Kinetics of α-Globin Binding to α-Hemoglobin Stabilizing Protein (AHSP) Indicate Preferential Stabilization of Hemichrome Folding Intermediate*  

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Background: α-Hemoglobin stabilizing protein (AHSP) facilitates hemoglobin production.

Results: AHSP preferentially binds to ferric versus ferrous α subunits and induces reversible structural alterations within seconds of binding.

Conclusion: AHSP exerts its effects by stabilizing a ferric α folding intermediate and inhibiting its participation in hemoglobin assembly.

Significance: AHSP is a molecular chaperone for ferric α-globin.

Human α-hemoglobin stabilizing protein (AHSP) is a conserved mammalian erythroid protein that facilitates the production of Hemoglobin A by stabilizing free α-globin. AHSP rapidly binds to ferrous α with association (k’AHSP) and dissociation (kAHSP) rate constants of 10 μM⁻¹ s⁻¹ and 0.2 s⁻¹, respectively, at pH 7.4 at 22 °C. A small slow phase was observed when AHSP binds to excess ferrous αCO. This slow phase appears to be due to cis to trans prolyl isomerization of the Asp²⁹-Pro³⁰ peptide bond in wild-type AHSP because it was absent when αCO was mixed with P30A and P30W AHSP, which are fixed in the trans conformation. This slow phase was also absent when met(Fe³⁺)-α reacted with wild-type AHSP, suggesting that met-α is capable of rapidly binding to either Pro³⁰ conformer. Both wild-type and Pro³⁰-substituted AHSPs drive the formation of a met-α hemichrome conformation following binding to either met- or oxy(Fe²⁺)-α. The dissociation rate of the met-α-AHSP complex (kAHSP ≈ 0.002 s⁻¹) is ~100-fold slower than that for ferrous α-AHSP complexes, resulting in a much higher affinity of AHSP for met-α. Thus, in vivo, AHSP acts as a molecular chaperone by rapidly binding and stabilizing met-α hemichrome folding intermediates. The low rate of met-α dissociation also allows AHSP to have a quality control function by kinetically trapping ferric α and preventing its incorporation into less stable mixed valence Hemoglobin A tetramers. Reduction of AHSP-bound met-α allows more rapid release to β subunits to form stable fully, reduced hemoglobin dimers and tetramers.

Hemoglobin A (HbA)² is a highly conserved dioxygen transport protein present in red cells of all mature, healthy vertebrates (1). Because of its functional importance, abundance, and experimental tractability, this protein is a model system for a wide variety of biochemical and medical studies (1, 2). One important research aim is to better understand how HbA is synthesized and maintained in vivo because dysregulation of this process often results in serious anemias, including structural hemoglobinopathies and thalassemia syndromes (3, 4). HbA production includes α and β gene transcription and translation, subunit binding, heme binding to the apoprotein, redox regulation, and subunit assembly into tetramers (5–9). The end product, HbA, is a heterotetramer containing two pairs of non-covalently associated α and β subunits, each bearing one iron-containing protoporphyrin IX prosthetic group (heme (Fe²⁺) or heme/hemin (Fe³⁺)) (1, 10).

α-Hemoglobin stabilizing protein (AHSP) is an erythroid protein that rapidly and reversibly binds to monomeric forms of apo- and holo-α but not β, αβ dimers, or tetrameric HbA (11, 12). AHSP binds α in a 1:1 stoichiometry using a surface that excludes β (12–15), and it has been shown to induce numerous structural changes following binding (14–18). Current evidence suggests that AHSP is a molecular chaperone for HbA production (for reviews, see Refs. 19–21). However, questions remain regarding its role in α prosthetic group acquisition, oxidation state maintenance, subunit folding, and stabilization prior to incorporation into HbA.

In this work, we used optical absorbance and fluorescence emission spectroscopy in stopped-flow rapid mixing experiments to measure the rates of α binding to and release from AHSP. In agreement with work done by Gell et al. (16), we report that the affinity of AHSP is dependent on α oxidation state and for the first time show that the rate of met-α dissociation from AHSP is dramatically slower than that for reduced α dissociation. Experiments with clinically significant and rationally selected AHSP and α mutants revealed the functional wild-type AHSP with an N-terminal Gly-Ser dipeptide; βWT, wild-type β subunits; Hb, hemoglobin; heme, ferroprotoporphyrin IX; hemin, ferriprotoporphyrin IX; met, ferric oxidation state.
importance of the loop separating α-helices 1 and 2 of AHSP. Collectively, this work suggests that AHSP stabilizes α in vivo by preferentially binding an oxidized α hemichrome folding intermediate and temporarily impairing α assembly into HbA until reduction to the ferrous state has occurred.

**EXPERIMENTAL PROCEDURES**

**Recombinant Human AHSP Expression and Purification**—AHSP protein was obtained from pGEX-2T (GE Healthcare) with the full-length human AHSP gene inserted downstream of the *Schistosoma japonicum* glutathione S-transferase gene (pGEX-2T-AHSP) (GenBank™ accession number NM_016633.2) (11, 12). AHSP mutants were generated using a QuikChange II site-directed mutagenesis kit (Agilent Technologies, Inc., Santa Clara, CA) in accordance with manufacturer’s instructions and the following PCR primers: AHSPP30A, 5’-CTG AAT CAG CAG TTC TAT GGT CTC GTC TCT GAA GAA GAC-3’ and 5’-GTC TTC TCT AGG GTA TTC CTC GTC TCT GAA GAC-3’. AHSPP30W, 5’-ATCAGAGGTCTCATTGAGC-3’; AHSPQ25K, 5’-CAAGCAGCTCATTGAGCTGCCG-3’; AHSPD29R, 5’-CATGTTAACATCTGGTACG-3’. Primers were obtained from Integrated DNA Technologies, Inc. (Coralville, Iowa).

AHSP was expressed as a GST fusion protein using *Escherichia coli* BL21 cells (EMD Biosciences, Inc., San Diego, CA; Novagen brand) using methods developed previously (11, 12). The soluble GST-AHSP present in the supernatant was captured using 20 ml of glutathione-Sepharose FF medium and an ÄKTA FPLC system (GE Healthcare). During this process, PBS was used as a binding and wash buffer, and 50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0 at 25 °C was used as an elution buffer. GST was cleaved from AHSP using 500 units of thrombin obtained from GE Healthcare. Reduced glutathione, elution buffer. GST was cleaved from AHSP using 500 units of thrombin obtained from GE Healthcare. Reduced glutathione, pH 8.0 at 4 °C instead of overnight. Following this incubation, samples were rapidly buffer-exchanged into 10 mM Tris-HCl, pH 8.0 at 4 °C using a column containing 200 ml of Sephadex G-25 medium (Sigma-Aldrich). Samples were then applied to another column containing 100 ml of diethylaminoethyl cellulose medium that had been equilibrated with the same buffer (DE52 medium, Whatman). This column retains β and tetrameric HbA while allowing α to flow through. HbA was then eluted with 20 mM Tris-HCl, pH 7.4 at 4 °C, and β was eluted using 100 mM Tris-HCl, pH 7.0 at 4 °C. Rather than regenerating sulfhydryl groups using the methods of Geraci et al. (25), a final concentration of 5 ml/liter β-mercaptoethanol was added to each sample on ice after which the samples were immediately exchanged into 10 mM Tris-HCl, pH 8.0 at 4 °C using a column containing 200 ml of Sephadex G-25 medium. This entire process was done in less than 8 h, and all work was done in a room maintained at 4 °C. Regeneration of sulfhydryl groups was assayed by Boyer titration (27). Chain isolations were done in 4-ml batches of 50 mg/ml HbA.

**Protein Identity, Purity, and Stability Verification**—Plasmid DNA was isolated from each AHSP expression and sent for sequencing to Lone Star Laboratories, Inc. (Houston, TX) using manufacturer-specified sequencing primers to verify the absence of any unwanted mutations. These primers were 5’-GGG CTG CGA CAC GAC GTT TGG TG-3’ and 5’-CCG GGA GCT GCA TGT GTC AGA AGG-3’. SDS-PAGE gels stained with Coomassie Blue were used to confirm protein expression; GST cleavage; and GST, thrombin, and contaminant removal. MALDI-TOF spectrometry performed at Rice University (Houston, TX) confirmed the identity and purity of AHSP. HbA, α, and β purities and reassembly efficiency were verified by cellulose acetate electrophoresis (Helena Laboratories Corp., Beaumont, TX). Absorbance spectra and ligand binding kinetics were also verified to ensure that the samples retained normal function following purification and isolation (28). Heme protein concentrations were determined using extinction coefficients reported previously (29, 30). With the exception of AHSPP30W, AHSP concentrations were determined by optical absorbance at 280 nm using the extinction coefficient 11,460 M⁻¹ cm⁻¹, which was calculated using the ExPaSy Proteomics Server ProtParam Tool. The extinction coefficient used for AHSPP30W was 16,960 M⁻¹ cm⁻¹, which was calculated in the same manner.

**Instrumentation and Materials**—Manual mixing spectrophotometry was done in either a Cary 50Bio (Varian, Inc., Palo Alto, CA) or a UV2401PC spectrophotometer (Shimadzu, Inc., Columbia, MD) using cuvettes purchased from Starna Cells (Atascadero, CA). Stopped-flow spectrophotometry was done using either a modified Durrum Model D-110 (Palo Alto, CA) or an Applied Photophysics PiStar kinetic circular dichroism stopped-flow spectrophotometer (Leatherhead, Surrey, UK). Unless otherwise indicated, all stopped-flow fluorescence

**Native Human HbA Purification and Chain Isolation**—HbA was purified from units of human blood obtained from the Gulf Coast Regional Blood Center (Houston, TX) using established methods (24). Separated α and β chains were isolated using established methods (25, 26) that were modified as follows. Incubation of CO-liganded HbA with 4-(hydroxymercuri)benzoic acid was limited to ~4 h at 4 °C instead of overnight. Following this incubation, samples were rapidly buffer-exchanged into 10 mM Tris-HCl, pH 8.0 at 4 °C, and β was eluted using 100 mM Tris-HCl, pH 7.0 at 4 °C. Rather than regenerating sulfhydryl groups using the methods of Geraci et al. (25), a final concentration of 5 ml/liter β-mercaptoethanol was added to each sample on ice after which the samples were immediately exchanged into 10 mM Tris-HCl, pH 8.0 at 4 °C using a column containing 200 ml of Sephadex G-25 medium. This entire process was done in less than 8 h, and all work was done in a room maintained at 4 °C. Regeneration of sulfhydryl groups was assayed by Boyer titration (27). Chain isolations were done in 4-ml batches of 50 mg/ml HbA.

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RESULTS

Rates of AHSP Binding to Native α—Recombinant human wild-type AHSP (AHSPWT) exhibits intrinsic fluorescence from several aromatic side chains (32). Although wild-type human α and β also contain these residues, neither of these subunits exhibit strong intrinsic fluorescence due to highly efficient fluorescence resonance energy transfer to their heme prosthetic groups (33, 34). When α binds to AHSP, this energy transfer quenches the intrinsic fluorescence of AHSP. The key residue in this process is thought to be AHSP Trp44 (32), which is solvent-exposed in unbound AHSP and becomes buried when α is bound (14).

Baudin-Creuza et al. (32) were the first to report that fluorescence quenching occurs upon formation of a complex between α and AHSP. Their data are consistent with a simple one-step bimolecular association reaction (32).

\[
\frac{\alpha + \text{AHSP}}{k_{\text{AHSP}}} \rightarrow \alpha\text{-AHSP}
\]

REACTION 1

We confirmed and extended their work using a stopped-flow fluorometer to measure the rates of association (\(k'_{\text{AHSP}}\)) and dissociation (\(k_{\text{AHSP}}\)) (19), and our initial kinetic measurements were subsequently confirmed by Brillet et al. (35). As shown in Fig. 1A, mixing \(\alpha^{WT}\) with AHSPWT resulted in rapid fluorescence quenching on time scales of less than 100 s. The observed time courses for wild-type AHSP are biphasic with a fast phase showing a rate that is linearly dependent on \([\alpha^{WT}]\) and a smaller (20–30%) slow phase showing an observed rate that is concentration-independent and equal to 0.04 ± 0.01 s⁻¹ in 100 mM phosphate buffer at pH 7.4 at 22 °C. This slow phase was not present when the concentration of \(\alpha^{WT}\) was substantially less than that of AHSPWT.

The titration in Fig. 1A indicates that the fast phase of the binding reaction corresponds to a bimolecular association event, which is consistent with previous studies of the formation of reduced \(\alpha\)-AHSP complexes (11, 12, 14, 19). The associ-

experiments were performed using exit and entrance slit widths of 5 nm for the excitation monochromator, an excitation wavelength of 280 nm, and a cutoff filter fitted to the sample housing that allowed the measurement of total sample fluorescence emission above a wavelength of 302 nm. The volume of the cell was 20 μl, and the excitation pathlength of the incident light was 10 mm. The photomultiplier unit was positioned at a 90° angle from the incident light, and the fluorescence cell width was 1 mm (total cell dimensions were 10 × 2 × 1 mm). Shot volumes were between 100 and 200 μl, and mixing was performed using equal volumes of reactant solutions.

Unless otherwise noted, all experiments were performed using 50–150 mM potassium phosphate buffer, pH 7.4 at 22 °C, and all concentrations are given as postmixing. Glass syringes were used whenever possible in the stopped-flow experiments to prevent atmospheric gas contamination (Cadence Science, Northampton, MA). Controls reactions designed to account for the potential effects of photobleaching, precipitation, denaturation, and aggregation were performed, and no changes occurred on the time scales shown (data not shown; see Ref. 28). Determination of bimolecular association rate constant for AHSPWT binding to both reduced and oxidized \(\alpha^{WT}\) \((k'_{\text{AHSP}})\)) under pseudo-first-order conditions. The lines represent fits to an expression derived from Reaction 1 with the \(y\)-intercept fixed to values of \(k_{\text{AHSP}}\) measured directly by \(\beta\)-CO displacement (Table 1 and Fig. 5). Buffers were bubbled with the indicated gases prior to use. To generate ferric \(\alpha^{WT}\), a 5-fold excess of potassium ferricyanide was added to \(\alpha^{WT}\) prior to the experiment, and the samples were stored on ice to inhibit denaturation and precipitation of met-α. All concentration values are postmixing.

FIGURE 1. Time courses and association rate constants for \(\alpha\)-COWT binding to AHSPWT. A, fluorescence signal changes after rapidly mixing \(\alpha\)COWT with AHSPWT. Numbers in the right margin represent the postmixing nanomolar (nM) concentrations of \(\alpha\)-COWT. Control reactions designed to account for the potential effects of photobleaching, precipitation, denaturation, and aggregation were performed, and no changes occurred on the time scales shown (data not shown; see Ref. 28). B, determination of bimolecular association rate constant for AHSPWT binding to both reduced and oxidized \(\alpha^{WT}\) \((k'_{\text{AHSP}})\)) under pseudo-first-order conditions. The lines represent fits to an expression derived from Reaction 1 with the \(y\)-intercept fixed to values of \(k_{\text{AHSP}}\) measured directly by \(\beta\)-CO displacement (Table 1 and Fig. 5). Buffers were bubbled with the indicated gases prior to use. To generate ferric \(\alpha^{WT}\), a 5-fold excess of potassium ferricyanide was added to \(\alpha^{WT}\) prior to the experiment, and the samples were stored on ice to inhibit denaturation and precipitation of met-α. All concentration values are postmixing.
The association ($k'_{\text{AHSP}}$) and dissociation ($k_{\text{AHSP}}$) rate constants were obtained from kinetic data similar to those in Figs. 1, 2, and 5. The equilibrium dissociation constant ($K_{\text{AHSP}}$) was calculated from the ratio $k_{\text{AHSP}}/k'_{\text{AHSP}}$. The $K_{\text{AHSP}}$ values reported by Gell et al. (35, 36) are given in parentheses and were determined by isothermal titration calorimetry in 20 mM sodium phosphate buffer at pH 7.4 at 22 °C using 450 mM succrose in the case of ferric HbWT to mitigate precipitation. Gell et al. (16) did not report a $K_{\text{AHSP}}$ for αCO binding to AHSPP30W; the value for this reaction in the last row is for αO2. The association and dissociation rate constants ($k'_{\text{AHSP}}$ and $k_{\text{AHSP}}$, respectively) reported by Brlet et al. (35) are shown in brackets and were determined in PBS at 37 °C.

| Reaction | $k'_{\text{AHSP}}$ | $k_{\text{AHSP}}$ | $k_{\text{AHSP}}$ | $K_{\text{AHSP}}$ |
|----------|-------------------|-------------------|-------------------|-------------------|
| AHSPWT + αWT | 10 ± 1.9 [20] | 1.0 | 0.17 [0.35] | 17 [17] [93] |
| AHSPWT + αWT | 11 | 1.0 | 0.20 | 18 [98] |
| AHSPWT + μWT | 10 | 0.0017 | 1.0 | 0.17 [4.3] |
| AHSPP30A + αWT | 9.2 | 0.14 | 0.19 | 15 (38) |
| AHSPP30A + αWT | 11 | 0.0066bc | 0.5c | 0.60f |
| AHSPP30W + αWT | 13 | 0.0072 | 1.4 | 0.56 (7.7) |
| AHSPP30W + μWT | 17 | 0.0042bc | 0.5c | 0.24f |

* A slow second phase (~25% amplitude) is observed with a first-order rate equal to 0.04 s⁻¹ that is independent of [α] (Fig. 1A).
* The rate parameters for met-αWT binding and release from AHSPP30A and AHSPP30W were determined from a more limited set of data than those for the other reactions.
* Binding was estimated from one set of concentrations, and release was measured at one high [β] with the value of $k_{\text{AHSP}}$ fixed to the average value obtained by McGovern et al. (39). Time courses for these reactions are provided in Fig. 2 of Khandros et al. (36).
* The time course for met-α dissociation from AHSPP30A indicated two phases, and fitting to a two-exponential expression gave a fast phase with an amplitude of ~33% and $k_{\text{AHSP}}$ of ~0.04 s⁻¹ and a slow phase with an amplitude of ~67% and $k_{\text{AHSP}}$ of ~0.002 s⁻¹. The larger rate is still significantly slower than the rate of dissociation of reduced α from this mutant. The value in the table is calculated from the half-time of the reaction. Similar analyses of the met-α AHSPWT and AHSPP30W dissociation reactions indicate that if any fast phases exist their amplitudes are ≤15% of the total fluorescence changes (see Fig. 2B in Khandros et al. (36)).

**FIGURE 2. Effects of Pro30 mutation of AHSP on α binding.** A, time courses for the reaction of αWT with AHSPP30W. The postmixing [αWT] in mM is given beside each trace. Experiments with AHSPP30A also showed no detectable slow phase (see Fig. 2A in Khandros et al. (36)). The decreases in the fluorescence changes at high micromolar αWT concentrations were the result of light scattering, giving higher background signals. They are consistently observed in all experiments regardless of mutation at [αCO] ≥ 1 μM (28). B, determination of the bimolecular association rate constant for αCO binding to AHSPP30A and AHSPP30W under pseudo-first-order conditions. Lines represent fits to Reaction 1 with the y-intercept fixed to values of $k_{\text{AHSP}}$ measured directly by βCO displacement (Table 1 and Fig. 5).
of AHSP WT than the cis conformer (13, 14). Combined with our kinetic data, these structural data suggest that the fast phase shown in Fig. 1 is due to the association of ferrous AHSP WT with the trans Asp29-Pro30 AHSP WT conformer. These data also suggest that the slow phase represents a rate-limiting cis-to-trans isomerization of the Asp29-Pro30 peptide bond followed by rapid binding.

The reaction of AHSP WT with met-α WT also lacks a slow phase (Fig. 4). The total amplitude changes are similar to those for the AHSP reaction with either αO2 WT and αCO WT, suggesting that met-α can bind to either the cis or trans AHSP WT conformers during bimolecular association perhaps because of greater conformational flexibility.

To investigate further our interpretation of the slow phase, peptidylprolyl isomerases were added to the solutions of AHSP WT prior to mixing with αCO. We tried recombinant cyclophilin-A and FK506-binding protein 4 (Prospec Protein Specialists, East Brunswick, NJ). However, no detectable change in the amplitude or rate of the slow phase was observed after preincubation of AHSP WT with either of these enzymes (data not shown). Either the active sites of these enzymes have specificities that preclude interaction with AHSP WT, or the origin of the slow phase results from some other type of conformational isomerization at the Pro30 loop.

Rates of α Dissociation from AHSP Complex—Gel filtration chromatography and electrophoretic mobility shift assays have shown that β subunits are capable of competitively displacing α from AHSP (11, 12, 15). HbA formation occurs during this process because (a) α has a much higher affinity for β than for AHSP and (b) α cannot simultaneously bind to AHSP and β because both of these interactions involve the same set of interfacial α helices (14, 15). A scheme showing these interactions is given in Fig. 5A.

The only species in Fig. 5A that exhibits strong intrinsic fluorescence in solution is AHSP when it is not in complex with holo-α (19, 32). When α-AHSP complexes are mixed with β, displacement of α from AHSP increases fluorescence emission
as free AHSP is generated. The rate of this process allows measurement of $k_{\text{AHSP}}$ (19, 32, 35).

The observed rate constants for the reaction of $\beta$ with ferrous $\alpha$-AHSP complexes are monophasic at high concentrations, and the rate of displacement increases with increasing $[\beta]$. Representative time courses for a set of displacement reactions are shown in Fig. 5B, and a more detailed comparison of time courses for ferric and ferrous $\alpha$ dissociation from wild-type and the Pro30 mutants of AHSP is given in Fig. 2B of Khandros et al. (36). The amplitudes of the fluorescence increases for $\alpha$ displacement from the various AHSP complexes are slightly smaller in magnitude than the decreases observed in the association experiments (28). The smaller fluorescence increases are mostly likely due to the higher level of background absorbance and light scattering that occur with the addition of excess $\beta$. As expected, displacement of $\alpha$ from ASHPWT showed roughly twice the increase as from AHSPWT due to the presence of a second Trp.

At high AHSP and $\beta$ concentrations, the amount of free $\alpha$ during the displacement reaction is small throughout the reaction. Under these conditions, the rate of $\alpha$ displacement from AHSP by $\beta$ is given by the following equation, assuming a steady-state for free $\alpha$,

$$ r_{\text{obs}} = \frac{k_{\text{AHSP}}k'_{\alpha\beta}[\beta]}{k'_{\alpha\beta}[\beta] + k_{\text{AHSP}}[\text{AHSP}]} $$

(Eq. 1)

where $k_{\text{AHSP}}$ is the rate constant for dissociation of the $\alpha$-AHSP complex, $k'_{\text{AHSP}}$ is the association rate constant for complex formation, and $k'_{\alpha\beta}$ is the bimolecular rate constant for the association of free Hb subunits to form an $\alpha\beta$ dimer (38). By measuring the observed rate of replacement ($r_{\text{obs}}$) at increasing $[\beta]$, this equation can be used to obtain fitted values for $k_{\text{AHSP}}$ and the ratio of $k'_{\alpha\beta}$ to $k'_{\alpha\beta}$ (19). Representative fits to Equation 1 are shown in Fig. 5C.

In these studies, low concentrations of $\alpha$-AHSP complexes were mixed with 5-fold or greater concentrations of $\beta$. These concentrations introduce a departure from pseudo-first-order conditions, making Equation 1 approximate because the free concentration of AHSP is increasing from zero during the reaction. To address this, we fixed the free [AHSP] in Equation 1 to 50% of the postmixing total AHSP concentration (0.125 $\mu$m). Then $r_{\text{obs}}$ values were determined by either fitting the observed time courses to a single exponential expression or computed from the measured half-time ($r_{\text{obs}} = \ln2/t_{1/2}$). This simplified analysis provides fitted values for $k_{\text{AHSP}}$ that are identical to those obtained by numerical integration of the rate equations that allow the concentration of AHSP to increase with time (35), and good fits to plots of $r_{\text{obs}}$ versus $[\beta]$ were obtained (Fig. 5C).

For the fits to Equation 1 shown in Fig. 5C, the value of $k'_{\alpha\beta}$ was fixed to the value determined from the bimolecular association reactions shown in Figs. 1B and 2B, and $k_{\text{AHSP}}$ and $k'_{\alpha\beta}$ were allowed to vary. Table 1 summarizes the results of several displacement experiments involving the dissociation of ferric and ferrous $\alpha$WT from AHSPWT, ASHP30A, and AHSP30W. The fitted values of $k'_{\alpha\beta}$ were in the range of 0.2–1.0 $\mu$m$^{-1}$ s$^{-1}$, which are similar to the values reported for the association of deoxygenated $\alpha$ and $\beta$ (39). The equilibrium dissociation con-
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Although $k'_{\text{AHSP}}$ appears to be invariant, the rate of $\alpha$ dissociation from AHSP is strongly dependent on both the size of the amino acid at position 30 of AHSP and the oxidation state of $\alpha$. For $\alpha$CO WT, the P30A AHSP mutation had little effect on $k_{\text{AHSP}}$, but the P30W mutation caused a marked 25-fold decrease in $k_{\text{AHSP}}$ (Table 1). The value of $k_{\text{AHSP}}$ for the reduced forms of $\alpha$WT is $\sim0.2$ s$^{-1}$, but the value for met-$\alpha$ is 100-fold lower and gives rise to a subnanomolar value for $K_{d(\text{AHSP})}$. The time courses for met-$\alpha$ dissociation from AHSP(P30A) show heterogeneity (see Table 1, footnote c; see Fig. 2B in Ref. 36), suggesting multiple conformations for the mutant AHSP(P30A), met-$\alpha$ complex. Even, the rate of the most rapid phase is 5–10-fold smaller than $k_{\text{AHSP}}$ for the corresponding reduced $\alpha$ complex.

Effects of Other Mutations in AHSP Pro30 Loop and $\alpha$K99E Subunit Variant—Previous studies of AHSPWT have revealed detailed structural information regarding the $\alpha$-AHSP binding interface, including the identities of all the $\alpha$ chain amino acids that are thought to interact directly with AHSP (14, 15). In a previous study (40), we used this information as a basis for conducting literature-based searches for clinically significant $\alpha$ variants possessing mutations at these positions. It was hypothesized that the phenotypes associated with these mutations might be a result of aberrant $\alpha$-mutant AHSPWT interactions. Using a series of indirect binding studies, eight $\alpha$ missense mutations, mutations were investigated, and one was found to affect the $\alpha$-AHSP interface without detectably perturbing $\alpha$B subunit interactions (40). This mutation, called Hb Turriff, replaces $\alpha$ Lys99 with Glu (40).

To extend this work, we designed a series of AHSP mutants, which were hypothesized to restore binding to $\alpha$K99E (40). As shown in Fig. 6A, the $\alpha$WT Lys99 residue is positioned in close proximity to AHSPWT Pro30 when the two proteins are bound together. The $\alpha$K99E mutation is predicted to perturb this region of the $\alpha$-AHSP interface by introducing a negative charge and generating unfavorable electrostatic interactions with neighboring AHSPWT residues. To alter the charge on the complementary surface of AHSPWT and investigate the relative importance of electrostatic interactions in this region, Gln25 and Asp29 in AHSP were mutated to Lys and Arg, respectively. Hypothetical structures showing possible side chain rotamers in the mutant AHSP proteins are shown in Fig. 6B.

We expressed and purified these AHSP mutant proteins along with recombinant Hb Turriff. We then isolated the individual subunits of Hb Turriff to obtain $\alpha$K99E. All proteins were stable and found to behave similarly to their wild-type counterparts throughout the purifications. Although $\alpha$K99E exhibited altered mobility during diethylaminoethyl cellulose chromatography, this protein was still resolvable from both tetrameric Hb Turriff and $\beta$. The finding that Hb Turriff was expressed at the usual levels confirms our previous work, which suggested that the K99E mutation does not significantly perturb $\alpha$B subunit interactions.

The following sets of binding and dissociation reactions were investigated using reduced $\alpha$CO variants: 1) $\alpha$K99E binding to AHSPWT, 2) $\alpha$K99E binding to AHSPD29R, 3) $\alpha$K99E binding to AHSPD29R, 4) $\alpha$WT binding to AHSPQ25K, and 5) $\alpha$WT binding to AHSPD29R. Representational time courses as well as fits to the kinetic expressions for association ($k_{\text{obs}} = k'_{\text{AHSP}} + k_{\text{AHSP}}$) and dissociation (Equation 1) for these experiments are shown in Fig. 7, A–D. Longer time scales were investigated, and small slow phases with highly variable amplitudes and rates were observed for all these AHSP variants, which still have Pro at position 30. The rate and equilibrium constants for the major fast phases of these reactions are given in Table 2.

These results agree with our previous work (40) and with the data of Feng et al. (14), which showed that the $\alpha$ K99A mutation disrupts binding to AHSPWT. In the case of the $\alpha$ K99E mutation, no binding to AHSPWT was detected at all even at high micromolar concentrations of both proteins. Weak binding could be restored with the Q25K AHSP mutation, and moderate binding occurred with the D29R AHSP variant. Thus, it seems likely that the $\epsilon$-amino group of $\alpha$WT Lys99 participates in favorable electrostatic interactions with one or more polar side
chains on AHSP\textsuperscript{WT}. However, because \( \alpha^{WT} \) could still bind fairly strongly to AHSP\textsuperscript{Q25K} and AHSP\textsuperscript{D29R}, it also seems likely that the K99E mutation causes other conformational alterations in the globin structure.

\textbf{Rapid and Reversible AHSP-induced Hemichrome Formation—}HbA autooxidation is a spontaneous process in which either O\(_2\) bound to a ferrous heme group spontaneously dissociates as a superoxide radical, or O\(_2\) free in solution reacts with a transient aquo-deoxyheme, leaving the resulting iron in the ferric (Fe\(^{3+}\)) state (41–43). Following this reaction, the hemin iron is axially coordinated on one side by a histidine (the proximal or F8 histidine) and on the other by an H\(_2\)O or OH\(^-\) depending on pH (44, 45). If the hemin pocket unfolds, the distal histidine, His\(^58\)(E7) in \( \alpha \) (or other nearby basic amino acids on the distal side of the heme ring) can coordinate to the ferric iron atom (44–48). The resulting bishistidyl adduct is called a hemichrome (44, 45). Bishistidyl adducts can also form with ferrous heme groups, and the resulting products are termed hemochromes (44, 45). These species can be identified by characteristic peaks in the visible spectra of the corresponding metHb and deoxy-Hb derivatives (44, 45).

Previous work has shown that binding to AHSP accelerates the autooxidation of \( \alpha \)O\(_2\) to form a hemichrome with no readily identifiable intermediate aquo-met-\( \alpha \) state (14, 18). At neutral

\textbf{TABLE 2 Rates of \( \alpha^{WT} \) and \( \alpha^{K99E} \) binding to and dissociation from AHSP\textsuperscript{Q25K} and AHSP\textsuperscript{D29R}}

The association \( (k_{AHSP}^{\alpha}) \) and dissociation \( (k_{AHSP}^{-\alpha}) \) rate constants were obtained from the kinetic data in Fig. 7, and the equilibrium dissociation constants \( (K_{D(AHSP)}^{\alpha}) \) were calculated from the ratio \( k_{AHSP}^{\alpha}/k_{AHSP}^{-\alpha} \). The abbreviation “ND” signifies that a given value could not be determined. No binding of \( \alpha^{K99E} \)CO to AHSP\textsuperscript{WT} could be detected presumably because of very large \( k_{AHSP}^{\alpha} \) and \( K_{D(AHSP)}^{\alpha} \) values. In the case of \( \alpha^{K99E} \) binding to AHSP\textsuperscript{Q25K}, we were unable to measure displacement reactions, and \( k_{AHSP}^{-\alpha} \) was calculated based on the y-intercept of the association reaction measured under pseudo-first-order conditions (plot in Fig. 7B).

| Reaction                  | \( k_{AHSP}^{\alpha} \) (M \text{s}^{-1}) | \( k_{AHSP}^{-\alpha} \) (M \text{s}^{-1}) | \( K_{D(AHSP)}^{\alpha} \) (nM) |
|---------------------------|------------------------------------------|------------------------------------------|---------------------------------|
| AHSP\textsuperscript{WT} + \( \alpha^{WT} \) | 10 ± 1.9                                  | 0.17                                     | 0.26                            |
| AHSP\textsuperscript{WT} + \( \alpha^{K99E} \)CO | ND                                      | >1,000                                   | ND                              |
| AHSP\textsuperscript{Q25K} + \( \alpha^{K99E} \)CO | 2.5                                     | 17.5                                     | ND                              |
| AHSP\textsuperscript{Q25K} + \( \alpha^{WT} \)CO | 4.5                                     | 0.14                                     | 4.4                             |
| AHSP\textsuperscript{D29R} + \( \alpha^{WT} \)CO | 7.9                                     | 0.22                                     | 2.5                             |
| AHSP\textsuperscript{D29R} + \( \alpha^{K99E} \)CO | 5.7                                     | 0.20                                     | 9.0                             |

\textbf{FIGURE 7. Time courses and kinetics for \( \alpha^{K99E} \)CO binding to AHSP revertant mutants.} A, time courses for \( \alpha^{WT} \) and \( \alpha^{K99E} \)CO binding to AHSP\textsuperscript{WT}, AHSP\textsuperscript{Q25K}, and AHSP\textsuperscript{D29R}. B, determination of the association rate constants \( (k_{AHSP}^{\alpha}) \) under pseudo-first-order for the reactions in A. C, representative replacement reaction time courses for the displacement of \( \alpha^{WT} \)CO and \( \alpha^{K99E} \)CO from AHSP\textsuperscript{WT}, AHSP\textsuperscript{Q25K}, and AHSP\textsuperscript{D29R} by \( \beta \)CO. D, dependence of the replacement rates on [\( \beta \)CO]/[AHSP]. lines represent theoretical fits to Equation 1.
pH, rapid oxidation of free $\alpha_O_2$ by ferricyanide initially produces met-$\alpha$ with water coordinated to the sixth position of the iron atom. The spectral differences between $\alpha_O_2$ and met-$\alpha$ are shown in Fig. 8A with the ferrous oxygenated form exhibiting strong absorption peaks at 541 and 576 nm and aquo-met-$\alpha$ exhibiting large charge transfer bands peaking at ~505 and 635 nm (29, 30). Adding equimolar AHSP to met-$\alpha$ resulted in the immediate (~10 s) disappearance of the 505 and 635 nm bands and appearance of 535 and 565 nm bands, which are characteristic of hemichrome formation (Fig. 8A) (11, 44).

The kinetics of this spectral transition are shown in Fig. 8B. When 20 $\mu$M met-$\alpha$ was mixed with 20 $\mu$M AHSP, a large increase in absorbance was observed at 535 nm. The apparent bimolecular rate is 5–10 $\mu$M$^{-1}$ s$^{-1}$, which is identical to that observed when measuring binding by quenching of intrinsic AHSP fluorescence (Fig. 8B, gray time course). These results show that met-$\alpha$ hemichrome formation occurs simultaneously with binding to AHSP and that the conformational transition from aquo-met to the hemichrome form of $\alpha$ occurs at rates >100 s$^{-1}$ when bound to AHSP. If the hemichrome conformational change were slower, it would have been seen as a much slower 535-nm absorbance increase in Fig. 8B compared with the AHSP fluorescence decrease.

To investigate the reversibility of AHSP-induced hemichrome formation with respect to reduction, a solution of met-$\alpha$AHSP complex was rapidly mixed with excess sodium dithionite. This reagent is a reducing agent, which both consumes O$_2$ in aqueous solutions and rapidly converts met-HbA to ferrous deoxy-HbA (1, 30). As is shown in Fig. 9, rereduction of met-$\alpha$AHSP did not produce a spectrum indicative of a hemochrome, which would have had a moderate peak at ~530 nm and a very intense band at 558 nm (44, 49). Instead, reduction with dithionite produced a deoxy-$\alpha$ spectrum with a single peak at ~560 nm, which is characteristic of pentacoordinate deoxy-$\alpha$ in its native and fully folded form even though the subunit is still bound to AHSP (29). The time course of the reduction reaction was measured in a rapid scanning, stopped-flow spectrophotometer. The resulting time courses indicate that rereduction occurred rapidly (Fig. 9, inset) with an observed rate that is on the same order as the rate of reduction of free met-$\alpha$ (data not shown), and no hemochrome intermediates were observed. Thus, the $\alpha$ conformational changes after reduction are also very rapid even when the globin is bound to AHSP.

**DISCUSSION**

The two phases observed for the reaction of $\alpha^{WT}$ with AHSP$^{WT}$ (Figs. 1 and 4) seem to verify the structural heterogeneity first characterized by Santiveri et al. (13). The loss of the second, slow phase for the P30A and P30W AHSP mutations (Fig. 2) suggests strongly that the kinetic heterogeneity is due to peptidylprolyl cis-trans isomerization at the Asp$^{39}$-Pro$^{30}$ pep-
tide bond in AHSP. Based on the relative amplitude of the slow association phase, it appears that 20–30% of free \( \text{AHSP}^{\text{WT}} \) occupies a cis peptidylprolyl conformation that is relatively unreactive toward reduced \( \alpha \). Although this estimate differs from the 50% mixture reported by Santiveri \textit{et al.} (13), it is consistent with previous reports that at equilibrium 60–90% of model oligopeptides occupy the trans peptidylprolyl conformation and 10–40% occupy the cis conformation (for a review, see Ref. 50). Although more recent work has revealed that the exact ratios are dependent on which amino acid precedes Pro, in cases of Asp-Pro, it has been shown that 11–19% of model oligopeptides are dependent on which amino acid precedes Pro, in cases of Asp-Pro, it has been shown that 11–19% of model oligopeptides occupy the cis peptidyl-prolyl conformation (51). Additionally, the observed rate of the slow phase, 0.04 \( \pm \) 0.01 \( \text{s}^{-1} \) in 100 mM potassium phosphate buffer at \( \text{pH} 7.4 \) at 22 \( ^\circ \text{C} \), is consistent both with the rate assignment of AHSP conformational interconversion by NMR (13) and with other studies that show that peptidylprolyl cis-trans interconversions occur spontaneously with half-times of 10–100 s at 25 \( ^\circ \text{C} \) (50). The observations that the rate of the slow phase was independent of \( \alpha \) concentration and that the slow phase was not observed when \( \alpha \) was limiting are also supportive of this model. This mechanism implies that reduced \( \alpha \) reacts rapidly with the trans Pro\textsuperscript{30} conformer of AHSP in a simple bimolecular process (Reaction 1), whereas little or no binding occurs to the small remaining fraction of cis AHSP until it isomerizes to the trans conformation.

This interpretation is supported by the effects of the P30A and P30W AHSP mutations, which caused the formation of a single trans conformation at the 29–30 peptide bond (Fig. 3) (13). The time courses for \( \alpha \) binding to AHSP\textsuperscript{P30A} and AHSP\textsuperscript{P30W} are monophasic and bimolecular. The fitted values of \( k'_{\text{AHSP}} \) for these mutants are almost identical to that obtained from the fast phase for \( \alpha \) binding to AHSP\textsuperscript{WT}. This finding also suggests strongly that reduced \( \alpha \) binds rapidly to the trans peptidylprolyl Pro\textsuperscript{30} conformer of AHSP\textsuperscript{WT}. This idea is supported by the observation that the wild-type \( \alpha \)-AHSP complex was very difficult to crystallize, and formation of crystals was greatly facilitated by the P30A mutation (14–16).

However, none of these arguments for cis to trans peptidylprolyl isomerization are direct, and we did not observe an effect on the amplitude or rate of the slow phase when peptidylprolyl isomerases were added to the AHSP solution prior to mixing with \( \alpha \). Thus, it is possible that the slow phases observed for AHSP\textsuperscript{WT} reflect some other type of conformational isomerization that causes more quenching of intrinsic AHSP fluorescence after reduced \( \alpha \) is bound. Some autooxidation is occurring when \( \alpha \text{O}_2 \) is bound, but the AHSP-\( \alpha \text{CO} \) complex is stable with little or no UV-visible absorbance changes occurring on time scales of minutes to hours. The lack of a slow phase for met-\( \alpha \) binding to AHSP\textsuperscript{WT} suggests that the rapidly formed \( \alpha \)-hemichrome binds to either prolyl conformer.

The importance of the loop separating helices 1 and 2 of AHSP\textsuperscript{WT} is underscored by our studies of \( \alpha\text{K99F} \) (Hb Turriff). Our preliminary studies indicated that this variant is unstable \textit{in vivo} due to a loss of binding to AHSP\textsuperscript{WT} (40). Our new studies confirm that this mutant \( \alpha \) cannot bind AHSP even at micromolar concentrations and that the mild phenotype associated with Hb Turriff is caused by a loss of this interaction and not by disrupted assembly into \( \alpha \beta \) dimers and tetramers, which appears normal.

The results in Table 2 show that introduction of positively charged amino acids in the Pro\textsuperscript{30} loop of AHSP revertants can partially reverse the effects of the \( \alpha \) K99E mutation. However, the results are complex and indicate that, although favorable electrostatic interactions are important near the Pro\textsuperscript{30} loop, other conformational factors also play an important role.

The association and dissociation rate constants reported for \( \alpha \text{CO} \) and \( \alpha \text{O}_2 \) binding to AHSP in Table 1 agree well with those in our initial report (19) and with the parameters reported subsequently by Brillet \textit{et al.} (35). However, Brillet \textit{et al.} (35) reported that the “... dissociation rates were not found to depend strongly on the Hb oxidation state (oxy versus met versus deoxy).” Although our data also show that \( k'_{\text{AHSP}} \) is nearly the same for ferric and ferrous \( \alpha \) (Table 1), the rate of dissociation (\( k_{\text{AHSP}} \)) of the met-\( \alpha \)-AHSP\textsuperscript{WT} complex is \( \sim \)100-fold smaller than that for the reduced \( \alpha \)-AHSP complexes. The net result is a 100-fold higher affinity and a very small subnanomolar \( K_D \) for met-\( \alpha \) binding to AHSP.

Gell \textit{et al.} (16) observed a similar large relative decrease in \( K_D \) for met-\( \alpha \) versus \( \alpha \text{CO} \) binding to AHSP using isothermal titration calorimetry (Table 1). However, the absolute \( K_D \) values obtained by calorimetry are consistently higher than either we or Brillet \textit{et al.} (35) determined from the ratio of the kinetic parameters. The reasons for this discrepancy are not obvious. The cause may be related to systematic errors during curve fitting that arise because of the high concentrations of protein (1–5 \( \mu \text{M} \)) required by the isothermal titration calorimetry experiments and the low \( K_D \) values (\( \leq 0.1 \mu \text{M} \); Table 1).

Regardless of oxidation state or bound ligand, the bimolecular rate of \( \alpha \) binding to AHSP (\( k'_{\text{AHSP}} \approx 10 \text{ mm}^{-1} \text{s}^{-1} \)) is \( \sim 20 \)-fold greater than the rate constant for \( \alpha \) binding to \( \beta (k'_{\text{\beta\beta}} \approx 0.5 \text{ mm}^{-1} \text{s}^{-1} \) (Table 1 and Refs. 8, 37, 39, and 52–55)). However, the rate of \( \alpha \) dissociation from AHSP (\( k_{\text{AHSP}} \approx 0.2 \text{ s}^{-1} \) for ferrous \( \alpha \)) is roughly 200,000-fold greater than the rate of \( \alpha \) dissociation from \( \alpha \beta \) dimers (\( k_{\text{\beta\beta}} \approx 0.000001 \text{ s}^{-1} \) (52–54)). These findings led us to propose the model in Fig. 10 for facilitation of Hb assembly by AHSP (19).

Because AHSP binds to \( \alpha \) more rapidly than \( \beta \), it is likely that AHSP out-competes \( \beta \) kinetically for nascent free \( \alpha \) \textit{in vivo}. If the \( \alpha \) heme groups are in the ferrous form, they can be more readily displaced by \( \beta \) due to their dramatically higher affinity for \( \beta \) chains (\( K_D \approx 0.001 \text{ nm} \) (37, 52–54)) than for AHSP (\( K_D \approx 17 \text{ nm} \) (Table 1)) and the relatively high rate of ferrous \( \alpha \)-AHSP dissociation (\( k_{\text{AHSP}} \approx 0.2 \text{ s}^{-1} \)). However, if bound \( \alpha \) is in the oxidized hemichrome form, displacement by \( \beta \) chains is 100-fold slower. Thus, participation of ferric \( \alpha \text{AHSP} \) in Hba assembly is inhibited kinetically by AHSP, but disassembly of the resultant met-\( \alpha \)-AHSP complex is facilitated by hemin reduction.

Hemoglobin assembly requires apoglobin synthesis, partial folding, heme uptake, reduction, and \( \alpha \beta \) dimer formation. Although folding and assembly can occur without AHSP binding, we suggest that the highlighted pathway in Fig. 10 is favored in the presence of AHSP. AHSP first assists the folding of apo-\( \alpha \) (17) and then uptake of hemin to generate the met-\( \alpha \)-AHSP hemichrome complex. It has been shown that hemichrome forms are highly populated intermediates during the folding of
both holomyoglobin and holo-Hb (46–48). The α hemichrome folding intermediate is unstable when free in solution, loses hemin, and unfolds rapidly in the apoform, leading to precipitation (44–48). However, this met-hemin, and unfolds rapidly in the apoform, leading to precipitation.

The mechanism in Fig. 10 shows why the lack of K99E α binding to AHSP indirectly destabilizes HbA. When this variant is oxidized, it cannot be sequestered by AHSP, which allows ferric αK99E to either denature or react with β and be incorporated into relatively unstable mixed valency tetramers. Both the stabilization and quality control functions help explain the observation that disruption of AHSP<sup>WT</sup> in mice worsens the phenotypes of both α- and β-thalassemia syndromes (66, 67).

Although HbA assembly almost certainly occurs through several parallel pathways, AHSP binding appears to act as a shunt for ferric α until reduction can occur. Thus, even when β subunits are in excess (α-thalassemia), there will be more incorporation of unstable oxidized α into HbA when AHSP is absent, leading to more globin precipitates. In the case of excess α (β-thalassemia), the situation is even worse in the absence of AHSP when no chaperone is available to stabilize the excess met-α.

**CONCLUSIONS**

Cis-trans isomerization and electrostatic interactions in the Pro<sup>30</sup> loop between helices 1 and 2 of AHSP have marked effects on the rate and equilibrium constants for α binding and can help explain the phenotypes of α hemoglobinopathies that involve disruption of α binding to AHSP but not to β. Compared with ferrous α, met-α binds 100-fold more tightly to AHSP, resulting in a very low rate of dissociation of the ferric α-AHSP complex. Consequently, AHSP can trap met-α kinetically and inhibit its incorporation into unstable mixed valence HbA tetramers. This kinetic property coupled with AHSP stabilization of the hemichrome folding intermediate supports a dual role for AHSP. It acts both as a molecular chaperone to facilitate α folding and as a quality control protein to prevent assembly of unstable, partially oxidized Hb tetramers.

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