**α-Hemoglobin Stabilizing Protein (AHSP) Markedly Decreases the Redox Potential and Reactivity of α-Subunits of Human HbA with Hydrogen Peroxide**

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**Background:** α-Hemoglobin stabilizing protein (AHSP) modifies the redox properties of bound α-subunits.

**Results:** Isolated hemoglobin subunits exhibit significantly different redox properties compared with HbA. A significant decrease in the reduction potential of α-subunits bound to AHSP results in preferential binding of ferric α.

**Conclusion:** AHSP-α-subunit complexes do not participate in ferric-ferryl heme redox cycling.

**Significance:** AHSP binding to α-subunits inhibits subunit pseudoperoxidase activity.

α-Hemoglobin stabilizing protein (AHSP) is a molecular chaperone that binds monomeric α-subunits of human hemoglobin A (HbA) and modulates heme iron oxidation and subunit folding states. Although AHSP:αHb complexes autoxidize more rapidly than HbA, the redox mechanisms appear to be similar. Both metHbA and isolated met-β-subunits undergo further oxidation in the presence of hydrogen peroxide (H2O2) to form ferryl heme species. Surprisingly, much lower levels of H2O2-induced ferryl heme are produced by free met-α-subunits as compared with met-β-subunits, and no ferryl heme is detected in H2O2-treated AHSP:met-α-complex at pH values from 5.0 to 9.0 at 23 °C. Ferryl heme species were similarly not detected in AHSP:met-α-Pro-30 mutants known to exhibit different rates of autoxidation and hemin loss. EPR data suggest that protein-based radicals associated with the ferryl oxidation state exist within HbA α- and β-subunits. In contrast, treatment of free α-subunits with H2O2 yields much smaller radical signals, and no radicals are detected when H2O2 is added to AHSP-α-complexes. AHSP binding also dramatically reduces the redox potential of α-subunits, from +40 to −78 mV in 1 m glycine buffer, pH 6.0, at 8 °C, demonstrating independently that AHSP has a much higher affinity for Fe(III) versus Fe(II) α-subunits. Hexacoordination in the AHSP:met-α complex markedly decreases the rate of the initial H2O2 reaction with iron and thus provides α-subunits protection against damaging oxidative reactions.

HbA is a well-studied O2 transport protein that is known to participate in several biologically important redox reactions *in vivo* (1–4). This protein consists of two α-subunits and two β-subunits, with each subunit bearing a single, iron-containing protoporphyrin IX prosthetic group (5). Besides reversibly binding O2, these iron-containing groups and the surrounding residues are major sites of redox reactivity within HbA (6). The redox reactivity of HbA and isolated subunits leads in some cases to adverse effects due to radical-generating reactions, heme loss, and aggregation (7). To avoid these problems, Hb in red blood cells is found in a reducing environment. Furthermore, to deal with heme-related redox cycling outside of red blood cells, molecular chaperone proteins are naturally designed to bind and clear free Hb and its oxidation by-products from circulation (8).

AHSP represents a molecular chaperone protein (9–11). It rapidly and reversibly binds free α-subunits, but not β, αβ dimers, or tetrameric HbA (12, 13). Several studies have implicated AHSP as a modulator of α-subunit redox reactivity. For example, disruption of the *Ahsp* gene in mice leads to evidence of oxidative stress (14), and *in vitro* studies indicate that AHSP binding inhibits α-subunit reactions with oxidants such as hydrogen peroxide (H2O2) (15). Although these findings suggest that AHSP protects isolated α-subunits from oxidative damage and participation in harmful redox reactions *in vivo*, AHSP has also been shown to accelerate the rate of αO2 autoxidation to the ferric (met) state (16). MetHbA, isolated met-α-subunits, and isolated met-β-subunits are unstable due to accelerated rates of heme loss, denaturation, aggregation, and precipitation (9). Thus, it is puzzling why a molecular chaper-

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3 The abbreviations used are: AHSP, α-hemoglobin stabilizing protein; ferrous, Fe(II); ferric, Fe(III); ferryl, Fe(IV); hemichrome, endogenous hexacoordination of iron within HbA (bia-histidyl); Hb, hemoglobin; HbA, wild-type adult human Hb; heme, ferroprotoporphyrin IX; Hp, haptoglobin; met, ferric iron oxidation; O2•, superoxide radical.
modified (17). This method occasionally resulted in catalase contamination in β-subunit samples. Catalase could be removed by size exclusion chromatography using Superdex 200 media (GE Healthcare). In the case of HbA and subunits, previously determined extinction coefficients were used to determine protein concentrations in heme equivalents (23). The AHSP extinction coefficient utilized was 11,460 M⁻¹ cm⁻¹ at 280 nm, and was calculated using ExPASy Proteomics Server Protparam. Purified haptoglobin (Hp) solution was a kind gift from Bio Products Laboratory (Hertfordshire, United Kingdom). The isolation and fractionation of this protein from human plasma was done as previously reported (24). Size exclusion HPLC chromatograms of the Hp samples used in this study show the following molecular weight distribution: 60% αβ dimers (Hp 1–1), 21% αβ trimers (Hp 1–2), and 19% larger polymers (Hp 2–2).

Spectrophotometry—Autoxidation studies were performed at 37 °C using air equilibrated 0.05 M potassium phosphate, pH 7.0, at 37 °C (25, 26). Spectra were recorded every 1 min for 4 h, using an integration time of 0.5 s and an interval of 1 nm. AHSP and α-subunit concentrations were fixed at 10 μM in heme equivalents. Where indicated, 10 mg/ml of superoxide dismutase and 200 units/ml of catalase were added to the cuvettes prior to the start of the reactions (25). Co-oxidation of epinephrine was followed under the same buffer conditions by monitoring absorbance at 475 nm using 600 μM epinephrine and the same concentrations of superoxide dismutase and catalase (25).

To detect the ferryl heme oxidation state, ferric subunits were first generated by addition of a 10-fold molar excess of potassium ferricyanide to O₂-bound ferrous proteins, followed by brief incubation and removal of the potassium ferricyanide using Sephadex G-25 chromatography media. These steps were performed as quickly as possible at 4 °C because ferric subunits are highly unstable at room temperature. Materials were used within minutes of preparation. Ferryl heme detection studies were completed by manual mixing and stopped-flow spectrophotometry. In our stopped-flow experiments, 30 μM protein in heme equivalents was mixed with 3 mM H₂O₂ (post-mixing concentrations given) at 8 °C. In the manual mixing experiments, 60 μM protein in heme equivalents was mixed with 90 μM H₂O₂ at 22 °C. Both sets of experiments utilized 10 mM potassium phosphate buffer, pH 7.0. In the stopped-flow experiments, higher H₂O₂ concentrations were chosen to accelerate the time courses and to mitigate sample denaturation stemming from necessarily longer sample preparation times. Lower H₂O₂ concentrations were used in benchtop spectrophotometer experiments to ensure that the reactions were not too rapid to be measured conventionally. Where indicated, 2 mM sodium sulfide (Na₂S) was used to derivatize the ferryl heme oxidation state as sulfinemoglobin using established methods (27). Buffers used for variable pH experiments include 200 mM potassium phosphate (pH 7.0 and 8.0 at 23 °C) and 200 mM sodium acetate (pH 5.0 and 6.0 at 23 °C) (28).

Spectroelectrochemistry—Intrinsic reduction-oxidation potentials were determined under anaerobic condition using a custom-built optically transparent thin layer electrode cell. The path length of this cell is 0.06 cm and [Ru(NH₃)₆]Cl₃ was used as a cationic mediator (29–31). Well resolved differences in Soret
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band absorption for Fe(II) and Fe(III) heme species were used to follow the extent of reduction at different applied potentials \( E_{\text{applied}} \), which is defined as follows.

\[
E_{\text{applied}} = E_o^{1/2} - \frac{RT}{nF} \ln \left( \frac{[\text{Hbred}]}{[\text{Hbrox}]} \right) 
\]  
(Eq. 3)

Where \( E_o^{1/2} \) is the midpoint potential when \([\text{Hb}_{\text{red}}] = [\text{Hb}_{\text{ox}}] \), \( R \) is the universal gas constant, \( T \) is the absolute temperature, \( n \) is the number of electrons transferred in the redox process for an ideal Nernstian system and is a measure of cooperativity for a non-Nernstian system, and \( F \) is the Faraday constant. All potentials are reported with respect to normal hydrogen electrode (31).

In a typical experiment, hemeproteins were placed in 1 M glycine buffer, pH 6.0, at 8 °C, and degassed gently on a salt/ice mixture and then purged using argon gas. Subunit concentrations were fixed at 10 μM, and free \( \alpha \)-subunit and AHSP-\( \alpha \)-subunit complexes were fixed at 80 μM (heme equivalents). The heme to mediator ratio was kept at 1:10 for all reactions. This anaerobic solution was then introduced into an optically transparent thin layer electrode cell fitted with a platinum mesh working electrode, platinum wire auxiliary electrode, and a silver/silver-chloride reference electrode. The potentials were controlled using an EG & G Princeton Applied Research Potentiostat Model 263 (AMETEK, Berwyn, PA) and the Soret bands for oxidized and reduced species were recorded using a Cary 100 spectrophotometer (Varian, Inc., Palo Alto, CA). Heme proteins were oxidized to the Fe(III) state by application of a positive potential, and spectra were recorded after a steady absorbance measurement was obtained at 405 nm for oxidized \( \alpha \)-subunits and 413 nm for oxidized AHSP-\( \alpha \)-subunit complexes. Once a stable absorbance was achieved for the Soret band at an oxidizing potential, ensuring complete conversion of the number of electrons transferred in the redox process for an ideal Nernstian system, and is a measure of cooperativity for a non-Nernstian system, and \( F \) is the Faraday constant. All potentials are reported with respect to normal hydrogen electrode (31).

\[
\frac{\Delta G}{nF} = R T \ln \left( \frac{[\text{Hb}_{\text{red}}]}{[\text{Hb}_{\text{ox}}]} \right)
\]

Results

Autoxidation—HbA oxidation from the ferrous (Fe(II)) to the ferric (Fe(III)) state occurs spontaneously in the presence of air-equilibrated aqueous buffer and produces superoxide anion (O2−) (33, 34). The released superoxide anion then undergoes dismutation to \( \text{H}_2\text{O}_2 \) and \( \text{O}_2 \), and the \( \text{H}_2\text{O}_2 \) produced in this step can react with the protein to produce several other species (6). This spontaneous conversion of ferrous to ferric state in hemeproteins under aerobic conditions and the subsequent

HbA was removed using a Superoxer 12 10/300 GL column (GE Healthcare). Samples were oxidized to the ferric state by addition of a 10-fold molar excess \( K_{\text{Fe(CN)}}_6^- \) and incubation for \( \sim 3 \) min on ice. Each sample was then passed through a 10-DG (Bio-Rad) desalting column to remove excess \( K_{\text{Fe(CN)}}_6^- \), after which concentrations were measured. Where indicated, 0.1 M sodium fluoride or a 1.5-fold molar excess of \( \text{H}_2\text{O}_2 \) were added to each sample. Concentrations in heme equivalents were: 1) \( \alpha \)-subunits in the presence of sodium fluoride = 247 μM; 2) \( \alpha \)-subunits in the presence of \( \text{H}_2\text{O}_2 = 523 \mu \text{M} \); 3) AHSP-\( \alpha \)-subunit complexes in the presence of sodium fluoride = 247 μM; 4) AHSP-\( \alpha \)-subunit complexes in the presence of \( \text{H}_2\text{O}_2 = 385 \mu \text{M} \); 5) \( \beta \)-subunits in the presence of sodium fluoride = 207 μM; 6) \( \beta \)-subunits in the presence of \( \text{H}_2\text{O}_2 = 403 \mu \text{M} \); 7) HbA = 1.2 mM for all spectra; 8) Hp-Hb complexes = 0.6 mM for all spectra. In the samples treated with \( \text{H}_2\text{O}_2 \) incubation occurred for 10 s prior to manual freezing in a dry ice/ethanol bath. Spectra were normalized to heme concentration in Figs. 6 and 7, and in the radical yield calculations given in Table 2.

Reagents and Instrumentation—Unless otherwise specified, all reagents and chemicals were obtained from Thermo Fisher Scientific (Waltham, MA) or Sigma. Chromatographic media, columns, and chromatography equipment were obtained from GE Healthcare Bio-Sciences Corporation (Piscataway, NJ) and Whatman International Ltd. (Maidstone, Kent, United Kingdom). UV-visible absorbance spectroscopy measurements were made using an Agilent 8453 diode-array spectrophotometer (Agilent Technologies, Inc., Santa Clara, CA) or a Cary 100 Spectrophotometer (Varian, Inc., Palo Alto, CA). In both cases, 1-cm path length cells were used. Stopped-flow measurements were made using an Applied Photophysics SX-18 microvolume stopped-flow spectrophotometer (Leatherhead, Surry, UK). The path length was 10 mm and the entrance and exit slit widths were set to 1 mm each to give 4.8-nm spectral band widths. The volume of the cell was 20 μl. Shot volumes were between 100 and 200 μl, and mixing was performed using equal volumes of reactant solutions.

Data Analysis—Microsoft Excel was used for nonlinear least square data fitting to a single exponent expression to obtain the observed rate constants (Microsoft Corp., Redmond, WA) (32). Fitting routines in Origin were also used to verify the values obtained from Excel (OriginLab Corp., Northampton, MA). Spectroelectrochemical and sulfhemoglobin measurements were repeated three times to obtain standard deviations, and autoxidation reactions were repeated four times. Structure images were created using the PyMOL Molecular Graphics System (Schrödinger, LLC, New York).
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In agreement with previous studies (16, 35), 10 μM AHSP-α-subunit complexes autoxidize with an apparent rate constant of $\sim 2.3 \pm 0.2 \text{ h}^{-1}$ (Fig. 1A). The addition of catalase reduced the apparent rate constant to $1.2 \pm 0.2 \text{ h}^{-1}$ under the same experimental conditions. Superoxide dismutase did not appreciably affect the apparent rate constant of autoxidation, which was measured to be $2.4 \pm 0.3 \text{ h}^{-1}$ in its presence. For comparison, Fig. 1B depicts the initial phases of the autoxidation plots for isolated α- and β-subunits, which show at least 10-fold slower apparent rate constants ($\leq 0.3 \text{ h}^{-1}$). AHSP binding to α-subunits induces conformational changes that facilitate superoxide dissociation and hemichrome formation. Because catalase converts $\text{H}_2\text{O}_2$ into $\text{H}_2\text{O}$ and $\text{O}_2$, these data suggest that the slower rate of autoxidation in the presence of catalase is due to the diminished availability of $\text{H}_2\text{O}_2$, which is capable of reacting with ferrous and ferric HbA to facilitate more rapid oxidation of the protein. Caughey and co-workers (26) observed a similar decrease in the rate of autoxidation of $\text{HbAO}_2$, due to addition of catalase. The absence of a significant effect of superoxide dismutase agrees with most previous work (25, 26). In this case, SOD catalyzes the rapid dismutation of $\text{O}_2^-$ into $\text{H}_2\text{O}_2$ and $\text{O}_2$, indicating that $\text{H}_2\text{O}_2$ and not $\text{O}_2^-$ accelerates autoxidation. Even in the case of catalase, the effect is very small compared with the dramatic effect of AHSP binding on the autoxidation of $\alpha\text{O}_2$ (Fig. 1B).

The absence of an effect in the case of superoxide dismutase, which facilitates the rapid dismutation of $\text{O}_2^-$ into $\text{H}_2\text{O}_2$ and $\text{O}_2$, agrees with most previous work (25), although at least one conflicting report exists (26). However, both effects are very small compared with the effect of AHSP binding to $\alpha\text{O}_2$.

We also examined $\text{H}_2\text{O}_2$ and $\text{O}_2^-$ production from AHSP-α-subunits during autoxidation. We recorded spectral changes during the co-oxidation of epinephrine to adrenochrome in the same buffer and at the same temperature. We recorded changes in absorbance at 475 nm in the presence and absence of catalase and superoxide dismutase (25). This process has previously been used to assay for $\text{H}_2\text{O}_2$ and $\text{O}_2^-$ production during HbA autoxidation (25). Representative data from these studies are shown in Fig. 1C. Both enzymes showed additive effects on the diminution of the observed rate of co-oxidation of epinephrine. Similar effects of catalase and superoxide dismutase are observed for the autoxidation of native HbA (25).

**Ferryl Heme Species**—In addition to the oxidation of ferrous heme to the ferric heme state, iron atoms within HbA can undergo further oxidation to the ferryl heme state in the presence of reactive oxygen species (6, 7). This process has been associated with radical formation both in vitro and in vivo (36, 37), and significant work on HbA shows that ferryl heme formation occurs following oxidation of both ferrous and ferric protein by $\text{H}_2\text{O}_2$ (6, 7, 36). Recently, unique tyrosine-mediated inter-subunit electron transfer pathways have been proposed for intact HbA (20). Because autoxidation of oxy-Hb produces $\text{O}_2^-$ which spontaneously dismutates to form $\text{H}_2\text{O}_2$, we sought to characterize the behavior of both subunits in isolation with respect to $\text{H}_2\text{O}_2$ reactivity.

HbA oxidation to the ferryl heme state can be induced in vitro by subjecting the protein to moderate excesses of $\text{H}_2\text{O}_2$ (36). Ferryl heme formation causes the emergence of two broad
optical absorbance peaks with maxima at ~545 and 585 nm (36). We first mixed ferrous oxygenated HbA, α, β, and AHSP-α-subunit complexes with H₂O₂ using conditions similar to those reported by Tomoda et al. (38). These reactions were spectrally monitored for up to 2 min in a benchtop optical absorbance spectrophotometer. In agreement with the previous report (38), we found that isolated ferrous αO₂- and βO₂- subunits form hemichromes and precipitate on this time scale. In contrast, we observed that aquo- or hydroxy-(met) α- and β-subunits form relatively stable, transient ferryl heme complexes, which are similar to those observed for tetrameric met-HbA.

As shown in Fig. 2A, mixing 30 μM ferric β-subunits with 3 mM H₂O₂ (post-mixing concentrations) results in the emergence of broad absorbance bands at ~547 and 585 nm, which are indicative of formation of a Fe(IV) = O complex. The reaction of H₂O₂ with ferric α-subunits under equivalent conditions results in similar spectral transitions, with the emergence of ferryl-hemichrome-like spectra with absorbance peaks at ~543 and ~582 nm (Fig. 2B). The red lines in Fig. 2 represent spectra obtained for the reaction of intact ferric HbA with H₂O₂ using identical experimental conditions.

Fig. 2C shows spectra for the reaction of AHSP-(met)-α-subunit complexes with H₂O₂ using identical experimental conditions. We observed no absorbance changes between 3 ms and 20 s post-mixing. These data suggest that AHSP completely inhibits ferryl heme formation on these time scales (Fig. 2C), even in the presence of large excesses of H₂O₂.

On longer time scales between 20 s and 2 min, isolated ferric subunits precipitated following exposure to H₂O₂, as evidenced by marked increases in solution turbidity (not shown). In agreement with other reports (13, 15), however, we find that AHSP binding to α-subunits inhibits this H₂O₂-induced precipitation.

We detect no spectral evidence for ferryl heme intermediates under any conditions examined.

Because the absorbance spectra of hemichrome and ferryl heme species are similar (compare red spectra in Fig. 2, A and B, with the black spectrum in C), we used sodium sulfide (Na₂S) to further characterize the extent of ferryl heme formation in isolated met-subunits and AHSP-(met)-α-subunit complexes. This reagent has previously been shown to react with ferryl heme groups in HbA and myoglobin to produce quantifiable derivatives that give an absorbance peak at ~620 nm (27). The iron in the ferryl heme is reduced to the ferrous state and the sulfur is incorporated onto the porphyrin (27). This reaction depends both on the kinetics of ferryl heme formation and its rate of reaction with Na₂S, and has widely been used to derivatize ferryl Hb in vitro and in biological samples (27, 39).

We added 90 μM H₂O₂ to 60 μM ferric α-subunits, β-subunits, HbA, and AHSP-α-subunit complexes, incubated the samples at room temperature for 60–120 s (see the legend of Fig. 3 for the precise conditions), and recorded spectra before and after this incubation. After recording spectra at the completion of each incubation, 2 mM Na₂S was rapidly added to each sample, and again spectra were recorded. Representative spectra of the absorbance peaks at ~620 nm are shown in Fig. 3, A, B, and D. These data indicate that H₂O₂-treated met-HbA and met-β form sulfheme species quite readily (Fig. 3). However, α-subunit sulfheme formation is more limited, due in part to a slower rate of ferryl heme formation and a greater rate of precipitation (Fig. 3D). In contrast, the AHSP-(met)-α-subunit complexes do not form any detectable sulfheme species, verifying the spectral measurements in Fig. 2 (see Fig. 3C). Using a previously published extinction coefficient (27), the calculated
sulphene concentrations in our HbA, α, β, and AHSP-α samples were 26.6 ± 0.4, 9.5 ± 0.4, 29.4 ± 0.7, and 0 μM, respectively, of a total of 60 μM heme in each sample.

To further investigate our findings, we altered the evolutionarily conserved AHSP proline 30 in recombinant AHSP to generate AHSP(P30A) and AHSP(P30W) mutant proteins (17, 40). Although these mutations do not detectably perturb erythrocyte AHSP(P30A) and AHSP(P30W) mutant proteins (17, 44). This result predicts that AHSP binding should mark-edly decrease the reduction potential of isolated α-subunits, provided the binding equilibrium is significantly dependent on the oxidation state of heme.

To verify this prediction, we measured the reduction potential of α-subunits in the presence and absence of AHSP under anaerobic conditions using a spectroelectrochemical method. As shown in Fig. 5, the Nernst plots for isolated α-subunits and AHSP-α-subunit complexes are linear and show evidence of a simple, noncooperative, single electron reduction process. Based on these data, we calculate an $E_\text{C}^0$ of +40 mV (versus normal hydrogen electrode) for free α-subunits, which agrees with the reports of both Abraham and Taylor (42) and Banerjee and Cassoly (43). Binding to AHSP lowers the midpoint potential to −78 mV. These values are listed in Table 1, along with the reduction potentials we determined for HbA and Hp-Hb complexes. Like AHSP, Hp binding also lowers the reduction potential of HbA, but to a lesser extent, from +120 to +54 mV (24).

Interestingly, the redox process involving AHSP-α-subunit complexes was completely reversible as observed by reversing the polarity of the working electrode at the end of each spectroelectrochemical experiment. This result indicates that reduction does not dissociate the AHSP-α complex at the micromolar protein concentrations used in the measurements, as predicted from previous measurements of the $K_d$ for binding of the ferric (0.0006 μM) and ferrous (0.017 μM) forms of α to AHSP (17).

$E_\text{C}^0$ values measured for isolated β-subunits were variable and differed substantially from those reported by other groups (42, 43). Although the reason for this is unclear, we found that irreversible β-subunit denaturation during our experiments prevented reliable measurement. Also, β-subunits readily self-associate into homotetramers (45). Neither our experiments nor those of Abraham and Taylor (42) or Banerjee and Cassoly (43) adequately controlled for this phenomenon.

Radical Formation—We also investigated the effects of AHSP on protein radical formation using low temperature EPR spectroscopy. We prepared ferric α-subunits, β-subunits, HbA,
AHSP-α-subunits, and Hb-Hp complexes using the methods described under “Experimental Procedures,” and recorded spectra before and following the addition of either 0.1 M sodium fluoride or a 1.5-fold molar excesses of H₂O₂.

Fig. 6A contains spectra recorded in the presence or absence of sodium fluoride, which normally converts aquo-met or hydroxy-met samples into completely high spin forms. All three ferric resting samples showed EPR spectra indicative of a mixture of high and low spin ferric iron. The g values of the low spin α-subunits (2.792, 2.248, and 1.687) and AHSP-(met)-α-subunit complexes (2.932, 2.247, and 1.737) indicate that both species have a histidine imidazole distal ligand according to modified Truth-Diagram analysis (46), and the g values for the low spin β (2.752, 2.253, and 1.830) indicate a hydroxide ligand (Fig. 6A). The amount of low spin ferric iron in all three samples is substantial and should not be underestimated by directly comparing the apparent amplitudes of the high and low spin heme signals. The extent of low spin character is revealed by comparing the apparent amplitudes of the high and low spin is substantial and should not be underestimated by directly comparing the apparent amplitudes of the high and low spin heme signals. The extent of low spin character is revealed by comparing the apparent amplitudes of the high and low spin heme signals. The extent of low spin character is revealed by comparing the apparent amplitudes of the high and low spin heme signals. The extent of low spin character is revealed by comparing the apparent amplitudes of the high and low spin heme signals.

Thus, the high spin heme signals of HbA, AHSP, and Hb-Hp complexes using the methods described under “Experimental Procedures,” and recorded spectra before and following the addition of either 0.1 M sodium fluoride or a 1.5-fold molar excesses of H₂O₂. This contrast to that of met-HbA, which is as high as 76.4%. This strong stabilization of bis-His coordination. We estimated the amount of high spin heme detected at g = 2.0 under these conditions. The H₂O₂-reacted met-α sample shows less signal, and the AHSP-(met)-α sample shows almost no detectable free radical peak g = 2.0.

EPR spectra of the radical species recorded in the 100 gauss range are presented in Fig. 7. The radical generated in the α-subunit is centered at g = 2.0338 with a symmetric line shape and an overall width of 20 gauss. The radical signal found for β-subunits, although with a similar center at g = 2.0039, is asymmetric with an additional low field component at 2.033 and clear hyperfine structures in the major signal, with an overall width of 24.5 gauss. The half-saturation power is also different, 2.4 versus 10.4 milliwatts for the α- and β-subunits, respectively, indicating the location and structure of the protein radical found in the α- and β-subunits are likely different.
challenged with excess H$_2$O$_2$

ical modifications to free Hb have been observed if proteins are

ety of oxidative reactions that affect nearby molecules due to

tion to the oxoferryl species (Eqs. 1 and 2) (6). Both the ferryl

H$_2$O$_2$ reacts with ferric Hb, a radical species is formed in addi-

process that results in the formation of ferryl heme iron (6). If

in vitro

in the tetrameric form to +54 mV in the Hp-bound

alpha-beta-dimeric form (Table 1) (24). Thus, both Hb-binding pro-

teins facilitate oxidation, AHSP by inducing hemichrome

metabolism and Hp by promoting dissociation into more easily

oxidized dimers. These two proteins also deal with peroxide-

induced radical chemistry in markedly different ways (see

Inhibition of H$_2$O$_2$ Reactions by AHSP—Early structural work on AHSP-alpha-subunit complexes led to the proposal that the bis-

histidyl conformation strongly inhibits ferric-ferryl redox

cycling following exposure to H$_2$O$_2$ (15, 52). It was estimated

that at least 33% of the heme in ferrous alpha-O$_2$:AHSP complexes

converts to the ferryl heme form following H$_2$O$_2$ exposure,

whereas less than 10% of the heme in ferric met-alpha-AHSP com-

plexes converts to the ferryl heme form following the same

exposure (15). A more recent report indicates that H$_2$O$_2$-in-

cropped covalent modification of alpha-subunits can be prevented

by AHSP binding, and that two exposed alpha-subunit Tyr residues

(Tyr-24 and Tyr-42) are unable to take part in electron transfer

and ferryl heme protein radical formation (Fig. 8) (18).

In this work, we focused on ferric-ferryl redox transitions

within the AHSP-alpha complex. We confirmed that AHSP-alpha

autoxidizes ~10-fold more rapidly than isolated alpha-subunits

(13, 16), and found that this process both produces and con-

sumes H$_2$O$_2$. We also found that in the presence of molar

excesses of H$_2$O$_2$, the ferric forms of Hb alpha, and beta can react to

form ferryl heme intermediates, which can be detected using

N$_2$.S. By contrast, AHSP-(met)-alpha-subunit complexes show no

evidence of H$_2$O$_2$-induced ferryl heme intermediates or species

(Figs. 2 and 3). This lack of peroxidase activity is likely a conse-

quence of low accessibility of H$_2$O$_2$ to the heme iron once

AHSP is bound and induces bis-His coordination (Fig. 8C).

Internal hexacoordination also explains the lack of conversion

from low spin to high spin Fe$^{3+}$ by addition of excess fluoride to

the met-alpha-AHSP complex (Fig. 6A). These findings are con-

firmed in our experiments using buffers with pH values varying

Preferred Binding of AHSP to Met-alpha—Current evidence suggests that AHSP protects free alpha-subunits from oxidative

degradation by preferentially binding to the ferric form and

rapidly inducing structural changes that generate a stable hexa-

coordinated species (12, 13, 15, 17). Our spectrophotometric

experiments confirm that AHSP binds preferentially to the

alpha-subunits. The midpoint reduction potential of free

alpha-subunits decreases from +40 to ~78 mV when the subunit is

bound to AHSP (Fig. 5B, Table 1). These values can be used to

independently estimate the ratio of the equilibrium dissociation

constants for AHSP binding to reduced deoxy-alpha and met-

alpha-subunits (i.e. K$_{d,red}$/K$_{d,ox}$): A complete derivation of the

effect of AHSP binding on the reduction potential of alpha-subunits

is given in the Supplemental Scheme 1 in Supplemental Deri-

vation. Using this method and the parameters in Table 1, the

computed ratio is ~130, which is almost identical to the ratio

computed from the bimolecular association and unimolecular
dissociation rate constants (i.e. 100) reported by Mollan et al.

(17).

Interestingly, under comparable experimental conditions, the

midpoint potential of Hb alpha was found to decrease from

+120 mV in the tetrameric form to +54 mV in the Hp-bound

alpha-beta-dimeric form (Table 1) (24). Thus, both Hb-binding pro-

teins facilitate oxidation, AHSP by inducing hemichrome

formation and Hp by promoting dissociation into more easily

oxidized dimers. These two proteins also deal with peroxide-

induced radical chemistry in markedly different ways (see

below).

DISCUSSION

The reaction between H$_2$O$_2$ and ferrous Hb is a two-electron

process that results in the formation of ferryl heme iron (6). If

H$_2$O$_2$ reacts with ferric Hb, a radical species is formed in addition

to the oxoferryl species (Eqs. 1 and 2) (6). Both the ferryl

heme and its associated protein radical can induce a wide vari-

ty of oxidative reactions that affect nearby molecules due to

their high midpoint redox potentials, ~1 V (48). Specific chemical

modifications to free Hb have been observed if proteins are

challenged with excess H$_2$O$_2$ in vitro. First, the heme vinyl

groups may become modified and covalently linked to the pro-

tein (48). Second, extensive globin chain cross-links and irre-

versible modifications of several amino acids, primarily in

beta-subunits, have been observed (49). Studies have also revealed

the presence of oxidative changes to the surrounding tissues

following exposure to extracellular Hb, both in animals infused

with these proteins and when Hb is released from human red

cells leading to kidney or brain injury (8, 50, 51). It is not sur-

prising that several pathways exist in mammals to control these

events. Two key globin-binding proteins that appear to inhibit

oxidative damage are: (a) AHSP, which provides protection

against oxidative damage to alpha-subunits and surrounding pro-

teins during erythropoiesis (12–14) and (b) Hp and the CD163

receptor on macrophages, which coordinate Hb dimer clear-

ance during hemolysis (50). Our results indicate that AHSP and

Hp exert these functions through distinct mechanisms.

![FIGURE 7. Close-up EPR spectra of the radical species before and after H$_2$O$_2$ treatment. Individual spectra are labeled accordingly. EPR conditions were: microwave power, 1 milliwatt; microwave frequency, 9.3 GHz; range 140 G; modulation amplitude, 2 G; temperature, 20 K. Spectra were measured using the same samples as were used to generate the data in Fig. 6.](image-url)
**Redox Chemistry of AHSP**

**FIGURE 8.** Model structure of ferrous \( \alpha \)-subunit, \( \beta \)-subunit, and ferric AHSP-\( \alpha \)-subunit complex and oxidation reactions at the heme vicinity. A, \( \alpha \)-subunit. B, \( \beta \)-subunit. C, AHSP-\( \alpha \)-subunit complex. D, oxidation scheme. AHSP, \( \alpha \)-subunit, and \( \beta \)-subunit structures are shown as ribbons in cyan, silver, and gold, respectively. Heme or heme groups are shown using pink stick structures, with \( \alpha \)-Tyr-42 and \( \beta \)-Phe-41 sticks shown in green and blue, respectively. Distal and proximal histidines are also shown using stick structures. Corey-Pauling-Koltun coloring is otherwise used throughout. Structure images were created using the PyMOL Molecular Graphics System and PDB files 1Z8U and 1H0Q (15, 58).

from 5.0 to 8.0 (Fig. 4). Fig. 8D outlines a scheme of AHSP function based on these findings.

**Differences between Met-\( \alpha \), Met-\( \beta \), and AHSP-(met)-\( \alpha \)-Subunits**—Although our data show that isolated \( \alpha \)-subunits behave markedly differently than isolated \( \beta \)-subunits with respect to ferryl heme and protein radical formation, it is clear that more work is needed to determine the underlying mechanistic differences, including more directed measurements of the rates of formation and decay of other intermediates and site-directed mutagenesis studies designed to determine the structural origin of the radical signals in Fig. 7.

For example, the smaller radical and ferryl heme signals for \( \alpha \)-subunits could be due to stronger water coordination to Fe(III), which inhibits the initial reaction with \( \text{H}_2\text{O}_2 \) (53). There is evidence that the distal histidine in \( \alpha \)-subunits stabilizes bound ligands and distal pocket water to a greater extent than in \( \beta \)-subunits because of a more rigid conformation and closer proximity of the imidazole side chain to the coordinated O atom (Fig. 8, A and B) (54, 55).

In addition, Reeder et al. (20) have shown that ferryl-\( \alpha \)-subunits both autoreduce or react with added reducing agents ~10 times more rapidly than ferryl-\( \beta \)-subunits, and that these differences are due in part to facilitation of electron transfer by the solvent-exposed Tyr-42 side chain at the CD corner of the \( \alpha \)-but not \( \beta \)-subunits (Fig. 8). However, the extent of \( \alpha \)-Tyr-42 participation in the peroxidase reaction is not fully defined and studies with isolated mutant subunits remain to be done.

By contrast, the mechanistic cause of the lack of reactivity of the AHSP-(met)-\( \alpha \) complex with \( \text{H}_2\text{O}_2 \) and formation of ferryl heme species is much clearer. A dramatic conformational change in the CD corner of \( \alpha \)-globin occurs on binding to AHSP and autoxidation, which results in hemichrome formation with both His-58(E7) and His-87(F8) coordinated to the Fe(III) atom (Fig. 8C). This hexacoordination appears to completely inhibit the reaction of AHSP-(met)-\( \alpha \) with \( \text{H}_2\text{O}_2 \) to form the initial intermediates observed in free isolated subunits.

**Summary and Physiological Relevance**—Our study provides three biochemical findings of relevance to erythroid physiology. First, met-\( \beta \)-subunits are more prone to the \( \text{H}_2\text{O}_2 \)-induced formation of ferryl heme species and protein-based radicals than met-\( \alpha \)-subunits. This may explain why \( \alpha \)- and \( \beta \)-thalassemic erythroid cells exhibit distinct membrane abnormalities and oxidative modifications (56, 57). It also suggests that Hp-based \( \text{O}_2 \) therapeutics should be designed with more attention directed at mitigating oxidative reactions of \( \beta \)-subunits. Second, AHSP binding renders met-\( \alpha \)-Hp nearly inert to oxidative degradation by \( \text{H}_2\text{O}_2 \), with no ferryl heme species or protein-based radicals detected by either optical absorbance or EPR. Third, AHSP binding dramatically lowers the redox potential of \( \alpha \) to a much more negative value, and thermodynamically favors the ferric over ferrous iron.

Although AHSP and Hp both protect against Hb-mediated oxidative damage, the biochemical mechanisms are distinct. AHSP stabilizes nascent \( \alpha \)-subunits by inducing the formation of a stable hemichrome that inhibits interactions with \( \text{H}_2\text{O}_2 \) and other ligands. By contrast, Hp-bound \( \alpha \beta \) dimers retain their pseudoperoxidase activity, although the resulting ferryl heme species appear to be less reactive due in part to the stabilization of a protein-based radical on \( \beta \)-Tyr-145 (47).

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