A two-state analysis of co-operative oxygen binding in the three human embryonic haemoglobins

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The binding of oxygen to the three human embryonic haemoglobins, at pH 7.4, has been shown to occur as a co-operative process. Analysis of oxygen-binding curves obtained in the absence of organic phosphate allosteric effectors shows that the process can be described quite accurately by the two-state model of allosteric action. In the presence of organic phosphates, the binding affinity for oxygen to the T-state of the $\alpha_2\epsilon_2$ and $\zeta_2\epsilon_2$ haemoglobins is significantly lowered. The values of the best-fit two-state parameters determined for each of the embryonic haemoglobins together with the temperature-dependence of the overall equilibrium binding process are discussed in terms of oxygen transfer from the maternal blood supply. Fast-reaction studies have been used to determine the rate constants of the oxygen association and dissociation processes occurring in the R-state and the rate of the allosteric R > T conformational transition. Analysis of these data suggests a likely reason for the high affinity and low co-operativity of the embryonic proteins and identifies the origins of the inability of equilibrium measurements to identify chain non-equivalence in the R-state.

INTRODUCTION

At the earliest stages of human development between weeks 4 and 14 of gestation, before the development of a fully functional placenta, the human embryo synthesizes a series of three distinct embryonic haemoglobins $\zeta_2\epsilon_2$ (Gower I), $\zeta_2\gamma_2$ (Portland) and $\alpha_2\epsilon_2$ (Gower II) [1,2]. These embryonic haemoglobins combine reversibly with oxygen [3] and respond to the presence of various allosteric effectors [4]. We have over the past few years developed yeast expression systems which have allowed us to produce reasonably large quantities of each of the haemoglobins in pure form [4,5]. Preliminary oxygen-binding studies have involved measurements of the effects of various substances on the overall equilibrium character of the oxygen binding of each haemoglobin. These findings have largely been interpreted in terms of the two-state or concerted model of co-operativity originally formulated by Monod et al. [6]. This two-state model of co-operativity has at its core the axiom that only a single R-state and T-state exist, and co-operative functioning and allosteric modulation occur simply by a switching between different population distributions of the R- and T-states. Over the years many studies on the equilibrium and kinetic properties of adult haemoglobin have been analysed within this formalism with varying degrees of consistency [7–9]. Most often when inconsistency has been identified between experiment and theory, an extension to the two-state scheme allowing for chain inequivalence has proven appropriate [10]. However, under certain circumstances it has been shown that the model requires further expansion to allow for the existence of various T-states to accommodate the experimental data [11]. As yet no direct data have been presented to validate the application of this model (in any of its forms) in the case of the human embryonic haemoglobins.

In this study we have endeavoured to obtain data that will resolve this problem. We report here the first extended oxygen-binding equilibrium measurements to be obtained for the human embryonic haemoglobins and their analysis in terms of the two-state model. As a complement to these equilibrium studies, we also report a set of kinetic investigations of some of the elementary steps formulated to occur within the concerted model of co-operativity.

These data are discussed in terms of the possible allosteric functioning of this set of embryonic haemoglobins, within the context of oxygen transport from the maternal to embryonic blood supply.

MATERIALS AND METHODS

The three human embryonic haemoglobins were separately produced in yeast employing plasmid-based expression systems [3]. The proteins were isolated and subsequently purified using previously published protocols [4].

Oxygen-binding equilibrium curves were measured at 25 °C, in the presence of the Met-haemoglobin reducing system of Hayashi et al. [12], using an oxygen-diffusion chamber modified for stepwise increases in oxygen concentration [13,14]. The chamber of the oxygen-diffusion apparatus was coupled to cascaded Wosthoff (type M201 a–f) gas-metering pumps which allow equilibration of the sample with various concentrations of oxygen. Gas mixtures were produced by mixing $> 99.998\,\%$ nitrogen, oxygen and atmospheric air in various proportions.

Oxygen-binding data were evaluated in terms of the Hill coefficient (h) and the p_{50} (the partial pressure of oxygen giving half saturation) from linear plots of $\log[Y/(1-Y)]$ versus $\log(pO_2)$ for fractional saturation levels (Y) between 0.3 and 0.7. Diphosphoglycerate (DPG) was added to protein samples in the form of small aliquots of a concentrated pH-equilibrated solution

Abbreviation used: DPG, diphosphoglycerate.

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of the ammonium salt of 2,3-diphosphoglycerate. Cl^- was added to all samples to a level of 100 mM as KCl. Cl^- concentrations were assayed using a CMT10 coulometric titrator (Radiometer, Copenhagen, Denmark).

Two-state analysis

Binding curves obtained over a wide range of oxygen concentrations were analysed in terms of the two-state model of co-operativity [6] by fitting the data to the equation:

$$Y = [LP/K_T(1+P/K_T)^3 + P/K_R(1+P/K_R)^3]/ [L(1+P/K_T)^4 + (1+P/K_R)^4] \quad (1)$$

where Y is the fractional saturation with oxygen at oxygen tension P, K_R and K_T are the equilibrium constants for oxygen binding to the R- and T-state forms of the haemoglobin and L is the allosteric equilibrium constant expressing the relative stability of the R- and T-states of the haemoglobin in the absence of oxygen. The data were fitted using proprietary non-linear least-squares fitting routines (Tablecurve; Jandel Scientific).

Derived parameters such as the free energy of haem–haem interaction, median oxygen tension (P_m) and maximal Hill coefficient (h_{max}) were determined from the best-fit two-state parameters using standard equations [15].

Rapid-mixing measurements were made using an Applied Photophysics Bio-Sequential DX.17MV stopped-flow apparatus using a 1 cm path-length cell. Flash photolysis experiments employed an Applied Photophysics laser photolysis system. This system employs a 6 ns laser pulse of 100 mJ energy produced from a Nd:YAG laser which has been frequency-doubled to produce light at 532 nm.

Reaction traces were analysed in terms of either single or double exponentials by non-linear least-squares fitting of the data to the appropriate equations.

R-state oxygen-dissociation rates were determined by rapidly mixing the oxygenated form of the haemoglobin with an excess of sodium dithionite and observing the reaction trace at 436 nm [16]. R-state association rates were determined from the reaction traces obtained on recombination of the deoxygenated form of the protein with oxygen subsequent to partial photolysis (2 %) of the oxygenated complex by the 6 ns laser pulse. These traces were measured at 436 nm where the R to T transition is isosbestic [17].

The rate of the R > T quaternary transition was measured at 425 nm after full photolysis of the carbonmonoxy form of the embryonic haemoglobins, in the presence of 100 μM CO. At this wavelength the ligand-rebinding reactions are isosbestic [17]. In the case of photolysis experiments, 20 reaction traces were measured and averaged to provide a better signal-to-noise ratio.

RESULTS

Equilibrium oxygen binding

Oxygen binds to the three human embryonic haemoglobins in a co-operative process. Haemoglobin $\zeta_2\epsilon_2$ shows a distinctly higher oxygen affinity ($p_{50} = 1.9$ mmHg) than haemoglobins $\alpha_2\epsilon_2$ and $\zeta_2\gamma_2$ ($p_{50} = 3.2$ and 2.8 mmHg respectively at pH 7.2). The oxygen affinity of the haemoglobins is also affected by pH in the range 7.0–7.5, with haemoglobin $\zeta_2\gamma_2$ showing a small Bohr effect [dlog(p_{50})/pH] of -0.15, and haemoglobins $\alpha_2\epsilon_2$ and $\zeta_2\epsilon_2$ showing larger effects with Bohr coefficients of -0.59 and -0.42 respectively. In the absence of organic phosphate allosteric effectors, the process of oxygen binding fits, to a good approximation, the two-state or concerted model of co-operativity [6]. By obtaining data at both high and low extremes of saturation (Figure 1) it is possible to evaluate the three main allosteric parameters associated with the two-state model, namely L, K_R and K_T. Fitting this model to the data yields the parameter values

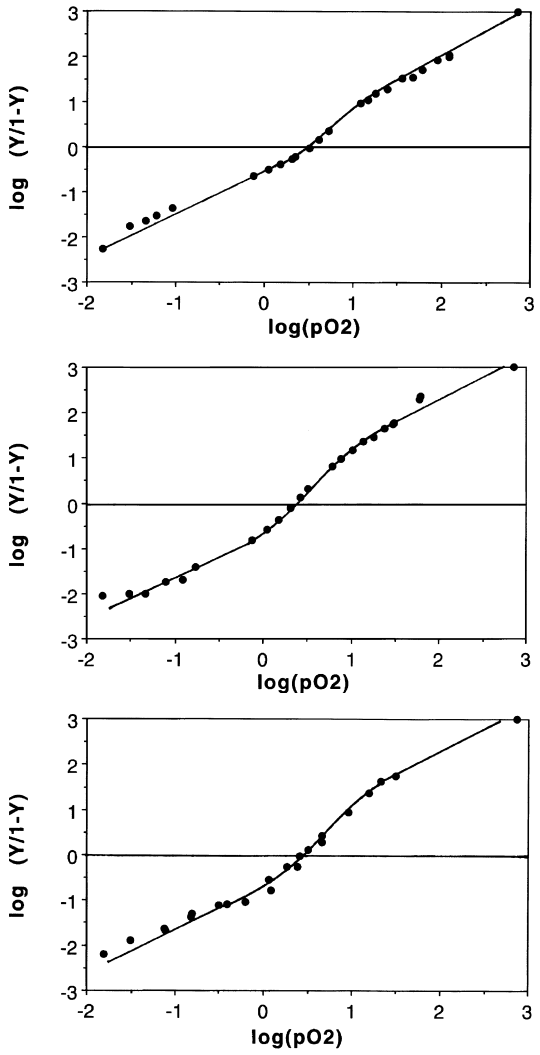

Figure 1 Oxygen-binding curves for human embryonic haemoglobins

The oxygen-binding data for human embryonic haemoglobins $\zeta_2\gamma_2$, $\alpha_2\epsilon_2$ and $\zeta_2\epsilon_2$ are shown (top to bottom). Binding curves were obtained in 0.1 M Hepes at pH 7.2 and 25 °C in the presence of 100 mM KCl. The haemoglobin concentration was 0.25 mM in all cases.

Table 1 Some two-state equilibrium properties of human embryonic haemoglobins

Where applicable, results are means ± S.E.M.

	p_{50} (mmHg)	h	K_T (μM)	K_R (μM)	L
$\alpha_2\epsilon_2$	2.7	1.9	10.3 ± 3	0.46 ± 0.04	2.0e4 ± 1e4
+ DPG	4.8	1.8	29	0.44	3.8e5
$\zeta_2\gamma_2$	2.5	1.4	7.9 ± 2	0.31 ± 0.04	6.3e4 ± 2e4
+ DPG	2.6	1.5	8.3	0.35	6.9e4
$\zeta_2\epsilon_2$	2.4	1.9	11.4 ± 3	0.53 ± 0.04	1.7e3 ± 1e3
+ DPG	4.5	2.0	23	0.6	3.4e3

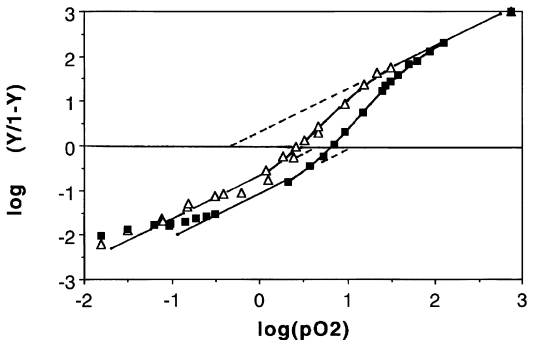

Figure 2 Effect of DPG on the oxygen-binding curves of human embryonic haemoglobin

The extended Hill plots for $\alpha_2\epsilon_2$ in the absence (Δ) and presence (■) of 2.5 mM DPG are shown. Other conditions were as in Figure 1. The broken lines show extrapolations of values fitted for K_R and K_T (upper and lower limbs respectively).

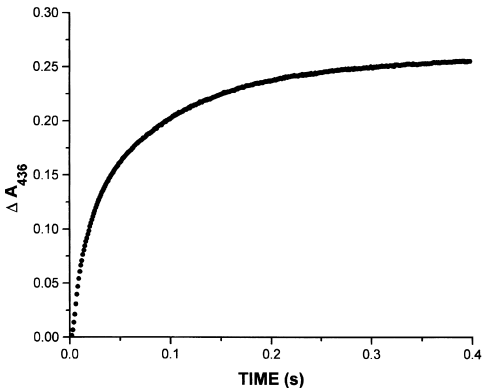

Figure 3 Oxygen dissociation from oxyhaemoglobin

The change in A_{436} is shown after rapid mixing of 10 μM human haemoglobin $\zeta_2\epsilon_2$ with an excess of sodium dithionite. The reaction occurred at 25 °C in 100 mM Hepes buffer containing 100 mM NaCl at pH 7.4.

presented in Table 1. In the absence of organic phosphates, P_m, calculated using eqn. (2)

$$P_m = (1/K_R)[Lc^4 + 1)]^{1/4} \quad (2)$$

(where $c = K_R/K_T$) was found consistently to be slightly higher than the p_{50} values, and the h_{max} to be slightly higher than the h_{50} values (Table 1). h_{max} was evaluated by solving for pO_2 in:

$$d^2\{\log[Y/(1-Y)]\}/d(\log pO_2)^2 = 0 \quad (3)$$

then calculating $d\{\log[Y/(1-Y)]\}/d(\log pO_2)$ at the value of pO_2 derived from eqn. (3).

The free energy of haem–haem interaction in each of the haemoglobins was also evaluated from:

$$\Delta G^0 = -2.303\ \boldsymbol{R}T\log(K_T/K_R) \quad (4)$$

The allosteric parameters yielded values of 7.7, 7.9 and 7.6 kJ/mol for haemoglobins $\alpha_2\epsilon_2$, $\xi_2\epsilon_2$ and $\xi_2\gamma_2$ respectively.

When oxygen-building equilibrium measurements were repeated in the presence of saturating concentrations of DPG [4], the binding curves were shifted to higher oxygen concentrations in the case of haemoglobins $\alpha_2\epsilon_2$ and $\zeta_2\epsilon_2$ (Figure 2) but were unaffected in the case of haemoglobin $\zeta_2\gamma_2$. Moreover it was found that the binding curves did not shift symmetrically. In the presence of saturating quantities of DPG, the value of K_R was essentially unchanged from that seen in the absence of DPG. In contrast, the lower limb of the binding curve, which reflects the value of K_T, is significantly shifted in the case of haemoglobins $\alpha_2\epsilon_2$ and $\zeta_2\epsilon_2$ (Figure 2 and Table 1).

Furthermore a closer examination of the lower limb of the binding curves of the Hill plots obtained in the presence of DPG shows that the slope of the Hill plot below a saturation of 3% may well be below 1.0.

Effect of temperature

Equilibrium oxygen-binding curves were also obtained in the absence of organic phosphate allosteric effectors over a range of temperatures at pH 7.4. Although it was not possible to obtain high-accuracy binding curves under all the conditions employed, it was possible to obtain accurate values for the parameter p_{50} over the temperature range 10–37 °C. This parameter and its temperature-dependence are useful, as they allow us to ascertain the effect of temperature on the overall process of oxygenation.

Table 2 Some kinetic properties of human embryonic haemoglobins

The data for $\alpha_2\beta_2$ are taken from ref. [12]

	O_2 k_R off (s^{-1})	O_2 k_R on ($\mu M^{-1}\cdot s^{-1}$)	$10^4 \times R > T$ (s^{-1})
$\alpha_2\epsilon_2$	49, 11	94, 28	2.5
$\zeta_2\gamma_2$	54, 11	92, 38	2.6
$\zeta_2\epsilon_2$	70, 11	94, 24	2.9
$\alpha_2\beta_2$	40, 12	140, 42	1.9

The data obtained fit a standard van't Hoff isochor dependence on temperature:

$$\Delta H = -4.574[(T1T2)/(T1{-}T2)]\,\Delta\log p_{50}/1000 \quad (5)$$

From this plot we obtain values of the overall enthalpies of oxygenation, corrected for the heat of solution of oxygen, of −43, −51 and −42 kJ/mol for the embryonic haemoglobins $\alpha_2\epsilon_2$, $\zeta_2\gamma_2$ and $\zeta_2\epsilon_2$ respectively.

Kinetic studies

The rate of oxygen dissociation from the R-state forms of the embryonic haemoglobins was obtained by rapidly mixing the fully oxygenated form of the protein with an excess of sodium dithionite in the stopped-flow apparatus. The reaction, when followed at 436 nm, proceeded as the sum of two exponential processes for each embryonic haemoglobin (Figure 3). The rates of each of these processes were found to be independent of oxygen concentration in the range 50–270 μM and dithionite concentration in the range 1 mg/ml to 10 mg/ml. The first-order rate constants are presented in Table 2.

The rates of oxygen association with each of the embryonic haemoglobins in the R-state were measured subsequent to partial photolysis (2%) of the oxygenated forms of the protein after initiation of the reaction by a 6 ns actinic laser pulse. The recombination reaction of oxygen with the triliganded R-state form of the protein was monitored at 436 nm. Analysis of the recombination time courses showed the presence of the sum of two exponential processes, with each process contributing equally

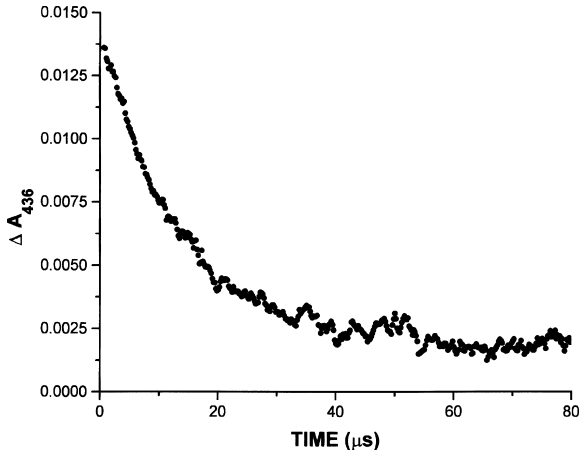

Figure 4 Oxygen association with embryonic haemoglobin

The change in A_{436} is shown for the recombination of 50 μM human embryonic haemoglobin $\zeta_2\gamma_2$ with oxygen in the presence of 1.25 mM O_2 after 2% photolysis of the oxygenated species produced by a 6 ns laser flash. Solution conditions were as in Figure 3.

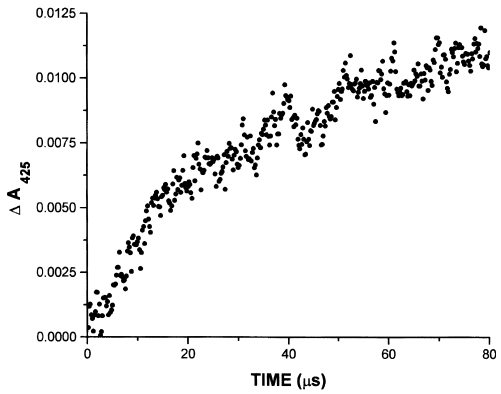

Figure 5 The R to T transition in embryonic haemoglobin

The time course for the change in A_{425} after full photolysis of a 50 μM sample of the carbonmonoxy form of human embryonic haemoglobin $\zeta_e\epsilon_2$ is shown. The reaction occurred in the presence of 100 μM CO in solution as described in Figure 3.

to the overall A_{436} change (Figure 4). The rate of each of these reactions was found to be dependent on oxygen in the range 300 μM to 1.25 mM. The second-order rate constants associated with each of the three human embryonic haemoglobins are presented in Table 2.

Full photolysis of the carbonmonoxy form of the embryonic haemoglobins in the presence of relatively low concentrations of CO leads to the initiation of two separate reactions. The deoxygenated form produced by photolysis, which is initially in the R-state, undergoes a very rapid quaternary change to the T-state (Table 2) which occurs in the microsecond time range and is accompanied by changes in A_{425} (Figure 5). The unliganded haemoglobin also recombines with CO in a process that is isosbestic at 425 nm but that exhibits a large change in A_{436}. By comparison with the R < T transition, the rate of recombination with CO is very slow, having a half-time of milliseconds in the presence of 100 μM CO [18].

DISCUSSION

In the absence of organic phosphates all the embryonic haemoglobins exhibited co-operative oxygen-binding curves, but with Hill coefficients lower than the value of 2.8 seen for the adult protein. At physiological pH, $\alpha_2\epsilon_2$ and $\zeta_2\gamma_2$ show very similar oxygen affinities, whereas $\zeta_2\epsilon_2$ shows a somewhat higher affinity. Non-linear least-squares fitting of the binding data to eqn. (1) shows that the two-state model of co-operativity is a reasonable model for the binding of oxygen to all the embryonic haemoglobins under these conditions. Within this formalism we see that the embryonic haemoglobins exhibit values for K_R and K_T similar to those previously reported for the adult protein of 0.3 and 10.7 μM for the R- and T-state respectively [19]. Significant for the higher affinity of $\zeta_2\epsilon_2$ is the finding that the relative stability of the unliganded T- and R-states, as expressed by the allosteric constant L, is an order of magnitude lower in $\zeta_2\epsilon_2$ than in $\zeta_2\gamma_2$. It should also be noted that the T-state in the case of all the embryonic haemoglobins is considerably less stable than in the adult case where $L = 1.2 \times 10^5$ under similar conditions [17]. With regards to the origins of the relative instability of the T-state, we can make some observations based on the amino acid sequences of the four proteins, even in the absence of three-dimensional structures of the embryonic haemoglobins. In the α-chain, the N-terminus of Val^1 stabilizes the T-state conformation by making a significant contribution to a Cl-bridged hydrogen-bonded network involving Argα^{141}, Valβ^{34}, Aspα^{126} and Valα^1 [20]. In the ζ-containing embryonic haemoglobins, the Valα^1 NH_2 is replaced by an N-terminally acetylated Ser, and thus the stabilizing contribution of the hydrogen-bonded network will be reduced. A naturally occurring high-affinity adult mutant, Hb Dunn, has been characterized in which Aspα^6 is replaced by Asn. In the ζ-chain, the Aspα^6 is replaced by a Gln residue and can thus, by analogy, be expected to contribute to the higher affinity of these proteins, although its exact role in stabilizing the T-state has not yet been identified. A comparison of the γ- and ϵ-chain amino acid sequences with some naturally occurring high-affinity β-chain mutants also identifies a number of amino acids that may stabilize the T-state. Hb Porto Alegre has a Serβ^9 to Cys mutation, and the γ- and ϵ-chains both have an Ala at the corresponding position. Hb Willamette has a Proβ_{51} to Arg mutation, and the γ-chain has an Ala at the same position, and this may well affect the structure of the D-helix of the protein. The two embryonic haemoglobin β-type chains also carry Glu (γ) and Lys (ϵ) changes at Thrβ^{87} which may affect the structure of the F-helix of the protein. The proposition that the higher oxygen affinity of the embryonic haemoglobins may arise from destabilization of the T-state is supported to some degree by the finding that the energy of haem–haem interaction is lower than in the case of the adult protein (9 kJ/mol) [19].

In the presence of DPG, the oxygen-binding curves for haemoglobins $\alpha_2\epsilon_2$ and $\zeta_2\epsilon_2$ show marked shifts (Figure 2) in the value of K_T, which is in contradiction to the pure form of the two-state model as first formulated by Monod et al. [6] in which allosteric effectors alter only the allosteric equilibrium position (L) while leaving the binding characteristics of the R- and T-state unaltered. Under these conditions clearly the two-state model breaks down for these haemoglobins and a complete analysis would need to take into account the additional properties of the DPG-bound T-state [21]. Interestingly the addition of DPG also changes the character of the T-state species. At oxygen saturations below 3%, it appears that the lower limb of the Hill plot (Figure 2) may well present a slope of less than unity. This may indicate differential binding of oxygen to the α- and β-type chains in the T- state and a further deviation from the pure two-

state model. Such chain heterogeneity is clearly evident in the kinetic characteristics of the R-state (see below) of the embryonic haemoglobins and has previously been reported to be present in the T-state of the adult protein [22].

We have previously shown that, at 37 °C in the complete absence of allosteric effectors, the intrinsic oxygen affinities of the embryonic haemoglobins and the adult protein are very similar [23]. The low level of response to allosteric effectors of the embryonic haemoglobins, however, guarantees that, under physiological conditions, the embryonic haemoglobins show a significantly higher affinity for oxygen. The enthalpy of oxygenation reported above for the embryonic haemoglobins is quite similar to that reported for maternal blood [24,25] and thus the process of oxygen transfer across the placenta, accompanied by the deoxygenation/oxygenation cycles of the adult and embryonic haemoglobins, is unlikely to be associated with a significant difference in heat exchange. This is in stark contrast with the situation that pertains in the fetal stage of development in which the considerable amount of heat generated by the fetus from metabolic activity [26] is transferred to the maternal circulation using a heat pump constituted by the fetal and maternal haemoglobins, in which the fetal haemoglobin enthalpy of oxygenation is 30% lower than the adult value [25,27]. We assume that the lack of a thermal pumping mechanism in the embryo simply reflects its low metabolic rate and high surface to weight ratio, which allows simple dissipation of any excess heat to the maternal tissues.

Our kinetic studies shed further light on the functioning of the human embryonic haemoglobins. Oxygen-dissociation rates measured for the R-state of the protein indicate the presence of two related kinetic processes, not resolved previously in preliminary studies [28]. A comparison of these data with those previously reported for the adult protein [18,29] leads us to assign the two processes to oxygen dissociation from the two types of chain present in the haemoglobins. We thus assign that faster rate to dissociation from the β-type chains as previously reported. In studies of oxygen binding to the R-state, we again observe two reaction processes which we, in common with previous reports [29,30], assign to differential reactivities of the α- and β-type chains, with the β-type chain exhibiting the higher rate of reactivity. Having made these assignments, we can now determine K_R values for each of the chain types in the R-state by evaluating the ratio of the two appropriate rate constants. Interestingly these calculations yield K_R values for the α- and β-type chains that are quite similar, within a single haemoglobin species, just as is the case in the adult protein [17]. Furthermore if we average these kinetically determined oxygen-binding equilibrium constants for the α- and β-chains within each of the three haemoglobins we obtain values of 0.45, 0.39 and 0.59 μM for haemoglobins $\alpha_2\epsilon_2$, $\zeta_2\gamma_2$ and $\zeta_2\epsilon_2$ respectively, which correlate very well with the values of the R-state binding constants of 0.45, 0.31 and 0.52 μM determined independently from our equilibrium binding curves. Thus, although we have kinetic evidence for chain inequivalence in the R-state of all the embryonic haemoglobins, in equilibrium measurements the close similarity of the K_R values for the α- and β-type chains means that binding curves do not distinguish these differences.

As pointed out above, in the case of equilibrium measurements in the presence of organic phosphates, it appears that chain inequivalence may also prevail in the T-state of these proteins, as has been reported to be the case in the adult protein [11]. Unfortunately the very high oxygen affinities shown by the embryonic haemoglobins have prevented us from making kinetic studies of the T-states of these proteins [22].

The kinetic data also show that the rate of the R > T transition is similar in both adult and embryonic proteins, even though the value of L differs by almost an order of magnitude between the adult and embryonic proteins. The large difference in the value of L between the adult and embryonic proteins thus arises predominantly from the difference in rate of the transformation of the T- to the R-state.

Our data suggest that the kinetic and equilibrium characteristics of the adult and embryonic haemoglobins in the R-state are rather similar and lead us to the conclusion that the high affinity and low co-operativity expressed in the human embryonic haemoglobins arises mainly from a relative destabilization of the T-state in the case of the embryonic proteins.

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