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Oxygen Binding of Monotreme Haemoglobins I. Oxygen Binding Parameters of the Haemoglobins of Platypus, *Ornithorhynchus anatinus*, and Echidna, *Tachyglossus aculeatus*

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Abstract

Oxygen binding parameters have been determined for echidna and platypus haemolysates. At pH 7·1 and 25°C platypus haemoglobin with an oxygen half-saturation pressure (P_{50}) of 2·4 kPa has a lower oxygen affinity than echidna haemoglobin with a P_{50} of 1·6 kPa. The ΔH^0 of oxygenation of platypus haemoglobin is −38·9 kJ/mol which is more exothermic than the −29·7 kJ/mol of echidna haemoglobin. Platypus haemoglobin has a steeper alkaline Bohr curve than echidna haemoglobin, while both haemoglobins show high levels of cooperativity at various temperatures and pH values. It appears that diphosphoglyceric acid modulates the affinity of echidna and platypus haemoglobins to different extents. This information is interpreted in terms of the steric effects of ligand binding originating in the amino acid sequence differences of these two species.

Introduction

The platypus, *Ornithorhynchus anatinus* Shaw and Nodder 1799, and echidna (spiny anteater), *Tachyglossus aculeatus* Shaw and Nodder 1792, are the only surviving members of the Monotremata. Morphologically both animals give the immediate impression of being uniquely adapted to their specialized environments. The streamlined platypus is well adapted to its semi-aquatic life, while the echidna is well adapted to digging and burrowing. The anatomical specializations of the monotremes have suggested to respiratory physiologists that these animals have specialized respiratory responses to the apparently asphyxial conditions which these animals regularly tolerate.

Johansen *et al.* (1966) examined the respiratory responses of the platypus during simulated dives. The blood showed a sigmoidal oxygen dissociation curve with an oxygen half-saturation pressure (P_{50}) of 4·0 kPa* and a pronounced Bohr effect. During submersion the platypus showed bradycardia. A further study on platypus blood by Parer and Metcalfe (1967*a*) gave a P_{50} value of 3·6 kPa at 31°C and pH 7·4, a relatively high oxygen capacity of 22·7% (v/v), and a Bohr effect factor of 0·56. A similar study on echidna blood (Parer and Metcalfe 1967*b*) gave a P_{50} value of 2·8 kPa at pH 7·4 and 31°C, an oxygen capacity of 21·9% (v/v) and a Bohr effect factor of 0·49. The effect of asphyxial conditions on respiratory responses of the echidna have been investigated by Bentley *et al.* (1967) and Augee *et al.* (1971). Under these conditions the animals developed bradycardia but the respiratory rate remained constant. Hypercapnia was more important than hypoxia in the development of bradycardia in burrowing echidnas.

* 1 kPa = 7·5 mm Hg.

The evolutionary development and radiation of these egg-laying mammals has been difficult to trace because of an inadequate fossil record. In an effort to understand the phylogenetic relationships of the monotremes the amino acid sequences of the β-chain (Whittaker *et al.* 1972) and α-chain (Whittaker *et al.* 1973) of echidna haemoglobin IB were determined as well as the sequences of the α-chain (Whittaker and Thompson 1974) and β-chain (Whittaker and Thompson 1975) of platypus haemoglobin. Calculations based on the differences in amino acid sequence of the α-chain suggested that monotremes diverged from other mammalian groups 211 million years ago, while calculations based on the β-chain gave 132 million years for the divergence date.

The unique phylogenetic relationships of the two monotremes, the contrast in habitat, and information regarding their haemoglobin sequences were the major reasons for the examination of the haemoglobins here reported.

Materials and Methods

Preparation of Haemolysates

Heparinized platypus and echidna blood (about 10 ml of each) was obtained from Dr M. E. Griffiths, Division of Wildlife Research, CSIRO, Canberra, and transported to the laboratory on ice.

The cells were collected by centrifugation at 3000 *g* at 4°C and washed three times with 0·9% (w/v) sodium chloride. The cells were lysed using 0·02 M tris–HCl, pH 8·0 at 0°C. Cell debris was removed by centrifugation at 20000 *g* for 10 min and the haemolysates converted to the carbon monoxide form by gently shaking under a barrier of carbon monoxide at 4°C. Carbon monoxide was removed from haemoglobin solutions just prior to determination of oxygen dissociation curves by gently shaking 0·2 mM haemoglobin solutions at 4°C under a barrier of oxygen and in the presence of a 500-W incandescent lamp until the spectrum of carbonmonoxyhaemoglobin was replaced by that of oxyhaemoglobin.

Electrophoresis

The composition of the haemoglobin solutions was examined using horizontal starch–gel electrophoresis in gels containing 2 M urea, 0·01 M KCN and 0·01 M EDTA. The discontinuous buffer system of Poulik (1957) at pH 8·6 was used. The echidna haemoglobin used was a mixture of Hb-IB and Hb-IIA (Cooper *et al.* 1973).

Oxygen Dissociation Curves

Oxygen dissociation curves were determined using a spectrophotometric method similar to that described by Rossi-Fanelli and Antonini (1958) and Benesch *et al.* (1965). The tonometer used consisted of a 1-cm spectrophotometer cell attached to an equilibrating chamber having a total volume of about 30 ml. A mobile glass spacer 9 by 8 by 50 mm was inserted in the spectrophotometer cell to give an effective optical path length of 2 mm and a solution volume of about 1·5 ml. A tap on the equilibration chamber allowed it to be evacuated so as to deoxygenate the haemoglobin solution in the tonometer. Complete deoxygenation was determined from the spectral properties of the haemoglobin solution. Small measured volumes of air were introduced to the haemoglobin solution by the tap on the equilibration chamber and the tonometer was equilibrated by rotation at 40 rev/min in a thermostated (0–40°C) water bath for 6 min. After equilibration of the haemoglobin solution with successive additions of air, the spectrum was determined between 400 and 750 nm, using a Unicam SP 800 recording spectrophotometer equipped with a thermostated cell compartment. Between 2 and 10 equilibrations were made between deoxygenation and complete oxygenation. Isosbestic points were obtained at 162, 546 and 567 nm, indicating insignificant methaemoglobin formation. Prolonged experiments at 30 and 35°C tended to give poor isosbestic points due to significant methaemoglobin formation and denaturation (Benesch *et al.* 1965), so experiments at these temperatures were of limited duration to avoid these problems.

The pressure of oxygen in the tonometer after each successive addition of air and the percentage saturation of the haemoglobin with oxygen were calculated using the method described by Rossi-Fanelli and Antonini (1958). Absorbance readings were taken at 472, 558 and 574 nm.

Oxygen dissociation curves were determined by plotting $\log p_{O_2}$ against fractional saturation Y. Plots of $\log p_{O_2}$ versus $\log Y/(1-Y)$ based on the Hill equation

$$Y = K(p_{O_2})^n/[1+K(p_{O_2})^n]$$

gave straight lines between $Y = 0{\cdot}1$ and $Y = 0{\cdot}9$, and from the line of best fit the $\log P_{50}$ value and Hill coefficient (n) were determined.

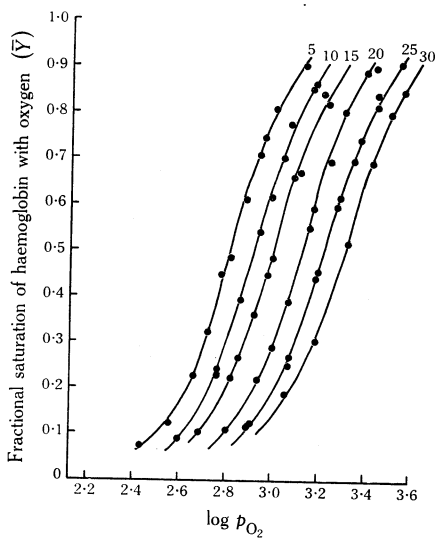

Fig. 1. Effect of temperature on oxygen dissociation curves of echidna haemoglobins in phosphate buffer, ionic strength 0·2 and pH 7·1. Plot of fractional saturation of haemoglobin with oxygen ($\bar{Y}$) versus $\log p_{O_2}$ (Pa). Temperatures (°C) are shown at the top of each graph.

Removal of Organic Phosphates

The removal of diphosphoglyceric acid (DPG) from haemoglobin was based on the method of Benesch *et al.* (1968). Haemolysates of carbonmonoxyhaemoglobin were dialysed against 0·1 M sodium chloride which was kept at a pH of between 8 and 8·5 using either 0·01 M tris or a few drops of NaOH. A 5-ml sample of carbonmonoxyhaemoglobin was loaded onto a Sephadex G25 column (25 by 900 mm) which had been equilibrated with water adjusted to pH 8·5; the column was then loaded with 5 ml of 0·1 M NaCl immediately prior to the application of the sample. The column was developed at pH 8·5 and the effluent collected. Phosphate estimations on the haemoglobin fractions, using the method of Ames and Dubin (1960), showed removal of most organic phosphate from the haemoglobin but occasionally traces (0·12 μmol phosphate per mole of haemoglobin) remained. The 'stripped' haemoglobin was dialysed against 0·05 M bis–tris buffer, pH 7·1, for the determination of oxygen dissociation curves.

Results

Thermodynamic Studies in Phosphate Buffer

Haemolysates of echidna and platypus haemoglobins were dialysed against phosphate buffer, ionic strength 0·2 and pH 7·1, and oxygen dissociation curves were determined at varying temperatures between 5 and 30°C. The plot of $\log p_{O_2}$ versus saturation (Y) yielded a series of sigmoidal curves for both platypus and echidna haemoglobins (Fig. 1). When these data were plotted according to the Hill plot a series of straight lines was obtained for $Y = 0{\cdot}1$ to $Y = 1{\cdot}0$, shown in Fig. 2.

The series of straight lines suggests that the degree of cooperativity is the same for each of the four binding steps. From the lines of best fit the $\log P_{50}$ value and Hill coefficient (n) were determined for both platypus and echidna haemoglobins at each temperature. Echidna haemoglobin had a Hill coefficient of $2{\cdot}93 \pm 0{\cdot}16$, whereas platypus haemoglobin had a Hill coefficient of $2{\cdot}85 \pm 0{\cdot}22$, indicating a high level of cooperativity in both monotreme haemoglobins.

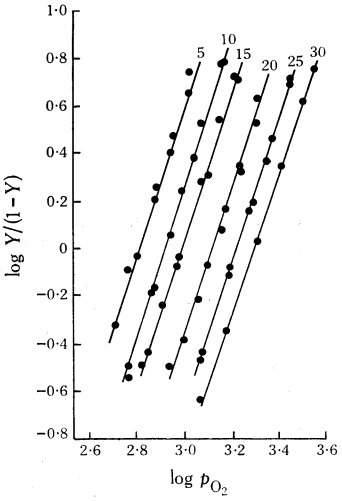

Fig. 2. Effect of temperature on the Hill plot of oxygen equilibria of echidna haemoglobins in phosphate buffer, ionic strength 0·2 and pH 7·1. Temperatures (°C) are shown at the top of each graph. Pressure was measured in pascals.

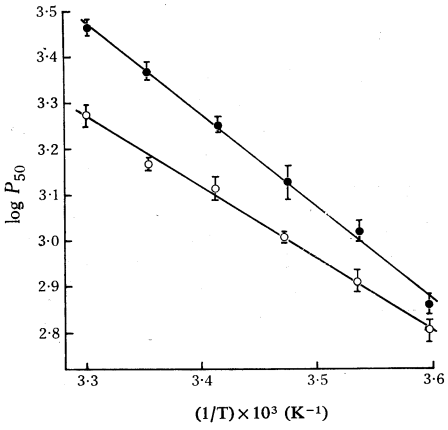

Fig. 3. Effect of temperature on the $\log P_{50}$ (Pa) of monotreme haemoglobins. Oxygen equilibria were determined in phosphate buffer, ionic strength 0·2 and pH 7·1.
● Platypus haemoglobin.
○ Echidna haemoglobin.

Plots of $(1/T) \times 10^3$ versus $\log P_{50}$ values for platypus and echidna haemoglobins yielded the lines of best fit shown in Fig. 3. The results in Fig. 3 show that at all temperatures echidna haemoglobin has a higher affinity for oxygen than platypus haemoglobin.

Values for ΔH^0, the overall heat per mole of oxygen for the reaction $Hb + O_2 \rightarrow HbO_2$, were calculated from these plots using the thermodynamic relation

$$\Delta H^0 = 2{\cdot}303R \times \mathrm{d} \log P_{50}/\mathrm{d}(1/T).$$

The heats measured using this relation include not only the intrinsic heat of oxygenation of the haems but also the heat of other linked processes including Bohr proton release, binding of DPG and chloride ions and the heat of solution of oxygen which is about 12·6 kJ/mol. The free energy ΔG^0 was determined from the relation $\Delta G^0 = -RT\ln K$ and the entropy ΔS^0 from $\Delta G^0 = \Delta H^0 - T\Delta S^0$. These values for platypus and echidna haemoglobins are shown in Table 1, together with control results based on experiments with human haemoglobin lysates.

The results in Table 1 indicate that echidna haemoglobins have a higher affinity for oxygen and a larger ΔH^0 value than platypus haemoglobins. Oxygen binding to platypus haemoglobin is therefore more exothermic than it is for echidna haemoglobin, and any change in temperature of the haemoglobins has a greater effect on the oxygen affinity of platypus haemoglobins than on that of echidna haemoglobins.

Table 1. Oxygen binding parameters of platypus, echidna and human haemoglobins
The oxygen half-saturation pressure, P_{50}, and the values for ΔH^0, ΔG^0 and ΔS^0 are shown

	Platypus	Echidna	Human
P_{50} (kPa at 25°C)	2·36	1·56	1·33
ΔH^0 (kJ/mol)	−38·91±1·26	−29·71±1·05	−40·17±1·26
ΔG^0 (kJ/mol)	7·11±0·08	6·07±0·08	5·77±0·13
ΔS^0 (J/deg)	−154·81±3·77	−119·66±3·56	−153·55±5·02

Both platypus and echidna haemolysates used in these thermodynamic studies contain multiple haemoglobins and the observed results represented the energetics of a variety of bonds being broken and formed. These bond changes accompany homotropic and heterotropic interactions which result from oxygen binding to the multiple haemoglobins. There has been no attempt to measure the separate Adair k_1, k_2, k_3 and k_4 values and therefore ΔH_1^0, ΔH_2^0, ΔH_3^0 and ΔH_4^0 values as has been described for human haemoglobin by Imai and Yonetani (1975).

Thermodynamic Studies of 'Stripped' Haemoglobins

Some difficulty was experienced in completely freeing the echidna and platypus haemoglobins of DPG and in some experiments up to 0·12 μmol phosphate per mole of haemoglobin remained. When freed of organic phosphates, oxygen dissociation curves of the stripped haemoglobins were determined in 0·05 M bis–tris buffer, pH 7·1. From the plot of $\log Y/(1-Y)$ versus $\log p_{O_2}$, the $\log P_{50}$ value was determined for several temperatures. The $\log P_{50}$ value for each temperature is shown in Table 2.

Stripped echidna haemoglobin showed a higher oxygen affinity than stripped platypus haemoglobin throughout the temperature range examined. When the data were plotted according to the thermodynamic relationships and the line of best fit was determined, it gave, for echidna haemoglobin, a P_{50} value at 25°C of 0·5 kPa and a ΔH^0 value of −39·7±5·4 kJ/mol; for platypus haemoglobin the P_{50} value at 25°C is 0·6 kPa and the ΔH^0 value is −42·5±5·0 kJ/mol.

Bohr Effect

The Bohr curves for echidna and platypus haemoglobins were prepared by dialysing haemoglobin haemolysates against phosphate buffer, ionic strength 0·2 and pH 7·1, and then adjusting the pH to values between 5·6 and 8·2, using 0·05 M NaOH or 0·05 M HCl. Oxygen dissociation curves were estimated at 20°C for each pH value and from the plots of $\log Y/(1-Y)$ versus $\log p_{O_2}$, the $\log P_{50}$ and Hill coefficient (n) at each pH value were determined. The Bohr curves were obtained by plotting the $\log P_{50}$ values against pH and are shown for echidna and platypus haemoglobins in Fig. 4.

Table 2. Effect of temperature on the oxygen affinity of echidna and platypus haemoglobins 'stripped' of organic phosphates

Oxygen affinity is shown as $\log P_{50}$, the pressure (in pascals) at which the haemoglobin is 50% saturated with oxygen. Oxygen equilibria were determined in 0·05 M bis–tris buffer, pH 7·1

	Temperature (°C)			
	15	20	25	30
Echidna	2·44	2·51	2·64	2·79
Platypus	2·54	2·61	2·77	2·91

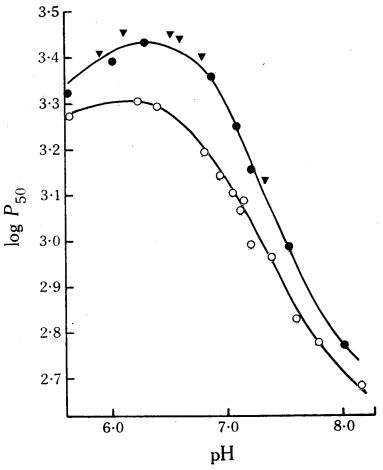

Fig. 4. Bohr curve for monotreme haemoglobins. Oxygen affinity is shown as $\log P_{50}$ (Pa), the pressure at which the haemoglobin is 50% saturated with oxygen, at measured pH values. Oxygen equilibria were determined at 20°C in phosphate buffer.
▼,● Platypus haemoglobin in phosphate buffer, ionic strength 0·1 (▼) and 0·2 (●).
○ Echidna haemoglobin in phosphate buffer, ionic strength 0·2.

Fig. 4 shows that echidna haemoglobin has a higher oxygen affinity throughout the pH range examined. The alkaline Bohr effect between pH 6·8 and pH 8·0 is expressed as $\Delta\log P_{50}/\Delta\text{pH}$, and values of 0·48 for platypus haemoglobin and 0·39 for echidna haemoglobin were obtained. Platypus haemoglobin therefore has a more pronounced Bohr effect than echidna haemoglobin and would have a greater response to carbon dioxide produced by respiring tissue. The Hill coefficient remained at about 2·9 for both echidna and platypus haemoglobins throughout the Bohr curve.

Discussion

The method described is suitable for determining oxygen binding parameters on small volumes of blood lysates. Human blood lysates were prepared as controls and at pH 7·1 and 25°C the P_{50} value was 1·3 kPa and the ΔH^0 value was −40·2 kJ/mol. This is comparable with P_{50} = 1·3 kPa and ΔH^0 = −41·8 kJ/mol reported by Antonini and Brunori (1971).

Previous studies by Parer and Metcalfe (1967*a*, 1967*b*) at pH 7·4 and 31°C gave P_{50} values of 2·8 and 3·6 kPa for echidna and platypus blood respectively. In this present study using a haemolysate dialysed against phosphate buffer, pH 7·1, P_{50} values at 31°C were 2·0 and 3·3 kPa for echidna and platypus haemoglobins respectively. The higher oxygen affinities reported here are attributed to the more limiting DPG levels present in the dialysed haemolysate and to different experimental procedures.

At pH 7·1 and 25°C the P_{50} values for echidna and platypus haemolysates were 1·6 and 2·4 kPa respectively. Hence the echidna has a relatively high oxygen affinity compared to eutherians and marsupials of the same size. The relatively low body temperature of the monotremes compared to eutherians and marsupials would accentuate these differences in P_{50} values.

The alkaline Bohr effect curve shows that platypus haemoglobin is more sensitive to H^+ than is echidna haemoglobin. This suggests that as the platypus dives, carbon dioxide produced by respiring tissue facilitates the unloading of oxygen from platypus blood to a greater extent than occurs for echidna haemoglobin under similar conditions. The burrowing echidna is more likely to be able to adjust to the more gradual asphyxia it is likely to encounter than would the diving platypus. Augee *et al.* (1971) showed that echidnas tolerate carbon dioxide levels of up to 10–12% before making behavioural adjustments.

Change in temperature affected the oxygen affinity of platypus haemoglobin to a much greater extent than that of echidna haemoglobin. The binding of oxygen to platypus haemoglobin with a ΔH^0 value of −38·9 kJ/mol is considerably more exothermic than the binding to echidna haemoglobin with a ΔH^0 value of −29·7 kJ/mol. The thermodynamics of oxygen binding have been determined under identical conditions for several marsupial species and in no case is the reaction more exothermic than that for platypus haemoglobin.

Smyth (1973) has shown that the platypus has poor thermoregulation outside the range 15–25°C and in water it may have fluctuations in body temperature of up to 6°C. A decrease in body temperature from 31 to 25°C would decrease the P_{50} value from 3·3 to 2·4 kPa, making delivery of oxygen to the tissues more difficult; this is possibly a factor in limiting diving excursions.

The removal of DPG reduced the P_{50} value of echidna haemoglobin at pH 7·1 and at 25°C from 1·6 to 0·4 kPa and for platypus haemoglobin it was reduced from 2·4 to 0·6 kPa. The binding of DPG therefore reduces the affinity of platypus haemoglobin more than that of echidna haemoglobin. The ΔH^0 values for echidna and platypus haemoglobins were smaller in the stripped condition than in the presence of DPG due to the absence of heat in the DPG interaction.

The amino acid sequence has been determined for the α- and β-chains of echidna Hb-IB (Whittaker *et al.* 1972, 1973) and platypus haemoglobin (Whittaker and Thompson 1974, 1975). These are the major haemoglobins present in the haemo-

lysates used in this study. The oxygen affinity of haemoglobin is related to the way in which the globin component affects the affinity of the haem iron for oxygen. Heterotropic allosteric modulators such as DPG and hydrogen ion interact with globin binding sites and the energetic constraints are transmitted through the molecule to proximal His F8 which in deoxyhaemoglobin displaces the haem iron slightly to the proximal side of the haem plane (Perutz 1976).

The monotreme haemoglobins are unusual in that they have a greater degree of amino acid variation in the α-chains than in the β-chains. The amino acid sequence studies show 16 amino acid differences between the α-chain of echidna Hb-IB and platypus haemoglobin and 14 amino acid differences between the β-chains. Goodman *et al.* (1975) have listed amino acid residues with defined functional roles in mammalian haemoglobin α- and β-chains. It might be expected that the marked differences in oxygen binding properties between echidna and platypus haemoglobins are due to amino acid substitutions at sites having known functional roles. However, the important $\alpha_1\beta_2$ contact sites, DPG binding sites, and residues involved in the Bohr effect are identical in platypus and echidna haemoglobin. Amino acid residues which are different in echidna and platypus haemoglobins and have a known function are shown in Table 3. The substitutions at the $\alpha_1\beta_1$ contacts and at the internal positions appear

Table 3. Significant amino acid differences in echidna and platypus haemoglobins
Sequence data are from Whittaker *et al.* (1972, 1973), and Whittaker and Thompson (1974). The functional significance is from Goodman *et al.* (1975)

Residue			Echidna Hb-IB	Platypus	Significance
α	1	NA1	Val	Met	αα contact, Bohr effect
α	34	B15	Leu	Gln	$\alpha_1\beta_1$ contact
α	35	B16	Ser	Ala	$\alpha_1\beta_1$ contact
α	46	CD4	Met	Phe	Haem contact internal position
α	55	E4	Val	Ile	Internal position
α	61	E10	Arg	Lys	Haem contact
α	105	G12	Phe	Ile	Internal position
β	44	CD3	Ser	Ala	Haem contact
β	123	H1	Thr	Ser	$\alpha_1\beta_1$ contact

to have sufficiently similar stereochemical properties that they are unlikely to have a significant effect. In the α-chains the substitutions CD4 Met → Phe and E10 Arg → Lys are involved in important haem contacts (Perutz 1976), and although the residues have similar chemical properties their steric differences, although minor, could well modify the relation between the haem and the CD and E helices. This may distort the αF8 His N_ε–Fe bond leading to a change in oxygen affinity as well as directly affecting the entry of oxygen to the haem which is sterically hindered by methyl γ_2 of E11 Val and N_ε of E7 His (Heidner *et al.* 1976).

In the β-chain the substitution at the haem contact CD3 Ser → Ala involves residues capable of forming salt links or H bonds with the haem propionate group. However, Perutz (1976) suggests that the β-chain haem propionate side chain is free to allow movement of the β-haem during oxygenation, and thus while the substitution is significant, the effects are difficult to assess.

The substitution at αNA1 Val → Met is unusual. αNA1 valine is responsible for 20% of the alkaline Bohr effect and this is related to the positioning of an inorganic anion (chloride) between the α-amino group of NA1 valine and the positively charged guanidinium of α141 arginine (Kilmartin 1976). During oxygenation the anion is apparently released as the oxygen affinity of the haemoglobin increases. Because selection appears to have conserved valine at this position the slight steric differences in the side chains at valine and methionine are apparently significant and may contribute to the greater sensitivity of platypus haemoglobin to hydrogen ions.

While it is difficult to evaluate the contributions of all the amino acid differences between echidna and platypus haemoglobins, it appears that differences in physiological properties can largely be accounted for by the differences at αNA1, αCD4, αE10 and βCD3. Closely related eutherian species usually have major amino acid variations in the haemoglobin β-chains but few variations in the α-chains (Dayhoff 1976). In contrast the monotremes have important variations in the haemoglobin α-chains which have been selected for and may account for the adaptation of the platypus and echidna to their specialized physiological and ecological requirements.

Acknowledgment

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