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OXYGEN BINDING PROPERTIES OF HEMOGLOBIN FROM THE WHITE RHINOCEROS ($|\beta_2$-GLU$|$) AND THE TAPIR

R. BAUMANN, G. MAZUR and G. BRAUNITZER

Zentrum Physiologie, Medizinische Hochschule, Hannover, and Max-Planck Institut für Biochemie, München – Martinsried, F.R.G.

Abstract. The β-chain of rhinoceros hemoglobin contains glutamic acid at position β_2, an important site for the binding of organic phosphates. We have investigated the oxygen binding properties of this hemoglobin and its interaction with ATP, 2,3-diphosphoglycerate, CO_2 and chloride. The results show that the presence of GLU at position β_2 nearly abolishes the effect of organic phosphates and CO_2, whereas the oxygen-linked binding of chloride is not affected. Thus rhinoceros hemoglobin has only protons and chloride anions as major allosteric effectors for the control of its oxygen affinity. From the results obtained with hemoglobin solutions it can be calculated that the blood oxygen affinity of the rhinoceros must be rather high with a P_{50} of about 20 torr at pH 7.4 and 37 °C, which conforms with observations obtained for other large mammals.

Allosteric factors
Bohr effect
Chloride
Organic phosphates
Oxygen affinity

At the present time, the small mammalian order Perissodactyla includes three families: the Equidae, which are the largest group, the Tapiridae and the Rhinocerotidae. Hemoglobin sequences and functional studies have only been obtained for hemoglobins of the Equidae group (cf. Mazur and Braunitzer, 1982; Matsuda *et al.*, 1980). All species of this group have glutamine at position β_2, one of the binding sites for 2,3-diphosphoglycerate (2,3-DPG), which in human hemoglobin is occupied by histidine (Arnone, 1972). However, experiments with horse hemoglobin have shown that the substitution is apparently of little consequence for the effect of organic phosphates (Bunn and Kitchen, 1973; Braunitzer *et al.*, 1978).

In contrast to the above results, the recently published primary structure of the hemoglobin from the white rhinoceros (*Cerathotherium sinum*) shows a glutamic acid residue at position β_2 (Mazur *et al.*, 1982). Glutamic acid at position β_2 has not been demonstrated in any other mammalian hemoglobin, but is present in

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some fish hemoglobins, notably the β-chains of carp, goldfish and trout hemoglobin type I (Braunitzer and Rodewald, 1980; Grujic-Injac *et al.*, 1980; Barra *et al.*, 1983). In view of the unusual character of the substitution found in rhinoceros hemoglobin, we have investigated the functional properties. The results show that the substitution has a profound effect on the interaction of rhinoceros hemoglobin with most allosteric effectors. In addition, data are presented for tapir hemoglobin function.

Materials and methods

Preparation of hemoglobin solutions. Samples of rhinoceros and tapir hemoglobin were shipped on ice. Any methemoglobin present in the samples was converted to hemoglobin following the method described by Bauer and Pacyna (1975). The hemoglobin solutions were equilibrated with 0.1 M NaCl on a 1.5 × 90 cm column of Sephadex G-25 fine. Stock solutions of hemoglobin with a concentration of 120 g/L were stored in liquid nitrogen until use.

Oxygen equilibrium curves of hemoglobin solutions (40 g/L) were determined spectrophotometrically (Niesel and Thews, 1961; Sick and Gersonde, 1969). For the determination of the Bohr effect ($\Delta \log P_{50}/\Delta pH$) the pH was varied using 0.05 M Bis-Tris or Tris buffers with a total concentration of 0.1 M Cl^-, unless otherwise indicated.

For experiments done in the presence of CO_2, bicarbonate buffers were used and the total concentration of bicarbonate and chloride adjusted to 0.1 M. The pH was measured with pH-meter 64 (Radiometer), and ATP and 2,3-diphosphoglycerate were purchased from Boehringer Co. Mannheim. The oxygen half-saturation pressure P_{50} and n-value between 20 and 80% saturation were determined from the Hill plot. The hemoglobin pattern was determined by isoelectric focussing (Drysdale *et al.*, 1971) using ampholine pH 6–8 (LKB, Bromma) and the isoelectric points of the separate fractions were determined as described elsewhere (Petschow *et al.*, 1977). Quantitative analysis of the gels was carried out by densitometry on a Gelman AC D 15 densitometer.

Photomicrographs of oxyhemoglobin crystals from rhinoceros and tapir hemoglobin were taken by phase contrast on a Leitz Stereoplan microscope.

Results

Hemoglobin pattern and solubility. Tapir blood has 4 hemoglobin components, each accounting for 20–30% of the total hemoglobin, while rhinoceros blood has a major component (80%) and one minor fraction of greater electrophoretic mobility (fig. 1 and table 1). Note that the estimated isoelectric points for all hemoglobin fractions are up to 0.3 pH units lower than for human hemoglobin A.

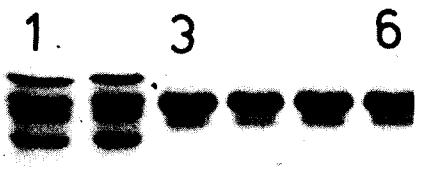

Fig. 1. Hemoglobin pattern of tapir blood (samples 1 and 2) and rhinoceros blood (samples 3 to 6).

TABLE I

Hemoglobin pattern of tapir and rhinoceros blood. pI estimated at 20 °C; corresponding value for major fraction of human adult hemoglobin pI = 7.33

Fraction number	Tapir Hb		Rhinoceros Hb	
	Estimated pI	% of total Hb	Estimated pI	% of total Hb
1	7.295	21	7.214	81
2	7.187	19.7	7.142	19
3	7.114	27.4		
4	7.009	31.9		

During the preparation of the hemoglobin solutions we found that oxyhemoglobin solutions from tapir and rhinoceros crystallize spontaneously at room temperature, physiological ionic strength and hemoglobin concentrations well below those present in the red cell. Thus, for rhinoceros hemoglobin one finds a concentration of 7.03 g/dl in the soluble phase at pH 6.97 and 22 °C. Figure 2 is a photomicrograph of crystalline precipitate removed from an oxygenated solution of rhinoceros hemoglobin with 0.1 M Cl^- and pH 7 at 22 °C.

Oxygen affinity and Bohr effect. At pH 7.2 and 37 °C the oxygen half-saturation pressures (P_{50}) of purified hemoglobin from tapir and rhinoceros are almost identical with 17.1 torr (2.28 kPa) and 17.4 torr (2.32 kPa). These values are higher than the P_{50} for human Hb under the same condition, which is 12 torr (1.6 kPa). The Bohr effect is −0.62 for rhinoceros hemoglobin and −0.58 for tapir hemoglobin in the physiological pH range (figs. 3 and 4, table 2).

Influence of organic phosphates and CO_2 on oxygen affinity. The effect of 2,3-DPG and ATP on rhinoceros hemoglobin is small. With 10 mol 2,3-DPG/mol Hb_4 the P_{50} increases from 17.1 torr (2.28 kPa) to 19.5 torr (2.6 kPa) and to 25.3 torr (3.38 kPa) with a 50-fold excess of 2,3-DPG; the same P_{50} value is obtained with 50 mol ATP/mol Hb_4. However, ATP is more effective than 2,3-DPG at intermediate concentrations (fig. 5). With 10 mol ATP/mol Hb_4 the P_{50} increases to

Fig. 2. Phase-contrast photomicrographs of oxyhemoglobin crystals from rhinoceros hemoglobin. Magnification 400 ×.

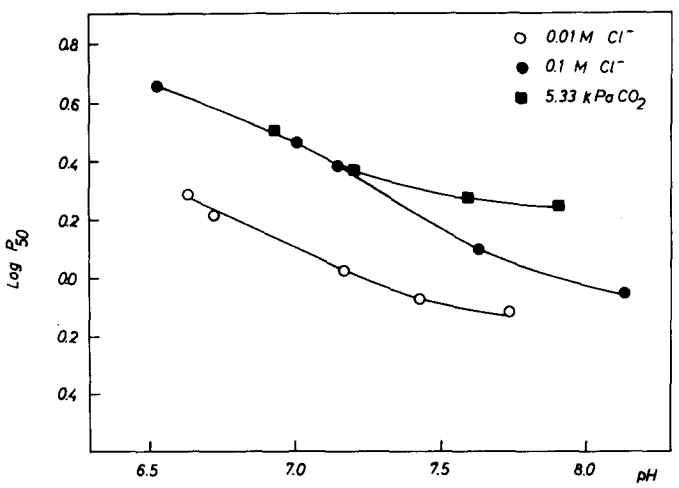

Fig. 3. Bohr effect and influence of chloride and CO_2 on oxygen affinity of rhinoceros hemoglobin. 37 °C, hemoglobin concentration 40 g/L. Log P_{50} given for kPa_{O_2}. For experiments in the presence of CO_2 the total concentration of Cl^- and HCO_3^- was adjusted to 0.1 M.

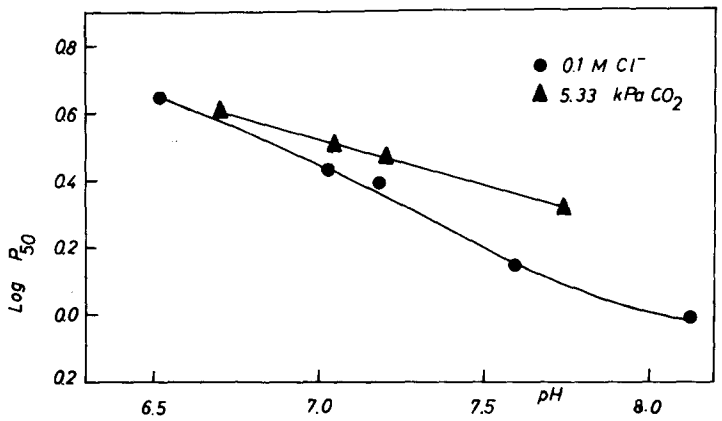

Fig. 4. Bohr effect and influence of CO_2 on oxygen affinity of tapir hemoglobin. Conditions as in fig. 3.

TABLE 2

P_{50} of rhinoceros and tapir hemoglobin solutions under various experimental conditions; values in brackets give P_{50} in torr; 37 °C; $[Hb_4] = 40$ g/L.

Condition	pH 7.2				pH 7.5			
	Rhino Hb P_{50}		Tapir Hb P_{50}		Rhino Hb P_{50}		Tapir Hb P_{50}	
	kPa	torr	kPa	torr	kPa	torr	kPa	torr
0.01 M Cl^-	1.01	(7.58)	n.d.*		0.82	(6.16)	n.d.	
0.1 M Cl^-	2.28	(17.1)	2.32	(17.4)	1.48	(11.1)	1.55	(11.61)
+ 5.33 kPa CO_2	2.32	(17.4)	2.92	(21.9)	1.97	(14.79)	1.97	(14.78)
+ 2 mol 2,3-DPG/mol Hb	2.32	(17.4)	2.72	(20.4)	n.d.		n.d.	

* n.d. = not determined.

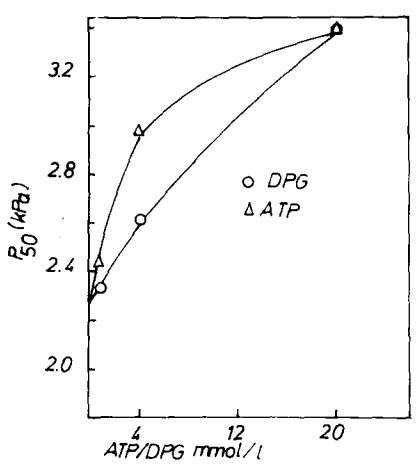

Fig. 5. Influence of ATP and 2,3-DPG on oxygen affinity of rhinoceros hemoglobin at pH 7.2, 37 °C. The chloride concentration was 0.1 M.

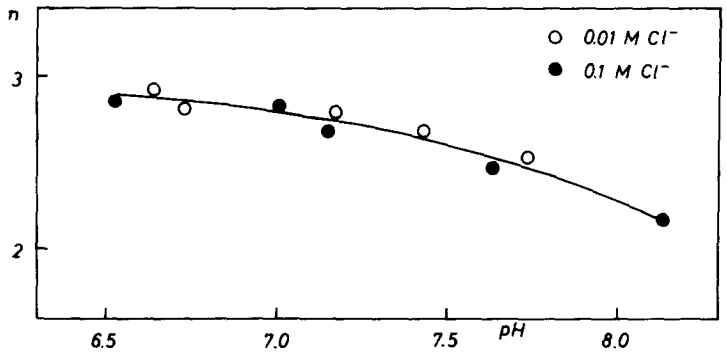

Fig. 6. pH dependence of the n-value of rhinoceros hemoglobin. Temperature was 37 °C.

21.9 torr (2.9 kPa). In the presence of 40 torr (5.33 kPa) CO_2 one observes a decrease of the oxygen affinity only at pH >7.2 (fig. 3 and table 2).

Compared to rhinoceros Hb, the response of tapir Hb to both CO_2 and 2,3-DPG is considerably increased, since 10 mol 2,3-DPG/mol Hb_4 raises the P_{50} by 8.4 torr (1.12 kPa) at pH 7.2 and 40 torr (5.33 kPa) CO_2 increases the P_{50} by 4.5 torr (0.6 kPa) at the same pH.

Effect of chloride on rhinoceros hemoglobin. Because of the small specific effects of 2,3-DPG and CO_2 on rhinoceros hemoglobin we determined the influence of chloride (fig. 3). At pH 7.2 the P_{50} increases from 7.6 torr (1.01 kPa) to 17.1 torr (2.28 kPa), when the chloride concentration is raised from 0.01 M to 0.1 M, and the Bohr effect decreases to -0.33 with only 0.01 M Cl^-. The n-values are dependent on pH but not the chloride concentration (fig. 6). Between pH 6.5 and 8.1 the n-value decreases from 2.9 to 2.1.

Discussion

Mammalian hemoglobins are customarily classified into two groups (Bunn, 1980; Perutz and Imai, 1980): (i) hemoglobins with high intrinsic oxygen affinity, which depend on 2,3-DPG for the regulation of their oxygen affinity, (ii) hemoglobins with low intrinsic oxygen affinity, like ruminant and felidae hemoglobins, which are independent of organic phosphates. This classification does not cover hemoglobins of those mammalian species that have a high oxygen affinity *in vivo, i.e.* diving mammals, species habitually exposed to hypoxia, or excessively large animals (Schmidt-Nielsen and Larimer, 1958; Bartels *et al.*, 1963; Bauer *et al.*, 1980; Jelkmann *et al.*, 1981). A common finding for all these hemoglobins is that their intrinsic oxygen affinity is relatively high, *i.e.* comparable to that of human hemoglobin, while the interaction with 2,3-DPG is considerably reduced. This may be

the result of a substitution at the organic phosphate binding site, as in elephant and rhinoceros hemoglobin (Braunitzer *et al.*, 1982; Bauer *et al.*, 1980; Mazur *et al.*, 1982) or due to altered tertiary/quaternary structures of the binding site that result from substitutions elsewhere in the molecule, as for instance in the hemoglobin of the mole (Jelkmann *et al.*, 1981). Since these species belong to very different orders of mammals, the loss or reduction of organic phosphate binding is a striking example of convergent evolution.

The substitution of β_2-HIS by glutamic acid has drastically reduced the affinity of rhinoceros hemoglobin for 2,3-DPG. A tenfold excess of 2,3-DPG over Hb increases the P_{50} only by 0.4 kPa at pH 7.2. Although the concentration of 2,3-DPG in the red cells of the rhinoceros is unknown, it is reasonable to assume that even if present in concentrations that represent the upper limit for mammalian red cells (10–12 mol 2,3-DPG/L RBC), it would not exert an important direct effect on oxygen affinity, except for the lowering of the intracellular pH. In this respect it is noteworthy that the estimated pI of rhinoceros hemoglobin is considerably lower than that of human hemoglobin, so that a normal intracellular pH (*e.g.* around pH 7.2 at pHe 7.4) could be maintained even in the absence of 2,3-DPG. That ATP is more effective than 2,3-DPG in reducing the oxygen affinity of rhinoceros hemoglobin is in accord with the recently proposed structure of the ATP-binding site in fish hemoglobins, where GLU β_2 is thought to interact with the adenine (Perutz and Brunori, 1982). In absolute terms, the effect of ATP is still small, since even with a tenfold excess of ATP over hemoglobin the P_{50} is raised by less than 5 torr.

An effect of CO_2 on the oxygen affinity of rhinoceros hemoglobin could not be demonstrated at pH <7.2. The primary structure of the globin chains shows that all N-termini are free (Mazur *et al.*, 1982). One obvious explanation for the reduced effect of CO_2 would be an increased ionization of the N-terminal amino group of the β-chain (which is responsible for most of the oxygen-linked CO_2 binding of mammalian hemoglobin), which could be caused through interaction of the amino group with the gamma-carboxyl group of GLU β_2.

The effect of chloride on the oxygen affinity of rhinoceros hemoglobin is of the same magnitude as that observed in human hemoglobin. At the β-chains chloride is bound to LYS β_{82} (Bonaventura *et al.*, 1976), but apparently the presence of GLU β_2 does not interfere. Bonaventura *et al.* (1980) have suggested that a reduction of the positive charge density in the central cavity should decrease the intrinsic oxygen affinity. However, our data show that the intrinsic oxygen affinity of rhinoceros hemoglobin as indicated by the P_{50} measured at low (10 mM) chloride concentration is not much lower (7.5 torr at pH 7.2) than that of human hemoglobin (5.3 torr) under the same conditions (Baumann, 1980). This result supports the conclusion of Perutz and Imai (1980) that mammalian hemoglobins with a low intrinsic oxygen affinity are characterized by a hydrophobic residue at position β_2 rather than a negatively charged one. From the data obtained in hemoglobin solutions it can be extrapolated that the P_{50} of rhinoceros blood under standard

conditions should be around 20 torr, which is in keeping with results for other large mammals like the elephant or hippopotamus (Bartels *et al.*, 1963; Leivestad *et al.*, 1973). Although we did not separate the two hemoglobin fractions, large functional differences are not to be expected since the rhinoceros hemoglobins differ only at two positions of the β-chain (β_{62} and β_{116}; Mazur *et al.*, 1982). Less is known about the structure of the four tapir hemoglobins, which have different alpha and beta-chains. At position β_2 one finds GLU as well as GLN (Mazur *et al.*, unpublished observation), which explains the larger effect of both CO_2 and 2,3-DPG on the unfractionated hemoglobin solution.

Conclusion: Rhinoceros hemoglobin represents an interesting example of a mammalian hemoglobin whose functional control is almost exclusively dependent on inorganic anions, *i.e.* chloride. The fact that the oxygen-linked binding of chloride is not changed through the presence of glutamic acid at position β_2 underlines the highly specific nature of substitutions at strategic sites in the hemoglobin molecule and is a further indication for the high degree of independence existing between organic and inorganic anion binding sites.

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