The Journal of Experimental Biology 206, 2693-2702

doi:10.1242/jeb.00475

Hemoglobin function in deep-sea and hydrothermal-vent endemic fish: *Symenchelis parasitica* (Anguillidae) and *Thermarces cerberus* (Zoarcidae)

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Accepted 6 May 2003

Summary

Deep-sea hydrothermal vents probably provide the harshest physico-chemical conditions confronting metazoan animals in nature. Given the absence of information on hemoglobin (Hb) function in hydrothermal-vent vertebrates, and the complex molecular and functional adaptations observed in hydrothermal-vent invertebrates, we investigated the oxygenation reactions of Hbs from the vent-endemic zoarcid *Thermarces cerberus* and the deep-sea anguillid *Symenchelis parasitica* from adjacent habitats.

Electrophoretically cathodic and anodic isoHbs from *S. parasitica* exhibit radical differences in O_2 affinity and pH and organic phosphate (ATP) sensitivities, reflecting a division of labor as in other 'class II' fish that express both Hb types. Remarkably, the cathodic Hb (I) lacks chloride sensitivity, and the anodic Hb (II) shows anticooperativity near half-saturation at low temperature. *T. cerberus* isoHbs exhibit similar affinities and pH sensitivities ('class I' pattern) but much higher O_2 affinities than those observed in Hbs of the temperate, shallow-water zoarcid *Zoarces viviparus*, which, unless compensated, reveals markedly higher blood O_2 affinities in the former species. The temperature sensitivity of O_2 binding to *T. cerberus* Hbs and the anodic *S. parasitica* Hb, which have normal Bohr effects, is decreased by endothermic proton dissociation, which reduces the effects of ambient temperature variations on O_2 affinity. In the cathodic *S. parasitica* Hb, similar reduction appears to be associated with endothermic conformational changes that accompany the oxygenation reaction.

Key words: hemoglobin, oxygen binding, enthalpy, deep-sea fish, hydrothermal vent, anguillid, zoarcid, *Thermarces cerberus*, *Symenchelis parasitica*.

Introduction

Deep-sea hydrothermal vents provide some of the harshest aquatic conditions that metazoans are exposed to in nature. Here, hot (350°C), anoxic waters bearing high levels of H_2S (4–8 mmol l^{-1}) heavy metals, arsenic compounds and CO mix chaotically with cold (2°C) deep-sea, O_2-laden (0.11 mmol l^{-1}) water at extremely high pressures (~25.3 MPa at 2500 m depth; Von Damm, 1990, 1995; Magemheim and Gieskes, 2002). Hemoglobins (Hbs) from invertebrates colonizing these habitats display striking adaptations, including high affinities for oxygen and pronounced Bohr effects, compared with those living in well-oxygenated environments (Terwilliger and Terwilliger, 1984; Arp et al., 1990; Toulmond et al., 1990; Hourdez et al., 1999, 2000a,b) and additionally may participate in the detoxification of sulfide (cf. Weber and Vinogradov, 2001). A well-documented example is the Hb of the giant vestimentiferan tube-worm *Riftia pachyptila*, which binds sulfide reversibly with a high affinity and transports the reduced compound to symbiotic chemoautotrophic bacteria that oxidize sulfide and fix CO_2 inside the body (Arp et al., 1987).

Fish show pronounced, well-documented adaptations in blood O_2-binding characteristics in response to environmental stresses (Weber, 1996) that comprise intraspecific adaptations (such as the decreases in the red cell levels of organic phosphates, ATP and guanosine triphosphate (GTP), that increase Hb–O_2 affinity and occur in individual specimens) as well as interspecific adaptations (between-species differences that are genetically coded and commonly involve differences in Hb structure and heterogeneity).

Fishes show marked isoHb differentiation and may be categorized accordingly. Whereas 'class I' species, such as cyprinids and ciclids (Gillen and Riggs, 1972), possess only electrophoretically anodic Hb components with relatively low O_2 affinities and pronounced Bohr and Root effects (decreases in O_2 affinity and carrying capacity, respectively, at low pH that promote O_2 unloading in the respiring tissues and in the

swimbladder), 'class II' fish (including eels, salmonids and some catfish) additionally have cathodic Hbs that exhibit high intrinsic O_2 affinities and low pH sensitivities (Powers and Edmundson, 1972; Gillen and Riggs, 1973; Weber et al., 1976a, 2000; Pellegrini et al., 1995) and may function as a reserve transport system when oxygenation of the anodic components is compromised by acidification and hypoxia (Weber, 1990, 2000a).

With no information available on Hb function in hydrothermal-vent vertebrates, we investigated isoHb differentiation, together with Hb–O_2 binding and its sensitivity to pH, temperature and red cell effectors (organic phosphate and chloride ions), in the anguillid pugnose eel *Symenchelis parasitica* and the zoarcid *Thermaces cerberus* and carried out some comparative measurements on the Hb of the temperate, shallow-water zoarcid *Zoarces viviparous*. Compared with *S. parasitica*, which shows highly cosmopolitan distribution at depths of up to 3000 m and lives at the borders of hydrothermal vents that it casually visits for food (Desbruyères and Segonzac, 1997), *T. cerberus* is endemic to hydrothermal-vent environments, where it intertwines with, and feeds on, *Riftia* (Dahlhoff et al., 1990; Geistdoerfer and Seuront, 1995), which requires sufficient quantities of free sulfide to support robust bacterial endosymbiosis (Luther et al., 2001).

Materials and methods

The pugnose eel *Symenchelis parasitica* (Goode and Bean 1879) (Synaphobrachidae, Anguilliform) was captured using fish traps at a depth of 1690 m at the 'Lucky Strike' vent field of the Mid-Atlantic Ridge (MAR) hydrothermal vents (37°17′ N and 32°16′ W) during the MARVEL'97 expedition. *S. parasitica* is a benthopelagic, necrophagus fish encountered on continental slopes and at upper abyssal ocean depths and was previously believed to be parasitic due to its sucker-shaped mouth. Specimens of the vent-endemic eelpout *Thermarces cerberus* (Rosenblatt and Cohen 1986) (Gadiformes, Zoarcoidei, Zoarcidae) were captured in *Riftia* clumps at 2500–2650 m depth at the East Pacific Rise (12°49′ N, 103°56′ W and 9°46′ N, 104°21′ W) during the HOT'96 cruise. The deep-sea fish used were approximately 30 cm long. The north-European eelpout *Zoarces viviparus* Linnaeus 1758, which was used for comparative measurements, originated from Aarhus Bay, Denmark.

Blood samples from the deep-sea species were drawn into EDTA-containing syringes, frozen and stored at –80°C until use. Hb solutions were prepared by addition of approximately two volumes of 0.02 mol l^{-1} Tris buffer, pH 7.6 and stripped of organic phosphates on MB-1 mixed ion-exchanger or by preparative isoelectric focusing. Other preparatory steps were carried out at 0–5°C, as previously described (Weber et al., 1987, 2000). IsoHb composition was investigated by electrophoresis on cellulose acetate strips (Gelman Science, Ann Arbor, MI, USA) and isoelectric focusing in 110 ml (LKB) columns containing Pharmacia (Biotech AB, Uppsala, Sweden) ampholines, pH 3.5–10 (0.55%) and 5–8 (0.18%). pH values of retrieved fractions were measured at 22°C using a BMS Mk2 Blood Micro System (Radiometer, Copenhagen, Denmark). *Z. viviparus* Hb was prepared from washed red cells and stripped by filtration through a column of Sephadex G25 (fine) gel. All Hb solutions were dialysed against three changes of 0.01 mol l^{-1} Hepes buffer, pH 7.67 containing 0.5 mmol l^{-1} EDTA and, where necessary, concentrated by ultrafiltration (Millipore 10 000 NMWL Ultra-free-4 filters). The Hb was frozen in 50 µl or 100 µl aliquots that were freshly thawed for O_2 equilibrium measurements.

Oxygenation equilibria of ultrathin (≤0.05 mm) layers of Hb solutions were recorded using a modified O_2-diffusion chamber (Weber, 1981; Weber et al., 1987) in the absence or presence of ATP (assayed using Sigma test chemicals) and 0.1 mol l^{-1} KCl (assayed using a Radiometer CMT10 chloride titrator). The pH was varied using Hepes buffers (final concentration 0.1 mol l^{-1}; Weber, 1992). The overall heat of oxygenation $\{\Delta H' = R\cdot\log_e[\Delta\log P_{50}/(T_1^{-1}–T_2^{-1})]$, where P_{50} is the half-saturation O_2 tension, R is the gas constant, and T_1 and T_2 are different absolute temperatures; Wyman, 1964$\}$ was investigated by measuring P_{50} values at 5°C, 25°C and 35°C and at pH values near 6.8 and 7.5 and interpolating the P_{50} at these two pH values from the linear $\log P_{50}$ *vs* pH regressions.

Results

Hb multiplicity

Isoelectric focusing (Fig. 1A) resolved *S. parasitica* Hb into a major component with an isoelectric point (pI) of 8.0 (Hb II), a minor cathodic component, Hb I (pI ~9.0), and two small anodic components, Hb III (pI ~6.5) and Hb IV (pI ~5.2, not shown). Planimetric analysis indicates relative proportions of approximately 13:82:5 for Hbs I, II and III, respectively. Mindful of the differentiation between cathodic (pI>8.6) and anodic (pI<8.6) fish Hbs, functional analyses focused on Hbs I and II. *T. cerberus* Hb resolved into three major anodic components (Hbs I, II and III) with pI values of approximately 7.0, 6.8 and 6.5, respectively, and five minor components with pIs of <6.5 and >7.0 (Fig. 1B).

O_2-binding characteristics

S. parasitica Hbs I and II exhibit radically different O_2-binding properties. Stripped Hb I has a markedly higher affinity than Hb II [P_{50} at pH 7.2 = 14 Torr (1.87 kPa) and 33 Torr (4.40 kPa), respectively, at 25°C] and higher cooperativity at half-saturation (n_{50}=1.7 and 1.1, respectively; Figs 2, 3).

Hb I shows a slight, reverse Bohr effect ($\varphi=\Delta\log P_{50}/\Delta$pH=0.08) that is unaffected by chloride but changes to a slight, normal Bohr effect (φ=–0.14) in the presence of Cl^- + ATP (Fig. 3A). By contrast, stripped Hb II has a pronounced Bohr effect (φ=–0.39) that is potentiated by both Cl^- (φ=–0.58) and Cl^- + ATP (φ=–0.74) in accordance with increased anionic binding at low pH. Hb II shows a reverse 'acid' Bohr effect below pH 6.7 (Fig. 3A), which thus extends to higher pH values than those occurring below pH 6.0 in anodic Hbs of

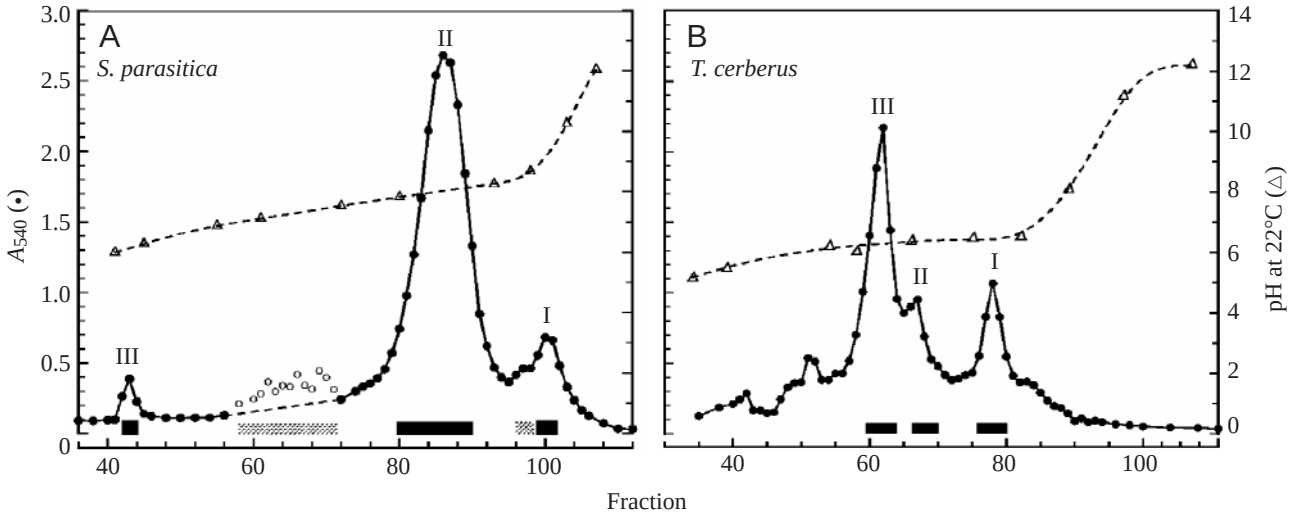

Fig. 1. Isoelectric focusing of erythrolysates of (A) *Symenchelis parasitica* and (B) *Thermarces cerberus* (described in Materials and methods). Circles, absorption at 540 nm; triangles, pH values at 22°C; solid rectangles, Hb-containing fractions that were pooled and dialysed for Hb–O_2 binding studies; shaded rectangles, fractions that contained greyish precipitated material. I, II and III refer to hemoglobins (Hbs) I, II and III, respectively.

trout, carp and catfish Hbs (Binotti et al., 1971; Gillen and Riggs, 1977; Weber et al., 2000). The P_{50}, n_{50} and φ values of the stripped hemolysate (Fig. 3A) are intermediate between those of Hb I and Hb II, indicating an absence of interaction between the components.

In contrast to the moderate Cl^- and ATP sensitivity of Hb II, Hb I lacks Cl^- sensitivity (Figs 2, 3) but exhibits strong Cl^- + ATP sensitivity. The persistence of a pronounced ATP effect in Hb I at high pH (Fig. 2A) is intuitively consistent with its high pI value, reflecting positively charged sites. The induction of a normal Bohr effect of Hb I by ATP reflects preferential phosphate binding at low pH and thus is analogous to the increased normal Bohr effect in Hb II in the presence of ATP. The data at 5°C (Fig. 3B) confirm the functional differentiation between Hb I and Hb II but show a larger Cl^- + ATP effect than at 25°C ($\Delta\log P_{50}$ at pH 7.5 = ~0.6 and ~0.4, respectively). Curiously, a distinct reverse Bohr effect (confirmed repeatedly) is manifest in Hb II at 5°C in the presence of Cl^- + ATP, although a large (normal) Bohr effect is seen in the absence of the phosphate (Fig. 3B). With no information on the structure of the Hbs, a molecular explanation cannot be offered.

Given that cytoplasmic domains of the red cell membrane protein Band 3 (cd-B3) bind at the phosphate-binding site of human deoxyHb (Walder et al., 1984), we investigated the effect of trout cd-B3, a synthetic 10-mer peptide corresponding to the amino terminus of trout Band 3 protein (Jensen et al., 1998), on *S. parasitica* Hb but found no detectable effect over a wide range of pH conditions (6.6–7.5; Fig. 3A).

Extended Hill plots (Fig. 4) of the major *S. parasitica* isoHb (Hb II) indicate negative cooperativity at extreme, low O_2 tensions and positive cooperativity only above ~60% O_2 saturation, indicating that the molecules remain frozen in the

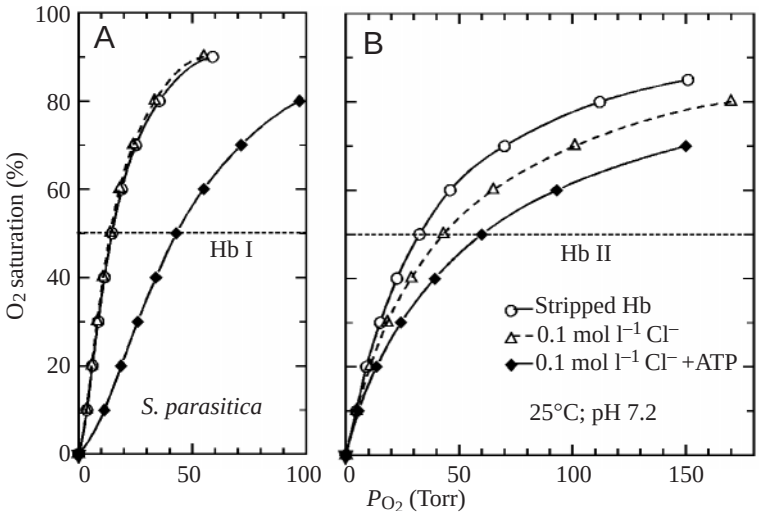

Fig. 2. O_2 equilibria of (A) Hb I and (B) Hb II of *Symenchelis parasitica* measured in 0.1 mol l^{-1} Hepes buffer at pH 7.2 in the absence of added anions (circles) and in the presence of either 0.1 mol l^{-1} Cl^- (triangles) or 0.1 mol l^{-1} Cl^- + saturating ATP concentration (ATP/tetrameric Hb ratio >100; diamonds), illustrating a large ATP effect and no Cl^- effect on Hb I and distinct effects of both effectors on Hb II. Heme concentrations were 0.10 mmol l^{-1} (Hb I) and 0.05 mmol l^{-1} (Hb II). (7.50 Torr=1 kPa.)

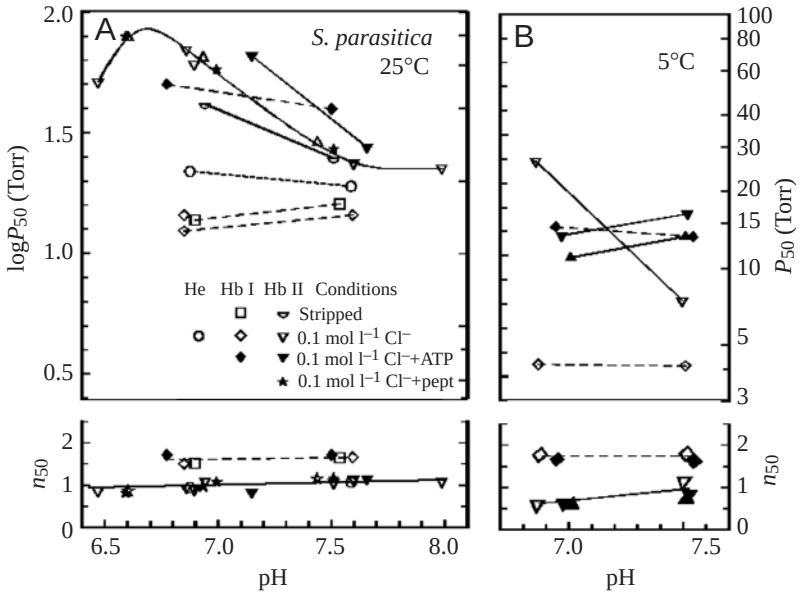

Fig. 3. P_{50} (O_2 tension at half O_2 saturation) and n_{50} (Hill cooperativity coefficient at P_{50}) values of *Symenchelis parasitica* hemolysate (He, dotted line), Hb I (dashed lines) and Hb II (continuous lines) and their pH dependence in the absence of effectors and in the presence of 0.1 mol l^{-1} KCl, saturating ATP levels and peptide corresponding to the 10-mer amino-terminal segments of the cytoplasmic domain of Band 3 protein (cd-B3) from trout red cell membranes (peptide/Hb tetramer molar ratio=5) measured at 25°C (A) and 5°C (B). Other conditions are as described in the legend to Fig. 2. (7.50 Torr=1 kPa.)

deoxygenated 'tense' conformation at low O_2 saturations. As is evident from Fig. 4, increased temperature as well as decreased pH lower O_2 affinity of Hb II predominantly by decreasing K_T without significantly impacting K_R (the O_2 association constants of the low-affinity deoxy and the high-affinity oxy states of the molecules, respectively), revealing greater Bohr effect and $\Delta H'$ values in the deoxygenated compared with the oxygenated state. Curiously, at low pH (7.0) and 5°C, Hb II shows distinct negative cooperativity (n<1) at 30–50% O_2 saturation in the absence of ATP (Fig. 4), which correlates with the high P_{50} value (and Bohr factor) found under these conditions (Fig. 3B).

T. cerberus Hb exhibits a strikingly higher O_2 affinity than does *Z. viviparous* hemolysate [P_{50}=9 Torr (1.2 kPa) and 30 Torr (4.0 kPa), respectively, at pH 7.0] and a lower pH Bohr factor (Fig. 5), whereby the affinity difference between the two species increases with decreasing pH. The Bohr factor decreased with increasing temperature (φ at pH 7.0–7.5=–0.62, –0.56 and –0.37 at 15°C, 25°C and 35°C, respectively), indicating temperature dependence of ionization groups as reported in other vertebrate Hbs (Antonini and Brunori, 1971), and falls drastically at high pH (>8; Fig. 5A). In contrast to the marked functional differentiation between *S. parasitica* isoHbs, *T. cerberus* isoHbs (I, II and III) show similar O_2 affinities [P_{50}=6–8 Torr (0.8–1.07 kPa) at pH 7.0 and 25°C], similar low chloride sensitivities, similar pronounced ATP effects (Fig. 6) and similar cooperativities (n_{50}=~1.5).

Oxygenation enthalpies

The O_2 affinities of *S. parasitica* and *T. cerberus* Hbs at 5°C, 25°C and 33–35°C yield essentially linear van't Hoff plots (Fig. 7), indicating temperature independence of the oxygenation enthalpies ($\Delta H'$), and similar heat capacities in the oxygenated and deoxygenated states of the Hb (cf. Fago et al., 1997a). The oxygenation enthalpies comprise the exothermic intrinsic heat of heme oxygenation (ΔH^0), the heat of solution of oxygen (ΔH^{sol}=~–13 kJ mol^{-1}) and endothermic contributions that include the oxygenation-linked dissociation

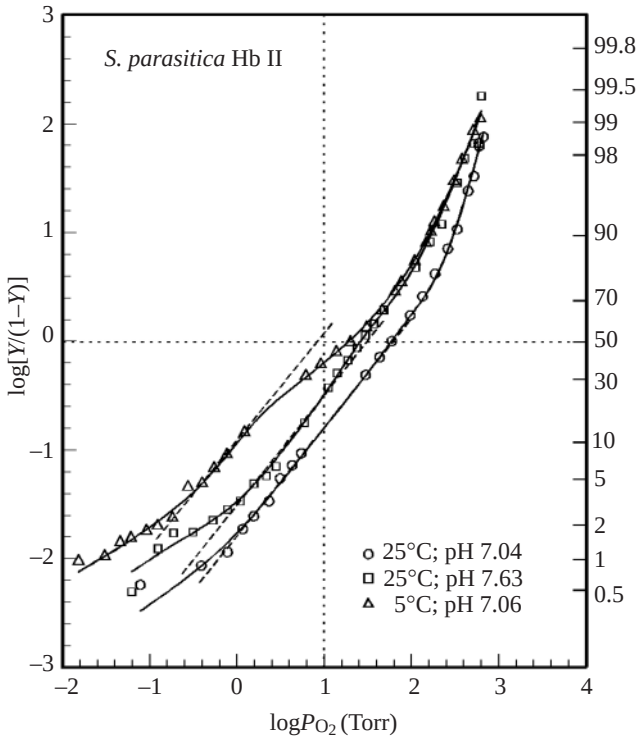

Fig. 4. Extended Hill plots (where Y is the fractional O_2 saturation) of *S. parasitica* Hb at pH 7.04–7.06 and either 5°C (triangles) or 25°C (circles) and at pH 7.63 and 25°C (squares). The intercepts of the asymptotes to the lower parts of the curves (broken lines with slopes of unity) with the vertical axis at logP_{O_2} indicate the K_T (the O_2 association constant of the low-affinity deoxy state of the molecules) values. Heme concentration, 0.80 mmol l^{-1}. (7.50 Torr=1 kPa.)

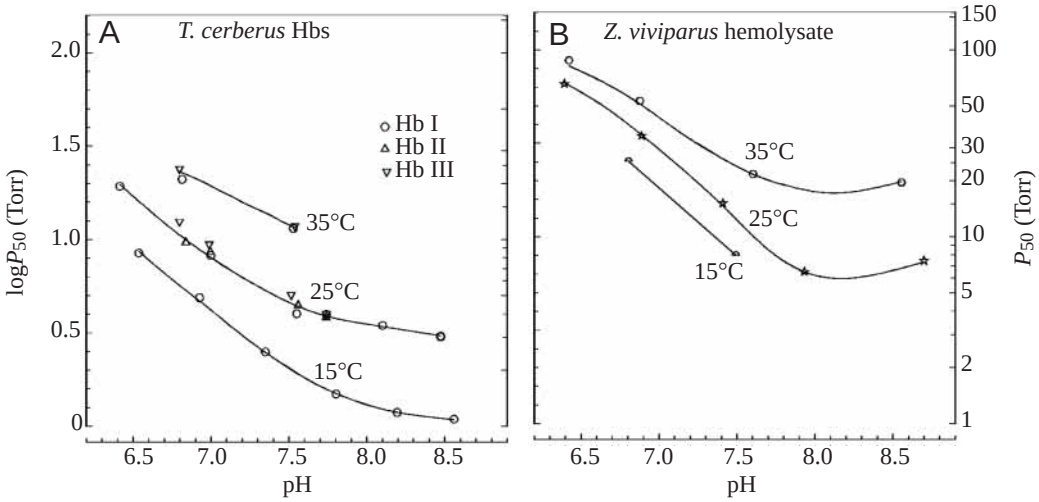

Fig. 5. P_{50} (O_2 tension at half O_2 saturation) values of (A) *Thermarces cerberus* Hbs I (circles), II (triangles) and III (inverted triangles) and (B) stripped *Zoarces viviparus* hemolysate, and their pH dependence at 15°C (semi-circles), 25°C (stars) and 35°C (circles). Heme concentrations, 0.16 mmol l^{-1}; Cl^- concentration, 0.1 mol l^{-1}. (7.50 Torr=1 kPa.)

of hydrogen ions, organic phosphate or chloride ions (ΔH^H, ΔH^P and ΔH^{Cl}, respectively). It follows that the Bohr effect and phosphate binding reduce $\Delta H'$ and that ΔH^0 can experimentally be assessed in the absence of oxygenation-linked ion binding under pH conditions where there is no Bohr effect.

S. parasitica Hb II shows high temperature sensitivity ($\Delta H'$=–52 kJ mol^{-1}) at pH 8.0, where there is almost no Bohr effect, and a lower sensitivity (–31 kJ mol^{-1}) at pH 6.8, where the Bohr factor is pronounced, suggesting that the intrinsic value (in the absence of proton and anion binding) exceeds –52 kJ mol^{-1}. As may be interpolated from Fig. 4, the heat of oxygenation is higher in the deoxygenated state than at half saturation (ΔH_T=~–70 kJ mol^{-1} and ~–40 kJ mol^{-1}, respectively, at pH 7.0). The cathodic Hb I, which has a slight, reverse Bohr effect (cooperativity between proton and O_2 binding), shows lower enthalpies ($\Delta H'$=–46 kJ mol^{-1} and –49 kJ mol^{-1}, respectively, at pH 6.8 and 7.5).

The temperature sensitivities of Hbs from the vent-endemic and temperate zoarcids *T. cerberus* and *Z. viviparus* show corresponding patterns (Fig. 7B) despite the large difference in O_2 affinities. At pH 8.4, where these Hbs only show slight Bohr effects (normal and reverse, respectively; Fig. 5A,B), the respective enthalpies of –72 kJ mol^{-1} and –78 kJ mol^{-1} suggest that the intrinsic heat for the reaction with O_2 is intermediate (~75 kJ mol^{-1}). The lesser reductions seen in *T. cerberus* Hbs compared with *Z. viviparus* Hbs under physiological conditions ($\Delta H'$=–63 kJ mol^{-1} compared with –42 kJ mol^{-1}, respectively, at pH 7.5, and –48 kJ mol^{-1} and –31 kJ mol^{-1}, respectively, at pH 6.8; Fig. 7B) tally neatly with the smaller Bohr factors in the former species.

Discussion

Extreme environmental conditions mandate integrated adaptations at systemic, cellular and molecular levels of biological organisation. Environmental factors that challenge Hb–O_2 transport in animals living at or near hydrothermal vents include hypoxia and low pH, which reduce O_2 binding, and high CO and H_2S levels, which potentially block O_2 transport and aerobic metabolism. Deep-water fish species exposed to high pressure generally exhibit lower oxygen consumption rates and enzyme activities and higher membrane fluidities than do shallow-water fish species (Sebert, 2002). The hematological characteristics of deep-sea fishes similarly reflect adaptations to low metabolic and activity rates: *Lycodes esmarkii*, *Antimora rostrata* and *Macrurus berglax* from a depth of 280–1400 m have low blood Hb levels, large erythrocytes and hyperbolic blood O_2 curves (Graham et al., 1985).

A relevant consideration pertaining to deep-sea habitats is how high hydrostatic pressure influences water O_2 tension and the O_2 affinity of blood and Hb. Thermodynamic considerations indicate that the solubility of O_2 decreases whereas its partial pressure increases with increasing pressure and depth (Fenn, 1972). Increasing hydrostatic pressure to 1000 atmos (101.3 MPa) raises the affinity of human and menhaden (*Brevoortia tyrannus*) Hb approximately 2-fold without disturbing the transition between the deoxygenated, low-affinity (T) state and the oxygenated, high-affinity (R) states of the Hb (Carey et al., 1977). Increasing pressure from 1 atmos (0.1 MPa) to 126 atmos (12.8 MPa) raises O_2 affinity of human whole blood, red cell suspensions and hemolysate without affecting the sensitivity to 2,3-diphosphoglycerate (DPG; Reeves and Morin, 1986). Similar affinity increases have been reported in most earlier studies, although these often suffer from pitfalls associated with the use of buffers with pressure-sensitive pK values and the pressure sensitivities of gas solubility and spectral absorbances (Reeves and Morin, 1986).

O_2 affinities: adaptive variation

Distinct adaptations to ambient conditions are seen. Thus Hb–O_2 affinities are much higher in vent-endemic *T. cerberus*, which grazes amongst sulfide-metabolising *Riftia*, than in *S. parasitica*, which frequents cold, O_2-laden deep-sea water and only casually visits the organic-rich vent areas. Also, whereas

Hb–O_2 affinities in *T. cerberus* are higher than in *Z. viviparous*, those in *S. parasitica* are lower than in the eel *Anguilla anguilla* (Fig. 8). However, in contrast to the striking adaptations encountered amongst Hbs of hydrothermal-vent invertebrates, the *Thermarces* and *Symenchelis* Hb systems exhibit the same basic functional differentiations as encountered in shallow-water class I and class II fish, respectively. This aligns with the view that the regulatory burden for environmental adaptations in vertebrates is shifted to higher (e.g. cellular and organismic) levels of organisation than in invertebrates (Weber and Vinogradov, 2001).

In humans, glycolytic enzymes compete with deoxyHb for binding cd-B3, providing a mechanism whereby Hb oxygenation can govern red cell glycolytic processes (Giardina et al., 1995; Messana et al., 1996). The insensitivity of *S. parasitica* Hb to trout cd-B3 (Fig. 3A) suggests that *S. parasitica* Hb does not play a transducer role. This agrees with

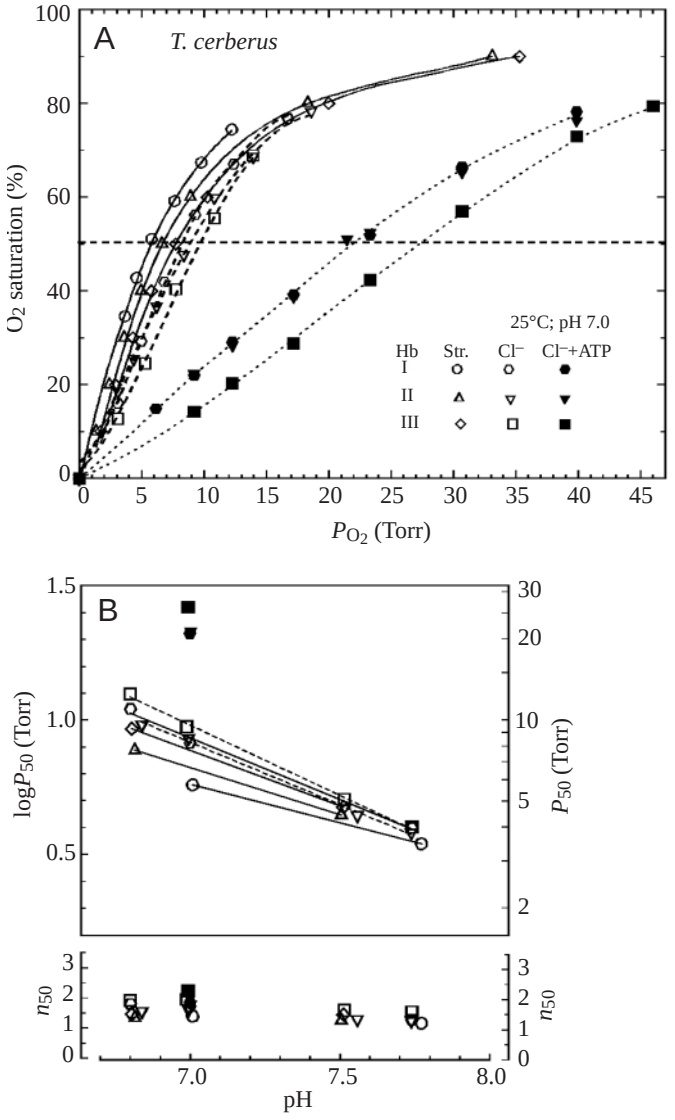

Fig. 6. (A) O_2 equilibrium curves of *Thermarces cerberus* Hbs I, II and III in the absence of added anions (solid lines), the presence of 0.1 mol l^{-1} Cl^- (dashed lines) and the presence of 0.1 mol l^{-1} Cl^- and saturating ATP concentration (20-fold excess over Hb tetramers; dotted lines). (B) Bohr effect plots. Buffer, 0.1 mol l^{-1} Hepes; heme concentration, 0.16 mmol l^{-1}. (7.50 Torr=1 kPa.)

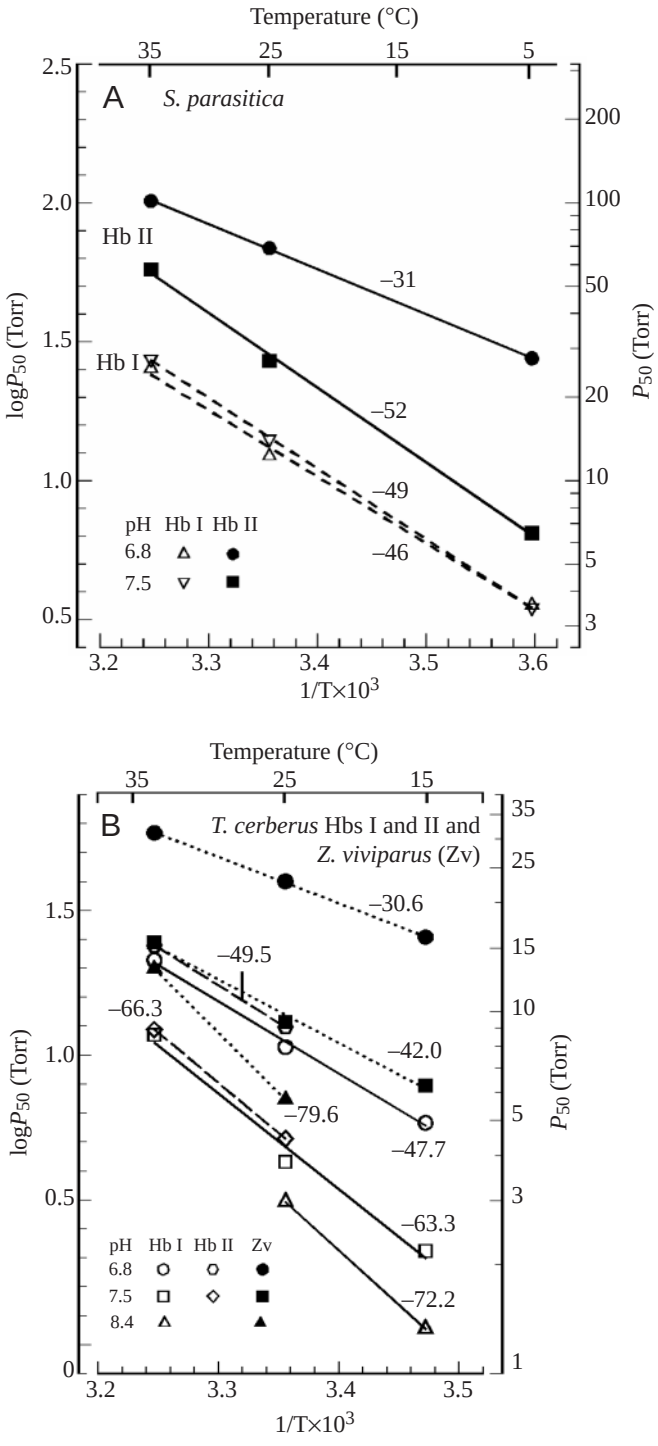

Fig. 7. van't Hoff plots of (A) *Symenchelis parasitica* Hbs I and II and (B) *Thermarces cerberus* Hbs I and II and *Z. viviparus* hemolysate. The data points at the indicated pH values were interpolated from logP_{50} *vs* pH curves at 5°C, 25°C and 35°C (Figs 3, 6) as described in Materials and methods. Numbers next to the lines are ΔH (heat of oxygenation) values in kJ mol^{-1}. (7.50 Torr=1 kPa.)

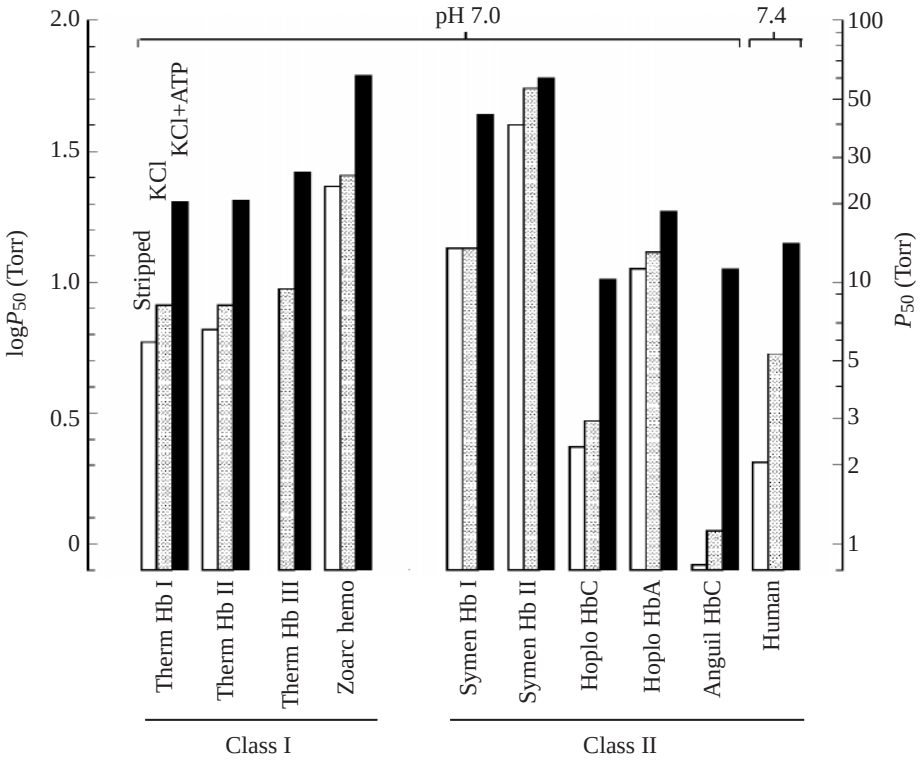

Fig. 8. P_{50} (O_2 tension at half O_2 saturation) values of *Thermarces cerberus* (Therm) Hbs I and II and *S. parasitica* (Symen) Hbs I and II compared with those for *Zoarces viviparus* hemolysate (Zoarc), *Hoplosternum littorale* cathodic and anodic Hbs (Hoplo HbC and Hoplo HbA; Weber et al., 2000) and eel *Anguilla anguilla* cathodic HbC (Anguil HbC; Fago et al., 1995) at pH 7.0 and human Hb at pH 7.4 (Imai, 1982). Open columns, stripped Hbs; shaded columns, Hbs in the presence of 0.1 mol l^{-1} KCl; solid columns, Hbs in the presence of 0.1 mol l^{-1} KCl + saturating concentrations of ATP (fish Hbs) or 2,3-diphosphoglycerate (DPG; human Hb). Other conditions: 0.1 mol l^{-1} Hepes buffer (fish Hbs) or 0.05 mol l^{-1} Bis-Tris (Human Hb) and 25°C (20°C for Anguil HbC). (7.50 Torr=1 kPa.)

data for salmonid (trout) isoHbs (Jensen et al., 1998; Weber, 2000a) but contrasts with human and catfish (*Hoplosternum littorale*) Hbs (Walder et al., 1984; Weber et al., 2000; Weber, 2000a), whose O_2 affinities are lowered by cd-B3, possibly implicating these Hbs in regulating cellular metabolism in an oxygenation-dependent manner (Weber, 2000b).

IsoHb differentiation

The Hb systems of *T. cerberus* and *S. parasitica* appear to be typical representatives of fish classes I and II, respectively (Fig. 8).

IsoHbs from the vent-endemic eelpout *T. cerberus* have similar O_2 affinities and pronounced, normal Bohr effects that decrease with increasing pH, where the proton-binding sites become neutralized. A significant adaptation in *T. cerberus* Hbs appears to be the high O_2 affinity compared with that in the temperate eelpout *Z. viviparus* [P_{50}=~8 Torr (1.07 kPa) and ~24 Torr (3.2 kPa), respectively, at pH 7.0 and 25°C]. The affinity difference will be further amplified in the presence of the natural compliment of red cell effectors [P_{50} values become ~20 Torr (2.67 kPa) and ~60 Torr (8 kPa) in the presence of ATP and 0.1 mol l^{-1} Cl^-; Fig. 8]. Although the exact mechanism of high affinity in *T. cerberus* must await the solution of their molecular structures, reduced sensitivities to chloride and phosphate effectors appear not to be involved (Fig. 8).

S. parasitica typifies class II fish, having cathodic Hb with relatively high O_2 affinity, a slight, reversed Bohr effect in the absence of organic phosphate and a large effect of ATP (that normalizes the Bohr effect) as well as an anodic Hb with relatively low affinity and marked Bohr and ATP effects – as found in other anguillids (Weber et al., 1976a; Fago et al., 1995; Tamburrini et al., 2001) and catfish (Garlick et al., 1979; Powers and Edmundson, 1972; Weber et al., 2000; Fig. 8). Unexpectedly, the affinities of stripped *S. parasitica* Hbs [P_{50}=14 Torr (1.89 kPa) and 33 Torr (4.4 kPa) for Hb I and II, respectively, at pH 7.2 and 25°C] are low compared with those obtained by the same technique in eel and catfish cathodic and anodic Hbs [P_{50}=≈2 Torr (0.23 kPa) and ≈8.5 Torr (1.13 kPa), respectively; Weber et al., 1976a; Fago et al., 1995, 1997b; Weber, 2000a] but are similar to those in trout Hbs [P_{50}=~17–20 Torr (2.27–2.67 kPa) at 20°C; Weber et al., 1976b] – all of these species are classified as class II.

The pH insensitivity of the cathodic Hbs favors O_2 binding under acidotic conditions (burst activity, acid influx or lactate secretion in the swimbladder; Powers, 1972; Weber, 1990). In *S. parasitica*, however, the division of labor between the cathodic Hb I and anodic Hb II may be of limited physiological significance due to the low abundance of Hb I (cf. Fig. 1). The higher sensitivity to ATP in cathodic Hb I compared with Hb II aligns with Hbs of the eel *Anguilla* (Weber et al., 1976a; Fago et al., 1995) and the Amazon fishes *Myllossoma*, *Pterygoplicthys* and *Hoplosternum* (Martin et al., 1979; Weber and Wood, 1979; Weber et al., 2000) but contrasts with rainbow trout (*Oncorhynchus mykiss*), whose cathodic Hb I is insensitive to phosphates. This confirms that the ‘model’ trout Hb system is exceptional rather than prototypical. An intermediate situation appears in the South African mudfish *Labeo*, where the phosphate sensitivity of cathodic Hb I is markedly lower than those of the anodic isoHbs (Frey

et al., 1998). In cathodic eel and *Hoplosternum* Hbs, the β-chain amino acid residues implicated in phosphate binding are Val1(NA1), Glu/His2(NA2), Lys82(EF6) and Lys/Ser143(H21), and the marked reverse Bohr effects (φ=0.2 and 0.38, respectively) in the absence of phosphates are attributed to the close proximity of these positively charged residues in the T-state, which reduces their affinity for protons. The smaller reversed Bohr effect (φ=0.08) in cathodic Hb I of *S. parasitica* may thus indicate a lower density of positive charges at the phosphate-binding site.

A significant finding is the low Cl^- sensitivity of *S. parasitica* Hb I (Fig. 3). Based on studies of mammalian/human Hb, two schools of thought exist as regards the molecular mechanism of the Cl^- effect: (1) oxygenation-linked Cl^- binding at specific sites (Fronticelli et al., 1994) [one between Val1(NA1) and Ser131(H14) on the α chain and another between Lys82(EF6) and Val1(NA1) on the β chain; Fantl et al., 1987; Riggs, 1988] and (2) a general neutralization of excess positive charges that destabilize the T-state in the central cavity (Perutz et al., 1994). Although the Cl^- sensitivities of abnormal human Hbs support the latter view, the Hb of the high-altitude Andean frog *Telmatobius peruvianus* [where loss of Cl^- sensitivity correlates with acetylation of NA1 of the α chains (as in fish) and replacement of polar α chain Ser131(H14) by nonpolar Ala] provides evidence for the implication of specific α chain sites (Weber et al., 2002). Elucidation of the primary structures of *S. parasitica* Hb I and Hb II promises valuable insight into the molecular basis for differentiated Cl^- sensitivities (Fig. 3).

Cooperativity

Another striking observation is the anticooperativity (n_{50}=~0.6) observed in the major *S. parasitica* isoHb at low temperature (5°C) and neutral pH (7.0) (Fig. 3B), which correlates with anticooperativity (n=~0.64) at 30–50% O_2 saturation (Fig. 4). Correspondingly low values (n_{50}=0.6) observed for CO and O_2 binding in Hbs of the deep-sea *Antimora rostrata* (Noble et al., 1975) and *Coryphaenoides acrolepsis* (R. E. Weber and F. C. Knowles, unpublished), respectively, raise the possibility that allosteric interactions may be differently expressed at atmospheric pressures compared with high hydrostatic pressures. However, no shape changes are seen in O_2-binding curves of human blood and Hb with hydrostatic pressures of 1–126 atmos (Reeves and Morin, 1986). n_{50} values below unity at low pH are diagnostic of Root effect Hbs that secrete O_2 into the swimbladders and retinae of fish (Pelster and Weber, 1991). Values of <1 can result from two populations of dissimilar heme groups, which could represent different isoHbs or α and β chains of the sets of Hbs (Noble et al., 1986). Obviously, the former possibility cannot explain the n<1 regions in the oxygenation curves of purified Hb II (Fig. 4).

Heats of oxygenation

The linear van't Hoff plots (Fig. 6) indicate that the enthalpy of oxygenation is temperature independent, unlike in the Antarctic fish *Dissostichus mawsoni*, where convex van't Hoff plots reflect increased temperature sensitivities at decreasing temperatures and marked changes in the heat capacity difference upon oxygenation (Fago et al., 1997a).

Given that *S. parasitica* Hb I lacks significant oxygenation-linked Cl^- binding and only shows a slight Bohr effect, its $\Delta H'$ in the absence of anions may be expected to approximate ΔH^0. However, the values found (–46 kJ mol^{-1} to –49 kJ mol^{-1}) are low compared with those for zoarcid Hbs (~–75 kJ mol^{-1}) and human Hbs (–78 kJ mol^{-1}) (Weber, 1992) at high pH, where the Bohr effect (oxygenation-linked proton dissociation) is almost zero. Curiously also, the overall heat of oxygenation of Hb I was not reduced by ATP addition (at pH 7.0, $\Delta H'$=–44 kJ mol^{-1} to –45 kJ mol^{-1} in the absence and presence of ATP, respectively; not shown). Given the small variability of the intrinsic heats of oxygenation of metal-containing gas-binding proteins (Klotz and Klotz, 1955), these observations indicate the presence of other endothermic reactions that reduce $\Delta H'$. A possible candidate is endothermic allosteric transitions, as in cathodic trout Hb I, where the low temperature sensitivity for CO binding is attributed to conformational changes conditioned to the molecules in the T-state (Wyman et al., 1977). In *S. parasitica* Hb II, however, the decreased temperature sensitivity is seen at high saturation (Fig. 4). In effect, this is analogous to bluefin tuna (*Thunnus thynnus*) Hb, where temperature insensitivity (which now reduces outward transport of heat and helps to maintain warm bodies) is attributable to the dissociation of a large number of Bohr protons late in the oxygenation process (Ikeda-Saito et al., 1983; Weber and Wells, 1989), and with tench (*Tinca tinca* red blood cells, where endothermic proton dissociation occurs at high saturation (Jensen, 1986). In each case, the resultant reduction temperature sensitivity will limit O_2 affinity variations in the Hb in the face of variable environmental temperatures.

We thank Anny Bang for expert technical assistance and Dr Angela Fago (Aarhus) for critically reading the manuscript. We also thank the crews of the research vessels *N/O Nadir* and *N/O Atalante* and of the submersible *Nautile*, as well as Françoise Gaill and Daniel Desbruyères, chief scientists for the cruises during which the samples were collected. Supported by the Danish Natural Science Research Council.

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