the FEBS Journal

Oxygen binding properties of non-mammalian nerve globins

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Keywords
neuroglobin; nerve hemoglobin; oxygen-binding; heme coordination

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(Received 5 December 2005, revised 23 January 2006, accepted 27 January 2006)

doi:10.1111/j.1742-4658.2006.05158.x

Oxygen-binding globins occur in the nervous systems of both invertebrates and vertebrates. While the function of invertebrate nerve haemoglobins as oxygen stores that extend neural excitability under hypoxia has been convincingly demonstrated, the physiological role of vertebrate neuroglobins is less well understood. Here we provide a detailed analysis of the oxygenation characteristics of nerve haemoglobins from an annelid (*Aphrodite aculeata*), a nemertean (*Cerebratulus lacteus*) and a bivalve (*Spisula solidissima*) and of neuroglobin from zebrafish (*Danio rerio*). The functional differences have been related to haem coordination: the haem is pentacoordinate (as in human haemoglobin and myoglobin) in *A. aculeata* and *C. lacteus* nerve haemoglobins and hexacoordinate in *S. solidissima* nerve haemoglobin and *D. rerio* neuroglobin. Whereas pentacoordinate nerve globins lacked Bohr effects at all temperatures investigated and exhibited large enthalpies of oxygenation, the hexacoordinate globins showed reverse Bohr effects (at least at low temperature) and approximately twofold lower oxygenation enthalpies. Only *S. solidissima* nerve haemoglobin showed apparent cooperativity in oxygen binding, suggesting deoxygenation-linked self-association of the monomeric proteins. These results demonstrate a remarkable diversity in oxygenation characteristics of vertebrate and invertebrate nerve haemoglobins that clearly reflect distinct physiological roles.

Invertebrate haemoglobins (Hbs) exhibit an astonishingly large variation in structure (molecular masses ranging from 12 to 3600 kDa) and functions that, apart from transporting and storing O_2, involve sensing and scavenging O_2, transporting NO and sulfide, regulating buoyancy and acting as enzyme, optical pigment and as catalyst of redox reactions [1].

The histological sites where intracellular invertebrate Hbs are encountered vary accordingly and include muscle, gill, gamete and nerve cells [1]. Nerve haemoglobins have been known for decades to occur in invertebrates [2], where they are mainly found in glial cells, often at high (mM) concentrations. In the absence of O_2 or other external ligands some invertebrate nerve Hbs show UV-visible absorbance spectra that resemble those of cytochrome *b* type pigments [3] rather than those typical of Hbs. In these so-called hexacoordinate globins, such as those of the bivalves *Spisula solidissima* and *Tellina alternata*, the distal HisE7 coordinates the sixth position of the haem iron in the absence of external ligands. Other nerve globins, such as those of the polychetous annelid *Aphrodite aculeata* and the nemertean worm *Cerebratulus lacteus,* show a pentacoordinate haem geometry when deoxygenated, as found

Abbreviations
Cygb, cytoglobin; Hbs, haemoglobins; Mb, myoglobin; Ngb, neuroglobin.

in Hb and myoglobin (Mb) of vertebrates. The nerve Hb of *C. lacteus* is the smallest globin protein known so far, with only 109 amino acid residues [4] instead of the standard ≈140–150 residues of globins.

A function in O_2 delivery to the highly metabolically active nerves is well established for invertebrate nerve Hbs [3–6] The seminal study by Kraus and Colacino [6] showed that nerve activity in the clam *T. alternata* persisted for ≈30 min after the induction of anoxia and correlated with the oxygenation state of nerve Hb, whereas nerve activity ceased upon O_2 removal in a related species (*T. plebeius*) lacking nerve Hb [6]. Similar studies on *S. solidissima* [3] have shown that the presence of nerve Hb can prolong nerve activity during anoxic episodes by functioning as an O_2 store. The same functional role has been proposed for the pentacoordinate nerve Hbs of *A. aculeata* [5] and *C. lacteus* [4].

Until the recent discovery of neuroglobin (Ngb) in neurons of the brain [7], the peripheral nervous system [8] and the retina [9], nerve Hbs were not known to occur in vertebrates. The physiological function of vertebrate Ngb is, however, less clear. Ngb displays greater sequence similarity (30%) with annelid *A. aculeata* nerve Hb than with vertebrate Hbs and Mbs (< 25 and < 21%, respectively), suggesting a common ancestry of invertebrate nerve Hbs and vertebrate Ngbs [5,7]. It has been proposed that vertebrate Ngb may play a role in O_2 supply of neurons, similar to invertebrate nerve globin [7,9]. Recent data, however, argue rather for a role of Ngb in scavenging of reactive oxygen and nitrogen species, including peroxynitrite [10].

Although the role of invertebrate nerve Hbs in supplying O_2 is clear, the available data on their O_2 equilibrium properties are fragmentary. We report here the oxygenation characteristics and their dependence on pH and temperature of pentacoordinate nerve Hbs of the annelid *A. aculeata* and the nemertean *C. lacteus*, of hexacoordinate nerve Hb of the bivalve mollusc *S. solidissima* and of hexacoordinate Ngb of the zebrafish, *Danio rerio*, and find basic functional differences between pentacoordinate and hexacoordinate nerve Hbs and vertebrate Ngbs.

Results

The nerve globins studied here exhibit markedly different O_2 affinities, *A. aculeata* nerve Hb having the lowest O_2 affinity (highest half-saturation oxygen tension, P_{50}) and *S. solidissima* nerve Hb the highest affinity (P_{50} = 1.1 and 0.3 torr, respectively, at 20 °C, pH 7.0) (Fig. 1, Table 1). The distinction was valid at all temperatures (12–30 °C) and pH values (≈ 6.5–8.0) investigated (Fig. 2).

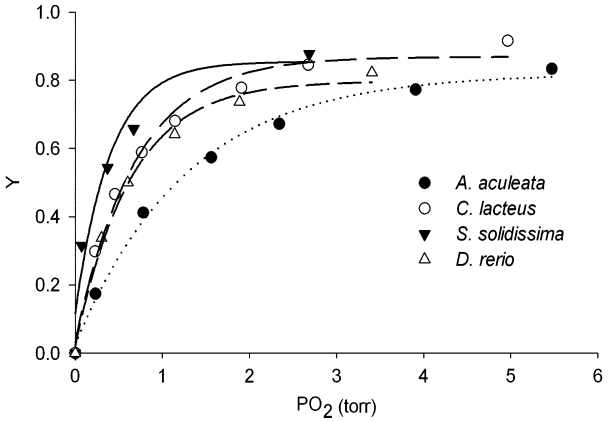

Fig. 1. Fractional O_2 saturation (Y) as a function of O_2 partial pressure for the nerve Hbs of *A. aculeata*, *C. lacteus*, *S. solidissima* and Ngb of *D. rerio* at 20 °C and pH 7.0, in 0.1 M Tris, 0.5 mM EDTA, 0.07–0.1 mM heme.

Table 1. P_{50} values (at 20 °C and pH 7.0) and overall ΔH-values (pH 7.4) for nerve Hbs from the four species.

Species	P_{50} (torr)	ΔH (kcal·mol^{-1})	Reference
A. aculeata	1.1	−21.1	This study
	1.2		[5]
C. lacteus	0.6	−19.7	This study
	0.6		[14]
S. solidissima	0.3	−11.0	This study
	0.6		[12]
D. rerio	0.7	−11.6	This study
	0.9		[13]

The globins showed either a reverse Bohr effect (P_{50} decreases with falling pH) or no pH sensitivity of O_2 affinity. A reverse Bohr effect was observed in the hexacoordinate globins of *S. solidissima* (Fig. 2C) and *D. rerio* (Fig. 2D), albeit only at low temperature (10 °C) and pH (< 6.5) in the latter species. In contrast, O_2 affinity was pH insensitive in the pentacoordinate nerve Hbs of *A. aculeata* (Fig. 2A) and *C. lacteus* (Fig. 2B). Except for *S. solidissima* nerve Hb, the Hill coefficients (n_{50}) were close to unity and independent of temperature and pH, as expected for noninteracting monomeric proteins (Fig. 2). The cooperativity coefficient of *S. solidissima* nerve Hb increased with increasing pH and decreasing temperature, attaining 1.5 (Fig. 2C).

The apparent heat of oxygenation for each globin was calculated from the slope of the van't Hoff plot, using P_{50} values obtained at pH 7.4. As illustrated by the negative slopes of the plots (Fig. 3, Table 1), all

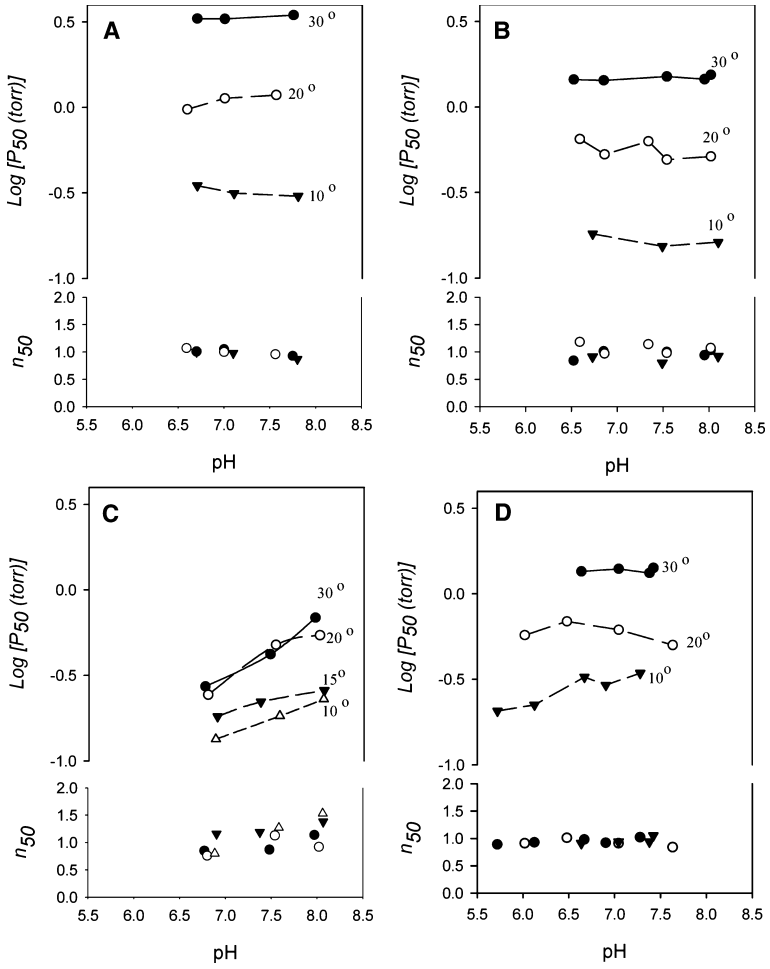

Fig. 2. O_2 affinity (log P_{50}) and Hill's coefficient (n_{50}) values of (A) *A. aculeata*, (B) *C. lacteus*, (C) *S. solidissima* nerve Hbs and (D) *D. rerio* Ngb in 0.1 M Tris, 0.5 mM EDTA as a function of pH at different temperatures as indicated. *D. rerio* globin solutions contained MetHb reducing reagents [28].

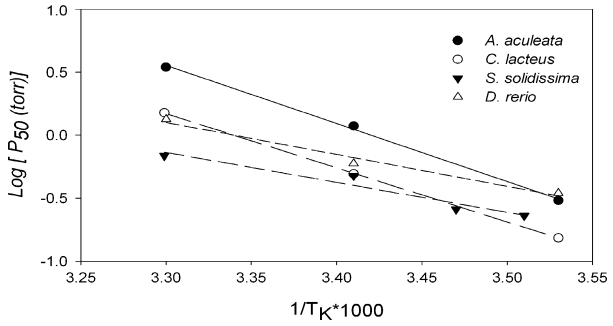

Fig. 3. Van't Hoff plots of *A. aculeata*, *C. lacteus*, *S. solidissima* nerve Hbs and *D. rerio* Ngb at pH 7.4. The log P_{50} values at various temperatures were interpolated from Fig. 1. The negative slope indicates exothermic O_2 binding.

the globins exhibited exothermic oxygenation reactions (O_2 affinity decreased with increasing temperature). Interestingly, the pentacoordinate nerve Hbs of *A. aculeata* and *C. lacteus* showed markedly higher overall heat release upon O_2 binding ($\Delta H = -21.1$ and -19.7 kcal·mol^{-1}, respectively) than the hexacoordinate globins of *S. solidissima* and *D. rerio* ($\Delta H = -11.0$ and -11.6 kcal·mol^{-1}, respectively). In turn, these latter globins showed slightly lower temperature sensitivities than sperm whale Mb (-14.9 kcal·mol^{-1}) [11], human Ngb (-15.7 kcal·mol^{-1} at temperatures > 18 °C) [10] and human cytoglobin (Cygb) (-14.3 kcal·mol^{-1}) [10]. Inspection of Fig. 2C showed that whereas a temperature increase from 12 to 20 °C decreased O_2 affinity of *S. solidissima* nerve Hb strongly at widely different pH values, a further temperature increase to 30 °C had essentially no effect. This contrasts with findings for the other globins studied here, all of which showed a steady decrease in O_2 affinity with increasing temperature.

A comparison of the functionally important amino acid residues located in the haem pocket (Table 2) shows that the pentacoordinate nerve globin of *C. lacteus* differs markedly from the other globins as it has Tyr, Gln and Thr at positions B10, E7 and E11, respectively. The hexacoordinate globin of *D. rerio*

Table 2. Functionally important amino acid residues in the haem pocket of human Mb and Ngb, *D. rerio* Ngb, *S. solidissima* nerve Hb, *C. lacteus* nerve Hb and *A. aculeata* nerve Hb.

Species	B10	E7	E10	E11	F8
Human Mb	Leu	His	Thr	Val	His
Human Ngb	Phe	His	Lys	Val	His
D. rerio Ngb	Phe	His	Lys	Val	His
S. solidissima Hb	Phe	His	Asn	Phe	His
C. lacteus Hb	Tyr	Gln	Lys	Thr	His
A. aculeata Hb	Phe	His	Lys	Phe	His

differs from the other globins here investigated in having Val at position E11 whereas only *S. solidissima* has Asn at position E10 instead of Lys (Table 2).

Discussion

Cooperative and noncooperative oxygen binding in nerve globins

The nerve Hbs of the invertebrate species investigated, *A. aculeata, C. lacteus* and *S. solidissima* and the Ngb of the zebrafish *D. rerio* show markedly different O_2 affinities (Fig. 1, Table 1). The here-reported P_{50} values are in good agreement with those inferred from previous kinetic studies (Table 1). In the high affinity nerve Hb of *S. solidissima* the dissociation rate for O_2 is low ($k_{off} = 30\ s^{-1}$) and the association rate is high, almost diffusion-limited ($130 \times 10^6\ M^{-1}{\cdot}s^{-1}$) [12], whereas a faster dissociation ($k_{off} = 360\ s^{-1}$) and similarly high association rate ($k_{on} = 170 \times 10^6\ M^{-1}{\cdot}s^{-1}$) correlate with the lower O_2 affinity found for the nerve Hb of *A. aculeata* [5].

With the exception of *S. solidissima*, the globins here investigated bind O_2 in a noncooperative manner as expected for monomeric structures [5,13,14]. Interestingly, the pentacoordinate nerve Hb from *A. aculeata* also binds O_2 noncooperatively despite the homodimeric structure previously observed by gel filtration [5], indicating that the two identical subunits are functionally independent. In contrast, the cooperativity coefficients above unity found in *S. solidissima* nerve Hb are consistent with haem–haem interactions, possibly within the proposed dimeric structure [3]. The dependence of cooperativity and O_2 affinity on pH and temperature may moreover reflect changes in the association state of the nerve Hb, where low pH and elevated temperatures would favour dissociation into monomers. The *in situ* P_{50} values of ≈ 2.3 and ≈ 2.9 torr found for Hb in intact nerves from *S. solidissima* and *C. lacteus* at 15 °C [3,4], respectively, are significantly higher than those found here at low globin concentrations (Fig. 1). Moreover, the *in situ* studies [3,4] showed cooperative O_2 binding that is not seen under our *in vitro* conditions. Vandergon *et al.* [4] assigned the cooperative O_2 binding seen in *C. lacteus* nerves to self association of the deoxygenated globin to at least tetramers, favoured by the high protein concentration found in the nerves (2–3 mM haem), suggesting that oxygenation and *in vitro* dilution cause dissociation into high-affinity dimers and monomers. The high O_2 affinity and the lack of cooperativity observed in this study for purified *C. lacteus* nerve Hb agree with earlier conclusions of a monomeric structure at low protein concentrations [15]. However, the existence of allosteric cofactors or interacting proteins that can modulate affinity and cooperativity *in vivo* cannot be excluded. Also for hexacoordinate *S. solidissima* nerve Hb the mechanisms that control O_2 affinity and cooperativity appear complex and deserve further study.

Absence of normal Bohr effect in nerve globins

The nerve globins here studied show reverse Bohr effects or no pH sensitivity at all. Given the absence of a Bohr effect in pentacoordinate vertebrate Mbs, the lack of pH sensitivity in pentacoordinate *C. lacteus* and *A. aculeata* nerve Hbs is not surprising. This result is in agreement with previous studies showing absence of a Bohr effect *in situ* in *C. lacteus* Hb in the pH range 7.3–7.9 [4]. In contrast, hexacoordinate *S. solidissima* nerve Hb clearly shows a reverse Bohr effect (Fig. 2C) as also is observed in *D. rerio* Ngb at low temperature and pH (Fig. 2D). Human Ngb similarly displays a reverse Bohr effect at temperatures below ≈ 18 °C [16] and, as with *D. rerio* Ngb, this effect disappears at higher temperatures, suggesting that temperature dependence of the pH sensitivity is a common character of vertebrate Ngbs. The Bohr effect in human Ngb depends primarily on the presence of the HisE7 distal residue [16], which is present in the hexacoordinate globins as well as in the pentacoordinate globin of *A. aculeata* (Table 2). The reverse Bohr effect in hexacoordinate nerve globins can be ascribed to protonation at the HisE7 at low pH, which increases O_2 affinity as the residue swings out of the pocket [16]. In human and mouse Ngb this opening of the haem pocket also involves the rupture of the bond between a haem propionate and the side chain of LysE10, that blocks access to the haem for external ligands [16,17]. Consistently the reverse Bohr effect is more pronounced in *S. solidissima* nerve Hb having Asn at position E10 than in *D. rerio* Ngb having LysE10, which will bind a negatively charged propionate more strongly than Asn. Overall, the haem pocket of

S. solidissima nerve Hb appears to be more accessible to solvent than that of other hexacoordinate globins studied [12], which contributes to the high O_2 affinity observed. A different mechanism operates in *C. lacteus* nerve Hb, where the ThrE11 residue is a major factor controlling O_2 affinity. In this Hb, TyrB10 and GlnE7 in the distal haem pocket may strongly stabilize the bound O_2 as seen in the Hb of the nematode *Ascaris suum* that exhibits an extremely high O_2 affinity [18]. However, in *C. lacteus* nerve Hb the presence of polar Thr rather than Val in position E11 (as in *A. suum* Hb) modifies the orientation of TyrB10 and partly disrupts the H-bond network that stabilizes the bound O_2, which reduces the O_2 affinity [15]. Evidently an interplay between several key functional residues in the haem pocket (Table 2) is responsible for ligand affinity modulation in the globins here studied.

Divergent temperature sensitivities of penta- and hexacoordinate nerve globins

An interesting finding is the clear difference between penta- and hexacoordinate globins in the temperature sensitivity of their O_2 affinity (Fig. 3). The globins studied here show essentially linear van't Hoff plots and temperature-independent heats of oxygenation, similar to vertebrate Mb, Hb and hexacoordinate Cygb [10,11,16]. The large enthalpy of oxygenation of *C. lacteus* nerve Hb may reflect the relatively large exothermic contribution of H-bonds stabilizing the bound O_2 in the haem pocket compared to the other globins investigated in this study, as *C. lacteus* Hb has GlnE7 and TyrB10 instead of the usual HisE7 and PheB10 (Table 2). The causes of the large heat of oxygenation in *A. aculeata* nerve Hb, that also has HisE7 and PheB10 in the distal haem pocket, are not obvious, and may include formation of weak bonds located elsewhere that are associated with binding of O_2.

The markedly lower overall heat of oxygenation in the hexacoordinate nerve globins of *S. solidissima* and *D. rerio* than in the pentacoordinate globins of *A. aculeata* and *C. lacteus* supports the view that hexacoordinate binding of the distal HisE7 to the haem in globin proteins not only decreases haem-O_2 affinity but also reduces temperature sensitivity of ligand binding [19]. The numerically lower ΔH values in hexacoordinate globins reflects endothermic dissociation of the distal HisE7 from haem upon oxygenation [19]. Additionally, other factors are likely to contribute to the temperature effects of the O_2 affinity. As discussed above, temperature-dependent O_2-linked association and dissociation of monomers may occur in *S. solidissima* nerve Hb. Such effects might contribute to the decreased temperature sensitivity at high temperatures (Fig. 2C). Temperature-dependent enthalpy of oxygenation is not unusual among globins. It has previously been shown for monomeric human Ngb [10] and tetrameric Antarctic fish Hbs [20], and related to non-negligible changes with temperature in the content of O_2-linked H-bonds and salt bridges [21].

The variability of metazoan nerve haemoglobins

The universal occurrence of globins in the nervous systems of vertebrates and several invertebrate taxa had been considered as support for a common evolutionary origin and similar functions of these proteins [7,22,23]. However, recent sequence analyses have demonstrated that at least *S. solidissima* nerve Hb derived from a 'normal' blood Hb [12], whereas the phylogenetic relationships of *C. lacteus* nerve Hb has not been resolved. In contrast, *A. aculeata* nerve Hb and *D. rerio* Ngb may share a common ancestry [24], whereas, for example, haem-coordination and oxygenation heat are markedly different. The diversity of evolutionary history is accompanied by an astonishing variability of several oxygen binding parameters in nerve Hbs, such as apparent cooperativity, Bohr effect and heat of oxygenation. Overall oxygen affinities (P_{50}) of invertebrate nerve Hbs are similar to that of a Mb. Mb mainly acts as intracellular oxygen supply protein, and such a function has been convincingly demonstrated for several invertebrate nerve Hbs [6], including those studied here [5,3,4]. The physiological role of Ngb from *D. rerio* and other vertebrates is less certain [25]. Ngb has been proposed to be involved in oxygen transport or storage [7,9] or in the detoxification of reactive oxygen or nitrogen species [10,16,26]. It should, however, be borne in mind that the globins may assume distinctive functional characteristics in their respective *in vivo* cellular environments.

Experimental procedures

Globin extraction

Approximately 0.5 g of dissected *A. aculeata* nerve cord tissue was placed in 1 mL 20 mM Tris buffer pH 8.0, homogenized, vortexed in multiple short bouts and centrifuged for 10 min at ~200 ***g***. The supernatant was saved and the procedure was repeated until the nerve tissue became colourless.

The globin was purified by FPLC using a Waters 15Q anion-exchange column equilibrated with 10 mM Tris buffer and eluted in a 0–0.5 M NaCl gradient. Absorbance was recorded simultaneously at 280 and 576 nm. Purity was

checked by thin layer IEF using Phast gels (pH 3–9; Amersham Biosciences, Piscataway, NJ, USA), which indicated the absence of other major protein components. Isolated *A. aculeata* nerve Hb was dialysed against CO-equilibrated 10 mM Hepes pH 7.7, containing 0.5 mM EDTA and stored at −80 °C until use.

Recombinant *S. solidissima, C. lacteus* nerve Hbs and *D. rerio* Ngb were expressed and purified as earlier described [27]. Briefly, the cDNA of the globins were cloned into the expression vector pET3a. After expression of the globins in the *Escherichia coli* BL21(DE3)pLysS cells, the *S. solidissima, C. lacteus* nerve Hbs and *D. rerio* Ngb were each purified to homogeneity. For the *S. solidissima* and *C. lacteus* nerve Hbs the purification procedure included ammonium sulphate precipitation (40–90% saturation), where the 90% pellet was redissolved and dialysed against 5 mM Tris/HCl pH 8.5, followed by DEAE–Sepharose fast flow ion exchange chromatography (step elution in 5 mM Tris/HCl pH 8.5, 200 mM NaCl) and gel filtration on a Sephacryl S200 column in 5 mM Tris/HCl pH 8.5. The globin fractions from *C. lacteus* and *S. solidissima* were each pooled and concentrated. For *D. rerio* Ngb a 60% ammonium sulphate precipitation procedure was followed by elution through a DEAE–Sepharose fast flow column (step elution in 5 mM Tris/HCl pH 8.5, 500 mM NaCl) and a Sephacryl S200 gel filtration column in 5 mM Tris/HCl pH 8.5. The Ngb fractions were then pooled and concentrated. After purification the samples were reduced by dialysis under anaerobic conditions against N_2- and CO-equilibrated 10 mM BisTris buffer pH 7.5, containing 0.5 mM EDTA, 1 $mg{\cdot}mL^{-1}$ dithiothreitol and 2 $mg{\cdot}mL^{-1}$ sodium dithionite, followed by exhaustive dialysis against N_2- and CO-equilibrated buffer to eliminate unreacted dithiothreitol and dithionite, as described [10]. Samples were stored under an atmosphere of CO in cryo vials placed in liquid N_2.

Oxygen equilibrium studies

O_2 equilibrium curves were recorded as described [10]. In brief, ultrathin (< 0.05 mm) layers of 4-μL globin solutions were placed in a modified thermostatted diffusion chamber and stepwise equilibrated with mixtures of humidified O_2 or air and ultra pure (> 99.998%) N_2 using precision Wösthoff gas-mixing pumps. Changes in absorbance were monitored continuously at 428 nm for the hexacoordinate and at 436 nm for the pentacoordinate globins using a UV-visible Cary 50 Probe spectrophotometer equipped with optic fibres. Each equilibrium curve consisted of five or more points of which four typically were within the 40–60% O_2 saturation range. Among the globins investigated, only *D. rerio* nerve globin showed significant autoxidation during O_2 binding recordings. In order to counter autoxidation the enzymatic MetHb-reducing system [28] was added to the samples with the following composition: glucose 6-phosphate (15 mM); glucose 6-phosphate-dehydrogenase (0.0073 $mg{\cdot}mL^{-1}$); NADPH (1 mM); ferredoxin NADPH reductase (0.0017 mM); ferredoxin (0.0038 mM); and catalase (0.0015 mM), O_2 tensions and Hill coefficients at half-saturation (P_{50} and n_{50}) were interpolated from the zero-intercept and the slope, respectively, of Hill plots, log [Y/(1–Y)] vs. log PO_2, where Y is the fractional O_2 saturation.

The apparent heat of oxygenation (ΔH) was calculated from the van't Hoff equation as:

$$\Delta H = 2.303R(\delta \log P_{50})/(\Delta[1/T])$$

where R is the gas constant (1.987 cal $mol^{-1}{\cdot}K^{-1}$) and T is absolute temperature.

A BMS2 MK2 thermostatted microelectrode (Radiometer, Copenhagen, Denmark) was used to measure pH in 100-μL subsamples. O_2 binding measurements were carried out using globin samples dissolved in 0.1 M Tris buffers containing 0.5 mM EDTA. Final globin concentrations were 0.07–0.1 mM (haem-basis).

Acknowledgements

This study was supported by EU grant QLG3-CT-2002-01548, the Danish Natural Science Research Council, the Aase & Ejnar Danielsens Fund, the Augustinus Foundation, the 'Direktør Dr techn. A. N. Neergaards og Hustru' Fund, the 'Maskinfabrikant Jochum Jensen og Hustru' Fund, the Novo Nordisk Foundation and the Deutsche Forschungsgemeinschaft (Bu956/5; Ha2103/3). SD is a postdoctoral fellow of the Fund for Scientific Research of Flanders (FWO).

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