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Alpha-haemoglobin stabilizing protein (AHSP) stabilizes apo- α -haemoglobin in a partially folded state

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SYNOPSIS

To produce functional haemoglobin, nascent α -globin (α^0) and β -globin (β^0) chains must each bind a single haem molecule (to form α^h and β^h) and interact together to form heterodimers. The precise sequence of binding events is unknown, and it has been suggested that additional factors might enhance the efficiency of Hb folding. The α -haemoglobin stabilizing protein (AHSP) has previously been shown to bind α^h and regulate redox activity of the haem iron. Here, we use a combination of classical and dynamic light scattering and NMR spectroscopy to demonstrate that AHSP forms a heterodimeric complex with α^0 that inhibits α^0 aggregation and promotes α^0 folding in the absence of haem. These findings indicate that AHSP may function as an α^0 -specific chaperone, and suggest an important role for α^0 in guiding Hb assembly by stabilizing β^0 and inhibiting off-pathway self-association of β^h .

INTRODUCTION

Haemoglobin (Hb) is one of the best studied of all proteins, and a system that has established many paradigms in the relationship between protein structure and function [1]. However, an area that remains comparatively poorly understood is Hb folding and assembly [2]. A specific protein chaperone that may assist Hb folding has recently been proposed [3], but the mechanism has not been extensively explored.

Adult human haemoglobin (Hb A) is a tetramer of two α -haemoglobin (α^h) and two β -haemoglobin (β^h) subunits [4]. The apo globin chains (α^0 and β^0) lack the haem prosthetic group and precipitate readily, indicating that haem plays a key role in stabilizing the α^h and β^h structures [5]. The haem-bound chains also require stabilization by binding to each other, as evidenced by increased rates of precipitation [6], elevated haem/iron release [7] and

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AUTHOR CONTRIBUTIONS

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accelerated autoxidation [8] of the free chains compared to Hb A. The α^h and β^h subunits form a strong $\alpha^h\beta^h$ heterodimer, which exists in equilibrium with the $\alpha^h_2\beta^h_2$ tetramer [9]. There is evidence that $\alpha^h\beta^h$ assembly proceeds through semi-Hb intermediates, $\alpha^0\beta^h$ or $\alpha^h\beta^0$ [10, 11], which may serve to increase the haem-affinity of apo-subunits [7]. These species confer enhanced stability and possess many functional properties of the intact Hb A [12].

Although Hb can be reconstituted from apo globins and haem *in vitro*, it is possible that other factors are involved in establishing an ordered assembly pathway *in vivo* to ensure maximum efficiency by, for example, ensuring that haem becomes inserted in the correct orientation [13]. Recently, we obtained genetic and biochemical evidence that α -haemoglobin stabilizing protein (AHSP) may have a role in Hb assembly [3]. AHSP has been proposed to sequester free α^h that is produced during normal Hb synthesis, and to inhibit its ability to cause oxidative damage [14–17]. This mechanism permits a slight overproduction of α^h in order to competitively inhibit the formation of deleterious β^h_4 tetramers [18]. More recently, we have found that knocking out AHSP expression in both α - or β -thalassaemia mice leads to an exacerbated anaemia [3, 15]. This result suggests that AHSP plays an active role in the process of Hb chain assembly, irrespective of the final Hb chain balance. Here, we characterize the effects of AHSP binding on α^0 structure and aggregation state, and compare these with the effects of β^h/β^0 binding.

EXPERIMENTAL

Protein sample preparation

AHSP, Hb A, α^h and β^h were produced as previously described [19]. AHSP obtained from this procedure contained the full-length native sequence (102 residues) with an additional Gly-Ser dipeptide at the N-terminus. Hb A, α^h and β^h were obtained from human blood. To generate α^0 and β^0 , purified Hb A was applied to a C4 reversed-phase HPLC column in 35% acetonitrile, 55% H₂O, 0.1% trifluoroacetic acid and developed with a 35–55% acetonitrile gradient applied over 30 minutes, similar to the method of Masala *et al.* [20]. The elution was monitored at 280 and 400 nm and three peaks were eluted in the order: haem, β^0 and α^0 . Alternatively, apo-globins were prepared from de-salted α^h and β^h subunits by acid-acetone extraction, in which the haem is solubilised and the protein fraction is precipitated, collected, washed and dialysed into H₂O [21]. Fractions were lyophilized and stored at –80 °C prior to use. Haem contamination was determined by UV-visible absorption spectroscopy in 6 M guanidine HCl, 10 mM Tris. HCl, pH 8.0: haem concentration was calculated using an absorption coefficient at 390 nm (ϵ_{390}) of 37800 M^{–1} cm^{–1} [19] and polypeptide concentration was obtained using ϵ_{280} values calculated from the amino acid sequence (neglecting the small contribution from residual haem). Both methods yielded a typical haem contamination less than 1% (mol/mol).

To produce ¹⁵N-labelled α^0 for NMR we used the Hb A expression plasmid pHE7, kindly provided by Dr. Chien Ho, and purified Hb A according to established protocols [22, 23]. This method yields Hb A subunits with native N-termini. The ¹⁵N-labelled Hb A was purified subject to the same procedures as native Hb A [19] to obtain α^0 .

Circular dichroism (CD) spectroscopy

Spectra were collected on a Jasco J-720 spectropolarimeter, using a 1-mm path length cell, and the temperature was controlled using a water-jacketed cell holder and calibrated by means of a thermocouple placed inside the CD cell. Camphor-10-sulfonic acid (CSA) was used as a calibration standard. A 1 mg ml⁻¹ solution of CSA was prepared using the absorbance extinction coefficient of 34.6 M⁻¹ cm⁻¹ [24]. Using a 0.1 cm path of CSA standard the raw ellipticity at 290.5 nm was adjusted to be 33.5 [25], after correction for the unobstructed beam. The total absorbance was monitored to ensure adequate signal strength across the spectrum (HT < 600). Final spectra were the sum of a minimum of three scans. For the thermal denaturation measurements, the ellipticity at 222 nm was monitored over a temperature range of 15–88°C and a temperature gradient of 1°C min⁻¹.

Dynamic light scattering (DLS)

DLS measurements were made in 20 mM sodium phosphate, pH 6.3, using a DynaPro™ instrument (Protein Solutions). The time-dependent fluctuation in scattering intensity was fit to one or more intensity autocorrelation functions to extract the corresponding translational diffusion coefficients (D) using the manufacturer's software. The Stokes radius (R_S ; the radius of the corresponding hard sphere with the same diffusion coefficient) was calculated according to

$$R_S = \frac{RT}{6\pi N_A \eta D}$$

The viscosity of the solution (η) was taken to be 1.005×10^{-2} poise at 20 °C and temperature corrected using the manufacturers software. Molecular weight (M) was estimated from R_S according to

$$M = \frac{4\pi N_A R_S^3}{3(\bar{v} + \delta)}$$

where N_A is Avogadro's constant, $\bar{v}$ is the partial specific volume (assumed to be 0.73 cm³ g⁻¹), δ is a hydration factor (assumed to be 0.3 g H₂O per 1 g protein).

Multi-angle light scattering (MALS)

Samples were applied to a Superose 12 column (GE Healthcare) and eluting protein fractions were monitored by in-line MALS and refractive index (RI) measurements. MALS intensity was measured at three angles (41.5°, 90.0° and 138.5°) using a mini-DAWN (Wyatt Technology Corp., Santa Barbara CA) equipped with a 690 nm laser. Voltages for the three detectors are normalised using BSA monomer (Sigma) as a standard isotropic scatterer. Absolute MALS intensity was calibrated against a standard sample of HPLC grade toluene. Protein concentrations are obtained from in-line RI measurements (Optilab differential refractometer, Wyatt Technology Corp.), calibrated against standard salt solutions. The refractive index increment with respect to mass concentration (dn/dc) was taken to be 0.19 ml g⁻¹ for all proteins/complexes. Weight-average molecular weight (M_w)

of the solute was calculated for every 5 μ l volume across each eluting peak (a single data “slice”) using the Rayleigh-Debye-Gans scattering model for a dilute polymer solution, as implemented by the ASTRA software (Wyatt Technology Corp.).

NMR

All NMR measurements were conducted on Bruker Avance 600 and 800 MHz spectrometers equipped with 5 mm triple-resonance (^1H , ^{13}C , ^{15}N) cryoprobes. Samples were prepared at 0.2–0.4 mM protein concentration in water and adjusted to 10 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 6.3 (95% $^1\text{H}_2\text{O}$, 5% $^2\text{H}_2\text{O}$; pH values were uncorrected for the presence of $^2\text{H}_2\text{O}$) with 40 μM 5,5-dimethylsilapentanesulfonate (DSS) included as a 0 ppm ^1H chemical shift reference. The ^{15}N chemical shift scale was referenced indirectly from the DSS. Backbone resonances for α^{h} in complex with AHSP were obtained from HNCA, CBCA(CO)NH, HNCACB and ^{15}N NOESY-HSQC experiments.

RESULTS

α° preparations adopt a variable α -helical content

To investigate the structure of α° , haem was extracted from purified α^{h} in ice-cold acid-acetone [21], or by reversed-phase HPLC purification of Hb A [20]. Both methods achieved haem contamination levels of < 1% (Figure 1A). At pH 3.2, the far-UV circular dichroism (CD) spectrum of α° indicated a largely unfolded conformation. Upon raising the pH to 6.3 a spectrum characteristic of α -helical secondary structure was obtained (Figure 1B), consistent with numerous previous studies [5, 19, 21, 26, 27]. At 8 $^{\circ}\text{C}$, CD spectra indicate that α° retains a significant fraction of the native secondary structure compared to α^{h} (Figure 1C, solid and dashed lines). Raising the temperature to 30 $^{\circ}\text{C}$ induced a significant reduction in α -helical content of α° and an apparent increase in unfolded species, as evidenced by a reduction in ellipticity at 192 and 222 nm and a blue shift in the minimum below 208 nm. By comparison the CD spectra of α^{h} and AHSP showed little variation within this temperature range (not shown). Unusually, the CD spectrum of α° obtained from different preparations showed significant variation in mean residue ellipticity at 222 nm, and in the 208/222 nm ellipticity ratio. These observations suggest that α° samples readily undergo an irreversible change during purification and storage.

α° contains regions of well-ordered tertiary structure

To investigate the tertiary structure of α° we produced ^{15}N -labeled Hb A in an *E. coli* expression system [22] and purified the ^{15}N -labelled α° subunits for NMR studies. As shown in Figure 1D, the ^1H , ^{15}N heteronuclear single quantum coherence (HSQC) spectrum of α° at pH 3.2 is indicative of an unfolded state, displaying sharp NMR signals with most H_{N} resonances clustered in the range 8–8.7 ppm. The number and position of signals is as expected for the 141 residue sequence. Upon raising the pH to 6.3 we saw a general increase in chemical shift dispersion indicating the appearance of some well-ordered tertiary structure (e.g., Figure 1E, hatched box 1). A number of signals from the pH 3.2 spectrum remain (e.g., hatched box 2). Thus the sample appears to contain both folded and unfolded α° polypeptides. The distribution between the folded and unfolded forms was variable between preparations. A comparison with the ^{15}N -HSQC of α^{h} (Figure 1F) does not reveal any

perfectly overlapping signals; however this is not unexpected considering the large chemical shifts that would be induced by the haem group. Due to sample instability over a wide range of solution conditions it was not possible to obtain resonance assignments for the folded form of α^0 . The average ^{15}N linewidths for both folded and unfolded α^0 conformers at pH 6.3 are similar at ~ 18 Hz, and are significantly broadened compared to spectra of α^h (14.5 ± 1 Hz), which undergoes monomer-dimer equilibrium, and acid unfolded α^0 (8.0 ± 0.5 Hz, presumed monomer), suggesting that folded and unfolded α^0 species are aggregated at pH 6.3.

Unfolding and aggregation of α^0 is inhibited by AHSP

To investigate the influence of AHSP on the stability of α^0 we performed CD thermal melts, monitoring ellipticity at 222 nm. AHSP undergoes a completely reversible thermal unfolding process, with the mid-point temperature of unfolding (T_m) of 57.1 ± 1.7 °C (Figure 2A, dotted line, and Table 1). In contrast, free α^0 showed a complex, non-cooperative and non-reversible denaturation curve (Figure 2A, solid). Addition of AHSP conferred a significant stabilization of α^0 at physiologically relevant temperatures below 40 °C (Figure 2A, dashed). Indeed, AHSP was almost as effective at stabilizing α^0 in this assay as haem (Figure 2B, solid). By comparison, AHSP confers little thermal stabilization of α^h (Figure 2B, dot-dashed line). Hb A is considerably more stable than any of the others species, presumably reflecting the effect of the very high-affinity $\alpha^h:\beta^h$ dimer.

To investigate that basis for α^0 stabilization we monitored the temperature-induced formation of soluble aggregates using dynamic light scattering (DLS) to determine Stokes' radius (R_S). The R_S measured for α^0 at 10 °C was considerably larger than expected for a folded globular monomer (Table 1), instead being consistent with aggregated or denatured protein – the empirical relationship $R_S = 0.221N^{0.57}$ nm, relating R_S to protein chain length (N) for a denatured protein [28] yields $R_S = 3.7$ nm for α^0 , close to the measured value. AHSP had a R_S of 1.7 ± 0.1 nm, consistent with the expectation for a monomeric protein of 12 kDa (Table 1). Addition of 1 molar equivalent of AHSP to α^0 gave rise to a single species with $R_S = 2.6 \pm 0.1$ nm, consistent with dissociation of α^0 aggregates and/or compaction of the α^0 structure. On raising the temperature, aggregates were seen to develop in free α^0 solutions (Figure 2C, filled triangles). Addition of AHSP inhibited this aggregation up to ~ 35 °C (Figure 2C, open triangles, dashed line), beyond which the sample rapidly converted to large (>100 nm) particles, with visible precipitation. Similar results were obtained for a α^h :AHSP complex (Table 1 and Figure 2C). Hb A and AHSP gave no indication of aggregation up to the temperature limit of the DLS apparatus (60 °C; not shown).

The stabilizing effect of AHSP on α^0 precipitation was further demonstrated in a turbidity assay. Proteins were incubated at 25 °C and absorbance at 700 nm was monitored as an indirect measure of light scattering by insoluble material. Precipitate formed rapidly in solutions of α^0 (Figure 2D, closed triangles). Addition of AHSP completely abolished α^0 precipitation over the 80-minute time course of the experiment (Figure 2D, open triangles).

α^o binds AHSP, β^h or β^o to form dimeric complexes

To characterize the interactions of α^o with potential binding partners in Hb assembly we performed multi-angle classical light scattering (MALS) in-line with size exclusion chromatography (SEC). MALS yields the weight-average molecular weight (M_w) of proteins in solution, independent of shape or frictional properties. Figure 3A shows the use of this method to characterize the interaction between AHSP and α^h , known to be a dimer from crystallographic studies. The measured M_w of free α^o (18 kDa) is higher than expected for a monomer (15.7 kDa), as a result of weak concentration-dependent self-association (data not shown and ref [29]). AHSP has $M_w = 11.8 \pm 0.2$ kDa (independent of loading concentration) but elutes considerably earlier than α Hb from SEC due to a more elongated shape (greater R_s). Mixing of a 1:1.5 molar ratio of AHSP: α^h , followed by SEC, yielded a monodisperse complex with $M_w = 25.3 \pm 1.2$ kDa, close to the value expected for a 1:1 complex (Figure 3A, solid line) and a smaller peak corresponding to unbound α^h .

At pH 6.3, α^o eluted from SEC in a broad asymmetric peak with a broad distribution of particle sizes ranging from ~20 kDa to over 100 kDa (Figure 3B and Table 1), indicative of non-specific self-association. Addition of an equimolar quantity of AHSP caused a dramatic shift to a discrete α^o :AHSP dimer with a M_w of 26 ± 0.20 kDa (Figure 3C). Using this approach we found that α^o also forms a dimeric complex with β^h and in doing so effectively inhibits the formation of β^h tetramers (Figure 3D). Even more surprisingly, α^o and β^o , which are both unstable and aggregated in isolation, interact to form a more stable $\alpha^o\beta^o$ dimer that elutes as a single peak (Figure 3E). These data highlight α^o :AHSP, $\alpha^o\beta^h$, and $\alpha^o\beta^o$ as potentially important intermediates in Hb A assembly.

AHSP and β^h stabilize folded conformations of α^o

To directly probe how α^o structure is influenced by AHSP and β^h , we recorded ^{15}N -HSQC spectra of complexes labelled only on the α^o subunit. The α^o sample used for this experiment gave an NMR spectrum corresponding to the unfolded species (Figure 4A). Addition of 1 molar equivalent of unlabeled β^h resulted in a dramatic reduction in signals associated with this unfolded form, and the appearance of a new set of resonances more characteristic of a well-ordered structure, albeit with a significantly smaller number of peaks than expected for the full α^o sequence (Figure 4B). Addition of AHSP produced a similar stimulation of α^o folding (Figure 4C, main panel, black). In comparison, addition of AHSP to labelled α^h yielded a well-dispersed spectrum with the expected number of peaks (Figure 4C, main panel, red). Backbone ^{15}N , $^1\text{H}_\text{N}$, $^{13}\text{C}_\alpha$ shifts were obtained to 87% completeness for the α^h subunit of α^h :AHSP using standard triple-resonance methods. With this data in hand, HNCA and ^{15}N NOESY-HSQC spectra were acquired for α^o :AHSP, labelled on the α^o subunit. Fewer than 80 backbone H^N groups were found that gave rise to detectable nuclear Overhauser effects (NOEs). Short-range NOEs and H_N , C_α scalar connectivities were observed for α^o residues 14–18 and 20–26 in helices A–B, residues 111–116 linking helices G–H and residues 34–36, and 38 in the helix B–C corner. Chemical shift similarities with the corresponding residues in α^h :AHSP indicated that these regions adopt similar conformations in the two structures. Some additional α^o residues could be assigned on the basis of distinctive H_N , N , C_α chemical shifts and NOE patterns when compared to α^h :AHSP spectra. In total, backbone resonances were assigned for 38 α^o residues and

mapped onto a previously determined crystal structure of α^h :AHSP [30] (Figure 4D, spheres). It is notable that assignments were not obtained in helix F, or for the majority of helices G–H, indicating that these signals are very weak/absent or have chemical shifts too dissimilar to those from α^h :AHSP to be identified. The greatest chemical shift similarities mapped close to the AHSP interface (Figure 4D, blue and yellow spheres) with larger differences closer to the haem pocket (red spheres), as might be expected.

To probe the α^o :AHSP interface further, we reversed the labelling strategy and recorded a ^{15}N -HSQC of labelled AHSP in complex with unlabelled α^o (Figure 4D, insert, black) and compared this to a spectrum of AHSP bound to unlabelled α^h (red), assigned in a previous study [30]. Overall the two spectra were very similar, with coinciding peaks for 73 of the 99 non-proline residues in AHSP (Figure 4D, blue shading on AHSP ribbon, the remaining residues are shaded grey). Most of these peaks were within 0.03 ppm combined weighted ^1H , ^{15}N chemical shift, with the maximum difference of 0.1 ppm (Supplemental Figure 1). This indicates that each of these residues lies in a similar physiochemical environment in the α^o :AHSP and α^h :AHSP complexes. The similarities extended to the subunit interface (Supplemental Figure 1), indicating that the complementary surfaces of α^o/α^h , comprising the G–H helices and helix B–C linker, adopt similar structures in each complex.

DISCUSSION

The structure of α^o

Currently, the Hb folding process is not well characterized and in particular the potential role of chaperones is not well established. Here we use a range of biophysical methods to demonstrate that AHSP can promote folding and inhibit aggregation of α^o , suggesting key roles for AHSP and α^o in regulating Hb assembly.

NMR data indicate the presence of variable proportions of ‘folded’ and ‘unfolded’ forms of α^o in typical preparations. Even samples displaying substantially unfolded NMR spectra at pH 6.3 retained α -helical CD signals, suggesting that α -helical conformations persist in α^o aggregates not visible by NMR.

Addition of AHSP leads to dissociation of soluble α^o aggregates, formation of a stable α^o :AHSP dimer, and a dramatic shift from unfolded to partially folded forms of α^o . In complex with AHSP, regions of the A, B, C, and E helices of α^o adopt a stable state that at least partially resembles the native structure on the basis of chemical shifts. In general, assigned residues are surface exposed suggesting that buried residues may exhibit weak/absent NMR signals as a result of exchange between alternate packing arrangements. The F helix of α^o is particularly notable for the absence of any assigned resonances. The significant degree of α -helical character observed by CD for α^o and α^o :AHSP, coupled with the large number of broad/absent NMR signals in the ‘folded’ spectra, suggests that regions of α^o may sample an ensemble of α -helical conformations on the chemical shift timescale (μs). The G–H helices are presumed to be well-folded in the α^o :AHSP complex based on AHSP binding data, and thus the absence of clear NMR signals in this case may reflect exchange between free and bound α^o in addition to internal conformational dynamics.

Thus, AHSP can stabilize α^0 in solution whilst still retaining a large degree of conformational flexibility in the α^0 subunit. An attractive possibility is that this serves a functional role by allowing the bulky haem group to access its binding pocket. Such a mechanism has been proposed previously for apo-myoglobin, which retains a near-native fold, except for the F helix, which is disordered until after haem binding [31]. It remains to be determined if AHSP favourably influences haem-binding kinetics of α^0 or improves selectivity for the correct haem orientation. In general, globins traverse very different folding pathways, despite high degrees of similarity in the final protein folds [32], hence further study of α^h and β^h folding is required to understand Hb assembly.

The HSQC spectra of α^0 in complex with AHSP or β^h , or in the free folded form, share a number of similar (but not identical) peak patterns, such as peaks arising from Gly residues 15, 18, 22, 25, 57 and Thr 41 (Supplementary Figure 2). The suggestion is that AHSP and β^h stabilize a pre-existing conformation of α^0 . Interestingly, peptides corresponding to residues 1–86 of α^0 have been found to be capable of haem-binding [33], supporting the view that the N-terminal region of α^0 can fold independently from the rest of the protein.

The importance of α^0 in Hb assembly

Our study of α^0 provides some insights into potential pathways of Hb assembly. Assembly is thought to proceed through a series of partially haem-saturated intermediates including semi-Hb species, $\alpha^h\beta^0$ and $\alpha^0\beta^h$, which exist as dimers in solution [12, 34]. Semi-Hb may form through the interaction of α^h and β^h chains with nascent β^0 and α^0 chains, or by incorporation of haem into an $\alpha^0\beta^0$ species [2]. However, although dimeric $\alpha^0\beta^0$ can be produced *in vitro* by removal of haem from tetrameric Hb A [5, 35, 36], purified α^0 and β^0 chains were found not to recombine [5]. The role of $\alpha^0\beta^0$ as a significant species in Hb assembly is still debated [2, 36]. In our hands, purified α^0 and β^0 , each unstable in isolation, do combine spontaneously to form an $\alpha^0\beta^0$ dimer that stabilizes both partners, forming what appears to be a discrete 1:1 dimer. In addition, we provide direct evidence that free α^0 outcompetes β^h oligomerization to yield a dimeric semi-Hb $\alpha^0\beta^h$. These data support previously under-appreciated roles for α^0 in Hb assembly, including inhibiting the off-pathway self-association of β^h and β^0 .

Due to the general instability of free Hb subunits, the efficiency of Hb assembly may be enhanced by the action of specific chaperones [2]. Defects in Hb A production in mouse knockout models [3, 14, 15] and the finding that *Ahsp* gene expression is up-regulated under iron-limiting conditions [37], when free apo-globin chains accumulate, suggest that AHSP plays an important role in Hb A production. Our current findings that AHSP stabilizes α^0 in a partially folded conformation and dramatically increases its resistance to thermal induced denaturation/aggregation provide biochemical support for a role of AHSP as an α^0 chaperone. We propose that stabilization of α^0 by AHSP facilitates the formation of $\alpha^0\beta^0$ as an important intermediate in HbA assembly, and that α^0 :AHSP and α^0 : β^0 complexes might constitute significant routes of entry for haem.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Bettati S, Viappiani C, Mozzarelli A. Hemoglobin, an "evergreen" red protein. *Biochim Biophys Acta*. 2009; 1794:1317–1324. [PubMed: 19361581]
2. Mollan TL, Yu X, Weiss MJ, Olson JS. The role of alpha-hemoglobin stabilizing protein in redox chemistry, denaturation, and hemoglobin assembly. *Antioxid Redox Signal*. 2010; 12:219–231. [PubMed: 19659437]
3. Yu X, Kong Y, Dore LC, Abdulmalik O, Katein AM, Zhou S, Choi JK, Gell D, Mackay JP, Gow AJ, Weiss MJ. An erythroid chaperone that facilitates folding of alpha-globin subunits for hemoglobin synthesis. *J Clin Invest*. 2007; 117:1856–1865. [PubMed: 17607360]
4. Perutz MF, Rossmann MG, Cullis AF, Muirhead H, Will G, North AC. Structure of haemoglobin: a three-dimensional Fourier synthesis at 5.5-Å resolution, obtained by X-ray analysis. *Nature*. 1960; 185:416–422. [PubMed: 18990801]
5. Yip YK, Waks M, Beychok S. Influence of prosthetic groups on protein folding and subunit assembly. I. Conformational differences between separated human alpha- and beta- globins. *J. Biol. Chem*. 1972; 247:7237–7244. [PubMed: 4638545]
6. Fessas P. Inclusions of hemoglobin erythroblasts and erythrocytes of thalassemia. *Blood*. 1963; 21:21–32. [PubMed: 13944958]
7. Hargrove MS, Whitaker T, Olson JS, Vali RJ, Mathews AJ. Quaternary structure regulates heme dissociation from human hemoglobin. *J Biol Chem*. 1997; 272:17385–17389. [PubMed: 9211878]
8. Rachmilewitz EA, Peisach J, Blumberg WE. Studies on the stability of oxyhemoglobin A and its constituent chains and their derivatives. *J Biol Chem*. 1971; 246:3356–3366. [PubMed: 4324898]
9. Turner BW, Pettigrew DW, Ackers GK. Measurement and analysis of ligand-linked subunit dissociation equilibria in human hemoglobins. *Methods Enzymol*. 1981; 76:596–628. [PubMed: 7329280]
10. Kawamura-Konishi Y, Chiba K, Kihara H, Suzuki H. Kinetics of the reconstitution of hemoglobin from semihemoglobins alpha and beta with heme. *Eur. Biophys. J*. 1992; 21:85–92. [PubMed: 1396406]
11. Vasudevan G, McDonald MJ. Spectral demonstration of semihemoglobin formation during CN-heme incorporation into human apohemoglobins. *J. Biol. Chem*. 1997; 272:517–524. [PubMed: 8995292]
12. Tsuneshige A, Kanaori K, Samuni U, Danstker D, Friedman JM, Neya S, Giangiacomo L, Yonetani T. Semihemoglobins, High Oxygen Affinity Dimeric Forms of Human Hemoglobin Respond Efficiently to Allosteric Effectors without Forming Tetramers. *J. Biol. Chem*. 2004; 279:48959–48967. [PubMed: 15361521]
13. Ishimori K, Morishima I. Study of the specific heme orientation in reconstituted hemoglobins. *Biochemistry*. 1988; 27:4747–4753. [PubMed: 3167013]
14. Kihm AJ, Kong Y, Hong W, Russell JE, Rouda S, Adachi K, Simon MC, Blobel GA, Weiss MJ. An abundant erythroid protein that stabilizes free alpha-haemoglobin. *Nature*. 2002; 417:758–763. [PubMed: 12066189]
15. Kong Y, Zhou S, Kihm AJ, Katein AM, Yu X, Gell DA, Mackay JP, Adachi K, Foster-Brown L, Loudon CS, Gow AJ, Weiss MJ. Loss of alpha-hemoglobin-stabilizing protein impairs erythropoiesis and exacerbates beta-thalassemia. *J Clin Invest*. 2004; 114:1457–1466. [PubMed: 15545996]

16. Feng L, Zhou S, Gu L, Gell DA, Mackay JP, Weiss MJ, Gow AJ, Shi Y. Structure of oxidized alpha-haemoglobin bound to AHSP reveals a protective mechanism for haem. *Nature*. 2005; 435:697–701. [PubMed: 15931225]
17. Feng L, Gell DA, Zhou S, Gu L, Kong Y, Li J, Hu M, Yan N, Lee C, Rich AM, Armstrong RS, Lay PA, Gow AJ, Weiss MJ, Mackay JP, Shi Y. Molecular mechanism of AHSP-mediated stabilization of alpha-hemoglobin. *Cell*. 2004; 119:629–640. [PubMed: 15550245]
18. Luzzatto L, Notaro R. Haemoglobin's chaperone. *Nature*. 2002; 417:703–705. [PubMed: 12066171]
19. Gell D, Kong Y, Eaton SA, Weiss MJ, Mackay JP. Biophysical characterization of the alpha-globin binding protein alpha-hemoglobin stabilizing protein. *J Biol Chem*. 2002; 277:40602–40609. [PubMed: 12192002]
20. Masala B, Manca L. Detection of globin chains by reversed-phase high-performance liquid chromatography. *Methods Enzymol*. 1994; 231:21–44. [PubMed: 8041252]
21. Ascoli F, Fanelli MR, Antonini E. Preparation and properties of apohemoglobin and reconstituted hemoglobins. *Methods Enzymol*. 1981; 76:72–87. [PubMed: 7329287]
22. Simplaceanu V, Lukin JA, Fang TY, Zou M, Ho NT, Ho C. Chain-selective isotopic labeling for NMR studies of large multimeric proteins: application to hemoglobin. *Biophys. J*. 2000; 79:1146–1154. [PubMed: 10920044]
23. Shen TJ, Ho NT, Simplaceanu V, Zou M, Green BN, Tam MF, Ho C. Production of unmodified human adult hemoglobin in *Escherichia coli*. *Proc Natl Acad Sci U S A*. 1993; 90:8108–8112. [PubMed: 8367471]
24. Miles AJ, Wien F, Wallace BA. Redetermination of the extinction coefficient of camphor-10-sulfonic acid, a calibration standard for circular dichroism spectroscopy. *Anal Biochem*. 2004; 335:338–339. [PubMed: 15556575]
25. Tuzimura K, Konno T, Meguro H, Hatano M, Murakami T. A critical study of the measurement and calibration of circular dichroism. *Anal Biochem*. 1977; 81:167–174. [PubMed: 900476]
26. Leutzinger Y, Beychok S. Kinetics and Mechanism of Heme-Induced Refolding of Human alpha-globin. *Proc. Natl. Acad. Sci. U S A*. 1981; 78:780–784. [PubMed: 6940147]
27. Waks M, Yip YK, Beychok S. Influence of prosthetic groups on protein folding and subunit assembly. Recombination of separated human alpha-and beta-globin chains with heme and alloplex interactions of globin chains with heme-containing subunits. *J. Biol. Chem*. 1973; 248:6462–6470. [PubMed: 4730328]
28. Wilkins DK, Grimshaw SB, Receveur V, Dobson CM, Jones JA, Smith LJ. Hydrodynamic Radii of Native and Denatured Proteins Measured by Pulse Field Gradient NMR Techniques, Å†. *Biochemistry*. 1999; 38:16424–16431. [PubMed: 10600103]
29. Valdes R Jr, Ackers GK. Thermodynamic studies on subunit assembly in human hemoglobin. Self-association of oxygenated chains (alphaSH and betaSH): determination of stoichiometries and equilibrium constants as a function of temperature. *J Biol Chem*. 1977; 252:74–81. [PubMed: 833131]
30. Gell DA, Feng L, Zhou S, Jeffrey PD, Bendak K, Gow A, Weiss MJ, Shi Y, Mackay JP. A cis-proline in alpha-hemoglobin stabilizing protein directs the structural reorganization of alpha-hemoglobin. *J Biol Chem*. 2009; 284:29462–29469. [PubMed: 19706593]
31. Dyson HJ, Wright PE. Unfolded proteins and protein folding studied by NMR. *Chem Rev*. 2004; 104:3607–3622. [PubMed: 15303830]
32. Nishimura C, Prytulla S, Jane Dyson H, Wright PE. Conservation of folding pathways in evolutionarily distant globin sequences. *Nat Struct Biol*. 2000; 7:679–686. [PubMed: 10932254]
33. Komar AA, Kommer A, Krashennnikov IA, Spirin AS. Cotranslational folding of globin. *J Biol Chem*. 1997; 272:10646–10651. [PubMed: 9099713]
34. Cassoly R. Preparation of globin-hemoglobin hybrids: artificially prepared and naturally occurring semihemoglobins. *Methods Enzymol*. 1981; 76:121–125. [PubMed: 7329255]
35. Rossi-Fanelli A, Antonini E, Caputo A. Light scattering and sedimentation of native human adult and fetal globin. *J Biol Chem*. 1959; 234:2906–2910. [PubMed: 14439277]

36. Boys BL, Konermann L. Folding and Assembly of Hemoglobin Monitored by Electrospray Mass Spectrometry Using an On-line Dialysis System. *J. Am. Soc. Mass Spectrom.* 2007; 18:8–16. [PubMed: 16979901]
37. dos Santos CO, Dore LC, Valentine E, Shelat SG, Hardison RC, Ghosh M, Wang W, Eisenstein RS, Costa FF, Weiss MJ. An iron responsive element-like stem-loop regulates alpha-hemoglobin-stabilizing protein mRNA. *J Biol Chem.* 2008; 283:26956–26964. [PubMed: 18676996]

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Figure 1. The α^0 protein displays structural characteristics of the native α^h state

(A) UV-visible absorbance spectra for α^0 (100 μM) and oxy- α^h (10 μM). For comparison of characteristic haem absorption bands, the 350–700 nm region of the α^0 spectrum is also shown with absorbance magnified ten times (dashed line). (B) α^0 adopts an α -helical conformation upon neutralization of an acidic solution. α^0 (5 μM) was prepared in water at pH 3.2. The pH was raised by addition of 2 mM acetate (pH 4.4), or 2 mM sodium phosphate (pH 5.3, 6.3), or 5 mM Tris. HCl (pH 8.0). (C) Comparison of the far-UV CD spectra for: α^0 , α^h and AHSP at 8 $^\circ\text{C}$ and α^0 at 30 $^\circ\text{C}$. (D) ^{15}N -HSQC spectrum of α^0 (300 μM) at 288 K in water at pH 3.2. (E) Sample from D following addition of 10 mM (final)

sodium phosphate to a final pH of 6.3. (F) ^{15}N -HSQC spectrum of α^{h} (500 μM) in 10 mM sodium phosphate, pH 7.0 at 288 K.

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Figure 2. AHSP stabilizes α^0 against thermal induced denaturation and aggregation

(A) Thermal denaturation curves, representative of 3–6 measurements each for α^0 , AHSP and α^0 :AHSP monitored by CD at 222 nm, expressed as mean residue ellipticity (MRE). (B) Thermal denaturation curves for haem-bound globin chains, compared to that of the α^0 :AHSP complex. (C) Thermally induced formation of soluble aggregates monitored by DLS for α^0 (closed triangles), α^0 :AHSP (open triangles), α^h (closed circles), α^h :AHSP (open circles) and Hb A (open squares). Above 35 °C, α^0 :AHSP, α^h and α^h :AHSP rapidly converted to large ($R_s > 100$ nm) particles (not shown). Errors are the standard deviation of

three measurements. **(D)** Thermal induced formation of insoluble aggregates at 25 °C, detected by absorbance measurements at 700 nm (symbols have the same meaning as in B).

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Figure 3. Light scattering analysis of α^o complexes

(A) Elution traces from SEC (refractive index voltage, axis left-hand side) for 0.5 mg α^h (dotted line), 0.5 mg AHSP (dashed) and 0.5 mg AHSP mixed with a 1.5 molar excess of α^h (solid). MALS data was used to calculate M_w for every 5 μ l volume across each peak (open spheres, axis right-hand side) and the M_w calculated across the whole peak is shown in brackets. (B) SEC-MALS analysis of α^o (1 mg load). (C–E) SEC-MALS analysis for purified AHSP, β^h , and β^o samples (upper panels), and following mixing with an equimolar ratio of α^o (lower panels). Different SEC columns were used for A and B–E.

Figure 4. AHSP and β^h stabilize a folded conformation of α^o

(A) ^{15}N -HSQC spectrum of α^o (200 μM) in 10 mM sodium phosphate, pH 6.3, 288 K, showing no evidence of the folded conformation. (B) ^{15}N -HSQC spectrum of an $\alpha^o:\beta^h$ complex (150 μM) formed by addition of unlabelled β^h to the sample shown in A. (C) *Main panel*, ^{15}N -HSQC spectra of: labelled α^o bound to unlabelled AHSP (black; 200 μM complex, 10 mM sodium phosphate, pH 6.3, 298 K); labelled α^h bound to unlabelled AHSP (red; 370 μM , 10 mM sodium phosphate, pH 7.0, 298 K). *Inset*, ^{15}N -HSQC spectra of labelled AHSP bound to unlabelled α^o (black; 250 μM complex, 10 mM sodium phosphate

pH 6.9, 298 K), or α^h (red; 500 μ M complex, 10 mM sodium phosphate, pH 6.9, 298 K). (**D**) Chemical shift analysis of the α^o :AHSP and α^h :AHSP complexes. Per-residue, combined N and H_N chemical shift differences (δ_{N,H_N}) between the α^o and α^h subunits mapped onto the α^h :AHSP structure [30]: $\delta_{N,H_N} < 0.1$ ppm (blue spheres), $0.1 \text{ ppm} \leq \delta_{N,H_N} < 0.2$ ppm (yellow); $0.2 \leq \delta_{N,H_N} < 0.5$ ppm (red). AHSP residues that exhibit similar N, H_N chemical shifts in the α^o :AHSP and α^h :AHSP complexes are shown in blue (see text).

TABLE 1

Thermal stabilities and light scattering measurements.

Sample	Denaturation midpoint temp (°C)*	R_s (nm) [M_w predicted (kDa)] [†]	M_w (kDa) from MALS	Theoretical monomer M (kDa)
α^o	–	3.4 ± 0.2 [80–114]	$22\text{--}120$ [‡]	15.1
α^o :AHSP	53.7 ± 1.1	2.6 ± 0.1 [38–48]	26.0 ± 0.2	27.1
AHSP	57.1 ± 1.7	1.7 ± 0.1 [10–14]	11.8 ± 0.2	12.0
α^h	56.4 ± 1.0	2.0 ± 0.3 [12–30]	23 [‡]	15.7
α^h :AHSP	56.8 ± 1.0	2.5 ± 0.1 [34–43]	25.3 ± 1.2	27.7
α^o : β^h	–	–	37.0	31.6
α^o : β^o	–	–	31.4 ± 0.6	31
HbA	65.5 ± 0.9	2.9 ± 0.1 [54–66]	61 [‡]	64.5

Errors are ± 1 standard deviation for three measurements

* With the exception of AHSP, these samples undergo irreversible unfolding

[†] Measured at 3–5 mg/ml protein. M_w calculated for a spherical molecule with hydration at 0.3 g water per 1 g protein.[‡] These samples show a clear concentration dependence in M_w . Values are given for ~1 mg of injected protein.