Role of dimerization in the control of the functioning of the human haemoglobin mutant haemoglobin Howick (β_{37} Trp $\rightarrow$ Gly)

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Haemoglobin Howick shows a high oxygen affinity ($p_{50} = 1$ mmHg) and a low co-operativity ($n = 1.3$). Equilibrium studies show the protein to be essentially totally dimeric in the oxygenated form. A wide range of rapid kinetic experiments indicate that the deoxygenated form of the protein exists in a tetramer $\rightleftharpoons$ dimer equilibrium with an associated equilibrium constant of $3 \mu\text{M}$. These kinetic data also indicate that the oxygenated form of the protein exists in a tetramer $\rightleftharpoons$ dimer equilibrium with an associated equilibrium constant of 35 mM, and furthermore clearly identifies a large increase in the rate of

the tetramer-to-dimer dissociation process as the origin of the vastly increased dissociation equilibrium constants. Simulations of the protein-concentration-dependence of the oxygen-binding properties of haemoglobin Howick, based on the measured equilibrium parameters, closely fits the experimental data. The change in dimerization constant for the deoxygenated form of the protein corresponds remarkably well to the free-energy change predicted for the simple transfer of the amino acid side chain at position β_{37} from a hydrophobic to a hydrophilic environment during the dimerization process.

INTRODUCTION

Early crystallographic studies on haemoglobin (Perutz et al., 1968) showed that, during the process of oxygenation, a significant quaternary structural change occurs in the protein. This structural change predominantly involves a sliding motion between the $\alpha_1\beta_2$ contact surface of the protein (Fermi, 1975). It is at this surface that the protein is cleaved when it dimerizes under conditions of low concentration or high ionic strength (Ackers and Thompson, 1965; Guidotti, 1967; Chiancone et al., 1968; Park, 1970). Thus any change in the amino acid side chains that form this surface can be expected to alter drastically the characteristics of the haemoglobin with regard to oxygen binding and dimerization (Imai, 1982).

Although recent advances in molecular biology have made possible the production of site-directed mutants in haemoglobin (Vallone et al., 1993), much of our knowledge of the functioning of this important protein has arisen from the study of naturally occurring mutants (Lehman and Huntsman, 1974; Fermi and Perutz, 1981). One such recently discovered natural mutant is haemoglobin Howick (Owen et al., 1994). This mutant contains a glycine residue at position β_{37} in place of the normally occurring tryptophan. This tryptophan side chain normally forms a hydrogen-bonding network with five other amino acids in the deoxygenated form of the protein (Fermi and Perutz, 1981) and represents a major stabilizing influence on the important $\alpha_1\beta_2$ contact interface. Haemoglobin Howick thus presents an interesting molecule for study in that its properties are likely to give an insight into the relative significance of the tryptophan side chain to the overall stability of the dimer/dimer interface. To this end we have undertaken a series of equilibrium and rapid reaction studies to investigate the effect of this mutation on the characteristics of this haemoglobin. The outcome of these studies is a clearer understanding of the role of protein dimerization in controlling the functioning of this mutant and an independent estimation of the contribution the tryptophan side chain makes to the stability of the $\alpha_1\beta_2$ interface in the native protein molecule.

MATERIALS AND METHODS

Haemoglobin Howick was purified from a sample of whole blood by chromatography on a column of DEAE-Sephadex

using a linear pH gradient of 8.3 to 7.0 of 50 mM Tris/HCl buffer containing 0.1 mM EDTA as described by Owen et al. (1993). Haemoglobin concentration was estimated using the reduced carbonmonoxy form by measuring the absorption at 419 nm and employing the millimolar absorption coefficient of 191 cm^{-1} (Antonini and Brunori, 1971). Oxygen dissociation curves were determined at 25 °C using a HEMOX analyser (TSC, Medical Products, PA, U.S.A.). The molecular mass of the oxygenated derivative of the haemoglobin was determined by chromatography on a column (1 m $\times$ 2.5 cm) of Superose 12 previously calibrated with known molecular-mass standards.

Stopped-flow and flash-photolysis experiments were performed as previously described (Brittain, 1980, 1984). Analysis of reaction traces was as reported previously (Brittain and Greenwood, 1982; Brittain, 1986).

Simulations of oxygen-binding curves for a system undergoing oxygen-binding-linked dissociation were made using the approach first outlined by Imai and Yonetani (1977). In this scheme the following relationship applies:

$$Y = fY_2 + (1 - f)Y_4$$

where

$$f = (K_{4,2}Y_4/2CY_2^4)\{\sqrt{[1 + (4CY_2^4/K_{4,2}Y_4)]} - 1\}$$

and

$$Y_4 = (K_4p)^n/[1 + (K_4p)^n]$$

$$Y_2 = (K_2p)/[1 + (K_2p)]$$

Y is the fractional saturation of the system at an oxygen concentration of p ; f is the fraction of haemoglobin present as dimers at a total concentration of C ; $K_{4,2}$ is the tetramer–dimer equilibrium constant; Y_4 and Y_2 are the fractional saturations of tetramers and dimers and n is the Hill coefficient associated with the co-operative binding of oxygen to tetramers. The dimers are treated as non-co-operative in their binding of oxygen (Hewitt et al., 1972). In the simulations presented in this paper $K_{4,2}$ was assigned the value determined indirectly from the kinetic experiments described below. K_4 , K_2 and n were determined from the data of Mills et al. (1976), corrected to a temperature of 25 °C

using the temperature-dependence data contained in Antonini and Brunori (1971).

Unless stated otherwise, all reactions were performed at 25 °C in 50 mM Tris/HCl buffer containing 1 mM EDTA and 100 mM NaCl at pH 7.5.

RESULTS

Equilibrium studies

At a protein concentration of 95 μM and a pH of 7.5 haemoglobin Howick was found to exhibit a high oxygen affinity ($p_{50} = 1 \text{ mmHg}$) and a low degree of co-operativity ($n = 1.3$) (Figure 1).

When the oxygenated form of the protein was chromato-

Figure 1 A comparison of experimental and simulated oxygen-binding curves

Experimentally obtained oxygen-binding curves at 95 μM (●) and 250 μM (○) haemoglobin concentrations are compared with simulated binding curves (—) calculated as described in the text. Curves were obtained at 25 °C in 50 mM Tris/HCl buffer containing 100 mM NaCl at pH 7.5. Y represents the fractional saturation of haemoglobin with oxygen.

Figure 2 Kinetic difference spectra for the deoxygenation of haemoglobin Howick

The kinetic difference spectra are shown for the slow (○) and fast (△) deoxygenation reactions observed when 27 μM haemoglobin Howick is rapidly mixed with a solution containing 1 mg/ml sodium dithionite at 25 °C prepared in a sample of deoxygenated 50 mM Tris/HCl buffer containing 100 mM NaCl at pH 7.5.

Figure 3 Kinetic determination of the dimerization equilibrium constant for deoxyhaemoglobin Howick

The proportions (%R) contributed by the fast (○) reaction observed when haemoglobin Howick is rapidly mixed with 20 μM carbon monoxide and the slow association of deoxygenated dimers (△) observed when haemoglobin Howick is rapidly mixed with a solution containing 1 mg/ml sodium dithionite are shown as a function of haemoglobin concentration. —, Calculated best-fit haemoglobin-concentration-dependence of the proportion of dimers (%R) present in a system undergoing tetramer $\rightleftharpoons$ dimer equilibrium assuming an equilibrium constant of 3 μM .

graphed, under the same solution conditions as employed in the oxygen-binding studies, on a calibrated column of Superose 12, the protein was eluted in a symmetric peak at a volume indicative of an apparent molecular mass of 32 kDa. Unfortunately the very high oxygen affinity of the protein prevented us from obtaining reproducible data on the deoxygenated form of the protein.

Kinetic studies

When the oxygenated form of the protein (at 10 μM) was exposed to a short intense pulse of light, the recombination of the partially liganded form of the protein with oxygen occurred as a single process with an associated second-order rate constant of $4.9 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$.

If the carbonmonoxy derivative of the protein was used in similar experiments, because of its higher quantum yield, it proved possible to observe the recombination reaction with both the partially liganded and deoxygenated forms of the haemoglobin. After both complete and partial photolysis the recombination reaction with carbon monoxide occurred as a single step with an associated second-order rate constant of $8 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$. In contrast, when the deoxygenation process was monitored at 437.5 nm, after rapid mixing of the oxygenated protein with dithionite, the deoxygenation reaction occurred as the sum of two separate processes. At this wavelength the two deoxygenation reactions were not followed by any slower process and could easily be associated with first-order rate constants of 40 s^{-1} and 12 s^{-1} respectively. By following the deoxygenation reactions at different wavelengths, it was possible to obtain kinetic difference spectra for each of the individual deoxygenation processes (Figure 2). If the reaction of oxygenated haemoglobin with dithionite was monitored for extended time periods (many seconds), particularly at 430 nm, a much slower process was observed subsequent to the deoxygenation reactions. This slower process, often formerly referred to as a 'drift' process, has previously been assigned to the slow association of deoxygenated dimers [produced by the reaction of dithionite with oxygenated dimers (Antonini et al., 1968; Kellet and Gutfreund, 1970)] to form deoxygenated tetramers. Furthermore it was observed that this association of recently deoxygenated dimers contributed in increasing proportion of the total absorbance change as the

haemoglobin concentration was decreased (Figure 3). When the deoxygenated protein was rapidly mixed with a solution containing carbon monoxide in the stopped-flow apparatus, the subsequent reaction observed at 437.5 nm occurred as two processes. The second-order rate constants for these processes were evaluated at $3 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $3.2 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$. The relative contribution each process made to the overall absorbance change was found to depend on the haemoglobin concentration employed in the reaction (Figure 3).

DISCUSSION

The tryptophan residue normally present at position β_{37} in adult human haemoglobin has been identified as playing a crucial role in the maintenance of the $\alpha_1\beta_2$ subunit interface (Fermi and Perutz, 1981). Mutations at this interface have been shown to significantly alter the oxygen affinity and co-operativity of haemoglobin (Fujita, 1972; Sasaki et al., 1975).

In the case of the naturally occurring mutant, haemoglobin Howick, the tryptophan at position β_{37} is replaced by a glycine residue. Recent studies have shown an increased affinity for oxygen and a reduced co-operativity in this system (Owen et al., 1993). Such changes in haemoglobin function are consistent with an enhanced dimerization of this protein. The apparent molecular mass of the oxygenated form of this protein described above clearly indicates that the liganded form is essentially completely dimeric. The absence of any acceleration in the time courses observed in any of the recombination reactions also give strong support to the equilibrium evidence of a predominance of $\alpha\beta$ -dimers in solution at micromolar concentrations of protein.

The rapid deoxygenation process which occurs as two reactions can be rationalized in terms of different reactivities of the α - and β -subunits within an $\alpha\beta$ -dimer. The slower reaction exhibits a rate essentially identical with that previously reported for the deoxygenation of α -chains within the normal haemoglobin molecule (Olson et al., 1971; Mathews et al., 1989; Vandegriff et al., 1991). The faster reaction which occurs at a rate approximately 50% faster than that reported for the normal β -chain is then assigned to the mutant β -chain in haemoglobin Howick. This assignment is further supported by the kinetic difference spectra associated with each of the two reactions [Figure 2, see also Brittain et al. (1986)]. Unfortunately the very high degree of dissociation apparent in the oxygenated form of the protein even at high protein concentrations precludes any direct determination of the equilibrium constant for this process (see below). An evaluation of the equilibrium constant for the dimerization of the deoxygenated form of the haemoglobin is, however, possible. In a system in which the oxygenated form of haemoglobin is essentially totally dimeric whereas the deoxygenated form is only partially dissociated, it is possible to determine the equilibrium constant for the dissociation process of the deoxy form using a number of kinetic measurements (Turner et al., 1981). In this system the rapid deoxygenation of a dimeric sample of oxyhaemoglobin should be followed by an association reaction when the deoxy dimers associate to deoxy tetramers. This process has been identified previously and correlated with the slow phase which follows the rapid deoxygenation of haemoglobin (Antonini et al., 1968; Kellet and Gutfreund, 1970). The data obtained for the slow phase after the deoxygenation of haemoglobin Howick are shown in Figure 3 and fit well to a process of dimer-tetramer association with an associated equilibrium constant of $3 \mu\text{M}$. Furthermore, having shown that this process is indeed correlated with the aggregation of deoxygenated dimers, further analysis of the time courses for

this second-order association process yields a range constant of $2.7 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ and a first-order rate for the dissociation of deoxy tetramers to deoxy dimers of 0.8 s^{-1} . It is interesting to note at this stage that, for normal human haemoglobin, the reported equilibrium constant is $5 \times 10^{-11} \text{ M}$ (Atha and Riggs, 1976), whereas the rate constants for association and dissociation are $7.3 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $2.2 \times 10^{-5} \text{ s}^{-1}$ respectively (Turner et al., 1981). Thus the very large increase in the equilibrium constant for the dimerization of haemoglobin Howick arises almost completely from the enhancement of the dissociation rate constant. This pattern is also apparent in the reactivity of another naturally occurring mutant haemoglobin Kempsey ($\beta_{99} \text{ Asp} \rightarrow \text{Asn}$) which shows a dissociation rate of 0.7 s^{-1} (Turner et al., 1981) and exhibits a high oxygen affinity and low co-operativity (Bunn et al., 1974; Pettigrew et al., 1982; Coletta et al., 1986).

The validity of the estimate of the deoxy tetramer dissociation equilibrium constant can be proven by determining this constant indirectly by monitoring the ratio of dimers and tetramers in a solution of deoxyhaemoglobin. As described above, a solution of deoxyhaemoglobin Howick combines with carbon monoxide in a reaction consisting of two processes: the faster process can be assigned to the reaction of dimers whereas the slower reaction arises from the ligation of tetramers (Andersen et al., 1971; Sasaki et al., 1978). If a large excess of ligand is employed, then during the ligand-binding step it can be assumed that no re-equilibration of the dissociation reaction occurs. Under these circumstances, a plot of the percentage fast process against haemoglobin concentration yields an estimate of the equilibrium constant. Figure 3 shows that the carbon monoxide-binding data are also consistent with an equilibrium constant of $3 \mu\text{M}$ for the dimerization reaction of haemoglobin Howick.

If in the haemoglobin Howick system we make the assumption that the dimers and tetramers retain the same oxygen-binding properties as normal haemoglobin and only the ratio of their concentration is changed, it is then possible to estimate the equilibrium constant for the oxygenated form of haemoglobin Howick as approximately 35 mM. Armed with this estimate, it is then possible using the method of Imai and Yonetani (1977) to simulate oxygen-binding curves for haemoglobin Howick under conditions of different protein concentration. Such simulations are shown compared with the experimentally determined oxygen-binding curves in Figure 1 and account for the change in oxygen-binding characteristics from $p_{50} = 1 \text{ mm Hg}$, $n = 1.3$ at $95 \mu\text{M}$ haemoglobin to $p_{50} = 1.5$, $n = 1.6$ at $250 \mu\text{M}$ haemoglobin concentration. The closeness of the fit appears to validate the assumptions outlined above and the estimate of 35 mM for the equilibrium constant for the oxygenated form of the protein.

Finally, from our data it is now possible to quantitatively consider some of the energetics of the $\alpha_1\beta_2$ surface interface. When tryptophan β_{37} is replaced by serine as in haemoglobin Hirose, the difference in free energy between the normal and mutant has been estimated from the change in the deoxy equilibrium constant to be approximately 33 kJ/mol (Sasaki et al., 1978). By comparison when tryptophan β_{37} is replaced by glycine in haemoglobin Howick the difference in free energy is 27.2 kJ/mol. Interestingly if the dimerization process is considered simply as moving the side chain at position β_{37} from a hydrophobic environment in the tetramer to a hydrophilic environment in the dimer the differences in free energy would be 31 kJ/mol and 28.5 kJ/mol respectively (Nozaki and Tanford, 1971). This simple analysis thus suggests that much of the total free energy of 60 kJ/mol associated with the assembly of tetramers in normal haemoglobin (Mills et al., 1976) derives from the hydrophobic character of the $\alpha_1\beta_2$ interface.

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