ATP-induced Reverse Temperature Effect in Isohemoglobins from the Endothermic Porbeagle Shark (Lamna nasus)*

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The evolutionary convergence of endothermic tunas and lamnid sharks is unique. Their heat exchanger-mediated endothermy represents an interesting example of the evolutionary pressure associated with this specific characteristic. To assess the implications of endothermy for gas transport and the possible contribution of hemoglobin (Hb), we investigated the effect of temperature on the oxygen equilibria of purified isohemoglobin components V and III from the porbeagle shark (Lamna nasus). In the absence of ATP the effect of temperature on oxygen affinity is normal in both Hb III (P50 = 0.9 and 2.2 torr at 10 and 26 °C, respectively) and Hb V (P50 = 1.5 and 2.5 torr at 10 and 26 °C, respectively). In the presence of this effector P50 decreases with increasing temperature in both components (P50 at 10 and 26 °C = 9.9 and 8.4 torr (Hb III), respectively, and 9.6 and 7.4 torr (Hb V), respectively). The reverse temperature effect in the presence of ATP will reduce the risk of oxygen loss from the arterial to the venous blood by lowering the oxygen tension gradient between the blood vessels. The mechanism behind the reverse temperature effect resembles that found in the bluefin tuna (Thunnus thynnus), an endothermic teleost, thus evidencing further convergent evolution.

True tunas and lamnid sharks are the only fish that have developed endothermy. The fact that this characteristic has evolved independently in two such different families (Scombridae and Lamnidae, respectively) makes these species choice subjects for comparison of the traits associated with endothermy (1–4). Endothermic fishes maintain body temperatures of up to 20 °C above ambient water temperatures, depending on species, water temperature, and activity level (5–7). The consequences of high core body temperatures for gas transport, ion balance, oxygen consumption, aerobic capacity, and muscle performance have been the subject of extensive studies in endothermic tuna species (for review, see Refs. 8–11; however, little is known about these relationships in elasmobranchs. The porbeagle shark (Lamna nasus) has a core body temperature of 8–10 °C above ambient water temperature (12) and has one of the highest body temperatures among the Lamnidae (13–15). As in tunas its endothermy is brought about by a number of countercurrent heat exchangers, retae mirabilia, placed in series with the heat-producing organ (16–18). The lateral heat exchangers are placed between the surroundings and the internally located red muscle, and the major blood supply to the red muscle is directed through these via large cutaneous arteries and veins. By minimizing the heat loss via the blood, metabolically produced heat is retained within the active organ (5, 6).

One consequence of heat exchanger-mediated endothermy is that factors other than heat may be exchanged. The smaller the arteries and veins constituting the heat exchanger are, the more efficient the heat retention and the warmer the fish can be relative to the surrounding water. However, decreased dimensions of the heat exchanger increase the risk of oxygen diffusing from the cold arterial blood to the warm venous blood.

Already in 1960 Rossi-Fanelli and Antonini (19) showed that temperature has virtually no effect on oxygen binding of R-state crystalline Hb1 from the endothermic bluefin tuna (Thunnus thynnus) at pH values between 6.45 and 8.7. Further studies revealed that although temperature does not influence P50 in the 10–30 °C temperature range, it does alter the shape of the oxygen equilibrium curve (20, 21), resulting from a normal temperature effect at low oxygen saturation and a reversed one at higher saturations.

The heat exchanger-mediated endothermy represents a curious incidence of convergent evolution. In this context it is interesting to investigate whether endothermy in the porbeagle shark is associated with adaptations in the temperature sensitivities of Hb, as documented in Hb I of bluefin tuna (19–21). This paper reports the effects of temperature, ATP, and pH on isolated Hb components III and V of the porbeagle shark.

Preparation of Isohemoglobins and Oxygen Binding Studies—Isolation of the individual Hbs was performed using preparative isoelectric focusing (column, type 8102, Amersham Biosciences) at 3.9–4.5 °C (Fig. 1) after CO equilibration of the hemolysate. The ampholytes (Amer- sham Biosciences AB) used were pH 6–8 (40%) and pH 6.7–7.7 (60%) in a sucrose gradient. The seven separated components were dialyzed against three changes of CO-equilibrated, 10 mM Hepes buffer, pH 7.7, containing 0.5 mM EDTA.

Three Hb components (Hb V, IV, and III) accounted for far more of the Hb (Fig. 1). Their isoelectric points at 16 °C were 7.58, 7.62, and

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The abbreviations used are: Hb, hemoglobin; MWC, Monod-Wyman-Changeux.
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Saturation were extrapolated from the data in the fitting procedure as described by Fago et al. (25).

$P_{50}$, the oxygen tension at half-saturation of the Hb, was calculated as the $P_{50}$ value at \log(S/(1−S)) = 0, and $n_{50}$ was calculated as $\log(S/(1−S))/\log P_{50}$ at that PO$_2$. The median oxygen tension $P_{m}$ was calculated from

$$P_{m} = (1/K_R)(1/(L + 1/(Lc^s + 1)^{1/4}))$$

(Eq. 2)

where $c = K_\varnothing/K_R$ (28, 29).

The free energy of heme-heme interaction $\Delta G$ was calculated according to Wyman et al. (30).

$$\Delta G = RT\log((L + 1)/(Lc^s + 1)/(Lc^{-s} + 1))$$

(Eq. 3)

The difference in bond energies in the R and T states between the absence and presence of ATP was calculated as $\Delta G = RT\log(K_{ATP}/K_R)$, where $x$ denotes the T or R state.

The heat of oxygenation was calculated according to the following relationship

$$\Delta H = 2.303 \times R \times \log \left( \frac{1}{P_{50}} \right) \left( \frac{1}{T_1} - \frac{1}{T_2} \right)$$

(Eq. 4)

where $R$ is the gas constant, $x$ is $P_{50}$ of the fitted curves was similar to $P_{m}$, and the fitted curves were symmetrical around $P_{50}$.

Both Hbs exhibit high intrinsic oxygen affinities (low $P_{50}$ values) (Table I) and small, normal temperature effects at half-saturation ($\Delta H = -40.1$ and $-20.7 \text{ kJ mol}^{-1}$ in Hbs III and V, respectively) at pH 7.3 (Table II). Increasing temperature from 10 to 26 $^\circ$C increases $P_{50}$ from 0.9 to 2.2 torr (Hb III) and from 1.5 to 2.5 torr (Hb V) (Table I). The addition of ATP reduces the oxygen affinity in Hbs III and V, but less so at higher temperatures, resulting in a small reverse temperature effect in both Hbs ($\Delta H = 12.2$ and 11.6 $\text{kJ mol}^{-1}$ in Hbs III and V, respectively) (Table II).

Fig. 3 shows the temperature dependence of $P_{50}$ (the van't Hoff plot) and $n_{50}$ with and without ATP present. A reverse temperature effect in the presence of ATP is seen. ATP additionally increases cooperativity expressed as $n_{50}$ (Fig. 3, lower panel), particularly at high temperatures. As evident, Hb III and V show very similar oxygenation characteristics.

Mechanisms of the Temperature Effect—The MWC parameters $L$, $K_T$, and $K_R$ provide a mechanistic basis for the effects of temperature on the Hbs (Fig. 4 and Table I). At increasing temperature $L$ decreases, indicating a destabilization of the T (tense) state relative to the R (relaxed) state (Fig. 4a). ATP stabilizes the T state (increases $L$) but only slightly affects the temperature sensitivity.

Increasing temperature decreases oxygen affinity of the R and T states in Hb III and V, but more so in the R state (Fig. 4, b and c). ATP reduces the oxygen affinities of both the T and R states and their temperature sensitivities, making $K_R$ of both Hbs insensitive to temperature change, whereas $K_T$ is somewhat reduced by increasing temperature. The effect of ATP on $K_T$ decreases with increasing temperatures, indicating that ATP also binds to the R state, particularly at low temperatures. The T state becomes less stable at high temperatures ($L$ decreases), and the overall oxygen binding to the T state accordingly decreases.

**RESULTS**

**Effects of Temperature and ATP—**Extended Hill plots of the oxygen equilibria of Hbs III and V at pH 7.3 and at four temperatures (10, 16, 21, and 26 $^\circ$C) each in the presence or absence of ATP are shown (Fig. 2). The MWC model was successfully fitted to the data. In all instances $P_{50}$ of the fitted curves was similar to $P_{m}$, and the fitted curves were symmetrical around $P_{50}$.

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FIG. 2. Hill plots of oxygen equilibria of porbeagle shark at pH 7.3. a, Hb V, b, Hb III. Lines represent the fitting of the MWC model to the data. Diamonds, at 10 °C; squares, at 16 °C; circles, at 21 °C; triangles, at 26 °C; open symbols, in the absence of ATP; filled symbols, in the presence of ATP.

Table I

| Hb sample | Temperature (°C) | P₅₀ (torr) | n₅₀ | P₅₀ (torr) | Log L | S.E.* | Log K₅ | S.E.* | Log K₉ | S.E.* | ΔG | S.E.* |
|-----------|------------------|------------|-----|------------|-------|-------|--------|-------|--------|-------|-----|------|
| V         | 10               | 1.50       | 1.93| 1.94       | 1.44  | 2.66  | 0.219  | -0.622| 0.0417 | 0.505 | 0.0561 | 1.42 |
|           | 16               | 1.39       | 1.63| 1.66       | 1.47  | 1.25  | 0.271  | -0.814| 0.300  | 0.150 | 0.0699 | 1.10 |
|           | 21               | 1.46       | 1.28| 1.28       | 1.50  | 0.725 | 0.362  | -0.570| 0.165  | 0.0211| 0.0745 | 0.562|
|           | 26               | 2.54       | 1.22| 1.26       | 2.68  | 0.330 | 0.0994 | -1.075| 0.183  | -0.304| 0.0215 | 0.513|
| V + ATP   | 10               | 9.65       | 2.34| 2.38       | 9.04  | 3.95  | 0.219  | -1.514| 0.0272 | 0.0313| 0.0790 | 1.98 |
|           | 16               | 8.52       | 2.52| 2.53       | 8.39  | 3.33  | 0.271  | -1.65 | 0.0426 | -0.907 | 0.0636 | 2.12 |
|           | 21               | 7.81       | 2.30| 2.30       | 7.66  | 3.00  | 0.362  | -1.52 | 0.0319 | -0.134 | 0.0510 | 1.89 |
|           | 26               | 7.44       | 2.14| 2.15       | 7.70  | 2.18  | 0.0994 | -1.62 | 0.0710 | -0.340 | 0.0448 | 1.76 |
| III       | 10               | 0.883      | 1.82| 1.84       | 0.923 | 1.65  | 0.148  | -0.614| 0.0939 | 0.446 | 0.0376 | 1.30 |
|           | 16               | 1.45       | 1.53| 1.53       | 1.44  | 1.69  | 0.128  | -0.516| 0.0285 | 0.263 | 0.0321 | 0.920|
|           | 21               | 1.94       | 1.48| 1.49       | 2.02  | 1.10  | 0.0462 | -0.809| 0.0283 | -0.0225| 0.0126 | 0.884|
|           | 26               | 2.16       | 1.16| 1.19       | 2.27  | 0.993 | 0.0791 | -1.05 | 0.188  | -0.268 | 0.0135 | 0.384|
| III + ATP | 10               | 9.89       | 2.36| 2.39       | 9.33  | 3.84  | 0.299  | -1.54 | 0.0292 | -0.0106| 0.0757 | 1.98 |
|           | 16               | 10.9       | 2.33| 2.36       | 10.34 | 3.70  | 0.334  | -1.59 | 0.0266 | -0.0910| 0.0868 | 1.98 |
|           | 21               | 7.06       | 2.13| 2.13       | 7.07  | 2.47  | 0.150  | -1.48 | 0.0327 | -0.0232| 0.0395 | 1.69 |
|           | 26               | 8.38       | 2.08| 2.08       | 8.35  | 2.44  | 0.161  | -1.51 | 0.0249 | -0.0311| 0.0434 | 1.65 |

* Standard error of the fitted parameters.

Table II

| Hb sample | ΔH_Happ | L | T | R | P₅₀ |
|-----------|---------|---|---|---|-----|
| V         | -222    | -27.1 | -81.5 | -20.7 |
| V + ATP   | -182    | -4.97 | -38.0 | 11.6  |
| III       | -184    | -66.6 | -77.8 | -40.1 |
| III + ATP | -152    | 5.51  | -31.5 | 12.2  |

The apparent heat of reaction, ΔH_Happ, for L, the T and R states, and at half-saturation oxygen tension (P₅₀) in Hb III and V in the absence or presence of ATP.

The reverse temperature effect in the presence of ATP thus stems from the combined effects of the reduction of L with increasing temperature and reduced temperature sensitivity of the T state and especially of the R state. These effects are reflected by the temperature invariance of the lower asymptotes of the extended Hill plots in Fig. 2 (constant K₅ values) and a change in the slope of the curve around log[(S)/(1 - S)] = 0 as L falls at higher temperatures. The change in K₉ is evident from the greater stability of the upper asymptotes of the curves in the presence of ATP than in its absence.

Free Energies and Enthalpies of Reaction—In the absence of ATP the free energy of heme-heme interaction, ΔG, falls with increasing temperature (Table I). By stabilizing the T state, as evidenced by the temperature invariance of K₅ and the increase in L (Fig. 4), ATP increases the free energy of cooperativity but does so more at high than at low temperature, thus resulting in a low ATP sensitivity at high temperature. The change in ΔG mirrors the effect of temperature on L and n₅₀ and reflects the ATP-induced increased cooperativity.

In both Hb III and V, ATP increases the apparent (overall) heat of oxygenation, ΔH_Happ (ΔH at P₅₀), which becomes positive (Table II). This effect is consistent with the elevation of ΔH₅₀, ΔH₉, and ΔH_R by ATP (Table II).

The displacement of the lower asymptote of the Hill plots in the presence of ATP reflects the additional bond energies that stabilize the T state in the presence of the polyanionic phosphate anion (Fig. 2). The ATP-induced bond energy differences are greater in the T state than in the R state (4.8–5.0 kJ/mol...
of heme)\(^{-1}\) compared with 2.5–2.6 kJ·(mol of heme)\(^{-1}\) for both Hbs at 10 °C and pH 7.3; Table III). Additionally, the bond energy difference decreases with increasing temperature in both the T and R states. The difference in bond energy, \(\Delta G_T\) and \(\Delta G_R\), with and without ATP is presented in Table III.

Effect of Changes in pH—Hill plots of oxygen equilibria of Hb III at 10 and 26 °C with and without ATP and at different pH values were fitted using the MWC model (Figs. 5 and 6). The pH sensitivities of \(P_{50}\) and \(n_{50}\) are shown in Fig. 7. At 10 °C Hb III shows a reverse Bohr effect between pH 8.3 and 7.5 (\(\varphi \approx -0.5\)) that becomes normal at pH <7.5 (\(\varphi \approx -0.6\)). At 26 °C the phosphate-free Hb show no Bohr effect in the experimental range of pH values. In the presence of ATP the Bohr effect becomes normal at both temperatures. Noticeably, the reverse temperature dependence with ATP present is marked at low pH and phases out at high pH (Fig. 7). The Bohr effect at pH ~7.0–7.3 in the presence of ATP is larger at 10 °C (\(\varphi \approx -0.7\)) than at 26 °C (\(\varphi \approx -0.3\)).

The cooperativity coefficient \(n_{50}\) is negatively affected by lowering pH at low temperature, whereas it is positively affected at high temperature (Fig. 7) and invariably higher in the presence of ATP than in phosphate-free solution. The \(n_{50}\) values at 10 °C exceed those at 26 °C at all pH values when ATP is absent and above pH 7.3 with ATP.

Mechanisms of the Bohr Effect—The allosteric control mechanisms of the Bohr effect are illustrated in Fig. 8. At 10 °C without ATP \(L\) decreases from >10\(^4\) at pH 8.0 to ~10\(^2\) at a pH of 7.6, indicating a destabilization of the T state at low pH. The reverse Bohr effect at 10 °C in the absence of ATP described previously concurs with the large fall in \(L\) as pH is lowered, whereas \(K_T\) and \(K_R\) remain unchanged (Fig. 8). With ATP present this effect is shifted to a lower pH (6.7); the pH effect becomes normal, \(K_T\) decreases, and \(K_R\) drops drastically as pH falls below 7.5. At 26 °C the effect of pH on \(L\), \(K_T\), and \(K_R\) is negligible in the absence of ATP. At 26 °C ATP elevates \(L\) by stabilizing the T state, in part because of a stabilizing effect of additional proton binding to the T state, its effect being larger at low pH. Concurrently, \(K_T\) becomes lower and slightly decreases at lower pH values, and \(K_R\) remains low and stable irrespective of the presence ATP (Fig. 8).

Free Energies of Reaction—The free energy of heme-heme interaction (\(\Delta G\)) is slightly higher at 10 °C than at 26 °C over the entire pH range and in the presence of ATP than in stripped Hb (Table IV). In the absence of ATP, \(\Delta G\) varies only.
slightly with pH. With ATP $\Delta G$ decreases at low pH (<7.5 at 10 °C) and increases at high pH (>7.0 at 26 °C) (Table IV).

**DISCUSSION**

The MWC model, which assumes functional homogeneity between the subunits comprising the tetrameric Hb, successfully describes the oxygen binding characteristics of porbeagle shark Hb. This indicates either that the chain heterogeneity found in porbeagle shark hemolysate (23) is not found in the purified Hb components investigated in this study or that it does not influence oxygen binding.

The reverse temperature effect on isolated Hb III and V from porbeagle shark resembles that in bluefin tuna Hb I in phosphate buffer (21). In Hbs from both species low temperature stabilizes the T state, and a reverse temperature effect on $K_R$ is seen at low pH. The difference between the two species is qualitative. In the bluefin tuna the stabilization of the T state at low temperature (high $L$) abolishes the $T \rightarrow R$ transition. Therefore, oxygen binds in the T state even at high oxygen saturation levels, revealing the presence of two functionally distinct subunits in the T state (21). In porbeagle shark the $T \rightarrow R$ transition does occur, and we find no evidence for differences in the two subunits comprising the Hb. Our results thus agree with those of Andersen and co-workers (23), who found a reverse temperature effect in hemolysate in the 5–15 °C range and a decrease in $L$ with rising temperature. Thus it appears that the evolutionary convergence of endothermic tunas and lamnid sharks includes changes in the functional mechanisms of their main Hb components.

In porbeagle shark Hb the reverse temperature effect is only seen at pH values below 7.5 and in the presence of ATP (Fig. 7). We find that the reverse temperature effect is caused largely by an increase in the allosteric constant, $L$, with decreasing temperature (Fig. 4). Although acting in the opposite direction, the concurrent increase in $K_R$ at pH 7.3 in the presence of ATP is
The overall reverse temperature effect. The effect of ATP on filled symbols open symbols) of ATP. In the absence (circles, △) or presence (filled symbols, □) at different pH values in °C, and in the presence and absence of ATP.

**Table IV**

| Temperature | pH | 10 kJ (mol of heme) \( \Delta G \) | pH | 10 kJ (mol of heme) \( \Delta G \) |
|-------------|----|-----------------------------------|----|-----------------------------------|
| °C          |    |                                   |    |                                   |
| 10          | 7.046 | 0.70 | 6.919 | 1.24 |
|             | 7.039 | 1.30 | 7.133 | 1.98 |
|             | 7.645 | 1.30 | 7.656 | 2.37 |
|             | 7.793 | 1.38 | 8.141 | 2.5 |
|             | 8.302 | 1.85 | 8.282 | 2.6 |
|             | 8.640 | 1.93 | 8.470 | 2.05 |
| 26          | 6.563 | 0.77 | 6.452 | 2.19 |
|             | 6.717 | 0.47 | 6.689 | 2.08 |
|             | 7.055 | 0.37 | 6.984 | 1.81 |
|             | 7.305 | 0.38 | 7.349 | 1.65 |
|             | 7.610 | 0.48 | 7.593 | 1.33 |
|             | 7.919 | 0.69 | 7.986 | 1.60 |
|             | 8.208 | 0.84 | 8.234 | 1.55 |
|             | 8.592 | 0.79 | 8.234 | 1.55 |

The Bohr effect in porbeagle shark Hb at 10 °C and in the presence and absence of ATP. The free energies of cooperativity, \( \Delta G \), at 10 °C and 26 °C with ATP (Hb III) are comparable to those found in tench (6.1 kJ (mol of heme) \( \Delta G \)) (34). Human Hb has a higher \( \Delta G \) at 25 °C (8.8 kJ (mol of heme) \( \Delta G \)) at pH 7.4, close to values found at 10 °C with ATP at high pH (Hb III, Table IV) and at 16 °C with ATP (Hb V, Table I) (35).

The Bohr effect in porbeagle shark Hb at 10 °C with ATP (φ = −0.76) is large and similar to values for other active fishes. Albacore tuna (Thunnus alalunga) and striped marlin...
have Bohr factors of −1.2 and −1.0, respectively (36, 37), whereas those obtained from the moderately active blue shark (Prionace glauca) stripped hemoglobin with and without added inositol hexaphosphate are −1 and −0.4, respectively (38). It is notable that the reverse Bohr effect at low temperature and in the absence of ATP extends to high pH values (7.5–8.3). The reverse Bohr effect is seen in other fish Hbs but is usually restricted to cathodic Hbs (for review, see Ref. 39), where it also disappears when ATP is present. This suggests that upon oxygenation alkaline Bohr groups change pKv value in the reverse direction from normal.

Possible Implications for Oxygen Transport—Allosteric co-factors such as organic phosphates and protons lower the intrinsically high oxygen affinity of Hb to values that ensure oxygen unloading at oxygen tensions that are sufficiently high for physiological needs. Compared with small Bohr effects reported in sluggish elasmobranch species (40), active ones, like the porbeagle and mako sharks, have substantial Bohr effects at low temperature and in the presence of ATP (23). The pH insensitivity on Kp has been interpreted as advantageous under changing environmental oxygen tensions (34). By maintaining a constant high affinity for oxygen in the R state, loading is protected during hypoxia and during exercise when the blood may become acidic. In porbeagle shark Kp falls at low temperature when pH is low, an effect not present at high temperature when environmental, dissolved oxygen concentration can be assumed to be lower and loading may become compromised.

The concentration of ATP and other organic phosphates in nucleated fish red cells decreases under conditions of decreased oxygen availability, forming a regulatory mechanism that favors oxygen loading through the resulting increase in oxygen affinity. During exercise the affinity gain may, however, be offset by the decreased blood pH (Bohr effect). In elasmobranchs the presence of high urea levels may hinder oxygen affinity modulation by dampening the ATP effect as seen in stripped hemolysate of spiny dogfish (33).

The presence of countercurrent heat exchangers in the porbeagle shark imposes a risk of oxygen loss from the arterial to the venous blood. However, as in the endothelial bluefin tuna, the presence of a reverse temperature effect in isolated Hb indicates that this risk is reduced. The increased oxygen affinity with rising temperature reduces the oxygen tension gradient between the arterial and venous blood. This adaptation to an efficient heat conservation mediated by heat exchangers may only be necessary in cases in which the heat exchangers are very efficient. As such the bluefin tuna and the porbeagle shark are among the warmest species in their distantly related families, respectively, each representing very efficient countercurrent heat exchangers (6, 13–16, 41–43).

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