A cis-Proline in α-Hemoglobin Stabilizing Protein Directs the Structural Reorganization of α-Hemoglobin

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α-Hemoglobin (αHb) stabilizing protein (AHSP) is expressed in erythropoietic tissues as an accessory factor in hemoglobin synthesis. AHSP forms a specific complex with αHb and suppresses the heme-catalyzed evolution of reactive oxygen species by converting αHb to a conformation in which the heme is coordinated at both axial positions by histidine side chains (bis-his-tidyl coordination). Currently, the detailed mechanism by which AHSP induces structural changes in αHb has not been determined. Here, we present x-ray crystallography, NMR spectroscopy, and mutagenesis data that identify, for the first time, the importance of an evolutionarily conserved proline, Pro30, in AHSP Pro30 adopts a cis-peptidyl conformation and makes contact with the N terminus of helix G in αHb. Mutations that stabilize the cis-peptidyl conformation of free AHSP, also enhance αHb conversion activity. These findings suggest that AHSP loop 1 can transmit structural changes to the heme pocket of αHb, and, more generally, highlight the importance of cis-peptidyl prolyl residues in defining the conformation of regulatory protein loops.

Mammalian adult hemoglobin (HbA) is a tetramer of two αHb and two βHb subunits, which is produced to extremely high concentrations (~340 mg/ml) in red blood cells. Numerous mechanisms exist to balance and coordinate HbA synthesis in normal erythropoiesis, and problems with the production of either HbA subunit give rise to thalassemia, a common cause of anemia worldwide. Previously, we identified α-hemoglobin stabilizing protein (AHSP) as an accessory factor in normal HbA production (1). AHSP forms a dimeric complex with αHb (see Fig. 1A) (2) but does not interact with βHb or HbA. AHSP also binds heme-free (apo) αHb (3) and may serve functions in both the folding of nascent αHb (4) and the detoxification of excess αHb that remains following HbA assembly (2, 5). Mice carrying an Ahsp gene knock-out display mild anemia, ineffective erythropoiesis, and enhanced sensitivity to oxidative stress (1, 6), features also observed in β-thalassemia patients due to the cytotoxic effects of free αHb.

Free αHb promotes the formation of harmful reactive oxygen species as a result of reduction/oxidation reactions involving the heme iron (7, 8). Reactive oxygen species can damage heme, αHb, and other cellular structures, resulting in hemoglobin precipitates and death of erythroid precursor cells (9–12). The presence of AHSP may explain how cells tolerate the slight excess of αHb that is observed in normal erythropoiesis, which is postulated to inhibit the formation of non-functional βHb tetramers, thus providing a robust mechanism for achieving the correct subunit stoichiometry during HbA assembly (13).

Structural and biochemical studies have begun to elucidate the molecular mechanism by which AHSP detoxifies αHb. AHSP binds to oxygenated αHb to generate an initial complex that retains the oxy-heme, as evidenced by a characteristic visible absorption spectrum (see Fig. 1B, middle) and resonance Raman spectrum (5). This initial oxy-αHb-AHSP complex then converts to a low spin Fe3+ complex (2), in which the heme iron is bound at both axial positions by the side chains of His58 and His87 from αHb (see Fig. 1B, right). The formation of this complex inhibits αHb peroxidase activity and heme loss (2). Bis-histidyl heme coordination is becoming increasingly recognized as a feature of numerous vertebrate and non-vertebrate globins (14) and has been shown previously to confer a relative stabilization of the Fe3+ over the Fe2+ oxidation state (15–17). Although bis-histidyl heme coordination has previously been detected in solutions of met-Hb, formed through spontaneous autodestruction of Hb (18–21), the bis-his-αHb-AHSP complex provides the first evidence that the bis-histidyl heme may play a positive functional role in Hb biochemistry by inhibiting the production of harmful reactive oxygen species.

Despite its potential importance, the mechanism by which AHSP influences heme coordination in its binding partner is still unknown. As shown in Fig. 1A, AHSP binds αHb at a sur-
face away from the heme pocket, and thus structural changes must somehow be transmitted through the αHb protein. It is intriguing that the free AHSP protein switches between two alternative conformations linked to cis/trans isomerization of the Asp29-Pro30 peptide bond in loop 1 (22) and that, in complex with αHb, this loop is located at the αHb-AHSP interface (see Fig. 1A). Peptide bonds preceding proline residues are unique in that the cis or trans bonding conformations have relatively similar stabilities (23), allowing an interconversion between these conformations that can be important for protein function (24, 25). Previous x-ray crystal structures of αHb-AHSP complexes have been obtained only with a P30A mutant of AHSP, in which isomerization is abolished and the Asp29-Ala30 peptide bond adopts a trans conformation, leaving the potential structural and functional significance of the evolutionarily conserved Pro30 undisclosed. Here, we demonstrate a functional role for AHSP Pro30 in conversion of oxy-αHb to the bis-histidyl form and identify a specific structural role for a cis Asp29-Pro30 peptide bond in this process. From a mechanistic understanding of how AHSP promotes formation of bis-histidyl αHb, we may eventually be able to engineer AHSP function as a tool in new treatments for Hb diseases such as β-thalassemia.

EXPERIMENTAL PROCEDURES

Protein Production—Full-length human AHSP (102 residues), and a construct with the C-terminal unstructured residues deleted (AHSP residues 1–90; AHSP-(1–90)), were expressed in Escherichia coli BL21 and purified as described previously (3). The final purified proteins contained an additional Gly-Ser at the N terminus as a consequence of the expression system. αHb from human blood was purified as described previously (3). Isotopic labeling of ASHP was achieved by expressing in shaker flasks with 13NH4Cl and 13C-glucose as sole nitrogen and carbon sources (26). Met-αHb was prepared from oxy-αHb by oxidation with 5 molar eq of K3Fe(CN)6 and filtered over Sephadex G-25 (Amersham Biosciences).

NMR Spectroscopy—AHSP and αHb-AHSP complexes were prepared at ~1 mm concentration in 10–20 mM Na2HPO4/NaH2PO4, pH 6.9 (95% 2H2O and 5% 4H2O) and 2 μM 5,5-dimethylsilacontanesulfonate. Data were collected at 288 K (AHSP-(1–90)), 298 K (AHSP) and 303 K (αHb-AHSP complexes) on Bruker Avance 600 and 800 MHz spectrometers, processed in TOPSPIN (Bruker), and analyzed using SPARKY.6 Backbone assignments were obtained using standard triple-resonance solution NMR methods (27). Side chain 13C assignments were made from the CC(CO)NH experiment (28). The ratio of the cis/trans Xaa29-Pro30 peptide bond isomers was determined from 15N-HSQC peak volumes.

X-ray Crystallography—A complex between oxy-αHb and AHSP residues 1–91 (AHSP-(1–91)) was produced as described (5) and oxidized by 4 molar eq of K3Fe(CN)6. Crystals were grown at 4 °C using the hanging drop vapor diffusion method. The well buffer contains 0.1 M MES, pH 6.5, 4% acetonitrile, and 14.5% (w/v) PEG3000. The crystals, with a typical dimension of 0.05 × 0.05 × 0.1 mm3, belong to the space group P6122 and contain two complexes per asymmetric unit. The unit cell has a dimension of a = b = 65.23 Å and c = 439.66 Å. Crystals were equilibrated in buffer containing 0.1 M MES, pH 6.5, 15% (w/v) PEG3000, and 21% (v/v) glycerol, and were flash frozen under a cold nitrogen stream. The native data sets were collected at the National Synchrotron Light Source X25 at the Brookhaven National Laboratories. The data were processed with Denzo and Scalepack (29). The structure was solved by molecular replacement using AMoRe (30). The atomic models of AHSP and αHb were built using O (31) and refined using CNS (32).

RESULTS

Mutation of Pro30 Inhibits the Conversion of Oxy-αHb to Bishis-αHb—To investigate the function of Pro30 in AHSP, we generated a number of point mutants at this position and assayed their ability to convert oxy-αHb to the protective bis-histidyl form. As shown in Fig. 1B, the oxy-αHb-AHSP and bishis-αHb-AHSP complexes display characteristic visible absorption spectra that allow the conversion process to be conveniently monitored. A series of representative spectra obtained during conversion of oxy-αHb-AHSP are shown in Fig. 2A. Each spectrum can be fit as a sum of the pure oxy- and bishis-αHb-AHSP spectra, and the series displays isosbestic points at 529 and 592 nm. The change in absorbance at 576 nm was fit to a single exponential function (Fig. 2B, closed circles) to obtain a rate constant. In comparison to the oxy-αHb-AHSP complex, the spectra of oxy-αHb alone displayed only a minor change at 576 nm (Fig. 2B, open squares) and lacked the isosbestic points at 529 and 592 nm, indicating a process distinct from conversion to bis-histidyl αHb (precipitation was evident).

Upon mutation of Pro30 to any of the residues Ala, Phe, Gly, Val, or Trp, we observed a ~4-fold reduction in the rate constant (p < 0.001) (Table 1 and Fig. 2C, lanes 5–9). The same isosbestic points were observed in each reaction. In contrast, alanine mutations introduced at positions 29 or 31 had minor effects (Fig. 2C). These results indicate that efficient conversion

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Oxy-αHb to the Bis-histidyl Form—Introduction of the P30A mutation abrogates the cis/trans isomerization of AHSP, resulting in a trans Asposta-Ala peptide bond (5, 22). The remaining Pro mutants also adopt a single conformation, assumed to be trans (as judged by one-dimensional NMR; data not shown), and all display a similar 4-fold reduction in activity. Prolyl cis/trans isomerization is known to modulate the function of numerous proteins (24, 25), raising the possibility that the cis/trans conformation of the Asposta-Pro peptide bond might be important for AHSP function. To investigate this hypothesis, we made substitutions to amino acids surrounding Pro. Previous work on peptides (33, 34) and proteins (35, 36) has established that the ratio of cis to trans prolyl conformers is strongly influenced by the identity of the preceding residue.

We found that D29F and D29W mutations resulted in enhanced αHb conversion activity compared with wild-type AHSP (Fig. 2C). In contrast, D29A, L31A, and L31W mutations in AHSP had little effect on the αHb conversion rate. To control for effects of the D29W mutation that might be unrelated to its influence on the conformation of the Xaaosta-Xaa peptide bond, we generated a double mutant, D29W, P30A. Significantly, this mutant had the same low activity as AHSP-(P30A) (Fig. 2C, lane 12), confirming that the stimulating effect of the D29W mutation relies upon the presence of P30A.

To investigate the structural consequences of these mutations, we assigned backbone 15N, H, and 13Cα resonances for the D29A, D29F, D29W, L31A, and L31W mutants. All mutants displayed evidence of distinct cis/trans isomers (supplemental Fig. S2). To assign these isomers, we used proline side chain carbon resonances. The chemical shift difference δ(13Cα) - δ(13Cα) for proline is diagnostic for the conformation of the preceding peptide bond, with average values of 4.5 ± 1.2 and 9.6 ± 1.3 ppm (± 1 S.D.) observed for trans and cis isomers, respectively (37). The relative abundances of the cis and trans isomers were then calculated from 15N-HSQC peak volumes. Overall, there was positive correlation between the fraction of cis peptide in free AHSP and the conversion rate of the corresponding oxy-αHb-AHSP complex (Fig. 2D).

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The Conformation of AHSP Is the Same in Oxy-αHb and Bishis-αHb Complexes—To gain insight into the function of AHSP Pro in oxy-αHb conversion, we carried out structural analyses of the initial oxy-αHb-AHSP and final bishis-αHb-AHSP complexes. Although free AHSP exists as a mixture of two conformers, only a single conformer can be detected in complex with αHb by NMR methods (5, 22). Using a construct of AHSP in which the C-terminal unstructured residues were deleted (AHSP residues 1–90; AHSP-(1–90)), we could obtain complete backbone and proline side chain carbon assignments in a complex with αHb. We confirmed that AHSP-(1–90), and mutants thereof, showed the same pattern of activity and conformational preference as the full-length proteins (Table 1 and Fig. 2, C and D).

To prevent conversion of the initial αHb-AHSP complex to the bis-histidyl complex, we used carbonmonoxy-αHb (CO-αHb) in the place of oxy-αHb. Importantly, the 15N-HSQC spectra of CO-αHb-AHSP-(1–90) and oxy-αHb-AHSP-(1–90) are essentially identical (supplemental Fig. S3). The chemical shift difference δ(13Cα) - δ(13Cα) for Pro in the
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CO-αHb-AHSP-(1–90) complex was 9.3 ppm, indicating a cis Asp29-Pro30 conformation. In comparison, Pro60 and Pro30 yield values of 3.7 and 2.6 ppm, respectively, and are thus predicted to be in the trans-peptidyl conformation, consistent with our previous crystal structures (2, 5). A comparison of backbone chemical shifts between AHSP-(1–90) and AHSP-(1–90, P30A), both in complex with CO-αHb, reveals substantial differences in the conformation of loop 1 (Fig. 3A), consistent with the presence of a cis Asp29-Pro30 peptide bond in CO-αHb-AHSP-(1–90).

To determine whether AHSP undergoes any conformation changes concomitant with αHb conversion, we incubated unlabeled oxy-αHb with 15N-labeled AHSP-(1–90) at 30 °C and recorded 15N-HSQC spectra at 22-min intervals for 18 h. The evolution of the final bishis-αHb-AHSP complex was confirmed by UV-visible spectroscopy. Only small changes in the 15N-HSQC spectra were observed in this experiment (Fig. 3B, bars), indicating only minor changes in AHSP structure. These changes were much less pronounced than the differences between the cis/trans conformers of free AHSP-(1–90) (Fig. 3B, dashed line) or between the cis and trans conformations of AHSP-(1–90) and AHSP-(1–90, P30A) bound to CO-αHb (Fig. 3A). Backbone chemical shifts were also assigned for AHSP-(1–90, P30A), for which peptide bond isomerization has been shown not to occur (2, 5), in complex with CO-αHb and bishis-αHb. Small chemical shift differences were observed, and these were similar to those observed for AHSP-(1–90). Together, these data reveal that the structure of AHSP changes very little during αHb conversion, and that, in wild-type AHSP complexes, the Asp29-Pro30 peptide bond is likely to remain in a cis conformation throughout.

Crystal Structure of Wild-type bishis-αHb-AHSP Complex—To understand in more detail the influence of cis-peptidyl Pro30 on the structure and function of αHb-AHSP complexes, we turned to x-ray crystallography. Despite extensive efforts over three years, we were unable to produce crystals of oxy-αHb complexed with wild-type human AHSP. Finally, after screening ~8,000 different conditions, we eventually generated small crystals of bishis-αHb-AHSP-(1–91). The structure of this complex was determined by molecular replacement and refined to a 3.2 Å resolution (Fig. 4 and supplemental Table S2).

Aside from loop 1 of AHSP, the conformations of individual subunits within the bishis-αHb-AHSP-(1–91) complex are vir-

### Table 1

| AHSP sequence | Affinity constant ($K_a$)* | Oxy-αHb conversion rate constant* | Proportion of cis Xaa29-Xaa30 peptide |
|---------------|--------------------------|----------------------------------|-------------------------------------|
| Wild-type AHSP | 98 ± 10                  | 0.73 ± 0.14 (n = 12)            | 0.42 ± 0.04‡                         |
| P30A          | 33 ± 3.0                 | 0.15 ± 0.02 (n = 6)             | 0‡                                   |
| P30G          | 450 ± 60                | 0.11 ± 0.04 (n = 4)             | 0                                    |
| P30V          | 42 ± 7.0                 | 0.22 ± 0.04 (n = 4)             | 0                                    |
| P30F          | 15 ± 2.9                 | 0.19 ± 0.06 (n = 6)             | 0                                    |
| P30W          | 77 ± 2.3                 | 0.18 ± 0.05 (n = 5)             | 0                                    |
| D29A          | 250 ± 30                | 0.57 ± 0.02 (n = 5)             | 0.70 ± 0.05                         |
| D29F          | 98 ± 0.12               | 0.73 ± 0.04 (n = 5)             | 0.73 ± 0.04                         |
| D29W          | 53 ± 4.2                 | 1.12 ± 0.10 (n = 5)             | 0.77 ± 0.03                         |
| L31A          | 0.67 ± 0.18              | 0.25 ± 0.04 (n = 5)             | 0                                    |
| L31W          | 0.53 ± 0.14              | 0.52 ± 0.04 (n = 5)             | 0                                    |
| D29W,P30A     | 0.22 ± 0.07              | 0.68 ± 0.04 (n = 5)             | 0                                    |
| AHSP-(1–90)   | 93 ± 5                  | 0.59 ± 0.07 (n = 9)             | 0.39 ± 0.05                         |
| P30A-(1–90)   | 0.26 ± 0.08              | 0.60 ± 0.04 (n = 3)             | 0                                    |
| D29A-(1–90)   | 0.54 ± 0.01              | 0.61 ± 0.04 (n = 3)             | 0                                    |
| D29F-(1–90)   | 1.11 ± 0.11              | 0.66 ± 0.04 (n = 4)             | 0                                    |
| D29W-(1–90)   | 1.33 ± 0.22              | 0.70 ± 0.05 (n = 5)             | 0                                    |
| D29W,P30A-(1–90) | 0.15 ± 0.03              | 0                                    |

* Values from single isothermal titration calorimetry experiments, with fitting errors for unweighted non-linear least squares regression analysis using MicroCal Origin software. Representative data are shown in supplemental Fig. S1. AHSP binding to oxy- or CO-αHb yielded identical $K_a$ within the bounds of experimental error (supplemental Table S1).

a Determined from UV-visible absorption spectroscopy at 30 °C. The mean and S.D. for n independent measurements are reported.

b Calculated from 15N-HSQC peak volumes at 25 °C: a mean value (± 1 S.D.) for all residues where amide signals from both the cis and trans Xaa29-Pro30 isomers can be resolved. Data from two independently prepared samples were combined.

c P30X mutants adopted a single conformer in the basis of one-dimensional NMR.
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![Figure 3](image)

**Figure 3.** AHSP-(1–90) contains a cis Asp-Pro peptide bond in complex with CO-αHb and bishis-αHb, A, mean weighted chemical shift differences (15N, HN, and 13C, nuclei; ΔδHN,HC) between AHSP-(1–90) and AHSP-(1–90,P30A) in their respective complexes with CO-αHb. Structural differences are limited to the Pro30 loop. B, mean weighted chemical shift changes for residues in AHSP upon conversion from the CO-αHb-AHSP-(1–90) complex to the bishis-αHb-AHSP-(1–90) complex (bars). Shift changes in the Pro30 loop are small compared with the differences between the cis and trans Asp-Pro isomers of free AHSP-(1–90) (dashed line).

In contrast, a marked difference is observed in the organization of AHSP loop 1 in the two bis-histidyl complexes (Fig. 4, B and C) that mirrors our NMR findings for the CO-αHb complexes (Fig. 3A). The x-ray structure confirms the existence of a cis Asp-Pro amide bond in AHSP (Fig. 4D) and reveals the significant effect of this bond on the molecular structure of loop 1. In bishis-αHb-AHSP-(1–91,P30A) the backbone amides of loop 1 residues Asp39 and Ala30 form hydrogen bonds with the backbone carbonyls of Val26 and Phe27, respectively, giving rise to a single helical turn in AHSP loop 1 (Fig. 4E). The same hydrogen-bonding pattern is precluded in the wild-type AHSP because proline lacks an amide proton. Instead, the Pro30 loop projects out further toward αHb (Fig. 4F). In this position, the carbonyl of Pro30 forms a hydrogen bond with the Lys39 side chain of helix G in αHb, and the Pro30 side chain makes van der Waals contacts with the backbone of Pro95 in helix G. The Ala30 side chain faces away from αHb and makes no contacts with αHb. Thus, the distinguishing feature of the wild-type bishis-αHb-AHSP interface, which could not be inferred from prior crystallography studies, is the packing of the rigid Pro30 side chain against helix G of αHb.

**DISCUSSION**

Recently, we obtained the first evidence that bis-histidyl heme coordination may have a positive functional role in detoxification of αHb, by inhibiting the production of harmful reactive oxygen species (2). Defining the role of bis-histidyl heme coordination in erythroid biology should also provide insights into the functional properties of bis-histidyl heme structures in other systems, such as the hemoglobins of some arctic fish species (38) and other mammalian globins such as neuroglobin and cytoglobin (39). Ultimately, understanding how and why AHSP promotes formation of bis-histidyl αHb may allow us to design experimental strategies to modify AHSP function in new treatments for thalassemia (40).

![Figure 4](image)

**Figure 4.** Structural features of the wild-type bishis-αHb(chemp)AHSP-(1–91) complex. Backbone overlays of the individual subunits of the bishis-αHb-AHSP-(1–91) (yellow; PDB code 3A3) and bishis-αHb-AHSP-(1–91,P30A) (blue; PDB code 1Z8U) complexes showing αHb subunits (root mean square deviation of 0.4 Å over 135 aligned backbone Cα atoms) with conserved bis-histidyl heme coordination (A) and AHSP-(1–91) with Ala30 and Pro30 side chains shown in space-fill (B). C, plot of the distance between Cα positions for the AHSP alignment shown in B. D, stereo view of the cis Asp-Pro conformation of the Pro30 loop in the bishis-αHb-AHSP-(1–91) complex. The electron density 2Fobs - Fcalc map, contoured at 1.0 α, is shown in green. E, detail of the bishis-αHb-AHSP-(1–91,P30A) complex showing intramolecular backbone hydrogen bonding in the loop. F, detail of the bishis-αHb-AHSP-(1–91) complex showing the Pro30 side chain positioned close to the backbone of Pro95 (spheres) in helix G of αHb (αG).
Our current findings indicate that loop 1 of AHSP plays an important role in the transmission of structural changes to the heme pocket of αHb. A cis Asp29-Pro30 bond is an important structural and functional feature of AHSP in complex with αHb. This conclusion was not evident from previous studies, which were expedited by the favorable effects of a P30A mutation upon crystallization.

The Mechanism of oxy-αHb Conversion—Conversion of oxy-αHb to the bis-histidyl coordination involves a significant reorganization of the αHb subunit, an rms shift of 3.2 Å over 135 Cα atoms compared with HbA (2). Despite this, we find that AHSP retains a remarkably similar conformation throughout the conversion process, making it unlikely that the reorganization of the αHb subunit is driven by a conformational change in AHSP. Rather, we liken the initial binding of AHSP to oxy-αHb to the setting of a “molecular mousetrap” in which some type of strain is introduced into the structure of oxy-αHb that is subsequently released in a structural rearrangement of the αHb subunit alone. Several lines of evidence suggest that this rearrangement is coupled with autoxidation of the heme iron. First, AHSP can bind to deoxy-αHb but cannot convert the Fe2+ heme to a bis-histidyl structure (41, 42). A preference for Fe3+ over Fe2+ has previously been observed in other bis-histidyl heme proteins (15–17). Second, conversion of Fe3+ met-αHb, which at physiological pH contains only a weakly bound water molecule in the distal heme pocket, occurs simultaneously with (or very rapidly after) binding to AHSP, suggesting that dissociation of superoxide is the rate-limiting step in the conversion of oxy-αHb-AHSP.

In the mousetrap mechanism, the function of AHSP appears to be 2-fold: to enhance the rate of heme autoxidation and to reduce the barrier to bishistidyl coordination imposed by the protein matrix. Presumably, the key to understanding how AHSP potentiates autoxidation lies in the structure of the initial oxy-αHb-AHSP complex. Although we have been unsuccessful in attempts to crystallize this complex, we have been able to obtain an x-ray crystal structure of the AHSP-(1–91,P30A) mutant bound to oxy-αHb (5), possibly because of the slow conversion of the P30A mutant complex at 4 °C. The presence of a detergent molecule in the heme pocket warrants some caution in interpreting this structure. Nevertheless, it provides a fortuitous opportunity to gain possible insights into the AHSP mechanism. Strikingly, the F-helix of αHb, which carries the proximal His67 in native αHb, is disordered in this crystal. A predicted increase in hydration of the heme (43), and/or a greater mobility of the heme-pocket histidines (18, 44) are plausible explanations for the comparatively rapid autoxidation of the oxy-αHb-AHSP complex. By way of comparison, it is known that even relatively minor changes in the heme pocket structure can have significant effects on the stability of oxy-heme. For example, an Mb mutant with the side chains of His64 and Val68 interchanged adopts a bis-histidyl heme coordination geometry and undergoes very rapid autoxidation in the presence of oxygen (45).

The current study suggests one mechanism by which AHSP may destabilize the oxy-αHb structure. In bis-his-αHb-AHSP, the Pro30 side chain makes contact with helix G of αHb, and NMR data indicate that a similar conformation of the Pro30 loop is present in the initial oxy-αHb-AHSP complex. Mutation and thermodynamic data indicate that Pro30 destabilizes the oxy— not the bis-his-αHb-AHSP complex, leading to the hypothesis that Pro30 introduces a steric clash with the N terminus of helix G in oxy-αHb. Such an interaction might be one factor promoting a strained mousetrap conformation in αHb. Indeed, comparison of the oxy-αHb-AHSP-Hb (1–91,P30A) and bis-his-αHb-AHSP-(1–91) crystal structures shows that the Pro30 loop conformation could not be accommodated in the former structure without some displacement of helix G. Distortion of helix G in αHb is a plausible route through which AHSP might disrupt the structure of the adjacent F-helix and consequently the heme pocket. Clearly AHSP-(P30A) retains some ability to stabilize bishis-αHb indicating that additional contacts must also be involved.

In the αHb-AHSP complex, bonding of a second histidine side chain to the ferric heme iron stabilizes a switch in the conformation of αHb to a less redox active state. Mousetrap models have been invoked to describe other systems in which a transition between two alternate protein conformations is triggered in response to a highly localized bond rearrangement (46–48). The serpin class of serine protease inhibitors is one notable example. Cleavage of a serpin by its targeted protease triggers a large conformational change that traps the protease in an irreversible complex (46).

Evolutionary Conservation of Pro30—The importance of Pro30 for AHSP function is highlighted by its conservation across species. Interestingly, AHSP from the South American opossum Monodelphis domestica is one of the very few AHSP sequences to lack a proline residue in loop 1. The opossum αHb sequence is also highly unusual in lacking a distal histidine, precluding the adoption of bis-histidyl coordination geometry and providing indirect support for the hypothesis that Pro30 is present to optimize the formation of the bis-histidyl complex. In addition to sequestering and detoxifying excess αHb, mouse knock-out studies suggest that AHSP may also function as an αHb-specific chaperone during Hb folding (4). The function of AHSP in vivo might well involve a subtle balance between these activities, and it may be that Pro30 has been adopted during evolution to optimize the rate of free αHb conversion. Such relatively small changes in protein activity can be highly significant at the level of cell and organism function. A familiar example is haploinsufficiency, in which inheritance of one defective allele reduces the expression of the respective protein ~2-fold and results in disease (49).

A Stable cis-Peptidyl Proline Motif That Defines an Active Loop Conformation—Proline residues are unique in that the cis or trans conformations of a preceding peptide bond have relatively similar stabilities (23). Interconversion between these conformations can influence protein folding (50) and function (24, 25). Alternatively, our results indicate that a stable cis peptide bond can confer important structural and functional properties, in this case driving a conformational change in a partner protein.

The cyclic structure of proline restricts the phi backbone dihedral angle, and, consequently, proline residues are associated with restricting protein loop mobility (51–53). Furthermore, a cis Xaa-Pro bond introduces more severe steric con-
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Constraints on $\phi$ and $\psi$ backbone angles than occurs for trans Xaa-Pro bonds (54). Consequently, cis and trans Xaa-Pro motifs have distinct structural preferences: a survey of the PDB reveals that cis Xaa-Pro is most commonly found in bend structures (regions where the backbone changes direction by $>70^\circ$ in the absence of backbone hydrogen bonding) (55) and least likely to appear in helical conformations (56). In addition, the lack of an amide proton means that proline cannot act as a hydrogen bond donor to stabilize regular secondary structure. Accordingly, loop 1 in bishis-\alpha-Hb-AHSP crystal structure contains a bend that is replaced by a helical segment in the bishis-\alpha-Hb-AHSP-(P30A) structure. This helical turn compacts loop 1 and prevents contact with helix G of \alpha-Hb.

Cis Xaa-Pro motifs have been found to play precise structural roles in several protein folds (57–59), active sites (60–65) and protein interaction surfaces (66–68). Here we provide structural data to show that, within the \alpha-Hb-AHSP complex, the cis Asp$^{29}$–Pro$^{30}$ motif introduces intersubunit contacts that potentiate autooxidation of oxy-\alpha-Hb. We envisage that cis-peptidyl proline may play a general role in defining the structure of regulatory protein loops.

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