Insights into Hemoglobin Assembly through in Vivo Mutagenesis of \( \alpha \)-Hemoglobin Stabilizing Protein*§

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Background: The \( \alpha \)-hemoglobin-stabilizing protein (AHSP) facilitates hemoglobin assembly.

Results: AHSP mutations that enhance binding affinity for \( \alpha \)-globin or slow its rate of autooxidation in vitro do not affect normal or stressed erythropoiesis in mice.

Conclusion: AHSP exhibits robust molecular chaperone activity in vivo even when its biochemical interactions with reduced \( \alpha \)-globin are perturbed.

Significance: Our findings support new models for AHSP activities in vivo.

\( \alpha \)-Hemoglobin stabilizing protein (AHSP) is believed to facilitate adult Hemoglobin A assembly and protect against toxic free \( \alpha \)-globin subunits. Recombinant AHSP binds multiple forms of free \( \alpha \)-globin to stabilize their structures and inhibit precipitation. However, AHSP also stimulates auto-oxidation of \( \alpha \)-O2 subunit and its rapid conversion to a partially unfolded bishistidyl hemichrome structure. To investigate these biochemical properties, we altered the evolutionarily conserved AHSP proline 30 in recombinantly expressed proteins and introduced identical mutations into the endogenous murine \( \alpha \)\( \text{H}_{\text{sp}} \) gene. In vitro, the P30W AHSP variant bound oxygenated \( \alpha \) chains with 30-fold increased affinity. Both P30W and P30A mutant proteins also caused decreased rates of \( \alpha \)-O2 autooxidation as compared with wild-type AHSP. Despite these abnormalities, mice harboring P30A or P30W \( \text{A}_{\text{hsp}} \) mutations exhibited no detectable defects in erythropoiesis at steady state or during induced stresses. Further biochemical studies revealed that the AHSP P30A and P30W substitutions had minimal effects on AHSP interactions with ferric \( \alpha \) subunits. Together, our findings indicate that the ability of AHSP to stabilize nascent \( \alpha \) chain folding intermediates prior to hemin reduction and incorporation into adult Hemoglobin A is physiologically more important than AHSP interactions with ferrous \( \alpha \)-O2 subunits.

Adult Hemoglobin A (HbA)\(^3 \) production in vivo is a highly coordinated process requiring balanced synthesis of individual \( \alpha \) and \( \beta \)-globin chains and their assembly with ferroprotoporphyrin IX (heme) prosthetic groups to form \( \alpha_2\beta_2 \) tetramers (1–3). Fully assembled HbA is very stable in comparison with isolated \( \alpha \) and \( \beta \) subunits in their heme (Fe\(^{3+}\))- or hemin (Fe\(^{3+}\))-containing (holo) and heme-lacking (apo) forms (4, 5). Free subunits, especially the Fe\(^{3+}\) (ferric) form, tend to catalyze the production of reactive oxygen species (ROS), denature, and form damaging precipitates (4, 6, 7).

Erythroid cells contain protective mechanisms to balance HbA synthesis and reduce the toxicity of free globin chains (8). For example, \( \alpha \)-hemoglobin stabilizing protein (AHSP) binds \( \alpha \) but not \( \beta \) chains or intact HbA (9, 10) and inhibits denaturation of free \( \alpha \)-globin in vitro (9, 10). Targeted deletion of the \( \text{A}_{\text{hsp}} \) gene in mice causes globin precipitation, mild hemolytic anemia, and enhanced sensitivity to oxidant stress (10, 11). Loss of AHSP also aggravates both \( \beta \)-thalassemia (11) and \( \alpha \)-thalassemia (12) in mice. In humans, several naturally occurring \( \alpha \)-globin missense mutations and AHSP variants appear to cause anemia by inhibiting the ability of \( \alpha \)-globin to interact with AHSP (13–17).

Recent studies have helped to define the biochemical and structural features of AHSP/\( \alpha \) interactions (15, 18–23). AHSP is comprised of three antiparallel \( \alpha \) helices with 11 unstructured amino acids at the carboxyl terminus (21–23). AHSP helix 1, helix 2, and the interconnecting loop interact with helices G and H of \( \alpha \)-globin at a surface that overlaps with the interface for \( \beta \)-globin binding. However, \( \beta \) subunits bind \( \alpha \) sub-

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3 The abbreviations used are: HbA, adult Hemoglobin A; apo, heme-lacking; bishistidyl conformation, ferric heme iron hexacoordinated with the proximal (E7) and distal (F8) histidines bound axially; DCFH, 2′,7′-dichlorofluorescein diacetate; ferric, Fe\(^{3+}\); ferrous, Fe\(^{2+}\); Hb, hemoglobin; heme, ferroprotoporphyrin IX; hemichrome, ferric bishistidyl hemoglobin; hemin, porphyrin group containing ferric iron; holo, heme (Fe\(^{3+}\))-or hemin (Fe\(^{3+}\))-containing; met, methemoglobin (ferric hemoglobin); ROS, reactive oxygen species; Mb, myoglobin; 5-FU, 5-fluorouracil; AHSP, \( \alpha \)-hemoglobin stabilizing protein.
units with higher affinity and displace α from AHSP complexes in solution and in vivo (9, 10, 14, 24, 25). These observations indicate that AHSP can act as a molecular chaperone to bind nascent α-globin and stabilize its folding prior to incorporation into HbA during normal erythropoiesis (12, 14, 24, 25).

AHSP promotes dramatic biochemical changes to bound αO₂ (for review, see Ref. 26). Normally, α-globin heme iron is axially coordinated by His87(F8), termed the proximal histidine. The opposite axial position binds dioxygen (O₂), which is stabilized by interaction with the distal histidine, His58(E7) (27). AHSP binding to αO₂ accelerates spontaneous heme iron oxidation (autooxidation) from an Fe²⁺ (ferrous) to an Fe³⁺ (or met) state. Autooxidation is followed immediately by internal coordination of His58(E7) to the heme iron, resulting in a hexacoordinate bishistidyl or hemichrome structure (18, 21, 22, 28). Bishistidyl hemoglobins occur as α-globin degradation products in β-thalassemia (4, 6, 29, 30), during normal degradation of hemoglobin, and in some naturally occurring globins in mammalian tissues, plants, certain bacteria, and cold water fish (31–40). Binding of His58 to heme iron appears to inhibit the ability of met-α to catalyze ROS production (39, 41). Thus, AHSP-mediated conversion of αO₂ to a bishistidyl state has been postulated to protect erythroid cells against oxidative damage during β-globin deficiency and/or oxidative stress. Although this possibility is supported by in vitro studies (22, 28), the in vivo relevance of AHSP-mediated conversion of bound αO₂ to the bishistidyl met-α state had not been examined until this study.

An evolutionarily conserved proline residue within loop 1 of AHSP regulates binding to ferrous αO₂ and its subsequent autooxidation (18). In the structure of the ASHP:met-α complex, the cis conformation at the ASHP Asp29-Pro30 peptide bond projects loop 1 toward α-globin helix G where AHSP Pro30 promotes backbone hydrogen bonding interactions. Pro30 amino acid substitutions in AHSP convert the 29-30 peptide bond to a completely trans conformation, which generally enhances the affinity of AHSP for ferrous αCO and αO₂ and reduces the rates of autooxidation and subsequent conversion to the bishistidyl form (18).

Because of these large effects on AHSP affinity for reduced α subunits and on the redox and coordination properties of bound α subunits, we chose to examine the in vivo phenotypes of P30A and P30W variants of murine AHSP (18). Although these mutations significantly altered AHSP/αO₂ interactions in vitro, they surprisingly produced no detectable consequences in mice. These observations led us to re-examine α subunit binding, autooxidation, and hemin loss from the mutant AHSP complexes in vitro. Our combined in vitro and in vivo findings refine and revise current models for AHSP function during erythropoiesis and emphasize the physiological importance of high affinity met-α binding to AHSP.

**EXPERIMENTAL PROCEDURES**

**Rates of a Subunit Binding and Release from Pro30 Variants**—Detailed descriptions of measurements of α subunit binding to and release from AHSP variants are given in the accompanying paper by Mollan et al. (42). Time courses for association of reduced and oxidized α subunits with AHSP were measured in an Applied Photophysics PiStar kinetic stopped-flow spectrophotometer (Leatherhead, Surrey, UK), monitoring the intrinsic fluorescence of AHSP. The binding of holocomplexes quenches the fluorescence of AHSP, and its release to β subunits restores the fluorescence emission. A detailed discussion of these signal changes and analysis of the observed time courses are also given in Mollan et al. (42).

**Autooxidation Rates of AHSP:αO₂ Complexes**—Autooxidation reactions were initiated by diluting a cold concentrated solution of αO₂ into an equimolar solution of AHSP in 0.1 M sodium phosphate, pH 7.0 at 37 °C and then recording optical absorbance visible spectra. Spectra were recorded using a Cary50Bio instrument (Varian, Inc., Palo Alto, CA). First-order rate constants for autooxidation to the hemichrome met-α were obtained by fitting the absorbance change time courses to a single exponential expression using the Microsoft Excel Solver plug-in (Microsoft Corp., Redmond, WA).

**Rate of Hemin Loss from oxidized α Subunits**—Hemin dissociation time courses were measured by mixing the ferric form of α or α-AHSP with the apoglobin form of H64V/V68F sperm whale myoglobin (Mb) as described previously for assaying hemin dissociation from metHb and metMb (43–46). Hemin loss from free ferric α generates α-apoglobin, which immediately precipitates at 37 °C, causing a dramatic increase in solution turbidity. Increasing solution turbidity interferes with monitoring the absorbance time courses at 600 nm for hemin transfer from α-globin to the apoMb reagent. To obtain analyzable time courses, the temperature was lowered to 10 °C, and 0.6 M sucrose was added to stabilize the apoprotein and inhibit precipitation (46). In addition, the reaction pH was lowered to 5.5 to speed up the reaction at the low temperature, which also helps prevent interference from apo-α-globin precipitation, particularly for free met-α subunits.

Hargrove et al. (44–46) have shown that hemin loss rates increase markedly at low pH as the proximal histidine becomes protonated. However, the relative effects of mutagenesis and oligomer dissociation for both Hbs and Msbs remain the same as that at higher, more physiological pH values. For the free met-Hb and met-α-AHSP complexes, the hemin loss reaction mixtures contained 6 μM met-α, 40 μM H64V/V68F apoMb, 30 μM potassium ferricyanide to ensure oxidation, and 6 μM either WT, P30A, or P30W AHSP. Spectra were recorded every minute for ~12 h, and the increase at 600 nm is due to formation of the “green” H64V/V68F holo-metMb complex as the α subunits lose hemin. Data were plotted as the normalized absorbance change at 600 nm versus time.

**Animals—** Ahsp P30W- and P30A-targeting constructs were generated by modification of a previously described Ahsp-targeting construct (supplemental Fig. 1) (10). Site-directed mutagenesis was used to replace the CCT codon for proline (Pro) with either GCT for alanine (P30A) or TGG for tryptophan (P30W). The targeting plasmids were linearized with SacI and electroporated into 129S5 embryonic stem cells. Clones with stable integration of the targeting vector were selected with G418 and ganciclovir and further screened for homologous recombinants by Southern blotting and karyotyping. Selected clones were injected into C57BL/6 blastocysts to produce chimeric mice. These were bred with C57BL/6 mice (The...
Jackson Laboratory, Bar Harbor, ME) to produce AhspP30A/+ or AhspP30W/+ heterozygotes. Neomycin selection cassettes were removed by interbreeding with E2A-Cre mice (47). Mice were genotyped using the primer set AHSP-KI-F (5′-AGA ACG GGG GGA TGC AAA TCC AAA ACA AAG-3′) and AHSP-KI-R (5′-CAA GTT CAC CCA GTC ATG AAC CAC AAT CAC-3′), generating a 190-base pair product for the wild-type allele and a 236-base pair product for the mutant allele. All mice used for experiments were backcrossed onto a C57BL/6 background for five to seven generations. AhspP1−/− mice were generated as described previously (10). β-Thalassemic (β-globinTrk3+/+) mice were kindly provided by Oliver Smithies (University of North Carolina, Chapel Hill, NC) (48). The Ahsp allele is genetically linked to the β-globin (Hbb) locus (11); Ahsp/β-globinTrk3−/− double mutant mice were generated as described previously (11). Animal protocols were approved by the Institutional Animal Care and Use Committee of The Children’s Hospital of Philadelphia (Philadelphia, PA).

**Hematological Analysis**—Mice were analyzed at 2 and 8 months of age. Blood was collected by submandibular bleeding, anticoagulated with EDTA, and analyzed on a Hemavet HV950FS analyzer (Drew Scientific, Dallas, TX). Reticulocyte counts were done using Retic-COUNT reagent according to the manufacturer’s protocol using a FACSCalibur flow cytometer (BD Biosciences). Flow cytometry data were acquired using equipment maintained by the Flow Cytometry Core Laboratory of The Children’s Hospital of Philadelphia Research Institute.

**Intracellular ROS Detection**—107 circulating erythrocytes were washed with Dulbecco’s PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.76 mM KH2PO4, pH 7.4 at 25 °C), loaded with 2′,7′-dichlorofluorescein diacetate (DCFH; Invitrogen) at 10 μM in PBS at 37 °C for 30 min, and washed again with PBS. Where indicated, H2O2 (Sigma-Aldrich) was added after DCFH loading at 10 or 100 μM in PBS, and cells were incubated at 37 °C for 30 min and washed again with PBS. DCFH mean fluorescence intensity was measured by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences).

**Detection of Globin Precipitates in Erythroid Cells**—Analysis of globin precipitates in erythrocyte membrane skeletons was performed as described (11, 12, 49, 50). Briefly, 10 μl of washed red blood cells were lysed, and pellets were extensively washed in ice-cold 0.05% PBS (6.85 mM NaCl, 0.135 mM KCl, 0.41 mM Na2HPO4, 0.088 mM KH2PO4, pH 7.4 at 4 °C). Membrane lipids were extracted with 56 mM sodium borate, pH 8.0 at 4 °C. Precipitated globins were dissolved in 8 M urea, 10% acetic acid, 10% β-mercaptoethanol, and 0.04% pyronin; fractionated by Triton-acetic acid-urea gel electrophoresis; and stained with Coomassie Brilliant Blue. Soluble hemoglobin fractions were analyzed as loading controls. Gel electrophoresis was performed using a Mini-PROTEAN Tetra Cell apparatus (Bio-Rad).

**AHSP Expression Analysis**—Erythrocytes were lysed with 40 volumes of water with 1 mM dithiothreitol and 1:500 protease inhibitor mixture (Sigma-Aldrich). Bone marrow erythroid precursors were purified from mice of the indicated genotypes by immunomagnetic positive selection using the EasySep PE Selection Kit (StemCell Technologies, Vancouver, British Columbia, Canada) with Ter119-PE antibody (Biolegend, San Diego, CA) according to the manufacturer’s instructions (51). Protein lysates from purified erythroblasts were prepared using radioimmune precipitation assay buffer with 1 mM DTT and 1:500 protease inhibitor mixture (Sigma-Aldrich). Protein concentrations were determined using a Pierce bicinchoninic acid assay kit (Thermo Scientific, Rockford, IL), and 10 μg of protein were resolved by 15% SDS-PAGE. After transfer to 0.2-μm PVDF membranes (Whatman), AHSP was detected using a rat polyclonal antibody raised against purified mouse AHSP protein and HRP-conjugated goat anti-rabbit secondary antibody. HRP-conjugated anti-β-actin antibody was used as a loading control. Western blots were developed using SuperSignal West Pico chemiluminescent reagent. Western blotting secondary antibodies, markers, and reagents were obtained from Thermo Scientific.

**5-Fluorouracil (5-FU) Treatment to Induce Erythropoietic Stress**—Mice were injected with 150 mg/kg 5-FU (Sigma-Aldrich) in normal saline intraperitoneally on day 0. On days 3, 9, 12, 15, 18, and 30, 15 μl of blood were collected from the tail vein into heparinized glass microhematocrit tubes (Globe Scientific, Paramus, NJ) for manual spin hematocrits and reticulocyte analysis.

**Isoelectric Focusing of 35S-Labeled Reticulocyte Extracts to Detect Globins and AHSP-αHb Complexes**—10 μl of freshly collected erythrocytes and reticulocytes (normalized to reticulocyte percentage and hematocrit) were incubated in methionine/cysteine-free DMEM (Invitrogen), 2 mM l-glutamine, 10% dialyzed PBS, and 0.1 mM non-essential amino acids at 37 °C for 1 h. 50 μCi of 35S-labeled methionine and cysteine (PerkinElmer Life Sciences) were added, and the cells were labeled for 10 min at 37 °C. The cells were washed with PBS and exposed to carbon monoxide to stabilize ferrous heme groups. The cells were lysed in 5 volumes of water, and the lysate was clarified by centrifugation. Lysates were resolved by isoelectric focusing (pH 6–8) on precast agarose gels from the Hemoglobin Test kit on an LKB 2117 Multiphor apparatus at 1500 V for 50 min (PerkinElmer Life Sciences). For autoradiography, gels were fixed in 10% trichloracetic acid for 10 min, washed in deionized water, and dried. Images were acquired using a phosphor screen and Storm 865 scanner (GE Healthcare). Bands were quantified using National Institutes of Health ImageJ software. For analyzing band globin and AHSP content, isoelectric focusing gel bands were excised, boiled in 2× Laemmli sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue, 0.125 M Tris HCl, pH 6.8; Sigma-Aldrich), and proteins were resolved by 15% SDS-PAGE. Gels were either dried for autoradiography or used for mouse AHSP Western blotting as described above.

**Statistical Methods**—Statistical analysis was performed using GraphPad Prism 4.0 software (GraphPad Software, La Jolla, CA). All group comparisons were done using one-way analysis of variance.

**Sequence Alignments**—AHSP protein sequences were obtained using the Basic Local Alignment Search Tool (BLAST) at the NCBI using the human AHSP sequence (NCBI accession number NM_016633.2) as the query, and using the Ensembl genome browser. After manual inspection for sequence quality,
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31 sequences were trimmed to include only helices 1 and 2 and aligned using the ClustalW algorithm with Strap multiple sequence alignment software (52).

RESULTS

AHSP Pro30 Mutations Alter α Chain Binding, Autooxidation, and Hemin Loss—The evolutionarily conserved proline at position 30 in loop 1 of AHSP contacts helix G of α-globin at the edge of the protein-binding interface (Fig. 1). To examine the function of this amino acid, Gell et al. (18) made a series of mutations that convert AHSP Pro30 to Gly, Ala, Val, Phe, and Trp and screened the resultant protein interactions with α-globin. Except for the P30G mutant, all AHSP variants showed enhanced affinity (lower \(K_D\) values) for ferrous \(\alpha\)-CO or \(\alpha\)-O2 as measured by isothermal titration calorimetry. All of these mutants also showed 3–4-fold decreased rates of autooxidation and concomitant hemichrome formation (18).

In concert with our mutagenesis studies of the endogenous Ahsp gene, the \(\alpha\) in vitro effects of P30A and P30W AHSP substitutions were reevaluated for recombinantly produced proteins. Direct measurements of the association and dissociation rate constants for the binding of these AHSP variants to native \(\alpha\)CO and met-\(\alpha\)O2 as measured by isothermal titration calorimetry. All of these mutants also showed 3–4-fold decreased rates of autooxidation and concomitant hemichrome formation (18).

In contrast, the rate of dissociation of \(\alpha\) subunits from the AHSP complexes in the presence of excess \(\beta\) subunits depended markedly on both the oxidation state of the \(\alpha\)-hemes and in the case of reduced \(\alpha\)CO on the amino acid at position 30. As shown in Fig. 2B, reduced \(\alpha\)CO was released from WT and P30A AHSP with half-times of roughly 2–4 s, whereas the half-time for dissociation from P30W AHSP was roughly 25-fold larger. In contrast, WT and both Pro30 AHSP mutants released oxidized \(\alpha\) subunits very slowly with half-lives on the order of 200–400 s, similar to that for the release of reduced \(\alpha\) subunits from the P30W mutant (Fig. 2B).

As a result of these differences in dissociation rate constants, the equilibrium dissociation constant (\(K_D\)) for \(\alpha\) subunit binding depended strongly on the heme oxidation state. When the heme was reduced, the P30W AHSP mutant showed a 30-fold higher affinity for \(\alpha\) subunits than WT and P30A AHSP, which showed \(K_D\) values of roughly 20 nM. However, when the \(\alpha\) subunits were oxidized, WT AHSP and the two Pro30 mutants showed the same high affinity with \(K_D\) values equal to 0.2–0.6 nM. Similar relative \(K_D\) differences for the AHSP variants were observed by isothermal titration calorimetry, although the isothermal titration calorimetry values were all 5–10-fold larger than those calculated from the association and dissociation rate constants. The cause of this discrepancy is discussed in Mollan et al. (42). The remarkably high affinity of AHSP for ferric \(\alpha\) subunits and the lack of significant differences between the

FIGURE 1. Evolutionary and structural significance of AHSP proline 30. A, structure of AHSP (yellow) bound to \(\alpha\)-globin (gray) showing Pro30 at the interaction interface. Proline residues are shown in purple. The figure was generated using PyMOL software and Protein Data Bank code 1Z8U. B, ClustalW alignment comparing primary AHSP helix 1-loop 1-helix 2 amino acid sequences in different species, showing conservation of proline 30.
Pro30 mutants were discovered as a result of our efforts to understand the results of the in vivo mutagenesis studies.

Autooxidation of WT, P30A, and P30W AHSP-αO2 complexes was also re-examined. As shown in Fig. 3, the rates of oxidation were 3–4-fold smaller than that for the wild-type complex as was observed by Gell et al. (18), but even these rates were still 2–3-fold greater than that for free αO2 (Table 1). As estimated by increases in turbidity, the overall resistances of the AHSP-αO2 complexes to precipitation were roughly the same for the wild type, P30A, and P30W AHSP (data not shown) even though the latter mutant-αO2 complexes autooxidized more slowly. This observation and the lack of phenotype observed for mice expressing the mutant Ahsp genes (described in subsequent sections) led us to examine the rates of hemin loss from the oxidized hemichrome met-α-AHSP complexes.

Time courses for hemin loss were obtained by mixing AHSP-met-α complexes with excess H64Y/V68F apoMb as a hemin-scavenging reagent (Fig. 4). The ultrastable apoMb mutant turns “green” as indicated by the appearance of a large absorbance peak at 600 nm when it accepts hemin from a donor (44 – 46). Unexpectedly, the rates of hemin loss from the P30A and P30W AHSP-met-α complexes were 2–7-fold greater than that from the wild-type AHSP-met-α complex (Fig. 4 and Table 1). Unfortunately, the apo-α-globin product rapidly precipitates at 37 °C at pH 7, particularly in the absence of AHSP, and the resultant turbidity increases make quantitative analyses of hemin dissociation time courses impossible. However, the relative order of the initial rates of the absorbance increases at 600 nm for the wild-type and two variant AHSP-met-α complexes under these more physiological conditions was the same as the order of the rate constants determined quantitatively at low temperature and pH (not shown).

As shown in Fig. 4B, binding to wild-type AHSP enhanced the rate of hemin loss from met-α by a factor of 2. Binding to the Pro30 AHSP mutants accelerated hemin dissociation even further. These increases in k – hemin seem to suggest increased exposure of the heme pocket to water in the AHSP complex. Lioug et al. (53) have shown that exposing the proximal histidine to water causes dramatic (~1000-fold) increases in the rate of hemin loss from metMb variants. Thus, even partial unfolding of the heme pocket in the AHSP-met-α complexes to form the hemichrome would be expected to increase the rate of hemin loss markedly.

One key result in Table 1 is that the net resistance of the variant AHSP-αO2 complexes to apoglobin formation, unfolding, and precipitation may be the same for all three variants.
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**TABLE 1**

In vitro properties of AHSP P30A and P30W variants

| αCO            | k<sup>−1</sup><sub>AHSP</sub> | k<sup>−1</sup><sub>AHSP</sub> | K<sub>D</sub> | k<sub>meta K<sub>D</sub></sub> | k<sub>meta pH 7.0, 37 °C</sub> | k<sub>−hemin pH 5.5, 10 °C</sub> |
|----------------|-----------------|-----------------|----------|-----------------|-----------------|-----------------|
| AHSP WT        | 10.0            | 0.17            | 17 (93)  | 0.17 (4.3)      | 0.93            | 0.89            |
| AHSP P30A      | 9.2             | 0.14            | 15 (38)  | 0.60            | 0.29            | 1.9             |
| AHSP P30W      | 13.6            | 0.0072          | 0.56 (7.7) | 0.24            | 0.24            | 6.2             |
| No AHSP        | 0.13            | 0.50            |          |                 |                 |                 |

*The numbers in parentheses indicate isothermal titration calorimetry data reported previously (18). These measurements are consistently higher than the values calculated from the rate constants. This discrepancy is discussed in the accompanying paper by Mollan et al. (42).*

**FIGURE 4. Effects of AHSP mutations on rate of hemin loss from oxidized α subunits.** A: spectral changes for the reaction of 6 μM AHSP-met-α complexes with excess (40 μM) H64Y/V68F apoMb. The increase in absorbance at 600 nm indicates hemin gain by the H64Y/V68F Mb reagent. B: time courses for hemin dissociation from AHSP-met-α complexes plotted as the normalized absorbance change. (A) and (B) show that the AHSP-P30W mutant has reduced stability and a longer half-life compared to the wild-type and mutant AHSP-P30A. These experiments were conducted in 0.6 M sucrose, 200 mM sodium acetate buffer, pH 5.5 at 10 °C.

Even though the Pro<sup>30</sup> mutant AHSP-αO<sub>2</sub> complexes autooxidized more slowly, the resultant AHSP-met-α complexes lost hemin more rapidly so that net rates of apo-α-globin appearance are probably similar for the Pro<sup>30</sup> mutants and wild-type AHSP.

**Generation of AHSP Pro<sup>30</sup> Substitutions in Mice**—We created Ahsp<sup>L30A</sup> and Ahsp<sup>W30W</sup> missense mutations in the endogenous murine gene (supplemental Fig. 1A). We reasoned that comparing hematologic parameters in these mutant strains would allow us to distinguish between effects caused by altered AHSP affinity for αO<sub>2</sub> and reduced rates of autooxidation and bishistidyl conversion. Mutant animals were born at normal Mendelian ratios, similar to what we observed previously for Ahsp<sup>−/−</sup> mice (10, 11). Erythroblast AHSP protein levels were normal in the mutant animals (supplemental Fig. 1B).

**Analysis of AHSP-α-Globin Complexes in Vivo**—Endogenous AHSP-α-globin complexes are more easily detected under conditions of α-globin excess (12, 48). Thus, we crossed the Ahsp<sup>W30W</sup> and Ahsp<sup>L30A</sup> mutant mice onto a β-thalassemia intermediate background (β-globin<sup>Th3/4</sup>) (48) and examined the formation of mutant AHSP-α-globin complexes by isoelectric focusing of radiolabeled reticulocyte extracts (12). We detected several globin-containing bands that could potentially be an AHSP-α-globin complex (supplemental Fig. 2A). We used Western blotting of proteins isolated from these bands to identify the band that contained both α-globin and AHSP (supplemental Fig. 2B), allowing us to quantify the relative amount of labeled α-globin in the complex in subsequent experiments. Accumulation of the AHSP-α-globin complex was significantly increased in Ahsp<sup>P30W/P30W</sup> β-globin<sup>Th3/4</sup> mice consistent with biochemical studies showing that the mutant protein had increased affinity for α-globin (Fig. 5). AHSP-α-globin complexes were present at wild-type levels in Ahsp<sup>P30A/P30A</sup> β-globin<sup>Th3/4</sup> mice. Thus, it was possible to increase AHSP-α-globin complex formation in vivo through targeted mutagenesis of Ahsp gene as predicted from the higher affinity of P30W AHSP for reduced αO<sub>2</sub> or αO<sub>2</sub> in vitro (Fig. 5B and Table 1). However, our assay did not allow us to measure the fraction of the AHSP-α-globin complexes, which were in the bishistidyl hemichrome form in vivo.

**Hematological Features of Ahsp<sup>P30A</sup> and Ahsp<sup>P30W</sup> Knock-in Mice**—We first compared baseline erythropoiesis in AHSP Pro<sup>30</sup> substituted and control mice (Table 2A and supplemental Table 1). Ahsp<sup>−/−</sup> mice exhibited reticulocytosis and elevated red cell distribution width consistent with mild hemolysis. These parameters were entirely normal in Ahsp<sup>P30A</sup> and Ahsp<sup>P30W</sup> mutant mice (heterozygotes and homozygotes) at ages 2 (Table 2) and 8 months (not shown). Ahsp<sup>P30A</sup> and Ahsp<sup>P30W</sup> mouse erythrocytes exhibited normal morphology, whereas Ahsp<sup>−/−</sup> erythrocytes exhibited shape abnormalities and eosinophilic inclusions (Refs. 10 and 11 and data not shown). Of note, Ahsp mutant mice used in this study were backcrossed onto a C57BL/6 genetic background. In this pure strain, loss of Ahsp caused minimal anemia in contrast to Ahsp<sup>−/−</sup> mice with a mixed 129SV/C57BL/6 genetic background (10, 11).

Unstable α-globin resulting from β-thalassemia or loss of AHSP generates excessive ROS and membrane-bound Hb precipitates (Heinz bodies) (10, 11, 54–60). In Ahsp Pro<sup>30</sup>-substituted mice, membrane-bound insoluble globins were barely detectable in erythrocytes, similar to wild-type controls (Fig. 6A). Moreover, in contrast to Ahsp<sup>−/−</sup> mice, erythrocytes from Ahsp<sup>P30W/P30W</sup> and Ahsp<sup>P30A/P30A</sup> animals did not show increased ROS generation either at baseline (Fig. 6B) or after treatment with hydrogen peroxide (Fig. 6C). We also examined the abilities of Ahsp mutant mice to respond to increased erythropoietic demands after treatment with 5-FU, a nucleotide analog that destroys hematopoietic precursors, resulting in transient suppression of circulating blood cells (Fig. 7) (61, 62).
TABLE 2

Key erythroid indices of AHSP Pro30 mutant mice at age 2 months in wild-type and β-thalassemia intermedia backgrounds

A. erythroid indices for mice with the indicated *Ahsp* genotypes. Data are shown as mean ± S.D. ***, p < 0.001; **, p < 0.01; *, p < 0.05 versus *Ahsp* −/− by one-way analysis of variance. No statistically significant differences were found between wild-type and Pro30 mutant *Ahsp* mice. B. erythroid indices of AHSP Pro30 mutant/β-globin (HbB) Th3/+ (β-thalassemia intermedia) mice. AHSP Pro30 missense mutations had no significant effects on erythroid indices of β-thalassemic mice. p < 0.01 for all β-globin −/+ versus β-globin −/− in all *Ahsp* genotypes by one-way analysis of variance. Additional hematological data from these experiments are summarized in supplemental Tables 1, 2, and 3.

**DISCUSSION**

Prior studies indicate two potential functions for AHSP during normal and pathologic erythropoiesis (8, 26, 63). First, AHSP acts as a molecular chaperone to bind and stabilize nascent α-globin folding intermediates prior to reaction with β-globin and assembly into HbA (Fig. 9). This function is gov-
erned by the rates of holo- and apo-α-globin binding to and release from AHSP (18, 25, 42, 64). Thus, AHSP/α-globin interactions during HbA synthesis must be optimized to promote α-globin stability yet not interfere significantly with release of α-globin for binding to β-globin during HbA assembly.

Second, AHSP forms stable complexes with excess free α-globin under conditions of β-globin deficiency to reduce the toxicity associated with α-globin oxidation, hemin loss, and precipitation. Mitigation of excess α-globin toxicity was postulated to occur in part through AHSP-mediated conversion of bound α subunits to a bishistidyl, hemichrome state, which limits ROS production. Biochemical studies support this model, although it has been difficult to test in vivo. Pro30 regulates both the affinity of AHSP for reduced α-globin and the rate of conversion of bound αO2 to the ferric bishistidyl met-α form (i.e. autooxidation).

FIGURE 6. AHSP Pro30 mutations do not destabilize α-globin or induce ROS production in vivo. A, detection of membrane-bound insoluble globins. Equal numbers of circulating erythrocytes from mice with the indicated Ahsp genotypes were lysed, and insoluble fractions were analyzed for globin precipitates by Triton-acetic acid-urea gel electrophoresis. Migration positions of α- and β-globin are indicated in the marker (M) lane. Soluble fractions (1%) were included as loading controls. Erythrocytes from Ahsp+/− and β-thalassemic (β-globinTh3+/−) (Th) mice represent positive controls. B, circulating erythrocytes from mice with the indicated genotypes were stained with the ROS indicator DCFH and analyzed by flow cytometry. Ahsp+/− and β-thalassemic (β-globinTh3+/−) mice were analyzed as controls. Primary data are shown in the left panel. The right panel summarizes DCFH mean fluorescence intensities (MFI) for a group of mice (n = 4–6 for each genotype). ***p < 0.001 versus Ahsp+/− genotype.

C, circulating erythrocytes from mice with the indicated Ahsp genotypes were stained with DCFH, incubated with various concentrations of hydrogen peroxide (H2O2), and analyzed for ROS production by flow cytometry. Four to six mice were analyzed from each group. Error bars show standard deviation. ***p < 0.001 versus Ahsp+/−.
This lack of phenotype for the Ahsp mutants requires the re-evaluation of current models for the physiological functions of AHSP during erythropoiesis and led us to re-examine the hypothesis that a major function of AHSP is to stabilize excess $\alpha$O$_2$ by oxidizing it to a hemichrome species to prevent ROS generation. More generally, our work also highlights the importance of using in vivo approaches to test hypotheses generated by in vitro biochemical experiments, which in this case suggested that there should be marked physiological differences between the P30A and P30W variants and wild-type AHSP.

An alternative and perhaps complementary function of AHSP is to act as a molecular chaperone for $\alpha$-globin during HbA assembly. This role is supported by kinetic studies showing that both ferrous and ferric holo-$\alpha$ bind $\sim 20$ times more rapidly to AHSP than to ferrous $\beta$ subunits (14, 25, 42). However, $\beta$ subunits have a 10,000-fold higher affinity for $\alpha$-globin than AHSP due to an extremely small $\alpha$1$\beta$1 dissociation rate constant, which is on the order of $10^{-5}$ s$^{-1}$ (25, 65). In comparison, the rate of $\alpha$ dissociation from AHSP is $10^2$–$10^4$ larger for all the AHSP variants listed in Table 1. These kinetic results suggest that newly synthesized $\alpha$-globin either with or without hemin binds initially to AHSP and subsequently is released for interaction with $\beta$-globin to form HbA (Fig. 9). At high AHSP and $\beta$-globin concentrations, the rate, but not the extent, of HbA assembly could be limited by the rate of release of $\alpha$-globin from AHSP (Fig. 9) (25).

The AHSP concentration in late stage erythroblasts is $\sim 0.1$ mM (10). The concentration of HbA subunits is $\sim 10$–20 mM, although the concentrations of free, non-tetrameric $\alpha$ and $\beta$ subunits are unknown (9, 10). One question is whether the ratio of AHSP to the pool of newly synthesized free $\beta$ chains is sufficiently low for optimal speed of HbA assembly and how easily this process can be perturbed under physiological conditions. For example, increased AHSP concentration and/or enhanced affinity for $\alpha$-globin could interfere with the rate of HbA tetramer formation. However, the $\sim 30$-fold increased affinity of AHSP P30W for $\alpha$-globin either with or without hemin binds initially to AHSP and subsequently is released for interaction with $\beta$-globin to form HbA (Fig. 9). At high AHSP and $\beta$-globin concentrations, the rate, but not the extent, of HbA assembly could be limited by the rate of release of $\alpha$-globin from AHSP (Fig. 9) (25).

To examine the importance of the latter properties in vivo, Pro$^{30}$ missense mutations (P30A and P30W) were introduced into endogenous murine Ahsp loci. As shown in Table 1, these mutations significantly altered the biochemical properties of AHSP in vitro. However, in contrast to the complete loss of AHSP, the P30A and P30W substitutions produced no detectable in vivo effects on hemoglobin synthesis or stability at base line, during erythropoietic stress, or under conditions of $\alpha$-globin excess ($\beta$-thalassemia) in mice.
The formation, spectral properties, and structure of AHSP-bishistidyl met-α complexes have been examined extensively in vitro (18, 21, 22, 28). The physiological function of this complex in vivo was examined in the current study by introducing endogenous Ahsp mutations that altered its rate of αO₂ autooxidation. AHSP amino acid substitutions that reduced this rate by 3–4-fold did not alter hemoglobin formation during normal erythropoiesis. Moreover, these mutations did not worsen β-thalassemia as might be expected if AHSP neutralizes the toxicity of free αO₂ by inducing formation of a stable and ROS-inhibiting bishistidyl structure. One explanation is that the lower autooxidation rates of the mutant AHSP αO₂ complexes are still sufficient to generate a stable bishistidyl complex that prevents ROS formation and α-globin precipitation in vivo.

It seems more likely that the toxicity of excess αHb is due to hemin loss, apoglobin denaturation, and Heinz body formation, which is a major source of ROS in red cells. This explanation is supported by the lack of effect of the Pro³⁰ mutations on erythropoiesis in vivo. Although the Pro³⁰ mutant AHSP αO₂ complexes autooxidized more slowly than the wild-type complex (Fig. 3 and Table 1), the mutant AHSP-met-α complexes lost hemin much more rapidly (Fig. 4). Thus, the resistance of the initial ferrous αO₂ complex to apo-α-globin formation is roughly the same for wild-type AHSP and the two variants, which could account for a lack of phenotype in the Ahsp mutant mice.

Finally, our findings and those presented in the accompanying paper (42) are also relevant to an unsolved problem concerning HbA synthesis. The redox state of heme during its insertion into apoglobins is not known. We suggest that during normal erythroid HbA assembly ferric heme is incorporated into AHSP-bound apo-α-globin, rapidly forming a hemichrome holo-α species with the heme pocket partially collapsed (Fig. 9). Subsequently, this intermediate is reduced by endogenous methemoglobin reductases to generate ferrous α-globin, which is released rapidly from AHSP for binding to β-globin.

Several lines of evidence support this model. Free ferrous heme (Fe⁴⁺) autooxidizes rapidly in milliseconds to hemin (Fe³⁺) even at low levels of O₂. Gell et al. (18) and others (64) have shown that AHSP binds and stabilizes apo-α-globin in vitro. Labeling studies in reticulocytes suggest that hemoglobin is initially synthesized with the heme in a ferric state (67,
68). As shown in Fig. 2B and Table 1, AHSP bound ferric met-α-globin with a 100-fold higher affinity than that for various reduced forms of α chains (18, 42). Culbertson and Olson (69) have shown that hemichrome species similar to that of the met-α-AHSP complex are key folding intermediates in the formation of holo-Hbs and holo-Mbs. Hemichrome α-globin bound to AHSP can be reduced anerobically in vitro to generate fully folded, pentacoordinate deoxy-α with no evidence of a hemichrome species (bishistidyl coordination of ferrous iron) (28, 42, 69). In vitro, bishistidyl hemoglobins have faster reduction kinetics than pentacoordinate forms (70), suggesting that the α-globin hemichrome intermediate state induced by AHSP may facilitate reduction of the ferric heme iron in vivo.

Our new in vitro data also support this model of AHSP chaperone activity involving stabilization of an α ferric, hemichrome folding intermediate. The AHSP P30A and P30W mutations did not significantly affect the uptake and release of met-α-globin (see Fig. 2 and Table 1). Thus, although αO2 autooxidation is impaired, the Pro30 mutations may have no detectable effects in vivo because the key function of AHSP is to prevent the precipitation of apo-α-globin conformers, facilitate uptake of hemin, stabilize the holo-met-α-globin intermediate, and prevent release for binding to β-globin until the α-globin is reduced. These biochemical chaperone properties of AHSP have been exploited to enhance α-globin synthesis in bacterial expression systems (20) and to quantify the pool of free α chains in β-thalassemia (19). Although in vivo experiments have been valuable for establishing models of hemoglobin assembly and the role of AHSP, here we show that in vitro studies are required to test and refine these models. Further mutagenesis and in vitro studies will improve our understanding of AHSP/α-globin interactions and enhance the use of AHSP as a diagnostic and biotechnological tool.

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