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Molecular Basis for ATP/2,3-Bisphosphoglycerate Control Switch-Over (Poikilotherm/Homeotherm) An Intermediate Amino-Acid Sequence in the Hemoglobin of the Great Indian Rhinoceros (*Rhinoceros unicornis*, Perissodactyla)*

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In memoriam of Rheinhold Benesch (1919–1986)

Summary: The complete primary structure of the two hemoglobin components of the Great Indian Rhinoceros *(Rhinoceros unicornis)* is presented. The ratio for the two components $B(\alpha_2\beta^I_2)$: $A(\alpha_2\beta^{II}_2)$ is 6:4. Polypeptide subunits were separated by chromatography on CM-cellulose in a buffer containing 8M urea. The sequence was studied by degradation of the tryptic and hydrolytic cleavage products in a liquid phase sequencer.

At position βNA2 component B has Asp, whereas component A has Glu, an ATP-binding site in fish and reptilian hemoglobins. The other phosphate binding sites i.e. βNA1 Val, βEF6 Lys and βH21 His are identical with 2,3-bisphosphoglycerate-(DPG)binding sites in mammalian hemoglobins, whereby rhinoceros hemoglobin resembles both ATP-sensitive poikilotherm hemoglobin and DPG-sensitive mammalian hemoglobin. The two components (β^I/β^{II}) additionally differ by exchange of Glu → Gly at position βA3 and Gln → Lys at position βGH3. The significance of these changes is discussed. Oxygenation properties of the two hemoglobins components and their dependence on ATP and DPG are given. The structure and function of Rhinoceros hemoglobin may give an insight into the evolution of the organic phosphate binding in vertebrate hemoglobins.

Die molekularen Grundlagen für die ATP- und 2,3-Bisphosphoglycerat-Kontrolle (poikilotherm-homöotherm) der Sauerstoffaffinität des Hämoglobins vom Panzernashorn (Rhinoceros unicornis, *Perissodactyla)*

Zusammenfassung: Die vollständige Primärstruktur der beiden Hämoglobine des Panzernashorns *(Rhinoceros unicornis)* wird angegeben. Die beiden Komponenten $B(\alpha_2\beta^I_2)$: $A(\alpha_2\beta^{II}_2)$ stehen in einem Verhältnis von 6:4. Die Peptidketten wurden über CM-Zellulose in einem Puffer mit 8M Harnstoff getrennt. Die Sequenzen wurden durch Abbau der tryptischen und hydrolytischen Spaltprodukte im Flüssigphasen-Sequenator bestimmt.
In Position βNA2 wird bei der Komponente B Asp, bei der Komponente A Glu gefunden, beides ATP-Bindungsstellen, wie sie bei Fischen, Amphibien und Reptilien gefunden werden: Die anderen Bindungsstellen für Polyphosphate sind βNA1 Val, βEF6 Lys und βH21 His, also 2,3-Bisphosphoglycerat-(DPG)-Bindungsstellen der Säuger. Die Rhinozeros-Hämoglobine zeigen beides, ATP-Sensitivität der Hämoglobine poikilothermer Tiere, aber auch DPG-Sensitivität der Hämoglobine der homöothermen Säuger. Die beiden Komponenten (β^I/β^{II}) unterscheiden sich zusätzlich durch Austausch von Glu → Gly in Position βA3 und Gln → Lys in Position βGH3.

Abbreviations:
Quadrol = *N,N,N′,N′*-tetrakis(2-hydroxypropyl)ethylenediamine; Reagent I = 4-(isothiocyanato) benzene sulfonic acid, sodium salt; Reagent IV = 7-(isothiocyanato) naphthalene-1,3,5-trisulfonic acid; trisodium salt; Hepes = 2 [4-(2-hydroxyethyl) 1-piperazino -ethane sulfonic acid, sodium salt; Hp = hydrolytic peptide; DPG = 2,3-bisphosphoglycerate; IPP = inositol pentakisphosphate; Tos-Phe-CH_2Cl = *N*-tosyl-L-phenylalanyl-chloromethane.

* 103rd Communication on hemoglobins; for the 102nd communication see ref.[1].

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Die Bedeutung dieser Unterschiede wird diskutiert und die physiologischen Daten und Eigenschaften der beiden Hämoglobine in ihrer Abhängigkeit von ATP und DPG wiedergegeben. Struktur und Funktion der Rhinozeros-Hämoglobine könnten Einblick in die Evolution der Bindung und Wechselwirkungen der organischen Polyphosphate bei Wirbeltier-Hämoglobinen geben.

Key words: Homeotherms, polyphosphate control, Rhinoceros, hemoglobin.

Hemoglobin is the oxygen carrier in the blood of all vertebrates except for a few antarctic, deep-sea fishes. Its oxygen-binding efficiency is regulated by allosteric cofactors like GTP (mainly found in fish), ATP (mainly found in fish, amphibians and reptiles), IPP (mainly in birds) and DPG (mainly in mammals). Studies on the primary structure of hemoglobin and its interaction with these anionic organic effectors have led to the identification of positively charged key amino-acid residues involved in the regulation. Thus, while DPG as well as ATP/GTP bind at position β1 Val and β82 Lys, DPG binds to β2 His and β143 His of mammalian hemoglobin molecules, whereas the nucleoside triphosphates specifically interact with β2 Glu/Asp and β143 Arg of fish hemoglobin[2,3].

Some members of the mammalian order Perissodactyla (families Rhinocerotidae and Tapiridae) are unique in having glutamic acid in position β2, one of the binding sites for ATP/GTP. We have extended our studies on the Rhinocerotidae and report the primary structures of the two hemoglobin components from *Rhinoceros unicornis*, which show aspartic or glutamic acid, respectively, at position β2 and present oxygenation properties and the sensitivities to DPG and ATP for the two hemoglobin components. The different allosteric control mechanisms in homeotherms and poikilotherms are examined on the basis of the new sequence data.

Material and Method

Preparation of hemolysate and electrophoresis

Hemolysate was prepared as described earlier[4]. The number of hemoglobin components was determined by polyacrylamide gel electrophoresis[5] using 7.5% gels and Tris/glycine buffer, pH 8.5 (Fig. 1a). The number of polypeptide subunits was determined in the presence of 8M urea and Triton X-100 according to Alter et al.[6] (Fig. 1b).

Separation of hemoglobin components

Whole hemolysate was subjected to ion exchange chromatography in a column (1.6 × 15 cm) of DEAE-Sephacel (Pharmacia, preswollen), equilibrated with 50mM Tris/HCl buffer pH 8.5. Elution was carried out with a gradient of 0.02–0.2M NaCl. Flow rate was maintained at 21 ml/h and fractions of 5 ml each were collected. The absorbance was monitored at 415 and 280 nm.

Separation of globin chains

A globin sample reduced with dithiothreitol for 3 h under nitrogen was applied to a 1.6 × 15-cm column of carboxymethyl cellulose[7] (Whatman CM-52) equilibrated with 8M urea containing, 0.2% mercaptoethanol and 25mM sodium acetate pH 5.7. A salt gradient of 0.02–0.08M NaCl was employed for eluting the polypeptides. Purity of the subunits was examined by polyacrylamide gel electrophoresis in the presence of urea and Triton X-100 (Fig. 1b).

Enzymatic cleavage

Native or oxidized polypeptide chains were digested with TPCK treated trypsin[8] (Worthington) at pH 10.7 and 9.5 for 3 h and an enzyme to substrate ratio of 5:100. The hydrolysate was then titrated to pH 4.5, centrifuged and subjected to gel filtration.

Fractionation of peptides

The enzymatic hydrolysate was prefractionated on Sephadex G-25 fine (2.6 × 140 cm) equilibrated and eluted with 0.1M acetic acid at a flow rate of 21 ml/h. Individual peaks were then rechromatographed by high-performance liquid chromatography on Lichrosorb RP-2 using an ammonium acetate/acetonitrile system[9].

Chemical cleavage

Hydrolytic cleavage of the Asp-Pro bond[10] was performed to obtain the C-terminal part of the polypeptide. The sample was dissolved in 6M guanidinium hydrochloride/70% formic acid and maintained at 42 °C for 55 h. The hydrolysis products were chromatographed on Sephadex G-50 fine (2.6 × 160 cm) with 8M urea in 10% formic acid. The C-terminal peptide was desalted over Sephadex G-25 in 1M acetic acid.

Amino acid analysis

Peptides were hydrolysed in constant boiling HCl (5.7M) at 110 °C for 20 h. Cysteine and methionine were estimated after performic acid oxidation. Tryptophan was estimated after hydrolysis in the presence of 6% thioglycollic acid. The analyses were performed on a Biotronic auto analyser LC 5000.

Sequence analysis

Sequence studies were carried out by automatic degradation[11] of tryptic and hydrolytic cleavage products in the liquid phase sequencers 890B & 890C (Beckman Instruments). Intact polypeptide or small lysine peptides were sequenced with Quadrol (0.25 and 1.0M) after coupling with reagent I[12] or reagent IV[13] whereas 3-diethylamino propyne[14] was employed in the case of arginine peptides and the C-terminal prolyl-peptide. Conversion of the thiazolinone to phenylthiohydantoin was performed with 3M trifluoroacetic acid at 80 °C. Identification of the phenylthiohydantoin derivatives of amino acids was followed either on thin-layer precoated silica gel plates (E. Merck)[15] or by high-performance liquid chromatography[16].

Oxygen equilibrium measurements

Solutions of the two major components showing some oxidation were reduced by addition of a trace of sodium dithionite, saturation with carbon monoxide and dialysis against several changes of CO-saturated, 0.05M Tris buffer containing 5×10^{-4}M EDTA. The hemoglobin solutions were concentrated by pressure dialysis through a molecular sieve membrane with cut-off at 10 kDa (nucleopore Corp., Pleasanton, California).

Oxygen equilibria were determined using a modified oxygen diffusion chamber technique, in which oxygen tensions in the gas mixtures passing through the chamber were increased stepwise using Wösthoff gas mixing pumps (D-4630 Bochum) to mix air and pure nitrogen (99.998%)[17]. The equilibria were determined at 37 and 25 °C at different pH values (obtained by buffering to 0.1M with Na Hepes, in the presence of 0.1M KCl and in the absence or presence of DPG, ATP and GTP. The concentrations of these organic phosphates in the stock solution used were assayed enzymatically using Boehringer test chemicals.

Results

Polyacrylamide gel electrophoresis of the hemolysate showed the presence of two components, A and B, occuring in a ratio of 4:6. Electrophoresis of the hemolysate under dissociating conditions revealed three polypeptide subunits (Fig. 1).

DEAE-Sephacel chromatography of the hemolysate resulted in the isolation of the two components in pure form (Fig. 2). Separation of the crude globin on CM-cellulose in the presence of 8M urea revealed four well-defined peaks (Fig. 3a), whereas only three peaks were identified on high-performance liquid chromatography of the hemolysate with Nucleosil C-4 using 0.1% trifluoroacetic acid and acetonitrile gradients (Fig. 3b). All four peaks from CM-cellulose chromatography were therefore analysed. Tryptic peptides were prefractionated on Sephadex G-25 and then chromatographed on Lichrosorb RP-2

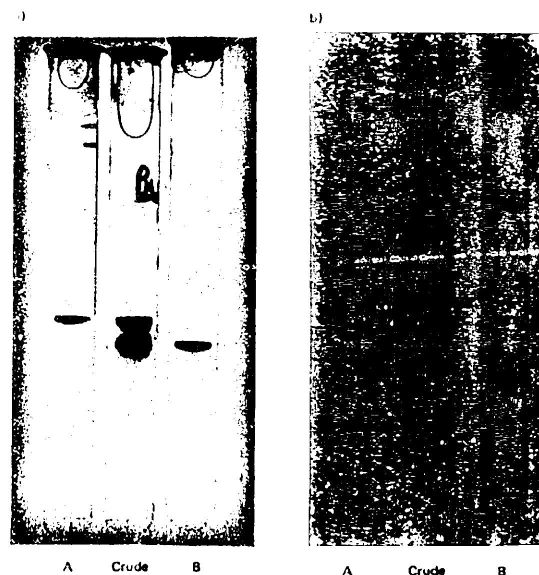

Fig. 1. Electrophoretic pattern of *Rhinoceros unicornis* hemolysate.

In the middle lane the crude hemolysate was used. A = Component A; B = component B. Components A and B have been separated by DEAE-Sephacel chromatography. a) Non-dissociating condition, with Tris/glycine buffer pH 8.5. b) Under dissociating conditions, in the presence of 8M urea and Triton X-100.

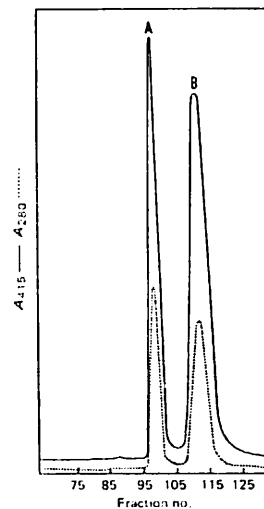

Fig. 2. Separation profile of the whole hemolysate on a DEAE-Sephacel column (1.6 × 15 cm) with 50mM Tris/HCl buffer, pH 8.5, and a gradient of 0.02–0.2M NaCl.

with 50mM ammonium acetate and 0–40% acetonitrile gradients (Fig. 4).

Sequence studies on the intact polypeptides resulted in the identification of the first 42 N-terminal residues. Tryptic peptides revealed the position of the remaining amino acids. Their amino-acid compositions are presented in Tables 2–4 (see supplementary material). Overlapping sequence was provided by the hydrolytic C-terminal prolyl-peptide. The complete amino-acid sequence of the α- and β-chains is presented in Fig. 5. Alignment of the sequence with human hemoglobin leads to 18 exchanges in the case of α- and 22 exchanges each for the β^{I} and β^{II} subunits requiring a minimum of 21 nucleotide substitutions (3 of which arise from two-point mutations) for the α-chain and 27 nucleotide substitutions for β^{I} and β^{II} (5 of these as a result of two point mutations). These substitutions alter the $\alpha_1\beta_1$ subunit cooperativity at 4 points, i.e. at β55 Met → Leu, β112 Cys → Val, β116 His → Gln, β125 Pro → Glu, $\alpha_1\beta_2$ contact at β43 Glu → Asp, and haem contacts at β70 Ala → Ser and β85 Phe → Tyr. In addition to this important DPG binding site β NA2 His has been mutated to Asp or Glu, respectively. Within the two β-chains three differences were located at βNA2 Asp/Glu, βA3 Glu/Gly and βGH3 Gln/Lys.

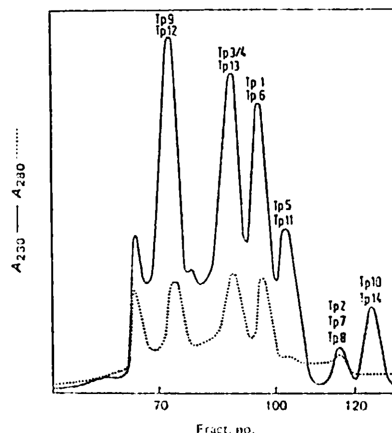

Fig. 4. Prefractionation of the tryptic peptides from α-globin chain (peak α^{II} of Fig. 3a) on a Sephadex G-25 column (fine, 2.6 × 140 cm), eluted with acetic acid. Flow rate, 20 ml/h.

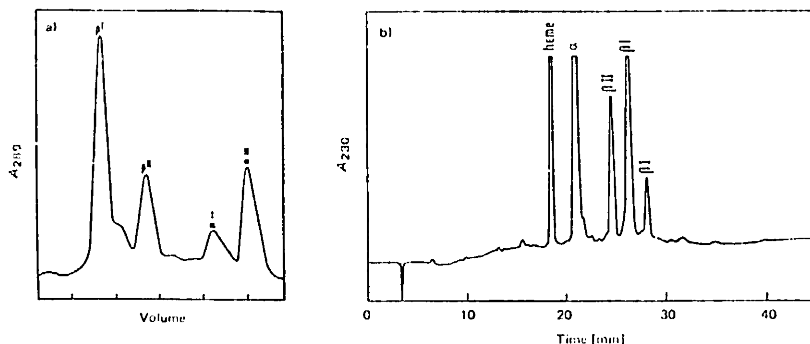

Fig. 3. Separation of the hemoglobin chains from a crude *Rhinoceros unicornis* hemolysate.

a) Profile on a CM-cellulose column (1.6 × 15 cm) eluted with 8M urea containing 25mM sodium acetate/0.2% mercaptoethanol using a gradient of 0.02–0.08M NaCl. b) High-performance liquid chromatography of a hemolysate on Nucleosil C-4 with 0.1% trifluoroacetic acid and 37–60% acetonitrile gradient.

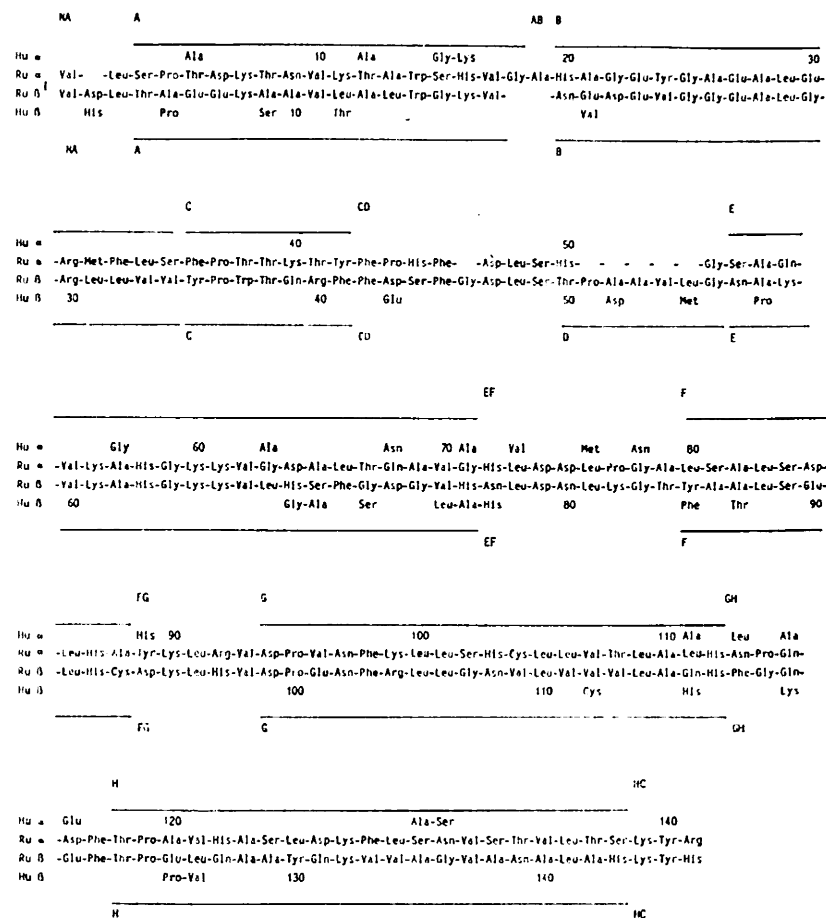

Fig. 5. Amino-acid sequence of *Rhinoceros unicornis* hemoglobin.

The sequences of the α- and β-chains are written in full (Ru α, Ru β^{I}). Exchanges as compared to adult human α- and β chains are (Hu α, Hu β) indicated. A, B, C etc. (bars) and AB, CD, GH show the helical and interhelical regions, respectively. The β^{II}-chain differs from the βI-chain at β2 Asp/Glu, β6 Glu/Gly and β119 βGH3 Gln/Lys.

Figs. 6 and 7 show the effect of DGP, ATP and GTP on the hemoglobin oxygenation properties of the isolated Rhinoceros hemoglobin components. At pH 7.4 and 37 °C components A and B display similar P_{50} values (13.8 and 12.0 mmHg) and the Bohr factors (= lg P_{50}/pH = – 0.60 and – 0.58, respectively). At 25 °C the P_{50} values for HbA and HbB were 6.3 and 6.0, whereas were – 0.72 and – 0.67 respectively. These data reflect values for the overall heats of oxygenation of approximately – 46 and – 42 kJ/mol O_2 (about – 11 and – 10 kcal/mol) for components A and B, respectively. The inverse relationship between ϕ and temperature accords with findings for human hemoglobin[18] and shows a qualitatively similar relationship between temperature and the pK values for ionization in the Rhinoceros hemoglobin. At pH 7.0 to 7.5 which may be expected to encompass physiological conditions, the cooperativity coefficient, n was about 2.0.

Both hemoglobins showed only slight sensitivity to organic phosphates at physiological pH conditions. Accordingly, DPG, ATP and GTP increased the lg P_{50} of stripped Hb A by only 0.05 (Fig. 6). Component B, however, exhibited slightly greater sensitivities to DPG (Δlg P_{50} approximately 0.06 and 0.13 for ATP and DPG respectively (Fig. 7).

Discussion

Rhinocerotidae together with Tapiridae (tapirs) and Equidae (horses) constitute the order Perissodactyla, which was one of the richest orders in the tertiary period some 65 million years ago.

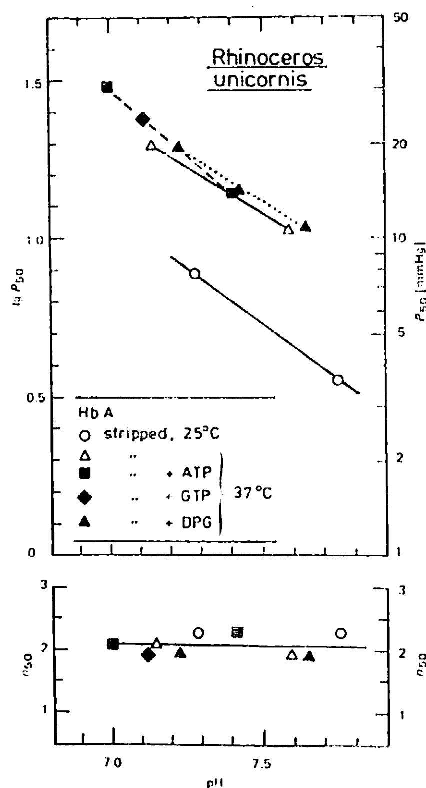

Fig. 6. Oxygen tensions and cooperativity coefficients at half saturation (P_{50} and n_{50}) and their pH dependence of *Rhinoceros unicornis* hemoglobin A measured in the presence of 0.1M KCl and 0.1M NaHepes buffers. ○, stripped Hb at 25 °C; △, stripped Hb at 37 °C; ■, ATP/tetrameric Hb molar ratio ~ 97; ◆, GTP/Hb ~ 65; ▲, DPG/Hb ~ 90. Tetrameric Hb concentration, 0.07mM.

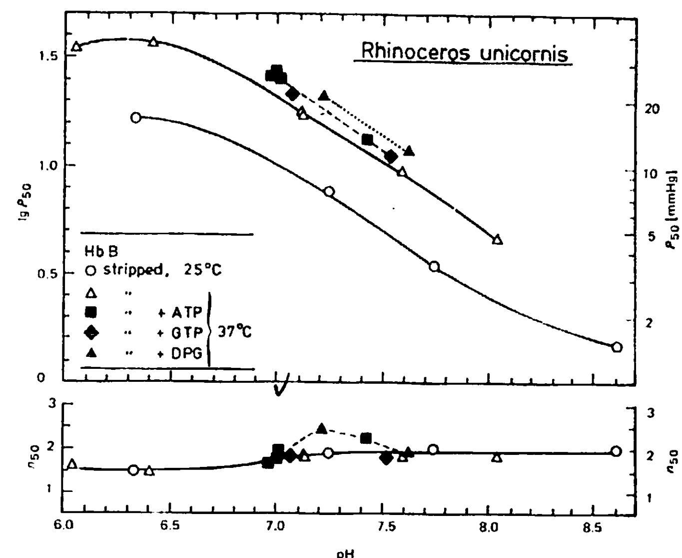

Fig. 7. P_{50} and n_{50} values of *Rhinoceros unicornis* Hb B.
Details as in legend to Fig. 6. Tetrameric Hb concentration = 0.08mM. ATP/Hb ~ 80, GTP/Hb ~ 55, DPG/Hb ~ 80.

Today the order is restricted to six species of horses, four species of tapirs and five species of rhinoceros. Studies on the order Perissodactyla have been of particular interest owing to the fact that their fossil record is well documented and have contributed much to present-day understanding of mammalian evolution.

Studies on the various members of this order aimed at unravelling the primary structure of hemoglobins undertaken by Braunitzer and colleagues[19-21] show the presence of glutamine at βNA2 in the case of equidae and glutamic acid in the case of Tapiridae and Rhinocerotidae. Since interaction of βNA2 His with DPG in mammals is well documented, these results evoked much interest as to whether these sequences reflect an intermediate mechanism that might have occured during the course of evolution. ATP is the most effective organic modulator of oxygen affinity in fishes, amphibians and reptiles, and a stereochemical model for its interaction has been proposed by Perutz and Brunori[22]. Table 1 shows the various sites of its interaction. The hemoglobin of the Great Indian Rhinoceros evidences an intermediate control.

With regard to oxygen binding the two hemoglobin components exhibit similar characters. The half-saturation oxygen tensions for HbA and HbB (17.8 and 15.8 mmHg, respectively at pH 7.2) correspond accurately with that (17.1 mmHg) given at this pH for the whole hemolysate from the White-Mouthed Rhinoceros (*Ceratotherium sinum*) by Baumann and coworkers[23], despite the fact that their measurements were carried out at about 8 times greater hemoglobin concentrations. This indicates that molecular dissociation did not influence the oxygen affinities recorded here. In contrast to the findings that ATP and DPG had the same effect on the P_{50} at 20-fold molar excess over hemoglobin tetramers in *Ceratotherium*, our data show a greater DPG than ATP effect in the most abundant Rhinoceros component (HbB) at saturating cofactor concentrations (cf. Fig. 7).

Although the two components are structurally different with β^{I} NA2 Asp, A3 Glu, GH3 Gln compared to β^{II} NA2 Glu, A3 Gly, GH3 Lys a "division of labour" such as that observed in some teleost hemolysates, where the cationic component exhibits higher affinities, smaller

Table 1.

a) Heterotropic control of oxygen affinity in vertebrates: The binding sites in β-chains.

Regulator	NA1 β1	NA2 β2	EF6 β82	H13 β135	H17 β139	H21 β143	H22 β144
DPG	Val	His	Lys	–	–	His	·
ATP/GTP	Val	Glu	Lys	–	–	Arg	–
IPP	Val	His	Lys	Arg	His	Arg	–
HCO_3^-	Ac-Ala	–	Lys	–	–	–	Glu

b) ATP/DPG switch-over mechanism (poikilotherm/homeotherm).

Sequence	β1	β2	β82	β143		Affinity
Helix	NA1	NA2	EF6	H21		
Poikilotherm	Val	His	Lys	Arg		ATP
Intermediate	Val	Glu	Lys	His	Rhinoceros Hb A	ATP < DPG
Intermediate	Val	Asp	Lys	His	Rhinoceros Hb B	ATP < DPG
Homeotherm	Val	His	Lys	His		DPG

Bohr factors and different temperature effects compared to the anionic component is not seen. The sensitivities of the Rhinoceros hemoglobins to phosphates are low, with the values of $\Delta \lg P_{50}$ (the difference between values in the absence and presence of the phosphates) equalling 0.05–0.13, compared to the values of about 0.4–0.6 found for fish hemoglobins with ATP and mammalian hemoglobins with DPG[24,25]. This indicates that the substitution of βNA2 His in human hemoglobin A for βNA2 Asp or Glu in rhinoceros drastically reduces DPG binding, while nucleoside triphosphate binding is also hindered by the persistence of the other DPG sites.

The temperature effect (as expressed in terms of the overall heat of oxygenation, ΔH) is similar to that recorded in other mammals, despite the higher variable body temperature (between 30 and 42 °C, P.G. Wright, personal communication) in the Rhinoceros, particularly in its extremities.

Given the monophyletic origin of mammals, the occurence in Rhinoceros hemoglobins of amino-acid residues that are involved in nucleoside-triphosphate binding in homeotherm vertebrates, cannot be considered an evolutionary relict, whereby it must reflect a reversal of evolution in terms of oxygen affinity control. The question of the adaptive significance of such secondary development in Rhinocerotidae and Tapiridae remains speculative.

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Supplementary Material

Table 2. Amino-acid composition of peptides from α-globin chain.

Peptide	T-1	T-2	T-3/4	T-5	T-6	T-7	T-8	T-9	T-10	T-11	T-12	T-13	T-14	Globin
pos.	1-7	8-11	12-31	32-40	41-56	57-60	61	62-90	91-92	93-99	100-127	128-139	140-141	
Asp	1.05	0.95	-	-	1.03	-	-	4.3 (4)	-	2.08	3.1 (3)	1.01	-	13.46 (13)
Thr	0.95	0.95	1.03(1)	1.95	1.05	-	-	1.0 (1)	-	-	1.85(2)	1.96	-	10.59 (11)
Ser	0.96	-	1.02(1)	0.96	1.87	-	-	1.90(2)	-	-	1.84(2)	2.89	-	11.37 (12)
Glu	-	-	2.88(3)	-	0.97	-	-	1.06(1)	-	-	1.06(1)	-	-	6.5 (6)
Pro	0.99	-	-	0.98	0.93	-	-	1.04(1)	-	0.95	1.95(2)	-	-	7.1 (7)
Gly	-	-	2.95(3)	-	1.05	1.04	-	3.4 (3)	-	-	-	-	-	8.36 (8)
Ala	-	-	4.85(5)	-	1.02	1.03	-	5.15(5)	-	-	3.1 (3)	-	-	15.46 (15)
Cys*	-	-	-	-	-	-	-	-	-	-	0.94(1)	-	-	0.81 (1)
Val	0.84	0.96	1.01(1)	-	0.93	-	-	2.1 (2)	-	2.05	1.93(2)	1.92	-	12.40 (12)
Met*	-	-	-	0.92	-	-	-	-	-	-	-	-	-	0.78 (1)
Leu	0.94	-	1.24(1)	1.1	1.2	-	-	6.2 (6)	1.1	-	7.2 (7)	2.2	-	19.86 (20)
Tyr.	-	-	0.91(1)	-	0.83	-	-	1.02(1)	-	-	-	-	0.83	4.05 (4)
Phe	-	-	-	1.95	2.1	-	-	-	-	1.01	0.89(1)	0.97	-	6.91 (7)
His	-	-	1.87(2)	-	1.80	0.94	-	2.1 (2)	-	-	2.67(3)	-	-	9.98 (10)
Trp	-	-	(1)	-	-	-	-	-	-	-	-	-	-	0.58 (1)
Lys	1.04	1.1	-	0.98	0.99	1.0	1.0	1.0 (1)	-	1.01	1.04(1)	0.98	-	9.82 (10)
Arg	-	-	1.02(1)	-	-	-	-	-	1.0	-	-	-	1.0	2.78 (3)
Sum	7	4	20	9	16	4	1	29	2	7	28	12	2	141

* Determined after performic acid oxidation

Table 3. Amino-acid composition of peptides from β^{I}-globin chain.

Peptide pos.	T-1 1-8	T-2 9-17	T-3 18-30	T-4 31-40	T-5 41-59	T-6 60-61	T-7 62-65	T-8 66	T-9 67-82	T-10 83-95	T-11 96-104	T-14 133-144	T-15 145-146	Hp	Globin
Asp	0.97	-	1.97	-	3.05	-	-	-	3.86	0.98	2.09	1.1	-	3.19	15.5 (15)
Thr	0.96	-	-	1.05	0.91	-	-	-	-	0.89	-	-	-	1.1	5.25 (5)
Ser	-	-	-	-	1.87	-	-	-	0.88	0.99	-	-	-	-	4.2 (4)
Glu	1.93	-	2.85	1.1	-	-	-	-	-	0.97	1.02	-	-	6.3(7)	14.09 (14)
Pro	-	-	-	1.1	1.01	-	-	-	-	-	1.05	-	-	1.97	4.1 (4)
Gly	-	1.09	2.99	-	2.1	-	0.98	-	2.2	1.0	-	1.04	-	3.08	13.06 (13)
Ala	0.89	2.87	1.04	-	2.91	-	0.97	-	-	1.98	-	3.94	-	6.98	18.08 (18)
Cys*	-	-	-	-	-	-	-	-	-	0.97	-	-	-	-	0.92 (1)
Val	1.0	1.1	1.93	1.73	1.01	0.95	-	-	1.8	-	1.06	1.97(3)	-	5.76(7)	16.2 (18)
Leu	0.99	2.06	1.3	2.2	1.97	-	-	-	2.86	1.98	1.2	1.1	-	6.3	19.72 (20)
Tyr	-	-	-	1.05	-	-	-	-	-	1.05	-	-	0.81	1.54(2)	4.1 (4)
Phe	-	-	-	-	2.71	-	-	-	0.96	-	1.03	-	-	2.81	7.1 (7)
His	-	-	-	-	-	-	0.96	-	1.93	1.1	1.08	1.02	1.02	2.76	8.9 (8)
Trp	-	(1)	-	(1)	-	-	-	-	-	-	-	-	-	-	1.24 (2)
Lys	0.98	0.87	-	-	1.0	1.05	1.1	1.0	1.1	1.02	-	1.0	-	2.1	10.3 (10)
Arg	-	-	0.95	0.88	-	-	-	-	-	-	1.0	-	-	1.1	3.1 (3)
Sum	8	9	13	10	19	2	4	1	16	13	9	12	2	47	146

* Determined after performic acid oxidation

Table 4. Amino-acid composition of peptides from β^{II}-globin chain.

Peptide pos.	T-1 1-8	T-2 9-17	T-3 18-30	T-4 31-40	T-5 41-59	T-6 60-61	T-7 62-65	T-8 66	T-9 67-82	T-10 83-95	T-11 96-104	T-13 121-132	T-14 133-144	T-15 145-146	Hp 100-145	Globin
Asp	-	-	1.97	-	3.1	-	-	-	3.90	0.88	1.87	-	1.2	-	3.2	14.4 (14)
Thr	0.98	-	-	0.82	0.88	-	-	-	-	0.78	-	0.81	-	-	1.12	5.11 (5)
Ser	-	-	-	-	1.78	-	-	-	0.94	0.93	-	-	-	-	-	4.2 (4)
Glu	2.01	-	2.87	1.1	-	-	-	-	-	0.90	0.94	3.82	-	-	5.79	11.96 (12)
Pro	-	-	-	0.91	1.04	-	-	-	-	-	0.98	0.99	-	-	1.89	5.02 (5)
Gly	0.98	1.06	3.04	-	2.02	-	0.94	-	2.2	1.04	-	-	1.1	-	3.4	14.4 (14)
Ala	0.96	2.82	0.99	-	3.04	-	0.96	-	-	1.91	-	1.85	3.84	-	6.78	17.71 (18)
Cys*	-	-	-	-	-	-	-	-	-	0.96	-	-	-	-	-	0.85 (1)
Val	1.03	0.78	2.01	1.72	0.87	0.98	-	-	1.82	-	0.95	-	2.37(3)	-	5.03(7)	16.1 (18)
Leu	1.06	2.1	1.1	2.07	2.1	-	-	-	3.1	2.04	1.08	1.2	1.1	-	6.2	19.4 (20)
Tyr	-	-	-	0.76	-	-	-	-	-	1.02	-	1.0	-	0.72	1.35(2)	4.1 (4)
Phe	-	-	-	-	2.90	-	-	-	0.99	-	1.07	1.1	-	-	2.89	7.3 (7)
His	-	-	-	-	-	-	0.94	-	1.88	1.1	1.0	-	1.1	1.0	3.1	8.38 (8)
Trp	-	(1)	-	(1)	-	-	-	-	-	-	-	-	-	-	-	1.19 (2)
Lys	0.99	0.92	-	-	1.04	1.06	1.1	1.0	1.04	0.98	-	1.01	0.97	-	3.2	10.8 (11)
Arg	-	-	0.88	0.78	-	-	-	-	-	-	0.95	-	-	-	1.1	2.76 (3)
Sum	8	9	13	10	19	2	4	1	16	13	9	12	12	2	47	146

* Determined after performic acid oxidation.

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High-Altitude Respiration of Birds

The Primary Structures of the Major and Minor Hemoglobin Component of Adult Goshawk (*Accipiter gentilis*, Accipitrinae)*

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Summary: The primary structures of the hemoglobin components Hb A and Hb D of the adult Goshawk (*Accipiter gentilis*) are presented. The globin chains were separated on CM-Cellulose in 8M urea buffer. Component separation was achieved by FPLC-chromatography on a TSK SP-5PW column in phosphate-buffers with a linear gradient of NaCl. The amino-acid sequences were established by automated Edman degradation of the globin chains and of the tryptic peptides in liquid-phase and gas-phase sequenators. The sequences are aligned with those of European Black Vulture (*Aegypius monachus*). Phylogenetic aspects and physiological properties for Goshawk hemoglobin are inferred from sequence data. A detailed evaluation of the oxygen-binding properties has been carried out during a prolonged study of the noteworthy ability of Falconiformes to cope with extremely low oxygen partial pressures, and will be the subject of a forthcoming paper.

*Zur Höhenatmung der Vögel: Die Primärstrukturen der Haupt- und Nebenkomponente der Hämoglobine des adulten Habichts (*Accipiter gentilis, *Accipitrinae)*

Zusammenfassung: Die Primärstrukturen der Hämoglobine Hb A und Hb D des adulten Habichts (*Accipiter gentilis*) wurden ermittelt. Die Ketten ließen sich in 8M Harnstoffpuffer über CM-Cellulose trennen. Die Isolierung der Komponenten geschah mittels FPLC über eine TSK SP-5PW-Säule in Phosphatpuffern. Eluiert wurde in einem linearen Gradienten aus NaCl.

Die Sequenzen wurden durch automatischen Edman-Abbau der Globinketten und der tryptischen Peptide nach der Film- oder Gasphasen-Methode ermittelt. Sie werden mit denen des Mönchsgeiers (*Aegypius monachus*) verglichen. Die Globinsequenzen des Habichts werden hinsichtlich ihrer phylogenetischen und atmungsphysiologischen Eigenschaften ausgewertet. Die Physiologie dieser Hämoglobine wird im Rahmen einer erweiterten Studie zur ungewöhnlichen Fähigkeit der Greifvögel, sehr niedrige Sauerstoffpartialdrücke zu ertragen, dargestellt werden.

Key words: High-altitude respiration, primary structure, hemoglobin, evolution of Falconiformes, FPLC.

Enzyme:
Trypsin (EC 3.4.21.4).

Abbreviations:
Quadrol, *N,N,N',N'*-Tetrakis-(2-hydroxypropyl)ethylenediamine; reagent IV, trisodium 7-(isothiocyanato)naphthalene-1,3,5-trisulphonate; TosPheCH$_2$Cl, (*N*-tosyl-L-phenylalanyl)chloromethane; RP-HPLC, reversed-phase high-performance liquid chromatography; Hb, hemoglobin; FPLC, fast-protein liquid chromatography.

* 104th Communication on hemoglobins, for 103rd communication see ref. 1.

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