α-Hemoglobin-stabilizing Protein (AHSP) Perturbs the Proximal Heme Pocket of Oxy-α-hemoglobin and Weakens the Iron-Oxygen Bond*§

Claire F. Dickson†, Anne M. Rich§, William M. H. D’Avigdor¶, Daniel A. T. Collins®, Jason A. Lowry‡, Todd L. Mollan¶, Eugene Khandros**, John S. Olson‖, Mitchell J. Weiss**, Joel P. Mackay¶, Peter A. Lay†, and David A. Gell††

* From the Menzies Research Institute Tasmania, University of Tasmania, Hobart, TAS 7000, Australia; § School of Molecular Bioscience, University of Sydney, NSW 2006, Australia; ¶ School of Chemistry, University of Sydney, NSW 2006, Australia; ** Cell and Molecular Biology Group, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Background: α-Hemoglobin stabilizing protein (AHSP) is a hemoglobin chaperone.

Results: AHSP causes a subtle perturbation of the proximal heme pocket of O₂-α-hemoglobin, lengthening the Fe-O₂ bond and enhancing O₂ dissociation.

Conclusion: Pro-30 in wild-type AHSP promotes αHb autooxidation by introducing strain into the proximal heme pocket.

Significance: αHb-AHSP complexes are intermediates in Hb assembly and achieve αHb detoxification.

α-Hemoglobin (αHb)-stabilizing protein (AHSP) is a molecular chaperone that assists hemoglobin assembly. AHSP induces changes in αHb heme coordination, but how these changes are facilitated by interactions at the αHb-AHSP interface is not well understood. To address this question we have used NMR, x-ray absorption spectroscopy, and ligand binding measurements to probe αHb conformational changes induced by AHSP binding. NMR chemical shift analyses of free CO-αHb and CO-αHb-AHSP indicated that the seven helical elements of the native αHb structure are retained and that the heme Fe(II) remains coordinated to the proximal His-87 side chain. However, chemical shift differences revealed alterations of the F8, G8, and H helices and the heme pocket of CO-αHb bound to AHSP. Comparisons of iron-ligand geometry using extended x-ray absorption fine structure spectroscopy showed that AHSP binding induces a small 0.03 Å lengthening of the Fe-O₂ bond, explaining previous reports that AHSP decreases αHb O₂ affinity roughly 4-fold and promotes autooxidation due primarily to a 3–4-fold increase in the rate of O₂ dissociation. Pro-30 mutations diminished NMR chemical shift changes in the proximal heme pocket, restored normal O₂ dissociation rate and equilibrium constants, and reduced O₂-αHb autooxidation rates. Thus, the contacts mediated by Pro-30 in wild-type AHSP promote αHb autooxidation by introducing strain into the proximal heme pocket. As a chaperone, AHSP facilitates rapid assembly of αHb into Hb when βHb is abundant but diverts αHb to a redox resistant holding state when βHb is limiting.

Adult human hemoglobin (Hb) is a tetramer of two αHb and two βHb subunits. The αHb and βHb chains share a common fold, and each binds a single, iron-containing protoporphyrin IX (heme) molecule in a deep pocket, protected from solvent. The central iron atom in each heme is the site of physiological dioxygen (O₂) binding. α-Hemoglobin stabilizing protein (AHSP) is a molecular chaperone that binds free αHb in a heterodimeric complex (see Fig. 1A) (1–3) and is essential for normal erythropoiesis (4–12).

One role of AHSP is to protect erythroid cells from oxidative damage arising from the action of the heme iron in free αHb (6). In aqueous solutions containing air-equilibrated buffer, the Fe(II) atoms of heme groups spontaneously oxidize to the ferric (Fe(III)) state, which results in the production of superoxide anions. This process is termed autooxidation. Autooxidation is inhibited by the physicochemical properties of the heme pocket that allow reversible oxygen binding. However, spontaneous oxidation is not blocked completely, and the superoxide anions generated undergo rapid enzymatic and non-enzymatic disproportionation to H₂O₂ and O₂. H₂O₂ is a key mediator of oxidative stress that can react with either reduced or oxidized globins to produce highly reactive ferryl or oxo-ferryl (Fe(IV)) heme groups (13, 14), and subsequent reactions lead to destruction of Hb as well as production of potent pro-oxidants that damage membranes and other cellular components (15–17). Additionally, autooxidation to the ferric state renders Hb incapable of O₂
transport and greatly enhances the rates of hemin loss and globin denaturation.

Compared with mature Hb, isolated αHb and βHb chains produce higher levels of reactive oxygen species (18–20), presumably linked to a loss of structural integrity of the heme pocket. Oxidative damage occurs in erythroid cells of β-thalassemic mice that contain elevated levels of unpaired αHb (21–24). Kong et al. (6) showed similar increases in reactive oxygen species in AHSP⁻/⁻ mice, suggesting that AHSP has a role in limiting reactive oxygen species production during normal erythropoiesis. In a purified system AHSP inhibits the reaction of Fe(III) αHb with H₂O₂ (2, 20), and recently Mollan et al. (20) demonstrated that AHSP binding prevents the production of Fe(IV)═O heme and associated protein radicals when Fe(III) αHb is exposed to H₂O₂.

The low reactivity of Fe(III) αHb in the presence of AHSP correlates with a change in αHb heme pocket structure. In native Hb, the αHb heme group is coordinated through a single His-87 side chain, termed the proximal histidine (see Fig. 1B), allowing exogenous ligands in the distal heme pocket to interact with iron. In the ferric αHb-AHSP complex, Fe(III) becomes coordinated by both the proximal and distal histidine side chains (bis-histidyl heme coordination; see Fig. 1C) (1, 2, 11, 25–27). Coordination of low-spin Fe(III) with six strong ligands blocks the binding of exogenous ligands and associated electron transfer reactions involving oxygen species. The switch from native to bis-histidyl heme coordination is accompanied by a change to a non-native αHb tertiary structure (r.m.s deviation of 3.3 Å over 135 Cα atoms), which dissociates from AHSP ~100-fold slower than Fe(II) αHb (20, 25, 27).

As well as inducing changes in the structure of Fe(III) αHb, AHSP binding causes a >10-fold increase in the autooxidation rate of Fe(II) αHb (11, 20, 25, 28, 29), also resulting in formation of bis-histidyl Fe(III) αHb. To understand how promoting αHb autooxidation is compatible with reduced reactive oxygen species production in erythroid cell, it is important to appreciate that repeated movement between different iron oxidation states can occur for a single heme group, and this redox cycling increases the probability of irreversible changes in the globin and other cellular components. In contrast, autooxidation of αHb to the bis-histidyl αHb structure dramatically lowers the Fe(III)/Fe(II) redox potential (20) and inhibits interactions with exogenous ligands, thus inhibiting redox cycling. However, the structural mechanism by which AHSP accelerates αHb autooxidation and, more generally, how AHSP promotes the protective bis-histidyl structure is not well understood.

Previous work from our group has suggested that AHSP induces strain into the O₂-αHb structure that is relieved upon autooxidation by reorganization of the αHb protein fold (25). Mutation of Pro-30 to Ala (AHSPP30A) in loop 1 of AHSP (Fig. 1A) leads to an ~4-fold reduction in αHb-AHSP autooxidation rates, suggesting that Pro-30 contributes to the strained conformation. However, contacts at the αHb-AHSP interface are transmitted to the αHb heme pocket and the nature of the strained O₂-αHb structure are unknown. Difficulties in characterizing the O₂-αHb-AHSP complex arise from its propensity to spontaneously convert to a bis-histidyl structure. A previous x-ray crystallographic study suggested that the strained O₂-αHb structure involved large-scale changes in the globin polypeptide structure and heme coordination geometry (1).

To explore these issues, we have used NMR and EXAFS to probe the complexes of CO- and O₂-ligated αHb with AHSP in solution. The results show that the tertiary structure of CO/O₂-αHb in these complexes closely resembles that of CO/O₂-αHb in the native HbA tetramer and, therefore, that our previous model of O₂-αHb-AHSP (1) is unlikely to represent the initial AHSP binding event in solution. We show that mutation of Pro-30 in AHSP leads to NMR chemical shift perturbations in the F-helical region of CO-αHb, and the concomitant changes in O₂ and CO binding reactivity help identify how this strain introduced into the proximal portion of the heme pocket promotes autooxidation of O₂-αHb.

**EXPERIMENTAL PROCEDURES**

**Protein Production**—The structured region, residues 1–90 of human AHSP, and mutants thereof were expressed and purified as described previously (25). CO-αHb was purified from human blood (3). O₂-αHb was generated by gently blowing oxygen across the surface of CO-αHb under a concentrated 10-watt halogen light source. The procedure was carried out on ice, and the formation of the O₂-αHb was monitored by UV-visible spectroscopy (Shimadzu UV-1601). Isotopically labeled αHb for NMR studies was co-expressed with βHb and methionine aminopeptidase in *Escherichia coli* from the previously described plasmid pHE7 (30). Expression was carried out in the *E. coli* strain JM109. One liter of overnight culture in DM-4 minimal media as described by Looker et al. (31) containing D-glucose (5 g liter⁻¹), NH₄Cl (0.7 g liter⁻¹), thiamine (30 mg liter⁻¹), ampicillin (100 mg liter⁻¹), and trace metal solution (32) was inoculated into a final volume of 3.5 liters of DM-4 medium containing thiamine (30 mg liter⁻¹), ampicillin (100 mg liter⁻¹), trace metal solution, yeast extract (50 μl of 10% w/v solution), and antifoam (Sigma) in a 5-liter fermentor vessel (New Brunswick BioFlo). Growth was maintained at 37 °C, pH...
AHSP Perturbs the Proximal Heme Pocket of O₂-α-Hemoglobin

6.8, with aeration and monitored in real time based on dissolved O₂ concentration. The culture exhausted the nitrogen supply at an absorbance of ~1 (600 nm) and was provided with 2 g of ¹⁵NH₄Cl. The culture exhausted the glucose supply at an absorbance of ~3, and 1.5 g of D-[U(99%)-¹³C]glucose was provided, and the temperature was shifted to 32 °C. The addition of further 2 g of ¹⁵NH₄Cl, 1.5 g of D-[U(99%)-¹³C]glucose occurred at an absorbance of ~3.5, and expression was induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside and 15 μM hemin. Cells were collected by centrifugation after 5 h, and the resulting cell pellets were washed once in 10 mM Tris-HCl, 0.5 mM EDTA, pH 8.0. Cell pellets were resuspended in the same buffer and saturated with CO before lysis by sonication (Ultrasonic Processor 500 W, Sonics & Materials Inc.). After clarification by centrifugation, recombinant Hb was purified as previously described (3). Sonicates (pMB) and purified as previously described (3).

Size Exclusion Chromatography (SEC) and In-line Light Scattering—SEC chromatography was carried out on a 24-ml Superose 12 column (GE Healthcare) in 0.1 M sodium phosphate, pH 7.0 (21 °C). Protein concentrations were determined based on UV-visible absorbance. The concentrations of AHSP and mutants thereof were obtained after unfolding in 6 M guanidine-HCl using molar extinction coefficients calculated based on the amino acid compositions (34). CO/O₂-αHb concentrations were determined based on absorption peaks arising from π → π* transitions in the porphyrin ring using molar extinction coefficients for the corresponding CO/O₂ forms of Hb (35). The weight-average molecular weight (Mₐ) of particles in solution was determined directly from measurements of light scattering and protein concentration (36). In-line light scattering measurements were made at 41.5°, 90.0°, and 138.5° with respect to the incident beam using a mini-DAWN with 690 nm laser (Wyatt Technology Corp., Santa Barbara CA), calibrated against toluene. Protein concentrations were determined based on the refractive index measured by an Optilab differential refractometer (Wyatt Technology Corp.) calibrated against NaCl standards. Molecular weight calculations were performed for each 50-μl fraction across the elution peaks using the ASTRA software (Wyatt Technology Corp.) with the assumption of a standard value for the specific refractive index increment with respect to sample concentration (dn/dc) of 0.19 ml g⁻¹. The presence of the heme and pMB groups upon the value that reported by Jameson et al. (43). The constraints and restraints used for the bond lengths and bond angles in the porphyrin ring and the imidazole ligand and for the Debye-Waller factors were as described previously (41). Constraints and restraints and multiple-scattering (MS) paths used in the fitting calculations are given in supplemental Tables.

EXAFS Data Analysis—The model-fitting calculations were performed by means of the program XFIT (42). The values of the parameters that were varied to optimize the agreement between the observed and calculated EXAFS were the coordinates x, y, and z of all atoms in the model in relation to an arbitrary set of Cartesian axes, the Debye-Waller factors, σ², a scale factor, S₀², and E₀. The value of S₀² was initially restrained to be 0.9 with an estimated standard deviation (e.s.d.) 0.1 for O₂-αHb and 0.92 (e.s.d. 0.02) for O₂-αHb:AHSP (i.e. to typical values for low-spin O₂-myoglobin and O₂-leghemoglobin). Both the observed and calculated EXAFS were Fourier-filtered (42). The k windows used for the EXAFS analyses are shown in the figures. The goodness-of-fit parameter R was calculated as described by Ellis and Freeman (42). The model used in the XFIT analyses is that described previously (41). The sixth coordination site was a dioxygen molecule where the terminal oxygen was arbitrarily constrained to lie in the plane perpendicular to the heme and passing through the meso-Cs. The O²⁻O² distance was restrained to be 1.22 Å (e.s.d. 0.01 Å), consistent with that reported by Jameson et al. (43). The constraints and restraints used for the bond lengths and bond angles in the porphyrin ring and the imidazole ligand and for the Debye-Waller factors were as described previously (41). Constraints and restraints and multiple-scattering (MS) paths used in the fitting calculations are given in supplemental Tables.

O₂/CO Binding Measurements—Rate constants for O₂ binding to and dissociation from ferrous αHb:AHSP complexes as well as rate constants for CO binding were measured using methods developed for relatively unstable Fe(II) O₂-Hb complex.
AHSP Perturbs the Proximal Heme Pocket of O$_2$–α-Hemoglobin

FIGURE 2. Conformational exchange in AHSP is dependent on the position of proline residues in loop 1. A, shown is the sequence of the first two $\alpha$-helices and intervening loop 1 of wild-type human AHSP and AHSP mutants used in this study. Conserved positions relative to the wild-type sequence are marked with dashes. B, shown is the structure of the bis-histidyl $\alpha$Hb-AHSP interface (white, PDB 3IA3; the Pro-30 side chain is shown in space-fill) with the AHSP$^{30A}$ structure overlaid (black, PDB 1Z8U). The positions of side chains mutated to proline in AHSP$^{30A}$ are shown in E–H. Portions of the assigned $^{15}$N-HSQC spectra of AHSP (800 $\mu$m) and AHSP$^{30A}$ (900 $\mu$m), E–H, portions of the $^{15}$N-HSQC spectra of AHSP$^{30A/N28P}$ (400 $\mu$m), AHSP$^{30A/D29P}$ (550 $\mu$m), AHSP$^{30A/L31P}$ (900 $\mu$m), and AHSP$^{30A/S33P}$ (330 $\mu$m) are shown. All $^{15}$N-HSQC spectra were recorded at 15 °C in 20 mM sodium phosphate buffer, pH 6.9–7.0.

RESULTS

A Proline Residue Is Precisely Positioned in Loop 1 of AHSP to Promote Autooxidation of $\alpha$Hb—The Asp-29–Pro-30 peptide bond in loop 1 of AHSP undergoes cis-trans isomerization that is coupled with small changes in the packing of the three $\alpha$-helices in AHSP (1, 46). Mutation of AHSP Pro-30 to a range of other residue types abolishes cis-trans peptidyl isomerization of AHSP and leads to a consistent 4-fold reduction in the rate of O$_2$–$\alpha$Hb autooxidation (11, 25). We previously proposed that cis-peptidyl Pro-30 makes physical contact with O$_2$–$\alpha$Hb that promotes autooxidation (25). However, it is possible that other changes in AHSP conformation related to cis-trans isomerization might explain the role of Pro-30 in $\alpha$Hb autooxidation. To investigate this possibility, we introduced proline mutations into AHSP$^{30A}$ at position 28 (AHSP$^{30A/N28P}$), 29 (AHSP$^{30A/D29P}$), 31 (AHSP$^{30A/L31P}$), or 33 (AHSP$^{30A/S33P}$) as shown in Fig. 2A. We argued that proline residues in these positions might still allow
AHSP Perturbs the Proximal Heme Pocket of O₂-α-Hemoglobin

AHSP to undergo conformational exchange, but the proline side chains would presumably not preserve contacts with αHb that are made by the native Pro–30. The locations of the introduced proline mutations at the αHb-AHSP interface are shown in Fig. 2B. We did not mutate Val–32 because this side chain packs into the hydrophobic core between the AHSP helices.

The propensity of the mutant AHSP proteins to undergo cis-trans isomerization was assessed from 15N-HSQC NMR spectra. A portion of the 15N-HSQC spectrum of wild-type AHSP is shown in Fig. 2C, with peaks arising from N-HN groups in the same residue in cis-prolyl and trans-prolyl conformers of AHSP labeled and joined by a dashed line. In the AHSP/D29A spectrum, a single N-HN peak was observed for each residue, consistent with a single AHSP conformer (Fig. 2D and Table 1) (1, 46). The spectrum of AHSP/D29A/N28P contained an additional set of weaker HSQC signals compared with AHSP/D29A (Fig. 2E, arrows, and Table 1) that was consistent with ~20% of molecules adopting an alternative conformation, most likely due to isomerization of the Phe-27–Pro-28 peptide bond. The 15N-HSQC spectra of AHSP/D29A/D29P, AHSP/D29A/L31P, and AHSP/D29A/S33P (Fig. 2, F–H) were all consistent with a single protein conformation, which indicated that prolines at positions 29, 31, and 33 do not result in measurable cis/trans isomerization. Chemical shift analyses were consistent with minor conformational changes in AHSP proximal to loop 1 and indicated that none of the mutations conferred gross structural changes (not shown). These results suggest that only cis-peptide bonds before Pro–30 or Pro–28 result in a significant amount of cis/trans isomerization.

The effect of AHSP proline mutations on αHb autooxidation is shown in Fig. 3. Autooxidation of O₂-αHb to bis-histidyl αHb was measured by changes in the visible absorption spectrum of αHb (Fig. 3, A and B). The absorbance time courses fit to single exponential expressions and yielded first-order rate constants for autooxidation (kₐut oxid; Fig. 3C and Table 1). AHSP/D29A showed a 4-fold reduction in the value of the rate constant compared with that of wild-type AHSP, as previously described (1, 25). All introduced-proline mutants had the same low activity as the AHSP/D29A (Fig. 3C and Table 1). A control mutation AHSP/D29A had the same activity as wild-type AHSP. The results indicate that the positioning of Pro–30 in loop 1 is critical for function. In addition, the observation that free AHSP/D29A/N28P showed cis/trans isomers in solution, yet conferred the same slowed rate of O₂-αHb autooxidation as AHSP/D29A, suggests that the propensity of free AHSP to undergo conformational changes does not in itself contribute to O₂-αHb-AHSP autooxidation activity. We have shown previously that stabilization of the cis-peptidyl Asp–29–Pro–30 AHSP conformation does correlate with increased autooxidation activity (25). Together the above data are consistent with the hypothesis that the effects of Pro–30 are mediated through direct contact between loop 1, containing the Pro–30 residue, and αHb.

Free Liganded αHb Monomer Adopts Native-like Secondary Structure—We next used NMR spectroscopy to probe for structural differences between αHb in the native Hb tetramer and free αHb in solution. The aim of these studies was to probe for structural changes in αHb that could explain the more rapid

---

**TABLE 1**

| Protein            | kₐut oxid (h⁻¹) | Residues | N-HN peaks |
|--------------------|-----------------|----------|------------|
| AHSP               | 0.59 ± 0.07     | 90       | 121        |
| AHSP/P30A          | 0.14 ± 0.01     | 90       | 80         |
| AHSP/D29A          | 0.53 ± 0.02     | 90       | 134        |
| AHSP/D29A/N28P     | 0.13 ± 0.02     | 90       | 113        |
| AHSP/P30A/D29P     | 0.14 ± 0.03     | 90       | 84         |
| AHSP/P30A/L31P     | 0.14 ± 0.05     | 90       | 85         |
| AHSP/P30A/S33P     | 0.14 ± 0.03     | 90       | 81         |

* Spin systems with characteristic chemical shifts for A3n, G3n, Arg, and Trp side chains were excluded.

* The AHSP construct used comprises residues 1–90 with the C-terminal unstructured residues removed (1). Approximately 50% of residues gave rise to two sets of signals in triple resonance NMR spectra, corresponding to the cis/trans AHSP conformers. Differences in signal overlap in HSQCs results in a total of 121 resolved N-HN peaks for WT AHSP and 134 resolved peaks for AHSP/D29A.

---

**FIGURE 3. The position of proline in AHSP loop 1 is important for function.**

A, conversion of O₂-αHb-AHSP to bis-histidyl αHb-AHSP was monitored by a decrease in intensity of the absorption band at 575.5 nm. B, time courses for conversion to bis-histidyl αHb in the presence of AHSP or selected AHSP mutants are shown, with fits to an exponential function. C, rate constants for O₂-αHb conversion reactions using AHSP and AHSP mutants shown in Fig. 1B are shown (average ± S.D. for 3–9 repeat assays). Mutation of Pro–30 to Ala (AHSP/D29A) reduces the rate constants by a factor of 4. The control mutation (AHSP/D29A) had no significant effect. Introducing Pro at position 28, 29, 31, and 33 failed to rescue any activity.
autoxidation observed in vitro and provide a suitable reference point for subsequent NMR studies of αHb-AHSP complexes. We found that sequential backbone assignments could not be obtained for residues 104–126 (corresponding to helices G and H), as also reported previously by Martineau and Craescu (47). The absence of NMR signals corresponding to these residues suggests that they might be in intermediate chemical exchange, perhaps as a result of αHb self-association.

In line with that possibility, we noted a marked decrease in SEC peak elution time with increasing αHb load concentration. This behavior is typical of protein self-association and is consistent with previous studies of αHb (48, 49). We reasoned that treatment of αHb with pMB, which covalently modifies Cys-104 of αHb and inhibits Hb chain homo (48) and hetero interactions (50), would allow more complete NMR data to be obtained. It has previously been shown that pMB treatment has no effect on the O2 and CO binding parameters of isolated αHb (51). The αHb self-association interface is clearly identified by the NMR data (Fig. 4C, red/orange) and is the same face that interacts with βHb (Fig. 4C, surface) to form the αHb–βHb heterodimer.

To obtain information about the secondary structure of monomeric CO-αHbPMB, we used TALOS+ (52) to derive φ and ψ backbone angle predictions from NMR chemical shift data. Seven helical secondary structure elements could be clearly identified corresponding to the A-C and E-H helices (Fig. 5). The angles derived by TALOS+ were very similar to angles determined from crystal structures of native CO-Hb (PDB 2DN3) (53), with correlation coefficients of 0.82 (φ) and 0.87 (ψ) over 127 residues. One difference was that TALOS+ consistently assigned the C helix region as α-helix rather than the 3_10-helix that is identified by the program DSSP (54) based on crystal structure coordinates (PDB 2DN3).

To obtain information about the heme pocket, we assigned His side chain resonances for CO-αHbPMB (Fig. 6 and Table 2). His-58 and His-87 side chain resonances displayed large upfield deviations from random coil chemical shifts consistent with positions above or below the plane of the heme ring. The chemical shifts were very similar to values previously obtained for CO-αHb at pH 5.3–5.7, 36 °C, (55, 56), and the αHb subunit of intact CO-Hb at pH 6.9, 29 °C (30, 57), strongly supporting the conclusion that free αHb adopts a heme pocket structure similar to that in Hb tetramers, with the heme iron bound through N^2 of His-87. In summary, preparation of monomeric αHb through treatment with pMB allowed us to obtain backbone and His side chain chemical shift data suitable for comparative NMR studies with αHb-AHSP complexes.

FIGURE 4. Identification of the αHb homodimerization interface. A, self-association of αHb was inhibited by treatment with pMB. SEC traces are shown for CO-αHb (red) or CO-αHbPMB after reaction with a 4-fold molar excess of pMB (black). Molecular weight from light scattering measurements is plotted across each peak (red/black symbols). B, portions of the 15N-HSQC for CO-αHb (red) and CO-αHbPMB (black) were recorded at 15 °C in 20 mM sodium phosphate, pH 6.9. Assignments are shown for new peaks in the CO-αHbPMB spectrum that are not present in the CO-αHb spectrum. C, shown are a ribbon (αHb) and surface (βHb) representation of the αHb/βHb dimer from CO-Hb (PDB 2DN3). Residues missing in the CO-αHb spectrum (red) or experiencing significant chemical shift difference between CO-αHb and CO-αHbPMB (orange) map to the tight βHb-interaction face, which is, therefore, also identified as the αHb homodimerization face.
AHSP Perturbs the Proximal Heme Pocket of O$_2^{-}\alpha$-Hemoglobin

CO-$\alpha$Hb Retains Near-native Secondary Structure and Heme Ligation in Complex with AHSP—To understand how AHSP promotes autooxidation of $\alpha$Hb, we examined chemical shift differences between free CO-$\alpha$Hb$^\text{PMB}$ and the CO-$\alpha$Hb-AHSP complex. Widespread changes in the $^{15}$N-HSQC of CO-$\alpha$Hb were observed upon binding of AHSP (Fig. 7A) despite the complex remaining in the CO-ligated form, as confirmed by UV-visible spectroscopy (not shown). A plot of backbone chemical shift differences between CO-$\alpha$Hb$^\text{PMB}$ and CO-$\alpha$Hb-AHSP reveals that the B-C corner and the F, G, and H helices of $\alpha$Hb are most affected (Fig. 7B). The B-C corner, helix G, and helix H comprise the AHSP binding face of $\alpha$Hb (1, 2, 25), and chemical shift changes are expected here as a result of the interface forming (Fig. 7B, gray background shading). In contrast, chemical shift changes in helix F cannot be explained by proximity to the AHSP surface, which implies that they arise from an induced structural change in $\alpha$Hb. However, NMR signals arising from the F helix region had similar line widths to signals from other regions of the $\alpha$Hb subunit, and TALOS$^+$ derived $\phi$ and $\psi$ angles indicated that the F helix region remains well ordered with similar secondary structure in the absence and presence of AHSP (Fig. 5).

His-87 side chain resonances remained strongly shifted in the complex with AHSP (Fig. 8A, red), with similar chemical shift values to the free CO-$\alpha$Hb$^\text{PMB}$ form (Fig. 8A, black). This provides strong evidence that His-87 remains the heme-ligating residue in CO-$\alpha$Hb-AHSP. In summary, AHSP binding to CO-$\alpha$Hb results in small but significant conformational changes in helix F even though the $\alpha$-helical character and heme binding function are retained. As such, these findings contrast with a previous x-ray crystallographic study in which we reported that O$_2^{-}\alpha$HbAHSP$^{P30A}$ has a highly unusual heme

| His residue | N$^\text{H1}$ | N$^\text{H2}$ | H$^\text{H1}$ | H$^\text{H2}$ |
|-------------|---------------|---------------|--------------|--------------|
| 20          | 191.0         | 182.7         | 7.20         | 8.21         |
| 45          | 239.7         | 175.0         | 7.27         | 7.99         |
| 50          | 201.4         | 181.2         | 7.06         | 8.07         |
| 58          | 250.6         | 153.8         | 4.47         | 8.30         |
| 72          | 188.1         | 162.8         | 7.44         | 8.38         |
| 87          | 163.3         | 218.2         | 1.92         | 1.47         |
| 89          | 218.0         | 182.0         | 6.92         | 7.74         |
| 112         | 187.3         | 179.1         | 6.76         | 8.38         |
| 58 (with AHSP) | 250.1       | 153.8         | 4.48         | 8.05         |
| 87 (with AHSP) | 163.2       | 218.6         | 1.10         | 1.43         |

Note that the heme binding function are retained. As such, these findings contrast with a previous x-ray crystallographic study in which we reported that O$_2^{-}\alpha$HbAHSP$^{P30A}$ has a highly unusual heme...

FIGURE 5. NMR chemical shift-derived $\psi$ angles for $\alpha$Hb in the absence and presence of AHSP. Backbone $\psi$ and $\phi$ angle predictions were calculated from $^{1}H$, $^{15}$N, $^{13}$C, and $^{12}$C chemical shifts using the program TALOS$^+$ (52). Predictions for CO-$\alpha$Hb$^\text{PMB}$ CO-$\alpha$Hb-AHSP, and CO-$\alpha$HbAHSP$^{P30A}$ are displayed as bars with a height $\pm$ 1 S.D. The $\phi$ and $\psi$ angles from the $\alpha$Hb subunit of a CO-HbA crystal structure (PDB 2DN3) are superimposed (filled circles) with $\alpha$-helix (black bars) and $\beta$ helix (white bars) secondary structure elements as assigned by DSSP (54).

TABLE 2

NMR resonances of His side chains for CO-$\alpha$Hb$^\text{PMB}$ and CO-$\alpha$Hb-AHSP

Resonances for CO-$\alpha$Hb$^\text{PMB}$ in 10 mM sodium phosphate, pH 7.0, at 288 K and CO-$\alpha$Hb-AHSP in 10 mM sodium phosphate, pH 7.0, at 288 K.

FIGURE 6. Histidine region of the $^{15}$N-HSQC spectrum of CO-$\alpha$Hb$^\text{PMB}$. A, shown is a portion of $^{15}$N-HSQC spectrum of CO-$\alpha$Hb$^\text{PMB}$ (0.8 mM) in 20 mM sodium phosphate, pH 7.0, at 15 $^\circ$C. The heme pocket His-58 and His-87 spin systems contain highly shifted resonances (linked by solid lines). Note that axes are discontinuous to remove large empty regions of the spectrum. B, shown is the central region of the spectrum shown in A with peaks assigned to surface histidine side chains.
AHSP Perturbs the Proximal Heme Pocket of O$_2$-α-Hemoglobin

CO-αHb-AHSP complex but, as described below, that now seems unlikely at least for the O$_2$-αHb-AHSP complex in solution.

**AHSP Pro-30 Perturbs the Proximal Heme Pocket of αHb—** Because mutation of AHSP Pro-30 to Ala or other residue types leads to a 4-fold decrease in the rate of O$_2$-αHb-AHSP autooxidation, we reasoned that CO-αHb-AHSP and CO-αHb-AHSP$_{P30A}$ complexes would display structural differences that give insight into the mechanism underlying AHSP-mediated αHb autooxidation. We, therefore, obtained backbone resonance assignments for CO-αHb in complex with AHSP$_{P30A}$ using standard triple resonance methods. Compared with wild-type AHSP, the AHSP$_{P30A}$ mutant induced more limited perturbations of CO-αHb (Fig. 7C). A direct comparison of CO-αHb chemical shifts in the CO-αHb-AHSP and CO-αHb-AHSP$_{P30A}$ complexes shows that the wild-type AHSP causes significantly larger chemical shift perturbations to the N-terminal fragment of αHb helix G and the whole of helix F (Fig. 7D), which suggests that AHSP Pro-30 makes contacts that are transmitted through the αHb structure and lead to perturbations of helix F.

Interestingly, we could not obtain backbone assignments for residues 135–138 of CO-αHb in complex with wild-type AHSP, and signals for these residues appeared to be absent from the NMR spectra. In NMR spectra of CO-αHb-AHSP$_{P30A}$, residues 135–138 were assigned and predicted as the regular α-helix by TALOS+ (Fig. 5). Residues 135–138 of native αHb are in a region of helix H that contacts helix F (Fig. 7E), which suggests that loss of NMR signals could reflect a destabilization of helix...
AHSP Perturbs the Proximal Heme Pocket of O$_2$-α-Hemoglobin

![EXAFS analysis of the heme sites of O$_2$-αHb and O$_2$-αHb-AHSP.](image)

FIGURE 9. EXAFS analyses of the heme sites of O$_2$-αHb and O$_2$-αHb-AHSP. Shown are EXAFS (A) and Fourier-transform amplitude (B) of EXAFS of O$_2$-αHb. Shown are EXAFS (C) and Fourier-transform amplitude (D) of EXAFS of O$_2$-αHb-AHSP. Shown are the observed data (solid line), data calculated from the best fit to the model (dashed line), residual data (dotted line), and window used in the Fourier filter (dot-dash). Molecular structures and Fe-ligand bond lengths and Fe-O–O bond angle for O$_2$-αHb (E) and O$_2$-αHb-AHSP (F) were obtained from EXAFS data.

H in αHb-AHSP related to changes in structure of the F helix. Overall the results here indicate that AHSP Pro-30 makes contacts that perturb helices F, G, and H of αHb, which together constitute the proximal heme pocket of αHb (Fig. 7E).

Comparison of the Iron-Ligand Structures in O$_2$-αHb and O$_2$-αHb-AHSP—NMR spectroscopic data indicated that His-87 is retained as the iron binding His residue in CO-αHb-AHSP, but these data do not provide detailed information about the exact geometries of the axial iron ligands. In addition, we could not determine NMR structures for the O$_2$-αHb-AHSP complexes due to autooxidation during NMR data collection. To probe the electronic structure and coordination environment around the heme iron more directly, we acquired x-ray absorption spectra at the iron K-edge for the physiologically more relevant O$_2$-αHb and O$_2$-αHb-AHSP complexes. Spectra were recorded at 10 K to minimize photo-damage and increase the importance of multiple-scattering contributions to the EXAFS (58, 59). Samples were prepared in buffer containing 0.88 M sucrose, which allowed the formation of a homogeneous glass upon freezing (59). This procedure permitted measurements on the unstable O$_2$-αHb-AHSP complex to be performed over extended time periods without conversion to the bis-histidyl ferric αHb form. The x-ray absorption near edge structure (XANES) spectra of O$_2$-αHb and O$_2$-αHb-AHSP (not shown) show no irradiation damage, and the similarity to spectra of the low-spin Fe(III) proteins metleghemoglobin, oxymyoglobin, and oxyhemoglobin (60) suggest that all have a low-spin Fe(III) center, which is consistent with an Fe(III)$^+$-O$_7^-$ species (61). This classification is also supported by the diagnostic oxidation- and spin-state resonance Raman marker bands (1, 62). The slightly lower edge energy of O$_2$-αHb-AHSP compared with free O$_2$-αHb suggests a lengthening of the Fe-O$_2$ bond in the AHSP complex.

EXAFS data obtained from O$_2$-αHb and O$_2$-αHb-AHSP were fitted to models for the iron sites using restrained and constrained multiple-scattering models (58, 63–65). The observed and calculated EXAFS and the corresponding Fourier transforms are shown for O$_2$-αHb and O$_2$-αHb-AHSP in Fig. 9, A–D, and the derived molecular structures for O$_2$-αHb and O$_2$-αHb-AHSP are shown in Fig. 9, E and F, respectively. The
bond length/angle and other fitted parameters obtained are listed in Table 3.

The errors in the iron-ligand bond lengths determined by EXAFS were estimated as the r.m.s. of contributions from both random and systematic errors. The random (statistical) errors due to noise in the data were estimated by Monte-Carlo calculations (42), and the systematic errors were assigned a conservative consensus value, 0.02 Å (44). For O2-αHb, the calculated statistical errors were 0.002 Å for Fe-NP, 0.008 Å for Fe-Ns, and 0.005 Å for Fe-O2. For O2-αHb-AHS, Monte-Carlo calculations yielded r.m.s. errors of 0.003 Å for Fe-NP, 0.012 Å for Fe-Ns, and 0.006 Å for Fe-O2. These statistical errors were combined with the maximum systematic error to obtain the estimated maximum r.m.s. errors in the reported bond lengths (Table 3). Monte-Carlo calculations yielded an r.m.s. error of 1.7° and 2.3° for the bond angles in O2-αHb and O2-αHb-AHS, respectively. These errors were combined with the respective r.m.s. errors of 2.1° and 3.2°, resulting from varying the O-O distance by ±0.03 Å (Table 4) to give respective estimated maximum r.m.s. errors of 2.7° and 3.9° for O2-αHb and O2-αHb-AHS. Therefore, the error in this bond angle estimated by two independent methods is 3–4°. The resulting Fe-O-O angles determined from restraining the starting angle between 115° and 175° and allowing the fit to go to convergence then removing the restraint were 130° (for 6 minima and 143° for 1 minimum); the latter was a local minimum with a higher R value and a low S2 value for a heme protein) in O2-αHb (Table 5) and 135° (for 6 minima and 101° for 1 minimum) in O2-αHb-AHS (Table 6). The fit obtained for the Fe-O-O bond angle of 101°, when the angle was initially restrained to be 115° and then this restraint was removed and the fit allowed to go to convergence, is likely to be a false minimum. This is because it is not chemically reasonable and is not consistent with the similarity in the shape of the XANES of O2-αHb and O2-αHb-AHS (not shown), as XANES is sensitive to bond angles. These analyses show that the bond lengths are independent of all of these changes in starting models and constraints/restraints, and the bond angles relax to the non-constrained values shown in Table 3. These detailed analyses of parameter space provide confidence that the EXAFS fits are robust and are not trapped in false minima. Similarly, varying the tilt or rotation of the proximal imidazole did not significantly alter the fitted bond length parameters (not shown).

Overall the iron-site geometry of O2-αHb and O2-αHb-AHS is similar to that derived from high resolution (1.25 Å) crystal structures of O2-Hb (53). However, the Fe-Ns and Fe-O2 bond lengths were shorter in O2-αHb than in O2-αHb-AHS (2.04 Å for Fe-N and 1.83 Å for Fe-O) (Table 3). Although these differences are within the conservative estimates of accuracy given in Table 3 (0.02 Å), the systematic error contributions are expected to be the same for both proteins and, hence, the errors obtained from Monte Carlo calculations are a better indication of the relative errors. For the Fe-O2 bond these errors were 0.005 Å and 0.006 Å in O2-αHb and O2-αHb-AHS, respectively, indicating that the 0.03 Å lengthening of Fe-O2 in O2-αHb-AHS is significant. It should be emphasized that the Fe-O2 bonds are substantially shorter than those of the other heme-ligand bonds, which is why the precision is so high. This is due to the rapid increase in the importance of single and multiple-scattering contributions as the bond length decreases.

The O2-αHb-AHS data were also fitted to a model where the iron-ligand bond lengths and Fe-O-O bond angle were constrained to be that in the O2-αHb-AHS structure (PDB 1Y01). The ability of this methodology to determine precise bond length and angle information on axial ligands has been established in studies of porphyrin model complexes and by the determination of precise bonding parameters in the heme environments of met- and deoxymyoglobin (41, 66) before the publication of the high resolution protein crystallographic structures (67, 68). Even taking the small differences between O2-αHb-AHS and O2-αHb into account, it is clear that the initial solution complex of O2-αHb-AHS has a “normal” coordination geometry similar to that in free O2-αHb chains.

**AHSP Perturbs the Proximal Heme Pocket of O2-α-Hemoglobin**

### Table 3

A comparison of Fe-ligand geometry from EXAFS and X-ray crystallographic analyses

| Species      | Method | Fe-Ligand distances | Fe-O-O angle (°) | Debye-Waller factors, σ2 | Other fit parameters |
|--------------|--------|---------------------|-----------------|--------------------------|---------------------|
|              |        | N°                  | N°              | O°                       | σ2                 |
|              |        | O°–O°               | N°              | O°                       |                     |
|              |        |                     |                 |                         | Eσ                  |
|              |        |                     |                 |                         | S0                  |
|              |        |                     |                 |                         | R                   |
| O2-αHb       | Crystal | 0.02 (8)            | 0.02 (8)        | 1.22 (8)                 | 0.002               |
|              |         |                     |                 |                         |                     |
|              |         |                     |                 |                         | Eσ                  |
|              |         |                     |                 |                         | S0                  |
|              |         |                     |                 |                         | R                   |

*PDB 2DN1 data at 1.25 Å resolution. Bond length errors are Cruckshank diffraction-data precision indicator (DPF) as calculated by REFMAC multiplied by 2-1/2 to convert from average coordinate error to average bond length error.

This work; 21-scan average. Error estimates in parentheses are calculated as described under “Comparison of the Iron-Ligand Structures.”

**AHSP Perturbs the Proximal Heme Pocket of O2-α-Hemoglobin**
AHSP Perturbs the Proximal Heme Pocket of $O_2$-$\alpha$-Hemoglobin

TABLE 4

| Effect of varying the O-O bond distance restraint on the Fe-O-O angle in human O$_2$-$\alpha$Hb and O$_2$-$\alpha$Hb-AHSP |
|---|
| The quantities enclosed by the brackets ([]) are the values of $\sigma_{rel}$ used by XFIT, which is analogous to e.s.d. |
| | Restraint on O-O bond | Fe-Ligand distances | Debye-Waller Factors, $\sigma^2$ | |
| | $\sigma_{rel}$ | N$^0$ | N$^1$ | O$^1$ | O$^2$ | Fe-O-O angle | $\sigma_{rel}$ | N$^0$ | N$^1$ | O$^1$ | O$^2$ | S$_0^2$ | R |
| O$_2$-$\alpha$Hb | 1.19 [0.01] | 2.00 | 2.04 | 1.83 | 1.18 | 131.4 | 0.001 | 0.001 | 0.003 | 0.920 | 14.8 |
| 1.25 [0.01] | 2.00 | 2.04 | 1.83 | 1.25 | 133.5 | 0.002 | 0.001 | 0.003 | 0.906 | 15.4 |
| 1.22 [0.03] | 2.00 | 2.04 | 1.83 | 1.21 | 130.0 | 0.002 | 0.001 | 0.003 | 0.92 | 15.9 |
| O$_2$-$\alpha$Hb-AHSP | 1.19 [0.01] | 2.00 | 2.08 | 1.86 | 1.19 | 137.6 | 0.001 | 0.002 | 0.002 | 0.916 | 16.8 |
| 1.25 [0.01] | 2.01 | 2.08 | 1.86 | 1.25 | 134.0 | 0.001 | 0.002 | 0.002 | 0.916 | 16.8 |
| 1.22 [0.03] | 2.01 | 2.08 | 1.86 | 1.22 | 135.0 | 0.001 | 0.002 | 0.002 | 0.916 | 16.8 |

TABLE 5

| Effect of restraining the Fe-O-O bond angle in human O$_2$-$\alpha$Hb on the MS XAFS analysis |
|---|
| The quantities enclosed by the brackets ([]) are the values of $\sigma_{rel}$ used by XFIT, which is analogous to e.s.d. |
| | Restraint on Fe-O-O bond angle | Fe-Ligand distances | Debye-Waller factors, $\sigma^2$ | |
| | $\sigma_{rel}$ | N$^0$ | N$^1$ | O$^1$ | O$^2$ | Fe-O-O angle $^a$ | $\sigma_{rel}$ | N$^0$ | N$^1$ | O$^1$ | O$^2$ | S$_0^2$ | R |
| 115° [1] | 2.00 | 2.05 | 1.83 | 1.22 | 115.0 | 0.001 | 0.001 | 0.003 | 0.939 | 15.9 |
| 125° [1] | 2.00 | 2.04 | 1.83 | 1.22 | 126.6 | 0.001 | 0.001 | 0.003 | 0.902 | 15.2 |
| 135° [1] | 2.00 | 2.04 | 1.83 | 1.21 | 134.9 | 0.001 | 0.001 | 0.003 | 0.909 | 15.3 |
| 145° [1] | 2.00 | 2.05 | 1.83 | 1.22 | 144.6 | 0.001 | 0.001 | 0.003 | 0.886 | 15.6 |
| 155° [1] | 2.00 | 2.05 | 1.83 | 1.22 | 155.0 | 0.001 | 0.001 | 0.003 | 0.895 | 15.8 |
| 165° [1] | 2.00 | 2.05 | 1.83 | 1.22 | 165.0 | 0.001 | 0.001 | 0.003 | 0.894 | 15.9 |
| 175° [1] | 2.00 | 2.05 | 1.83 | 1.22 | 175.0 | 0.001 | 0.001 | 0.003 | 0.894 | 15.9 |
| No constraint | 2.00 | 2.05 | 1.83 | 1.21 | 129.0 | 0.001 | 0.001 | 0.003 | 0.919 | 14.1 |
| 115° [b] | 2.00 | 2.04 | 1.83 | 1.21 | 129.7 | 0.001 | 0.001 | 0.003 | 0.922 | 15.0 |
| 125° [b] | 2.00 | 2.04 | 1.83 | 1.21 | 129.7 | 0.001 | 0.001 | 0.003 | 0.922 | 15.0 |
| 135° [b] | 2.00 | 2.04 | 1.83 | 1.21 | 129.7 | 0.001 | 0.001 | 0.003 | 0.922 | 15.0 |
| 145° [b] | 2.00 | 2.06 | 1.84 | 1.22 | 143.4 | 0.001 | 0.001 | 0.003 | 0.884 | 15.6 |
| 155° [b] | 2.00 | 2.06 | 1.84 | 1.22 | 143.4 | 0.001 | 0.001 | 0.003 | 0.884 | 15.6 |
| 165° [b] | 2.00 | 2.06 | 1.84 | 1.22 | 143.4 | 0.001 | 0.001 | 0.003 | 0.884 | 15.6 |
| 175° [b] | 2.00 | 2.06 | 1.84 | 1.22 | 143.4 | 0.001 | 0.001 | 0.003 | 0.884 | 15.6 |

Absence of AHSP variants. The ratio of $K_{O2}/K_{O2}$ provides the O$_2$ association equilibrium or affinity constant ($K_{O2}$). As reported by Vasseur-Godbillon et al. (28), AHSP binding to O$_2$-$\alpha$Hb causes a small decrease in the rate of O$_2$ binding and a larger 3–4-fold increase in the rate of O$_2$ dissociation. The net result is an ~4-fold decrease in O$_2$ affinity (Table 7). These results support the XAFS data and provide direct evidence that AHSP binding leads to weakening of the Fe-O$_2$ bond, which in turn leads to a significant increase in the susceptibility of O$_2$-$\alpha$Hb to autooxidation.

AHSP binding to $\alpha$Hb also slows the rate of bimolecular CO binding by a factor of ~3–4-fold, as also reported previously by Vasseur-Godbillon et al. (28) (Table 7). CO binding is limited primarily by the rate of internal bond formation and, therefore, provides a sensitive measure of iron reactivity that is primarily determined by interactions between the heme iron and the
proximal histidine. The ∼4-fold decrease in $k'_{CO}$ and $O_2$ affinity are consistent with conformational strain introduced on the proximal side of the heme pocket of αHb-AHSP due to interactions with AHSP Pro-30. This interpretation is strongly supported by the effects of Pro-30 mutations on ligand binding. As shown in Table 7, AHSPP30A and AHSPP30W mutations diminish the effects of AHSP binding on both $O_2$ affinity and the rate of CO association, and in the case of AHSPP30W, the observed ligand binding parameters are similar to those of free αHb. Thus, all our structural, spectroscopic, and functional data suggest that structural changes in the proximal heme pocket are induced by AHSP Pro-30, causing a weakening of the Fe-O$_2$ bond and an increase in the rate of autooxidation.

DISCUSSION

AHSP promotes autooxidation of $O_2$-αHb to prevent it from reacting with $H_2$O$_2$ under conditions of oxidative stress. The resultant bis-histidyl heme does not react with $H_2$O$_2$ and, therefore, cannot generate reactive ferryl species and protein radicals (2, 20). The bis-histidyl αHb-AHSP structure has been well characterized by x-ray crystallographic and spectroscopic methods (1, 2, 25–27), but the formation of this structure from $O_2$-αHb has been less well understood. The data presented here provide key insights as to how AHSP facilitates formation of the bis-histidyl state by making specific interactions at the αHb-AHSP interface and fit a model, explained below, in which αHb-AHSP complexes can adopt one of two well ordered conformations that are intermediates in Hb assembly and achieve $O_2$-αHb detoxification (Fig. 10).

Structure of αHb—By reacting pMB with Cys-104 and blocking αHb self-association, we obtained chemical shift-derived backbone dihedral angles and chemical shift analysis of His side chains, which together show that the conformation of free αHb is very similar to its structure in the native Hb tetramer. This conclusion is consistent with previous findings that heme pocket structure is well preserved in isolated subunits, based on far-UV (69, 70) and near-UV CD (69–72) and homonuclear NMR studies of ferrous and ferric αHb (47, 55, 56, 73, 74). None of these data provide any hints as to why free αHb is more prone to oxidation and subsequent rapid loss of the heme moiety and aggregation. Similar observations have been made in other systems. For example, the monomeric point mutants of the carrier protein transthyretin display identical x-ray crystal structures to the wild-type protein and yet have substantially reduced thermodynamic stabilities (75). In such cases, a native-like conformation still represents the lowest energy state available; however, the free energy well in which this conformer sits is shallower due to a reduction in the number of stabilizing interactions. In the case of αHb, stabilization is provided by multiple subunit interactions in the tetramer.

$CO$- and $O_2$-Hb Have Native-like Conformations in Complex with AHSP—A previous x-ray crystallographic study from our group suggested that $O_2$-αHb underwent large conformational changes upon binding AHSP (1), and it was assumed that these changes were responsible for the accelerated autooxidation. In that study residues Asp-74–Leu-91 of αHb (helix F) appeared disordered (as judged from a lack of interpretable electron density), and the heme-ligating residue was switched from the usual His-87 in helix F to His-58 in helix E. However, NMR line shape and TALOS+ chemical shift data presented here show that, in the solution state, helix F retains an ordered α-helical structure in CO-αHb-AHSP. In addition, analysis of His side chain resonances strongly suggests that His-87 retains the heme binding role. In the crystal structure, the Fe-O$_2$ bond is unusually long (2.7 Å) (1). Such a long Fe-O$_2$ bond is inconsistent with visible absorption and XANES and EXAFS spectroscopic data (Table 3), which indicate that the iron coordination...
AHSP Perturbs the Proximal Heme Pocket of $O_2\alpha$-Hemoglobin

geometries of $O_2\alpha$Hb and $O_2\alpha$Hb-AHSP are very similar. The EXAFS data do indicate a slight lengthening of the Fe-O$_2$ bond from 1.83 Å in $O_2\alpha$Hb to 1.86 Å in $O_2\alpha$Hb-AHSP, providing structural evidence that AHSP promotes superoxide dissociation and a decrease in O$_2$ affinity (Table 7).

Several possible explanations exist for the differences between the crystal structure described previously and the solution structure reported here. A detergent molecule located close to the heme group in the crystal might have influenced the structure; however, the detergent makes very few direct contacts with the protein. Perhaps more significantly, the crystals were grown under a standard atmosphere making it likely that partial autooxidation of $\alpha$Hb would occur. In addition, the intense x-ray beams used for protein crystallography can promote reduction of ferric proteins (59). A mixture of heme states arising from these processes would introduce static disorder in the crystal, complicating analysis of the x-ray data. Nevertheless, the crystal structure does provide evidence that helix F of AHSP is susceptible to conformational distortion in the presence of AHSP.

AHSP Pro-30 Promotes Changes in the Proximal Heme Pocket of $O_2\alpha$Hb That Enhance Autooxidation—We have previously shown that mutation of Pro-30 to a range of other residues leads to a 4-fold reduction in autooxidation of $\alpha$Hb. Here we show that introducing Pro at other positions in loop 1 did not restore native $O_2\alpha$Hb autooxidation and, although the AHSP$^{30A/N28P}$ may undergo cis/trans isomerization, $O_2\alpha$Hb autooxidation was not enhanced above the rate observed for AHSP$^{30A}$, which is only 2–3-fold greater than that of free $O_2\alpha$Hb (27). These data emphasize that the position of Pro-30 in AHSP loop 1 is critical to enhancing autooxidation.

AHSP Pro-30 is positioned adjacent to the beginning of helix G in the bis-histidyl $\alpha$Hb-AHSP complex. However, in the absence of crystallographic data, the degree of interaction between Pro-30 and helix G in $O_2\alpha$Hb-AHSP is unknown. Based on mutation and binding data, which show that Pro-30 destabilizes the $O_2\alpha$Hb-AHSP interface but not the bis-histidyl $\alpha$Hb-AHSP interface (11, 25, 27), we previously proposed that Pro-30 introduces a steric clash at the $O_2\alpha$Hb-AHSP interface, which is relieved upon transition to the bis-histidyl $\alpha$Hb-AHSP structure. Here we show that AHSP binding induces NMR chemical shift perturbations in F, G, and H helices of CO-$\alpha$Hb, which constitute the proximal heme pocket, and that mutation of Pro-30 markedly reduces these disturbances. These findings are consistent with a steric interaction between AHSP Pro-30 and $\alpha$Hb helix G that is propagated through packing interactions of the F, G, and H helices (represented as orange shading of these helices in Fig. 10, top row). Bonding interactions between the heme iron and proximal His-87 in helix F and the geometry of the proximal pocket are important factors influencing ligand reactivity of the iron (76, 77). It is, therefore, significant that AHSP decreases O$_2$ affinity and CO association rates of $\alpha$Hb and that mutation of Pro-30 restores ligand reactivity of the heme iron to near-normal levels, which suggests strongly that Pro-30-induced disturbance of the proximal heme pocket contributes to enhanced $O_2\alpha$Hb autooxidation and lower ligand reactivity in the presence of wild-type AHSP.

$O_2\alpha$Hb-AHSP and Bis-histidyl-AHSP Intermediates in Hb Assembly—Our data suggest a model in which AHSP can bind $\alpha$Hb in one of two well ordered conformations depending on the oxidation/ligand state of the heme (Fig. 10). $O_2\alpha$Hb is bound in a near-native conformation (Fig. 10, top), whereas Fe(III) $\alpha$Hb adopts a non-native globin conformation and bis-histidyl heme coordination (Fig. 10, bottom). We expect that Fe(II) deoxy $\alpha$Hb and CO-$\alpha$Hb will also bind to AHSP with near-native protein conformations on the basis that these forms of $\alpha$Hb all show the same affinity for AHSP as $O_2\alpha$Hb, which is 100-fold weaker than the interaction of AHSP with the non-native bis-histidyl Fe(III) $\alpha$Hb conformation (20, 25, 27). Notably, deoxy-Fe(II) $\alpha$Hb does not adopt bis-histidyl heme coordination (26) despite the availability of an open iron axial coordination site. These studies emphasize that specific coordination between the distal His-58 and the Fe(III) iron center and the structural changes in the $\alpha$Hb polypeptide chain occur in a concerted fashion during oxidation of the $\alpha$Hb-AHSP complex and do not occur when the iron atom is reduced.

Based on kinetic data, Khandros et al. (11) and Mollan et al. (27) proposed that $\alpha$Hb synthesized in the Fe(III) form in the presence of AHSP (78) proceeds through a bis-histidyl $\alpha$Hb-AHSP folding intermediate before heme reduction and incorporation into Hb (Fig. 10, solid black arrows). Bis-histidyl ferric $\alpha$Hb can in principle be transferred to $\beta$Hb and subsequently reduced (Fig. 10, dashed arrows); however, the rate of ferric $\alpha$Hb-AHSP dissociation is probably too slow to be physiologically relevant, and reduction almost certainly occurs more rapidly (27). Based on autooxidation rates measured in vitro (11, 20, 25, 28, 29), newly reduced Fe(II)/$O_2\alpha$Hb should be rapidly incorporated into Hb before autooxidation can occur if the concentration of free $\beta$Hb is high. However, under conditions of limiting $\beta$Hb that are known to occur during normal erythropoiesis (79, 80), the likelihood increases for the conversion of Fe(II)/$O_2\alpha$-AHSP species to bis-histidyl ferric complexes (Fig. 10, red arrows). The more rapid autooxidation of $\alpha$Hb in the presence of AHSP may be advantageous, as it provides a means to trap $\alpha$Hb in a relatively unreactive Fe(III) state that inhibits redox cycling and reactions with reactive oxygen species that could otherwise occur with free $\alpha$Hb. In addition, both free $O_2\alpha$Hb and met-Hb subunits are more prone to $H_2O_2$-induced ferryl and protein radical formation and precipitation (20, 81), making it advantageous to remove free $O_2\alpha$Hb and stabilize it as bis-histyl met-$\alpha$Hb-AHSP under conditions of oxidative stress.

To investigate the biological importance of autooxidation rate, Khandros et al. (11) performed gene knock-in experiments in mice. Mutations of AHSP Pro-30 to Ala or Trp did not disturb erythropoiesis (11), which suggested that a 4-fold decrease in $\alpha$Hb-AHSP autooxidation rate is not sufficient to elicit changes in erythropoiesis in the normal mouse model (11, 27). However, it is important to recognize that $O_2\alpha$Hb-AHSP$^{30A}$ still autoxidizes more quickly than does free $O_2\alpha$Hb (11, 25, 27) and that other interactions across the $\alpha$Hb-AHSP interface, in addition to those with Pro-30, are important for autooxidation and hemichrome formation.

A transition between two metastable $\alpha$Hb-AHSP structures, as outlined in Fig. 10, avoids persistent disordered states that
AHSP Perturbs the Proximal Heme Pocket of O₂-α-Hemoglobin

would favor unregulated reactions with an exposed heme and rapid heme dissociation. In this regard, it is interesting that mutations of Pro-30 to Ala or Trp decrease O₂-αHb autooxidation rates but enhance heme dissociation rates (11). In conclusion, it appears that the AHSP sequence has evolved to achieve a fine balance of distorting αHb sufficiently to promote changes in heme coordination that inhibit reactions with H₂O₂ without inducing an unacceptably high risk of heme loss or other irreversible denaturation events.

A Physiological Role for O₂-αHb·AHSP beyond the Red Blood Cell—Recently Straub et al. (82) reported that αHb and AHSP (but not βHb) are expressed in arterial endothelial cells at sites where the endothelial cells make direct contact with underlying smooth muscle (myoendothelial junctions) and that αHb expression regulates NO signaling from the endothelium to smooth muscle. NO signaling is important for maintaining vascular tone and modulating the response of smooth muscle to vasodilator and vasoconstrictor messengers. Importantly, the effect of heme proteins on NO signaling is dependent on the iron oxidation and ligand state. Fe(III) heme interacts only very weakly with NO, providing no barrier to NO diffusion. In contrast, O₂-heme reacts rapidly with NO to form nitrate (NO₃⁻) and Fe(III) heme, thus quenching NO signaling (83–85). In the endothelium, the reaction of NO with O₂-αHb·AHSP is expected to generate bis-histidyl αHb·AHSP. Therefore, in endothelial cells the balance between O₂-αHb·AHSP and Fe(III) bis-histidyl αHb·AHSP will impose restrictive/permis- sive states for NO signaling, respectively. In this regard, autooxidation and the interaction of Fe(III) bis-histidyl αHb·AHSP with reductase systems in endothelial cells are critical processes. Understanding these processes is now necessary for a full understanding of NO signaling in the vasculature.

Acknowledgments—We thank Dr. Chien Ho for the kind gift of the pHE7 plasmid and Drs. Aviva Leviya, Hugh Harris, Matthew Latimer, and Allyson Soo Hou for assistance at Stanford Synchrotron Radiation Lightsource. This work was performed with support from the Australian Synchrotron Research Program, which was funded by the Commonwealth of Australia under the Major National Research Facilities Program. Portions of this research were carried out at the Stanford Synchrotron Radiation Lightsource. The Stanford Synchrotron Radiation Lightsource Structural Molecular Biology Program is supported by the Department of Energy, Office of Biological and Environmental Research, and by the National Institutes of Health, National Center for Research Resources, Biomedical Technology Program.

REFERENCES
1. Feng, L., Gell, D. A., Zhou, S., Gu, L., Kong, Y., Li, J., Hu, M., Yan, N., Lee, C., Rich, A. M., Armstrong, R. S., Lay, P. A., Gow, A. J., Weiss, M. J., Mackay, J. P., and Shi, Y. (2004) Molecular mechanism of AHSP-mediated stabilization of α-hemoglobin. Cell 119, 629–640
2. Feng, L., Zhou, S., Gu, L., Dell, A. M., Mackay, J. P., Weiss, M. J., Gow, A. J., and Shi, Y. (2005) Structure of oxidized α-hemoglobin bound to AHSP reveals a protective mechanism for heme. Nature 435, 697–701
3. Gell, D., Kong, Y., Eaton, S. A., Weiss, M. J., and Mackay, J. P. (2002) Biophysical characterization of the α-globin binding protein α-hemoglobin stabilizing protein. J. Biol. Chem. 277, 40602–40609
4. Khim, A. J., Kong, Y., Hong, W., Russell, J. E., Rouda, S., Adachi, K., Simon, M. C., Blobel, G. A., and Weiss, M. J. (2002) An abundant erythroid protein that stabilizes free α-hemoglobin. Nature 417, 758–763
5. dos Santos, C. O., Duarte, A. S., Saad, S. T., and Costa, F. F. (2004) Expression of α-hemoglobin stabilizing protein gene during human erythropoiesis. Exp. Hematol. 32, 157–162
6. Kong, Y., Zhou, S., Khim, A. J., Katein, A. M., Yu, X., Dell, D. A., Mackay, J. P., Adachi, K., Foster-Brown, L., Louden, C. S., Gow, A. J., and Weiss, M. J. (2004) Loss of α-hemoglobin-stabilizing protein impairs erythropoiesis and exacerbates β-thalassemia. J. Clin. Invest. 114, 1457–1466
7. Lai, M. I., Jiang, J., Silver, N., Best, S., Menzel, S., Mijovic, A., Colella, S., Ragoussis, J., Garner, C., Weiss, M. J., and Their, S. L. (2006) α-Hemoglobin stabilising protein is a quantitative trait gene that modifies the phenotype of β-thalassaemia. Br. J. Haematol. 133, 675–682
8. Giordano, P. C., Zweegman, S., Akkermans, N., Arkesteijn, S. G., van Delft, P., Verteeh, F. G., Wajcman, H., and Hartveit, C. L. (2007) The first case of Hb Groene Hart [α119(H2)Pro→Ser, CCT→TCT (α1)] homozygosity confirms that α thalassemia phenotype is associated with this abnormal hemoglobin variant. Hemoglobin 31, 179–182
9. Yu, X., Kong, Y., Dore, L. C., Abdalmalki, O., Katein, A. M., Zhou, S., Choi, J. K., Gell, D., Mackay, J. P., Gow, A. J., and Weiss, M. J. (2007) An erythroid chaperone that facilitates folding of α-globin subunits for hemoglobin synthesis. J. Clin. Invest. 117, 1856–1865
10. Nasimuzzaman, M., Khandros, E., Wang, X., Kong, Y., Zhao, H., Weiss, D., Rivella, S., Weiss, M. J., and Persons, D. A. (2010) Analysis of α-hemoglobin stabilizing protein overexpression in murine β-thalassemia. Am. J. Hematol. 85, 820–822
11. Khandros, E., Mollan, T. L., Yu, X., Wang, X., Yao, Y., D’Souza, J., Gell, D. A., Olson, J. S., and Weiss, M. J. (2012) Insights into hemoglobin assembly through in vivo mutagenesis of α-hemoglobin stabilizing protein. J. Biol. Chem. 287, 11325–11337
12. Yu, X., Mollan, T. L., Butler, A., Gow, A. J., Olson, J. S., and Weiss, M. J. (2009) Analysis of human α globin gene mutations that impair binding to the α-hemoglobin stabilizing protein. Blood 113, 5961–5969
13. Giulivi, C., and Davies, K. J. (1994) Hydrogen peroxide-mediated ferrylhemoglobin generation in vitro and in red blood cells. Methods Enzymol. 231, 490–496
14. Nagababu, E., and Rikkind, J. M. (2000) Reaction of hydrogen peroxide with ferrylhemoglobin. Superoxide production and heme degradation. Biochemistry 39, 12503–12511
15. Reeder, B. J., Svistunenko, D. A., Cooper, C. E., and Wilson, M. T. (2004) The radical and redox chemistry of myoglobin and hemoglobin. From in vitro studies to human pathology. Antioxid. Redox. Signal 6, 954–966
16. Svistunenko, D. A. (2005) Reaction of haem containing proteins and enzymes with hydroperoxides. The radical view. Biochim. Biophys. Acta 1707, 127–155
17. Reeder, B. J. (2010) The redox activity of hemoglobin. From physiologic functions to pathologic mechanisms. Antioxid. Redox. Signal 13, 1087–1123
18. Joshi, W., Leb, L., Piotrowski, J., Fortier, N., and Snyder, L. M. (1983) Increased sensitivity of isolated α subunits of normal human hemoglobin to oxidative damage and crosslinkage with spectrin. J. Lab. Clin. Med. 102, 46–52
19. Scott, M. D., van den Berg, J. J., Repka, T., Rouyer-Fessard, P., Hebbel, R. P., Beuzard, Y., and Lubin, B. H. (1993) Effect of excess α-hemoglobin chains on cellular and membrane oxidation in model β-thalassemic erythrocytes. J. Clin. Invest. 91, 1706–1712
20. Mollan, T. L., Banerjee, S., Wu, G., Parker Siburt, C. J., Tsai, A. L., Olson, J. S., Weiss, M. J., Crumbliss, A. L., and Alyash, A. I. (2013) α-Hemoglobin stabilizing protein (AHSP) markedly decreases the redox potential and reactivity of α subunits of human HbA with hydrogen peroxide. J. Biol. Chem. 288, 4288–4298
21. Amer, J., Goldfarb, A., and Fibach, E. (2003) Flow cytometric measurement of reactive oxygen species production by normal and thalassemic red blood cells. Eur. J. Haematol. 70, 84–90
22. Srinoun, K., Svasti, S., Chumworathayee, V., Vadolas, J., Vattanaviboon, P., Fucharoen, S., and Winchagoo, P. (2009) Imbalanced globin chain synthesis determines erythroid cell pathology in thalassemic mice. Haematologica 94, 1211–1219
23. Leechaoenchai, A., Wannatung, T., Litanathudom, P., Svasti, S., Fucha-
AHSP Perturbs the Proximal Heme Pocket of O₂-α-Hemoglobin

eroen, S., Chokchaichamnanakit, D., Srisomsap, C., and Smith, D. R. (2011) Increased oxidative metabolism is associated with erythroid precursor expansion in β-thalassemia/Hb E disease. *Blood Cells Mol. Dis.* 47, 143–157

24. De Franceschi, L., Bertoldi, M., De Falco, L., Santos Franco, S., Ronzoni, L., Turrini, F., Colancecco, A., Camaschella, C., Cappellini, M. D., and Iolascon, A. (2011) Oxidative stress modulates heme synthesis and induces peroxiredoxin-2 as a novel cytoprotective response in β-thalassemic erythropoiesis. *Haematologica* 96, 1595–1604

25. Gell, D. A., Feng, L., Zhou, S., Jeffrey, P. D., Bendak, K., Gow, A., Weiss, M. J., Shi, Y., and Mackay, J. P. (2009) A cis-proline in α-hemoglobin stabilizing protein directs the structural reorganization of α-hemoglobin. *J. Biol. Chem.* 284, 29462–29469

26. Hamdane, D., Vasseur-Godbillon, C., Baudin-Creusa, V., Hoa, G. H., and Marden, M. C. (2007) Reversible hexacoordination of α-hemoglobin-stabilizing protein (AHSP)/α-hemoglobin versus pressure. Evidence for protection of the α-chains by their chaperone. *J. Biol. Chem.* 282, 6398–6404

27. Mollan, T. L., Kandros, E., Weiss, M. J., and Olson, J. S. (2012) The kinetics of α-globin binding to α-hemoglobin stabilizing protein (AHSP) indicate preferential stabilization of a hemichrome folding intermediate. *J. Biol. Chem.* 287, 11338–11350

28. Vasseur-Godbillon, C., Hamdane, D., Marden, M. C., and Baudin-Creusa, V. (2006) High-yield expression in *Escherichia coli* of soluble human α-hemoglobin complexed with its molecular chaperone. *Protein Eng. Des. Sel.* 19, 91–97

29. Zhou, S., Olson, J. S., Fabian, M., Weiss, M. J., and Gow, A. J. (2006) Biochemical fates of a hemoglobin bound to α-hemoglobin-stabilizing protein AHSP. *J. Biol. Chem.* 281, 32611–32618

30. Simpleanu, V., Lukin, J. A., Fang, T. Y., Zou, M., Ho, N. T., and Ho, C. (2000) Chain-selective isotopic labeling for NMR studies of large multimeric proteins. Application to hemoglobin. *Biophys. J.* 79, 1146–1154

31. Looker, D., Mathews, A. J., Neway, J. O., and Sterett, G. L. (1994) Expression of recombinant human hemoglobin in *Escherichia coli*. *Methods Enzymol.* 231, 364–374

32. Cai, M., Huang, Y., Sakaguchi, K., Clore, G. M., Gronenborn, A. M., and Craigie, R. (1998) An efficient and cost-effective isotope labeling protocol for proteins expressed in *Escherichia coli*. *J. Biol. Chem.* 273, 97–102

33. Shen, T. J., Ho, N. T., Zou, M., Sun, D. P., Cottam, P. F., Simpleanu, V., Tam, M. F., Bell, D. A., Jr., and Ho, C. (1997) Production of human normal adult and fetal hemoglobins in *Escherichia coli*. *Protein Eng.* 10, 1085–1097

34. Gill, S. C., and von Hippel, P. H. (1989) Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Chem.* 182, 319–326

35. Eaton, W. A., and Hofrichter, J. (1981) Polarized absorption and linear dichroism spectroscopy of hemoglobin. *Methods Enzymol.* 76, 175–261

36. Folta-Stogniew, E., Weiss, M. J., and Williams, K. R. (1999) Determination of molecular masses of proteins in solution. Implementation of an HPLC size exclusion and laser light scattering service in a core laboratory. *Anal. Biochem.* 279, 517–520

37. Robbins, D. A., Northrop, H. L., and Kellor, J. F. (1993) Determination of Fe-ligand bond lengths and angles in hemeproteins using x-ray absorption Spectroscopy. *Appl. Spectroscopy* 47, 1780–1784

38. Heinlen, R. M., Armstrong, R. S., and Lay, P. A. (2005) Three-dimensional structure determination using multiple-scattering analysis of XAFS. Applications to metallopeptides and coordination chemistry. *Coord. Chem. Rev.* 249, 141–160

39. George, G. N., Pickering, J. I., Pushie, M. J., Nienaber, K., Hackett, M. J., Ascone, I., Hedman, B., Hodgson, K. O., Aitken, J. B., Levine, A., Glover, C., and Lay, P. A. (2012) X-ray-induced photo-chemistry and X-ray absorption spectroscopy of biological samples. *J. Synchrotron. Radiat.* 19, 875–886

40. Rich, R. M. (1997) Determination of Fe-ligand bond lengths and angles in hemeproteins using x-ray absorption Spectroscopy. A Ph.D thesis, School of Chemistry, The University of Sydney, Sydney

41. Reed, C. A., and Cheung, L. S. K. (1977) On the bonding of FeO₂ in hemo globin and related dioxygen complexes. *Proc. Natl. Acad. Sci. U.S.A.* 74, 1780–1784

42. Spiro, T. G., and Strekas, T. C. (1974) Resonance Raman spectra of heme proteins. Effects of oxidation and spin state. *J. Am. Chem. Soc.* 96, 338–345
AHSP Perturbs the Proximal Heme Pocket of O₂-α-Hemoglobin

63. Binsted, N., Strange, R. W., and Hasnain, S. S. (1992) Constrained and restrained refinement in EXAFS data analysis with curved wave theory. *Biochemistry* **31**, 12117–12125

64. Chance, M. R., Miller, L. M., Fischetti, R. F., Scheuring, E., Huang, W. X., Sclavi, B., Hai, Y., and Sullivan, M. (1996) Global mapping of structural solutions provided by the extended x-ray absorption fine structure ab initio code FEFF 6.01. Structure of the cyrogentic photoprotein of the myoglobin-carbon monoxide complex. *Biochemistry* **35**, 9014–9023

65. Hasnain, S. S., and Hodgson, K. O. (1999) Structure of metal centres in proteins at subatomic resolution. *J. Synchrotron Radiat.* **6**, 852–864

66. Rich, A. M., Armstrong, R. S., Ellis, P. J., and Lay, P. A. (1998) Determination of the Fe-ligand bond lengths and Fe-N-O bond angles in horse heart ferric and ferrous nitrosylmyoglobin using multiple-scattering XAFS analyses. *J. Am. Chem. Soc.* **120**, 10827–10836

67. Vojtechovský, J., Chu, K., Berendzen, J., Sweet, R. M., and Schlichting, I. (1999) Crystal structures of myoglobin-ligand complexes at near-atomic resolution. *Biophys. J.* **77**, 2153–2174

68. Kachalova, G. S., Popov, A. N., and Bartunik, H. D. (1999) A steric mechanism for inhibition of CO binding to heme proteins. *Science* **284**, 473–476

69. Li, R., Nagai, Y., and Nagai, M. (2000) Changes of tyrosine and tryptophan residues in human hemoglobin by oxygen binding. Near- and far-UV circular dichroism of isolated chains and recombined hemoglobin. *J. Inorg. Biochem.* **82**, 93–101

70. Beychok, S., Tyuma, I., Benesch, R. E., and Benesch, R. (1967) Optically active absorption bands of hemoglobin and its subunits. *J. Biol. Chem.* **242**, 2460–2462

71. Geraci, G., and Li, T. K. (1969) Circular dichroism of isolated and recombined hemoglobin chains. *Biochemistry* **8**, 1848–1854

72. Geraci, G., and Parkhurst, L. J. (1981) Circular dichroism spectra of hemoglobins. *Methods Enzymol.* **76**, 262–275

73. Tran, A. T., Kolczak, U., and La Mar, G. N. (2003) Solution 1H NMR study of the active site molecular structure and magnetic properties of the cyanomet complex of the isolated α-chain from human hemoglobin A. *Biochim. Biophys. Acta* **1650**, 59–72

74. Tran, A. T., Kolczak, U., and La Mar, G. N. (2004) Solution 1H NMR study of the active site molecular structure and magnetic properties of the cyanomet complex of the isolated, tetrameric β-chain from human adult hemoglobin. *Biochim. Biophys. Acta* **1701**, 75–87

75. Azevedo, E. P., Pereira, H. M., Garratt, R. C., Kelly, J. W., Foguel, D., and Pálnano, F. L. (2011) Dissecting the structure, thermodynamic stability, and aggregation properties of the A25T transhysterin (A25T-TTR) variant involved in leptomeningeal amyloidosis. Identifying protein partners that co-aggregate during A25T-TTR fibrillogenesis in cerebrospinal fluid. *Biochemistry* **50**, 11070–11083

76. Olson, J. S., and Phillips, G. N., Jr. (1997) Myoglobin discriminates between O₂, NO, and CO by electrostatic interactions with the bound ligand. *J. Biol. Inorg. Chem.* **2**, 544–552

77. Capece, L., Marti, M. A., Crespo, A., Doctorovich, F., and Estrin, D. A. (2006) Heme protein oxygen affinity regulation exerted by proximal effects. *J. Am. Chem. Soc.* **128**, 12455–12461

78. Schulman, H. M., Martinez-Medellin, J., and Sidoli, R. (1974) The oxidation state of newly synthesized hemoglobin. *Biochem. Biophys. Res. Commun.* **56**, 220–226

79. Gill, F. M., and Schwartz, E. (1973) Free α-globin pool in human bone marrow. *J. Clin. Invest.* **52**, 3057–3063

80. Vasseur, C., Pissard, S., Domingues-Handi, E., Marden, M. C., Galactéros, F., and Baudin-Creuza, V. (2011) Evaluation of the free α-hemoglobin pool in red blood cells. A new test providing a scale of β-thalassemia severity. *Am. J. Hematol.* **86**, 199–202

81. Tomoda, A., Sugimoto, K., Suhara, M., Takeshita, M., and Yoneyama, Y. (1978) Haemichrome formation from haemoglobin subunits by hydrogen peroxide. *Biochem. J.* **171**, 329–335

82. Straub, A. C., Lohman, A. W., Billaud, M., Johnstone, S. R., Dwyer, S. T., Lee, M. Y., Bortz, P. S., Best, A. K., Columbus, L., Gaston, B., and Isakson, B. E. (2012) Endothelial cell expression of haemoglobin α regulates nitric oxide signalling. *Nature* **491**, 473–477

83. Doherty, D. H., Doyle, M. P., Curry, S. R., Vali, R. J., Fattor, T. J., Olson, J. S., and Lemon, D. D. (1998) Rate of reaction with nitric oxide determines the hypertensive effect of cell-free hemoglobin. *Nat. Biotechnol.* **16**, 672–676

84. Eich, R. F., Li, T., Lemon, D. D., Doherty, D. H., Curry, S. R., Aitken, J. F., Mathews, A. I., Johnson, K. A., Smith, R. D., Phillips, G. N., Jr., and Olson, J. S. (1996) Mechanism of NO-induced oxidation of myoglobin and hemoglobin. *Biochemistry* **35**, 6976–6983

85. Kim-Shapiro, D. B., Schechter, A. N., and Gladwin, M. T. (2006) Unraveling the reactions of nitric oxide, nitrite, and hemoglobin in physiology and therapeutics. *Arterioscler. Thromb. Vasc. Biol.* **26**, 697–705