Biochemical Fates of α Hemoglobin Bound to α Hemoglobin-stabilizing Protein AHSP*

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Alpha hemoglobin-stabilizing protein (AHSP), also called erythroid-associated factor or ERAF, is an abundant erythroid protein originally identified in a screen for genes that are regulated by the essential hematopoietic transcription factor GATA-1 (1). Previously, we have shown that AHSP specifically binds and stabilizes the α subunit of hemoglobin and reduces its cytotoxic effects in vivo, at least partly by inhibiting its ability to generate reactive oxygen species (2). Certain characteristics of the AHSP-αHb interaction are established (3–6). 1) AHSP binds αHb via the αβ dimer interface. Therefore, AHSP and βHb cannot bind αHb simultaneously. 2) βHb binds αHb more tightly than AHSP. Hence, βHb easily displaces AHSP from αHb. 3) AHSP can induce oxidation of the heme iron of αHb. 4) AHSP binding induces significant structural rearrangements in the globin chain of αHb.

The initial binding of AHSP to free oxy-αHb causes disordering of the globin F helix, movement of the heme group, displacement of the F8 histidine, coordination of the Fe(II) heme iron by the distal histidine (E7), and binding of O2 to the proximal side of the heme group (5). In this unique structure, the oxygen binding site is exposed to solvent, which predisposes it to the spontaneous loss of H2O2 and oxidation of the heme iron (auto-oxidation) (7). Indeed, the Fe(II) oxy-αHb-AHSP complex oxidizes much more rapidly at ambient temperature and oxygen pressure than free Fe(II) oxy-αHb alone. Concomitantly, there are further structural changes, and the heme iron becomes coordinated by both the F8 (proximal) and the E7 (distal) histidines (6). This AHSP-bound Fe(III) bis-histidyl or hemichrome structure is more resistant to denaturation and heme loss than oxidized free αHb. This configuration also inhibits the reaction of Fe(III) αHb with H2O2 and other oxidants, presumably because the hexacoordinate iron is less available for direct reaction with these substrates. Thus, hemichrome formation is one mechanism by which AHSP stabilizes αHb and renders it more chemically inert. Formation of AHSP-αHb complexes may be particularly important to limit erythrocyte damage in β thalassemia, a hemoglobinopathy in which reduction of β globin synthesis causes excessive free αHb to accumulate (2, 8). It is also likely that AHSP acts as a chaperone to stabilize either apo or holo αHb during normal HbA synthesis (1).

Although a number of αHb-AHSP interactions have been described by in vitro biochemical and structural studies, certain processes remain unclear. For example, we have shown that AHSP induces the formation of bis-histidyl αHb over several hours under ambient conditions, but how the rate of this reaction is affected by temperature and oxygen tension is unknown. βHb displaces AHSP from αHb to form αβ Hb-like com-

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4 The abbreviations used are: AHSP, α hemoglobin-stabilizing protein; Hb, hemoglobin; αHb, α hemoglobin (α globin plus heme); βHb, β hemoglobin (β globin plus heme); DTPA, diethylenetriaminepentaacetic acid; GST, glutathione S-transferase; EPR, electron paramagnetic resonance.

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FIGURE 1. Temperature dependence of AHSP-mediated changes in \( \alpha \text{Hb} \). Shown is the effect of AHSP on \( \alpha \text{Hb} \) spectra over time at different temperatures in 20 mM sodium phosphate, pH 7.4, 100 mM NaCl, and 10 \( \mu \)M DTPA: 2 °C (A), 22 °C (B), and 37 °C (C). The first traces of A–C were recorded immediately after the addition of AHSP to \( \alpha \text{Hb} \) at time 0. Subsequent traces were recorded every 45 min for A and B and every 2 min for C. The arrows indicate the direction of spectrophotometric changes over time. D, standard spectra for \( \text{Fe(II) } \alpha \text{Hb-AHSP complex (solid line) and Fe(III) } \alpha \text{Hb-AHSP complex (dashed line). Shown are fractional oxidation (F) and oxidation rate (F) of } \text{Fe(II) } \alpha \text{Hb-AHSP complex at different temperatures. The spectra in A–C were fitted to a linear regression model based on the two standard spectra in D, and the fraction of oxidized } \alpha \text{Hb was calculated.} \) Diamonds, 2 °C; squares, 22 °C; triangles, 37 °C; circles, 45 °C.

plexes, suggesting that AHSP can stabilize \( \alpha \) chains transiently prior to incorporation into HbA. However, AHSP binding causes significant structural rearrangements around the \( \alpha \text{Hb} \) heme group in both the \( \text{Fe(II) } \alpha \text{Hb-AHSP and Fe(III) bis-histidyl } \alpha \text{Hb-AHSP complexes. In this current work, we have shown that some of these structural changes are retained when the } \alpha \text{ subunits are displaced from AHSP by } \beta \text{Hb and that fully functional tetrameric HbA is not always formed. Determining whether or not fully active hemoglobin can be generated from } \alpha \text{Hb-AHSP complexes is critical for determining how AHSP functions in vivo. We conducted the present study to address two key questions. 1) How readily is the bis-histidyl } \text{Fe(III) } \alpha \text{Hb-AHSP complex formed under physiologic conditions? 2) Can AHSP-bound } \text{Fe(II)} \text{ or bis-histidyl } \text{Fe(III) } \alpha \text{Hb recombine with } \beta \text{Hb to form functional HbA? Our findings provide the basis for a model to explain how AHSP may function in erythrocytes to augment Hb assembly and stability.}

EXPERIMENTAL PROCEDURES

Interactions of \( \alpha \text{Hb and AHSP—} \)
Recombinant human AHSP, AHSP-glutathione S-transferase, \( \alpha \text{Hb, and } \beta \text{Hb, were prepared as previously described (2, 4). Fe(III) } \alpha \text{Hb was prepared by incubating oxy-} \alpha \text{Hb with a 4-fold excess of potassium ferricyanide followed by gel filtration chromatography using Sephadex G50 beads. Fe(II) } \alpha \text{Hb-AHSP complex was prepared by incubating 10 } \mu \text{M purified } \alpha \text{Hb with equimolar recombinant AHSP on ice for 30 min in 20 mM sodium phosphate, pH 7.4, 100 mM NaCl, and 10 } \mu \text{M diethylenetriaminepentaacetic acid (DTPA). Samples of this complex were incubated at various temperatures in a quartz cuvette and the spectral changes followed over time in a PerkinElmer Life Sciences Lambda 25 UV-visible spectrophotometer to monitor conversion to the } \text{Fe(III) bis-histidyl form. Deoxygenated Fe(II) } \alpha \text{Hb was generated from oxy-} \alpha \text{Hb by three cycles of vacuum and nitrogen purge in a Teflon sealed quartz cuvette followed by adding a molar excess of sodium dithionate. Equimolar AHSP was added using a gas-tight Hamilton syringe to generate deoxy-} \text{Fe(II) } \alpha \text{Hb-AHSP. Fe(III) } \alpha \text{Hb-AHSP complex was prepared by incubating Fe(II) } \alpha \text{Hb-AHSP for several hours and verifying the complete conversion spectrophotometrically. Standard extinction coefficient spectra of Fe(II) and Fe(III) complexes were prepared from a series of concentration-dependent spectra. The conversion rates of Fe(II) to Fe(III) complexes were calculated following linear regression of the experimental spectra to the two extinction coefficient spectra utilizing Sigma plot software (Systat Software) (9).}

Formation and Characteristics of Tetrameric Hb from \( \text{AHSP-} \alpha \text{Hb Complexes—} \)
To examine the displacement of AHSP from its complex with \( \alpha \text{Hb, equimolar } \beta \text{Hb was added and the mixture incubated at room temperature for 5 min. Cel-}

ulose acetate electrophoresis was performed using chromatography strips (Helena Laboratories, Beaumont, TX) according to the manufacturer’s instructions. The tetramers resulting from displacement of AHSP by \( \beta \text{Hb were examined for oxygen binding by standard tonometry (10). Briefly, 3 ml of the reaction mixtures were deoxygenated via three cycles of vacuum and nitrogen purge in a 300-ml tonometer. Known volumes of oxygen were added at room temperature and pressure, the samples rolled at 22 °C for 10 min, and spectra recorded. Semi-oxidized Hb
EPR Measurements—EPR spectra of Fe(III) αHb subunits free in solution, bound to AHSP, and bound to Fe(II) carboxy monoxy (CO) βHb subunits, were recorded with a Brucker EMX EPR spectrometer. The instrument conditions for the EPR measurements were: frequency, 9.60 GHz; power, 10 milliwatts; modulation amplitude, 10.9 G; modulation frequency, 100 kHz; and temperature, 4.5 K. The high spin signal at g = 6 (~1200 G) was quantified by double integration between 800 and 1700 G and comparison with the signal for α(FeIII)2β(FeII)2 Hb prepared by mixing newly oxidized αHb with an equimolar ratio of oxy-βHb (11). Rates of cyanomet Hb formation were monitored spectrophotometrically after the addition of a 4-fold molar excess of potassium cyanide to various Hb species. Reduction of oxidized Hbs was performed utilizing a ferredoxin reductase system, as previously described (12). Briefly, Hbs were premixed with glucose-6-phosphate, nicotinamide adenine dinucleotide phosphate, ferredoxin-nicotinamide adenine dinucleotide phosphate reductase, ferredoxin with or without superoxide dismutase and catalase (Sigma). Thereafter, the reaction was initiated by adding glucose-6-phosphate dehydrogenase (Sigma) at 37 °C and comparison with the signal for Hbs were premixed with glucose-6-phosphate, nicotinamide adenine dinucleotide phosphate reductase, ferredoxin with or without superoxide dismutase and catalase (Sigma). Thereafter, the reaction was initiated by adding glucose-6-phosphate dehydrogenase (Sigma) at 37 °C under aerobic conditions, and the accompanying spectral changes were recorded.

Results—AHSP converts to a Fe(III) bis-histidyl hemichrome complex (5). However, this reaction occurs over several hours at room air and temperature, which brings into question its biological significance, as erythropoiesis occurs at higher temperatures and lower oxygen tension. Therefore, we investigated the effects of these parameters on the rate of Fe(III) complex formation (Fig. 1). The conversion of αHb-AHSP from the oxygenated Fe(II) form to the Fe(III) bis-histidyl hemichrome is accompanied by characteristic spectrophotometric changes that allow the kinetics of this reaction to be monitored. The UV-visible spectrum of the Fe(II) complex was followed during incubation at a variety of temperatures (Fig. 1, A–C). The spectra were fitted to a two-component linear regression model utilizing standard spectra for the Fe(II) and Fe(III) αHb-AHSP complexes (Fig. 1D). From these fits, the proportion of oxidized heme was calculated and plotted against time (Fig. 1E). A dramatic increase in the rate of conversion to the Fe(III) complex with increasing temperatures from 22 to 37 °C occurred (Fig. 1F). This large temperature dependence was quantitated in terms of an activation energy (Ea) obtained from an Arrhenius plot of lnkautox versus 1/T. The observed value is quite large (121 kJ/mol) and is almost identical to that reported for the auto-oxidation of intact HbA (13), implying that both processes have a similar mechanism. The value of Ea for auto-oxidation is markedly higher than that for either O2 binding to (Ea ~ 20–30 kJ/mol) or dissociation from (Ea ~ 60–70 kJ/mol) mammalian Hbs and myoglobin (14).

AHSP-bound oxy-αHb contains a disorganized F helix on which the proximal histidine F8 resides, increasing exposure of the heme iron to solvent (5). This open heme pocket, with a lack of ligand coordination, is known to be associated with increased
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rates of auto-oxidation of the heme iron (7). Therefore, we reasoned that the conversion of αHb-AHSP from the Fe(II) to the Fe(III) form might occur through auto-oxidation. In this case, the conversion would be oxygen-dependent. We tested this by examining the Fe(II) αHb-AHSP complex after deoxygenation by sodium dithionite (Fig. 2). Anaerobic addition of one equivalent of dithionite induced a spectrum indicative of deoxygenated Fe(II) Hb (Fig. 2A, dashed line). This form of αHb-AHSP was stable, with minimal spectral changes after 1 h of incubation at 37°C (Fig. 2A, solid line). In contrast, 100% of fully oxygenated Fe(II) αHb-AHSP converted to the Fe(III) species upon similar incubation in room air (Figs. 1 and 2).

When Fe(II) αHb-AHSP complex was diluted from 15 to ~1 μM complex in air, the observed rate of auto-oxidation decreased markedly (Fig. 2, B and C). This result is the opposite of what is observed when intact oxyhemoglobin is diluted over the same concentration range. Zhang et al. (15) show that the rate of auto-oxidation increased from ~0.0001 min⁻¹ for Hb tetramers at high concentrations to 0.0007 min⁻¹ for Hb dimers observed at low concentrations. The most straightforward explanation for the decrease in the auto-oxidation rate of oxy-αHb-AHSP with dilution is that the complex is dissociating into AHSP and free oxy-αHb, which has a much smaller auto-oxidation rate. In the simplest model, the observed rate of auto-oxidation would be given by,

\[
k_{\text{autox,obs}} = k_{\text{autox,αHb-AHSP}} \cdot Y_{\text{complex}} + k_{\text{autox,freeαHb}} \cdot (1 - Y_{\text{complex}})
\]

(Eq. 1)

where \(Y_{\text{complex}}\) is the fraction of oxy-αHb bound to AHSP. \(k_{\text{autox,αHb-AHSP}}\) and \(k_{\text{autox,freeαHb}}\) are the first order rates of auto-oxidation of oxy-αHb bound to AHSP and free in solution, respectively. The value of \(Y_{\text{complex}}\) at equimolar αHb and AHSP is given by,

\[
Y_{\text{complex}} = \left( \frac{2 + \frac{K_d}{P_0}}{2} \right) - \sqrt{\left( \frac{2 + \frac{K_d}{P_0}}{2} \right)^2 - 4}
\]

(Eq. 2)

where \(P_0\) is the total concentration of αHb and \(K_d\) is the equilibrium dissociation constant for αHb binding to AHSP.

The rate of auto-oxidation of free oxy-αHb is roughly equal to that of Hb dimers, ~0.001 min⁻¹ (16). The dependence of the observed rates of auto-oxidation on total αHb (\(P_0\)) were fitted to Equations 1 and 2 (Fig. 2C). The best fit was obtained with \(K_d \approx 5 \mu M\) and a limiting rate at high protein concentration for auto-oxidation of oxy-αHb-AHSP equal to ~0.080 min⁻¹. This rate is ~80-fold greater than that for auto-oxidation of free αHb and Hb dimers and roughly 800-fold greater than that for tetramers. Very similar auto-oxidation rates were reported by Vasseur-Godbillon et al. (17): 0.0007 min⁻¹ for αHb, 0.0002 min⁻¹ for HbA, and 0.05 min⁻¹ for αHb-AHSP.

The fitted value of the \(K_d\) for oxy-αHb binding to AHSP is significantly higher than expected based on previous estimates by isothermal titration calorimetry (4). This discrepancy may be the result of using a simple one-step model to analyze both types of measurements, when it is clear that auto-oxidation and large secondary conformational changes occur after αHb binds to AHSP. Regardless of the exact interpretation, it is clear the oxy-αHb binding to AHSP causes a dramatic increase in the rate of auto-oxidation. Together, the data in Figs. 1 and 2 support a mechanism in which the formation of Fe(III) histidyl αHb-AHSP occurs through αHb auto-oxidation facilitated by association with AHSP. As auto-oxidation is accelerated under partially saturated conditions (18), this reaction is likely to be favored at the relatively low oxygen concentrations that occur normally in vivo.

Previously, we suggested that AHSP may stabilize αHb until free β chains become available for the formation of HbA. In support of this, both the Fe(II) and Fe(III) αHb-AHSP complexes form apparently tetrameric Hb species upon βHb addition (6). However, the biochemical properties of these HbA-like species are not well studied. We incubated both Fe(II) and Fe(III) αHb-AHSP with a freshly prepared excess of oxy-βHb and examined the resultant Hb complexes by cellulose acetate electrophoresis, which separates proteins according to size and charge (Fig. 3). For these experiments, we used AHSP fused to glutathione S-transferase, which permits eventual removal of AHSP and AHSP-bound Hbs by absorption to glutathione beads. Similar to AHSP, AHSP-GST binds free αHb and converts it to a more stable Fe(III) histidyl form (not shown). The addition of a 2-fold excess oxy-βHb to the Fe(II) αHb-AHSP complex produced four bands including the original complex, free AHSP, free βHb, and a new species with similar electrophoretic mobility to HbA (Fig. 3, lane 7). Reaction of βHb with Fe(III) αHb-AHSP produced similar bands, except that the newly formed HbA-like complex (slowest migrating species in Fig. 3, lane 8) had slightly reduced mobility.

FIGURE 3. Cellulose acetate electrophoresis analysis of Hb heterotetramers derived from αHb-AHSP complexes. AHSP was fused to glutathione S-transferase. The first four lanes show input protein controls. Fe(II) and Fe(III) αHb-AHSP complexes are shown in lanes 5 and 6, respectively. The addition of a 2-fold excess oxy-βHb to either complex resulted in a Hb species migrating similarly to HbA (lanes 7 and 8). Note that the Hb derived from Fe(III) αHb-AHSP migrates slightly slower (lane 8). The reactions represented in lanes 7 and 8 were depleted of free AHSP-GST and αHb-AHSP-GST by incubation with glutathione-agarose beads (lanes 9 and 10). Proteins were visualized by staining with Ponceau Red.
Passage of the AHSP-Hb reaction mixtures over glutathione beads removed most free AHSP and AHSP-αHb complexes (Fig. 3, lanes 9 and 10). However, the two complexes with similar mobility to HbA were unaltered, indicating that they did not contain AHSP. Together, these data indicate that the newly formed slow migrating bands in Fig. 3, lanes 7–10, represent α2β2 heterotetramers. However, the reduced mobility of the species resulting from reaction of βHb with Fe(III) αHb-AHSP suggests that this tetrameric Hb differs from normal HbA. For example, the oxidized αHb heme may retain the bis-histidyl conformation acquired during its interaction with AHSP and thus alter the mobility of the Hb tetramer.

To address these possibilities, we studied the biochemical features of the Hb heterotetramers generated from interaction between different forms of αHb-AHSP and βHb. First, we examined the oxygen binding properties of Hb resulting from the interaction of βHb with Fe(II) αHb-AHSP (Fig. 4A). This Hb exhibited similar electrophoretic mobility to oxy-HbA (Fig. 3, compare lanes 4, 7, and 9) and also displayed spectral properties identical to HbA at room temperature, pressure, and oxygen tension (Fig. 4A, solid line). Upon deoxygenation via three cycles of vacuum and nitrogen purge, a pure deoxygenated HbA spectrum was observed (Fig. 4A, dotted line). Sequential addition of oxygen resulted in progressive oxygenation with P50 = 1.85 mm Hg, slightly lower than that of normal HbA (~3 mm Hg). The resultant spectrum showed evidence of heme oxidation, as indicated by the ratio of peak absorbances at 542 and 577 nm. Based on deconvolution across the entire visible spectrum, ~30% of the final Hb was oxidized (Fig. 4A, dashed line), presumably via auto-oxidation during reoxygenation. However, the majority of AHSP-bound Fe(II) αHb appeared to be recycled into functional HbA.

Strikingly different results were obtained when similar experiments were performed utilizing Hb heterotetramers derived from the Fe(III) αHb-AHSP hemichrome complex. The initial spectrum of this Hb species was consistent with a 50:50 mix of oxygenated Fe(II) βHb and Fe(III) αHb heme moieties (Fig. 4B, solid line). Furthermore, deoxygenation of this Hb produced the expected 50:50 mix of deoxygenated Fe(II) and Fe(III) hemes (Fig. 4B, dotted line). However, sequential addition of oxygen produced minimal oxygenation and resulted predominantly in further heme oxidation and heme loss, as evidenced by the loss of absorbance in the 520–580 nm range and increased absorbance at 630 nm (Fig. 4B, dashed line). As mentioned previously, heme oxidation is autocatalytic, and thus, it is not surprising that a semi-oxidized Hb tetramer is unstable (18).

To examine further whether Fe(III) αHb retains its bis-histidyl hemichrome state in the Hb heterotetramers, we measured their reactivity toward cyanide, which binds Fe(III) heme iron to produce cyanomet Hb with a distinct spectrophotometric signature (Fig. 5A)
(14). Cyanide addition to purified Hb (αFe(III)2βFe(II)2) prepared by adding oxy-βHb to freshly prepared Fe(III) αHb resulted in rapid formation of cyanomet Hb, with complete conversion occurring within 5 min (Fig. 5, A and C). Conversion to cyanomet Hb appeared to occur at 100% of the hemes, suggesting that within this time period, Fe(II) βHb was oxidized to the Fe(III) form. Wallace et al. showed that anions such as cyanide and azide markedly accelerate auto-oxidation of native hemoglobin (19), and this marked acceleration also occurs for the partially oxidized Hb tetramers shown in Fig. 5A.

In contrast, when cyanide was added to Hb heterotetramers prepared from oxy-βHb and Fe(III) αHb-AHSP, the generation of cyanomet Hb was much slower (Fig. 5B and D). After 40 min of incubation with cyanide, less than 20% of the hemes were converted to the cyanomet form. Therefore, in this complex, the α subunits contain hemichromes that inhibit cyanide binding. Moreover, this hemichrome form also inhibits auto-oxidation of oxy-β subunits by cyanide, which we observed in the more native Hb valency hybrids shown in Fig. 5A.

We measured the electron paramagnetic resonance (EPR) spectra of frozen samples of free Fe(III) αHb, αHb bound to AHSP, and αHb reconstituted into Hb by addition of CO βHb to examine the spin states of the αHb Fe(III) atom. EPR is a standard method for assessing coordination of Fe(III) in terms of the spin states of the unpaired metal electrons. Concentrated samples of free Fe(III) αHb or Fe(III) αHb-AHSP were prepared and stored on ice. A portion of each was mixed with a 2-fold excess Fe(II) CO βHb and incubated at 4°C for 30 min to reconstitute αβ2, HbA tetramers. The CO form of βHb was used to prevent oxidation of these subunits and the appearance of β Fe(III) signals. Then, all samples were frozen for EPR analysis of the different AHSP or αβ2, HbA complexes.

As shown in Fig. 6A, there was virtually no g = 6 high spin signal in the 4.5 K spectrum of the Fe(III) αHb-AHSP complex. The low spin signal was complex and indicates multiple hemichrome conformers. Peisach and coworkers reported similar complex EPR spectra for Fe(III) HbA treated with urea or salicylate denaturants (20). Thus, AHSP rapidly converts all hemichrome-like spectra after the oxidation of free human oxy-αHb chains (20) and data not shown).

When CO βHb was added to free Fe(III) αHb to generate a mixed valence (αFe(III)1βFe(II)1) Hb, there was complete conversion to a simple 100% high spin EPR spectrum with a dominating peak at g = 6 (Fig. 6B). In contrast, reaction of Fe(III) αHb-AHSP with CO βHb produced a hybrid Hb with incomplete conversion of the Fe(III) αHb subunit to the simple aquomet form found in native Fe(III) HbA (Fig. 6C). Based on electrophoresis and gel filtration, all of the Fe(III) αHb subunits were removed from AHSP (not shown). However, the amplitude of the g = 6 signal and the optical spectra (Figs. 4 and 5) indicated that most (~70%) of the Fe(III) αHb subunits that were previously bound to AHSP still had a low spin hemichrome conformation in the hybrid tetramer. This result suggests that the AHSP-mediated rearrangement of αHb to a bis-histidyl structure is retained upon binding to βHb.

Cytochrome b4 metHb reductase converts oxidized Hb iron to the ferrous state in erythroid cells (21). We investigated whether a similar in vitro enzymatic system could reduce the Hb tetramer derived from Fe(III) αHb-AHSP (Fig. 7). We mixed CO βHb with either purified Fe(III) αHb (Fig. 7A) or Fe(III) αHb-AHSP (Fig. 7B) to generate semi-oxidized HbA heterotetramers (αFe(III)1βFe(II)1) (20). We used the more stable CO βHb in these experiments to minimize further breakdown of the
tetrameric complex from in vitro Hb auto-oxidation, as observed in Fig. 4B. We incubated the Hb tetramers with a reconstituted ferredoxin reductase system (12) in air and monitored reduction of the α subunit by visible light absorbance. Both semi-oxidized Hbs showed heme loss after treatment with the reductase system, as reflected by dampened absorbance through most of the visible spectrum (Fig. 7, A and B). As with any reduction system, it is possible for the ferredoxin reductase to generate accessory oxidants, which could account for the observed Hb instability. Therefore, we repeated these Hb reduction experiments in the presence of the antioxidant enzymes superoxide dismutase and catalase, both of which are relatively abundant in normal erythroid cells (Fig. 7, C and D). Under these conditions, the HbA tetramers derived from either free Fe(III) αHb or Fe(III) αHb-AHSP were both entirely reducible and bound oxygen, such that the resultant spectra matched that of HbA. The reaction was slower for the hemecontaining AHSP-derived Fe(III) αHb. Interestingly, this reduction system with catalase and superoxide dismutase was also capable of reducing the Fe(III) αHb-AHSP complex itself (data not shown). Taken together, our findings suggest that Fe(III) αHb derived from the AHSP complex is relatively stable and retains its hemechrom structure upon replacement of the AHSP with Hb. However, it appears that the resultant tetrameric Hb is functionally competent and that the hemechrom structure is reversible under appropriate reducing conditions.

**DISCUSSION**

Gene targeting studies demonstrate that AHSP is essential for normal Hb production. AHSP null mice have a compensated hemolytic anemia, and their erythrocytes exhibit increased reactive oxygen species, globin chain precipitates, and shortened life span (1, 2). It is likely that these effects are initiated by destabilized αHb that results from the loss of AHSP. Here we used biochemical approaches to study some of the factors that influence αHb-AHSP interactions. These findings refine and extend our model to explain AHSP actions in vivo (Fig. 8). We propose that newly synthesized oxygenated Fe(II) αHb is transiently bound and stabilized by AHSP during erythropoiesis. This interaction alters the structure of αHb reversibly, such that βHb can readily displace AHSP to generate functional HbA under reducing conditions.

If oxy-αHb remains bound to AHSP, the structure of the heme pocket is altered further, rapid auto-oxidation occurs, and additional conformational changes lead to the formation of a Fe(III) bis-histidyl complex. This hemechrom form of αHb is resistant to further oxidation and hemin loss, because the sixth coordinate position of heme iron is occupied and unable to participate in chemical reactions that generate reactive oxygen species. However, the stability of AHSP-bound αHb is achieved at an energetic cost, as the heme must be enzymatically reduced to generate functional HbA. This reduction
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can occur either before or after displacement by $\beta$Hb. The former would appear to represent a futile redox cycle in the presence of oxygen because of the rapid rate of auto-oxidation of bound oxy-$\alpha$Hb.

In vivo, the balance between Fe(II) $\alpha$Hb:AHSP and Fe(III) bis-histidyl $\alpha$Hb-AHSP is probably controlled by the redox environment, oxygen saturation, and most importantly, $\beta$Hb availability. Specifically, when $\beta$Hb is replete, less bis-histidyl complex is formed because AHSP-bound Fe(II) $\alpha$Hb is rapidly recruited into HbA. Alternatively, $\beta$Hb deficiency, as occurs in $\beta$ thalassemia, favors conversion of AHSP-bound $\alpha$Hb to the more inert bis-histidyl form. Hence, AHSP has at least two functions: to stabilize $\alpha$Hb transiently during normal HbA synthesis and to sequester $\alpha$Hb in a more stable form during conditions of $\alpha$Hb excess. Despite this, both $\alpha$Hb and $\beta$Hb are destabilized by AHSP deficiency. This likely reflects destabilization of $\alpha$Hb prior to interaction with $\beta$Hb, underscoring a physiologic role for the Fe(II) $\alpha$Hb-AHSP complex. Hence, AHSP probably has different roles in Hb synthesis according to globin chain synthesis ratios. It may be possible to examine this hypothesis further by measuring the levels of various AHSP-$\alpha$Hb complexes in normal and thalassemic strains of mice. Additional roles for AHSP might also exist. For example, AHSP binds apo (non heme-bound) $\alpha$ globin protein in vitro (4) and facilitates its folding and expression in Escherichia coli and in eukaryotic cells (1, 17). In this capacity, AHSP could interact with the nascent polypeptide chain to promote its stability, folding, or heme insertion during HbA synthesis.

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