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Differences between horse and human haemoglobins in effects of organic and inorganic anions on oxygen binding

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Despite the fact that the horse is one of the more common domesticated animals, there are few reports dealing with the properties of its blood, and no comprehensive study has been performed on the reactivity of horse haemoglobin towards organic and inorganic ions. Here we report data on the effects of the organic phosphates D-glycerate-2,3-bisphosphate (2,3-DPG) and InsP_6, and of chloride on the properties of horse haemoglobin. Thus the effect of saturating concentrations of 2,3-DPG on the oxygen affinity of horse haemoglobin is about 60% lower than with human adult haemoglobin under the same experimental conditions. The same applies also to InsP_6, whose effect on oxygen binding to horse haemoglobin is decreased by about 55% compared with human adult haemoglobin. On the whole, horse haemoglobin appears to be much less sensitive to organic phosphates than previously believed. These results are discussed in the light of the primary structure of the molecule.

INTRODUCTION

The oxygen affinity of mammalian haemoglobins is generally modulated by the binding of anionic cofactors, such as D-glycerate-2,3-bisphosphate (2,3-DPG) (Benesch & Benesch, 1967; Chanutin & Curnish, 1967). Binding of 2,3-DPG occurs at a specific site, which, at neutral pH, involves a cluster of eight positively charged amino acid residues (Val-NA1, His-NA2, Lys-EF6 and His-H21 of each β-chain) which are located on the dyad axis of the haemoglobin tetramer (Arnone, 1972). Besides 2,3-DPG, small anions such as Cl^- can also interact with specific residues of the protein moiety and are also effective in decreasing the oxygen affinity of haemoglobins (Chiancone *et al.*, 1975; Arnone & Williams, 1978; Amiconi *et al.*, 1981; Bucci & Fronticelli, 1985; Fronticelli *et al.*, 1988).

With regard to the allosteric interaction with 2,3-DPG, mammalian haemoglobins have been divided broadly into two groups: those with an intrinsically high oxygen affinity, modulated in red cells by interaction with 2,3-DPG, and those with intrinsically low oxygen affinities, which are almost insensitive to the presence of this organic phosphate (Bunn, 1971; Perutz & Imai, 1980). The haemoglobins of humans, horses, dogs, rabbits and rats are put forward as examples of the first group and those of sheep, goats and cows are examples of the second group (Perutz & Imai, 1980; Bunn, 1980, 1981). In the haemoglobins from this latter group, it has been found that the β-chain *N*-terminal residue is not present, and the adjacent His-NA2 is replaced by methionine. The replacement of a hydrophilic by a hydrophobic residue at β-NA2 has been suggested to be the basis of the low intrinsic oxygen affinity displayed by these haemoglobins (Perutz & Imai, 1980; Perutz, 1983).

Although the horse is one of the more common domesticated animals, there are few reports dealing with the properties of its blood and, to our knowledge, no comprehensive study has been published on the reactivity of horse haemoglobin towards organic and inorganic anions. Moreover, in the course of a previous investigation on haemoglobin from reindeer (Condò *et al.*, 1988; Giardina *et al.*, 1989), we performed, for comparison, a set of experiments on haemoglobin from horse. The results obtained were not as expected on the basis of the data available in the literature and called for further experiments, which are reported here. In fact, the effects of organic phosphates (2,3-DPG and InsP_6) and Cl^- revealed some peculiar features which are discussed in the light of the primary structure of the horse haemoglobin molecule.

MATERIALS AND METHODS

Horse (*Equus caballus*) blood samples were obtained from animals bred in Scuderia Giovenale (Capannelle, Rome, Italy), and fresh human blood samples were from a blood bank.

Blood samples were collected from the jugular vein of the horse without anaesthesia into an iso-osmotic NaCl solution containing 2-mM-EDTA. The cells were washed three times by centrifugation at 1000 *g* with iso-osmotic NaCl solution and the packed cells were lysed by adding 2 vol. of cold 7.5 mM-sodium phosphate (pH 7.4)/1 mM-EDTA/0.2 mM-phenylmethanesulphonyl fluoride. Stroma were removed by centrifugation at 12000 *g* for 30 min.

Electrophoretic analysis of haemoglobin components was performed by alkaline polyacrylamide-gel electrophoresis. Haemoglobin components were purified from

Abbreviations used: 2,3-DPG, D-glycerate-2,3-bisphosphate; P_{50}, pressure required to give 50% saturation; h_{50}, Hill coefficient at 50% saturation.

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total haemolysate by chromatography on a CM-52 column using a pH gradient from 6.0 to 8.0 in 0.01 M-phosphate buffer. Stripped haemoglobin was obtained by passing the haemolysate 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 501 X8).

Concentrated stock solutions of 2,3-DPG were prepared by dissolving the sodium salt (Sigma) in water or in buffer solutions. 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.

Oxygen dissociation curves were determined spectrophotometrically (Giardina & Amiconi, 1981) at a protein concentration of 3–5 mg/ml. Spectrophotometric measurements were carried out using a Kontron 860 Uvikon spectrophotometer.

RESULTS

Electrophoretic analysis of the haemolysate from horse revealed two different haemoglobin components, one of which represented about 80% of total pigment, with an isoelectric point of approx 6.5. The isoelectric point of the second haemoglobin component, which accounted for the remaining 20% of the pigment, was between 6.7 and 6.8.

An initial set of experiments performed in parallel on the purified major haemoglobin component and on the whole horse haemolysate showed no detectable differences. Nevertheless, the functional characterization reported below refers to the purified major haemoglobin component.

The oxygen-binding properties of horse haemoglobin were investigated within the pH range 5.8–9.0, both in the absence and in the presence of saturating concentrations of 2,3-DPG and InsP_6 (Fig. 1). In the absence of organic phosphates, but in the presence of 0.1 M-NaCl, horse haemoglobin displays an oxygen affinity which is lower, over the entire pH range examined, than that of human adult haemoglobin reported for comparison. The data obtained are in good agreement with those reported by Bunn (1971).

It has been reported previously (Yousef *et al.*, 1971) that, in spite of their higher levels of 2,3-DPG (8–9 mM), horse erythrocytes display an oxygen affinity very similar to that of human erythrocytes, whose 2,3-DPG content is significantly lower (5 mM). This observation is supported by the results shown in Fig. 1. Thus the effect on the oxygen affinity of horse haemoglobin of saturating concentrations of 2,3-DPG was about 60% lower ($\Delta\log P_{50} = 0.16$ at pH 7.6) than that on human adult haemoglobin oxygen affinity under the same experimental conditions ($\Delta\log P_{50} = 0.40$). This observation is in good agreement with that reported by McLean & Lewis (1975).

The same results were also found with InsP_6, whose effect on the oxygen affinity of horse haemoglobin, in comparison with that on human adult haemoglobin, was decreased by about 55% ($\Delta\log P_{50} = 0.42$ vs $\Delta\log P_{50} = 0.94$ at pH 7.6). Therefore, on the whole, horse haemoglobin appears to be much less sensitive to organic phosphates than believed previously (Bunn, 1971).

In an attempt to gain a better understanding of this phenomenon, a number of experiments have been performed in Hepes buffer (0.1 M) in order to investigate the effects of Cl^- and 2,3-DPG independently from one another. Fig. 2 shows that, in spite of the large change in the position of the titration curves along the log [effector] axis, the total change in log P_{50} induced by 2,3-DPG was almost identical to that induced by Cl^-.

Table 1 compares human adult haemoglobin and horse haemoglobin under stripped conditions in the presence

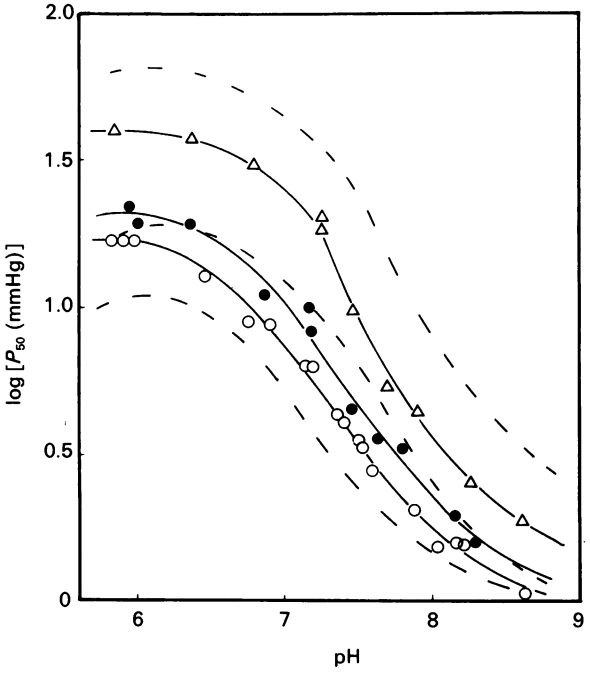

Fig. 1. Oxygen Bohr effect of horse haemoglobin at 20 °C

Incubations were carried out in 0.1 M-Bistris or Tris buffer plus 0.1 M-NaCl. ○, Stripped haemoglobin alone; ●, plus 3 mM-2,3-DPG; △, plus 3 mM-InsP_6. Broken lines refer to human adult haemoglobin stripped and in the presence of 2,3-DPG or of InsP_6 (going from the bottom to the top) reported for comparison. 1 mmHg = 133.3 Pa.

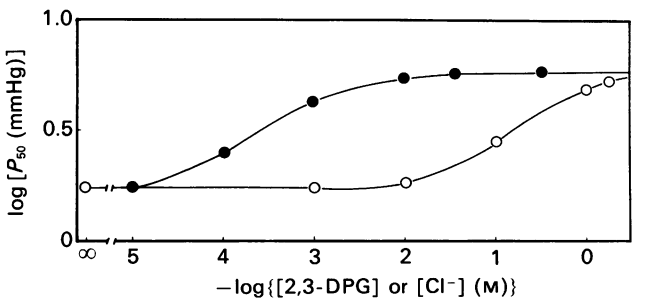

Fig. 2. Effect of 2,3-DPG (●) and Cl^- (○) concentration on the oxygen affinity of horse haemoglobin

Incubations were carried out in 0.1 M-Hepes at pH 7.6 and 20 °C; 1 mmHg = 133.3 Pa. It should be noted that even at the highest Cl^- concentration, the Hill coefficient was almost constant over the entire concentration range examined ($h_{50} = 2.3$–2.4). On this basis we may reasonably exclude dissociation of haemoglobin tetramers into dimers, since this process is known to result in both a decrease in co-operativity and an increase in oxygen affinity.

Table 1. Oxygen affinity of stripped human adult and horse haemoglobins in the absence and in the presence of Cl^- and/or 2,3-DPG

Conditions were 100 mM-Hepes buffer at pH 7.6 and 20 °C. 1 mmHg = 133.3 Pa.

Conditions	P_{50} (mmHg)	
	Human adult haemoglobin	Horse haemoglobin
100 mM-Hepes buffer	1.05	1.78
100 mM-Hepes buffer + 200 mM-NaCl	2.51	3.31
100 mM-Hepes buffer + 200 mM-NaCl + 3 mM-2,3-DPG	5.37	4.07

of Cl^- (200 mM) and/or 2,3-DPG (3 mM). The values reported outline once again the different effects of 2,3-DPG displayed by the two haemoglobin systems. Moreover, it should be stressed that, in the absence of both Cl^- and 2,3-DPG, horse haemoglobin displays an oxygen affinity which is about half that of human adult haemoglobin.

DISCUSSION

The primary structure of the polypeptide chains of horse haemoglobin has been deduced (Matsuda *et al.*, 1980; Clegg *et al.*, 1984). Comparison with the corresponding human chains shows 20 and 25 substitutions in the α- and β-chains respectively. The great majority of these substitutions have been found in other mammalian haemoglobins without any measurable effect. Consequently, it does not seem probable that they could play a role in the effect of anions on oxygen affinity. In this respect, an interesting substitution is found at the level of the 2,3-DPG pocket, where His-NA2 of the β-chain is replaced by a glutamine.

Table 2 reports the differences found in the *N*-terminal β-chain segments in some mammalian haemoglobins which display altered interactions with 2,3-DPG in comparison with human adult haemoglobin (Kleinschmidt & Sgouros, 1987). It has been proposed (Perutz & Imai, 1980) that the intrinsically low oxygen affinity which is characteristic of these haemoglobins could be due to the substitution of a hydrophilic residue (His) by a hydrophobic residue (Leu, Met or Phe), which would have to point into the interior of the protein, thereby mimicking 2,3-DPG action in stabilizing the tertiary deoxy structure of the β-subunits. However, llama haemoglobin, which is characterized by a low oxygen affinity even in the absence of organic phosphates, has at position β-NA2 the hydrophilic replacement His → Asn (Braunitzer *et al.*, 1977; Bauer *et al.*, 1980). Interestingly, horse haemoglobin, in which the same residue is substituted by a glutamine, has been described as a high-affinity haemoglobin with a normal reactivity towards 2,3-DPG. This contradiction has been reconciled by a structural analysis based on molecular model fitting (Perutz, 1983). The amido group of β-chain Gln-NA2 in horse haemoglobin can form a hydrogen bond with the phosphate of 2,3-DPG, whereas this interaction is not possible with the shorter side-chain of asparagine in llama haemoglobin.

However, the contradiction reported above has now been revealed to be only apparent, since our data show that horse haemoglobin in fact displays an intrinsic low oxygen affinity, and is characterized, in the presence of physiological concentrations of Cl^-, by a small effect of 2,3-DPG. We could try to reconcile all of these observations on the basis of what has been proposed in the case of human fetal haemoglobin. In fact, the lower affinity of human fetal haemoglobin for 2,3-DPG (Bauer *et al.*, 1968) has been attributed, at least in part, to a substitution in position β-NA3 (Leu → Phe), which, by displacing the NA segment, would lengthen the distance between the phosphate and His-NA2 (Frier & Perutz, 1977). A similar intramolecular distortion could be responsible for the low binding of 2,3-DPG observed in both horse and llama haemoglobins, although in these latter cases the substitution involves the position β-NA2.

Other important substitutions are illustrated by the case of mole (*Talpa europaea*), whose high blood oxygen affinity has been attributed to a weak interaction of its haemoglobin with 2,3-DPG (Jelkmann *et al.*, 1981), even if all of the binding residues for 2,3-DPG are conserved. Those authors, in fact, have attributed the decreased effect of 2,3-DPG to a displacement of the A helix, due to the changes in position β4 (Thr in human → Ser in

Table 2. Primary structures of the *N*-terminal segment of β-chains of some mammalian haemoglobins which show altered 2,3-DPG interactions compared with human adult haemoglobin

Residue	Human adult	Mole	Llama	Horse	Bovine	Sheep	Lemur
NA 1	Val	–	–	–			Thr
2	His	–	Asn	Gln	Met	Met	Leu
3	Leu	–	–	–	–	–	–
A 1	Thr	Ser	Ser	Ser	–	–	Ser
2	Pro	Gly	Gly	Gly	Ala	Ala	Ala
3	Glu	–	Asp	–	–	–	–
4	Glu	–	–	–	–	–	–
5	Lys	–	–	–	–	–	Asn
6	Ser	Gly	Asn	Ala	Ala	Ala	Ala

mole) and $\beta 5$ (Pro → Gly), which are next to the NA segment. In fact, as a result of this difference, the position of the contact sites for 2,3-DPG at NA1 ($\beta 1$) and NA2 ($\beta 2$) could be altered. It is interesting to note that identical substitutions are present in horse haemoglobin in addition to that in β-NA2 (His → Gln).

At physiological concentrations of Cl^-, the absence of Val-NA1 and the substitution of His-NA2 by a hydrophobic residue practically abolish the allosteric effect of 2,3-DPG, as seen in bovine haemoglobin, whereas when the substitution involves a hydrophilic residue, the effect of this phosphate is drastically decreased, but not completely abolished, by the competition of Cl^-, as seen in horse haemoglobin.

We may therefore consider that residues Val-NA1 and His-NA2 of β-chains appear to play a key role in the modulation of haemoglobin oxygen affinity by organic phosphates. However, the role of amino acid residues at positions A1 ($\beta 4$) and A2 ($\beta 5$) in determining the stereochemistry of the 2,3-DPG binding site cannot be disregarded. In fact, it is suggested that, not only in mole haemoglobin but in all of the haemoglobins characterized by a small effect of organic phosphates, these positions are occupied by different amino acid residues (Ser–Gly or Ser–Ala; see also Table 2) than in mammalian haemoglobins such as human adult haemoglobin, which show a significant effect of 2,3-DPG.

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