Biophysical Characterization of the α-Globin Binding Protein α-Hemoglobin Stabilizing Protein*

Received for publication, June 19, 2002, and in revised form, August 18, 2002
Published, JBC Papers in Press, August 20, 2002, DOI 10.1074/jbc.M206084200

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α-Hemoglobin stabilizing protein (AHSP) is a small (12 kDa) and abundant erythroid-specific protein that binds specifically to free α-(hem)globin and prevents its precipitation. When present in excess over β-globin, its normal binding partner, α-globin can have severe cytotoxic effects that contribute to important human diseases such as β-thalassemia. Because AHSP might act as a chaperone to prevent the harmful aggregation of α-globin during normal erythroid cell development and in diseases of globin chain imbalance, it is important to characterize the biochemical properties of the AHSP-α-globin complex. Here we provide the first structural information about AHSP and its interaction with α-globin. We find that AHSP is a predominantly α-helical globular protein with a somewhat asymmetric shape. AHSP and α-globin are both monomeric in solution as determined by analytical ultracentrifugation and bind each other to form a complex with 1:1 subunit stoichiometry, as judged by gel filtration and amino acid analysis. We have used isothermal titration calorimetry to show that the interaction is of moderate affinity with an association constant of 1 × 10^7 M^-1 and is thus likely to be biologically significant given the concentration of AHSP (~0.1 mM) and hemoglobin (~4 mM) in the late pro-erythroblast.

Mammalian hemoglobin, the red blood cell oxygen transport molecule, is a tetramer of two α- and two β-globin chains. Exquisitely coordinated expression of these α- and β-globin chains is required during erythropoiesis to generate high concentrations of hemoglobin, without production of either chain in excess; any disruption of normal globin gene expression patterns can lead to serious human disease (1, 2). One such disease is β-thalassemia, a common genetic disorder caused by mutations in one or more of the β-globin gene loci that result in reduced β-globin production. In addition to the direct effects of reduced β-globin synthesis, many of the symptoms of this disorder appear to be consequences of the resulting cytotoxic buildup of free α-globin (1, 2). Free α-globin is highly unstable and readily precipitates, damaging membrane structures and triggering the apoptotic cell death of erythroid precursors (1). The effects of dysregulated expression of individual globin chains can be severe, and consequently, it has long been thought that additional factors within the cell may assist with the processing of free globin chains and their assembly into mature hemoglobin (3). The identification of any such factors proved elusive for a long time.

Recently however, Kihm et al. (4) identified α-hemoglobin stabilizing protein (AHSP), a small 102-residue protein that may act to neutralize the harmful effects of any free α-globin generated during either normal erythropoiesis or in circumstances of disease (4). Expression of the AHSP gene is strongly up-regulated by the essential erythroid transcription factor GATA-1 such that AHSP accumulates to relatively high concentrations (~0.1 mM) in late erythroid precursor cells (4, 5). AHSP mRNA is present specifically in all hematopoietic tissues of the fetus and adult mouse, consistent with a role in the regulation of hemoglobin production throughout pre- and postnatal life.

AHSP specifically binds to the α-chain of hemoglobin, but not to the β-chain or to tetrameric hemoglobin, making it an ideal candidate for an α-globin-specific chaperone. Consistent with a role for AHSP in regulating coordinated globin expression, gene-targeting studies in mice showed that ablation of AHSP function leads to erythrocyte abnormalities that are also observed in β-thalassemia. The staining of erythrocytes from these mice with crystal violet reveals that they contain inclusion bodies of denatured hemoglobins, known as Heinz bodies (4). AHSP also prevents the precipitation of α-globin both in vitro and in COS cells, further supporting the idea that AHSP may prevent pathological aggregation and precipitation of α-globin in vivo.

Little further is currently known about the action of AHSP or the molecular details of its interaction with α-globin. Interestingly, however, the protein was previously identified (and named EDRF, for erythrocyte differentiation related factor) as a marker for transmissible spongiform encephalopathies (6), although the significance of this finding is currently unclear. Nothing is known about the physical or conformational properties of AHSP. Little information is available from sequence comparisons, because AHSP displays no clear homology to any protein of known structure. Here, we present the first biophysical analysis of AHSP and its specific interaction with α-globin. We demonstrate that AHSP is primarily α-helical in conformation, probably with an extended C-terminal region, and that it is slightly elongated. We also show that AHSP is monomeric in solution at concentrations of up to 1 mM and that the AHSP-α-
globin complex has a 1:1 stoichiometry with an association affinity constant ($K_a$) of $1.0 \times 10^7$ M$^{-1}$ at 20 °C. The formation of this complex does not appear to involve large-scale structural rearrangements of either component, judging from circular dichroism data, although small changes in either one or both components may take place. In addition, we find that the heme group of α-globin is not required for AHSP interaction. Finally, the behavior of truncation mutants of AHSP indicates that at least six amino acids from the N terminus and 17 from the C terminus are dispensable for α-globin binding activity.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—Full-length human AHSP cDNA (GenBank accession number AF147435) was sub-cloned into the bacterial expression vector pGEX-2T (Amersham Biosciences). Expression from this plasmid in *Escherichia coli* BL21 cells yielded full-length AHSP as a protein fusion with glutathione S-transferase (GST). AHSP expression was performed in shaker flasks overnight at 25 °C. Following cell lysis, the fusion protein was captured on a reduced glutathione-agarose column (Amersham Biosciences), and the AHSP polypeptide was subsequently released from the column by treatment with thrombin (Sigma), which acted at the engineered cleavage site. The thrombin-treated product was purified by reversed-phase HPLC. For each aliquot, the molecular mass of the purified protein as determined by electrospray mass spectrometry was 11,984 ± 0.5 Da, in agreement with the predicted mass of 11,984 Da (including an additional Gly-Ser dipeptide at the N terminus of the protein, which remains after thrombin cleavage). The final yield was ~26 mg of purified AHSP per liter of bacterial culture.

Purified α- and β-globin were obtained from human blood. All purification steps were carried out at 4 °C, where possible. Human red blood cells were obtained from whole blood by centrifugation (3000 rpm, 10 min) and washing with 0.9% (w/v) NaCl. Carbon monoxide was bubbled through the cell suspension to form CO-ligated hemoglobin, a stable form more resistant to oxidation-induced precipitation. Hemolysates were generated by addition of five cell volumes of deionized water on ice, and membrane fractions were removed by centrifugation (7000 rpm, 15 min) after addition of NaCl to a final concentration of 0.9% (w/v). The α- and β-globin chains were separated using the well-established method of reaction with β-hydroxymercuribenzoate (PMB) (7). In this case, 2.4 g of hemoglobin was incubated overnight with 143 mg of PMB. The fractions were separated on a Bio-Rad chromatography column using 150 mM NaCl, 20 mM sodium phosphate, pH 7.0, and applied to an UNO-Q anion exchange column (Bio-Rad). Isocratic elution of AHSP in 50 mM NaCl, 20 mM sodium phosphate buffer, pH 7.0, yielded protein of >95% purity as determined by reversed-phase HPLC (Phenomenex C18) and SDS-PAGE analysis. A final gel filtration step on Superose 12 (Amersham Biosciences) produced a single protein peak corresponding to AHSP. The molecular mass of the purified protein as determined by electrospray mass spectrometry was 11,984 ± 0.5 Da, in agreement with the predicted mass of 11,984 Da (including an additional Gly-Ser dipeptide at the N terminus of the protein, which remains after thrombin cleavage). The final yield was ~26 mg of purified AHSP per liter of bacterial culture.

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**Isothermal Titration Calorimetry**—As a direct measurement of the stability of AHSP, we performed isothermal titration calorimetry (ITC) experiments using a MicroCal VP-ITC titration calorimeter. Protein solutions were buffer-exchanged into 20 mM sodium phosphate buffer (pH 7.0) containing 150 mM NaCl by gel filtration chromatography. Heats of reaction were determined by multiple injections of 100 μM AHSP into 10 μM α-globin at 20 °C, with stirring at 310 rpm and a delay of 250 s between each injection. The reverse experiment (i.e. injections of 100 μM α-globin into 10 μM AHSP) was also performed. Heats of dilution for all experiments were measured from injection of each protein and subtracted from the raw data. Titration curves were analyzed using Origin 5.0 software (MicroCal) by fitting to various simple models provided in the software.

**Circular Dichroism Spectropolarimetry**—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. Spectra were collected at 20 °C over the wavelength range 184–280 nm, with a resolution of 0.5 nm and a bandwidth of 1 nm. Final spectra were the sum of three scans accumulated at a speed of 20 nm min$^{-1}$ with a response time of 1 s. For the thermal denaturation measurements, the ellipticity at 222 nm was monitored over a temperature range of 10–85 °C, using a resolution of 0.2 °C, a bandwidth of 1 nm, and a temperature gradient of 1 °C min$^{-1}$. CD spectra were analyzed, essentially by viewing the data. A small correction for the CD of the apo-globin was used when fitting the data. Circular dichroism spectra of AHSP were collected in a 0.1-mm path-length cell, and the temperature was controlled using a water-jacketed cell holder. Spectra were collected at 20 °C over the wavelength range 184–280 nm, using a resolution of 0.5 nm and a bandwidth of 1 nm. Final spectra were the sum of three scans accumulated at a speed of 20 nm min$^{-1}$ with a response time of 1 s.
For sedimentation velocity experiments, absorbance data were collected in two-channel centerpieces in continuous mode with a step size of 0.003 cm and at time intervals of 900 s, with no signal averaging. Preceding each run, the samples were spun at 3000 rpm for at least 3 h so that they reached thermal equilibrium in the rotor. Data were analyzed using the program Sedfit (12) to simultaneously determine the apparent molecular weight ($M_{app}$) and sedimentation coefficient for a given buffer and temperature ($s_{b}$).

In the above analyses, the partial specific volumes ($\upsilon$) of each protein were determined from the amino acid sequences (13) and adjusted for temperature (14) using the program SEDNTERP. $^2$ The buffer density was taken to be 1.0068 g ml$^{-1}$ and the viscosity 0.0105 Poise at 20 °C. For other temperatures, buffer density ($\rho_{b}$) and viscosity ($\eta_{b}$) were calculated using the SEDNTERP program, assuming the bulk of the temperature dependence to be contributed by the major component, water. Values for $s_{b}$ were transformed to standard sedimentation coefficients in water at 20 °C ($s_{20,w}$) using the following equation (16),

$$s_{20,w} = \frac{s_{b}(1 - \rho_{b}/\rho_{w})(\eta_{w}/\eta_{b})}{(1 - \rho_{w}/\rho_{b})} \left(\frac{\eta_{b}}{\eta_{w}}\right)$$  (Eq. 1)

where the density of water at 20 °C ($\rho_{w}$) is 0.9982 g ml$^{-1}$ and the viscosity ($\eta_{w}$) is 0.01002 Poise. For consideration of theoretical spherical molecules, the anhydrous radius ($R$) was calculated from molecular weight

$$R = 6.72 \times 10^{-2} M^{1/3}$$  (17),

and values for the frictional coefficient ($f_c$) and $s$ were calculated as described previously (16, 18).

RESULTS

AHSP and the AHSP-a-Globin Complex Are Predominantly $\alpha$-Helical—A secondary structure prediction carried out on the amino acid sequence of AHSP revealed that the protein may contain substantial amounts of $\alpha$-helix (Fig. 1A). We therefore sought to confirm this experimentally. A far-UV circular dichroism spectrum of AHSP (Fig. 1B) displays minima at 209 and 222 nm and a maximum at ~190 nm, indicating that the protein is folded and adopts a largely $\alpha$-helical conformation. Analysis of the CD spectrum using the CDPsR software package suggests that AHSP contains ~70% $\alpha$-helix. A thermal denaturation experiment, monitored using the ellipticity at 222 nm, showed that AHSP unfolds reversibly with a $T_m$ of 60 °C. All of these data are consistent with the protein adopting a well-defined, largely $\alpha$-helical conformation.

Given that free oxy-a-globin has a propensity to aggregate and precipitate, we investigated the possibility that the protein might be partially unfolded and that the binding of AHSP might stabilize a-globin by inducing substantial conformational change. A far-UV CD spectrum (Fig. 1C), deconvoluted using CDPsR, reveals that free a-globin contains ~80% $\alpha$-helix. Notably, a-globin within the hemoglobin tetramer is ~75% $\alpha$-helix, suggesting that free a-globin retains similar levels of secondary structure even in the absence of its $\beta$-globin partner, a conclusion in line with previous studies (19). In addition, thermal denaturation of a-globin, carried out as described above, revealed a single highly cooperative unfolding transition with a $T_m$ of 59 °C, consistent with a well-defined fold.

Formation of the AHSP-a-globin complex appears to result in a small reduction in total $\alpha$-helical content (Fig. 1C), but there is little evidence for large-scale structural rearrangement, suggesting that a-globin stabilization by AHSP occurs through an alternative mechanism. It should be borne in mind, however, that changes in the spatial organization of $\alpha$-helices might not be detected in this experiment.

Both AHSP and a-Globin Are Monomeric in Solution—We next sought to define the aggregation states of both AHSP and a-globin in solution. As noted previously, oxy-a-hemoglobin is unstable, largely as a result of oxidation of the iron (II) heme group to iron (III), and subsequent reactions leading to protein precipitation (20, 21). In contrast, the CO-liganded form of a-globin is much more resistant to this oxidation-induced precipitation but retains the ability to bind AHSP (4). Thus, we have used the CO-liganded form of a-globin to study the physical properties of the molecule and its interaction with AHSP.

Gel filtration chromatography of purified AHSP indicated that the protein forms a single species of discrete molecular size, with no evidence of aggregation into high molecular weight forms, even at concentrations up to 1 mM (AHSP is estimated to attain a concentration of 0.1 mM in pro-erythroblasts (4)). Calibration of the column with molecular weight

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$^2$ D. B. Hayes, J. P. Philo, and T. M. Laue (1994) sedNTerp: Interpretation of Sedimentation Data Version 1.0, 2000 lines of Visual Basic code, written for Windows 3.0, www.bibi.org/rasmb/rasmb.html.
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Fig. 2. Analytical ultracentrifugation analysis of AHSP. A, sedimentation equilibrium data for 7 μM AHSP at 30,000 and 42,000 rpm (open circles). Experiments were carried out at 20 °C. Absorbance at 280 nm (A_{280}) is plotted against r^2/2, where r is the radial position from the center of the rotor. The best fit to the full data set (7 and 14 μM AHSP at 18,000, 30,000, and 42,000 rpm) was a single non-interacting species of M_{n,app} = 11,200 Da, represented by the solid line. Residuals from this fit are shown in the top two panels. B, sedimentation velocity analysis of AHSP over a range of protein concentrations fitting a non-interacting single species with M_{n,app} = 11,800 ± 460 Da (n = 14). Experiments were performed at both 20 °C (open circles) and 4 °C (filled circle) in buffer and sedimentation coefficient (s) values converted to values in water at 20 °C (s_{20,w}; see “Experimental Procedures”). The sedimentation coefficient extrapolated to infinite dilution (s_{20,w}) is 1.52 S.

standard indicated that AHSP migrates in a position consistent with a globular protein of ~23 kDa, roughly twice the mass predicted by the primary amino acid sequence. By comparison, α-globin ran close to its expected monomeric size of ~14 kDa. However, because the elution properties of proteins in size exclusion chromatography are shape-dependent, we chose to characterize the aggregation state of both AHSP and α-globin using sedimentation equilibrium experiments. The data in Fig. 2A show clearly that AHSP is monomeric, suggesting that its gel filtration profile may reflect anisotropy in the shape of the protein. A single species fit was observed for all protein concentrations examined, up to 0.1 mM (the higher protein concentrations were monitored at 250 nm; not shown).

To investigate the hydrodynamic properties of AHSP further, we performed a series of sedimentation velocity experiments to measure the sedimentation coefficient (s) of AHSP (Fig. 2B). The model that best fitted the experimental data incorporated a non-interacting single species with M_{n,app} = 11,800 ± 460 Da (n = 14) and a sedimentation coefficient, extrapolated to infinite dilution (s_{20,w}), of 1.52 S (Fig. 2B). This result is in close agreement with the mass determined from equilibrium experiments and with a theoretical mass of 11,986 Da for the monomeric protein. Calculation of s for a theoretical sphere of the same mass yielded values in the range 1.56 ± s = 1.58, depending upon the level of hydration (ranging from anhydrous to hydration at 0.41 g of H_2O per 1 g of protein based on amino acid composition (22)). Hence, our data are consistent with AHSP assuming a globular fold, but, because the rate of sedimentation observed was slower than predicted by a spherical model, we suggest that AHSP may have a slightly elongated shape, consistent with the behavior of the molecule by gel filtration.

A similar analysis of CO-ligated α-globin indicated that it is also monomeric in solution (Fig. 3). Some small systematic deviations between the non-interacting monomer model and the experimental data were observed (Fig. 3A, residuals), but introducing either self-association or non-ideality into the model could not reduce these deviations. One possibility is that small amounts of oxidized material form aggregates during the course of the experiment, although the majority of α-globin was in the CO-ligated form as judged by spectrophotometric measurements in the region 540–550 nm (not shown). Given the small magnitude of the residuals, we propose that the monomer model is valid. Sedimentation velocity experiments yielded a value for s_{20,w} of 1.97 S (Fig. 3B). As before, s was calculated for a theoretical sphere of the same mass and yielded values in the range 2.09 ≥ s ≥ 1.64 (ranging from anhydrous to hydration at 3.9 g of H_2O per 1 g of protein). These values suggest that α-globin is rather spherical, in accordance with the gel filtration data. Note that the possible effects of the heme prosthetic group were ignored for the purpose of this analysis. Taken together, the results from gel filtration and sedimentation analysis indicate that purified AHSP and α-globin are both monomers in solution, but that AHSP is probably more elongated than α-globin.

The AHSP-α-Globin Complex has a 1:1 Stoichiometry—To examine the AHSP-α-globin complex in detail, we titrated AHSP into a solution of α-globin and monitored the formation of complex by gel filtration chromatography (Fig. 4A). These experiments showed that the interaction between AHSP and α-globin is sufficiently tight that, when mixed together, free monomers only occur in measurable quantities when one of the two proteins is in excess, even at protein concentrations below 10 μM (suggesting a K_D > 10^8 M^-1 for formation of the complex). Upon addition of 1 molar equivalent of AHSP to a sample of α-globin, a single species eluted from the gel filtration column with an apparent molecular size of ~30 kDa, suggesting the formation of a heterodimeric AHSP-α-globin complex. To confirm the stoichiometry, gel filtration fractions corresponding to the AHSP-α-globin complex were collected and subject to amino acid analysis. This method does not rely on estimates of protein concentration derived from optical absorbance measurements and, therefore, provides an independent measurement of subunit concentrations in the complex. The experimentally derived amino acid composition was compared with values calculated for theoretical AHSP-α-globin complexes of 1:1, 2:1, and 1:2 subunit stoichiometry. Analysis of the relative levels of amino acids characteristic of either AHSP or α-globin indicated a 1:1 subunit stoichiometry for the complex (Fig. 4B). Global analysis of the data by determination of S_{residuals} between observed and calculated amounts of every residue gave values of...
0.8, 8.5, and 9.8 for the 1:1, 2:1, and 1:2 models, respectively, confirming the 1:1 model to be the best fit. Samples of AHSP and \( \alpha \)-globin alone were also analyzed, and the expected amino acid compositions were confirmed. In summary, all the data indicate the AHSP-\( \alpha \)-globin complex is a heterodimer.

**AHSP and \( \alpha \)-Globin Form a Moderately Tight Complex**—We next used isothermal titration calorimetry to measure thermodynamic parameters for the AHSP-\( \alpha \)-globin interaction. Fig. 5 shows that formation of the complex is exothermic, and the data fitted well to a simple 1:1 binding model (Fig. 5, lower panel) with \( \Delta H = 8.75 \pm 0.07 \text{ kcal mol}^{-1} \) and \( K_A = 1.0 \times 10^7 \text{ M}^{-1} \) at 20°C.

We note that this interaction affinity is greater than that observed for the \( \alpha_1\beta_1 \) (dimer) \( \leftrightarrow \alpha_1\beta_1\alpha_2\beta_2 \) (tetramer) equilibrium state of fully oxygenated hemoglobin. Hence, AHSP binding could have consequences for oxygen transport if it contacted...
the residues of α-globin that mediate this dimer ↔ tetramer association (i.e., those residues at the α1β2 interface). To investigate this possibility, we performed binding competition experiments using AHSP and an equimolar mixture of CO-ligated α- and β-subunits (these display virtually identical dimer ↔ tetramer equilibrium to oxygenated subunits; $K_a = 0.8 \times 10^6$ M$^{-1}$ at 20°C (23)). We observed that α-globin became exclusively incorporated into a complex with β-globin, with no evidence of a ternary complex with AHSP (Fig. 6B). This result was observed irrespective of the order of addition of components, hence, the pre-formed α-globin-AHSP complex was not protected from dissociation. Furthermore, addition of recombinant AHSP to purified hemoglobin A had no effect on the Hill coefficient for oxygen binding, indicating that the cooperativity of oxygen binding is unaffected by AHSP (not shown). Together these results suggest that AHSP is unlikely to interact with α-globin at the α1β2 interface of hemoglobin.

AHSP Binds α-Globin in the Absence of the Heme Prosthetic Group—To begin to probe the structural determinants of the AHSP-α-globin interaction, we first investigated the role of the heme prosthetic group. Removal of the heme group from α-globin was achieved by reversed-phase HPLC chromatography at low pH. Apo-α-globin fractions were lyophilized and then taken up in buffer already containing AHSP. These observations suggest that the

![Graph](image-url)

**Fig. 5.** Determination of the AHSP-α-globin affinity using isothermal titration calorimetric. A 100 μM solution of α-globin was titrated into 10 μM AHSP in 7-μl increments. Differential power signal between sample and reference cell is shown in the upper panel. Negative power indicates an exothermic reaction. In the lower panel, the heat change per mole of α-globin for each injection is plotted against the ratio of α-globin to AHSP, after subtraction of the heats of dilution. Fitting of this curve using non-linear least squares methods indicated a single-site binding model with the following values: apparent enthalpy, $-8.75 \pm 0.07$ kcal mol$^{-1}$; association constant $K_a = 1.0 \pm 0.1 \times 10^7$ M$^{-1}$; stoichiometry, $n = 1.0$. Experiments were carried out at 20°C.

β-globin out-competes AHSP for α-globin binding, as determined by gel filtration. Mixing of equimolar quantities of α- and β-globin results in formation of a single complex (grey-dashed line). Mixing of AHSP, α-globin, and β-globin in equal quantities (solid line; the globins have greater absorption at 280 nm due to the heme group) produces the same αβ complex and monomeric AHSP (compare the same amount of AHSP injected alone, dashed line). B, AHSP binds α-globin in the absence of heme. Gel filtration data recorded after injection of a 1:1 molar ratio mixture of apo-α-globin and AHSP (solid line). The elution profile for AHSP alone (dashed line) is shown for comparison. Fractions 14–22 from each run were collected (corresponding to elution volumes of 12.5–17 ml) and analyzed by SDS-PAGE (insets).

![Graph](image-url)

**Fig. 6.** Characterization of the AHSP-α-globin interaction. A, β-globin out-competes AHSP for α-globin binding, as determined by gel filtration. Mixing of equimolar quantities of α- and β-globin results in formation of a single complex (grey-dashed line). Mixing of AHSP, α-globin, and β-globin in equal quantities (solid line; the globins have greater absorption at 280 nm due to the heme group) produces the same αβ complex and monomeric AHSP (compare the same amount of AHSP injected alone, dashed line). B, AHSP binds α-globin in the absence of heme. Gel filtration data recorded after injection of a 1:1 molar ratio mixture of apo-α-globin and AHSP (solid line). The elution profile for AHSP alone (dashed line) is shown for comparison. Fractions 14–22 from each run were collected (corresponding to elution volumes of 12.5–17 ml) and analyzed by SDS-PAGE (insets).
Fig. 7. Analysis of α-globin binding by deletion mutants of AHSP. A, schematic diagram of deletion mutants of AHSP with reference to their predicted secondary structure and a summary of their α-globin binding properties, as determined in B. B, gel filtration data showing the capacity for AHSP deletion mutants to bind to α-globin. Recombinant AHSP (10 μM) deletion mutants were mixed with purified human α-globin (5 μM) and run on a TSK-GEL gel filtration column. The retention time for α-globin was determined by monitoring absorbance at 540 nm, indicating the level of incorporation into the complex with AHSP.

apo-α-globin may somehow be stabilized by AHSP binding, although CD measurements of apo-α-globin in the presence or absence of AHSP did not reveal substantial changes in secondary structure. Apo-β-globin showed no binding to AHSP in our assay (not shown).

The N and C Termini of AHSP Are Not Required for α-Globin Binding—To further define structural requirements for the AHSP-α-globin interaction, we generated a series of truncation mutants of AHSP and assayed their ability to bind α-globin by gel filtration (Fig. 7). Deletions from the C terminus of up to 17 amino acids (mutant ΔC17) had no significant effect on α-globin binding. In addition the ΔC17 mutant adopted an α-helical conformation similar to the full-length protein as determined by CD (not shown), supporting the hypothesis that this region may not contribute to the folded globular region of AHSP. Deletion of a further 6 residues (mutant ΔC23) completely abrogated the interaction in this assay. Thus, the C-terminal region that is not required for the formation of a complex with α-globin corresponds closely to the unstructured C-terminal tail predicted from sequence analysis (Fig. 1A). Likewise, an N-terminal truncation of 6 residues had no effect on binding, whereas removal of 18 residues abolishing binding (Fig. 7). An intermediate effect was seen for the ΔN12 mutant: binding was observed but with a substantially reduced affinity.

DISCUSSION

Given that the precipitation of α-globin in vivo carries severe medical consequences, it is reasonable to suppose that an organism might contain proteins to assist in the regulation of correct globin assembly. AHSP is a small globular protein that has recently been shown to bind specifically to α-globin and prevent its precipitation in vitro. Moreover, loss of AHSP through gene ablation in mice causes hemoglobin precipitates to accumulate in red blood cells, demonstrating that AHSP is required for normal hemoglobin production in animals. Further investigations into the structure of AHSP and the nature of its interaction with α-globin are likely to provide the basis for new therapeutic strategies to inhibit the formation of toxic hemoglobin precipitates in disorders of globin chain imbalance, such as β-thalassemia. Here we show that AHSP is a globular α-helical protein with a somewhat asymmetric shape and with flexible N and C termini that are not required for α-globin binding. AHSP exists as a monomer in solution and binds α-globin with only minor changes in the secondary structure content of either protein. Interestingly, the α-globin heme group is not required for interaction with AHSP, despite the fact that α-globin cannot attain a native fold in the absence of this prosthetic group. Indeed, we find that interaction with AHSP stabilizes apo-α-globin in solution, suggesting that AHSP may also prevent aggregation of α-globin from which the heme group has been lost. AHSP might bind either to the regions of residual structure in apo-α-globin or induce structure in regions that are otherwise less well defined. In this regard, CD analysis of α-globin proteolytic fragments has suggested that the apo-α-globin N terminus is a region that maintains residual structure (27).

The AHSP-α-globin complex forms with a 1:1 subunit stoichiometry and an association constant in the sub-micromolar range (1 × 10^7 M^-1). With the observed affinity, the AHSP-α-globin interaction is likely to be biologically significant, given the concentrations of AHSP (0.1 mM) and hemoglobin (4 mM) found in red blood cells. In addition there are consequences regarding the site of AHSP binding on α-globin. Hemoglobin exists in a dimer ↔ tetramer equilibrium, with each subunit contributing to an intra-dimer α1β1 interface and an interdimer α1β2 interface. The α1β1 interface is essentially unchanged in all ligand states of hemoglobin and constitutes a very high affinity interaction (KΑ ≫ 10^10 M^-1). It is the α1β2 interface that becomes reorganized upon oxygen binding, thereby giving rise to the well-described allosteric effects. For deoxyhemoglobin, the equilibrium constant for tetramer formation through the α1β2 interface is 4.2 × 10^10 M^-4 at 21.5 °C (28), well above the binding affinity of AHSP. Upon full oxygenation (or CO binding), this association constant is reduced substantially to ~1 × 10^6 M^-1 (23), potentially making the α1β2 interface accessible to AHSP, if it were to bind this region. However, we have shown that β-globin out-competes AHSP, even under conditions of CO saturation. These findings suggest that the AHSP binding site might overlap with the high affinity α1β1 interface rather than the α1β2 face. Alternatively, a conformational change in α-globin upon binding β-globin may be responsible for masking the AHSP interaction site, although we favor
the former possibility; CD measurements have demonstrated that there is no significant change in the conformation of α-globin upon binding to β-globin (19). It is likely that high resolution structural information will ultimately distinguish these possibilities. In particular, it will be interesting to determine whether the AHSP-α-globin interface structurally mimics the α,β, interface, especially given that AHSP is, like the globins, a predominantly α-helical protein.

Regardless of the site of interaction, our finding that AHSP binding is effectively excluded upon formation of αβ complexes is consistent with the proposed role for AHSP in erythroid cells as an α-globin-specific chaperone. Aβ1 limits the toxicity of free HbA, not associated with α1, which might enhance the catabolism of excess free α1-globin. Other outcomes are also possible. For example, AHSP interacts with protein degradation machinery (32), which is consistent with the proposed role for AHSP in erythroid cells (Harding, S. E., Rowe, A. J., and Horton, J. C., eds) pp. 90–125, The Royal Society of Chemistry, Cambridge

Methylated α-globin can be released for assembly into hemoglobin A tetramers. Other outcomes are also possible. For example, AHSP might enhance the catabolism of excess free α-globin through interactions with protein degradation machinery (32), which are known to occur with other chaperone proteins. It is also possible that AHSP plays other roles in erythrocyte maturation, for example, by acting as a monitor of free α-globin levels that feeds back to globin gene regulation. These potential functions could be mediated through the non-helical N- and C-terminal domains of AHSP, which we have demonstrated to be dispensable for α-globin binding.

Based on our current and previous findings, AHSP appears to represent a novel type of molecular chaperone with distinct characteristics. It most closely resembles members of the small heat shock protein family in its size and ATP independence, although, unlike small heat shock proteins, AHSP does not form higher order oligomeric complexes (33). Also, in contrast to most other chaperones, which are widely expressed and relatively promiscuous with respect to substrate interactions, AHSP appears to be highly tissue- and substrate-specific. Our current studies on the biochemistry of the AHSP-α-globin interaction provides fundamental information that is relevant to understanding its biological function. Future studies to define the structure of the AHSP-protein complex should lead to an improved understanding of hemoglobin biology and elucidate novel approaches for minimizing its pathological precipitation in various disease states.

Acknowledgments—We thank Jacqui Matthews and Frank Bunn for helpful suggestions.

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