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# COMPARATIVE ANALYSIS OF AUTOXIDATION OF HAEMOGLOBIN

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## Summary

**Autoxidation of oxyhaemoglobin (oxyHb) to methaemoglobin was measured at different temperatures in haemoglobin solutions from Atlantic hagfish, river lamprey, common carp, yellowfin tuna and pig. The aims were to evaluate the impact of the absent distal histidine in hagfish haemoglobin, the importance of oxyHb being either monomeric (hagfish and lamprey) or tetrameric (carp, tuna and pig) and to gain information on the temperature-sensitivity of autoxidation. The rate of autoxidation was lower in hagfish than in carp, yellowfin tuna and lamprey haemoglobins at any given temperature. Substitution of the distal histidine residue (His E7) with glutamine in hagfish haemoglobin was therefore not associated with an accelerated autoxidation, as might be expected on the basis of the normal protective role of His E7. Glutamine may have similar qualities to histidine and be involved in the low susceptibility to autoxidation. The low oxidation rate of hagfish haemoglobin, together with an oxidation rate of lamprey haemoglobin that did not differ from that of carp and yellowfin tuna haemoglobins, also revealed that autoxidation was not accelerated in the monomeric oxyhaemoglobins. Pig haemoglobin was oxidised more slowly than fish haemoglobins, demonstrating that fish haemoglobins are more sensitive to autoxidation than mammalian haemoglobins. The rate of autoxidation of hagfish haemoglobin was, however, only significantly greater than that of pig haemoglobin at high temperatures. Autoxidation was accelerated by rising temperature in all haemoglobins. Arrhenius plots of carp and yellowfin tuna haemoglobin revealed a break at 25 °C, reflecting a lower temperature-sensitivity between 5 and 25 °C than between 25 and 40 °C.**

Key words: haemoglobin, oxidation, methaemoglobin, temperature, hagfish, lamprey, carp, yellowfin tuna, pig, *Myxine glutinosa*, *Lampetra fluviatilis*, *Cyprinus carpio*, *Thunnus albacares*, *Sus scrofa domesticus*.

## Introduction

Haemoglobin (Hb) undergoes spontaneous oxidation (autoxidation) of the iron in its haem groups, forming methaemoglobin (metHb), which cannot transport oxygen. Autoxidation of oxygenated haemoglobin (oxyHb) can be written schematically as (Equation 1):

$$\mathrm{Hb(Fe^{2+})O_2} \xrightarrow{k} \mathrm{metHb(Fe^{3+}) + O_2^-}\,, \qquad (1)$$

where $k$ is the observed first-order rate constant. The reaction not only forms metHb, which is functionally inert with respect to $O_2$ transport, but also a superoxide radical $O_2^-$, which can lead to further oxidation reactions. It is therefore important that autoxidation be minimised under physiological conditions and that the red blood cells contain enzymes that can reduce metHb to functional haemoglobin and remove superoxide (for a recent review, see Faivre et al., 1998).

The rate of autoxidation of haemoglobin and myoglobin is several orders of magnitude lower than for free haem groups or for haem groups that have become exposed to the solvent by unfolding of the globin moiety (Sugawara et al., 1995). Thus, the embedding of the haem group in the globin provides significant protection against autoxidation. The haem group lies in a hydrophobic pocket made by the folds of the globin chain. The haem is covalently linked to the globin *via* the proximal histidine residue (His F8) and is wedged into its pocket by a phenylalanine residue (Phe CD1). These two amino acid residues are common to all haemoglobins (Perutz, 1990). Most haemoglobins also have a histidine residue on the distal side of the haem group (Perutz, 1990). This distal histidine residue (His E7) is involved in protection against autoxidation. Its role has been probed by site-directed mutagenesis in sperm whale myoglobin. Substition of His E7 with a number of different amino acid residues was associated with a 40- to 350-fold increase in the rate of autoxidation (Springer et al., 1989). On this basis, it is of interest to evaluate autoxidation in the haemoglobin of the Atlantic hagfish *Myxine glutinosa*, which is one of the few species that contain a haemoglobin without the distal histidine residue. In *Myxine glutinosa*, the E7 position is occupied by a glutamine residue (Liljeqvist et al., 1982). Hagfish are ancient animals and share with another ancient group of vertebrates, the lampreys, an additional peculiarity: the haemoglobin is monomeric in

the oxygenated state and aggregates into oligomers upon deoxygenation (Nikinmaa et al., 1995; Fago and Weber, 1995). In mammals, both monomeric oxymyoglobin and isolated α- and β-chains of haemoglobin are more readily oxidised than tetrameric oxyhaemoglobin (Tsuruga et al., 1998). This provides an additional stimulus to study autoxidation in hagfish and lamprey haemoglobin to clarify whether the monomeric nature of their oxygenated haemoglobin is associated with increased susceptibility to autoxidation compared with the tetrameric haemoglobins of teleosts fishes and mammals.

Information on autoxidation in lower vertebrates is limited. Fish haemoglobins are quite sensitive to oxidation (Riggs, 1970; Sharp, 1973), but experiments evaluating rate constants under standardized and comparable conditions in different species are scarce. It has been suggested that fish haemoglobins may have minimal autoxidation rates at the temperatures to which species are adapted (Wilson and Knowles, 1987). Information on the influence of temperature is indeed required in fish because, as ectotherms, they experience quite variable body temperatures and because autoxidation is highly temperature-sensitive (Wilson and Knowles, 1987; Jensen et al., 1998).

The present study evaluates the rate of autoxidation and its temperature-dependence in the composite haemoglobins from two agnathans (Atlantic hagfish and river lamprey), two teleosts fishes (common carp and yellowfin tuna) and one mammal (domestic pig).

## Materials and methods

Atlantic hagfish (*Myxine glutinosa*) were kept in full-strength sea water at Kristineberg Marine Biological Station, Sweden. River lamprey (*Lampetra fluviatilis*) and common carp (*Cyprinus carpio*) were held in 400 l freshwater tanks at Odense University, Denmark. Yellowfin tuna (*Thunnus albacares*) were maintained in circular outdoor tanks at the Kewalo Research Facility (National Marine Fisheries Service, Honolulu Laboratory), Hawaii, USA. Blood was sampled from anaesthetised animals [1:10000 MS 222, 3-aminobenzoic acid ethyl ester; Sigma]) *via* the caudal vessels (or caudal sinus in hagfish). Blood from domestic pig (*Sus scrofa domesticus*) was obtained from the Danish Crown slaughterhouse in Odense.

Red blood cells were separated by centrifugation and subsequently washed three times in physiological saline. Following the final wash and centrifugation, the packed red blood cells were frozen in liquid nitrogen. Hagfish red blood cells were transported to Odense while frozen in liquid $N_2$. Yellowfin tuna red blood cells were airshipped from Hawaii to Odense on dry ice. The samples were stored at −80 °C until use. Distilled water was added upon thawing, and the red blood cell debris was isolated by centrifugation. Cell solutes were removed from the supernatant by passing the haemolysate three times through a mixed-bed ion-exchange column (Amberlite MB1, BDH). The total haemoglobin concentration was measured by the cyanmethaemoglobin method (using an extinction coefficient of 11 mmol $l^{-1}$ $cm^{-1}$ at 540 nm). The stripped haemoglobin solution was diluted to a monomeric haemoglobin concentration of 0.16 mmol $l^{-1}$ (tetrameric concentration of 0.04 mmol $l^{-1}$) and buffered to pH 7.3 (a representative intraerythrocytic pH), using Hepes buffer at a final concentration of 0.1 mol $l^{-1}$. Measurements of pH were performed with the capillary pH electrode of a Radiometer (Copenhagen, Denmark) BMS 3 electrode set-up.

In each experiment, a sample (3 ml) of haemoglobin was equilibrated with humidified air in an Eschweiler (Kiel, Germany) tonometer thermostatted to a constant temperature. After 40 min (time zero in the experiments), a 1 ml subsample was transferred to a cuvette kept at the same temperature in a spectrophotometer (Milton Roy Spectronic 1201). The absorbance was measured at 560, 576 and 630 nm, allowing the concentrations of oxyhaemoglobin (oxyHb), deoxyhaemoglobin (deoxyHb) and methaemoglobin (metHb) (Benesch et al., 1973) to be calculated. The haemoglobin sample was then returned to the tonometer for continued equilibration. In most experiments, measurements were performed on an hourly basis up to 6 h. In some cases, a more frequent sampling regime was used, and the measurements were continued until 7.5 h. At high equilibration temperatures, precipitates of denatured haemoglobin occasionally developed at late stages of the equilibration. In these cases, only the absorbance data prior to the appearance of precipitation were used. Experiments were performed at 5, 15, 25, 30, 35 and 40 °C for hagfish, carp and yellowfin tuna haemoglobins. For lamprey haemoglobin, experiments were performed at 5, 15, 25 and 30 °C, and for pig haemoglobin, experiments were performed at 15, 25, 35 and 40 °C. The number of experiments for each species at each temperature was 4–9.

Autoxidation of oxyhaemoglobin can be described by equation 2:

$$[\mathrm{oxyHb}]_t = [\mathrm{oxyHb}]_0 \times e^{-kt} , \quad (2)$$

where $[\mathrm{oxyHb}]_t$ and $[\mathrm{oxyHb}]_0$ are the concentrations of oxyHb at times $t$ and zero, respectively, and $k$ is the first-order rate constant. Plots of $\ln([\mathrm{oxyHb}]_t/[\mathrm{oxyHb}]_0)$ *versus* time are accordingly linear, with slope $-k$, allowing determination of $k$ ($h^{-1}$) by linear regression. The significance ($P<0.05$) of differences in $\ln k$ was probed by two-factor (species and temperature) analysis of variance (ANOVA) followed by the Tukey multiple-comparison test. Since there were slight differences in the number of test temperatures among species, the statistical analysis proceeded in three steps. First, hagfish, carp and yellowfin tuna were compared using data from all six temperatures. Then data from hagfish, carp, yellowfin tuna and lamprey were compared at the four temperatures for which data on lamprey were available. Finally, hagfish, carp, yellowfin tuna and pig were compared at the four temperatures for which data for pig were available.

## Results

Examples of individual experiments (chosen as those at which the individual $k$ values were closest to the mean values at the given conditions) are shown for hagfish and carp in Fig. 1. The

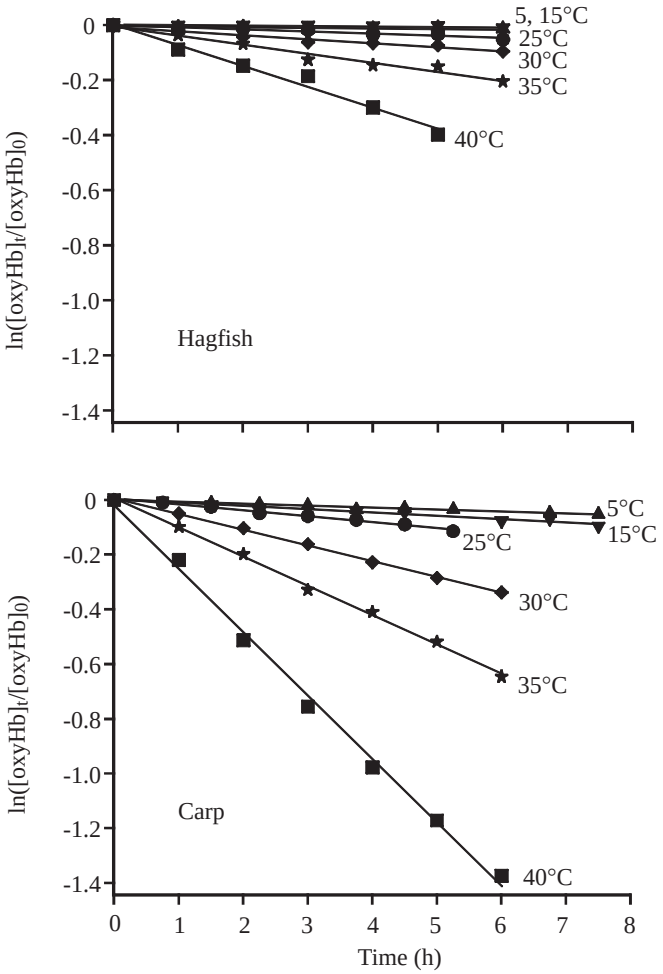


Fig. 1. Examples of the time-dependence of the autoxidation reaction for hagfish (*Myxine glutinosa*) and carp (*Cyprinus carpio*) oxyhaemoglobin (oxyHb) at various temperatures. The measurements were carried out in 0.1 mol $l^{-1}$ Hepes buffer (pH 7.3) at a monomeric haemoglobin concentration of 0.16 mmol $l^{-1}$. $[oxyHb]_t$ and $[oxyHb]_0$ are the concentrations of oxyHb at times $t$ and zero, respectively.

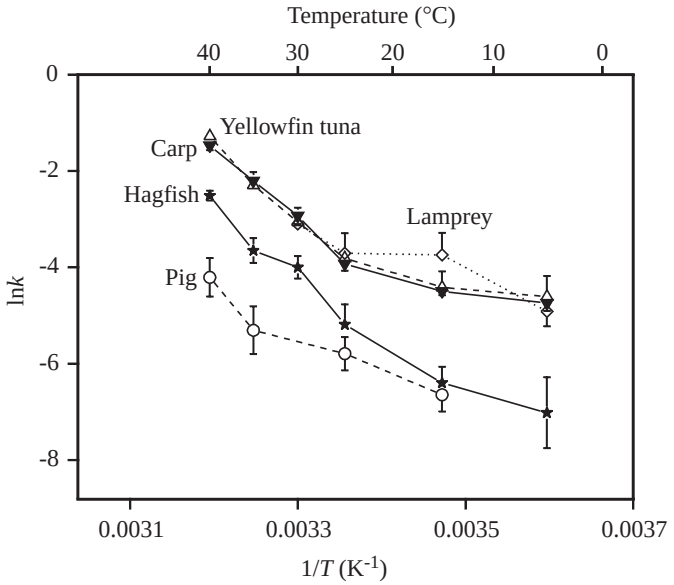


Fig. 2. Temperature-dependence of autoxidation of hagfish, carp, yellowfin tuna, lamprey and pig oxyhaemoglobins. The natural logarithm of the measured first-order rate constant ($k$, $h^{-1}$) is plotted as a function of the inverse absolute temperature ($T$, K) (i.e. Arrhenius plots), with temperatures in °C given on the upper $x$-axis. Values are means ± S.E.M. ($N$=4–9 at each point).

plots of $\ln([oxyHb]_t/[oxyHb]_0)$ *versus* time ($t$) were linear, revealing that the rate of autoxidation was first order with respect to [oxyHb]. The observed first-order rate constant $k$ (the negative slope of the curves) increased with temperature, reflecting an acceleration of autoxidation with increasing temperature (Fig. 1). Since the curves are drawn to the same scale, it is also evident that autoxidation was slower in hagfish haemoglobin than in carp haemoglobin at any given temperature (Fig. 1).

An overview of the data for all five species is given in Fig. 2, which shows ln$k$ as a function of the inverse absolute temperature ($T$, K). For convenience, a scale in °C is included at the top of Fig. 2. There was a significant increase in ln$k$ with increasing temperature in all five species. Values of ln$k$ were significantly lower in hagfish than in carp, yellowfin tuna and lamprey at any given temperature, but there were no significant differences between carp, yellowfin tuna and lamprey. Values of ln$k$ for pig haemoglobin were significantly lower than for carp, yellowfin tuna and lamprey haemoglobin at all temperatures tested, and they were significantly lower than for hagfish haemoglobin at 35 and 40 °C but not at 15 and 25 °C.

Rather than revealing an overall linearity, the ln$k$ *versus* 1/$T$ relationships (Arrhenius plots) for carp and yellowfin tuna seemed to contain two linear segments: one between 5 and 25 °C and a second between 25 and 40 °C (Fig. 2). Linear regression on these segments and subsequent calculation of the activation energy ($E_a$=−slope×$\boldsymbol{R}$, where $\boldsymbol{R}$ is the gas constant) accordingly suggested $E_a$ values for carp and yellowfin tuna, respectively, of 27.9 and 27.5 kJ $mol^{-1}$ between 5 and 25 °C and of 125.3 and 129.5 kJ $mol^{-1}$ between 25 and 40 °C. In hagfish, the $E_a$ value between 25 and 40 °C (129.9 kJ $mol^{-1}$) was rather similar to those of carp and tuna, but the tendency for two linear segments was less clear-cut. The overall $E_a$ in hagfish was 93.2 kJ $mol^{-1}$. The lamprey data gave an $E_a$ of 44.9 kJ $mol^{-1}$, and in pig the $E_a$ value was 66.0 kJ $mol^{-1}$.

## Discussion

The present data show that autoxidation of oxyhaemoglobin is strongly temperature-dependent and that the rate of autoxidation differs significantly among species. Autoxidation of hagfish haemoglobin attracts special interest, because its haemoglobin lacks the distal His E7 residue and is monomeric in the oxygenated state. Both these characteristics could potentially increase the susceptibility to autoxidation (see Introduction). The rate of autoxidation of hagfish oxyHb was, however, significantly lower than for the tetrameric and His-E7-containing haemoglobins of carp and yellowfin tuna.

Hagfish oxyHb was also more resistant to autoxidation than lamprey oxyHb, which is monomeric like hagfish haemoglobin but contains the distal histidine residue. Only in comparison with mammalian (pig) haemoglobin did hagfish oxyHb show an increased tendency to autoxidation, but only at high temperatures and not at low temperatures (Fig. 2).

*Mechanism of autoxidation and the role of the distal histidine*

The mechanism of autoxidation, as outlined for mammalian oxyHb and oxyMb, may involve a nucleophilic displacement of $O_2^-$ from $HbO_2$ by a water molecule or $OH^-$ that enters the haem pocket from the solvent (e.g. Shikama, 1990; Tsuruga and Shikama, 1997). The water molecule or $OH^-$ (the nucleophile, *X*) remains bound to the ferric iron to form aqua- or hydroxy-metHb (Equation 3):

$$Hb(Fe^{2+})O_2 + X \rightarrow Hb(Fe^{3+})X + O_2^- . \quad (3)$$

Autoxidation can also proceed *via* initial dissociation of dioxygen followed by binding of the nucleophile with subsequent oxidation of haem iron and formation of superoxide (Wallace et al., 1982) (Equation 4, Equation 5, Equation 6):

$$Hb(Fe^{2+})O_2 \leftrightarrow Hb(Fe^{2+}) + O_2 , \quad (4)$$

$$Hb(Fe^{2+}) + X \leftrightarrow Hb(Fe^{2+})X , \quad (5)$$

$$Hb(Fe^{2+})X + O_2 \rightarrow Hb(Fe^{3+})X + O_2^- . \quad (6)$$

In air-saturated solutions (as used in the present experiments), where $HbO_2$ saturation is high but less than 100 %, autoxidation is likely to involve both types of reaction (Abugo and Rifkind, 1994).

The distal histidine residue normally protects against autoxidation by stabilising bound $O_2$ and by restricting entry of water into the haem pocket (Springer et al., 1989). Its importance is illustrated by the 40- to 350-fold increase in the rate of autoxidation when His E7 in sperm whale myoglobin is replaced with the residues Gly, Val, Phe, Thr, Asp, Cys, Met, Arg, Lys or Tyr (Springer et al., 1989). Myoglobin from the mollusc *Aplysia* is one of the few natural myoglobin/haemoglobin pigments that lack the distal histidine. In *Aplysia*, the distal E7 position is occupied by a Val residue, which is associated with a much higher susceptibility to autoxidation than in mammalian myoglobins (Shikama and Matsuoka, 1994). This may be explained by the Val residue being much smaller in size than a His residue, rendering the haem pocket more open to attack by a solvent water molecule on the $FeO_2$ centre (Shikama and Matsuoka, 1994).

Glutamine, which occupies the E7 position in *Myxine glutinosa* haemoglobin, has similar qualities to histidine because it occupies a similar amount of tertiary space and has a side-chain nitrogen taking part in hydrogen bonding (Liljeqvist et al., 1982). Therefore, the His→Gln substitution may be less critical than the substitutions that were probed by Springer et al. (Springer et al., 1989). Certainly, the present species comparison reveals that the unusual absence of a distal histidine residue need not be associated with accelerated autoxidation. In addition to the similarity between His and Gln, the presence of Gln rather than His at position E7 could be ameliorated by compensatory substitutions at other sites in the protein so that the combination of amino acid residues in the haem cavity of hagfish haemoglobin provides it with a lower susceptibility to autoxidation than in teleosts and lamprey haemoglobins. Indeed, this is suggested by the present data (Fig. 2).

*Autoxidation in relation to aggregation state*

Monomeric myoglobin is normally more susceptible to autoxidation than tetrameric haemoglobin, and isolated α- and β-chains also oxidise more readily than the tetramer (Tsuruga et al., 1998), suggesting that monomeric pigments may be more sensitive to autoxidation than tetrameric pigments. The lower rate of autoxidation of hagfish oxyHb than of teleost oxyHb (Fig. 1, Fig. 2), however, shows that this need not always be the case. This supports the idea that the special amino acid arrangement in the haem cavity of *Myxine glutinosa* haemoglobin somehow shields against accelerated autoxidation. The rate of autoxidation of monomeric lamprey haemoglobin was higher than that of hagfish haemoglobin, but it was not significantly different from that of the two teleosts with tetrameric haemoglobin (Fig. 2).

The observed rate of autoxidation of pig haemoglobin at 35 °C is comparable with that reported at the same temperature (and pH) for human haemoglobin (Tsuruga et al., 1998), supporting that pig haemoglobin can be used as a mammalian control. Autoxidation of pig oxyHb was significantly lower than that of the fish haemoglobins (Fig. 2), corroborating earlier reports that fish haemoglobins are more sensitive to oxidation than are mammalian haemoglobins (Riggs, 1970; Sharp, 1973; Wilson and Knowles, 1987). Since the experiments were performed with dilute haemoglobin solutions, it is relevant to consider whether dissociation of some of the tetramers to dimers could be involved in the observed differences. Fish haemoglobins are, however, less liable to dissociate to dimers than are mammalian haemoglobins (Edelstein et al., 1976), and this proposal cannot explain the difference. Furthermore, at the chosen haemoglobin concentration (0.16 mmol haem $l^{-1}$) and pH, dissociation to dimers is minimal and of only marginal influence on rate constants for autoxidation even in mammalian haemoglobin (Tsuruga and Shikama, 1997). It appears that fish haemoglobins are inherently more susceptible to oxidation than mammalian haemoglobins.

*Temperature-sensitivity of autoxidation*

Increased temperatures accelerate the autoxidation of oxyhaemoglobin, but the extent to which this is of physiological importance varies among the species. *Myxine glutinosa* lives at low temperatures and does not tolerate high temperatures (Martini, 1998). At its typical habitat temperature (close to 5 °C), the rate of autoxidation is very low and indistinguishable from that of mammalian haemoglobin (Fig. 2). The carp, in contrast, is a eurythermal species and may experience temperatures covering most of the range examined. The carp used in the present experiments were acclimated to 15 °C. Around this temperature, the absolute *k* values are

relatively low, apparently supporting the idea that autoxidation rates are low at the temperatures to which given fish species are adapted (Wilson and Knowles, 1987). However, when given the choice, carp prefer higher temperatures, with a final temperature preference of 32 °C (Pitt et al., 1956). At this temperature, both absolute oxidation rates and the temperature-sensitivity of autoxidation are considerably increased (Fig. 2). It may be hypothesised, therefore, that the cellular enzymatic defence against haemoglobin oxidation is effective in carp, and that the temperature-dependence of metHb reduction is comparable with that for autoxidation. Otherwise, increasing temperatures *in vivo* would lead to a build-up of red blood cell metHb. Yellowfin tuna is a warm-water fish that spends most of its time in water at temperatures above 22 °C (Brill et al., 1999). Even though absolute *k* values are still relatively low at this temperature, the increased temperature-sensitivity above 25 °C (Fig. 2), together with the fact that tuna have deep core muscle temperatures above ambient temperatures, suggests that yellowfin tuna should also have an effective cellular defence against autoxidation.

Even though some reports have suggested that fish erythrocytes contain high levels of metHb (Graham and Fletcher, 1986), the conventional picture is that metHb levels are kept low (approximately 1–2 % of total haemoglobin) in circulating red blood cells of fish (Wells et al., 1997). Thus, spontaneous autoxidation, which occurs at higher rates in fish than in mammalian haemoglobins, appears to be effectively countered by enzymatic reduction inside the red blood cells. However, when oxidising agents are present in excess, the delicate balance between oxidation and reduction of haemoglobin is challenged, and high metHb levels develop (e.g. Jensen, 1990).

The $E_a$ values for carp and yellowfin tuna were lower in the temperature range 5–25 °C than in the range 25–40 °C. The apparent break at 25 °C in the Arrhenius plot for carp and yellowfin tuna haemoglobins could implicate a temperature-dependent conformational change. Absolute $E_a$ values for carp and yellowfin tuna haemoglobin in the temperature range 25–40 °C are comparable with that of hagfish haemoglobin and with the few values reported at similar temperatures for other fish haemoglobins (Wilson and Knowles, 1987) and for human haemoglobin (Zavodnik et al., 1992).

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## References

**Abugo, O. O. and Rifkind, J. M.** (1994). Oxidation of hemoglobin and the enhancement produced by nitroblue tetrazolium. *J. Biol. Chem.* **269**, 24845–24853.

**Benesch, R. E., Benesch, R. and Yung, S.** (1973). Equations for the spectrophotometric analysis of hemoglobin mixtures. *Analyt. Biochem.* **55**, 245–248.

**Brill, R. W., Block, B. A., Boggs, C. H., Bigelow, K. A., Freund, E. V. and Marcinek, D. J.** (1999). Horizontal movements and depth distribution of large adult yellowfin tuna (*Thunnus albacares*) near the Hawaiian Islands, recorded using ultrasonic telemetry: implications for the physiological ecology of pelagic fishes. *Mar. Biol.* **133**, 395–408.

**Edelstein, S. J., McEwen, B. and Gibson, Q. H.** (1976). Subunit dissociation in fish haemoglobins. *J. Biol. Chem.* **251**, 7632–7637.

**Fago, A. and Weber, R. E.** (1995). The hemoglobin system of the hagfish *Myxine glutinosa*: aggregation state and functional properties. *Biochim. Biophys. Acta* **1249**, 109–115.

**Faivre, B., Menu, P., Labrude, P. and Vigneron, C.** (1998). Hemoglobin autooxidation/oxidation mechanisms and methemoglobin prevention or reduction processes in the bloodstream. Literature review and outline of autoxidation reaction. *Art. Cells, Blood Subs. Immob. Biotech.* **26**, 17–26.

**Graham, M. S. and Fletcher, G. L.** (1986). High concentrations of methemoglobin in five species of temperate marine teleosts. *J. Exp. Zool.* **239**, 139–142.

**Jensen, F. B.** (1990). Nitrite and red cell function in carp: Control factors for nitrite entry, membrane potassium ion permeation, oxygen affinity and methaemoglobin formation. *J. Exp. Biol.* **152**, 149–166.

**Jensen, F. B., Fago, A. and Weber, R. E.** (1998). Hemoglobin structure and function. In *Fish Physiology*, vol. 17, *Fish Respiration* (ed. S. F. Perry and B. Tufts), pp. 1–40. San Diego, CA: Academic Press.

**Liljeqvist, G., Paléus, S. and Braunitzer, G.** (1982). Hemoglobins. XLVIIII. The primary structure of a monomeric hemoglobin from the hagfish, *Myxine glutinosa* L.: evolutionary aspects and comparative studies of the function with special reference to the heme linkage. *J. Mol. Evol.* **18**, 102–108.

**Martini, F. H.** (1998). The ecology of hagfishes. In *The Biology of Hagfishes* (ed. J. M. Jørgensen, J. P. Lomholt, R. E. Weber and H. Malte), pp. 57–77. London: Chapman & Hall.

**Nikinmaa, M., Airaksinen, S. and Virkki, L. V.** (1995). Haemoglobin function in intact lamprey erythrocytes: interactions with membrane function in the regulation of gas transport and acid–base balance. *J. Exp. Biol.* **198**, 2423–2430.

**Perutz, M. F.** (1990). Mechanisms regulating the reactions of human hemoglobin with oxygen and carbon monoxide. *Annu. Rev. Physiol.* **52**, 1–25.

**Pitt, T. K., Garside, E. T. and Hepburn, R. L.** (1956). Temperature selection of the carp (*Cyprinus carpio* Linn.). *Can. J. Zool.* **34**, 555–557.

**Riggs, A.** (1970). Properties of fish hemoglobins. In *Fish Physiology*, vol. IV (ed. W. S. Hoar and D. J. Randall), pp. 209–252. New York: Academic Press.

**Sharp, G. D.** (1973). An electrophoretic study of hemoglobins of some scombroid fishes and related forms. *Comp. Biochem. Physiol.* **44**B, 381–388.

**Shikama, K.** (1990). Autoxidation of oxymyoglobin: a meeting point of the stabilization and the activation of molecular oxygen. *Biol. Rev.* **65**, 517–527.

**Shikama, K. and Matsuoka, A.** (1994). *Aplysia* myoglobin with unusual properties: another prototype in myoglobin and haemoglobin biochemistry. *Biol. Rev.* **69**, 233–251.

**Springer, B. A., Egeberg, K. D., Sligar, S. G., Rohlfs, R. J., Mathews, A. J. and Olson, J. S.** (1989). Discrimination between oxygen and carbon monoxide and inhibition of autoxidation by myoglobin. Site-directed mutagenesis of the distal histidine. *J. Biol. Chem.* **264**, 3057–3060.

**Sugawara, Y., Matsuoka, A., Kaino, A. and Shikama, K.** (1995). Role of globin moiety in the autoxidation reaction of oxymyoglobin: effect of 8 M urea. *Biophys. J.* **69**, 583–592.

**Tsuruga, M., Matsuoka, A., Hachimori, A., Sugawara, Y. and Shikama, K.** (1998). The molecular mechanism of autoxidation for human oxyhemoglobin. Tilting of the distal histidine causes nonequivalent oxidation in the β chain. *J. Biol. Chem.* **273**, 8607–8615.

**Tsuruga, M. and Shikama, K.** (1997). Biphasic nature in the autoxidation reaction of human oxyhemoglobin. *Biochim. Biophys. Acta* **1337**, 96–104.

**Wallace, W. J., Houtchens, R. A., Maxwell, J. C. and Caughey, W. S.** (1982). Mechanism of autooxidation for hemoglobins and myoglobins. Promotion of superoxide production by protons and anions. *J. Biol. Chem.* **257**, 4966–4977.

**Wells, R. M. G., Baldwin, J. and Seymour, R. S.** (1997). Low concentrations of methaemoglobin in marine fishes of the Great Barrier Reef, Australia. *Mar. Freshwater Res.* **48**, 303–309.

**Wilson, R. R., Jr and Knowles, F. C.** (1987). Temperature adaptation of fish hemoglobins reflected in rates of autoxidation. *Archs. Biochem. Biophys.* **255**, 210–213.

**Zavodnik, I. B., Piletskaya, T. P. and Stepuro, I. I.** (1992). Auto-oxidation and oxygenation of human hemoglobin. *Mol. Biol.* **26**, 40–45.