A study of the kinetics of the reaction of ligands with the liganded states of mouse embryonic haemoglobins

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The reactivities of the liganded states of the embryonic haemoglobins of the mouse with both O₂ and CO were measured and compared with the reactivities of the adult protein. Laser-photolysis experiments on the recombination of O₂ with the partially oxygenated proteins indicates chain heterogeneity in the adult and embryonic EII and EIII species, with the difference in subunit reactivity being greatest in the embryonic species. Haemoglobin EI shows chain equivalence in these experiments. The homogeneous time courses observed for the O₂-dissociation reactions are consistent with chain equivalence within all the proteins with regard to this reaction. The specific values obtained for the respective rate constants from each of these studies indicates that the high O₂ affinity previously reported for haemoglobin EI is, in greatest part, due to its low O₂ dissociation rate. Flash-photolysis studies on the binding of CO with the partially liganded forms of the proteins show the same patterns of chain heterogeneity as seen in O₂-binding studies.

INTRODUCTION

The kinetics of the reaction of haemoglobin with various ligands have been a major field of study since the early pioneering work of Roughton (1934) and Gibson & Roughton (1955). Over the years a vast range of measurements have been made on both normal and mutant haemoglobins (Antonini & Brunori, 1971; Parkhurst, 1979) from a wide range of species. Even though such a wide range of experiments have been reported, kinetics measurements have been confined almost exclusively to the adult proteins.

The adult human protein, haemoglobin A, has been extensively characterized in terms of both its kinetic and equilibrium properties. Equilibrium properties have been measured for the human foetal protein, but no systematic study of the embryonic species has appeared. We have used the mouse embryonic system as a model for mammalian haemoglobin development. The mouse has been shown to produce three embryonic haemoglobins analogous in structure to the human proteins and also to show somewhat similar developmental patterns in terms of the synthesis of these three proteins (Purdie *et al.*, 1983; Brittain & Wells, 1983). Each of the embryonic haemoglobins has been isolated and purified and its equilibrium O₂-binding properties have been measured. These experiments have shown that each of the embryonic haemoglobins exhibits very high O₂ affinity, presumably as a necessary requirement for the scavenging of O₂ before the establishment of full placental function. An unexpected finding was the lack of any co-operative function in the mouse embryonic haemoglobin EI (Purdie *et al.*, 1983). These findings prompted us to undertake an examination of the kinetic properties of this embryonic system in order to probe further the functioning of this set of proteins and in particular to investigate any anomalous kinetic behaviour associated with the functioning of embryonic haemoglobin EI.

MATERIALS AND METHODS

Mice of the strain C57 BL/6J were time-mated by using the methods of Whitten (1958). This procedure involves presenting the females to a male for a period of only 12 h and so allows the gestational age of the embryos to be more precisely established. Pregnant females were killed and the embryos dissected directly into cold saline solution (0.9% NaCl). In the present study about 500 embryos of 13 days' gestation were used. The pooled embryonic blood was separated into its constituent components by ion-exchange chromatography as previously described (Purdie *et al.*, 1983).

Stopped-flow measurements were performed with the apparatus described by Brittain (1980). The replacement reactions used to determine the rates of O₂ dissociation from the liganded state were analysed according to the procedures of Andersen *et al.* (1973) and Olson *et al.* (1971). Flash-photolysis studies on the CO complex of the haemoglobins were performed with the apparatus described by Brittain (1984). Photolysis studies on the oxygenated complexes of the haemoglobins were performed by using a xenon-flash-lamp-pumped dye laser (Electro Photonics, Belfast, N. Ireland, U.K.). The light pulse produced by the laser had a duration of 1.3 μ s with an associated spectral bandwidth of 6 nm. The dye employed in the laser was Coumarin 153 at a concentration of 100 μ M in spectroscopic-grade methanol (Lambda Physics, Acton, MA, U.S.A.) tuned to the lasing maximum of 541 nm. The samples were housed in a 2 mm-pathlength cell.

Time courses for each of the reactions were subjected to both single-exponential and double-exponential non-linear least-squares-regression analysis. The quality of the fit to each type of time course was determined initially from the residual sum-of-the-squares of the deviations. Systematic deviations in the distribution of the residuals were quantified by using the procedures of Swed &

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Fig. 1. Time courses for the reaction of O_2 with partially oxygenated haemoglobins

The semi-logarithmic plots are shown for the reaction of adult mouse haemoglobin (○) and embryonic haemoglobins EI (△), EII (□) and EIII (●) with $300 \mu M$ O_2 after 10% photolysis of $40 \mu M$ protein in 50 mM-Tris/HCl buffer, pH 8.2. The curves for EII (□) and EIII (●) have been offset for clarity.

Eisenhart (1943). Where the residual sum-of-the-squares of the deviations for the single-exponential and double-exponential models were not sufficiently different so as to allow a clear choice to be made between these models, the mean sum-of-squares for each model was calculated. The ratio of these mean sum-of-squares for the two possible models was then used to decide on the most appropriate model, by employing the criteria suggested by Mannervik (1982).

RESULTS

Rate of O_2 binding to the liganded haemoglobins

The rate of O_2 binding to the liganded haemoglobins was measured by performing partial-photolysis experiments over a range of O_2 concentrations. When oxyhaemoglobin is partially photolysed (less than 10%) it is expected that the observed recombination reaction will be essentially that due to O_2 binding with $Hb(O_2)_3$ (where Hb represents haemoglobin) or in terms of the two-state model of co-operativity to the R-state haemoglobin. By using laser photolysis to provide a

Fig. 2. Kinetic difference spectra of the biphasic reaction traces observed in O_2 -recombination experiments with haemoglobins

The optical absorbance changes associated with the fast (◆) and slow (◇) phases observed in the reaction of adult mouse and embryonic haemoglobins EII and EIII with O_2 subsequent to partial photolysis are shown. Experimental conditions were as indicated in Fig. 1 legend.

Fig. 3. Time courses for the replacement of O_2 by CO for haemoglobins

Typical semi-logarithmic plots of the replacement of O_2 by CO for adult (○) and embryonic haemoglobins EI (△), EII (□) and EIII (●) are shown. The reactions were performed by rapidly mixing $20 \mu M$ protein, oxygenated with 1.38 mM O_2 , with 1 mM CO in 50 mM-Tris/HCl buffer, pH 8.2.

short-duration high-intensity light pulse the reaction of each haemoglobin with O_2 was monitored over the O_2 concentration range 60 – $300 \mu M$. The nature of the time course observed fell into two classes (Fig. 1). Embryonic haemoglobin EI showed monophasic reaction curves with semi-logarithmic plots that were accurately linear for at least 98% of the total excursion at all wavelengths investigated. Haemoglobins A, EII and EIII exhibited biphasic reaction curves in which the optical absorbance change associated with each of the phases was

wavelength-dependent. Fig. 2 shows the kinetic difference spectra measured from the wavelength-dependence of the optical absorbance contributions of the two phases.

Rate of O₂ dissociation from the liganded haemoglobins

The rate of O₂ dissociation from the liganded haemoglobins was determined by the stopped-flow replacement method. On rapidly mixing oxygenated haemoglobin, at various O₂ concentrations, with a high concentration of CO time courses of the replacement of O₂ by CO were determined. In the case of all the haemoglobins studied monophasic time courses were observed that were accurately mono-exponential for over 95% of the excursions (Fig. 3). In accordance with the analysis given by Andersen *et al.* (1973) for the kinetics of the replacement of O₂ by CO, on an otherwise fully liganded haemoglobin, under the conditions employed the rate of replacement observed ($k_{\text{obs.}}$) is related to the ligand concentrations as follows:

$$k_{\text{obs.}} = k_4[\text{Hb}] \left(\frac{l'_4[\text{CO}]}{l_4[\text{CO}] + k'_4[\text{O}_2]} \right)$$

where k_4 is the rate of oxygen dissociation from the fully liganded haemoglobin and l'_4 and k'_4 are the rates of CO and O₂ binding to the triply liganded species. Thus a plot of $1/k_{\text{obs.}}$ versus the O₂ concentration employed, at a fixed CO concentration, yields a straight line with an intercept of $1/k_4$ (Fig. 4).

Rate of CO binding to the liganded haemoglobins

The rate of CO binding to the liganded form of the haemoglobins was determined under conditions similar to those used for the measurement of O₂ binding.

Fig. 4. Dependence of replacement reaction rate constant for haemoglobins on O₂ concentration

The reciprocal of the rate of replacement of O₂ from oxygenated adult haemoglobin (○) and embryonic haemoglobins EI (△), EII (□) and EIII (●) by CO is shown. Experimental conditions were as indicated in Fig. 3 legend with the exception that O₂ concentration was varied.

Fig. 5. Time courses for the recombination of CO with partially liganded haemoglobins

Time courses are shown for the reaction of 45 μM-haemoglobins [EI (△), EII (□), EIII (●) and adult (○)] with 94 μM-CO in 50 mM-Tris/HCl buffer, pH 8.2, after 6% photolysis of the CO complex. The trace for EI (△) has been offset for clarity.

However, in CO-binding studies the high quantum yield for the photolysis process, together with the relatively low rate of ligand binding, allowed the use of a conventional flash-lamp photolysis apparatus. The recombination of the liganded haemoglobins with CO after partial photolysis (approx. 6%) showed the same characteristics as seen in the O₂-binding studies. Once again the haemoglobins A, EII and EIII exhibited markedly biphasic reaction traces in which the proportion of each phase was wavelength-dependent. Haemoglobin EI showed wavelength-independent monophasic recombination time courses (Fig. 5).

The values of the various kinetic constants for each type of haemoglobin are summarized in Table 1.

DISCUSSION

The binding of O₂ and CO to the partially liganded haemoglobins clearly shows the presence of two distinct kinetic phases for the reactions involving binding to the adult haemoglobin and embryonic haemoglobins EII and EIII. Although chain heterogeneity has not been observed in kinetic studies of the reaction of liganded human haemoglobin with O₂ (McCray, 1972; Sawicki & Gibson, 1977), chain heterogeneity has been reported for O₂ binding to the deoxy protein, with the larger spectral contribution at longer wavelengths being assigned to the β-chains (Sawicki & Gibson, 1977). The same spectral distribution has also been reported for the reaction of

Table 1. Rate constants for ligand reactions with mouse haemoglobins

$k_{\text{off}}(\text{O}_2)$ represents the measured dissociation rate for oxygen; $k_{\text{on}}(\text{O}_2)$ and $k_{\text{on}}(\text{CO})$ represent the measured rate of binding of the ligands O_2 and CO to the partially dissociated liganded haemoglobin.

Haemoglobin	$k_{\text{off}}(\text{O}_2)$ (s^{-1})	$k_{\text{on}}(\text{O}_2)$ ($\mu\text{M}^{-1} \cdot \text{s}^{-1}$)	$k_{\text{on}}(\text{CO})$ ($\mu\text{M}^{-1} \cdot \text{s}^{-1}$)
Adult	20.6	23.6, 57	0.4, 1.4
Embryonic EI	3.5	13	0.96
Embryonic EII	11.8	18, 60	0.7, 3.0
Embryonic EIII	14.5	12, 61	0.7, 2.6

human haemoglobin valence hybrids with CO (Cassoly & Gibson, 1972) and in the replacement reaction of O_2 by CO in human haemoglobin (Olson *et al.*, 1971). In other species chain heterogeneity in ligand binding has also been interpreted in terms of the β -chains making the larger contribution at longer wavelengths (Brittain *et al.*, 1982; Brittain, 1985). If this assignment is assumed to be applicable to the heterogeneous O_2 and CO binding to the embryonic haemoglobins, then clearly the rapid reaction phase is associated with the α -chains. This is in contrast with the reportedly higher reactivity of separated human β -chains (Noble *et al.*, 1969), but is in accord with the higher reactivity of the α -chains seen in shark haemoglobin (Brittain *et al.*, 1982).

The replacement method for the measurement of O_2 dissociation rates was chosen so as to avoid possible artifacts, which are often observed when rapid mixing with dithionite is used to measure this parameter (Dalziel & O'Brien, 1957). The dithionite method requires that the dissociation of the first molecule of O_2 from the fully liganded species is rate-limiting on subsequent dissociation, or else complex time courses are observed. In the case of the replacement method the molecule is always present in the fully liganded state and hence complications arising from deoxygenation of partially liganded species are eliminated. Under these conditions the adult haemoglobin and the embryonic species exhibited monophasic reaction traces. In the light of the reported heterogeneity in other mammalian haemoglobins for this reaction (Olson *et al.*, 1971), the results reported in the present study were subjected to rigorous analysis in an attempt to identify the presence of any heterogeneity. Firstly, the data were fitted to a single exponential and the distribution of the residuals was tested for randomness according to the procedures of Swed & Eisenhart (1943). The probability that the residual distribution was purely random in nature was > 0.99 in all cases. The residual sum-of-the-squares from a non-linear least-squares fitting to two exponentials was found to be insignificantly smaller than that of a one-exponential fit, by using the criteria of Mannervik (1982). Furthermore the best-fit double exponential was typically to a ratio of 10% fast to 90% slow at 427 nm, where any chain heterogeneity would be expected to lead to a best fit with 50% fast and 50% slow. On the basis of these analyses we consider the replacement data are most appropriately considered as arising from the reaction of a homogeneous system that does not show chain differences.

When analysed with the same rigour as outlined above, the kinetic data obtained for both the ligand-binding reactions and the replacement reactions exhibited by the embryonic haemoglobin EI all showed monophasic time courses.

The existence of heterogeneity in the ligand-recombination reactions of the partially liganded forms of the mouse adult haemoglobin and the embryonic haemoglobins EII and EIII is common to the reactions of many haemoglobins. Many lower vertebrate species are characterized by heterogeneity in their reactions with ligands in the partially liganded states (Morris & Gibson, 1982; Dickinson & Gibson, 1981; Brittain *et al.*, 1982; Brittain, 1985). Adult human haemoglobin, however, is reported to show heterogeneity only in the O_2 -dissociation reactions (Sawicki & Gibson, 1977; Olson *et al.*, 1971).

It may be noted that the value of the rate constant for the binding of O_2 to human haemoglobin, reported as $59 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$, is in very close agreement with the faster phase seen in O_2 binding to adult haemoglobin and embryonic haemoglobins EII and EIII. With regard to the comparison of the O_2 -dissociation rates the situation is completely reversed. Human haemoglobin, in common with other mammalian haemoglobins, shows chain heterogeneity in ligand-replacement reactions, although the differences in reported rates is as little as 40% (Olson *et al.*, 1971). In adult mouse haemoglobin replacement reactions yield a rate of O_2 dissociation identical with that of the human β -chain, whereas the haemoglobins EII and EIII show rates very similar to that seen for human α -chains (Sawicki & Gibson, 1977).

In the case of haemoglobin EI the rates of ligand reaction do not correlate with any values obtained for the adult human protein. It may be noted that the high O_2 affinity observed for haemoglobin EI (Purdie *et al.*, 1983) arises mainly from the low O_2 -dissociation rate and matches quite closely the value calculated for the high-affinity chains of adult mouse haemoglobin and embryonic haemoglobins EII and EIII.

In conclusion it is clear that both the adult and embryonic haemoglobins of the C57 BL/6J mouse show distinctive reactivity with ligands. The previously reported sensitivity of the embryonic components EII and EIII to pH and organic phosphates (Purdie *et al.*, 1983) undoubtedly modifies the reactivity pattern referred to above. The lack of heterogeneity seen with haemoglobin EI, under all the conditions explored, correlates well with the non-co-operative equilibrium data previously reported and must reflect a lack of kinetic distinction between the χ - and ϵ -chains present in this protein (Melderis *et al.*, 1974).

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