Haem disorder in recombinant- and reticulocyte-derived haemoglobins: evidence for stereoselective haem insertion in eukaryotes

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We have used NMR spectroscopy to measure haem disorder in adult human haemoglobin (HbA) obtained from mature erythrocyte cells and from yeast expressing recombinant HbA. Reticulocyte-derived HbA contained much higher levels of haem disorder (11% α - and 28% β -subunit disorder) than observed for HbA from mature erythrocytes (1.5% α - and 8% β -subunit disorder). Thus, unlike *in vitro* combination of haem and apoHb, biosynthetic haem insertion is not random with respect to orientation, but appears to show stereoselectivity. Recombinant HbA isolated from yeast showed 32% α - and 45% β -subunit

haem disorder. These levels relaxed to their equilibrium positions after incubating the Hb in the ferric form. Recombinant embryonic human Hbs showed less haem disorder than recombinant HbA. The levels of haem disorder in embryonic Hbs $\zeta_2\epsilon_2$ and $\zeta_2\gamma_2$ appear to have their equilibrium values. We propose that, in eukaryotes, *in vivo* haem insertion occurs via both co-translational mechanisms and insertion via semiHb- β .

Key words: adult, assembly, embryonic, NMR.

INTRODUCTION

The haem prosthetic group can be inserted into apomyoglobin and apohaemoglobin in two structurally distinct orientations ('haem disorder') related by a 180° rotation about the α - γ meso-carbon axis [1,2]. The distinction between haem orientations is possible because of the asymmetric placement of methyl and vinyl residues about the porphyrin macrocycle (see Figure 1 below). Haem disorder can be produced experimentally at high levels by *in vitro* combination of haem and apoglobins [2,3]. However, haem disorder also occurs naturally at low levels in sperm-whale (*Physeter macrocephalus*) myoglobin [1], and at high levels in myoglobin from yellowfin-tuna (*Thunnus albacurus*) muscle [4]. Haem disorder appears to have negligible functional consequences in sperm-whale myoglobin [5,6] – a finding consistent with the minor effects of reconstituting myoglobins with haems containing altered porphyrin substituents [7]. Haem disorder in Hb has been reported to somewhat increase autoxidation rates, and to alter subunit–ligand affinities [3].

Haem disorder is also observed at low levels (2% α - and 10% β -subunit disorder) in adult Hb (HbA) isolated from blood [2,3]. Fetal human Hb ($\alpha_2\gamma_2$) isolated from umbilical-cord blood shows 5% α - and 15% γ -subunit haem disorder [8]. For the adult and fetal Hbs these levels of haem disorder are thought to represent the equilibrium position *in vivo*. The extent and dynamics of haem disorder can be measured by NMR spectroscopy of the paramagnetically shifted haem peripheral methyl protons [2,3] and by CD spectroscopy of the haem Soret band [5,6,9]. Non-equilibrium levels of haem disorder in myoglobin and Hb relax to equilibrium levels on incubating the ferric Hbs at room temperature for 24 h [2,3]. Non-equilibrium haem disorder can be trapped by conversion of the ferric proteins into the low-spin cyanide or azide complexes, which show extremely slow haem reorientation and the best resolved haem methyl resonances [3].

Haem disorder is absent in myoglobins reconstituted with completely symmetric synthetic haems [10]. Along with nuclear-Overhauser-effect measurements [11], these experiments have demonstrated unambiguously that haem disorder arises from distinct haem orientations within the globin, rather than from different globin conformations around the haem.

The observation of a naturally occurring haem disorder in Hb raises the following questions about haem insertion during Hb biosynthesis. First, in the otherwise exquisitely regulated Hb biosynthetic pathway, why is haem insertion not stereospecific, and what is the nature of the apoglobin intermediates involved in haem insertion *in vivo*? Second, what are the levels of α - and β -subunit haem disorder at the completion of Hb synthesis *in vivo*, and do these levels relax to the observed equilibrium levels during the life of the erythrocyte cell as suggested previously? [2].

The mean lifetime of mature erythrocytes is 120 days [12]. Assuming steady-state erythrocyte production, the average age of Hb in mature erythrocytes is approx. 60 days. However, all Hb production occurs during maturation of the erythrocytic series and precedes formation of the mature erythrocyte. Maturation of the erythrocytic series occurs over approx. 7.6 days, during which time reticulocytes, the immediate precursors of erythrocytes, leave the marrow and enter the peripheral circulation, where they further mature into erythrocytes over approx. 1.3 days [12,13]. About 90% of the Hb synthesized is made during the 3-day lifetime of the reticulocyte and in the 2.3-day period preceding reticulocyte formation [12–14]. Thus, in contrast with the 60-day average age of mature erythrocyte Hb, peripheral reticulocyte Hb has an average age of approx. 2.7 days (65 h). Examination of reticulocyte Hb should therefore provide an estimate of the level of haem disorder arising during Hb biosynthesis.

Consequently, we have compared the levels of haem disorder in HbA obtained from erythrocytes whose reticulocyte content

Abbreviations used: HbA, adult human haemoglobin; DSS, 2,2-dimethyl-2-silapentane-5-sulphonate.

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was depleted (0% reticulocytes) with that of samples highly enriched in reticulocytes ($\geq 69\%$ reticulocytes). We have also measured the levels of haem disorder in recombinant adult ($\alpha_2\beta_2$) and embryonic human Hbs ($\alpha_2\epsilon_2$, $\zeta_2\epsilon_2$ and $\zeta_2\gamma_2$) expressed in yeast. In the yeast expression system, Hb synthesis is induced for only 12 h prior to harvest, and HB accumulates wholly in the ferrous state. This precludes any significant relaxation of haem disorder before Hb isolation, and allows comparison of haem disorder as a function of the biosynthetic machinery (in yeast and humans) and as a function of globin sequence.

EXPERIMENTAL

Blood was collected from a subject into EDTA-treated vacuum containers and used immediately. Leucocytes were removed from the whole blood by passage through a column of microcrystalline cellulose equilibrated with isotonic Hepes buffer, pH 7.4, containing KCl [15]. Reticulocytes were separated from aged erythrocytes by consecutive centrifugations on isopycnic density gradients constructed respectively from KCl- and NaCl-rich iso-osmotic arabinogalactan solutions [16]. Arabinogalactan (Larex UF) was obtained from Larex (St Paul, MN, U.S.A.). Cells from each density fraction were stained with Brecher's reticulocyte stain, and the percentage of reticulocytes was determined by microscopic examination at $1000\times$ magnification [16,17]. Recombinant Hbs were produced in yeast as described previously [18]. Hbs were purified in the carbonmonoxy (CO) form (in order to prevent any reorganization of the haems during processing) by a sequence of chromatographic steps on columns of carboxymethylcellulose CM52 (Whatman, Singapore), zinc-charged-chelating Sepharose Fast Flow, and Resource S or Resource Q ion-exchange columns (Pharmacia) [19,20]. Subunit stoichiometries were confirmed by reversed-phase HPLC analysis [19].

NMR measurements were made using a Bruker X 400 MHz NMR spectrometer (Acton, MA, U.S.A.) operated at 27°C , unless otherwise stated. Met-aquoHb samples were prepared from CO-Hb by oxidation with ten haem equivalents of potassium ferricyanide under intense light. Oxidation was followed by monitoring visible spectra, and was complete within 3 min. Met-azidoHb samples were prepared from CO-Hbs as above, but with the inclusion of 20 mM sodium azide to ensure immediate conversion into the stable met-azido complex [2]. Samples were exchanged into 25 mM Tris/HCl buffer, pH 8.0, containing 25 mM NaCl (with azide as required), by passage down a column of Sephadex G-25 (Fine grade). After concentration to 20–60 mg/ml, a small crystal of 2,2-dimethyl-2-silapentane-5-sulphonate (DSS) was added, and $^2\text{H}_2\text{O}$ added to 10% (v/v). These additions left the apparent pH unchanged (uncorrected for $^2\text{H}_2\text{O}$), and spectra were independent of pH in the range pH 7.5–8.0. Spectra were collected at an acceptable signal-to-noise ratio, typically requiring 1600–2000 transients. An acceptable spectrum of the reticulocyte Hb sample was obtained after 54000 transients. In all cases, spectra were collected with the following acquisition parameters: 20 MHz spectral width, 36000 data points, 300 ms water presaturation pulse, $6.5\ \mu\text{s}$ 90° pulse and a cycle time of 0.4 s. Chemical shifts are reported relative to DSS. Baseline-corrected NMR spectra were converted into JCAMP 5-column-XY format using Bruker software. These files were converted into 2-column-XY format and imported into PeakfitTM spectroscopy analysis software (SPSS, San Rafael, CA, U.S.A.) for determination of spectral components [3]. The experimental determination of haem disorder is based on the recognition that the two possible protein-

Figure 1 Molecular structure of the haem prosthetic group

The Figure shows the numbering system and rotational axis through the $\text{C}_\alpha\text{--C}_\gamma$ meso carbons.

bound haem rotamers (Figure 1) place the porphyrin peripheral methyl groups into different micro-environments within the haem-binding cleft. The methyl groups in each of these environments resonate at different chemical shifts. Thus, by determining the fraction of the haem methyl groups present in each rotamer (from the area of the NMR-spectral peak associated with the appropriate methyl group), it is possible to obtain a measure of haem disorder. As the resonance peaks overlap in most spectra, we employed a non-linear least-squares fit of the entire spectrum in order to obtain amplitude and width parameters for individual resonances from which we could calculate relative areas. Unsmoothed spectral data points were all simultaneously used to fit the spectrum to the sum of the appropriate number of resonances. Convergence was normally achieved in less than 20 iterations with amplitudes and widths varying freely. The coefficient of determination (r^2) was typically > 0.995 , and the fitted amplitudes and width parameters for any particular peak typically had a 95% confidence at $\pm 0.5\%$ to $\pm 1\%$ of their determined values. Comparison of values obtained from different pairs of resonance peaks within a single spectrum showed that the percentage-disorder values are consistent to within $\pm 1\text{--}2\%$. As no statistically rigorous determination of the absolute error associated with the percentage disorder values is available, we estimate that our reported values are likely to be accurate to within $\pm 1\text{--}2\%$. Percentage disorder was calculated from the following equation:

$$\text{Disorder (\%)} = \frac{(\text{area of minor signal})}{(\text{area of major} + \text{area of minor signal})} \times 100$$

In order to obtain a value for haem disorder in pure reticulocyte-derived haemoglobin, we need to correct our data for erythrocyte contamination using the expression

$$D_r = D_o + C_e V_e F_e (D_o - D_e) / C_r V_r F_r$$

where D_r , D_e and D_o are the pure reticulocyte Hb, contaminating erythrocyte Hb and the observed haem-disorder levels for a given

cell fraction respectively. F_e and F_r are the observed fraction of erythrocytes and reticulocytes in a given cell fraction respectively, and C and V are the Hb concentrations and cell volumes of the respective cell types. For cells isolated in the same density fraction we assume $C_e = C_r$, and the cell-volume ratio is known ($V_e/V_r = 0.86$ [12]). The disorder level of pure reticulocyte HbA for each cell fraction can thus be estimated by assuming the contaminating erythrocyte HbA has equilibrium haem-disorder levels (α 1.5%, β 8%). For the 69%-reticulocyte fraction we estimate pure reticulocyte HbA has 15% α - (cf. 11% observed) and 36% β -haem disorder. These values are probably overestimates, because the contaminating erythrocytes are the youngest mature erythrocytes [16,21], and their Hb may not have attained equilibrium haem-disorder levels.

RESULTS

Manipulation of reticulocyte cell density by incubation in potassium- or sodium-rich buffers, followed by density-gradient centrifugation [16], enabled us to isolate erythrocyte fractions containing < 0.1%–85% reticulocytes. Beginning with 100 ml of whole blood, the most enriched fraction contained only a few hundred micrograms of Hb. Experimental requirements dictated use of the fraction containing $\geq 69\%$ reticulocytes and approx. 5 mg of total Hb.

The downfield regions of the 400 MHz NMR spectra of HbA isolated from erythrocyte fractions containing $\leq 0.1\%$ and $\geq 69\%$ reticulocytes are shown in Figures 2(A) and 2(B), respectively. The spectral assignments and results of curve-fitting for all Hbs are shown in Table 1. The spectra exhibit well resolved hyperfine shifted haem methyl resonances in the 13–29 p.p.m. region. Analysis of the NMR spectrum of Hb from mature erythrocytes (Figure 2A) shows it is almost identical in form and the amount of haem disorder (1.5% α -, 8% β -haem disorder) with that reported previously for adult Hb [2,3]. Although clearly arising from the same signals as the spectrum of mature erythrocyte Hb (Figure 2A), the NMR spectrum of reticulocyte Hb (Figure 2B) shows markedly elevated levels of haem disorder (11% α -, 28% β). Even higher levels of haem disorder (32% α -, 45% β) were observed in recombinant HbA isolated from yeast after a 12 h expression period (Figure 2D and Table 1). The high levels of disorder in recombinant HbA returned to the low equilibrium values typical of erythrocyte HbA on incubation in the met-aquo form for 25 h (Figure 2C and Table 1).

The downfield regions of the NMR spectra of recombinant embryonic human Hbs $\alpha_2\epsilon_2$, $\zeta_2\epsilon_2$ and $\zeta_2\gamma_2$ are shown in Figure 3. Hbs $\zeta_2\epsilon_2$ and $\zeta_2\gamma_2$ show well resolved haem methyl resonances, but these signals are poorly resolved for $\alpha_2\epsilon_2$ at 27 °C. Spectral resolution for $\alpha_2\epsilon_2$ was improved greatly by data collection at 45 °C, enabling signal assignments for the spectrum collected at 27 °C. Signal assignments for the embryonic Hbs were made by analogy with the spectrum of adult Hb and sequential comparison of spectra from Hbs containing common subunits (i.e. $\alpha_2\beta_2 \rightarrow \alpha_2\epsilon_2 \rightarrow \zeta_2\epsilon_2 \rightarrow \zeta_2\gamma_2$). We also assumed signals arising from the reversed-haem orientation would occur downfield of the main signal, as found for HbA [2,3]. Assignment of γ -subunit haem methyl resonances was assisted by comparison with published spectra for fetal Hb ($\alpha_2\gamma_2$) [8]. For Hb $\zeta_2\epsilon_2$ and Hb $\zeta_2\gamma_2$, NMR spectroscopy was also used to monitor titrations of the ferric Hbs with sodium azide (Figure 4). This aided assignment of ζ - and γ -signals, because γ -subunits have higher affinity for azide, and signals arising from these subunits appear before ζ -subunit signals during the titration [8]. Titrations with azide followed by visible spectroscopy also confirmed that the haem groups were

Figure 2 NMR spectra of HbA from mature erythrocytes, reticulocytes and yeast

(A) The spectrum of HbA isolated from mature erythrocytes. (B) The spectrum of HbA isolated from a red-blood-cell fraction containing 69% reticulocytes and 31% mature erythrocytes. (C) The spectrum of recombinant HbA from yeast after 25 h incubation in the met-aquo form. (D) The spectrum of freshly isolated recombinant HbA from yeast. Signal assignments are shown in normal type and *italics* for crystallographic (major form at equilibrium) and non-crystallographic haem orientations respectively. All Hbs were in the ferric-azido form. Spectra were recorded at 27 °C and the sample buffer was 23 mM Tris/HCl, pH 8.0, containing 20 mM sodium azide, 23 mM NaCl and 10% $^2\text{H}_2\text{O}$. Signal assignments are indicated in the Figure, and also in Table 1. The calculated percentage haem disorder are recorded in Table 1.

fully saturated with azide under these conditions (results not shown).

Despite identical growth conditions and expression periods in yeast, the levels of haem disorder in $\alpha_2\epsilon_2$ (25% α -, 29% ϵ , Table 1), $\zeta_2\epsilon_2$ (17% ζ -, 22% ϵ) and $\zeta_2\gamma_2$ (22% ζ -, 23% γ) were lower than observed for recombinant HbA (32% α -, 45% β). In contrast with the result for recombinant HbA, incubation of ferric Hbs $\zeta_2\epsilon_2$ and $\zeta_2\gamma_2$ at neutral pH and room temperature for 48 h had no effect on their haem disorder levels, suggesting that, for these proteins, biosynthetic haem insertion yields the equilibrium product directly. Ferric Hb $\alpha_2\epsilon_2$ precipitated after several hours under these conditions preventing comparison of haem disorder in aged and freshly isolated protein. This also precluded the use of azide titrations to resolve signals arising from α and ϵ subunits in the NMR spectrum of Hb $\alpha_2\epsilon_2$ collected at 27 °C.

DISCUSSION

Haem disorder in HbA

Recently synthesized HbA isolated from reticulocytes (15% α -, 36% β -subunit disorder; calculated for pure reticulocytes as

Table 1 Signal assignments and chemical shifts for Hb spectra, and calculated percentage haem-disorder levels

Numbers and letters in **bold** and *italics* arise from the crystallographic (major form at equilibrium) and non-crystallographic haem orientations, respectively. Percentage-disorder values have been determined experimentally and have an estimated error of $\pm 1\text{--}2\%$ (see the Experimental section). The retic value corresponds to 15% α and 36% β in pure reticulocytes (see the text). Abbreviations: M, methyl group; retic, reticulocyte; SA, signal assignment; V, vinyl C_α proton.

(a)																
Hb	Haem disorder (%)			Chemical shift (p.p.m. from DSS)												
	α	β	SA ...	8M α	5M α	8M β	5M β	1M α	4V α	1M β	4V β	5M α	5M β	8M α	8M β	
$\alpha_2\beta_2$ (0% retic)	1.5	8		27.9	27.4	26.5	26.1	22.4	21.5	20.7	18.1	16.3	15.2	14.8	13.7	
$\alpha_2\beta_2$ (69% retic)	11	28		27.7	27.5	26.5	26.1	22.4	21.5	20.7	18.1	16.3	15.2	14.8	13.7	
$\alpha_2\beta_2$ (yeast, new)	32	45		27.9	27.5	26.6	26.1	22.4	21.5	20.8	18.2	16.3	15.2	14.9	13.7	
$\alpha_2\beta_2$ (yeast, aged)	2.9	10		28.1	27.4	26.9	26.1	22.4	21.5	20.7	18.1	16.3	15.2	14.8	13.7	
(b)																
Hb	Haem disorder (%)			Chemical shift (p.p.m. from DSS)												
	α	ϵ	SA ...	8M α	5M α	8M ϵ	5M ϵ	1M α	1M ϵ	4V α	5M α	5M ϵ	8M α	8M ϵ		
$\alpha_2\epsilon_2$	25	29		28.2	27.8	27.3	27	22.3	21.9	21.3	16.6	16.3	15.2	14.9		
(c)																
Hb	Haem disorder (%)			Chemical shift (p.p.m. from DSS)												
	ζ	ϵ	SA ...	8M ζ	5M ζ	8M ϵ	5M ϵ	1M ζ	1M ϵ	5M ζ	8M ζ	5M ϵ	8M ϵ			
$\zeta_2\epsilon_2$	17	22	29.3	28.3	27.2	26.6	23.2	22	18.8	17.7	16.3	14.5				
(d)																
Hb	Haem disorder (%)			Chemical shift (p.p.m. from DSS)												
	ζ	γ	SA ...	8M ζ	5M ζ	8M γ	5M γ	1M ζ	1M γ	5M ζ	8M ζ	8M γ				
$\zeta_2\gamma_2$	22	23		29.4	28.1	26.5	26.1	23.1	21.9	18.8	17.3	15

Figure 3 Comparison of the downfield hyperfine-shifted portions of the 400 MHz ^1H NMR spectra of human embryonic Hbs and HbA

The spectra of HbA and embryonic Hb are indicated. All Hbs were present as the ferric-azido form. Other conditions were as described in Figure 2. Indicated signal assignments are also recorded in Table 1. The protein used in each experiment is identified at the left of each trace.

described in the Experimental section) and yeast (32% α , 45% β) shows considerably higher levels of haem disorder than that found in mature erythrocyte HbA (1.5% α , 8% β), but lower

than that found in freshly reconstituted haemoglobin. The elevated levels of haem disorder in newly synthesized HbA apparently reach the equilibrium position within the 60-day average age of the erythrocyte. Thus, as suggested originally by La Mar and co-workers [2], our results show that haem disorder results from haem insertion during Hb biosynthesis, that Hb containing more extensive haem disorder does exist *in vivo*, and suggest that Hb containing haem disorder is a functional protein. Our results also show a significant asymmetry in haem-disorder levels between α - and β - subunits that is greater in reticulocyte HbA than in recombinant HbA [2,3]. These intermediate levels of haem disorder could arise in two ways. First, if biosynthetic haem insertion leads initially to Hb with equal levels of each haem orientation, the observed levels could reflect a partial approach to the equilibrium position between the time of Hb synthesis and isolation. Elapsed times ranged from an average of approx. 76 h for reticulocyte Hb (average time of synthesis plus isolation time), to approx. 6.5 h for recombinant Hb. Alternatively, if negligible haem reorientation has occurred, the intermediate levels of haem disorder could be a direct result of biosynthetic haem insertion and indicate that this is a stereoselective process. However, the results presented here suggest that haem insertion into apoglobins *in vivo* probably occurs by a mixture of stereoselective and random insertion mechanisms.

We cannot exclude the possibility that our reticulocyte HbA data reflect the occurrence of some haem reorientation (i.e. partial approach to equilibrium) between the time of Hb synthesis and isolation. The rates of haem reorientation in the products of

Figure 4 Titration of human embryonic ferric-Hb $\zeta_2\gamma_2$ with azide ions

Ferric-Hb $\zeta_2\gamma_2$ was titrated with sodium azide and the hyperfine-shifted haem methyl resonances were recorded at the azide/haem molar ratios indicated. Other conditions were as described in Figure 2. The signals marked with an asterisk arise from small amounts of contaminating free γ -subunits. Samples of Hb $\zeta_2\gamma_2$ used for measurement of % haem disorder were further purified by ion-exchange chromatography to remove free γ -subunits to trace levels. Indicated signal assignments are also recorded in Table 1.

Hb synthesis (deoxy- and oxy-Hb) are reported to be extremely low [2], but differences in experimental conditions prevent quantitative extrapolations to the results presented in this study. Equilibration of haem orientations occurs *in vivo* in the ferric subunits of mixed-valence met-aquo/ferrous Hb species which are at steady-state levels during the normal redox cycling of Hb. However, considering the low average age of reticulocyte HbA, significant relaxation of haem-disorder levels by this mechanism over only 3 days would require much greater turnover through metHb than the 0.5–3% per day observed for HbA in mature erythrocytes [22]. It is possible this could occur if the oxidative destruction of mitochondria during reticulocyte maturation exposes Hb to greater oxidative stress than in normal erythrocytes [23]. Elevated autoxidation rates could also account for the asymmetry in subunit haem-disorder levels. However, these levels contrast with those expected during haem reorientation in fully oxidized met-aquoHb, where β -subunit haem reorientation is 4-fold faster than in α -subunits at pH 7.4 [3]. At this pH, autoxidation of α -subunits is 30–50% faster than β -subunits [24,25], allowing for their faster approach to equilibrium haem disorder as suggested by the results in the present study. However, we do not know of any evidence for elevated redox cycling of reticulocyte Hb, and active synthesis of the metHb reductase system in reticulocytes argues against it [26]. Consequently, we consider it more likely that the asymmetric and intermediate haem-disorder levels in reticulocyte HbA are the direct result of

stereoselective haem insertion, rather than the consequence of partial haem reorientation.

Haem disorder in recombinant Hbs

Recombinant HbA was produced in yeast during a 12 h expression period. In this case there is only one cell type and the average Hb molecule is only 6 h old, precluding any haem reorientation between Hb synthesis and isolation. When HbA is produced under these circumstances, the initial product exhibits 32% α - and 45% β -subunit disorder. These levels relaxed to the equilibrium position when the Hb was maintained in the met-aquo form for 25 h (Figure 2C, 2.9% α , 10% β disorder). This clearly indicates that synthesis of HbA in yeast leads initially to a metastable form in which haem insertion is essentially random for β -globin, but partially stereoselective for α -globin. This observation is extended by the results for the three embryonic Hbs, where all subunit types show evidence for partially stereoselective haem insertion (Figure 3 and Table 1). In contrast with the asymmetry between the haem-disorder levels of α - and β -subunits within HbA, the embryonic Hbs show similar levels of haem disorder in each subunit type, both within and between Hbs. For the Hbs expressed in yeast the observed dependence of haem disorder on globin sequence is $\beta > \alpha \cong \epsilon \cong \zeta \cong \gamma$. Comparison of haem disorder levels between the α -subunits of recombinant $\alpha_2\beta_2$ and $\alpha_2\epsilon_2$ shows a modest effect of partner subunit on α -subunit haem disorder levels. It is possible that larger differences in haem disorder might exist between the embryonic Hbs when they are expressed in erythroid cells, as observed for HbA where disorder is correlated with the site of synthesis. However, this seems less likely for Hbs $\zeta_2\epsilon_2$ and $\zeta_2\gamma_2$, because they show equilibrium haem-disorder levels when isolated from yeast.

In contrast with the negligible effects of partner subunits on haem-disorder levels, the NMR spectra of the recombinant Hbs show there are small, but significant, differences in chemical shifts for haem methyl resonances from a particular subunit type within different tetramers, for example α in $\alpha_2\beta_2$ or $\alpha_2\epsilon_2$ (Table 1). These differences do not appear to arise from any major alteration in globin structure induced by the presence of different globin partners. Comparison of the three-dimensional structures of the α -subunit in $\alpha_2\beta_2$ and $\alpha_2\epsilon_2$ clearly indicates that the common α -subunit has, within experimental error, exactly the same tertiary structure which is independent of its partner [27]. The observed small chemical shifts for any particular subunit could well arise from small differences in axial nodal plane orientation of the proximal His residue. A rotation of approximately 0.5° of the proximal His would be quite sufficient to produce the small observed differences of < 0.4 p.p.m. observed for any particular chain [28,29], but would not be detectable from X-ray-diffraction studies of the protein. The larger differences in chemical shift for the same resonances from any pair of corresponding subunit types (e.g. ϵ and β), undoubtedly arise from a combination of the recognized tertiary structural differences between the subunits [27], and the differences in amino acid composition within their haem-binding cavities.

Nature of haem insertion in Hb biosynthesis

Although we have not identified a unique route for haem insertion during Hb biosynthesis, the results from the present study provide insight into the origin of haem disorder and the nature of the intermediates involved. There are large differences between the nature of haem insertion *in vivo* and that occurring during reconstitution of Hb *in vitro*. In rapid-mixing experiments, an excess of a haem species (usually ferric) is mixed with apoHb at

neutral pH, leading to formation of Hb containing randomly oriented haem groups in processes occurring in the millisecond to seconds time scale [3,30,31]. In contrast, *in vivo*, the cytoplasmically synthesized apoglobins obtain ferrous deoxyhaem from the outer mitochondrial membrane. The majority of the holoprotein is synthesized in the reticulocyte over a 3-day period, with carefully co-regulated synthesis of haem and apoglobins, which avoids formation of a large excess of either reaction partner. Analysis of radiolabelled cell lysate shows that pools of apo- α and apoHb (apo- $\alpha\beta$ dimers) are present in normal reticulocytes [32]. These species and the partially haem-saturated semiHbs can all bind haem *in vitro* [30,33–37]. *In vitro* experiments have shown that reaction of haem with the vacant β -site in semiHb- α yields a single haem orientation in the β subunit, i.e. 0% (*sic*) disorder, whilst the corresponding reaction with the semiHb- β yields a product with 40% disorder in the α -chain [38]. This difference in reactivity arises from the fact that, not only is the rate of initial binding of haem to the vacant β -site in semiHb- α reduced from that in the isolated chain [36,39] (unlike the case of the semiHb- β), but the subsequent rate-limiting structural rearrangement reactions provide a different overall mechanism for haem binding in the two semiHbs [39]. These kinetic and mechanistic differences themselves appear to have their origins in the relative structural flexibilities of the α - and β -chains and the differential modulation of this flexibility by the haem-bound partner chain [38,39]. These results, which show lower disorder in the α - than β -subunits, thus suggest that haem insertion *in vivo*, at least in part, occurs initially into the β -chain followed by haem insertion into the α -chain of semiHb. The absence of highly stereoselective haem insertion (as observed *in vitro* for purely semiHb species [38]) indicates a significant amount of haem insertion *in vivo* occurs co-translationally as well as into α -globin and apoHb, producing semiHb- α , as observed *in vitro* [31,37,40].

The haem-disorder levels observed in recombinant HbA expressed in yeast also suggest the major initial haem binding species are β -globin and semiHb- β , but the lower asymmetry and higher overall disorder levels indicate more initial haem insertion occurs in α -globin and semiHb- α in yeast than in reticulocytes. This could be the result of differences in expression conditions between the two cell types. In our yeast expression systems Hb is produced over a period of hours in cells which have been primed for maximal haem synthesis. In contrast with reticulocytes, the globin genes are located on a plasmid in the yeast system, and we do not expect tight co-regulation of haem and globin synthesis. Furthermore, the intracellular pH in yeast is considerably lower than in the reticulocyte [41,42]. Acidic pH has been shown to alter the stability of the subunit interface in apo-protein dimers, but not in holo-protein dimers [43]. This may well lead to a less structured apoglobin at low pH and hence less stereoselective haem insertion. Elevated levels of β -subunit haem disorder were also observed in recombinant HbA produced in *Escherichia coli* [44], although these levels were lower than we observed in the present study for HbA produced in yeast.

Interestingly, we found the recombinant embryonic Hbs $\zeta_2\epsilon_2$ and $\zeta_2\gamma_2$ contained equilibrium haem-disorder levels, as isolated, indicating they are a direct result of haem insertion. This is in contrast with the result for recombinant HbA, despite identical expression conditions. For the embryonic subunits, no results are available concerning the stereoselectivity of *in vitro* haem insertion into the corresponding globins. Thus we can only speculate as to whether the corresponding semiHbs are responsible for the observed stereoselectivity in the haem insertion reactions, or whether this is intrinsic to the isolated globins. However, we do note that the embryonic Hbs exhibit much stronger binding of haem in their β -type subunits [45], and this

phenomenon could well influence the selection of haem orientation in any semiHb intermediates. Further support for the primacy of β -type subunits in determining overall haem disorder levels comes from the observation of a decrease in recombinant α -subunit haem disorder when the partner subunit is changed from β to ϵ . Haem disorder thus appears to preserve a record of the ensemble of apoglobin tertiary and quaternary structures present at the time of haem insertion. The detailed nature of the mechanism whereby eukaryotes achieve stereoselective haem insertion into globin proteins remains to be investigated.

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