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The Role of Alpha-Hemoglobin Stabilizing Protein in Redox Chemistry, Denaturation, and Hemoglobin Assembly

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Abstract

Hemoglobin biosynthesis in erythrocyte precursors involves several steps. The correct ratios and concentrations of normal alpha (α) and beta (β) globin proteins must be expressed; apoproteins must be folded correctly; heme must be synthesized and incorporated into these globins rapidly; and the individual α and β subunits must be rapidly and correctly assembled into heterotetramers. These events occur on a large scale *in vivo*, and dysregulation causes serious clinical disorders such as thalassemia syndromes. Recent work has implicated a conserved erythroid protein known as Alpha-Hemoglobin Stabilizing Protein (AHSP) as a participant in these events. Current evidence suggests that AHSP enhances α subunit stability and diminishes its participation in harmful redox chemistry. There is also evidence that AHSP facilitates one or more early-stage post-translational hemoglobin biosynthetic events. In this review, recent experimental results are discussed in light of several current models describing globin subunit folding, heme uptake, assembly, and denaturation during hemoglobin synthesis. Particular attention is devoted to molecular interactions with AHSP that relate to α chain oxidation and the ability of α chains to associate with partner β chains. *Antioxid. Redox Signal.* 12, 219–232.

Introduction

Normal human blood contains ~15 g of the erythrocyte oxygen transporter hemoglobin (Hb) per 100 milliliters. About 90–95% of adult hemoglobin is a 64.4 kDa tetrameric protein called HbA (17). HbA consists of two holo-alpha (α^H) and two holo-beta (β^H) chains, each of which possesses a single iron-containing protoporphyrin IX (heme) group. A great deal is known about the synthesis, structural biology, and physiology of HbA (reviewed in refs. 17, 24, 91, and 95). However, there are many open questions regarding α and β globin folding, heme uptake, and subunit assembly, all interrelated events that may follow several pathways *in vivo* (reviewed in refs. 10, 16, 37, 95, and 106) (Fig. 1). Thus, heme insertion and globin folding appear to facilitate each other (55, 71, 107, 117) and subsequent post-translational HbA biosynthetic events may similarly be interconnected. It is important to understand the mechanisms of HbA assembly, both because it represents a model system for synthesis of an abundant biologically important multi-subunit protein and because disorders in this process are associated with common human diseases. Moreover, understanding how HbA forms *in vivo* should provide clues for optimizing the manufacture of recombinant Hbs to be used as blood substitutes. Recent characterization of the Alpha-Hemoglobin Stabilizing Protein (AHSP) has generated renewed interest in understanding the complete HbA assembly process and raises the issue of whether HbA requires chaperone or escort proteins to optimize subunit folding, heme uptake, and HbA formation and minimize Heinz body formation and oxidative stress *in vivo* (9, 38, 62).

AHSP is a conserved 102-amino-acid protein that is found in erythrocyte precursors of many mammals (27, 42, 62, 76). We have been unable to find homologous protein or DNA sequences in the other vertebrates, most of which have nucleated red cells. Because free α^H chains are unstable (14, 56, 92), an early hypothesis was that AHSP acts as a molecular chaperone for free α chains prior to their incorporation into HbA (38, 62). In support of this idea, it has been shown that: (a) AHSP associates with α^H chains in solution, but not with β^H chains or HbA; (b) HbA forms as β^H chains are titrated into solutions containing AHSP:α^H-chain duplexes; and (c) AHSP

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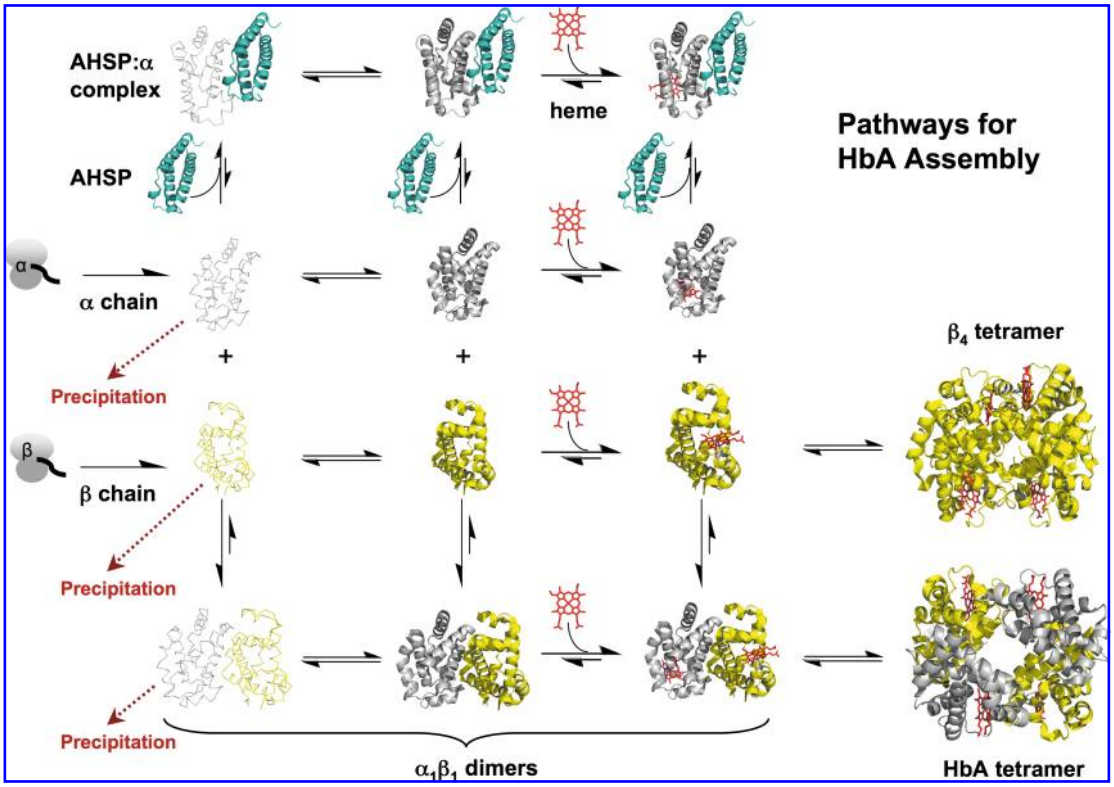

FIG. 1. Post-translational HbA biosynthesis events. Following subunit translation, HbA production may occur along several different pathways. The most likely pathways are summarized in this figure. Not depicted is the possibility that heme insertion into one chain drives subunit assembly and subsequent heme insertion into another. Also not shown is that heme insertion and certain association events may occur co-translationally. AHSP is shown in *teal*, heme in *red*, α chains in *gray*, and β chains in *yellow*. The use of *thin lines* and *ribbons* for the backbone structures indicates unfolded and folded states, respectively. Drawings were produced using PyMol and PDB entries 2DN1, 1CBM, 1LFL, and 1Z8U (11, 12, 35, 85). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

does not remain bound to HbA following the titration (62). Mice with disrupted *Ahsp* alleles exhibit microcytic anemia and possess: (a) erythrocytes with high Hb inclusion body content, shortened circulation life spans, and evidence of oxidative damage; (b) an increased amount of apoptotic erythroblasts; (c) elevated reticulocyte levels; (d) bone marrow with erythroid hyperplasia; and (e) enlarged spleens with extramedullary erythropoiesis (67). Other studies using isolated α^{H} chains have revealed that AHSP reduces α^{H} chain precipitation and reactive oxygen species production (34, 35, 62). Lastly, throughout erythrocyte maturation, *Ahsp* gene expression increases incrementally in a manner that coincides with α^{H} chain production and is controlled by GATA-1, which is a regulator of globin expression (27, 62).

Recent efforts to clarify the mechanism of AHSP action have led to several new insights in Hb biology, which include experimental evidence strongly supporting the idea that AHSP inhibits precipitation of free α subunits and acts as a molecular chaperone for the incorporation of α chains into HbA. The biology, genetics, and physiology associated with *Ahsp* gene expression have been reviewed previously. In this review, we provide a summary of the most recent biochemical and biophysical studies of AHSP-α^{H}-chain interactions related to HbA production, subunit redox chemistry, certain hematological pathologies, and areas for further research.

Pathways for Hemoglobin Assembly *In Vivo* and *In Vitro*

The folding, heme binding, and subunit assembly reactions required to generate fully functional hemoglobin tetramers are shown in Fig. 1 (reviewed in refs. 10, 16, 37, 43, 95, and 106). The first steps involve α and β globin gene translation, which takes place on the order of a few minutes per subunit (25, 51, 63, 72, 73). Given that α helices and other structural features are capable of forming spontaneously in less than a few microseconds (30, 36, 41, 111), it is plausible that α and β chains could acquire some of their secondary and tertiary structure co-translationally. Several studies using cell-free protein expression systems support this idea and suggest that heme or hemin insertion is also a co-translational process (65, 66, 95). Heme uptake causes helicity increases from 18% to 65% in α chains and 51–65% in β chains (107). The reaction of heme with $\alpha^{O}\beta^{O}$ subunit dimers and most other folded apoglobins is rapid and spontaneous, with second-order association rate constants on the order of 10^{7}–10^{8} $M^{-1}s^{-1}$ (89). However, several studies report that α^{O} and β^{O} globins only acquire heme after first dissociating from ribosomes, as depicted in Fig. 1 (8, 10, 33), and only free $\alpha^{O}\beta^{O}$ globin dimers and partially heme-saturated tetramers, but not folded and soluble α^{O} globins, have been reported *in vivo* (8, 33, 115).

Thus, it is not clear whether heme can react with unfolded subunits either post- or co-translationally.

In vitro reconstitution studies suggest that HbA formation occurs through a series of monomeric, dimeric, and tetrameric intermediates that are partially saturated with heme (55, 112, 114, 116) (Fig. 1, lower rows). Some workers have suggested that α^{H} chains may drive heme insertion into β^{O} globins following $\alpha^{H}\beta^{O}$ subunit dimer formation (1,2). Consistent with this hypothesis, α^{O} chains in apohemoglobin dimers have been shown to have a higher affinity for heme than β^{O} chains (18, 44, 55, 59, 70, 71, 84, 89, 104, 113, 114, 116). However, in our view, there is no consistent evidence to suggest that heme insertion into monomers drives dimer and tetramer assembly. Instead, we feel that assembly of partially unfolded α^{O} and β^{O} subunits into fully folded $\alpha^{O}\beta^{O}$ globin dimers drives rapid and high affinity heme uptake (Fig. 1, bottom row). However, the evidence in favor of this idea is indirect and based primarily on the observations that removal of heme from HbA results in $\alpha^{O}\beta^{O}$ globin dimers (117) and that any dissociation into monomeric α^{O} and β^{O} subunits leads to immediate precipitation of both subunits at temperatures above 5°C (107, 117). Although isolated and partially or completely unfolded α^{O} and β^{O} subunits do not readily recombine to form $\alpha^{O}\beta^{O}$ globin dimers *in vitro* (107, 117), indirect evidence for the existence of the dimeric species has been reported in some studies (8, 33, 115). Bucci and coworkers have shown that isolated β^{O} chains posses much more well-defined structural features than α^{O} chains (82, 83) and once heme is bound, $\beta_4{}^{H}$ tetramers can form, making isolated β chains much more stable than α chains, which do not self-associate into tetramers (99, 100). Thus, β chains may self-chaperone (Fig. 1, third row from top), and AHSP may perform an analogous function by generating stable AHSP:α^{H}-globin complexes (Fig. 1, first row from top) (38, 119).

The association of isolated α^{H} and β^{H} chains to form intact tetramers in solution has been studied extensively by rapid mixing and electrophoretic techniques (4, 6, 7, 13, 15, 21, 22, 33, 39, 52, 53, 58, 60, 74, 75, 78–81, 94, 99, 100, 107, 117, 118). When mixed together at low concentrations, α^{H} and β^{H} chains rapidly and spontaneously associate with each other to form HbA *in vitro* (reviewed in ref. 37), and the observed bimolecular association rate constants are on the order of 5×10^{5} $M^{-1}s^{-1}$ (5, 13, 75). Isolated β^{H} chains readily form homotetramers ($\beta^{H}{}_4$) at subunit concentrations $\geq 10\,\mu M$ (99, 100), whereas α^{H} chains remain monomeric at subunit concentrations $\leq 100\,\mu M$ and are more prone to autooxidation and precipitation (9, 14, 15, 56, 92, 99). The assembly of HbA measured *in vitro* by mixing high concentrations of isolated α^{H} and β^{H} subunits involves three steps: (a) dissociation of $\beta_4{}^{H}$ chain tetramers into β^{H} chain monomers, which occurs with rate constants $=0.25$–$0.001\,s^{-1}$, depending on the concentration of inorganic or organic phosphates present (75), followed by (b) association of monomeric α^{H} and β^{H} chains to form $\alpha^{H}\beta^{H}$ subunit dimers, and (c) association of these dimers to form tetrameric HbA (5, 37, 74, 75). At low subunit concentrations where the β subunits remain monomeric ($\leq 10\,\mu M$), only the latter two steps occur (75). The assembly reaction sequence appears to be the same for oxygenated and deoxygenated subunits (74). In the first bimolecular step, the α and β subunits combine to form the $\alpha_1\beta_1$ interface, which involves extensive hydrophobic and some electrostatic interactions between the G, H, and part of the B helices of the partner subunits, and then these dimers associate in the second bimolecular step to form two new interfaces, $\alpha_1\beta_2$, which are less extensive and involve more polar interactions (Figs. 1, 4, and 5A) (17, 24, 61, 86, 87).

Role of AHSP as a Chaperone During Hemoglobin Production

The initial work on HbA assembly described above did not include a role for chaperones; however, the discovery of AHSP and its ability to reversibly bind α^{H} chain monomers led to the classification of AHSP as a molecular chaperone (38, 62). A molecular chaperone is defined as "…a protein that binds to and stabilizes an otherwise unstable conformer of another protein—and by controlled binding and release of the substrate protein, facilitates its correct fate *in vivo:* be it folding, oligomeric assembly, transport to a particular subcellular compartment, or controlled switching between active/inactive conformations" (49). Mechanistically, these proteins work by blocking or undoing improper binding events and inhibiting the aggregation and precipitation of unfolded proteins (31, 32, 47, 48). Chaperones exist in diverse organisms (19, 96), and the inclusion of AHSP in this class of proteins is based on evidence that has been reviewed by Weiss *et al*. in 2005 (110) and 2009 (109). Early studies strongly suggest that AHSP protects α^{H} chains from precipitation *in vivo* and may act as an escort protein for fully folded free α^{H} chains by keeping them available for incorporation into HbA (34, 35, 62, 119). Newer studies, some of which are unpublished, are reviewed below and provide more detailed mechanistic insights into the molecular interactions of AHSP with α subunits, which in turn generate new models for HbA assembly.

Marden and co-workers constructed a co-expression system that can be used to investigate whether AHSP affects α chain production *in vivo* (101). Their system enables expression of either GST-tagged α chains alone or in conjunction with GST-tagged AHSP in *Escherichia coli.* Biochemical analyses indicated that the tagged α chains purified using this system have properties almost identical to those of native α chains (101). The researchers observed that AHSP significantly enhances soluble α^{H} chain production when the two proteins are co-expressed in the presence of added heme (101). When GST-α-chains were expressed alone, exposing the cell lysates to GST-AHSP during disruption did not recover any insoluble α chains that may have accumulated throughout the growth. In agreement, we find that expression of α subunits alone in *E. coli* does not lead to the accumulation of any protein, either in the supernatant or pellet of the initial lysate, indicating complete degradation (50, 108). Vasseur–Godbillon *et al.* (101) suggested that AHSP facilitates α chain production by binding newly synthesized globin and facilitating folding and heme uptake, as shown in the first row of Fig.1. Their study demonstrates that AHSP acts to prevent α chain aggregation, precipitation, and degradation (Fig. 1, red arrows), which is consistent with a molecular chaperone function.

Weiss and co-workers (119) have provided further evidence for AHSP chaperone activity by exploiting the adverse effects of *Ahsp* gene disruption in mice (67). Given that a small pool of free α chains is known to exist in erythroblasts (8, 20, 40, 64, 77, 93, 97), Yu *et al.* (119) hypothesized that the ill effects caused by *Ahsp* gene disruption might be mitigated by

lowering α *globin* gene dosage. This would reduce or eliminate the pool of free α chains that are unescorted by AHSP, and consequently might lessen the severity of the *Ahsp* knockout phenotype (119). The following strains of mutant mice were constructed to investigate this hypothesis: (a) mice lacking 1 of 4 α *globin* alleles (α-*globin*$^{*\alpha/\alpha\alpha}$); (b) homozygous null *Ahsp* (*Ahsp*$^{-/-}$) mice; and (c) *Ahsp*$^{-/-}$ α-*globin*$^{*\alpha/\alpha\alpha}$ double mutants. Surprisingly, the α-*globin*$^{*\alpha/\alpha\alpha}$ mutation did not rescue the *Ahsp*$^{-/-}$ phenotype. Rather, *Ahsp*$^{-/-}$α-*globin*$^{*\alpha/\alpha\alpha}$ mice exhibited more severe phenotypes than mice carrying either mutation alone (119). The mice possessing both mutations exhibited significant amounts of β^H chain precipitation (119). To try to understand this *in vivo* result, Yu *et al.* (119) conducted a series of *in vitro* assays using recombinant AHSP. They showed that AHSP renders α chains more resistant to trypsin digestion, and that it enhances HbA production yields in an *in vitro* wheat-germ transcription and translation system, possibly by facilitating α chain folding (119). These findings suggest that AHSP is more than just a stabilizer of excess free α chains and may be an active participant in HbA production.

Dos Santos *et al.* (26) discovered that human Ahsp gene expression is affected by the presence and absence of iron. They showed that the 3′-end of *Ahsp* mRNA contains a stretch of noncoding nucleotides that are predicted to form a stem-loop structure in solution (26). This sequence is similar to known iron responsive elements (IREs), and the investigators hypothesized that this stem-loop might interact with iron regulatory proteins (IRPs) in a way that makes *Ahsp* gene expression iron dependent (26). Upon investigation, IRP–IRE interactions were confirmed, and it was shown that iron disrupts these interactions and results in the destabilization of *Ahsp* mRNA (26). By contrast, iron depletion using the chelator desferrioxamine had the opposite effect (26). These data strongly suggest that *Ahsp* gene expression is upregulated when iron is scarce and downregulated when iron is abundant. These findings support the idea that AHSP stabilizes free α^O chains that are known to build up when iron and heme are in short supply (Fig. 1, first reaction in the top row) (26). Conversely, when intracellular iron and heme are abundant, there are fewer free α^O chains and therefore less of a need for AHSP, explaining IRE-mediated downregulation (*i.e.*, when the lower rows in Fig. 1 dominate in HbA assembly) (26).

Based on all of these studies, Yu *et al.* (119) suggested that AHSP may play a role in α chain folding and heme uptake. Such a role would be consistent with the observation that AHSP causes significant structural changes upon binding, particularly in the area of the heme binding pocket (see section below and refs. 34 and 35). It is plausible that AHSP modulates the rates and affinities for heme binding to α chains (35). Alternatively, AHSP might ensure that heme is inserted in the correct orientation or that iron-free protoporphyrin IX is excluded from the binding pocket (119). Notably, AHSP and β^H chains bind to α^H chains at the same interface (i.e., the α chain portion of the $\alpha^H{}_1\beta^H{}_1$ interface in HbA, see Fig. 5), making their interactions mutually exclusive (34, 35, 90). AHSP must dissociate from α^H chains before β^H chains can bind to form dimeric and tetrameric Hb (Fig. 1, third column) (9, 62). Thus, it appears that AHSP does not participate in the events that are downstream of $\alpha_1{}^H\beta_1{}^H$ dimer formation, but instead acts as a competitive inhibitor of HbA assembly starting from isolated α^H and β^H subunits (see discussion below). If AHSP does facilitate HbA production (101, 119), it must do so by either preventing α subunit precipitation and degradation or enhancing folding and heme insertion. In addition, AHSP must allow fairly rapid release of α chains for subsequent binding to β subunits during HbA formation in order not to significantly impede the rate of assembly.

Effects of AHSP on α^H Chain Redox Chemistry

AHSP significantly affects the redox chemistry of α^H chain heme groups (34, 35, 67). Its binding to isolated ferrous (oxy)α^H chains markedly accelerates the rate of heme iron oxidation and reduction of bound O_2 to superoxide and its subsequent dismutation to H_2O_2 (34). The spectral changes associated with these events were first investigated by Weiss, Mackay, Shi, Gow, and coworkers several years ago (Fig. 2A) (34, 35, 121). They found that the observed rate of autooxidation of isolated ferrous (oxy)α^H chains increases almost 80-fold when AHSP is present, going from 0.001 to 0.077 min^{-1} at 22°C (121). In addition, the final product of the autooxidation reaction when α^H chains are bound to AHSP has an optical spectrum similar to that of a low-spin, hexacoordinate hemichrome (Fig. 2A), indicating that both the distal and proximal histidines are coordinated to the iron atom (34, 35). This conclusion is corroborated by the crystal structure of the ferric AHSP:α^H-chain duplex that shows that His58 and His87 coordinate axially with the hemin iron atom (Fig. 2C, bottom panel) (34, 35).

The spectral and structural changes associated with autooxidation of AHSP:oxy-α^H-chain duplexes are complex and indicate multiple intermediates. Feng *et al*. (34, 35) observed what appears to be O_2 bound to iron on the proximal side of the heme, with His58(E7) coordinated directly to the iron atom in crystals of mutant AHSP(P30A):(oxy)α^H-chain duplexes kept in the cold (Fig. 2C, middle panel). The P30A AHSP mutation slows the rate of formation of the bound, ferric hexacoordinate α-chain complex. If these crystals are oxidized with $K_3Fe(CN)_6$, the resulting ferric AHSP(P30A):α^H-chain complex exhibits hexacoordination by both His58 (E7) and His87(F8) (Fig. 2C, bottom panel). For reference, the active site of α^H chains within fully oxygenated HbA is shown in Fig. 2C, upper panel. EPR spectra for the ferric forms of α^H chains in complex with β^HCO chains (*i.e.*, HbA), free in solution, and bound to AHSP are shown in Fig. 2B. In tetrameric hemoglobin [α(FeIII)βCO, Fig. 2B], the ferric α^H chains show an almost completely high spin, $g = 6$ EPR spectrum, indicative of water coordination at the sixth axial position (121). By contrast, free ferric α^H chains show less high spin $g = 6$ signal and some low spin signal, which is intrinsically much less intense in EPR derivative spectra. In contrast, no high spin, $g = 6$ signal is seen for the ferric AHSP:α^H-chain complex, which is completely low spin (Fig. 2B, inset) (121). Thus, it is clear that binding of AHSP to α^H chains does cause significant perturbations of the heme pocket, markedly affecting ligand coordination geometry. Marden and co-workers measured the effects of AHSP binding on the reactivity of α^H chains with carbon monoxide (CO) after laser photolysis (9). The recombination rate of α^H chains with CO was found to be much slower when bound to AHSP, which is also consistent with extensive heme pocket structural changes in the ferrous (deoxy)α-chain:AHSP complex (9).

The observation that AHSP increases the rate of autooxidation of α^H chains almost 100-fold seems to contradict a

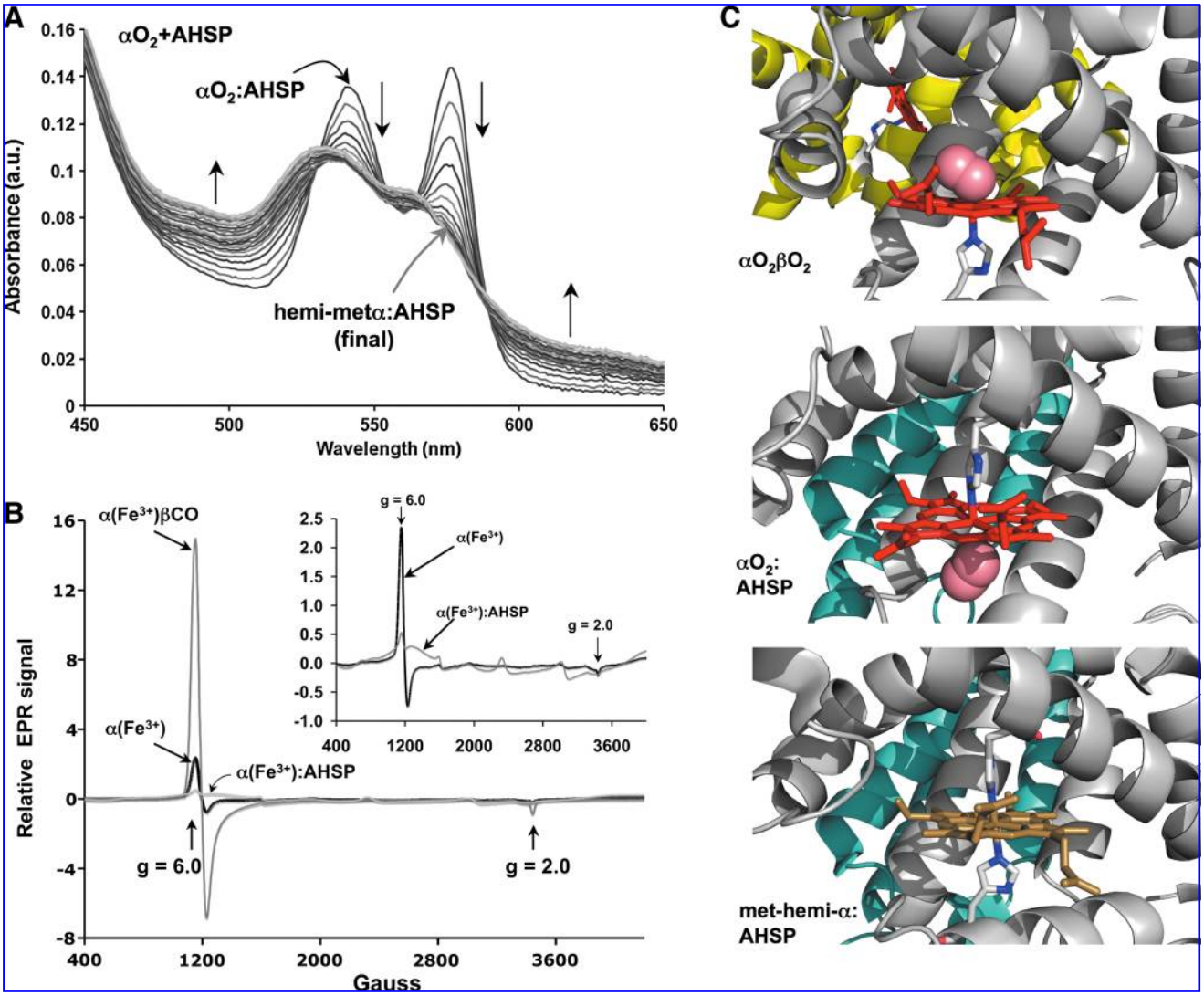

FIG. 2. AHSP-induced structural and redox chemistry changes. (**A**) Spectral changes induced by co-incubation of equimolar amounts of AHSP and oxy-α^{H} chains [see also Zhou *et al*. (121)]. Protein concentrations were 10 μM each in air equilibrated 100 m*M* potassium phosphate buffer, pH 7.0 at 25°C. Each *line* represents 30 min intervals recorded over ~18 h. (**B**) Electron paramagnetic resonance (EPR) spectra of ferric α^{H} chains in the presence and absence of AHSP and α^{H} CO chains (reproduced from Zhou *et al.* (121)). EPR spectra were recorded using the following parameters: frequency, 9.60 GHz; power, 10 milliwatts; modulation amplitude, 10.9 G; modulation frequency, 100 kHz; and temperature, 4.5 K. (**C**) Effects of AHSP on heme pocket structure of α^{H} chains. *Top*: heme pocket of α^{H} chains within fully oxygenated HbA (2DN1). *Middle*: heme pocket of α^{H} chains within the AHSP(P30A):(oxy)α^{H}-chain duplex (1Y01). *Bottom*: heme pocket of α^{H} chains within a fully oxidized AHSP(P30A):α^{H}-chain duplex (1Z8U). Dioxygen is depicted in *pink*, α chain helices in *gray*, β chains in *yellow*, heme in *red*, hemin in *brown*, and the proximal and distal histidines in Corey–Pauling–Koltun (cpk) coloring. Drawings were produced using PyMol and PDB entries 2DN1, 1Z8U, and 1Y01 (34, 35, 85). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

role in stabilizing α^{H} chains. Oxidation of hemoglobin is normally the first step in denaturation. Hemin dissociates much more readily from most globins in the oxidized form, and the resulting loss of hemin leads to rapid unfolding and irreversible precipitation of the apoglobin at physiological temperatures (45, 46). In addition, most methemoglobins react with H_2O_2 to form even more reactive oxygen species and protein free radicals. However, Feng *et al.* (35) showed that AHSP binding to both (oxy)α^{H} chains and met-α^{H} chains confers resistance to H_2O_2-induced heme damage and reactive oxygen species (ROS) production (35). This observation explains why ablation of *Ahsp* alleles in mice results in erythroid cells with increased amounts of reactive oxygen species, signs of oxidative stress, and a heightened sensitivity to oxidizing agents such as phenylhydrazine (67). These combined data prompted Feng *et al*. to hypothesize that "… AHSP stabilizes [α^{H} chains] by converting [them] into an oxidized but fully liganded low-spin, nonreactive state" (67). Because free α^{H} chains are cytotoxic (9, 14, 15, 56, 92), it seems likely that, despite promoting autooxidation, binding to AHSP inhibits this toxicity by inducing hexacoordination of bound ferric α chains, which prevents rapid hemin loss, generation of

ROS, and precipitation, all of which would lead to general oxidative stress and membrane damage *in vivo* in the absence of AHSP (54, 88).

AHSP, α^H Chain Binding and Dissociation Events

Changes in intrinsic fluorescence of AHSP in the presence of α^H and β^H chains were first examined in detail by Baudin–Creuza *et al.* (9). These manual mixing fluorescence studies revealed rapid quenching of AHSP Trp44 fluorescence during binding to α^H chains, which themselves do not fluoresce due to highly efficient fluorescence resonance energy transfer (FRET) to the heme prosthetic group (3, 9). We have extended this work over the last year to investigate the kinetics of α^H chain binding to and release from AHSP. Quenching of AHSP Trp44 fluorescence by bound α^H was used to measure the rate of AHSP:α^H-chain duplex formation in stopped-flow rapid mixing experiments, starting with the isolated proteins. Normalized time courses for the reaction of 1.0 μM AHSP with various concentrations of isolated α^H chains are shown in Fig. 3A. The binding reaction is clearly bimolecular, and a plot of the observed rate versus α^H chain concentration under pseudo first-order conditions gives an apparent association rate constant for AHSP binding of $k'_{AHSP} \approx 10\,\mu M^{-1}s^{-1}$. Remarkably, this value is ~20-fold greater than the association rate constant for (deoxy)α^H subunits binding to (deoxy)β^H subunits during *in vitro* HbA re-assembly experiments ($k'_{1,2} \approx 0.5\,\mu M^{-1}s^{-1}$, Fig. 4). This difference in rate of binding to α^H subunits is shown dramatically in Fig. 3A by the dashed line, which shows the α^H–β^H chain association reaction at 3.0 μM subunit concentrations as measured by optical absorbance changes at 445 nm (75).

β^H chains were used to displace α subunits from the AHSP complex, a reaction that was first reported by Kihm *et al.* (Fig. 4B) (62). Like α^H chains, β^H chains do not fluoresce in solution due to efficient energy transfer to their heme groups (3). Thus, the increase in fluorescence emission signal observed upon mixing β^H chains with AHSP:α^H-chain duplexes reports directly the extent of AHSP dissociation from α^H chains. Figure 3B shows a time course for the displacement of α^H from the AHSP complex by excess β^H subunits. The observed rate for this displacement reaction is given by:

$$k_{obs} = k_{AHSP}\frac{k'_{1,2}[\beta]}{k'_{AHSP}[\mathrm{AHSP}] + k'_{1,2}[\beta]} \qquad \text{Equation 1}$$

Using previously determined values for $k'_{1,2}$ and k'_{AHSP}, the best fitted value of k_{AHSP} for plots of k_{obs} versus [β]/[AHSP] is $\approx 0.1\,s^{-1}$. These kinetic parameters for AHSP binding to α^H chains suggest a dissociation equilibrium constant (K_d) of $\sim 10^{-2}\,\mu M$, which is similar to the value estimated by Gell *et al*. several years ago (38). By comparison, the estimated rate constant for dissociation of the $\alpha_1\beta_1$ duplex is $\sim 5\times10^{-5}\ s^{-1}$ and the K_d for dissociation of this Hb dimer is $\sim 10^{-6}\,\mu M$ (75, 79).

These experiments show that: (a) β^H chains have a 10,000-fold higher affinity for α^H chains than AHSP but (b) AHSP binds to α^H chains ~20 times faster than to β^H chains. Thus, in an equal mixture of AHSP, α^H chains, and β^H chains, AHSP

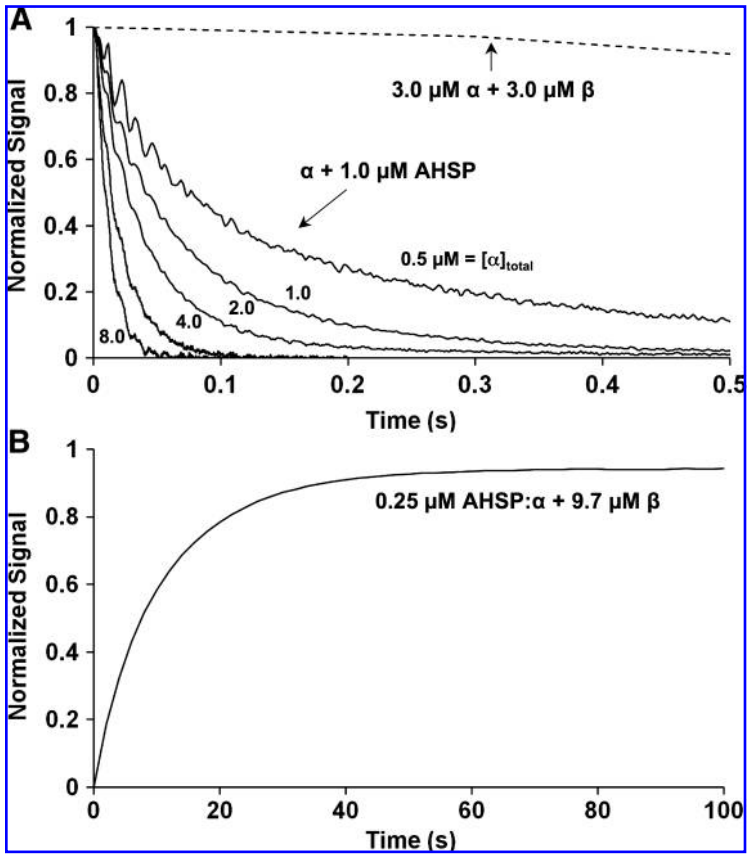

FIG. 3. AHSP-α^H-chain binding and dissociation events. (**A**) AHSP association with α^H (CO)-chains (*solid traces*) and (deoxy)α^H-chain association with (deoxy)β^H-chains (*dashed trace*). The reaction of AHSP with free α^H(CO)-chains was studied using an Applied Photophysics PiStar rapid mxing instrument. The buffer used was 50 mM potassium phosphate, pH 7.2 at 22°C and had been purged with 1 atm of pure CO prior to use. Excitation light was $\lambda = 280$ nm, entrance and exit slit widths were 10 nm each, path length was 10 mm, and a 302 nm cutoff filter was used to record total fluorescence upon symmetric mixing. The concentrations cited here are postmixing values, and the resulting traces were normalized to the fluorescence intensity decrease. The *dashed trace* is a normalized (deoxy)α^H- and (deoxy)β^H-chain association reaction followed by UV-visible absorbance spectroscopy using the method of McGovern *et al.* (75) (see Fig. 4). (**B**) Reaction of the AHSP:α^H(CO)-chain duplex with β^H (CO)-chains. Experimental conditions were the same as in (**A**), except that fluorescence intensity increases were observed and normalized.

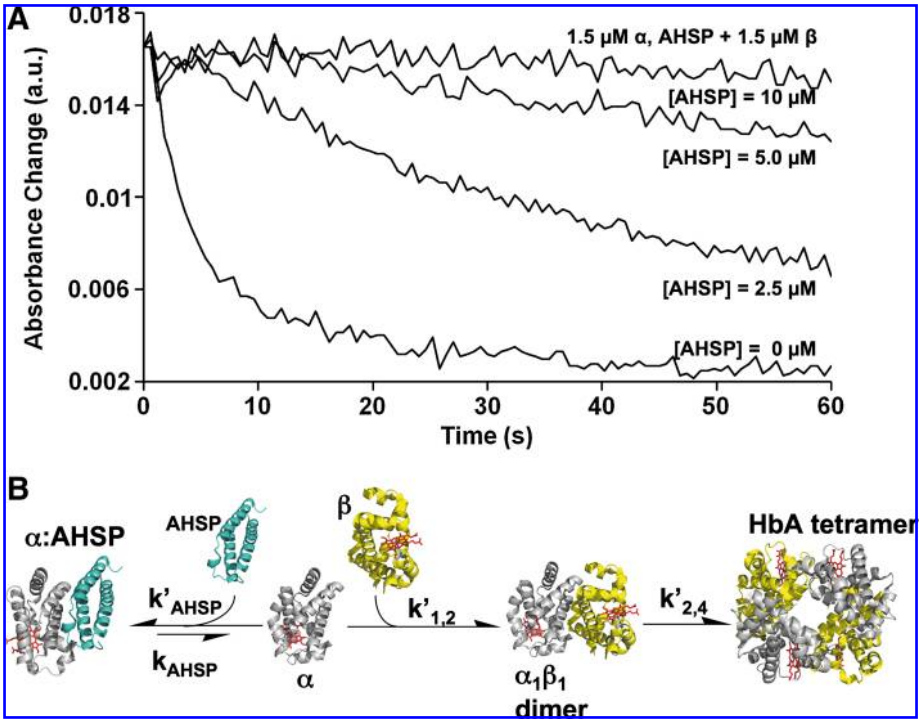

FIG. 4. The effect of AHSP on α^H and β^H chain assembly. (**A**) Measurement of deoxyHbA formation starting from isolated deoxy-α^H and β^H chains. Assembly was followed using the method of McGovern *et al*. (75). Separate solutions of isolated chains were mixed in a Gibson–Durrum stopped-flow apparatus, and absorbance decreases at 445 nm were followed as function of time. AHSP at the indicated concentration (after mixing) was incubated with α^H chains prior to reaction with β^H chains. The buffer used was 50 m*M* potassium phosphate with excess sodium dithionite, pH 7.4 at 20°C; this buffer was purged with nitrogen prior to use. All concentrations listed are postmixing values and the concentraion of AHSP was varied from 0 (*bottom trace*) to 10 μM (*top trace*). (**B**) Mechanism depicting the different binding events in which α^H chains participate and the mechanism of AHSP inhibition of HbA assembly. The colors, drawings, and structural data contained in this panel are identical to those in Fig. 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

will bind to α^H subunits first. However, because α^H subunits have a much higher affinity for β^H chains than AHSP, hemoglobin will eventually be formed, albeit more slowly than the initial AHSP association phase. The rate of α^H chain dissociation from the AHSP complex is ~50,000 times larger than its rate of dissociation from the $\alpha_1\beta_1$ dimer, and more importantly, the absolute value of k_{AHSP} ($\sim 0.1\ s^{-1}$) is large enough to allow rapid formation of HbA at high concentrations of β^H subunits and low concentrations of AHSP (Fig. 3). These kinetic properties of AHSP are what would be expected of a chaperone: rapid binding to α^H chains to prevent unfolding and precipitation but rapid release to β^H chains to allow Hb assembly. We are currently examining these reactions in greater detail to take into account the effects of key AHSP mutants on both α^H chain binding and release, and the effects of various naturally occurring α^H chain variations.

Effects of AHSP on the Rate of Hb Assembly

As described in Fig. 1 and the section above, high concentrations of AHSP will decrease the rate of HbA assembly by competing with β^H chains for binding to α^H chains. This effect is demonstrated directly in Fig. 4. In these experiments, deoxygenated α^H and β^H subunits are mixed in a stopped-flow apparatus, and when they assemble into tetrameric deoxyhemoglobin, there is a marked "sharpening" and increase in intensity of the Soret maximum, which results in small increases in absorbance at 430 nm and decreases at 445 nm of the heme group. This spectral transition is associated with further out-of-plane movement of the iron atom in the T or low affinity quaternary state of deoxyHbA, has been termed the R to T or Hb* to Hb spectral transition, and can be used to follow the assembly of deoxyHbA (13, 75). A normalized time course for the anaerobic reaction of 2.5 μM (deoxy)α^H chains with 2.5 μM (deoxy)β^H chains is shown in Fig. 4A (curve labeled [AHSP] = 0) and can be described by two consecutive second-order processes with rate constants equal to $0.5\ \mu M^{-1}s^{-1}$ because under these conditions the β^H subunits remain monomeric (Fig. 4B) (75). When AHSP is pre-incubated with α^H subunits, the reaction with β^H subunits is slowed markedly, with the rate of HbA assembly decreasing almost linearly with increasing [AHSP] (Fig. 4A).

At high AHSP and β^H chain concentrations, the rate of Hb assembly becomes first order, limited by the rate of α^H subunit displacement from AHSP by β^H chains, and equal to the expression in Equation 1. The observed rate will approach zero as the ratio [AHSP]/[β^H chains] becomes large as is shown in Fig. 4. However, if the ratio is small (*i.e.*, [β^H chains]>> [AHSP]), the process is limited by the rate of α^H subunit dissociation from AHSP. The value of k_{AHSP} is on the order of $0.1\ s^{-1}$, based on the results in Fig. 3, and similar to the rates of the bimolecular reaction of α^H with β^H chains in the micromolar protein concentration range. Thus, it is clear that high excess concentrations of AHSP will inhibit the rate of Hb formation, and to be an effective chaperone, AHSP must be expressed at concentrations that are lower than β^H subunits. However, it is important to note that even though the rate of Hb A assembly is slowed at high [AHSP], Hb formation always occurs, albeit slowly, because the affinity of β^H chains for α^H subunits is still 10,000-fold greater than that of AHSP.

Clinical Relevance of AHSP Function

The participation of AHSP in HbA biosynthesis prompted early speculation that dysregulation of this protein may play a causative role in certain diseases. The relationships between

AHSP and the thalassemia syndromes have been examined recently because these disorders involve HbA biosynthesis and α^H and β^H chain instability. Viprakasit *et al*. (105) investigated whether variations in the apparent clinical severity of Hb E β thalassemia could be explained by the presence of mutant *Ahsp* alleles among affected individuals. Several single nucleotide polymorphisms (SNPs) were identified, but none of them were found to correlate with the severity of the thalassemic phenotype (105). Other work has shown that both healthy and thalassemic individuals may possess an uncommon missense mutation that results in AHSP with an isoleucine at position 75 instead of an asparagine (N75I) (28). This mutation does not occur at the AHSP-α^H-chain interface, but initial work suggests that it is nonetheless functionally important (29). In another study, Lai *et al.* (69) have shown that *Ahsp* mRNA levels in reticulocytes vary considerably in healthy individuals, and several sequence variants have been identified which affect *Ahsp* transcription levels and may be linked to the phenotypic discordance observed in certain types of β thalassemia. Also, a variant in *Ahsp* intron 1 has been associated with altered AHSP expression levels and occurs commonly in healthy individuals (29). Interestingly, certain *Ahsp* alleles bearing SNPs that are thought to result in diminished AHSP expression have been linked to Heinz body-, drug-, and infection-induced hemolytic anemia in a preliminary report (57). Thus, *Ahsp* gene expression levels and function appear to be relevant in a variety of clinical contexts, and ongoing work in this area is likely to be informative.

Naturally occurring α^H chain mutations may result in impaired interactions with AHSP and be responsible for clinically observed hemoglobinopathy phenotypes. Marden and co-workers have suggested that AHSP-α^H-chain interactions are impeded by a proline to serine mutation at position 119 of α^H chains (P119S, Hb Groene Hart), which results in an α thalassemia phenotype (102). Another novel α^H chain mutation (F117S, Hb Foggia) also results in a phenotype typical of α thalassemia (68). This mutation is predicted to disrupt favorable interactions with AHSP (34, 68). Vasseur *et al.* (103) have recently evaluated a set of clinically relevant α^H chain mutations: H103L (Bronovo); C104Y (Sallanches); C104S (Oegstgeest); T108N (Bleuland); L109R (Suan Dok); L109Q; F117S (Foggia); P119S (Groene Hart); P119L (Diamant); and L129P (Utrecht). They co-expressed GST-tagged wild-type AHSP with GST-tagged mutant α^H chains in *E. coli*, and quantified expression levels of these α^H chain mutants. The detection of variable amounts of soluble α^H chains demonstrates that the selected amino acid replacements have significant effects on the stability of the AHSP-α^H-chain interface. However, in almost all of these cases, the mutations occur in a region of α^H chains that is part of the shared interface for binding to both AHSP and β^H chains (Fig. 5). Thus, these results are ambiguous, and it is unclear which disrupted interactions, AHSP:α^H-chain or $\alpha_1\beta_1$, give rise to the observed phenotypes (68, 103).

Yu *et al*. (120) recently investigated the interactions of AHSP, β, and α chains with eight different α chain mutations: R31S (Prato); K99E (Turrif); K99N (Beziers); H103Y (Lombard); H103R (Contaldo); F117S (Hb Foggia); P119S (Hb Groene Hart); and R31S (Prato). Using a variety of biochemical assays, all eight mutations were found to disrupt α chain interactions with AHSP, β chains, or both (120). The three most informative mutations were R31S (Prato), H103Y (Lombard), and K99E (Turrif). The positions of the native side chains are highlighted in the $\alpha_1\beta_1$ and AHSP:α^H-chain interfaces shown in Fig. 5. Arg31 plays a prominent role in stabilizing the interface with β^H subunits, but is not part of the interface with AHSP. As result, the α R31 mutation only inhibits co-expression of α^H chains with β^H chains, but has no effect on co-expression of α^H chains with AHSP because the mutant AHSP:α^H-chain complex can form to the same extent as the wild-type duplex (120).

The opposite situation occurs for mutations at the α K99 position. Lys 99 is not part of the $\alpha_1\beta_1$ interface, but does appear to interact with Asp29 of AHSP near the edge of the AHSP:α^H-chain interface (Fig. 5B). Mutations at this position inhibit co-expression of α^H chains with AHSP but not with β^H chains. Thus, formation of the AHSP:α^H-chain complex is impaired but not the $\alpha_1\beta_1$ interface in HbA. To prove that this effect was specific, Yu *et al.* (120) demonstrated that the loss of interaction due to the K99E mutation in α^H chains could be rescued by introducing a D29R mutation into AHSP (Fig. 5). Mutating the negatively charged AHSP D29 to a positively charged Arg re-established a favorable interaction with the negatively charged Glu side chain in the α K99E mutant. Because the K99E and K99N mutations in α^H chains do not disrupt the $\alpha^H{}_1\beta^H{}_1$ interface, it is very plausible that the clin-

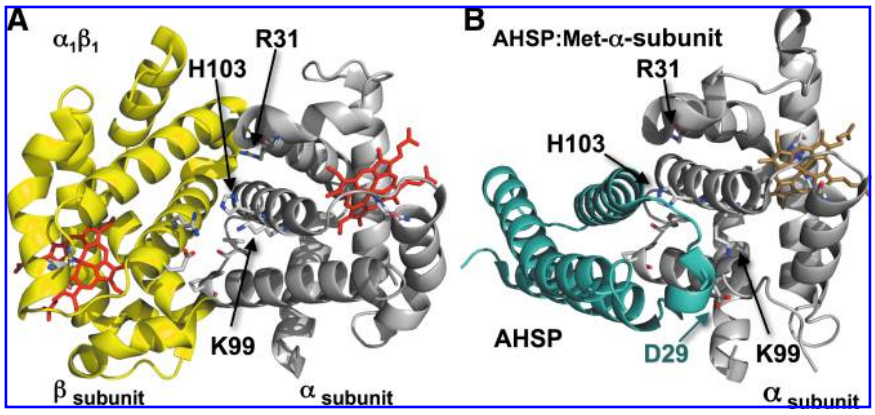

FIG. 5. Key residues within the $\alpha^1\beta^1$ and AHSP-α^H-chain interfaces. (**A**) Ribbon diagram of the $\alpha^1\beta^1$ dimer interface with selected residues depicted in CPK colors using stick format. The α^H R31 and H103 side chains both participate in strong interactions with the partner β^H subunit, whereas the side chain of α^H 99 K does not. (**B**) Ribbon diagram of the AHSP:met-α^H-chain interface with the same α chain amino acids highlight as CPK colored sticks as in **A**. The α^H chain K99 and H103 side chains participate in favorable interactions with AHSP, whereas R31 does not. In both panels, α chain helices are depicted in *gray*, β chains in *yellow*, heme in *red*, hemin in *brown*, and the proximal and distal histidines in *CPK colored sticks*. Drawings were produced using PyMol and PDB entries 1LFL and 1Z8U (11, 35). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

ical phenotypes associated with these mutations are caused exclusively by disrupted AHSP interactions. These results provide strong evidence that AHSP plays a role in facilitating HbA assembly and preventing native α^H chain precipitation *in vivo*.

The H103 mutations serve as examples of alterations that prevent formation of both the AHSP:α^H-chain and $\alpha_1\beta_1$ interfaces. The H103Y and H103R mutations inhibit co-expression of α^H chains with both AHSP and β^H chains, and both cause clinically relevant and more severe anemias than the α K99 mutations (120). Presumably the mutations studied by Vasseur *et al.* (103) show similar *in vitro* expression characteristics and clinical severities since most are found in both interfaces.

Concluding Remarks and Future Directions

Available evidence strongly suggests that AHSP promotes HbA production in maturing erythrocyte precursors through several mechanisms, all of which involve direct binding to α chains. Ongoing work in this area is aimed at clarifying the details of these molecular interactions. Of particular interest is whether and to what extent AHSP participates in α chain folding and heme uptake *in vivo.* Also, interactions between AHSP:α^H-chain duplexes and other proteins might reveal additional pathways in which AHSP participates, and questions regarding the role of AHSP in iron utilization have only recently been raised.

Additional research investigating the evolutionary history and conservation of the *Ahsp* gene will be informative. Although this gene appears to be present in most but not all mammals, it has not been found in the sequenced genomes of non-mammalian vertebrates such as amphibians, chickens, or lizards (26), and there are no reports of closely related homologs in microorganism or plants. These observations, along with the finding that murine *Ahsp* gene ablation results in relatively healthy animals with only mild anemia (67), support the suggestion by dos Santos *et al.* (26) that AHSP may be a late evolutionary development that "fine-tunes" certain erythroid processes in mammals, particularly those with nonnucleated red cells (reviewed in 23). Thus, perhaps AHSP is both an adaptation for HbA biosynthesis and for optimization of erythrocyte integrity in the absence of an intact nucleus and the ability to re-transcribe the α and β *globin* genes and replenish Hb. Chaperonin overexpression allows bacteria to tolerate more mutations in key proteins (98), and perhaps AHSP evolved to allow greater tolerance of α chain mutations in response to hematological disorders, infectious diseases, or environmental stresses specific to certain mammals.

It may be possible to exploit the properties of AHSP for therapeutic purposes. For example, increasing *Ahsp* gene expression might ameliorate the effects of certain thalassemia syndromes or other conditions that are associated with dysregulation of HbA production. Also, certain conditions may be tied more directly to AHSP malfunction, in which case restoring normal AHSP function might be a clinically useful approach. AHSP also represents a potentially useful tool for increasing the expression of recombinant hemoglobin (rHb) as the source material for Hb-based oxygen carriers or blood substitutes. For example, co-expressing α and β *genes* with low levels of AHSP in prokaryotic expression systems may lead to enhanced holo-hemoglobin production by preventing α globin precipitation. However, any favorable effects will be concentration dependent, because significant AHSP overexpression to levels similar to those of α^H and β^H chains will inhibit HbA assembly in addition to depleting cellular resources.

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Author Disclosure Statement

JSO and MJW declare inventorship in connection with U.S. Patent Application 11/685,986 entitled "Enhancing Recombinant Hemoglobin (rHb) Production by Co-expression with Alpha Hemoglobin Stabilizing Protein (AHSP)." JSO declares inventorship in connection with the following U.S. Patents: 08/381,175, 09/654,688, 10/107,871, and application 60/610,108 (under review), all of which involve engineering recombinant Hb for use as a Hb-based oxygen carrier (HBOC) or blood substitute. Aside from their associations with their respective research institutions, none of the authors are presently affiliated with any business entity that would create a commercial or financial interest regarding the information set forth in this manuscript. The authors are presently not paid consultants, nor do they serve in a managerial or advisory capacity for any business entity which would create an actual or potential conflict of interest regarding the information set forth in the manuscript.

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Abbreviations Used

AHSP = alpha-hemoglobin stabilizing protein
CO = carbon monoxide
EPR = electron paramagnetic resonance
GST = glutathione-S-transferase
Hb = hemoglobin
HbA = wild-type adult human Hb
Mb = myoglobin
rHb = recombinant Hb
ROS = reactive oxygen species
SNPs = single nucleotide polymorphisms
superscript H = containing heme or hemin; e.g., α^H
superscript O = lacking heme or hemin; e.g., α^o

Hereinafter, "chains" and "subunits" refer to Hb monomers irrespective of the presence or absence of heme or hemin, whereas "globin" specifically refers to monomers lacking heme or hemin. The use of CAPITAL ROMAN typeface refers to proteins, and the use of *lower case italicized* typeface refers to genes.

Abbreviations, conventions (partially derived from 38, 62, 107).

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