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Subunit dissociation and reassociation leads to preferential crystallization of haemoglobin Bart's (γ_4) from solutions of human embryonic haemoglobin Portland ($\zeta_2\gamma_2$) at low pH

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A variety of human haemoglobins (Hbs) are produced at different stages of human development, including three embryonic Hbs, foetal Hb and adult Hb. All are heterotetramers. During crystallization experiments on human embryonic Hb Portland ($\zeta_2\gamma_2$), it was discovered by crystallographic and biochemical analysis that the homotetramer Hb Bart's (γ_4) preferentially crystallizes from $\zeta_2\gamma_2$ solutions below pH 5. This results from dissociation of Hb Portland into γ_2 dimers and ζ monomers and has interesting implications for subunit interactions and tetramer stability in Hbs. It also makes possible a full crystallographic analysis of Hb Bart's, which is of considerable medical significance because of its presence in the red blood cells of millions of people worldwide who suffer from α-thalassaemia.

1. Introduction

Haemoglobin (Hb) is a classic example of an oligomeric protein in which the cooperative binding and dissociation of a ligand (oxygen) is coupled to changes in the interactions and orientations of the subunits. This requires that the subunit interactions are strong enough for the transmission of signals to and from remote binding sites, but not so strong as to lock the oligomer into one state. Five major haemoglobins are produced during human development, each of them optimized to satisfy the O_2-supply requirements at a particular stage of development. All are heterotetramers; three embryonic Hbs, Gower I ($\zeta_2\varepsilon_2$), Gower II ($\alpha_2\varepsilon_2$) and Portland ($\zeta_2\gamma_2$), foetal ($\alpha_2\gamma_2$) Hb and adult ($\alpha_2\beta_2$) Hb. These Hbs differ in their oxygen affinities, in their cooperative behaviour and in their tetramer stabilities; for example, the tetramer stability of liganded foetal Hb is nearly 100-fold greater than that of adult Hb (Manning *et al.*, 1999). They therefore offer fascinating natural systems through which to explore oligomer interactions and their contributions to structure and function.

The various structural states of adult Hb have been well characterized (Baldwin, 1980; Shaanan, 1983; Fermi *et al.*, 1984). This is not true of the other human Hbs, however. Although a number of studies of the functional role of foetal Hb ($\alpha_2\gamma_2$) have appeared (for example, see Allen *et al.*, 1953; Weatherall *et al.*, 1974; Huisman, 1981), only the structure of the deoxy form of this protein has been determined (Frier & Perutz, 1977). Likewise, although functional studies on the three embryonic Hbs have been carried out (Hofmann, Carrucan *et al.*, 1995; Hofmann, Mould *et al.*, 1995; Hofmann & Brittain, 1996), the only structure available is for the carbonmonoxy form of Gower II Hb ($\alpha_2\varepsilon_2$) (Sutherland-Smith *et al.*, 1998); no structure has been determined for Gower I Hb ($\zeta_2\varepsilon_2$) or Hb Portland ($\zeta_2\gamma_2$) and no structure at all is available for the ζ-chain.

Here, we report results that stem from our attempts to crystallize Hb Portland. High-quality crystals were obtained at pH values below 5.0, but remarkably these proved to be of a homotetrameric species, γ_4, known as Hb Bart's (Ager & Lehmann, 1958). The formation of this protein, which is of medical significance for its occurrence in serious human thalassaemias, implies that complex dissociation and reassociation events must have occurred, with interesting implications for the subunit interactions in these proteins.

2. Materials and methods

2.1. Preparation, crystallization and data collection

Recombinant Hb Portland was expressed in *Saccharomyces cerevisiae* and purified as previously described (Mould *et al.*, 1994; Hofmann, Mould *et al.*, 1995). The protein was oxidized to the met form based on the method of Yamamoto & La Mar (1986). First, met-aquo-Hb was prepared under a stream of O_2 with strong illumination in the presence of a twofold molar excess of potassium ferricyanide. The protein was separated from the residual reagents with a HiTrap (Pharmacia, Uppsala, Sweden) desalting column. Reconstituted met-azido-Hb was then prepared by

the addition of a threefold molar excess of sodium azide.

Initial crystallization conditions were found by a search procedure based on a polyethylene glycol orthogonal array (Kingston *et al.*, 1994). Crystals were grown by hanging-drop vapour diffusion at 294 K in 4 µl drops by mixing equal volumes of the protein solution (22 mg ml^{-1} in 23 m*M* Tris–HCl pH 8.0 with 23 m*M* NaCl and 5 m*M* NaN_3) with reservoir solution. Although crystals grew over a wide range of pH (4.9–8.5), the best diffracting crystals appeared at pH 4.9, the lower limit of the array. Subsequent miniscreens yielded large cube-shaped crystals (Fig. 1*a*) that grew to full size in 20–30 d when equilibrated over a reservoir solution of 200 m*M* acetic acid/KOH (pH 4.5–4.9) and 14–21%(*w*/*v*) methoxy-PEG 5000. For data collection, crystals were flash-frozen after the addition of 30%(*v*/*v*) glycerol to the mother liquor as a cryoprotectant. X-ray data were collected at 110 K using a MAR Research 345 image-plate detector with Cu $K\alpha$ radiation from a Rigaku RU-H3R rotating-anode generator equipped with focusing mirrors. Data were collected as a series of 1° oscillation frames and processed with the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

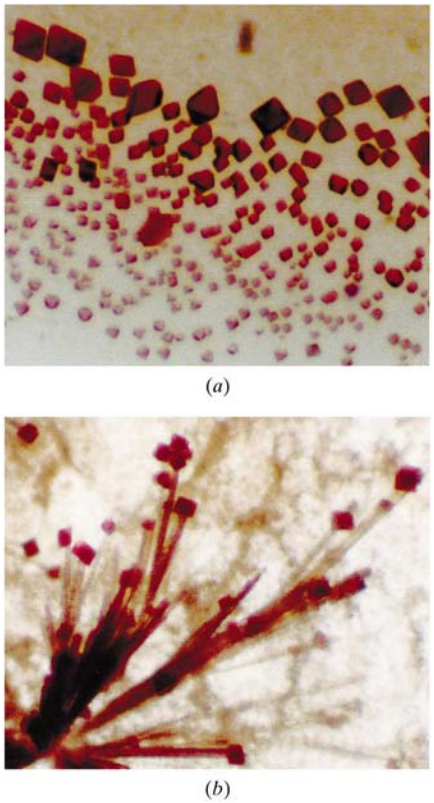

Figure 1
(*a*) Crystals of met-azide (NNN)-γ_4 haemoglobin. (*b*) Mixture of rod-shaped NNN-$\zeta_2\gamma_2$ crystals with cube-shaped NNN-γ_4 crystals growing near the ends of the rods. The sizes of the largest NNN-γ_4 cubes are approximately 0.15 × 0.15 × 0.15 mm.

2.2. Reverse-phase and size-exclusion HPLC analysis

The globin compositions of various crystals were analyzed by reverse-phase HPLC. Crystals were removed from hanging drops, washed extensively with mother liquor and dissolved in distilled water containing 0.5%(*v*/*v*) trifluoroacetic acid. The globin protein chains were separated on a C18 Jupiter (Phenomenex, Torrance, CA, USA) column using a 0–40%(*v*/*v*) acetonitrile gradient. Absorbance was measured at 214 nm.

Hb Portland at various pH values was analyzed by size-exclusion chromatography. Protein was diluted with either 100 m*M* NaCl, 50 m*M* sodium acetate (at pH 4.5 or 5.0) or 100 m*M* NaCl, 50 m*M* bis-tris propane (at pH 6.0 or 7.0) and injected onto a Superose 12 (Pharmacia, Uppsala, Sweden) column equilibrated at the appropriate pH. Absorbance of the eluent was measured at 280 nm. The column elution volumes were calibrated at neutral pH with a sample of authentic human haemoglobin (tetramer) and myoglobin (monomer). Fractions were collected and analyzed using reverse-phase HPLC as described above.

3. Results and discussion

Solutions of Hb Portland in both ferrous carbonmonoxy and met-(ferric)-azido forms were used in crystallization experiments. In both cases crystals were obtained under similar conditions and proved to be isomorphous; the details given below are for the met-azido form. Crystals were obtained initially over a wide pH range, 4.9–8.5, with the best (cube-shaped) crystals (Fig. 1) ultimately being obtained at pH 4.5–4.9. These crystals were orthorhombic, space group $P2_12_12$, with unit-cell parameters a = 60.54, b = 81.63, c = 53.04 Å. This gives a Matthews coefficient (Matthews, 1968) of V_M = 2.00 Å^3 Da^{-1} (40% solvent), assuming the presence of two globin subunits in the asymmetric unit. X-ray diffraction data were collected from these crystals to give a data set of 22 691 unique reflections that was 99.7% complete to 1.86 Å resolution, with an R_{merge} of 6.4%.

Attempts to solve the structure by molecular replacement ran into difficulties. In molecular-replacement calculations using *CNS* (Brunger *et al.*, 1998) and a truncated $\alpha\beta$ heterodimer as a search model a solution was readily obtained; however, attempts to refine this model or to obtain alternative solutions using different search models consistently indicated that the two subunits in the asymmetric unit were in fact both γ, rather than the expected $\zeta\gamma$ heterodimer. Model building with *O* (Jones *et al.*, 1991) from a 'generic' polyalanine globin model showed that for both subunits the γ sequence fitted much better than the ζ sequence. This was confirmed by automatic model building using *wARP* (Lamzin & Wilson, 1997). With the application of crystallographic symmetry elements, this implied that the crystal structure must contain γ_4 homotetramers. This was confirmed by crystallographic refinement, full details of which will be given elsewhere for both carbonmonoxy- and met-azido-γ_4 structures, and we turned our attention to characterizing the events that had occurred during crystallization.

In order to confirm the composition of the cube-shaped crystals grown at pH 4.9 and used for our diffraction studies, we dissolved several crystals and injected them onto a

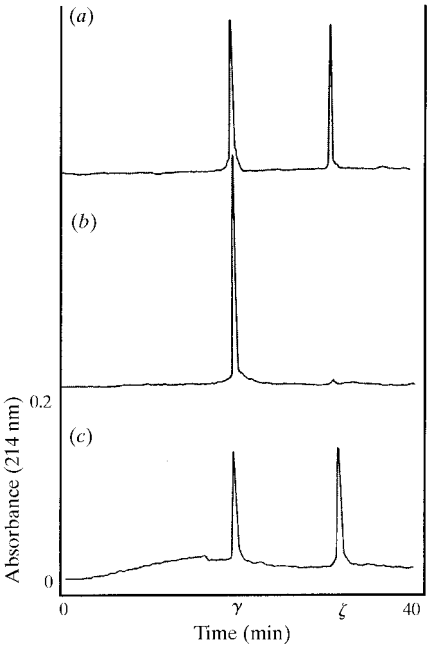

Figure 2
Reverse-phase HPLC chromatograms depicting the elution profiles of human Portland haemoglobin samples. Flow rate was 0.2 ml min^{-1}, sample volumes were 50 µl, gradient was 0–40%(*v*/*v*) acetonitrile and elution was monitored at 214 nm. Each chromatogram derives from an independent experiment; the amount of protein loaded is not directly comparable between them. (*a*) Protein solution prior to setting up crystallization trials. (*b*) Dissolved cube-shaped crystals as depicted in Fig. 1(*a*). (*c*) Dissolved rod-shaped crystals similar to those found in Fig. 1(*b*).

reverse-phase (RP) HPLC column. The results are shown in Fig. 2. The protein present in these crystals consists solely of Hb γ-chains (Fig. 2*b*). This is a striking result considering that the protein solution used for the crystallization drops was Hb Portland ($\zeta_2\gamma_2$) and thus comprised equimolar amounts of ζ- and γ-chains (Fig. 2*a*). Rod-shaped crystals that grew at pH 5.8 and 7.9 were also analyzed by RP-HPLC and were found to be composed of approximately 1:1 mixtures of ζ- and γ-chains (data not shown); these were presumably authentic Hb Portland. Interestingly, if the pH is lowered slightly below 4.9, we often obtain a mixture of thin rods and (all-γ) cubes, with the cubes growing at the ends of the rods (Fig. 1*b*). These rods can grow to 1 mm in length with a width of 0.05 mm but they are often hollow and unfortunately do not diffract beyond ~8 Å. When the rods were analysed by RP-HPLC, they were found to be composed of of ζ- and γ-chains (Fig. 2*c*). The rods grow to full length within 2–3 d of setting up the drops, whereas the cubes do not appear until 2–5 d later and continue to grow for another 20–30 d, suggesting that there is a slow dissolution of the Hb Portland crystals, dissociation of the $\zeta_2\gamma_2$ hetero-tetramer and reassociation of γ-chains to form the γ_4 homotetramer.

To help understand these dissociation/association events at low pH, solutions of Hb Portland were incubated at several conditions of different pH and injected onto a size-exclusion HPLC column. Fig. 3 shows the elution profiles of protein incubated at pH 7.0, 6.0, 5.0 and 4.5. At pH 7.0 (top panel), there is a single peak that elutes at the position expected for a Hb tetramer. In contrast, at pH 4.5 (bottom panel) two peaks have appeared, the larger peak at a position indicative of a dimer and a smaller peak at a position expected for a monomer. Reverse-phase HPLC analysis of collected fractions revealed that the dimeric species is almost exclusively γ_2 and the monomers are entirely ζ-chains (data not shown). Thus, under these conditions Hb Portland dissociates to γ_2 dimers and ζ monomers.

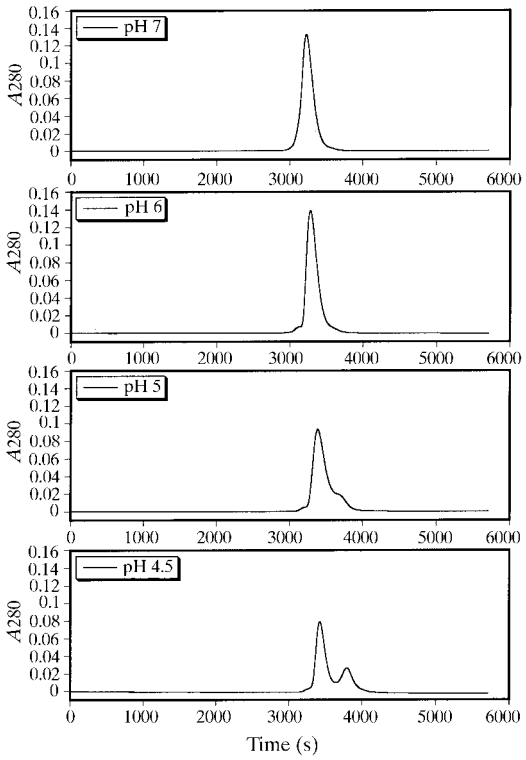

Figure 3
Typical size-exclusion chromatograms of Portland haemoglobin (Hb) incubated at different pH conditions. The pH varied, from top to bottom, between 7, 6, 5 and 4.5. Elution was monitored at 280 nm. Sample volumes and amounts were identical for each run: 180 µl and 97 µg Hb, respectively. The column was calibrated with human adult haemoglobin and myoglobin at neutral pH. The peak at pH 7 corresponds to tetramers. The peak at pH 6 may be a mixture of tetramers and dimers. At pH 5, the large peak corresponds to dimers and the shoulder at approximately 3700 s coincides with monomers. The larger peak at pH 4.5 elutes as dimers, while the smaller peak corresponds to monomers. The very small shoulders at approximately 3300 s for both pH 5 and 4.5 corresponds to tetramers. Fractions (0.3 ml) were collected for each run and further analyzed by reverse-phase HPLC.

Human adult haemoglobin is normally present in neutral solution almost exclusively in the heterotetrameric $\alpha_2\beta_2$ structure. However, under different conditions it is well recognized that the protein can dissociate into $\alpha\beta$ heterodimers (Svedberg & Pedersen, 1940; Rossi-Fanelli *et al.*, 1961; Kirshner & Tanford, 1964). The preservation of the $\alpha\beta$ heterodimer association is understandable in terms of the structure of adult Hb, since the $\alpha\beta$ dimer interface is the most extensive in the tetramer and hardly changes during the allosteric transition between deoxy (T) and liganded (R) states. At neutral pH, significant dissociation is normally only observable at low micromolar protein concentrations (Kellett, 1971). However, dissociation to dimers is favoured when the protein is present in the R state (usually ferrous or ferric with ligand bound) and at high or low pH where the dimerization constant is reported to reach values well into the millimolar concentration range (Lunelli *et al.*, 1994). Furthermore, at extreme pH values (pH < 5.0 and pH >10.0) dissociation of the protein can proceed towards the formation of monomeric forms of the protein.

By low-pH incubation of samples of human embryonic Hb Portland ($\zeta_2\gamma_2$), we have succeeded in preferentially crystallizing the γ_4 protein Hb Bart's. Our molecular fractionation studies have clearly shown that at low pH values Hb Portland dissociates into γ_2 homodimers and ζ monomers. Our studies confirm the recent results of Adachi *et al.* (2000), who showed that γ-chains form stable homodimers that do not readily dissociate into monomers. This contrasts with adult Hb, for which $\alpha\beta$ heterodimers are formed, and implies that in the embryonic protein there are distinct differences in the intersubunit interactions. We infer that the ζ–γ interactions are weaker than the γ–γ interactions and aim to explore this at the atomic level through crystal structure analyses of the $\zeta_2\gamma_2$ and γ_4 proteins.

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