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Temperature modulation of oxygen transport in a diving mammal (*Balaenoptera acutorostrata*)

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The functional properties of haemoglobin from the Lesser Rorqual whale (*Balaenoptera acutorostrata*) have been characterized as a function of the heterotropic effector concentrations and temperature. The results obtained suggest the existence of sophisticated modulation mechanisms based on the interplay of organic phosphates, carbon dioxide, lactate and temperature. These, together with the very small apparent heat of oxygenation (ΔH) of oxygen binding, have been physiologically interpreted on the basis of the specific metabolic needs of this diving mammal.

INTRODUCTION

We have recently shown (Condò *et al.*, 1988; Brix *et al.*, 1989*a*; Giardina *et al.*, 1989*a*,*b*) that haemoglobins from Arctic ruminants are characterized by a very low temperature sensitivity of oxygen binding. This has been interpreted to be beneficial to animals living in Arctic environments. In order to investigate how general this phenomenon could be, we have extended our study to the respiratory pigment from the Lesser Rorqual whale, *Balaenoptera acutorostrata* (Brix *et al.*, 1989*b*), two specimens of which were caught off the north coast of Norway on the Norwegian Scientific Whale Cruise 1988 arranged by the Biological Institute, Oslo, Norway.

In addition, whale haemoglobin could be an interesting molecule from other points of view, since the animal is specialized for prolonged dives, often in cold environments (water temperature down to 0 °C). In this respect, as other with diving mammals, the whale may have developed particular mechanisms for the maintenance of adequate oxygen supply to tissues in hypoxic conditions, and for the utilization of the additional lactic acid which is produced during anaerobic metabolism.

To test the above hypothesis, besides using D-glycerate-2,3-bisphosphate (2,3-DPG), we have investigated other heterotropic effectors of haemoglobin function, such as CO_2 and lactic acid, whose effects have been studied as a function of temperature. The results obtained indicate the existence of sophisticated modulation mechanisms which may be of great physiological importance and which have never been reported previously. The data are also discussed in the light of the primary structure of the molecule.

MATERIALS AND METHODS

The haemoglobin of the whale *Balaenoptera acutorostrata*, collected and prepared as previously described (Brix *et al.*, 1989*b*), was stripped by passing the haemoglobin solution first through a Sephadex G-25 column, equilibrated with 0.01 M-Tris buffer, pH 8.0, containing 0.1 M-NaCl, and then through a column of mixed-bed ion-exchange resin (Bio-Rad AG 501X8).

The oxygen equilibria were measured with a diffusion chamber technique (Lykkeboe *et al.*, 1975), except that the stepwise increases in oxygen tension were generated by cascaded gas-mixing pumps (Wosthoff), while absorbance changes between zero and full saturation were monitored on recorder with high sensitivity units (Radiometer). The application of small amounts of haemoglobin solution (layer thickness about 10 μm) minimized equilibration time and *m*-haemoglobin formation, which was always less than 1%. Alternatively, oxygen dissociation curves were obtained spectrophotometrically by the tonometric method (Giardina & Amiconi, 1981) at a protein concentration of 3–5 mg/ml. The oxygen binding data were fitted to the MWC model (Monod *et al.*, 1965) as previously described (Brix *et al.*, 1989*b*).

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

$$\Delta H \text{ (kcal/mol)} = \frac{-4.574[T_1T_2/(T_1-T_2)] \times \Delta \log P_{50}}{1000}$$

where T is temperature, P_{50} is the pressure required to give 50% saturation, and 1 kcal = 4.18 kJ. The pH was measured on oxygenated and deoxygenated blood under the same conditions as the oxygen equilibrium curves. The mean value has been used in the presentation of the data.

Concentrated stock solutions of 2,3-DPG were prepared by dissolving the sodium salt (Sigma) in water or in buffer. Concentrated stock solutions of InsP_6 (0.1 M) were prepared by dissolving the sodium salt of phytic acid (Sigma) in water and adjusting the pH to the desired value with concentrated phosphoric acid.

RESULTS

Dhindsa *et al.* (1974) have shown that haemoglobins from diving mammals are generally characterized by a slightly higher oxygen affinity than those from other mammals of comparable body weight. This observation has been confirmed with *B. acutorostrata* haemoglobin, but only at alkaline pH values (> 7.4). In fact, as shown in Fig. 1, in the more acidic pH range the oxygen affinity of whale haemoglobin in the presence of 3 mM-2,3-DPG is almost identical with that of human haemoglobin. As a consequence, the pH-dependence of the oxygen affinity is much more pronounced, with the Bohr factor ($\Delta \log P_{50}/\Delta$pH) being significantly higher in whale haemoglobin than in human haemoglobin A (−0.91 versus −0.74 in the presence of 3 mM-2,3-DPG).

Abbreviations used: 2,3-DPG, D-glycerate-2,3-bisphosphate; P_{50}, pressure required to give 50% saturation; ΔH, apparent heat of oxygenation.
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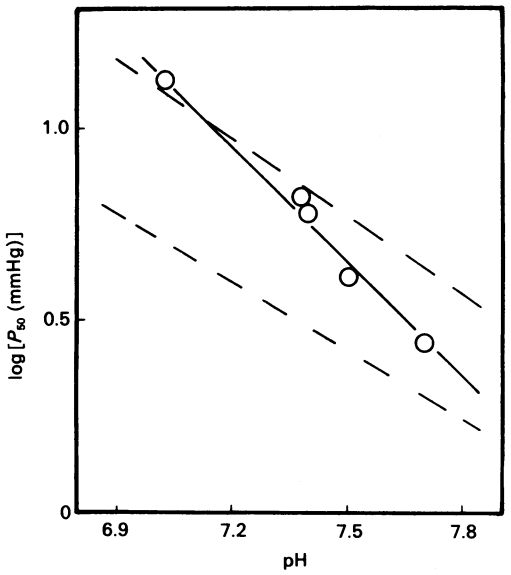

Fig. 1. Oxygen Bohr effect of whale haemoglobin in the presence of 3 mM-2,3-DPG

Conditions: 0.1 M-bistris or Tris buffer plus 0.1 M-NaCl at 20 °C. Broken lines show human haemoglobin A in the absence (lower line) and in the presence (upper line) of 3 mM-2,3-DPG.

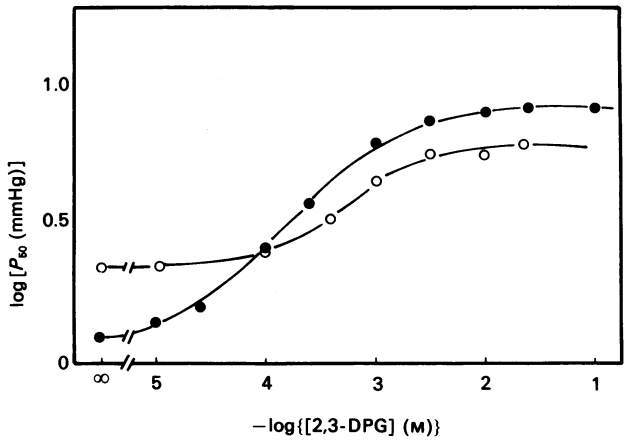

Fig. 2. Effect of 2,3-DPG concentration on haemoglobin oxygen affinity

Balaenoptera acutorostrata haemoglobin (○) and, for comparison, human haemoglobin A (●) are shown. Conditions: 0.1 M chloride-free Hepes buffer at pH 7.4 and 20 °C.

The results reported in Fig. 2 show the effect of increasing concentrations of 2,3-DPG on the oxygen affinity of whale haemoglobin in a chloride-free buffer at pH 7.4 and 20 °C. From the mid-points of the transitions it appears clear that, as compared with haemoglobin A, whale haemoglobin is characterized by a lower affinity constant (about 6 times) for 2,3-DPG with, in addition, the total amplitude of the effect being decreased by about 50%. This finding will be discussed later in the light of the primary structure of the molecule.

Figs. 3 and 4 show the effect of CO_2 on the oxygen affinity of whale haemoglobin both under stripped conditions and in the presence of InsP_6 at saturating concentrations. At 20 °C (Fig. 3) the experimental data follow a trend very similar to that obtained with human haemoglobin A (Kilmartin & Rossi-Bernardi, 1973) showing a substantial increase of oxygen release in the presence of CO_2. The effect is particularly evident under stripped con-

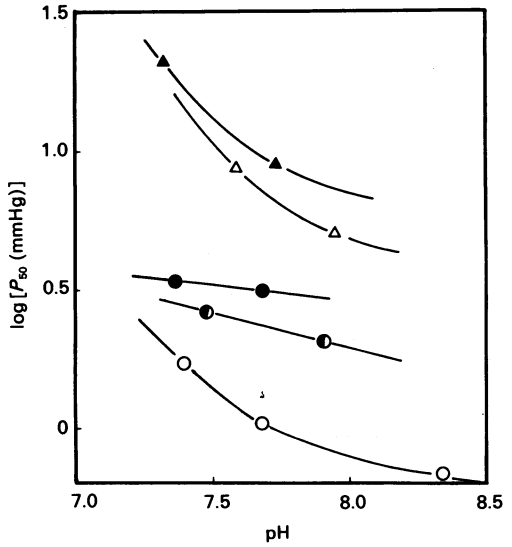

Fig. 3. Effect of CO_2 at 20 °C on the oxygen affinity of whale haemoglobin at different pH values

Assay conditions were 0.1 M-Tris buffer plus 0.1 M-NaCl. ○, Stripped haemoglobin; ◑, plus 1% CO_2; ●, plus 2% CO_2; △, plus 30 mM-InsP_6; ▲, plus 30 mM-InsP_6 and 2% CO_2.

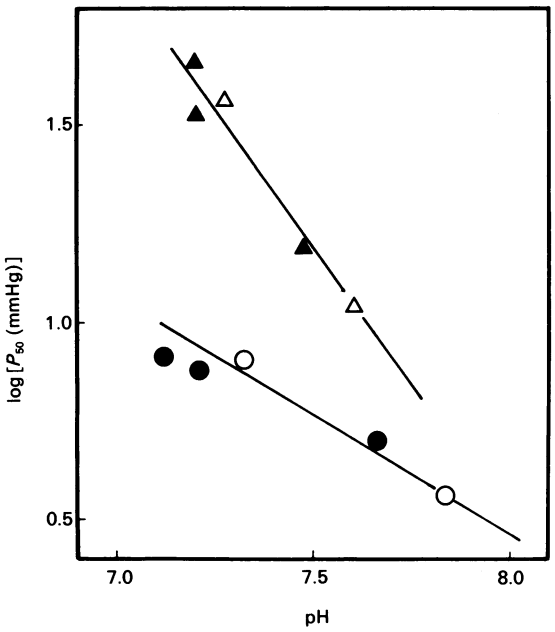

Fig. 4. Effect of CO_2 at 37 °C on the oxygen affinity of whale haemoglobin at different pH values

Assay conditions were 0.1 M-Tris buffer plus 0.1 M-NaCl. ○, Stripped haemoglobin; ●, plus 2% CO_2; △, plus 30 mM-InsP_6; ▲, plus 30 mM-InsP_6 and 2% CO_2.

ditions, but is also observed in the presence of 3 mM-2,3-DPG (results not shown) and also, though to a lesser extent, in the presence of a high concentration (30 mM) of InsP_6, which is the strongest phosphate effector for all these haemoglobins which are organic-phosphate-modulated (Fig. 2).

As expected, the specific effect of CO_2 increases at high pH values, where the amino groups involved (pK ~ 7.2) are mainly uncharged. Thus the alkaline Bohr effect of whale haemoglobin is substantially decreased in the presence of physiological con-

centrations of CO_2 as previously reported for haemoglobin A (Kilmartin & Rossi-Bernardi, 1973).

Completely different results were obtained at 37 °C (Fig. 4); at this temperature, the effect of CO_2 on the oxygen affinity of whale haemoglobin disappeared both in the absence and in the presence of organic phosphates over the whole pH range examined. It is particularly relevant to remark that, at this temperature and under the same experimental conditions, human haemoglobin was affected by CO_2 (Kilmartin & Rossi-Bernardi, 1973), whereas in the case of the mole (*Talpa europea*), a mammal adapted to a hypoxic environment, CO_2 has almost no effect on the oxygen affinity of its haemoglobin (Jelkmann *et al.*, 1981).

As far as the lactate effect is concerned, it is well known that this ion, like other anions, decreases the oxygen affinity of human haemoglobin A (Guesnon *et al.*, 1979; Amiconi *et al.*, 1981). This effect is particularly evident in the case of whale haemoglobin (see Tables 1 and 2), even if the experiments have been performed in the presence of $\sim$ 200 mM-Cl^- which would tend to mask the effect of lactate on haemoglobin oxygen affinity.

Moreover, from the data reported in Tables 1 and 2, it appears that, although at 20 °C (Table 1) the effect of lactate decreases in the presence of CO_2, being almost negligible at 2% CO_2, at 37 °C, where the effect of CO_2 is no more evident (Table 2), lactate increases the oxygen release in both the absence and the presence of 2% CO_2. Since it is known that, in the presence of organic phosphates in saturating amounts, the specific effect of lactate on human haemoglobin tends to disappear, we have measured the effect of this anion in the presence of 10 mM-2,3-DPG (Table 3). The experiments clearly show that, contrary to what has been generally observed for other mammalian haemoglobins, in the case of *B. acutorostrata* haemoglobin an increase in lactate concentration induces a decrease of the oxygen affinity, even in the presence of saturating 2,3-DPG.

The overall ΔH of oxygen binding represents the sum of the 'intrinsic' heat for the binding of the ligand and of all the other contributions which derive from ligand-linked processes. Thus the high ΔH value obtained under stripped conditions at pH 7.5 is decreased by addition of CO_2 (Fig. 5*a*) from -50 kJ/mol (-12 kcal/mol) (no CO_2) to -14.6 kJ/mol (-3.5 kcal/mol) (in the presence of 2% CO_2).

It should also be noted that the overall ΔH for oxygen binding to haemoglobin under stripped conditions decreases as the pH is brought towards acidic values, due to the endothermic con-

Table 1. Effect of lactate at 20 °C on the oxygen affinity of stripped whale haemoglobin in the presence or in the absence of CO_2

The assay conditions were 0.1 M-Tris buffer plus 0.1 M-NaCl. The P_{50} values, obtained as described in the Materials and methods section, had an error ±1%.

Conditions	pH	log[P_{50} (mmHg)]
No CO_2	7.67	0.02
No CO_2; 0.1 mM-lactate	7.67	0.20
1% CO_2	7.90	0.32
1% CO_2; 0.1 mM-lactate	7.90	0.41
2% CO_2	7.67	0.49
2% CO_2; 0.1 mM-lactate	7.67	0.52

Table 2. Effect of lactate at 37 °C on the oxygen affinity of stripped whale haemoglobin in the presence or the absence of CO_2

Assay conditions were 0.1 M-Tris buffer plus 0.1 M-NaCl. The P_{50} values should be considered ±1%.

Conditions	pH	log[P_{50} (mmHg)]
No CO_2	7.83	0.56
No CO_2; 0.1 mM-lactate	7.83	0.72
2% CO_2	7.66	0.70
2% CO_2 plus 0.1 mM-lactate	7.66	0.88

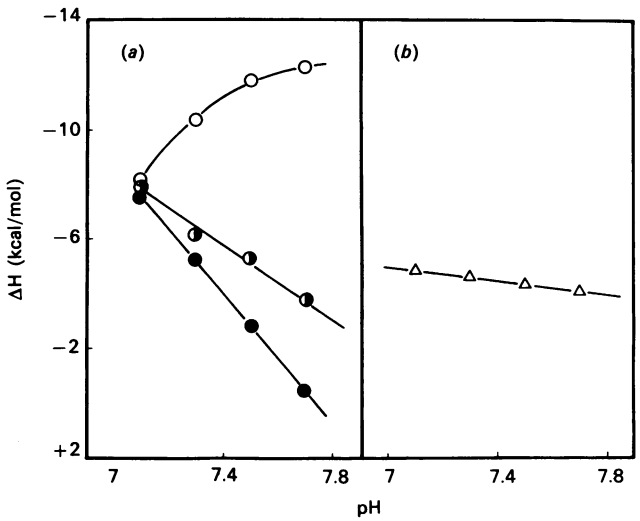

Fig. 5. Apparent heat of oxygenation for whale haemoglobin as a function of pH calculated from the integrated Van't Hoff equation

(*a*) Experiments were performed by the Lykkeboe method (Lykkeboe *et al.*, 1975). ○, Stripped haemoglobin in the absence of CO_2; ◑, plus 1% CO_2; ●, plus 2% CO_2. (*b*) Experiments performed by tonometric method in which the concentration of CO_2 is that present in air; △, stripped haemoglobin plus 3 mM-2,3-DPG. Conditions: 0.1 M-Bistris or -Tris buffer plus 0.1 M-NaCl. The values of ΔH are corrected for the heat contribution of oxygen in solution (-3 kcal/mol). 1 kcal = 4.18 kJ.

Table 3. Effect of lactate on the oxygen affinity of whale haemoglobin in the presence of 2,3-DPG

2,3-DPG was present in saturating amounts (10 mM) at pH 7.4 and 20 °C. The P_{50} values should be considered ±1%.

Conditions	log [P_{50} (mmHg)]
0.1 M-Hepes buffer + 3 mM-2,3-DPG (no chloride)	0.77
0.1 M-Tris buffer + 3 mM-2,3-DPG (200 mM-chloride)	0.74
0.1 M-Tris buffer + 3 mM-2,3-DPG + 0.1 mM-lactate	0.98
0.1 M-Tris buffer + 3 mM-2,3-DPG + 10 mM-lactate	1.06

tribution of the Bohr protons. However, in the presence of CO_2, the pH dependence is completely reversed (see Fig. 5*a*).

Moreover, in the presence of 2,3-DPG the overall ΔH of oxygen binding is substantially decreased (−16.7 kJ/mol; −4 kcal/mol) with respect to that measured under stripped conditions, and appears to be almost pH independent (Fig. 5*b*). The values obtained in the presence of CO_2 or 2,3-DPG are significantly lower than those measured for human haemoglobin A under the same experimental conditions.

DISCUSSION

The oxygen-binding behaviour of *B. acutorostrata* haemoglobin has revealed some unusual properties never seen before, which are probably linked to the peculiar diving behaviour of this mammal. The much more pronounced pH dependence of the oxygen affinity found in whale haemoglobin compared with human haemoglobin A (see Fig. 1) is probably a consequence of the reported lesser effect of 2,3-DPG on this haemoglobin. Thus the Bohr factor is high and markedly influenced by organic phosphate concentrations. Moreover, the data reported above suggest that lactate, apart from its indirect effect on pH, may even at a low concentration (0.1 mM) play an important role in oxygen unloading in the blood of *B. acutorostrata* after a long dive in hypoxic conditions.

As far as the temperature effect is concerned, it must be recalled that in mammalian haemoglobins the oxygen dissociation curves are strongly affected by this parameter. Thus the overall heat of oxygenation (ΔH) for human haemoglobin is approx. −44 kJ/mol (−10.5 kcal/mol) in the absence of organic phosphates. However, the temperature sensitivity is decreased under natural conditions by the endothermic displacement of heterotropic effectors. Hence the ΔH of human haemoglobin A decreases to −31.4 kJ/mol (−7.5 kcal/mol) in the presence of 3 mM-2,3-DPG (Benesch *et al.*, 1969; Antonini & Brunori, 1971). In this respect, whale haemoglobin shows, in the absence of organic phosphate, a ΔH of −46 kJ/mol (−11 kcal/mol) at pH 7.4, a value which is comparable with that reported for haemoglobin A under the same experimental conditions; this value appears to be significantly decreased in the presence of CO_2 (see Fig. 5). Moreover, in the presence of physiological amounts of 2,3-DPG, *B. acutorostrata* haemoglobin shows the very low sensitivity of oxygen binding which seems to be a characteristic of all Arctic mammal haemoglobins. Thus, at pH 7.4 and in the presence of 3 mM-2,3-DPG, the ΔH (corrected for the heat of oxygen in solution) is −18.8 kJ/mol (−4.5 kcal/mol), a value lower than that of human haemoglobin and comparable with that of reindeer (−16.7 kJ/mol; −4 kcal/mol) and musk ox haemoglobin (−15.5 kJ/mol; −3.7 kcal/mol) under the same experimental conditions (Brix *et al.*, 1989*a*; Giardina *et al.*, 1989*a*).

To look for a possible physiological significance of the very small ΔH of oxygen binding in this animal, we have to consider that, although the core of the organism is certainly isolated from the environment and maintains a constant temperature of 37–38 °C, the fins and tail may still be at a significantly lower temperature due to the presence of a countercurrent heat exchanger which, in order to save energy, allows the temperature to decrease in these isolated parts of the organism. This could drastically impair the unloading of oxygen if the protein were characterized by a higher ΔH of oxygen binding (Wood, 1980; Reeves, 1980).

The lower temperature of whale blood in the fins and tail, in comparison with the rest of the body, gives a rationale also to the temperature-dependence of the effect of CO_2. In fact, the data indicate that within the core of the body CO_2 does not display any allosteric effect, because at 37 °C the differential binding of this ligand with respect to oxy and deoxystructure is completely abolished. However, CO_2 does facilitate oxygen unloading in the fins and tail, which are the areas of great muscular activity. This is comparable with the situation in man. The allosteric property of CO_2 may come into operation also at the level of the lungs, depending on the temperature of the air breathed by the animal; of course, if this is true, elimination of CO_2 by the lungs would increase the oxygen affinity of haemoglobin, facilitating the loading of oxygen.

As far as the effect of lactate is concerned, although we were not able, due to the lack of material, to test the effect of lactate at 37 °C, the data obtained at 20 °C and reported in Table 3 show a significant effect of this anion even in the presence of 2,3-DPG in saturating amounts. On the whole, these data seem to indicate that lactate, even at a concentration as low as 0.1 mM, plays an important role in oxygen unloading in the blood of the whale *B. acutorostrata*. Due to the difficulty in obtaining blood samples from whales this conclusion may have to be substantiated in the future by further experiments on other diving mammals.

Finally, since the primary structure of both polypeptide chains (α and β) of *B. acutorostrata* haemoglobin is available (Abbasi *et al.*, 1984), we looked for molecular modifications which could explain the particular behaviour of whale haemoglobin. Comparison with the corresponding human chains shows the presence of 23 and 14 amino acid substitutions in the α and β chains respectively which are randomly distributed over the entire lengths of both chains. Exchanges of structural importance were not found, apart from four substitutions in the β chains which are involved in the $\alpha_1\beta_1$ contacts, and five in the α chains, three of which are involved in the same contact region and two in the $\alpha_1\beta_2$ contact. The possible influence of these substitutions on the functional properties of the molecule is not clear. However, it could be suggested that they may in some way contribute to the modulation of the heterotropic interactions. Thus it is well known that the intersubunit contacts play a key role in regulating several functional properties of haemoglobin as clearly outlined by the stereochemical model proposed by Perutz on the basis of crystallographic studies (Perutz, 1970; Baldwin, 1980) and by more recent data reported on the functional properties of hybrid haemoglobins obtained from human haemoglobin A and *Xenopus laevis* haemoglobin (Condò *et al.*, 1987). On the whole, it appears that the role of α–β intersubunit contacts is not only related to the stability of the tetrameric structure and to the display of homotropic interactions, but it may extend over the whole molecule also modulating the extent of heterotropic interactions.

With respect to the low affinity of whale haemoglobin for 2,3-DPG, the only substitution which may be involved in the phenomenon seems to be at position 5 (A2) of β chains where Pro (human) is replaced by Ala. It is interesting to note that the same substitution has been found in some mammalian haemoglobins (Perutz & Imai, 1980), characterized by altered interactions with 2,3-DPG compared with human haemoglobin A. In particular the weak interaction of mole (*Talpa europea*) haemoglobin with 2,3-DPG (Jelkmann *et al.*, 1981), even if all the binding residues for this organic phosphate are conserved, has been attributed to changes in positions β4 (Thr in human → Ser in mole) and β5 (Pro → Gly). The same applies to horse haemoglobin, in which the same types of amino acid substitutions are paralleled by an altered interaction with organic phosphates (Giardina *et al.*, 1990). On the basis of these results, the decreased effect of 2,3-DPG has been attributed (Jelkmann *et al.*, 1981; Giardina *et al.*, 1990) to a displacement of the A helix induced by changes in positions β4 and β5 which are next to the NA segment. In conclusion, the change at β5 (Pro → Ala) observed in hae-

moglobin from *B. acutorostrata* could alter the stereochemistry of the 2,3-DPG binding site and hence the functional effect of this organic phosphates.

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