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ATP-induced temperature independence of hemoglobin–O_2 affinity in heterothermic billfish

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SUMMARY

The inverse relationship between temperature and hemoglobin–O_2 affinity resulting from the exothermic nature of heme oxygenation favors O_2 unloading from blood to warm, metabolically active tissues. However, this temperature sensitivity is maladaptive, and commonly countered in regional heterotherms, where it may hamper unloading (e.g. in cold extremities of arctic mammals) or increase the diffusive arterio-venous short-circuiting of O_2 (e.g. in counter-current heat exchangers of warm swimming muscles of tuna). We hypothesized analogous blood specializations in heterothermic billfish, whose warm eyes and brains increase the temporal resolution of vision, and measured hemoglobin–O_2 binding properties in three species over a wide pH range, at two temperatures, and in the absence and presence of the major red cell effector, ATP, permitting detailed assessment of overall oxygenation enthalpies ($\Delta H'$) and contributions from oxygenation-linked proton and ATP dissociation. Billfish express multiple isohemoglobins with similar O_2 affinities and pronounced sensitivities to pH and ATP. Compared with the moderate effects associated with proton dissociation upon oxygenation, dissociation of ATP and coupled extra Bohr protons virtually obliterates the temperature sensitivities. At pH 7.4, where this effect is maximal, ATP changes $\Delta H'$ values of blue marlin, striped marlin and shortbill spearfish hemoglobins from –39, –49 and –44 kJ mol^{-1} O_2, respectively, to +26, +4 and –7 kJ mol^{-1}. Thus in addition to allosterically modulating hemoglobin–O_2 affinity, ATP diminishes its temperature sensitivity, reducing deleterious arterio-venous short-circuiting of oxygen in the cranial billfish heat exchangers. The mechanism underlying this reduction in oxygenation enthalpy differs fundamentally from that in tuna, supporting independent evolution of this trait in these scombroid lineages.

Key words: allosteric effectors, ATP, enthalpy, hemoglobin, marlin, oxygen binding, temperature effect.

INTRODUCTION

Temperature has long been recognized as a *de facto* effector of hemoglobin (Hb) function (Barcroft and King, 1909; Macela and Seliskar, 1925; Weber et al., 1989; Giardina et al., 1993; Samaja et al., 2003). This is because O_2 binding by the heme groups is exothermic (i.e. the oxygenation enthalpy, ΔH^O, is negative), which underlies the commonly observed reduction in Hb–O_2 affinity (increase in half-saturation O_2 tension, P_{50}) with rising temperature. This temperature effect is generally considered physiologically advantageous as it increases O_2 unloading from the blood perfusing warm (exercising) tissues in parallel with the temperature-induced increase in O_2 consumption. However, it is potentially maladaptive in regionally heterothermic animals, where it often is reduced or even reversed. Examples are cold-tolerant ungulates (Coletta et al., 1992; De Rosa et al., 2004), where normal temperature sensitivities (increased Hb–O_2 affinity at low temperature) would compromise O_2 unloading in peripheral tissues (skin, limbs) that are cool (curtailing heat loss). In heterothermic fish like tuna and lamnid sharks, that have core body temperatures of up to 20°C above ambient values (Carey et al., 1971; Dewar et al., 1994)], reduced temperature sensitivities of Hb oxygenation could decrease the overall oxygen partial pressure difference across the countercurrent, heat-exchanging retia mirabilia and thus reduce the potential oxygen loss from the arterial blood feeding the swimming muscle (Larsen and Malte, 2003).

The overall ΔH ($\Delta H'$) is the net result of several components. Apart from the intrinsic heat of heme–O_2 binding (ΔH^O), the heat of solution of O_2 (ΔH^{sol}=–12.6 kJ mol^{-1}) and possible contributions from the heat of conformational (structural) changes associated with O_2 binding (ΔH^{cc}), it importantly also includes contributions from oxygenation-linked, endothermic dissociation of allosteric effectors. In vertebrate Hbs, where the main effectors are protons, organic phosphates and chloride ions, these are predominantly ΔH^{H+}, ΔH^P and ΔH^{Cl}, respectively. The effect of pH (protons and CO_2) on O_2 affinity is known as the Bohr effect ($\varphi = \Delta \log P_{50}/\Delta pH$; where P_{50} is the half-saturation pressure of oxygen). In addition to the normal 'alkaline' Bohr effect (a decreased Hb–O_2 affinity with decreasing pH caused by proton binding to low-affinity, deoxygenated Hb that promotes O_2 unloading in metabolizing, acidic tissues) that is expressed at physiological pH, vertebrate Hbs may additionally exhibit a reverse 'acid' Bohr effect (increased O_2 affinity associated with oxygenation-linked proton binding) at very low pH (<~6.2 in human Hb). Compared with mammals where the major organic phosphate modulating red-cell O_2 affinity is 2,3-diphosphoglycerate (DPG), the major phosphate cofactor in fish is ATP, though some fish species additionally have high levels of guanosine triphosphate (GTP) that generally is a more potent allosteric effector than ATP (Weber, 2000).

The endothermic dissociation of red cell effectors from Hb upon oxygenation may thus be the primary source of adaptive reductions

in the numerical value of $\Delta H'$ in Hbs of heterothermic vertebrates. Indeed, in cold-tolerant ruminants, low temperature sensitivity of O_2 affinity correlates with a heightened oxygenation-linked, endothermic dissociation of chloride ions compared with that in other mammalian Hbs (Coletta et al., 1994; De Rosa et al., 2004). By contrast, the temperature insensitivity of tuna (*Thunnus thynnus*) Hb (Rossi-Fanelli et al., 1960) arises through a pH-dependent control of O_2 affinity (Yokoyama et al., 2004) associated with large Bohr effects (allosteric release of many protons upon oxygenation) (Jensen, 2001). In tuna Hb component I, the temperature insensitivity at half-saturation correlates with a normal temperature sensitivity below P_{50} and a reversed sensitivity above P_{50} (Ikeda-Saito et al., 1983) indicating that Bohr proton dissociation occurs predominantly at high O_2 saturations – as also applies to the Hbs of tench (Jensen, 1986), rainbow trout (Brauner et al., 1996) and yellowfin and skipjack tunas (Lowe et al., 1998). That organic phosphates may also be implicated is evident from the major Hb components (Hbs III and V) of the lamnid porbeagle shark, in which small Bohr effects mirror minor contributions from proton binding whereas the presence of ATP eliminates (and even reverses) the temperature sensitivity [changes $\Delta H'$ from –40 and –20 kJ mol^{-1}, respectively, to ~+12 kJ mol^{-1} (Larsen et al., 2003)].

A hitherto overlooked group of regional heterotherms as regards the thermodynamic contributions of allosteric effector binding to the Hb, is the billfish (marlins, sailfish and spearfish). These agile predators possess modified (non-contractile) muscle tissues in the cranium that warm the brains and eyes to temperatures up to 15°C above ambient water temperatures (Carey, 1982; Block, 1986; Fritsches et al., 2005). Cranial endothermy evolved independently several times and has also been documented in all five genera of tuna, in lamnid sharks and in opah, *Lampris guttatus* (and also may occur in butterfly mackerel and big-eye thresher sharks) making it the most widespread form of regional heterothermia among fishes (Sepulveda et al., 2007; Runcie et al., 2009).

As in tuna, billfish Hbs show high pH sensitivities of O_2 affinity [Bohr factors, φ=–0.74 and –1.0 in striped marlin Hb and blue marlin blood, respectively (Wells and Davie, 1985; Dobson et al., 1986)] that potentially signify major enthalpy contributions from oxygenation-linked proton dissociation. However, unlike tuna Hb where the enthalpies of O_2 binding to the T (tense) and R (relaxed) states have opposite signs (being exothermic and endothermic, respectively, reflecting the release of Bohr protons at high O_2 saturation) (Ikeda-Saito et al., 1983), the enthalpy associated with binding of CO (a heme ligand with a similar effects on Hb structure as O_2) to striped marlin Hb, is endothermic in both the R (carboxylated) and T (unliganded) states (ΔH=+23 and +17 kJ mol^{-1}, respectively) (Brittain, 1986).

With the aim of assessing the blood and molecular adaptations to heterothermy in blue marlin, striped marlin and shortbill spearfish, we measured Hb–O_2 equilibria under strictly controlled physicochemical conditions, i.e. at two temperatures, over a wide pH range and in the absence and presence of the red cell phosphate effector, ATP.

MATERIALS AND METHODS

Blood samples were taken from the gills of blue marlin (*Makaira nigricans* Lacepède 1802), striped marlin (*Tetrapturus audax* Philippi 1887) and shortbill spearfish (*Tetrapturus angustirostris* Tanaka 1915) captured at Kailua Kona, Hawaii. Washed, frozen red cells were shipped to Aarhus and kept at –80°C until use.

The relative contributions of ATP and GTP to the nucleoside triphosphate (NTP) pool in the red cells were estimated by thin layer chromatography as previously described (Johansen et al., 1976). The isoHb composition was investigated by preparative isoelectric focusing in 110 ml columns (Weber et al., 2002) in the presence of 0.7% ampholines (pH gradient 3.5–10).

Hb was prepared by admixing thawed erythrolysates with equal volumes of 0.1 mol l^{-1} Hepes buffer, pH 7.74, refreezing and thawing (to ensure complete hemolysis) and centrifugation for 20 min at 12,000 ***g*** to remove cell debris. The Hbs were stripped from endogenous ionic effectors on a column of Amberlite MB-1 resin. Where needed the Hb was reduced by adding a slight molar excess of sodium dithionite and dialysing against N_2-equilibrated 0.01 mol l^{-1} buffer. Absorption spectra of fully reduced, oxygenated Hbs showed α/β peak (A576.0 nm/A539.7 nm) absorption ratios of 1.06 as characterizes pure human oxyHb (Zijlstra and Buursma, 1997). O_2 equilibria were determined in 0.1 mol l^{-1} Hepes buffer using a modified diffusion chamber and Wösthoff gas mixing pumps as previously described (Weber, 1981; Weber et al., 2000). In the procedure, absorption of ultrathin layers of the Hb solution at 436 nm is recorded during stepwise increases in O_2 tension in equilibrating gas mixtures perfusing the chamber. O_2 tensions and Hill's cooperativity coefficients at half saturation (P_{50} and n_{50}, respectively) were obtained from linear regressions of Hill plots, log([oxyHb]/[deoxyHb]) *vs* logP_{O_2}, of four or more equilibration steps in the 25–75% O_2 saturation range, and showed coefficients of determination (r^2) >0.996. Fractional saturations were calculated by relating absorptions upon full equilibration at each step to those obtained for the same sample fully equilibrated with pure (>99.998%) N_2 and pure O_2. It follows that observed saturation at each step is expressed as a percentage of the maximum saturation obtainable under the actual same physico-chemical conditions. ATP and GTP were added as sodium salts and assayed using Sigma test chemicals. As tested with replicate applications of the same Hb sample (Weber, 1992), this method yields highly reproducible P_{50} values (4.73±0.04; mean ± s.e.m., n=6).

$\Delta H'$ values were calculated using the van't Hoff isochore (Wyman, 1964):

$$\Delta H' = 2.303\,R\,\Delta\log P_{50} / \Delta(1/T)\,,$$

where R is the gas constant and T the absolute temperature. The heat of oxygenation-linked reactions with allosteric effectors was assessed as the difference between $\Delta H'$ values in the presence and absence of these ligands (cf. Weber et al., 1985; Weber et al., 2008).

RESULTS AND DISCUSSION

Hb multiplicity and pH sensitivity

Red cell ATP/GTP ratios obtained for blue marlin (5.76±0.47; mean ± s.e.m., n=3), striped marlin (5.67±2.26; n=3) and shortbill spearfish (6.27±0.03; n=2) illustrate that ATP is the dominant phosphate effector of billfish Hb.

Isoelectric focusing showed pronounced Hb multiplicity in each of the three species. As illustrated for striped marlin (Fig. 1), all isoHbs are electrophoretically anodal (isoelectric points, pI<8]. As with anodal isoHbs from teleosts (Weber, 2000), the major (Hbs III and VI) as well as minor (Hbs I+II and IV) components of striped marlin exhibit similar O_2 affinities (Fig. 2). At pH 7.35 (the intraerythrocytic pH in tench at plasma pH 8.05) (Jensen and Weber, 1982) and 10°C, P_{50} values of the stripped Hbs are 2–3 mmHg. The isoHbs also exhibit similar Bohr effects and sensitivities to saturating levels of ATP and GTP (that raise P_{50} values by approximately one order of magnitude at pH 7.35; Fig. 2). These Hb properties assign these billfish to 'class I' fish – that differ from class II species (eels, salmonids and catfishes), which in

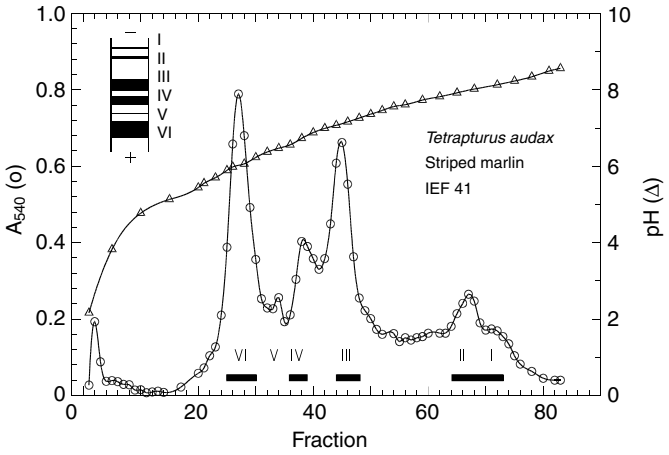

Fig. 1. Preparative isoelectric focusing of striped marlin Hb, showing two major isoHbs components (III and VI, with isoelectric points, pI, ~7.0 and ~6.0, respectively) and four minor components (I, II, IV and V, with pI ~8.0, 7.9, 6.8 and 6.5, respectively). Circles, absorption at 540 mn; triangles, pH values of eluted fractions. Horizontal bars show fractions pooled and dialyzed for O_2 equilibrium measurements (see Fig. 2). Inset, diagram of the isoelectric focusing column, showing the separated isoHbs.

addition to anodic isoHbs have 'cathodic' components that exhibit high pI value (>8.6), and reversed Bohr effects in the absence of anionic effectors (Weber et al., 1976; Weber, 1990).

The close correspondence in the O_2 affinities of the isolated isoHbs (Fig. 2) with that found in the composite hemolysate (see below) indicates a lack of functionally significant interactions between concurring isoHb components, that, as with Hbs from other ectothermic vertebrates (Brunori et al., 1974; Frische et al., 2001), may occur in the same red cells (as opposed to being segregated in distinct cells). The lack of interaction *in vitro* and the concordant functional properties of the Hb components justify the use of the composite hemolysates to assess the effector and temperature sensitivities of billfish isoHbs in their naturally occurring concentration ratios.

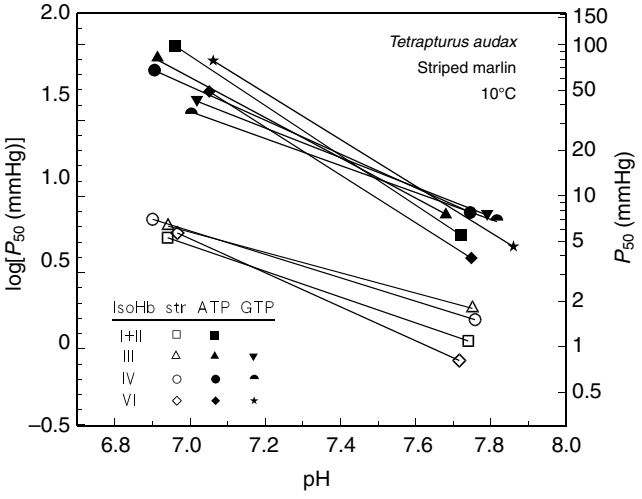

Fig. 2. O_2 tensions at half-saturation (P_{50}) of isoHb components I+II, III, IV and VI of striped marlin (isolated by preparative isoelectric focusing, see Fig. 1) measured in 0.1 mol l^{-1} Hepes buffer, in the absence of effectors (stripped; open symbols) and in the presence of saturating levels (molar phosphate:tetrameric Hb ratio >50) of ATP and GTP (solid symbols).

The stripped hemolysates of blue marlin, striped marlin and shortbill spearfish show similar O_2 affinities (P_{50}=3–4 mmHg at pH 7.4 and 10°C; Figs 3–5) combined with high pH sensitivities. [The data pertaining to striped marlin has previously appeared in a review paper (Weber and Jensen, 1988)]. As read from the slopes of the logP_{50} curves, the Bohr effects are absent at pH>8.2 but increase drastically at lower pHs. The Bohr factors are moreover radically increased in the presence of phosphate: at 10°C, φ ranges from –0.8 to –0.9 in the absence, and –1.6 to –2.1 in the presence of ATP (Table 1). Furthermore, whereas the maximal Bohr effect occurs at or below pH 7.0 in the absence of ATP, the pH of the maximum is shifted to natural physiological red cell pH values (7.2–7.6) in the presence of ATP (Table 1).

Cooperativity coefficients at half saturation (n_{50}) in the absence of ATP are invariant of pH between pH 8.5 and 7.0, and begin to decrease below this range (Figs 3–5). In the presence of ATP, the reduction in n_{50} initiates at a higher pH and is more pronounced, attaining a value of ~1 at 25°C, and ~0.7 at 10°C. Given that cooperativity values decreasing to <1 at low pH characterize the Root effect of fish Hbs (an extreme stabilization of the Tense state structure at low pH that facilitates O_2 unloading in the retia mirabilia of the eyes or the swimbladder), these results align with the observation that organic phosphates increase the Root effect and extends the pH range of its occurrence upwards and into the physiological range (Weber and de Wilde, 1975; Pelster and Weber, 1990). Moreover, the observation that n_{50} values in each species fall below unity only at 10°C but not at 25°C (Figs 3–5) suggests an inverse relationship between temperature and the magnitude of the Root effect.

The Bohr factors of the stripped billfish Hbs are markedly lower at 25°C than at 10°C (Table 1; Figs 3–5). This accords with the observed temperature dependence of the Bohr effect in human Hb (Antonini et al., 1965). The strong reverse temperature effect observed in *Thunnus maccoyii* (southern bluefin tuna) blood between 10°C and 23°C but not at higher temperatures (Clark et al., 2008) is reminiscent of the temperature dependence of oxygenation

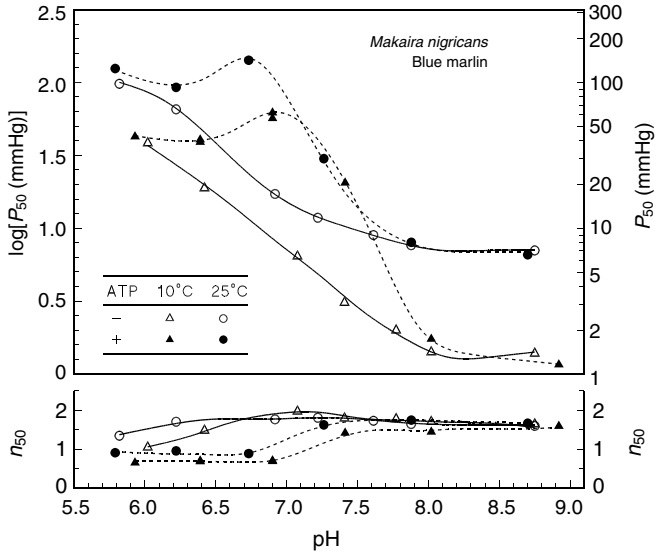

Fig. 3. O_2 tensions and Hill's cooperativity coefficients at half saturation (P_{50} and n_{50}) of stripped hemolysate of blue marlin and their pH dependence measured in 0.1 mol l^{-1} Hepes buffer at 10°C (triangles) and 25°C (circles) in the absence (open symbols) and presence (solid symbols) of ATP. [Hb] (tetrameric basis), 0.26 mmol l^{-1}; ATP/tetrameric Hb ratio, 1.81.

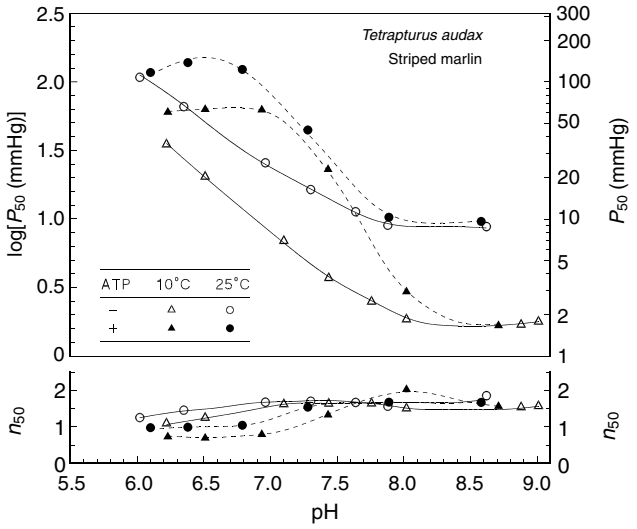

Fig. 4. P_{50} and n_{50} values and their pH and ATP dependence of striped marlin hemolysate. [Hb] (tetrameric basis), 0.31 mmol l^{-1}; ATP/tetrameric Hb ratio, 1.82. Other details as in legend to Fig. 3.

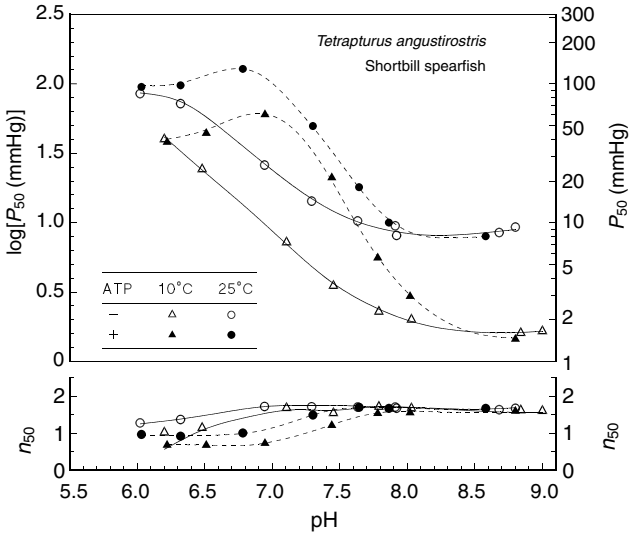

Fig. 5. P_{50} and n_{50} values and their pH and ATP dependence of shortbill spearfish hemolysate. [Hb] (tetrameric basis), 0.27 mmol l^{-1}; ATP/tetrameric Hb ratio, 1.75. Other details as in legend to Fig. 3.

enthalpies observed in the Hb from the Antarctic toothfish *Dissostichus mawsoni* (Fago et al., 1997) and illustrates the need for carrying out future studies on billfish and tuna Hbs at several, small temperature increments.

ATP sensitivity

ATP strongly reduces the O_2 affinities of billfish Hbs $[(\log P_{50})_{ATP}–(\log P_{50})_{str}]\approx 0.80$ at pH 7.4 (Table 1) reflecting a large capacity for adaptive modulation of P_{50} *via* changes in erythrocytic ATP levels. Moreover, the 'dose–response' curves (Fig. 6) reveal a high sensitivity to ATP at physiological ATP/Hb ratios (of 1 to 2). Logarithmic plots of P_{50} *vs* total [ATP] (Fig. 6B) show slopes (~0.44) that are considerably higher than the maximum value (0.25) predicted for the release of one ATP molecule per four O_2 molecules bound. Also, when plotting logP_{50} *vs* free [ATP], calculated as total [ATP]–0.5 [Hb_4], based on the assumption that at P_{50}, half of the tetrameric Hb molecules are ATP liganded, the maximum slopes (0.32) still exceed 0.25 (Fig. 6C). This deviation may reflect the absence of strict coupling between binding of heme ligands and ligand-induced T↔R conformational shifts, as seen with carp Hb that may be constrained in the low-affinity 'Tense' structure even when fully liganded (Tan et al., 1973). Alternatively, billfish Hb tetramers may bind more than one phosphate polyanion – as is also observed with GTP binding to cathodic Hbs of three species of eels, which, like billfish Hbs, exhibit high phosphate sensitivities (Olianas et al., 2005). Specifically, Hb of the eel *Conger conger* appears to harbor a second GTP binding site (apart from that between the β-chains) comprising four α-chain residues and one β-chain residue [Tyr-α(C1), Lys-α(G6), Asp-α(H9), Arg-α(HC3) and Asp-β(G10)]. Another case where the 1:1 stoichiometry between tetrameric Hb and organic phosphates may not apply is South polar skua (*Catharacta maccormicki*) Hb that has a cluster of six positive charges on the α-chain that may form a site where the phosphate polyanion docks before migrating to the main site (Riccio et al., 2001). Also, the biphasic nature of P_{50} *vs* log[DPG] plots suggests the presence of two DPG binding sites in dromedary Hb (Amiconi et al., 1985).

Table 1. Oxygen affinities of bill fish hemolysates and their pH and temperature sensitivities

	Blue marlin		Striped marlin		Shortbill spearfish	
***P_{50}* values, pH 7.4**	P_{50} (mmHg)	LogP_{50}	P_{50} (mmHg)	LogP_{50}	P_{50} (mmHg)	LogP_{50}
Stripped Hb 10°C	3.47	0.54	4.07	0.61	3.85	0.59
Stripped Hb 25°C	10.5	1.02	14.8	1.17	12.6	1.10
$\Delta_{\text{stripped Hb}}^{(20–10°C)}$		0.48		0.56		0.51
Hb+ATP 10°C	21.9	1.34	25.7	1.41	23.4	1.37
Hb+ATP 25°C	18.2	1.26	32.4	1.51	35.5	1.55
$\Delta_{\text{Hb+ATP}}^{(20–10°C)}$		–0.08		0.10		0.18
Bohr factors (φ)	φ_{max}	$pH_{\varphi max}$	φ_{max}	$pH_{\varphi max}$	φ_{max}	$pH_{\varphi max}$
Stripped Hb 10°C	–0.80	7.0	–0.81	6.8	–0.91	6.8
Stripped Hb 25°C	–0.88	6.6	–0.68	6.6	–0.77	6.8
Hb+ATP 10°C	–2.13	7.6	–1.70	7.6	–1.63	7.6
Hb+ATP 25°C	–1.69	7.2	–1.11	7.3	–1.36	7.5
$\Delta H'$ (kJ mol^{-1}), pH 7.4						
Stripped Hb	–39		–49		–44	
Hb+ATP	+26		+4		–7	
$\Delta H'_{[\text{stripped Hb} – (\text{Hb+ATP})]}$	+65		+53		+37	

P_{50}, oxygen affinities; φ_{max}, the maximal Bohr factors ($\Delta\log P_{50}/\Delta pH$), $pH_{\varphi max}$, the pH at this maximum, and $\Delta H'$, the apparent heats of oxygenation.

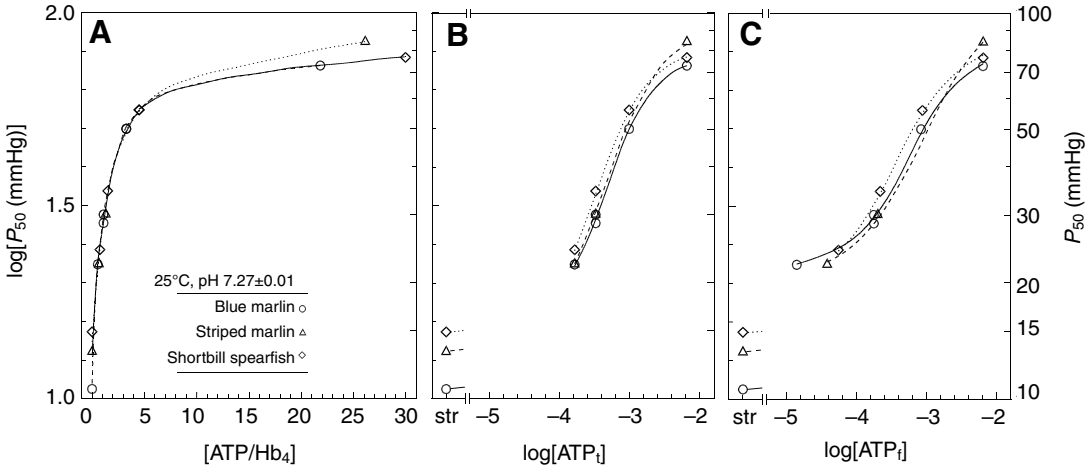

Fig. 6. ATP sensitivity of stripped hemolysates of blue marlin (circles), striped marlin (triangles) and shortbill spearfish (diamonds). Dependence of P_{50} on (A) molar ATP:tetrameric Hb ratio, and (B) total and (C) estimated free molar ATP concentrations. Temperature, 25°C; pH 7.27±0.01; tetrameric Hb concentrations: blue marlin, 0.30 mmol l^{-1}; striped marlin, 0.25 mmol l^{-1}; shortbill spearfish, 0.22 mmol l^{-1}.

$\Delta H'$ and its pH and ATP dependence

The temperature dependence of O_2 affinities of billfish hemolysates vary strikingly with pH and the presence of organic phosphates (Table 1; Figs 3–5). The $\Delta H'$ values interpolated over a wide pH range and in the absence and the presence of ATP permit calculation of the contributions of oxygenation-linked dissociation of protons and ATP to the overall enthalpic changes.

In the absence of anionic effectors and at high pH (~8) where the Bohr effect is absent (see Figs 3–5) and the heats of effector dissociation are negligible, the measured oxygenation enthalpies will approximate the intrinsic enthalpy of heme oxygenation, ΔH^O. The values obtained under these conditions are comparable for the three billfish hemolysates, ~–62 kJ mol^{-1} (Fig. 7), and correspond closely to that (ΔH^O=–59 kJ mol^{-1}) determined calorimetrically for human HbA (Atha and Ackers, 1974). Comparing the ΔH^O value of –62 kJ mol^{-1} with the overall $\Delta H'$ values of +26, +4 and –7 kJ mol^{-1} obtained for blue marlin, striped marlin and short-billed spearfish Hbs in the presence of ATP and at pH 7.4 reveals large (+88, +66 and +55 kJ mol^{-1}, respectively) enthalpic contributions of ATP and proton binding.

pH

As pH falls below 8 in the absence of ATP, the numerical values of overall $\Delta H'$ (Fig. 7) vary inversely with the Bohr effect (Figs 3–5). For blue marlin this change (from~–62 kJ mol^{-1} at pH 8.0 to ~–20 kJ mol^{-1} at pH 6.6; Fig. 7), indicates an overall ΔH^{H+} (enthalpy of proton release) of ~42 kJ mol^{-1} O_2 (~168 kJ mol^{-1} tetrameric Hb). In the absence of structural data on billfish Hbs the implicated ionization sites remain unknown. In human Hb, histidine residues are responsible for close to 90% of the total alkaline Bohr effect (Berenbrink, 2006), with the C-terminal histidines of the β-chains (that also are present in anodic fish Hbs) accounting for much of this effect. Moreover, in teleosts, the N-terminal α-chain residues – which previously had been considered to account for 25% of the Bohr effect in humans (Perutz, 1983; Berenbrink, 2006) – are blocked by acetylation. At 10°C and in the absence of ATP, the maximal Bohr factors observed in billfish hemolysates (φ=–0.80 to –0.91; Table 1) indicate O_2-linked release of 3.2–3.6 (integrally 4) protons per tetramer. In the presence of ATP the Bohr factors correspond to the release of 6.5–8.2 protons upon oxygenation (Table 1). Tuna Hb has a similarly large Bohr effect (maximal release of 4 mol H^+ per mol Hb tetramer in the absence of ATP) and have nine titratable 'neutral' residues, suggestive of seven histidine residues and two α-amino groups (Jensen, 2001). Based on the reported heats of protonation of imidazole groups of ~24 kJ mol^{-1} (Atha et al., 1974) and 16–37 kJ mol^{-1} (Bhattacharya and Lecomte, 1997)], the overall enthalpy of proton release of ~42 kJ mol^{-1} O_2 observed in blue marlin at pH 7.4 (above) indicates ionization of at least two imidazoles per O_2 molecule bound (eight per tetrameric Hb molecule). In this regard, however, it should be noted that X-ray crystal analysis of tuna Hb has identified a mechanism of pH-dependent control of ligand affinity that includes 'novel' proton binding sites (at His-α60, His-β69/Asp-β72 and Asp-$\alpha_1$96/Asp-$\beta_2$101) (Yokoyama et al., 2004) that may have different heats of ionization. Thus, the identification of the 'Bohr-proton' binding residues implicated in billfish Hbs must await elucidation of the structure of billfish Hbs.

The numerically higher Bohr factors observed in billfish Hbs in the presence of ATP may be attributable to the induction of protein basic groups by the proximity of the phosphate's negative charges, as described for human Hb in the presence of the potent allosteric effector inositol hexaphosphate (Gill et al., 1980). In the three billfish species studied here the ATP-induced increases in the observed maximal Bohr factors [(φ^{Hb+ATP}–$\varphi^{stripped\,Hb}$)=0.7–1.3] (Table 1), indicate the

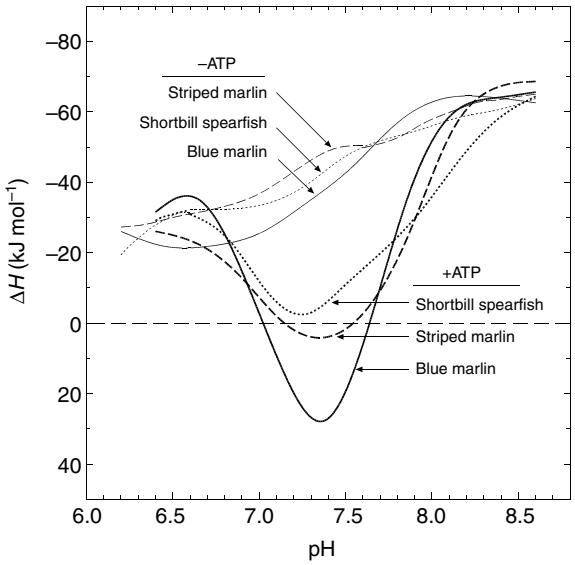

Fig. 7. Heats of oxygenation (excluding the heat of solvation of O_2) of stripped hemolysates of blue marlin, striped marlin and shortbill spearfish in the absence (thin lines) and presence (thick lines) of ATP, interpolated from logP_{50}/pH relationships (Figs 3–5).

oxygenation-linked dissociation of three to five *additional* protons per tetramer, in the presence (as compared with the absence) of ATP.

ATP

Strikingly, the ATP-induced reductions in $\Delta H'$ are greatest at physiological pH (Fig. 7). Explicitly, at pH 7.4 the markedly exothermic oxygenation reactions in the absence of ATP ($\Delta H'$=–39, –49 and –44 kJ mol^{-1} for blue marlin, striped marlin and short-billed spearfish Hbs), become endothermic or radically reduced ($\Delta H'$=+26, +4 and –7 kJ mol^{-1}, respectively) in the presence of ATP (Table 1).

Assessed as the difference between the oxygenation enthalpies in the absence and presence of ATP at pH 7.4 (Fig. 7), the overall heat of ATP binding and coupled reactions (ΔH^{P}) are +65, +53 and +37 kJ mol^{-1} O_2, respectively, for blue marlin, striped marlin and short-billed spearfish Hbs (Table 1). Being fourfold larger per tetrameric Hb, these enthalpy changes are high compared with corresponding values for O_2-linked reactions of ATP with human Hb (–50 kJ mol^{-1} ATP at pH 7.2) and carp Hb (–76 kJ mol^{-1} ATP at pH 7.4) (Greaney et al., 1980) – and of DPG with human Hb (–43 to –55 kJ mol^{-1} DPG) (Benesch et al., 1969; Bunn et al., 1971; Nelson et al., 1974; Hamasaki and Rose, 1974). As proposed for the reaction of human Hb with the potent effector inositol hexaphosphate (Gill et al., 1980), these large enthalpy changes may be ascribed to the oxylabile endothermic release of additional protons from binding sites induced by the proximity of the negative charges of ATP. Large contributions to the overall enthalpy of oxygenation from ionization reactions in billfish Hbs tally neatly with the large Bohr effects and their drastic augmentation by ATP. Phosphate-linked protonation has wide-ranging relevance. "The importance of protonation in the binding of organic phosphates to hemoglobin may well extend to the specific binding of other phosphate substrates to enzyme reaction sites" (Gill et al., 1980).

Biological implications

Endothermy, which is the ability to maintain elevated body temperature by metabolic means, has been documented in lamnid sharks and in a single major assemblage of large oceanic teleosts, the Scombroidei, where it is thought to have evolved independently in three lineages – the tunas, the billfish and the butterfly mackerel, *Gasterochisma melampus* (Block et al., 1993). Results of our study are consistent with the multiple origins of endothermy hypothesis, as distinctively different molecular mechanism underlies the adaptive reduction in thermal sensitivities of Hb–O_2 affinity in billfish and tuna. Although the reduction in both lineages are attributable to enthalpic contributions from oxygenation-linked effector dissociation, the main effector is ATP in billfish but protons in tuna [both strategies differing from those in regionally heterothermic ungulates, where chloride ions play an analogous role (Coletta et al., 1992)]. It should be borne in mind that these interspecific specializations (decreased temperature sensitivity resulting from structural differences in the Hb molecules that increase effector interaction) are distinct from the decreases in temperature sensitivity seen in temperature-acclimated fish that result from downregulation of red cell effector levels at high temperatures (cf. Grigg, 1969).

The observed temperature insensitivity in billfish Hbs has several physiological implications. Although the high ATP sensitivity and the coupled reduction in temperature sensitivity of Hb–O_2 affinity may hamper O_2 uptake in the gills and O_2 unloading in warm tissues, these potentially detrimental consequences are compensated for by parallel specializations. For instance, high hematocrits [43% in blue marlin (Dobson et al., 1986)] and large Bohr effects, which, combined with low blood buffering capacities that appear to characterize teleost fish generally (Jensen, 2001) should safeguard O_2 delivery to warm organs. As in lamnid sharks (Larsen et al., 2003a) the positive $\Delta H'$ may lower the arterio-venous P_{O2} gradient and thus arterial O_2 loss in the counter-current afferent and efferent arterioles of retia associated with the cranial heater organs. Since normal temperature sensitivities (endothermic deoxygenation in tissues and exothermic oxygenation in the gills) imply outward heat transport [that may account for about 9% of heat produced from glucose metabolism (cf. Weber and Wells, 1989)], the temperature insensitivity will reduce oxygenation-linked heat loss from the warm eyes and brains, thus helping to maintain the strong regional heterothermia. In the swordfish, *Xiphias gladius*, a futile cycle in the modified extraoccular muscle warms the eyes and brain 10–15°C above ambient water temperatures (Fritsches et al., 2005). The resulting retinal warming has been demonstrated to increase temporal resolution up to 12 times, giving these fast-swimming predators "a crucial advantage over their agile, cold-blooded prey" (Fritsches et al., 2005), which may be an important incentive for the evolution of 'temperature insensitive' Hbs in this group.

The evolution of heterothermy in large oceanic fishes may also be associated with niche expansion into cold, nutrient-rich seas, since the ability to warm the brain and retina is considered to have arisen independently in the three lineages in scombroid fish (tunas, billfish and butterfly mackerels), each time in association with movements from oligotropic tropical water into nutrient-rich colder water (Block et al., 1993).

That reduced and reversed temperature sensitivities encountered in fish Hbs may not be uniquely associated with regional heterothermy follows from its occurrence in the ectothermic scombrid, *Scomber japonicus*, which suggests that heterothermy may have evolved after the evolution of reduced temperature sensitivities in these Scombroidei lineages (Clark et al., 2010). Irrespective of the evolutionary origin of the temperature insensitivity in billfish Hbs, the high ATP sensitivities of their Hbs reveal high capacities for adaptive modulation of O_2 affinity and clearly underlie the drastic reductions in the observed temperature sensitivity of O_2 binding to billfish Hbs at physiological pHs. Moreover, dual or multiple incentives (e.g. improved visual acuity, niche expansion) for the evolutionary development of regional heterothermy within a particular lineage (e.g. billfish) need not have been mutually exclusive, but may have been cooperative.

LIST OF ABBREVIATIONS

Hb	hemoglobin
n_{50}	Hill's cooperative coefficient
pI	isoelectric point
P_{50}	half-saturation pressure of oxygen
ΔH	enthalpy of oxygenation
φ	Bohr factor (=$\Delta\log P_{50}/\Delta$pH)

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