Ligand binding kinetics and dissociation of the human embryonic haemoglobins

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The three human embryonic haemoglobins have been studied using a range of stopped-flow and flash photolysis experiments. The association and dissociation kinetics and equilibrium constants for the tetramer–dimer reactions of the deoxy and oxygenated forms have been investigated and found to be characterized by constants similar to those of the human adult protein. The rates of oxygen dissociation from the embryonic haemoglobins have been measured and appear to be responsible for the

high oxygen-binding affinity associated with the embryonic proteins compared with the adult protein. The pH dependence of the oxygen dissociation rate constants also accounts for the rather unusual, previously described, Bohr effects characteristic of the embryonic haemoglobins. A general scheme has been developed coupling both the dimer–tetramer equilibria and ligand-binding steps observed following photolysis of the liganded forms of the human embryonic haemoglobins.

INTRODUCTION

The embryonic stage of human development which occurs between weeks 1 and 12 of gestation is characterized as the phase during which the circulation is first established. Thus during this period the embryo must transform from an organism using purely diffusional transport of oxygen to its tissues to one employing a fully functional circulatory system optimized to scavenge oxygen from the mother via the placenta. This major morphological shift is associated, at the molecular level, with the sequential synthesis of three distinct haemoglobin (Hb) molecules, namely Gower I ($\zeta_2\epsilon_2$), Gower II ($\alpha_2\epsilon_2$) and Portland ($\zeta_2\gamma_2$).

Although the human embryonic haemoglobins were first described many years ago [1] until recently only very limited investigations of their functioning had been published. However, with the advent of recombinant techniques for the expression of human haemoglobins it has recently become possible to study these important oxygen transport proteins in detail. We have described the basic characteristics of the process of oxygen binding to the three embryonic haemoglobins and the modulation of this capacity by protons, organic phosphates and chloride ions [2–4]. The embryonic haemoglobins show very distinct Bohr effects and differential binding of organic phosphates. But in particular we have been able to identify that it is their response towards chloride ions in solution which is the major controlling factor governing the transport of oxygen from the interstitial fluid to the respiring tissues of the early embryo. All of these previous studies have employed equilibrium methods. In this study we report our survey of the kinetic characteristics of the human embryonic haemoglobins which we have undertaken in order to identify which of the elementary kinetic steps, if any, govern the overall equilibrium properties previously reported.

MATERIALS AND METHODS

The three human embryonic haemoglobins Gower I ($\zeta_2\epsilon_2$), Gower II ($\alpha_2\epsilon_2$) and Portland ($\zeta_2\gamma_2$) were expressed in yeast expression

systems, isolated and purified as described previously [3]. Adult haemoglobin was prepared as described by Antonini and Brunori [5].

Flash photolysis and stopped-flow kinetic experiments were performed as described earlier [6–8]. Ligand-binding experiments were performed under pseudo-first-order conditions with ligand in large excess. Time courses obtained in these experiments were fitted to single- or double-exponential functions using non-linear least squares procedures employing a commercially available program (Table Curve 2D; Jandel Scientific, San Rafael, CA, U.S.A.). The relative quality of the fit of the data to one or two exponentials was estimated by comparison of the sums of the squares of the residuals; no account was explicitly taken of the different degrees of freedom associated with the two expressions.

The rate of the transition from deoxy tetramers to deoxy dimers ($^4\rightarrow^2k_R$) was determined by measuring the rate of complex formation with haptoglobin [9–14]. In this reaction, in which only deoxy dimers react essentially irreversibly with the haptoglobin ($K = 10^{15} \text{ M}^{-1}$ [15]), the rate can be obtained from the time course of the absorbance change at 430 nm. The binding of haptoglobin to dimeric haemoglobin is associated with a decrease of 17% in the absorbance at 430 nm [14]. In this set of experiments deoxygenated haemoglobin in buffer at pH 9.0 was reduced by the addition of sodium dithionite and anaerobically transferred to a stoppered spectrophotometer cuvette. A small volume of concentrated deoxygenated haptoglobin solution, in the same buffer, was then injected anaerobically into the haemoglobin solution to give a final reaction mixture containing 11 μM haemoglobin, 20 μM haptoglobin and 1 mg/ml sodium dithionite. The subsequent reaction was monitored at 430 nm. Reaction rates were determined by fitting the resulting time courses to a single-exponential process.

The rate of the transition from deoxy dimers to deoxy tetramers ($^2\rightarrow^4k_R$) was determined by measuring the second-order rate constant for the ‘drift’ phase observed at 430 nm following the rapid mixing in the stopped-flow apparatus of a sample of oxygenated haemoglobin with an excess of sodium dithionite

Abbreviations used: Hb, haemoglobin; k_T and k_R , ligand-binding rate constants for the T and R states respectively; $^4k_{R-T}$, rate constant for the R to T transition; $^2\rightarrow^4k_R$, dimer association rate constant; $^4\rightarrow^2k_R$, tetramer dissociation rate constant; $K_{4,2}$, tetramer–dimer equilibrium constant; k_{onO_2} , oxygen-binding rate constant.

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[10,16,17]. The concentration of deoxygenated dimers produced immediately after deoxygenation of the sample was taken to be half the concentration of oxygenated dimers present in the oxygenated sample prior to mixing. This correction is necessary as the stopped-flow experiment involves the mixing of equal volumes of each of the reacting solutions. The concentration of the oxygenated dimers was determined using the value of the haemoglobin tetramer–dimer equilibrium constant ($K_{4,2}$) and the total haemoglobin concentration employed.

The oxygen dissociation rate as a function of pH was determined by rapidly mixing an oxygenated sample of the haemoglobins at 5 μ M with a deoxygenated sample of buffer at the same pH containing approx. 2 mg/ml of sodium dithionite and following the subsequent reaction at 437.5 nm [18]. The time courses were analysed using the procedures outlined above.

The oxygen association rate constant was determined by partial photolysis of an oxygenated sample of the haemoglobin at low oxygen concentration. The reassociation reaction was followed at 437.5 nm. It should be noted that using the apparatus available to us (Applied Photophysics, London, U.K.), which delivers approx. 200 J in 10 μ s, only approx. 5% photolysis of the haemoglobin–oxygen complex is achieved. The percentage photolysis is expressed as the fraction of the absorbance change observed between the oxygenated and deoxygenated haemoglobin obtained from static spectra. The low level of photolysis achieved for the oxygenated haemoglobins probably arises from a relatively low quantum yield for the process, as is normally the case for haemoglobins [5]; however, we have not explicitly determined the quantum yield for these systems. Thus the rate determined in these experiments is equivalent to the rate of binding of the fourth molecule of oxygen to the R state of the protein.

The carbon monoxide association rate constants were determined by monitoring the absorbance change associated with the recombination of carbon monoxide with the haemoglobin following photolysis. The reaction was monitored at 423 nm in all cases except for the recording of the kinetic difference spectra. Typically 3 μ M haemoglobin was used and the sample housed in a 1-cm-pathlength cylindrical cell. In the case of the carbon monoxide reaction studies full photolysis of the sample is easily achieved using the apparatus described above. In experiments designed to produce partial photolysis the sample was partially shielded from the flash tubes. In these cases the degree of photolysis is expressed as the percentage of the absorbance change associated with full photolysis of the same sample.

RESULTS

CO photolysis at high pH

When each of the ferrous carbon monoxide derivatives of the three human embryonic haemoglobins were subjected to rapid photolysis at pH 9.0 the recombination reaction produced a time course at 423 nm consisting of a fast and a slow exponential component (Figure 1; Table 1). By observing the recombination at different wavelengths it was possible to obtain a kinetic difference spectrum for the two component processes (Figure 2). At any of the wavelengths studied it was found that although the relative absorbance contribution from each process differed from that at any other wavelength the rate constant associated with each component was wavelength-independent. It was also found that at any particular wavelength the relative proportion of the absorbance change associated with each component was concentration-independent in the range 0.3–30 μ M protein. Identical properties were observed for each of the embryonic haemo-

Figure 1 Recombination reaction of human embryonic haemoglobin with CO

The time course is shown for the recombination of 3 μ M haemoglobin $\zeta_2\epsilon_2$ with 100 μ M CO at 20 °C in 100 mM Tris/HCl buffer at pH 9.0 (○) and 100 mM BisTris/HCl buffer at pH 6.5 (●) observed at 435 nm. The best fit double-exponential function is shown for both conditions (solid lines).

globins when they were subjected to partial photolysis in the range 10–50% photolysis.

CO photolysis at low pH

When the three human embryonic haemoglobins were subjected to photolysis at pH 6.5 the recombination reaction with carbon monoxide also showed the presence of two exponential processes. The faster process occurred at a rate very similar to that seen at high pH values while the slower process occurred at a much lower rate than any process observed at high pH (Figure 1, Table 1). Recombination following partial photolysis was associated with the production of only the faster-reacting species and the difference spectrum associated with each of the two processes observed at low pH values showed, within experimental error, the same form. It should be noted that at low pH values it proved impossible to convincingly decompose the faster process into the sum of two exponential processes as was possible in the high pH experiments.

Deoxyhaemoglobin dimerization

When each of the human embryonic haemoglobins were mixed with a small excess of haptoglobin in the presence of sodium dithionite the resulting complex formation produced a change in the Soret spectrum characterized by a decrease in the absorbance at 430 nm of 17% (Figure 3). The complex formation process followed a single-exponential time course at 430 nm (Figure 4). The rate constants obtained from the time courses were very similar in each case and are listed in Table 2.

Oxygen dissociation rate

When the oxygenated haemoglobins were rapidly mixed with an excess of sodium dithionite and the reaction monitored at 437.5 nm the time course followed a single-exponential process

Table 1 Rate constants for the binding of CO to human embryonic haemoglobins

Protein	$10^{-6} \times \text{Rate at pH 9.0}^a \text{ (M}^{-1} \cdot \text{s}^{-1}\text{)}$	$10^{-6} \times \text{Rate at pH 6.5}^b \text{ (M}^{-1} \cdot \text{s}^{-1}\text{)}$	$K_{4,2}^c \text{ (M}^{-1}\text{)}$
$\alpha_2\beta_2$	7.0	4.0	2.0
	2.9	0.2	
$\alpha_2\epsilon_2$	6.0	2.0	2.5
	1.0	0.25	
$\zeta_2\epsilon_2$	6.5	3.0	2.0
	1.4	0.2	
$\zeta_2\gamma_2$	5.0	3.5	3.4
	1.0	0.5	

^a The higher rate corresponds to reaction with the β chain.

^b The higher rate corresponds to binding to the R state, and the lower rate to binding to the T state.

^c $K_{4,2}$ was calculated from the % of fast component seen when $3 \mu\text{M}$ haemoglobin reacts at pH 6.5.

Figure 2 Kinetic difference spectra for the reaction of human embryonic haemoglobin with CO at high pH values

The kinetic difference spectra are shown for the fast (■) and slow (□) kinetic components observed for the reaction shown in Figure 1.

Figure 3 Complex formation between haemoglobin and haptoglobin

The Soret spectra are shown for $7 \mu\text{M}$ deoxygenated haemoglobin in the absence (broken line) and presence (solid line) of a small excess of haptoglobin at pH 9.0 in 100 mM Tris/HCl buffer.

Figure 4 Time courses for the reaction of deoxyhaemoglobin with haptoglobin

The time courses are shown for the reaction of deoxygenated haemoglobins $\alpha_2\beta_2$ (○); $\alpha_2\epsilon_2$ (△); $\zeta_2\epsilon_2$ (●) and $\zeta_2\gamma_2$ (▲) with haptoglobin observed at 430 nm at 20 °C in 100 mM Tris/HCl buffer at pH 9.0. A is the absorbance at any time, A_0 is the initial absorbance and A_f is the final absorbance at 430 nm. The concentration of haemoglobin and haptoglobin in the reaction mixtures was $11 \mu\text{M}$ and $20 \mu\text{M}$ respectively.

(Figure 5). When this reaction was performed at different pH values in the range pH 5–9 the rate for both the adult and embryonic haemoglobins was found to be pH-dependent. However, the degree of sensitivity of the rate of this reaction towards pH was considerably different for each of the proteins (Figure 6).

Association of deoxy dimers

When each of the oxygenated haemoglobins were rapidly mixed with a deoxygenated solution containing an excess of sodium dithionite and the reaction was monitored at 430 nm the time course showed the presence of two processes occurring on very different time scales (Figure 7). At this wavelength the rapid component consists of the rate process observed at 437.5 nm. The slow component corresponds to the association of deoxygenated dimers to form deoxygenated tetramers [16,17]. By measuring the rate of this process it is thus possible to determine the rate of association of deoxygenated dimers.

Oxygen binding rate

Partial photolysis (5%) of the oxygenated forms of the human embryonic haemoglobins leads to recombination reactions charac-

Table 2 Dimerization rates and oxygen binding rates at pH 9.0

$^{4,2}k_R$ was determined from the reaction of deoxyhaemoglobin with haptoglobin at pH 9.0. $^{2,4}k_R$ was determined at pH 7.4 from the drift phase observed at 430 nm. $^{\circ}K_{4,2}$ was determined at pH 9.0 assuming pH independence of $^{2,4}k_R$ as previously described for the adult protein [10]. $k_{on}O_2$ was determined at pH 7.4 by partial photolysis (the presence of two values indicates the rates determined for the binding to each chain; a single value indicates the presence of degenerate rate constants).

Protein	$10^{-3} \times ^{4,2}k_R$ (s ⁻¹)	$10^{-4} \times ^{2,4}k_R$ (M ⁻¹ · s ⁻¹)	$^{\circ}K_{4,2}$ (M ⁻¹)	$10^{-7} \times k_{on}O_2$ (M ⁻¹ · s ⁻¹)
$\alpha_2\beta_2$	1.3	19.5	15	7.7
$\alpha_2\epsilon_2$	1.5	18.4	12	11
$\zeta_2\epsilon_2$	1.9	14.5	7.6	8.8; 2
$\zeta_2\gamma_2$	1.1	2.2	2.0	8.4; 2.3

Figure 5 Time course for the dissociation of oxygen from haemoglobin

The time course is shown for the reaction of 2 mg/ml sodium dithionite with 5 μ M haemoglobin $\zeta_2\gamma_2$ observed at 437.5 nm in 100 mM Bis/Tris buffer at pH 8.7 at 20 °C.

Figure 6 pH dependence of the oxygen dissociation rate

The pH dependencies of the oxygen dissociation rate for the haemoglobins $\alpha_2\beta_2$ ($\square$), $\alpha_2\epsilon_2$ ($\bullet$), $\zeta_2\gamma_2$ ($\triangle$) and $\zeta_2\epsilon_2$ ($\circ$) are shown. The rates were measured under the conditions given in Figure 5 except that Tris buffers were used between pH 5 and 7 and Bis/Tris buffers between pH 8 and 9.

terized by single-exponential recombination time courses when observed at 437.5 nm in the case of the adult protein and embryonic haemoglobin Gower II ($\alpha_2\epsilon_2$). In the case of the

Figure 7 The time course for the association of deoxyhaemoglobin dimers

The time course is shown for the reaction of 5 μ M haemoglobin $\zeta_2\gamma_2$ with 2 mg/ml sodium dithionite observed at 430 nm in 100 mM Bis/Tris buffer at pH 7.4 and 20 °C.

human embryonic haemoglobins Gower I ($\zeta_2\epsilon_2$) and Portland ($\zeta_2\gamma_2$) the recombination reaction consisted of the sum of two exponential processes each of which contribute equally to the total overall absorbance change observed at 437.5 nm. The resulting rates of reaction of oxygen with the tri-ligated R-state forms of each of the proteins are given in Table 2.

DISCUSSION

CO reaction kinetics

As both the adult and embryonic haemoglobins exhibited qualitatively the same form of reactivity, in their recombination reactions following photolysis of the carbon monoxide derivative, the single Scheme 1 can be employed to rationalize the reactions observed under different experimental conditions:

Scheme 1

At high pH values and relatively low protein concentrations the resting system of molecules represented in Scheme 1 would be expected to be almost exclusively populated by the tetrameric and dimeric R-state carbon monoxide-bound species, namely ${}^4\text{Hb}(\text{CO})_4$ and ${}^2\text{Hb}_R(\text{CO})_2$. This situation is to be expected for the adult protein based on the previously derived values of the allosteric equilibrium constant for the fully ligated form [19] and a value of the tetramer–dimer equilibrium constant ($K_{4,2}$) of 2 μ M [20]. Also under the high-pH conditions employed in this study the value of the rate constant for the R to T transition (${}^4k_{R-T}$) can be estimated from the data of Gibson and colleagues [21,22] as approx. 300 s⁻¹. Thus assuming that the common form

of reactivity shown by the embryonic proteins arises from a commonality of mechanism, photolysis at pH 9.0 should initially form the deoxygenated tetrameric and dimeric species, $^4\text{Hb}_R$ and $^2\text{Hb}_R$. At the high concentrations of CO employed in these studies the rate of recombination with CO is approx. 1000 s^{-1} while the competing rate of conversion from the R-state deoxy tetramer ($^4\text{Hb}_R$) into the T-state deoxy tetramer ($^4\text{Hb}_T$) is estimated to be only approx. 300 s^{-1} . The assignment of the measured rates of recombination to the R state might thus at first appear uncertain. However, at the low protein concentrations used in these studies more than half of the haemoglobin is present as dimers which cannot undergo the R to T transition in the time scale of the measurements. However, most convincingly, the finding that the reaction rates determined at high pH on full photolysis are identical to those obtained at only 10 % photolysis (a condition under which the haemoglobin can confidently be assigned to the R state) clearly indicates that any R to T conversion which occurs during the recombination process does not contribute significantly to the measured rate constants. Thus we assign the measured recombination rates to the R form of the proteins. Under these conditions all the proteins studied in this work exhibit two kinetic processes. The kinetic difference spectra associated with these processes (Figure 2) are typical of those previously reported for reaction of CO with α - and β -type globin chains, with the β -type chains exhibiting the higher reactivity. At high pH values no protein concentration dependence was observed in the recombination reaction as a shift in protein concentration simply alters the initial ratio of the R-state tetrameric and dimeric forms both of which exhibit R-state reactivity [23] and are thus indistinguishable in these experiments. Partial photolysis of approx. 10 % yields predominantly $^4\text{Hb}_R(\text{CO})_3$ and $^2\text{Hb}_R(\text{CO})$ as the photo products [24,25] and recombination is indistinguishable in character from that observed for full photolysis.

At low pH values the main change in the kinetic values previously reported for the reactivity of the adult protein is an increase in the value of $^4k_{R-T}$. Under the conditions employed in this study the value of this rate constant is expected to approach 6500 s^{-1} [22]. Thus at low pH photolysis initially yields the same species as those produced at high pH, namely $^4\text{Hb}_R$ and $^2\text{Hb}_R$. However, at low pH the deoxy tetrameric R-state form $^4\text{Hb}_R$ is now effectively converted into the deoxy tetrameric form $^4\text{Hb}_T$ before it can recombine with CO. As the rate of interconversion between the deoxy R-state forms is very slow ($^2\text{--}^4k_R$ seconds and $^4\text{--}^2k_R$ many minutes ([10,17], see also below)) the recombination process now consists of the R-state rate of binding of CO to $^2\text{Hb}_R$ and the T-state rate of binding to $^4\text{Hb}_T$ (it should be noted that in this situation our failure to convincingly deconvolute the R-state recombination reaction into the α and β components probably reflects the ill-conditioned problem associated with the fitting of three exponential processes to a single time course [26]). Furthermore under these conditions the relative contributions to the total absorbance made by the recombination at the T-state rate and the R-state rate should give a measure of the relative proportions of the R-state tetramer and dimer initially present in the carbon monoxide derivatives. Using these data we can obtain values for the constant $K_{4,2}$ for each haemoglobin (Table 2). The value obtained for the adult protein using this analysis is quite similar to the value previously reported in the literature [20] and we take this as support for the validity for our general scheme of reactivity.

According to this scheme partial photolysis (approx. 10 %) would yield $^2\text{Hb}_R(\text{CO})_1$ and $^4\text{Hb}_R(\text{CO})_3$ [24,25]. The equilibrium constant for the R–T transition of the triply ligated form would be expected to overwhelmingly favour the R form [22] and

furthermore the rate of conversion of the small amount of $^4\text{Hb}_R(\text{CO})_3$ into $^4\text{Hb}_T(\text{CO})_3$ would be predicted to occur at a rate of only one-thousandth of that of the deoxy form [27]. Thus recombination would occur at the R-state rate only, as is observed.

Dissociation reactions

The rate of dimerization of deoxyhaemoglobin tetramers at pH 9.0 is remarkably similar for each haemoglobin and the adult protein in our hands yielded a rate of dimerization very similar to that reported previously at high pH [10]. It is also interesting to note that although a single point mutation in the adult protein can alter the dissociation equilibrium constant by a factor of 10^6 [28] the embryonic rate of dimerization of deoxyhaemoglobin tetramers at pH 9.0 is remarkably similar for each of the embryonic and adult proteins [10] despite the fact that the ζ -globin, γ -globin and ϵ -globin proteins share only a 57 %, 73 % and 75 % sequence similarity with the adult globins respectively [29–31].

Oxygen association rates

As shown in Table 2 the rates of oxygen association for adult haemoglobin and human embryonic haemoglobin Gower II ($\alpha_2\epsilon_2$) are very similar to those previously reported for the adult protein [27]. However, embryonic haemoglobins Gower I ($\zeta_2\epsilon_2$) and Portland ($\zeta_2\gamma_2$) exhibit two recombination rates one of which is similar to that of the adult protein and one significantly slower. If we make the assumption that in these two embryonic haemoglobins these two rates originate from chain equivalence then by analogy with previous analysis of the adult protein [23,27] we can assign the lower rate to the ζ chain.

General

The reactivity of the embryonic haemoglobins towards ligands and their tetramer–dimer equilibria show a remarkable similarity to the adult protein particularly considering their very different amino-acid compositions and sequences. The major distinction between the adult and embryonic haemoglobins highlighted in this study is their kinetics of ligand dissociation. Although at this stage we have not made any measurements of ligand dissociation from the T states of the embryonic proteins, it would appear from the pH-dependence of the oxygen dissociation rates, taken together with the similarity of the oxygen association rates, that both the high oxygen affinities and unusual Bohr effects characteristic of the human embryonic haemoglobins have their origins in the rate of oxygen dissociation from the proteins.

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