An investigation of the functioning of the two major haemoglobins of the *Sphenodon* using fast reaction kinetic methods

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The blood of the *Sphenodon* (*Sphenodon punctatus*) has been fractionated into two major and one minor haemoglobin components by ion-exchange chromatography. The two major haemoglobins have been studied in terms of their kinetic reactions with both O₂ and CO. The combination of flash photolysis and stopped-flow indicates kinetic differences between two states of the proteins identified with the allosteric T and R forms. The major kinetic findings show that (i) in these haemoglobins the T state is retained to a higher level of ligation than that commonly found in mammals, (ii) the rate of conversion from the R to the T state (k_0RT) is some three orders of magnitude lower in the *Sphenodon* haemoglobins than in mammalian systems. A comparison between the kinetic and equilibrium data for these haemoglobins indicates that the very weak cooperativity exhibited by these proteins arises from the close similarity of the affinities of the two allosteric states together with a very low value for the allosteric equilibrium constant, although very significant kinetic differences exist between the two states.

INTRODUCTION

The *Sphenodon* (Tuatara) is today found only on a few offshore islands of New Zealand. It is the sole remaining representative of the pre-dinosaur order of Rhynchocephalia reptiles and as such is not apparently significantly different to the reptilian fossils of the Mesozoic era (200 million years ago) [1]. The oxygen transport system of the *Sphenodon* has some features of note. The exceptionally large erythrocytes show an unusually low mean cell haemoglobin concentration, being significantly lower than in other reptiles [2–4]. The blood circulates at a temperature of 18–20 °C [5,6]. The large erythrocytes contain within them three tetrameric haemoglobins with two components each representing 40–45% of the total haemoglobin present [7]. Each of the stripped haemoglobin species shows very low Hill coefficients of ~1.1 and only a very small Bohr effect, which is associated with O₂ affinities of 133.3–200 Pa. In the presence of the natural intracellular allosteric effector, ATP, the O₂ affinity is reduced to approx. 1.07–4 kPa, whilst the degree of cooperativity remains very low [2,7].

The functioning of this allosteric system is thus of interest for a number of reasons. It represents one of the first land vertebrate organisms and exhibits an unusual allosteric pattern as outlined above. Despite the availability of equilibrium data as yet no significant kinetic measurements have been made on these haemoglobins. This study represents the first measurements of *Sphenodon* haemoglobin ligand-binding kinetics. Previously only a single observation on CO photolysis has appeared which indicated that unfractionated *Sphenodon* haemoglobins exhibit a unique correlation between the rate of geminate recombination and certain resonance Raman vibrational bands [8].

METHODS AND MATERIALS

Deep-frozen red blood cells were kindly provided by Dr. O'Dougherty, Victoria University, New Zealand. The red cells were lysed by the addition of 5 vol. of cold (4 °C) 0.05 M-Tris/HCl buffer at pH 8.5, which was saturated with CO. The resulting haemoglobin solution was separated from the nucleic acid gel produced on lysis by centrifugation at 2000 g for 10 min. The resulting haemoglobin solution was then centrifuged at 15000 g for 30 min to remove cell debris.

The CO-complexed haemoglobin solution was adsorbed on to a column of DEAE-Sephadex (2.5 cm × 20 cm) which was previously equilibrated with 0.05 M-Tris/HCl buffer at pH 8.5. The column was washed with 20 ml of the equilibration buffer and then with a gradient formed from mixing 150 ml of 0.05 M-Tris/HCl, pH 8.5, and 150 ml of 0.05 M-Bistris/HCl buffer at pH 5.5. All buffers used in these chromatographic steps were equilibrated with 101325 Pa CO gas. The flow rate was adjusted to ~10 ml/h and 2 ml fractions were collected. The elution profile was monitored at 541 nm.

The CO-complexed haemoglobin fractions were stored at –100 °C until required. When thawed they were passed through 3–5 cycles of evacuation and equilibration with O₂ in the presence of a strong light in order to convert the carbonmonoxy to the oxy complex. The deoxy form was prepared by 3–5 cycles of evacuation and equilibration with O₂-free nitrogen. In both cases the progress of the conversion was followed by absorption spectroscopy. For ligand-binding kinetics, gas-equilibrated buffer solutions were prepared by standard anaerobic syringe techniques. Flash photolysis and stopped-flow measurements were made using the

Fig. 1. Ion-exchange elution profile of Sphenodon haemoglobins

Separation of the haemoglobin components of whole haemolysate was achieved by using a 2.5 cm $\times$ 20 cm column of DEAE-Sephadex. The carbonmonoxy form of the haemoglobins were eluted by a gradient of 150 ml of 0.05 M-Tris/HCl buffer, pH 8.5, versus 150 ml of 0.05 M-Bistris/HCl buffer, pH 5.5 ($\square$). The elution was monitored at 541 nm ($\circ$).

apparatus previously described [9,10]. Data analysis and kinetic simulations using a fourth-order Runge-Kutta numerical integration procedure were carried out as previously reported [11,12] with the exception that in the present study a BBC microcomputer was employed to perform the appropriate calculations.

RESULTS AND DISCUSSION

The ion-exchange chromatography of the carbonmonoxy complexes of the Sphenodon haemoglobins on DEAE-Sephadex produced three haemoglobin peaks (Fig. 1). The initial small peak was not studied further. The two major peaks were collected and used in the kinetic studies presented below. For clarity the separated haemoglobins are referred to as T2 and T3, representing the order in which they were eluted. Over a number of preparations using pooled red blood cells from a number of individuals it was always found that the ratio of T1/T2/T3 was 5–10%:50–55%:40–45%. The chromatographic separation is in good accord with previous electrophoretic separation results for pooled blood reported earlier [7]. In most of the subsequent studies a common functionality was observed for haemoglobins T2 and T3 and hence the individual haemoglobins will only be referred to where necessary.

Flash photolysis at pH 7.5 of the CO-complexed haemoglobins produced recombination time courses consisting of two exponential processes (Fig. 2). This form of time course often arises from one of three common situations in an otherwise homogeneous haemoglobin system, (1) α/β -chain kinetic heterogeneity [13–16], (2) dimerization of haemoglobin tetramers [11,17,18], or else (3) the existence in solution of two differently reacting conformational forms of haemoglobin [19,20]. The origins of the form of the experimentally observed reaction curves in the present case were explored in a series of experiments using various conditions. An investigation of the wavelength-dependence of the contribution of each of the kinetic processes to the overall observed absorbance change (Fig. 3) indicated that, within experimental error, both

Fig. 2. Typical reaction trace of the recombination of Sphenodon haemoglobin with CO

A typical reaction trace is shown for the recombination of 3.3 μ M Sphenodon haemoglobin T2 with 1 mM-CO subsequent to photolysis. The experiment was carried out in 0.05 M-Tris/HCl buffer at pH 7.5 and 20 $^{\circ}$ C, and the reaction was monitored at 435 nm. The best-fit data ($\bullet$) to Scheme 1 is shown and was obtained using the reaction rates $k_1 = 1 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$, $k_2 = 600 \text{ s}^{-1}$ and $k_3 = 4 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$.

Fig. 3. Kinetic difference spectra for CO recombination with Sphenodon haemoglobin

The wavelength-dependence is shown for the total absorbance change ($\bullet$) and that due to the fast process ($\circ$) observed subsequent to photolysis of Sphenodon haemoglobin T2. For experimental conditions see Fig. 2.

processes had almost identical difference spectra, with each process contributing a constant proportion to the total absorbance change at all wavelengths, at a fixed CO concentration. This is not the case expected if α/β -chain heterogeneity is the source of the observed kinetic heterogeneity, as the α - and β -chain difference spectra are well resolved [13,14]. If the time courses arose from dimerization of the haemoglobin tetramers then the application of the equations of Antonini *et al.* [18]:

$$\frac{1-f}{f^2} = 2 K_{4,2} [\text{Hb}] \quad (1)$$

(where f is the fraction of fast process, $K_{4,2}$ is the dissociation constant and $[\text{Hb}]$ is the haemoglobin monomer concentration) to the above results would indicate a dimerization constant of approx. 2.5 μ M. In this case the proportion of fast phase, at a given CO concentration, could be expected to be sensitive to

Fig. 4. Ligand- and protein-concentration-dependence of the percentage of the fast process in CO recombination with Sphenodon haemoglobin

The observed ligand- (●) and protein- (□) concentration dependence of the proportion which the fast process contributes to the overall absorbance change observed in recombination with CO is shown together with that predicted by Scheme 1 using the parameters given in Fig. 2 (○).

changes in protein concentration in the μM range. When the haemoglobin concentration employed in photolysis experiments was varied from 0.3 to 22 μM , at a fixed CO concentration, the proportion of fast phase was found to be independent of protein concentration (Fig. 4), thus ruling out the possibility of dimerization as the source of the two kinetic processes. For the third possibility the reaction could be represented in the form originally presented by Gibson [19]:

Although this model can in principle be extended to encompass the allosteric transitions of both liganded and unliganded forms [13,25,26], the absence of accurate values for L and c (see below) in this system precludes its extension in this case.

If this is the case then the ligand-concentration dependence of the two processes observed in Fig. 2 should relate to the processes k_1 and k_3 . Fig. 5 shows the CO-concentration-dependence of the two processes. It was found that this model could be fitted to the observed time courses, using the appropriate rate expressions, in a fourth order Runge-Kutta simulation [12]. Such a fit yielded the points shown in Fig. 2 with a best fit value of $k_2 = 600 \text{ s}^{-1}$. However the fitting of a single time course is not sufficient to verify the operation of the model. The model also predicts that the fraction of fast phase should be a function of the CO concentration employed. This dependence was modelled and compared

Fig. 5. Concentration-dependence of the rates of reaction of Sphenodon haemoglobin with CO

The concentration-dependence of the rate of the fast process (□) and slow process (○) observed subsequent to flash photolysis is shown together with that observed by stopped-flow techniques (◆).

Fig. 6. pH-dependence of the proportion of the fast process observed subsequent to flash photolysis of the CO complexes of Sphenodon haemoglobins

The pH-dependence of the proportion of the total absorbance change contributed by the fast process subsequent to flash photolysis is shown for Sphenodon haemoglobins T2 (○) and T3 (□). The CO concentration used was fixed at 500 μM .

with that observed experimentally (Fig. 4). These data together with the kinetic spectra are taken as proof of the operation of a reaction mechanism such as that shown in Scheme 1 in the case of Sphenodon haemoglobin reaction with CO subsequent to photolysis of the bound CO. In the presence of IHP (inositol hexakisphosphate) the recombination time courses showed the same pattern as above except that the fraction contributed by the fast process, at a fixed CO concentration, was drastically reduced, typically being reduced from ~ 80 to $\sim 20\%$ at 1 mM-CO. It should be noted, however, that in the presence of IHP the ligand-concentration dependence of the two kinetic processes were not altered from their values in the absence of IHP.

The nature of the two kinetic processes was further investigated as a function of pH in the region of pH 5–9. At a fixed CO concentration it was found that the

Table 1. A comparison of ligand binding rates

All rates are $\text{M}^{-1} \cdot \text{s}^{-1}$ unless otherwise stated.

Ligand	Rate	Rate ($\text{M} \cdot \text{s}^{-1}$)		
		Sphenodon		Mammalian
		T2	T3	
CO	k_{on}			
	Fast	1×10^6	1.2×10^6	$6.9 \times 10^6 \ddagger$
	Slow	4×10^4	4×10^4	$4.5 \times 10^5 \ddagger$
	k_2	$600 \text{ s}^{-1}*$	600 s^{-1}	$> 10^5 \S$
CO + IHP	k_{on}			
	Fast	7×10^5	1.2×10^6	—
	Slow	2.5×10^4	5×10^4	—
O_2	k_{on}			
	Fast	7×10^6	8.2×10^6	$2 \times 10^7 \ddagger$
	Slow	2×10^6	1.3×10^6	$5 \times 10^6 \ddagger$
	k_2	$250 \text{ s}^{-1} \ddagger$	—	$> 7 \times 10^3 \ddagger$
O_2 + IHP	k_{on}			
	Fast	8.2×10^6	8.5×10^6	—
	Slow	1.3×10^6	1.1×10^6	—
O_2	k_{off}	14.5 s^{-1}	29 s^{-1}	$13.7 (22) \P$

* Represents $k_0 RT$; $\ddagger$ $k_2 RT$; $\ddagger$ from [24]; $\S$ from [27]; $\parallel$ from value of k_0 [27] using ligation-dependence of [25]; $\P$ from [16].

proportion of the fast process was sensitive to pH, following essentially a titration curve with an apparent pK of ~ 6.8 for T3 and ~ 7.5 for T2 (Fig. 6). Once again, although changes in proportions of the two phases were observed as a function of pH, the rates of the two processes were not pH-dependent.

On the basis of the above experimental findings it would appear that the Sphenodon haemoglobin-CO complexes, when exposed to rapid photolysis, yield as an initial product Hb* which then decays to the normal Hb species with a rate of $\sim 600 \text{ s}^{-1}$. This process is accompanied by recombination of both haemoglobin species with CO (see Table 1). This type of reactivity has been observed in other haemoglobin systems. As long ago as 1959, Gibson [19,20] reported a similar form of reactivity in sheep haemoglobin, although the pattern was observed only at high pH (9.1) and low temperature (1°C) with an associated value of $k_2 = 200 \text{ s}^{-1}$. Gibson suggested that, within the formalism of the two-state model of cooperativity [21], the species Hb* could be identified with the highly reactive R state. This proposal has since been verified [22,23] and Gibson's original results have been explained as arising from a non-equilibrium distribution of species maintained by an R to T conversion rate which is slow at 1°C and pH 9.1, as compared with the ligand-combination rates [24]. In more recent work employing dye-laser photolysis of human haemoglobin at 20°C and pH 8–9 the rate of conversion of the original photolysis product R state to T state has been measured at 6400 s^{-1} [13]. At pH 7 and 20°C the rate is $> 10^5 \text{ s}^{-1}$ [25–27].

Hence the outstanding difference between the Sphenodon and mammalian system lies in the much lower value of k_2 (expressed as $k_0 RT$ within the two-state formalism) in the former system. The pH-sensitivity of the proportion of the fast process follows a similar

Fig. 7. Typical reaction trace of the recombination of Sphenodon haemoglobins with O_2

A typical reaction trace is shown for the recombination of $9.8 \mu\text{M}$ Sphenodon haemoglobin T2 with $72.5 \mu\text{M}$ O_2 at pH 7.5. The reaction was monitored at 437 nm and the best-fit data ($\bullet$) to Scheme 1 are shown obtained using the reaction rates $k_1 = 7 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$, $k_2 = 250 \text{ s}^{-1}$ and $k_3 = 2 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$.

pattern to that seen in mammalian systems but in the Sphenodon the pH-sensitivity of k_2 ($k_0 RT$) is much lower, such that even at pH 5 the rate is still low enough to allow the recombination of significant amounts of R state (Hb*) before its conversion to the resting T state can occur. The effect of the addition of IHP on the rate of R to T conversion has also been seen in human haemoglobin, with the addition of the allosteric effector significantly increasing the value of k_2 ($k_0 RT$) and hence inducing an increase in the amount of slow-reaction T state observed at any particular CO concentration [25].

The oxy complexes of the Sphenodon haemoglobins exhibit a similar qualitative pattern of recombination kinetics to those seen for the CO complexes (Fig. 7). However, as a consequence of the low power available (200 J) and the low quantum yield of the oxy complexes, as compared with the CO complexes, only $\sim 12\%$ photolysis could be achieved. Under these conditions it is easy to show that the only significant recombining species produced by the flash is $\text{Hb}(\text{O}_2)_3$ [24]. Subsequent recombination with photolysed products yielded biphasic time courses with associated rate constants at $7 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $2 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ and a predominance of the fast process at $300 \mu\text{M}$ O_2 ($\sim 95\%$). The addition of IHP in saturating amounts (5 mM) led to a reduction in the proportion of the fast process ($\sim 75\%$ at $300 \mu\text{M}$ O_2) but produced a smaller effect than that seen in the case of CO recombination kinetics. The qualitative differences seen between O_2 and CO kinetics undoubtedly arise from only partial photolysis being achieved in the former case. Thus, whereas recombination with CO occurs to unliganded haemoglobin, recombination with O_2 occurs with $\text{Hb}(\text{O}_2)_3$ [24]. The biphasic character of the O_2 -recombination time courses, with a predominance of the fast process, might arise in two ways. It is possible that the allosteric equilibrium position for $\text{Hb}(\text{O}_2)_3$ contains a measurable percentage of the T-state form which is responsible for the slow process if allosteric equilibrium is achieved before recombination. Or it is possible that the allosteric equilibrium position for $\text{Hb}(\text{O}_2)_3$ represents a considerable population of the T state but the equilibrium position is not achieved experimentally due to the slow R to T transition at the

Fig. 8. Ligand-concentration-dependence of the rates and percentages of the fast process in O_2 recombination with Sphenodon haemoglobins

The ligand-concentration-dependence is shown for the rates of the fast ($\square$) and slow ($\blacksquare$) processes together with the observed concentration-dependence of the proportion of the fast process present ($\bullet$). The concentration dependence of the proportion of the fast process predicted by Scheme 1 is shown for comparison ($\circ$).

level of triple ligation, i.e. a low value of k_3RT , together with very rapid recombination rates with O_2 . As the switch-over point from the T to R state is not known for this system the former possibility cannot be ruled out. However, further analysis of the experimental data would favour the later proposition. The best fit to the O_2 kinetic data (Fig. 8) requires a value of $k_2 = 250 \text{ s}^{-1}$. This is a significant reduction from the value of 600 s^{-1} obtained for k_0RT and is in qualitative agreement with a reduction in the R to T conversion rate at increasing levels of ligation reported previously [25], although the ligation-dependence in the Sphenodon is clearly less than the 2.4^n dependence observed for human haemoglobin [25]. In line with normal allosteric theory the presence of IHP leads to an increased equilibrium concentration of T-state species at all ligation levels. More importantly, in the present study, it appears that IHP exerts this effect by increasing the rate of R to T transition as previously alluded to [25,26] and so leads kinetically to an increase in the slow process as the R to T transition becomes more competitive with the rate of R-state recombination with O_2 .

Some evidence for a late switch-over point for the allosteric equilibrium is indicated by the fact that stopped-flow combination of T2 and T3 with CO yields non-accelerating time courses (Fig. 9) which show identical rate constants for CO combination with those observed for the slower process observed in photolytic studies (Fig. 5). If an allosteric switch from predominantly T to predominantly R state occurred at approx. two ligands bound, as in mammalian haemoglobins, then accelerating time courses would be expected.

The amalgamation of these kinetic data with the previously published equilibrium data on Sphenodon haemoglobin gives a most revealing insight into the mechanism of action of these ancient haemoglobins.

Fig. 9. Time courses for CO combination with Sphenodon haemoglobin observed in the stopped-flow apparatus

Semi-logarithmic plots are shown for the time courses obtained when Sphenodon haemoglobin T2 ($\bullet$) at $3.8 \mu\text{M}$ reacted with $50 \mu\text{M}$ -CO and haemoglobin T3 ($\circ$) at $3.0 \mu\text{M}$ reacted with $250 \mu\text{M}$ -CO in the stopped-flow apparatus at 20°C . The upper time scale relates to the reaction of T2 and the lower to T3.

Sphenodon haemoglobins show very low co-operativity ($h \sim 1.1$, [2]) associated with binding curves of $P_{50} = 133.3\text{--}200 \text{ Pa}$. It has been clearly shown [28,29] that essentially non-cooperative binding curves can arise in a two-state system when either L approaches unity or L approaches $1/c^4$, for a fixed value of c (where L is the allosteric equilibrium constant representing the ratio of the concentration of T to R states in the absence of ligand and c is the ratio of the affinities of T and R states).

In the case where L approaches unity it has been shown that the observed p_{50} value is essentially identical with K_R and where L approaches $1/c^4$ the p_{50} value approaches K_T [28]. For the Sphenodon haemoglobins it has been found that the stripped proteins give essentially non-cooperative curves with $p_{50} = 200 \text{ Pa}$ and that addition of saturating amounts of organic polyphosphates produces non-cooperative curves with $p_{50} \sim 2 \text{ kPa}$. A value of K_R can also be obtained independently from the kinetic measurements presented in Table 1. The value of K_R should be equal to $k_{\text{off}}/k_{\text{on}}$, which from Table 1 is $\sim 2 \mu\text{M}$ for T2 and $\sim 3.5 \mu\text{M}$ for T3. These values represent pO_2 values of $133.3\text{--}266.6 \text{ Pa}$ in good agreement with the values obtained from the equilibrium binding curves. It therefore appears that the non-cooperative functioning of Sphenodon haemoglobin arises from the existence of the situation where L approaches unity.

This analysis can then be taken one step further. The kinetic data clearly show that the species $\text{Hb}(O_2)_3$ has a significant T-state contribution, as indicated by a measurable contribution from the slow process. Now,

the allosteric equilibrium constant governing the species $\text{Hb}(\text{O}_2)_3$ is given simply by $L \cdot c^3$. Thus if $L \cdot c^3$ is not far removed from unity and L is close to unity, so must be c . That is, the intrinsic affinities of the T and R states, are very similar. Indeed, if K_R is $\sim 2 \mu\text{M}$ as outlined above and as a first approximation it is assumed that the p_{50} value obtained from non-co-operative binding curves in the presence of saturating amounts of organic polyphosphates represents K_T then c would have a value of ~ 0.1 in this case.

The above findings thus indicate that these primitive haemoglobins exhibit their unusual equilibrium O_2 -binding properties as a consequence of the close similarity of their R- and T-state affinities coupled to a very low L value. Nevertheless, it is also clear that the R and T states exhibit quite distinct rates of reaction with ligands and the close similarity of the affinities of the T and R states is a consequence of the similarity of the ratios of their association and dissociation rate constants rather than the absolute values of these kinetic parameters.

The question remains as to why the *Sphenodon*, which apparently has the capability to produce an allosteric system, exhibits essentially non-co-operative oxygen binding. The answer may lie in the distinction between homotrophic and heterotrophic allosteric interactions. The blood of this animal is clearly non-co-operative in terms of its homotrophic O_2 binding but nevertheless it is also clear that its O_2 affinity is sensitive to the presence of heterotrophic allosteric effectors. It is possible that the very low metabolic demand of this animal can be met by a non-cooperative O_2 -binding system whilst the heterotrophic effects provide the capacity for adaptation to changing environmental conditions via modulation of haemoglobin O_2 affinity controlled by changing levels of intra-erythrocytic organic polyphosphates.

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