The role of amino acid α^{38} in the control of oxygen binding to human adult and embryonic haemoglobin Portland

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The role of the amino acid at position α^{38} in haemoglobin has been probed using site-directed mutagenesis. When the Thr residue at position α^{38} (which is totally conserved in all mammals) is changed to a Gln, the equilibrium properties of the protein are significantly altered. Equilibrium and kinetic data show that the R-state properties of the protein are essentially unaffected by the mutation whilst the allosteric equilibrium and T-state properties are changed. Mutation of the naturally occurring Gln³⁸ of the human embryonic haemoglobin ζ -chain (the only known non-Thr containing globin) to a Thr residue shows the converse change in properties produced by the adult mutation, although in this case the situation is complicated by significant chain

heterogeneity in the T state. An extension of the two-state model of co-operativity is presented to describe quantitatively the equilibrium ligand binding in the presence of T-state chain heterogeneity. A molecular model is described in which the putative interaction of α Gln³⁸ and β Tyr¹⁴⁵ is identified which make a significant contribution to the previously reported unusual ligand-binding properties of the ζ -chain containing human embryonic haemoglobins.

Key words: binding, embryonic, haemoglobin, mutation, oxygen.

INTRODUCTION

During the allosteric transition in haemoglobin the protein undergoes significant structural change involving the rearrangement of the intersubunit bonding between the α_1 - and β_2 -chains, at what has become known as the sliding contact surface [1,2]. This interface involves mainly the α -chain C helix and the β subunit FG corner. During the rearrangement His⁹⁷ β , which is interdigitated with Pro⁴⁴ α and Thr⁴¹ α in the deoxy structure, shifts to interdigitate between Thr⁴¹ α and Thr³⁸ α in the oxy structure [3]. Consequently, a great deal of attention has been focused on the functional roles played by these residues.

A relatively large number of naturally occurring point mutations of the $\alpha_1\beta_2$ interface residues have been characterized. All of the mutants involving residues α^{41} , α^{44} and β^{97} show increased oxygen affinity and reduced cooperativity [4–9]. The residue at position α^{38} by comparison is totally conserved in all adult mammalian species [10]. Two artificial mutants, Val³⁸ α and Ser³⁸ α , show increased oxygen affinity and decreased Hill coefficient together with a reduction in the allosteric constant L [10]. However, these mutations introduce residues rather similar in size to the naturally occurring Thr, and examination of molecular models does not suggest the possibility of any additional interactions as a consequence of substitution of this residue.

Of the three human embryonic haemoglobins, the two proteins ($\zeta_2\epsilon_2$, $\zeta_2\gamma_2$) produced at the earliest stages of human development contain an α -type ζ -chain. This chain contains a Gln residue at position 38. Molecular modelling of this protein shows possible hydrogen bonding of Gln³⁸ to Tyr¹⁴⁵ β in the deoxy structure, in one of its accessible rotamers. Such an interaction could be of major functional significance, since the breaking of the H-bonding of Tyr¹⁴⁵ β to the carbonyl function of Val⁹⁸ β is proposed to

function in the normal triggering of the allosteric transition. The stereochemistry of the putative interaction seen in models of the ζ -chain would be such that it would be expected to compete with the hydrogen-bonding of Tyr¹⁴⁵ β to the amide carbonyl of Val⁹⁸ β [11] and so destabilize the T-state structure. This being the case the naturally occurring Gln at position 38 of the ζ -chain would be expected to have two effects: (1) alteration of the energetics of the interdigitation change of His⁹⁷ β on going from the T to R state; common to the two previously studied artificial mutants described above and (2) destabilization of the T state by weakening of the Tyr¹⁴⁵ β –Val⁹⁸ β carbonyl interaction, which would be unique to the specific presence of the Gln residue. In order to investigate further these proposed functions we have produced the $\alpha_2\beta_2$ protein mutant Thr³⁸ α → Gln and the embryonic $\zeta_2\gamma_2$ mutant Gln³⁸ ζ → Thr. By comparison of the properties of these two mutant forms with those of the wild-type proteins we can establish the role played by the naturally occurring Gln³⁸ ζ residue in the wild-type human embryonic haemoglobin.

MATERIALS AND METHODS

The mutant forms of the proteins used in this study were produced using the methods previously described [12,13]. Mutant and wild-type protein production was confirmed by mass spectral analysis of the proteins isolated using reversed-phase HPLC as described earlier [14].

All oxygen-binding data were obtained at 25 °C using an oxygen diffusion chamber modified for stepwise increases in oxygen concentration [15]. Identical data were obtained from haemoglobin samples either in the presence or absence of the met-haemoglobin reducing system [16]. Oxygen-binding data were evaluated in terms of the Hill coefficient and p_{50} (the partial pressure of oxygen giving half saturation) from plots of log

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$(Y/1 - Y)$ versus $\log pO_2$. Binding curves obtained over a wide range of oxygen concentration were analysed in terms of the two-state model by nonlinear least squares fitting of the experimental data to:

$$Y = \frac{LP/K_T(1 + P/K_T)^3 + P/K_R(1 + P/K_R)^3}{L(1 + P/K_T)^4 + (1 + P/K_R)^4}, \quad (1)$$

where Y represents fractional saturation with oxygen, L is the allosteric constant, K_R and K_T represent the oxygen affinities of the R and T state and P is the partial pressure of oxygen corresponding to a particular saturation Y . For the cases of the wild-type and mutant forms of the embryonic haemoglobin, in which the low oxygen concentration limb of the Hill plot of the binding curve did not show a unitary slope, expected for a two-state model [17], the data were further analysed by nonlinear least squares fitting to eqn. (2), which allows for chain heterogeneity within the T state, and was derived from the generalized two-state model with chain heterogeneity derived by Ogata and McConnell [18].

$$Y = \frac{2\alpha(1 + \alpha)^3 + L\alpha(1 + \alpha c_\alpha)(1 + \alpha c_\beta)(c_\alpha + 2\alpha c_\alpha c_\beta + c_\beta)}{2(1 + \alpha)^4 + L(1 + \alpha c_\alpha)^2(1 + \alpha c_\beta)^2} \quad (2)$$

where Y represents fractional saturation with oxygen at a partial pressure P , L is the allosteric constant and $\alpha = P/K_R$, $c_\alpha = K_R/K_T\alpha$ and $c_\beta = K_R/K_T\beta$ where K_R is the oxygen affinity of the R state and $K_T\alpha$ and $K_T\beta$ represent the oxygen affinities of the two types of chain within the T state. It should be noted that the use of α and β in eqn. (2) merely represents the existence of two types of binding site within the T state and cannot at this stage be assigned uniquely to the α - and β -type chains.

All nonlinear least squares fitting was performed using proprietary nonlinear least squares fitting routines (Tablecurve, Jandel Scientific, CA, U.S.A.).

Flash photolysis experiments were performed as previously described [19]. Reaction traces were analysed in terms of either single or double exponentials by nonlinear least squares fitting to appropriate equations. R-state oxygen-binding rates were determined by analysis of the recombination time courses, obtained at 436 nm, subsequent to partial photolysis (approx. 2%) of the oxygenated form of the protein produced by the 6 ns laser pulse. The rate of the $R \rightarrow T$ transition was measured at 426 nm after full photolysis of the carbonmonoxy form of the protein in the presence of 100 μ M CO. At this wavelength the ligand-rebinding process is isosbestic [20]. In the case of photolysis experiments 20 reaction traces were averaged to provide an improved signal to noise ratio prior to exponential fitting.

RESULTS

Protein characterization

Expression of the mutant forms of the recombinant adult and embryonic haemoglobins was found to occur at levels very similar to those of the wild-type proteins [13] and electro-spray mass spectroscopy of both the mutant and wild-type chains gave molecular masses within 1 Da of that predicted.

Equilibrium oxygen binding

Oxygen-binding studies of the recombinant wild-type adult haemoglobin showed cooperative oxygen binding ($n = 2.3$) with a p_{50} of 7.2 mmHg. The mutant form of the adult protein exhibited a lower level of cooperativity ($n = 1.9$) and a higher oxygen affinity ($p_{50} = 4.4$ mmHg) (Figure 1). Recombinant wild-type haemoglobin Portland showed a modest degree of cooperativity ($n = 1.7$) and relatively high oxygen affinity ($p_{50} =$

Figure 1 Oxygen-binding curves for human adult haemoglobin

Oxygen-binding curves were obtained for the wild-type adult haemoglobin ($\square$) and the $\alpha\text{Thr}^{38} \rightarrow \text{Gln}$ mutant form of the protein ($\bullet$). Data were obtained at 25 °C in 0.1 M Hepes at pH 7.0 in the presence of 100 mM NaCl and 1 mM EDTA. The haemoglobin concentration was 0.25 mM.

Figure 2 Oxygen-binding curves for human embryonic haemoglobin Portland

Oxygen-binding curves were obtained for the wild-type human embryonic haemoglobin Portland ($\bullet$) and the $\zeta\text{Gln}^{38} \rightarrow \text{Thr}$ mutant form of the protein ($\circ$). Conditions were as in Figure 1.

3.3 mmHg). The mutant form of the embryonic protein showed a somewhat lower degree of cooperativity ($n = 1.5$) and exhibited only a slightly different oxygen affinity ($p_{50} = 3.1$ mmHg) (Figure 2). Two-state analysis of the oxygen-binding curves obtained for the wild-type and mutant forms of the adult haemoglobin indicated that the oxygen affinity of the R-state is unaffected by the mutation at position α^{38} , whilst the oxygen affinity of the T-state is raised and the value of the allosteric constant (L) is lowered by the $\text{Thr} \rightarrow \text{Gln}$ mutation. Two-state analysis of the effect of changing the Gln^{38} of the ζ -chain in haemoglobin Portland to a Thr indicates a very small shift in the value of K_T

Table 1 Oxygen-binding equilibrium parametersError estimates: (a) ± 0.1 , (b) ± 0.3 , (c) ± 0.3 , (d) ± 2.0 , (e) $\pm 1 \times 10^4$.

	n^a	$p_{50}(\text{mmHg})^b$	$K_R(\text{mmHg})^c$	$K_T(\text{mmHg})^d$	L^e
$\alpha_2\beta_2$	2.3	7.2	0.26	21	5
$\alpha'_2\beta_2$	1.9	4.4	0.22	10	1.6×10^5
$\zeta_2\gamma_2$	1.7	3.3	0.22	4.7	5.5×10^4
			0.14†	1†, 28†	4.0×10^4
$\zeta'_2\gamma_2$	1.5	3.1	0.19	4.1	1.5×10^5
			0.14†	2†, 72†	1.2×10^5

* Mutant forms of the proteins.

† Values derived from eqn. (2).

Figure 3 Oxygen re-association with adult haemoglobin

The time course is shown for the recombination of $10 \mu\text{M}$ adult haemoglobin with 1.1 mM oxygen at pH 7.2 in 0.1 M Hepes buffer containing 100 mM NaCl and 1 mM EDTA at 20°C following 2% partial photolysis. The reaction was monitored at 436 nm . The line through the points is the best fit double exponential. $\Delta \text{O.D.}$ values are expressed as a percentage.

and an increase in L compared with that seen in the wild-type protein. Closer examination of the fit to the two-state model in the case of both forms of haemoglobin Portland, however, reveals that at low oxygen saturation the fit is poor. Analysis of the oxygen-binding data for haemoglobin Portland, using a model which allows for chain heterogeneity in the T state, produces a very good fit. Such an analysis indicates a difference of approx. 30-fold in the oxygen-binding affinity of the ζ - and γ -chains in the T state (Table 1), with one of the chains exhibiting a significantly lower affinity in the mutant form of the protein.

Ligand-binding kinetics

When the oxygenated forms of the wild-type and mutant adult and embryonic haemoglobins were subjected to partial photolysis, the subsequent ligand recombination kinetics exhibited time courses best described by the sum of two exponentials, with each exponential contributing approximately half of the total optical density change (Figure 3). Within the ligand concentration range $100 \mu\text{M}$ to 1.1 mM both of the observed kinetic phases exhibited a linear dependence on oxygen concentration (Table 2).

When the carbonmonoxy forms of the proteins were fully

Table 2 Ligand-binding kinetics

k , oxygen-binding rates; l , CO-binding rates. Error estimates (a) ± 5 , (b) ± 5 , (c) ± 1 , (d) ± 0.2 .

	$k_1 (\mu\text{M}^{-1} \cdot \text{s}^{-1})^a$	$k_2 (\mu\text{M}^{-1} \cdot \text{s}^{-1})^b$	$l_1 (\mu\text{M}^{-1} \cdot \text{s}^{-1})^c$	$l_2 (\mu\text{M}^{-1} \cdot \text{s}^{-1})^d$
$\alpha_2\beta_2$	99	50	5.0	1.0
$\alpha'_2\beta_2$	78	59	7.0	1.5
$\zeta_2\gamma_2$	91	46	6.0	1.8
$\zeta'_2\gamma_2$	110	40	5.7	1.5

* Mutant forms of the protein.

Figure 4 CO re-association with haemoglobin

The time course is shown for the recombination of $15 \mu\text{M}$ $\zeta\text{Gln}^{38} \rightarrow \text{Thr}$ mutant embryonic human haemoglobin with $400 \mu\text{M}$ CO at pH 9.0 in 0.1 M Borate buffer at 25°C , following photolysis of the carbonmonoxy complex. The reaction was monitored at 436 nm . The line through the points represents the best fit double exponential. $\Delta \text{O.D.}$ values are expressed as a percentage.

Table 3 R $\rightarrow$ T transitionError estimates (a) ± 1.5 . $t = 30^\circ\text{C}$.

	$k (\times 10^{-4} \cdot \text{s}^{-1})^a$
$\alpha_2\beta_2$	5.1
$\alpha'_2\beta_2$	5.3
$\zeta_2\gamma_2$	8.4
$\zeta'_2\gamma_2$	9.4

* Mutant forms of the protein.

photolysed at pH 9.0, the time courses of the recombination reactions were best described by the sum of two exponentials (Figure 4 and Table 2). Partial photolysis (approx. 10%) of the carbonmonoxy form of the proteins produced recombination time courses indistinguishable from those observed on full photolysis.

R $\rightarrow$ T transition

When the carbonmonoxy forms of each of the haemoglobin were rapidly photolysed in the presence of moderate concentrations of

CO (100 μ M), at pH 7.2 the process of conversion of the R deoxy to the T deoxy forms of the protein followed a single exponential process occurring on the microsecond time scale (Table 3).

DISCUSSION

Previous studies on the human embryonic haemoglobins have identified distinct functional characteristics for the ζ -containing proteins which appear to arise from a change in the ease of transition from the T to R state [19]. The conversion of the T to R state involves rotation of the $\alpha\beta$ dimers about the intersubunit contact surface, which consists mainly of the α -chain C helix and the β -chain FG turn and requires disengagement of His⁹⁷ β from between Thr⁴¹ α and Pro⁴⁴ α , rotation of the $\alpha\beta$ dimers and reengagement of His⁹⁷ β between Thr⁴¹ α and Thr³⁸ α [2]. Within this context it is important to note that the Thr residue at position 38 of the human α -chain is conserved in all mammalian α -chains and no naturally occurring mutants have been identified [21]. These findings alone are usually considered to be sufficient to identify a residue with a significant functional role. However, examination of high resolution structures of adult haemoglobin does not identify any interactions for Thr³⁸ which could be considered major contributors towards the control of the T to R transition. Molecular modelling of the adult deoxy structure with Thr³⁸ replaced by Gln (as in the embryonic ζ -chain) places the amine function of the Gln residue 3.4 Å (1 Å = 10^{-10} m) from the centre of the Tyr¹⁴⁵ ring (Figure 5). This is predicted to be the optimum distance for NH–aromatic ring hydrogen bond formation [22]. Furthermore the hydrogen-bonding pattern suggested from the model of the deoxy structure would be in direct opposition to the Tyr¹⁴⁵–Val⁹⁸ β carbonyl hydrogen bond which stabilizes the deoxy quaternary structure of the protein [23]. It would thus be predicted that the mutation of Thr³⁸ α to Gln should lower the allosteric constant L (i.e. a shift towards the R state in the deoxy protein and also increase the oxygen affinity of the T state). The absence of any naturally occurring α ³⁸ mutants could be due to an inherent instability of such a protein, but the results reported above exclude this possibility.

The structure–function predictions, based on the model studies described above, are borne out by the experimental data. Mutation of Thr³⁸ α to Gln, in the adult protein, raises the affinity of the T state by a factor of two, whilst lowering the allosteric constant (Table 1). In the case of the haemoglobin Portland, analogous predictions can be made for the reverse of the mutation outlined above. However, in this case, as the structure of the deoxy form of the ζ -chain has not yet been obtained, such predictions must be more circumspect. Initial inspection of the data for the ζ Gln³⁸ $\rightarrow$ Thr mutation shows an increase in the allosteric constant, but a more detailed investigation shows a clear difference in oxygen affinity between the globin chains within the T state (Figure 2 and Table 1). As pointed out by Srinathan and Rose [24] such a heterogeneity could be expected to lower the cooperativity of haemoglobin and may well represent a necessary requirement for the haemoglobin to perform its normal physiological function prior to the establishment of a fully functional placenta.

In both the case of the adult and embryonic proteins kinetic data allow us to identify further the processes responsible for the functional changes subsequent to mutation. Both oxygen and carbon monoxide recombination kinetics can best be described as arising from recombination with the two different chains [25], each of which has a slightly different rate constant, which is essentially insensitive to the mutation at position 38. The rate of

Figure 5 Putative structure of α ³⁸ mutant human adult haemoglobin

Schematic representation of the β_1 FG corner– α_1 C helix interaction in adult deoxy haemoglobin in which α ³⁸ is mutated to Gln.

the R $\rightarrow$ T transition for the adult and embryonic proteins is essentially unaffected by mutation of residue 38. We interpret these data together with the change in the allosteric constant to indicate that the shift in allosteric constant arises primarily from a change in the ease with which the proteins move from the T to the R state.

In summary, comparison of the effects of mutation of residue 38 in both adult and embryonic proteins is consistent with Gln³⁸ in the embryonic protein interacting with Tyr¹⁴⁵ and manifesting itself in a decrease in the allosteric constant L . The weakening of the normal deoxy Tyr¹⁴⁵ H-bonding pattern is manifest in the destabilization of the T-state and an associated rise in the oxygen affinity of this state. The distinction between the effects of the mutation at residue 38 seen in the adult and embryonic proteins may well arise from the effects of other amino acid differences which occur in the region of the sliding contact between the α - and β -type chains. The amino acids in the FG helix are common to both the β - and γ -chains whilst a single difference occurs at position 36 (Phe α $\rightarrow$ His ζ). As outlined above, however, we propose that the significance of the Gln at position 38 of the ζ -chain lies in its potential interaction with the Tyr¹⁴⁵ of the β - or γ -chain. In the case of the haemoglobin Portland protein, although the Tyr¹⁴⁵ is retained, in the γ -chain, the three preceding residues are all altered (Ala¹⁴² $\rightarrow$ Ser, His¹⁴³ $\rightarrow$ Ser and Lys¹⁴⁴ $\rightarrow$ Arg) and we propose that these differences in sequence may well modify the interaction of the Gln³⁸ ζ with the Tyr¹⁴⁵ γ . Confirmation of this proposal will, however, need to await future structural investigations and mutation studies. We thus conclude that the presence of a Gln residue at position 38 of the ζ -chain, although not the sole origin of the unique properties of the embryonic human haemoglobins, is integral to the establishment of an oxygen-binding protein of high affinity and modest cooperativity, necessary to fulfil the unique oxygen-transporting role required of it at the earliest stages of human development.

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REFERENCES

- 1 Perutz, M. F. (1972) *Nature* **237**, 195–208
- 2 Baldwin, J. and Chothia, C. (1979) *J. Mol. Biol.* **129**, 175–220
- 3 Smith, F. R., Lattman, E. E. and Carter, C. W. (1991) *Proteins* **10**, 81–91
- 4 Ohba, Y., Imai, K., Uenaka, R., Ami, M., Fujisawa, K., Itoh, K., Hirakawa, K. and Miyaji, T. (1989) *Hemoglobin* **13**, 637–648
- 5 Honig, G. R., Vida, L. N., Shamsuddin, M., Mason, R. G., Schlumpf, H. W. and Luke, R. A. (1980) *Biochim. Biophys. Acta* **626**, 424–431
- 6 Harano, T., Harano, K., Ueda, S., Shibata, S., Imai, K., Ohba, Y., Shinohera, T., Horio, S., Nishioka, K. and Shirotani, H. (1982) *Hemoglobin* **6**, 379–390
- 7 Lorkin, P. A., Lehmann, H., Fairbanks, V. F., Berglund, G. and Leonhardt, T. (1970) *Biochem. J.* **119**, 68–73
- 8 Taketa, F., Huang, Y. P., Lubnoch, J. A. and Dessel, B. H. (1975) *Biochim. Biophys. Acta* **400**, 348–354
- 9 Ohba, Y., Imanaka, M., Matsuoka, M., Hattori, Y., Miyaji, T., Funaki, C., Shibata, S., Shimokata, H., Kuzuya, F. and Miwa, S. (1985) *Hemoglobin* **9**, 11–24
- 10 Hashimoto, M., Ishimori, K., Imai, K., Miyuzaki, G., Moritomo, H., Wada, Y. and Morishima, I. (1993) *Biochemistry* **32**, 13688–13695
- 11 Togi, A., Ishimori, K., Unno, M., Konno, T., Morishima, I., Miyazaki, G. and Imai, K. (1993) *Biochemistry* **32**, 10165–10169
- 12 Mould, R., Hofmann, O. and Brittain, T. (1994) *Biochem. J.* **298**, 619–622
- 13 Hofmann, O., Mould, R. and Brittain, T. (1994) *Prot. Eng.* **7**, 281–283
- 14 Hofmann, O., Mould, R. and Brittain, T. (1995) *Biochem. J.* **306**, 367–370
- 15 Weber, R. E. (1981) *Nature* **292**, 386–387
- 16 Hayashi, A., Suzuki, T. and Shih, M. (1973) *Biochim. Biophys. Acta* **310**, 309–316
- 17 Imai, K. (1982) *Allosteric Effects in Haemoglobin*, pp. 6–8, Cambridge University Press, U.K.
- 18 Ogata, R. T. and McConnell, H. M. (1971) *Cold Spring Harbor Symp. Quant. Biol.* **36**, 325–336
- 19 Brittain, T., Hofmann, O., Watmough, N. J., Greenwood, C. and Weber, R. E. (1997) *Biochem. J.* **326**, 299–303
- 20 Vandegriff, K. D., Le Tellier, Y. C., Winslow, R. M., Rohlf, R. J. and Olson, J. S. (1991) *J. Biol. Chem.* **266**, 17049–17059
- 21 Huismann, T. H. J., Carver, M. F. H. and Efremov, (1996) G. D. in *A Syllabus of Human Hemoglobin Variants* (1996) The Sickle Cell Anaemia Foundation, GA, U.S.A.
- 22 Levitt, M. and Perutz, M. F. (1988) *J. Mol. Biol.* **210**, 751–754
- 23 Bettati, S., Mozzarelli, A. and Perutz, M. F. (1983) *J. Mol. Biol.* **281**, 581–585
- 24 Srinathan, R. and Rose, G. D. (1994) *Proc. Natl. Acad. Sci., U.S.A.* **91**, 11113–11117
- 25 Matthews, A. J., Rohlf, R. J., Olson, J. S., Tame, J., Renaud, J. P. and Nagaki, K. (1989) *J. Biol. Chem.* **264**, 16573–16583

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