increases at higher osmolalities (Fig. 4, Table 1), indicating that a greater number of water molecules is released upon oxygenation. Solutes from the Ringer solution do not bind directly to hemoglobin in red blood cells, which is indicated by the dependence of the relative shifts in P_{50} as a function of $\log_e$Osm (Table 1).

Discussion

The O_2-binding properties of *Myxine glutinosa* hemoglobins are sensitive to changes in water activity. Given that water activity decreases O_2 affinity, water molecules bind preferentially to the oligomeric, low-affinity state, whereby water acts as an allosteric effector. This response is opposite to that of human hemoglobin, where water stabilizes the oxygenated, high-affinity state of the hemoglobin, but similar to that found in the dimeric hemoglobin from the gastropod mollusc *Scapharca inaequivalvis*, where 6–8 water molecules bind at the dimer interface in the deoxy conformation (Royer et al., 1996). Interestingly, the recently solved crystal structure of dimeric (deoxy) hagfish hemoglobin (Mito et al., 2002) indicates a subunit arrangement similar (but not identical) to that of lamprey and dimeric *Scapharca* hemoglobins, with the two heme groups in close contact with each other (Heaslet and Royer, 1999; Royer, 1994).

M. glutinosa possess a complex system of interacting and non-interacting monomeric hemoglobins that reversibly associate when deoxygenated to form dimers and tetramers (Fago and Weber, 1995; Fago et al., 2001). One would expect that an increase in water activity would favour dissociation into monomers as the protein surface area exposed to solvent

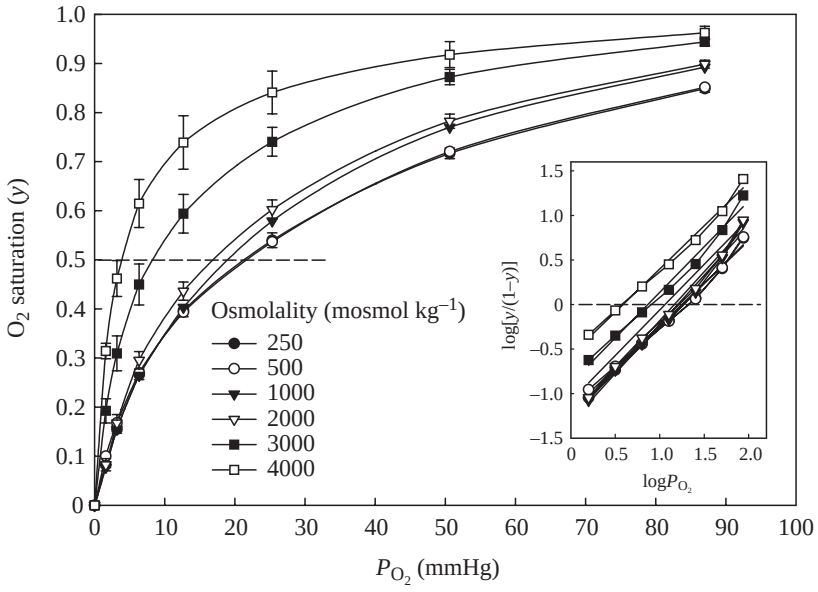

Fig. 3. Oxygen equilibrium curves for intact red blood cells equilibrated at various osmolalities (0.25–1.0 osmol kg^{-1}). Inset, Hill plots of the data. Values are means ± S.E.M. obtained from fitting the observed spectra.

increases, but in hagfish hemoglobin the opposite is true. Since water molecules stabilize the dimeric/tetrameric state, it appears that during the association between monomers, specific water binding sites become available that bridge individual monomers. Accordingly, the number of water molecules stabilizing the associated state neatly correlates with increasing tendency of the hemoglobins to associate, being highest in HbI+II in the presence of bicarbonate, where tetramers are formed upon deoxygenation, and lowest in HbII+III in the absence of bicarbonate, where the hemoglobin remains monomeric when deoxygenated, as observed by sedimentation velocity experiments (Fago et al., 2001). Bicarbonate increases the tendency to associate and favours formation of low-affinity oligomers (Fago et al., 1999, 2001), and consequently the number of water molecules involved in oligomer stabilization increases in the presence of CO_2 (Fig. 1, Table 1). In addition, bicarbonate and water may have a synergistic effect in stabilizing the dimeric and tetrameric states of the hemoglobin.

A major finding of this study is that O_2 affinity is similarly affected by changes in osmolalities (and water activities) both in red blood cells and in the purified hemolysate; in both cases it increases as water activity decreases. Moreover, around the physiological value of approximately 1000 mosmol kg^{-1} ($\log_e a_w$ approx. –0.02) the number of water molecules stabilizing the oligomeric state of the hemoglobin (that are liberated upon O_2 binding) is similar in the purified hemolysate and in the intact cells (Fig. 4, Table 1). This finding, which takes advantage of the exceptional ability of *M. glutinosa* red blood cells to withstand osmolality changes, reflects a true allosteric role of water in regulating O_2 binding at a cellular level. Since the monomer–monomer association constants are not known, it is not possible to evaluate the extent of association of the hemoglobin complexes formed either in hemolysate or in intact red blood cells at a specific protein concentration. However, at much lower protein concentrations (60 μmol l^{-1} heme) than those used in this study (500 μmol l^{-1} heme) and in the presence of bicarbonate, the deoxygenated hemolysate consists entirely of monomers and tetramers, as shown by gel filtration and ultracentrifugation experiments (Fago et al., 1999, 2001), indicating that formation of the tetrameric species HbI+II is favoured over the dimeric HbII+III species, and that water sensitivity in the hemolysate and in the red blood cells may thus be largely attributed to HbI+II, although this species represents only approximately 15% of the total hemoglobin (Fago et al., 2001). Moreover, since the dependence of the O_2 affinity on changes in water activity becomes larger in intact red cells than in pure hemolysate at high osmolalities ($\log_e a_w$ below –0.04), the formation of other types of monomer–monomer associations in shrunken cells (i.e. where protein concentration is high) than those hitherto identified *in vitro* cannot be excluded. Interestingly, the six monomeric hemoglobins from the sea lamprey *Petromyzon marinus* create a complex network of possible associations (Rumen and Love, 1963). Alternatively, the weak binding of solute to oxygenated hemoglobin (Table 1) may become strong at higher solute concentration.

Changes in red blood cell volume have opposite effects on intracellular water activity and protein concentration. Specifically, in swollen red blood cells, an increase in water

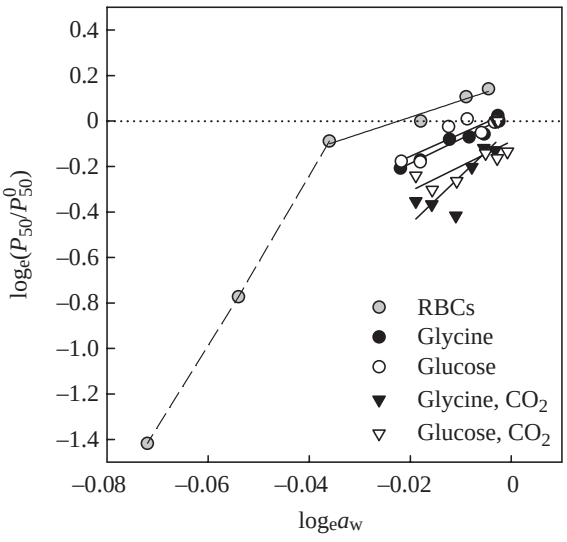

Fig. 4. Effect of solution water activity on the relative change in P_{50} ($\log_e P_{50}/P_{50}^0$) in red blood cells (RBC) (Fig. 3) (grey circles) and in stripped hemolysate (Fig. 1) with added glycine (filled symbols) or glucose (open symbols) in the absence (circles) and presence (triangles) of CO_2.

activity would shift the allosteric monomer–oligomer equilibrium towards oligomer formation and thus lower O_2 affinity, whereas protein dilution would favour monomer formation and thus increase O_2 affinity. Our results indicate that the water effect is strong enough to overshadow protein dilution and to be measurable during osmotic stress experiments. In lampreys, monomeric hemoglobins similarly associate upon deoxygenation, and erythrocyte shrinking (with the consequent increase in protein concentration) decreases O_2 affinity (Airaksinen and Nikinmaa, 1995); this is in contrast to hagfish red blood cells, suggesting that lamprey hemoglobins have little or no sensitivity to changes in water activity. Accordingly, no water molecules were detected at the dimeric interface of the deoxy form of the lamprey *Petromyzon marinus* HbV (Heaslet and Royer, 1999), although this could be due to the fairly low resolution of the crystallographic structure (2.7 Å).

Although the O_2 affinity of hagfish hemoglobin depends on water activity, blood O_2 binding is unlikely to be affected *via* this mechanism in the natural environment where salinity is constant. Presumably hagfishes have never been exposed to changes in water osmolality during evolution and they do not show the adrenergic red cell swelling upon exposure to hypoxia encountered in teleosts, despite the increased production of noradrenaline (Perry et al., 1993). The low cooperativity and the small Bohr and anion effects (except that of bicarbonate) observed in the hemoglobin of hagfish appear to secure O_2 transport under their natural conditions and mode of life (constant salinity and temperature, slow-moving behaviour; Wells et al., 1986).

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