Reversible Hexacoordination of \( \alpha \)-Hemoglobin-stabilizing Protein (AHSP)/\( \alpha \)-Hemoglobin Versus Pressure

EVIDENCE FOR PROTECTION OF THE \( \alpha \)-CHAINS BY THEIR CHAPERONE

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Using high hydrostatic pressure or hydrogen peroxide as perturbing agents, we demonstrate a protective effect of the chaperone AHSP for the \( \alpha \)-chains of Hb. High pressure induces an irreversible aggregation of the ferrous deoxy \( \alpha \)-chains, whereas the AHSP/\( \alpha \)-Hb complex shows reversible hexacoordination of the \( \alpha \)-Hb without protein aggregation. Upon pressure release, the relaxation kinetics of the transition from the hexacoordinated to pentacoordinated form of \( \alpha \)-Hb in the presence of AHSP exhibit a biphasic shape. High pressure did not induce dissociation of \( \alpha \)-Hb from its chaperone, as evidenced by the ligand binding kinetics that show a unique rate for the AHSP/\( \alpha \)-Hb complex. For both free \( \alpha \)-Hb and the AHSP/\( \alpha \)-Hb complex, the bimolecular rate constant of CO binding \( (k_{\text{vs}}) \) versus pressure exhibits a bell shape, attributed to the transition of the rate-determining step from the chemical barrier to the migration of CO within the protein matrix. These results reveal a plasticity of the \( \alpha \)-Hb active site in the presence of the chaperone and indicate that the AHSP was still active at 300 MPa. The ferric state of the AHSP/\( \alpha \)-Hb complex shows hexacoordination even at atmospheric pressures, indicating a His-Fe-His binding scheme as previously observed in neuroglobin and cytoglobin. The reaction with hydrogen peroxide of ferric \( \alpha \)-Hb within the complex also demonstrates a protection against aggregation.

The \( \alpha \)-hemoglobin-stabilizing protein (AHSP),\(^2\) an abundant protein in vertebrate erythrocytes, is described as the chaperone of the \( \alpha \)-hemoglobin (\( \alpha \)-Hb) chains, which acts to protect against the aggregation of \( \alpha \)-chains involved in \( \beta \)-thalassemia pathology. The \( \alpha \) and \( \beta \) hemoglobin subunits of human adult hemoglobin (HbA) form a heterotetramer \( (\alpha_2\beta_2) \) in red blood cells leading to a cooperativity of oxygen binding. Although the \( \alpha \) and \( \beta \) genes are localized in different chromosomes, there is a coordinated expression to generate a functional and stable allosteric tetramer. The consequence of an excess of either chain is a pathological disorder generally known as thalassemia. Moreover it is known that isolated \( \alpha \)-hemoglobin (\( \alpha \)-Hb) is unstable, poorly soluble, and subject to aggregation and formation of Heinz bodies, as found in \( \beta \)-thalassemia (1).

The discovery of a molecular chaperone, AHSP, indicates an additional protein-protein reaction involved in the mechanism of pathological disorders induced by an excess of \( \alpha \)-Hb (2). AHSP is a small protein of 102 residues, which adopts an elongated antiparallel three helical fold, as described by NMR studies (3). AHSP has the ability to bind free \( \alpha \)-Hb or apo-\( \alpha \)-globin, but not HbA or the \( \beta \)-hemoglobin chains (\( \beta \)-Hb) (2, 4). However the presence of \( \beta \)-Hb can replace AHSP from the \( \alpha \)-Hb/AHSP complex to form functional HbA. Recently, a crystallization of the \( \alpha \)-Hb/AHSP complex revealed the key elements of the interaction (5); AHSP specifically recognizes the G and H helices of \( \alpha \)-Hb and more precisely certain residues such as Lys-99, His-103, and Phe-117. The AHSP/\( \alpha \)-Hb interface (Fig. 1) is quite comparable to the \( \alpha 1\beta 1 \) interface in Hb dimers and can thus explain the competition of \( \beta \)-Hb and AHSP for binding \( \alpha \)-Hb (5). Isothermal titration calorimetry showed that the affinity of AHSP for \( \alpha \)-Hb is about 100 nM, which is compatible with a biological role of chaperone (4), as compared with the tighter interface between the \( \alpha \) and \( \beta \)-Hb characterized by a dissociation constant around 1 nM at pH 7 (6).

In hemoglobins, the heme prosthetic group normally exists in either the ferrous (\( \text{Fe}^{2+} \)) or ferric (\( \text{Fe}^{3+} \)) state and has octahedral coordination geometry with six potential binding sites. The pyrrole nitrogens occupy the four equatorial binding sites, and one of the proximal axial sites is coordinated by a highly conserved His side chain. This proximal coordinated-covalent bond stabilizes the protein structure by slowing heme dissociation and influences the reactivity of the heme iron on the distal side. The opposite, or distal side, is generally available for ligand binding.

The binding of AHSP to oxygenated ferrous \( \alpha \)-Hb induces a conformational change of the F helix near to the active site of \( \alpha \)-Hb, which may weaken the bond between the iron atom and the proximal histidine, while increasing the probability of interaction with the distal His (7). The results of such rearrangements of the heme pocket also provoke an increase in the oxidation rate of the heme iron and modification of the chemical reactivity of the heme iron of \( \alpha \)-Hb bound to AHSP (7, 8). The crystallographic structure (Fig. 1) as well as the absorption spectroscopy indicated a significant hexacoordination via the distal histidine in the ferric complex (7) whereas in the classical...
vertebrate globins, the distal site is free for the ferrous form but in the ferric form, the iron is bound to a water or OH molecule depending on the pH.

Two recently discovered globins in vertebrate species, neuroglobin (Ngb) found in neurons (9, 10) and cytoglobin (Cygb), expressed in all tissues (11, 12), have the peculiarity of being in a His-Fe-His-hexacoordinated form in both the ferric and ferrous iron states. Flash photolysis studies of these new hexacoordinated (Hx) globins revealed high recombination ($k_{on}$) and low dissociation ($k_{off}$) rates for $O_2$, suggesting a high intrinsic affinity for oxygen. However, because of competition with the internal ligand, the HisE7 residue, the observed oxygen affinity (near 1 torr) is typical of Mb (10, 13).

The high flexibility of the E-helix of the Ngb was described as a crucial structural element in the regulation of the HisE7 binding to the heme (13–15). Actually, much evidence tends to suggest that the regulation of the HisE7 binding is governed by the movement of the E-helix leading to the presence of two conformations of the protein. The fact that Ngb can switch between two forms, with and without the disulfide bond, may require a more flexible protein structure. For example, the affinity for the distal histidine decreases by nearly an order of magnitude once the disulfide bridge is reduced (13–15). The combination of temperature and high pressure studies clearly shows that the stability of these globins is correlated with the hexacoordination probably through the stabilization of the heme pocket environment (13, 14).

This study focuses on the AHSP/$\alpha$-Hb complex and the understanding of the mechanism of their interactions. High pressure constitutes an elegant approach in the study of protein-protein interactions. In fact, it has been shown that high pressure destabilizes weak interactions such as hydrophobic bonds and salt bridges that play important roles in maintaining the three-dimensional protein structure (16). Using this method, we demonstrate that high pressure induces a reversible bi-histidyl hexacoordination of the $\alpha$-Hb iron atom in the presence of its molecular chaperone. The results suggest a protective role of AHSP to avoid precipitation of the $\alpha$-Hb.

**Protection of $\alpha$-Hb by Its Chaperone AHSP**

reaction of ferric $\alpha$-Hb with hydrogen peroxide ($H_2O_2$) within the complex also shows a protective role of AHSP toward $\alpha$-Hb, possibly to prevent cellular damage initiated by the reaction of hemoproteins with $H_2O_2$.

**Materials and Methods**

Preparation of $\alpha$-Hb and AHSP/$\alpha$-Hb Complex—After reaction of human Hb with $p$-hydroxymercuribenzoic acid, the isolated $\alpha$-Hb chains were purified by ion exchange chromatography as described previously (17, 18) with slight modification. The isolated $\alpha$-Hb chains were then saturated with CO and stored at $-80^\circ C$.

AHSP was expressed as a GST fusion protein and purified as described previously (8, 19). The GST-AHSP/$\alpha$-Hb complex was obtained after adding an excess of carbonmonoxy $\alpha$-Hb chains to GST-AHSP fixed to glutathione-Sepharose 4B beads as previously described (8, 19).

The AHSP/$\alpha$-Hb complex was obtained after direct cleavage by thrombin of GST proteins bound to glutathione beads (19). The samples were also analyzed by size-exclusion chromatography using a Superose$^{TM}$ 12 10/300 GL column (GE Healthcare Biosciences, Uppsala, Sweden) eluted at 25 °C with a 150 mM Tris acetate buffer, pH 7.5, or with phosphate-buffered saline at pH 7.4 (19).

Spectral Measurements at High Pressure—All high pressure experiments were performed on isolated native $\alpha$-Hb and the AHSP/$\alpha$-Hb complex in 50 mM Tris-HCl buffer at pH 7.0, 25 °C. Tris buffer, rather than phosphate buffer, was chosen for experiments versus pressure because the variation of pH versus pressure of about 0.02 units per kilobar is much lower (20).

The globin concentration used for high pressure studies was typically 10 $\mu$M $\alpha$-Hb (on a heme basis) determined from the carbonmonoyx form, using an extinction coefficient of 190 $\text{M}^{-1}\text{cm}^{-1}$ of the Soret band. Spectral measurements under high pressure were obtained using a 700 Mpa high pressure optical cell interfaced to a Cary 3E spectrophotometer. The deoxy samples were obtained by equilibration under nitrogen following an adding of a 10-fold excess of sodium dithionite. The CO form of $\alpha$-Hb or the AHSP/$\alpha$-Hb complex was obtained after equilibration of the samples under 1 atm CO for about 15 min, followed by addition of an excess of dithionite. All absorption spectra were corrected for the compressibility of the solvent as described previously (15).

The optical pressure system, capable of generating a pressure of 700 MPa, has been described previously (15). The high pressure bomb fits snugly into a Cary 3E spectrophotometer sample compartment thus minimizing any movement relative to the light path. The sample previously equilibrated in the appropriate condition was introduced into the high pressure quartz optical cuvette of volume of 450 $\mu$l and an optical path of 5 mm. The globin solution was isolated from the compression fluid by a double Bridgman seal. The pressure was increased in 50 MPa increments and held for 3 min before the spectral measurements to assure a stability of the final pressure.
Protection of α-Hb by Its Chaperone AHSP

The temperature of the high pressure cell was maintained by a circulating water bath.

Kinetics of Relaxation of the AHSP/α-Hb Complex—Briefly, the kinetic experiments after pressure release were done after 3 min of equilibration of the sample in the pressure cell at 300 MPa and at the desired temperature. The kinetics were started by a pressure release and were followed by monitoring the absorbance changes at different wavelengths (536, 561 nm) of the ferrous AHSP/α-Hb spectrum. The rates were obtained by fitting the kinetics to the bi-exponential Equation 1,

$$\Delta A_{\text{obs}}(t) = A_1 \exp(-k_1 \times t) + A_2 \exp(-k_2 \times t) \quad (\text{Eq. 1})$$

where $A$ is the amplitude, and $k$ is the rate coefficient. The relaxation kinetics were recorded at different temperatures, and the activation energy of the transition low spin to high spin relaxation kinetics were recorded at different temperatures, of ferrous deoxy α-Hb and the complex of α-Hb with AHSP was typically 20 μM. The CO rebinding reaction for the pentacoordinated species was analyzed by fitting the time course of the absorbance change at 436 nm with a monoeXponential curve in Equation 2,

$$\Delta A_{\text{obs}} \exp(-k_{\text{obs}} \times t) \quad (\text{Eq. 2})$$

where $k_{\text{obs}} = (k_{\text{obs}}^\text{CO}) \times [\text{CO}]$. From the pressure dependence of ligand binding kinetics, the activation volume ($\Delta V^\text{a}$) of the ligand binding reaction (the volume change between the transition state with respect to the initial state) can be determined from the slope of the plot of $\ln k$ versus pressure shown in Equation 3,

$$\Delta V^\text{a} = -RT \frac{\partial \ln k}{\partial P} \text{RT} \quad (\text{Eq. 3})$$

where $R$ is the gas constant (8.314 J mol$^{-1}$ K$^{-1}$), $T$ is absolute temperature, and $k$ is the observed rate.

Reaction of Ferric α-Hb with $H_2O_2$—The reactions were performed by incubating free α-Hb or AHSP/α-Hb complex (30 μM heme basis) in 100 mM phosphate buffer at pH 7.0, 25 °C with different concentrations (15 μM to 3 mM) of $H_2O_2$. After 5 min of incubation, a 30-μl aliquot was withdrawn and mixed with 10 μl of concentrated SDS loading buffer. The sample was then analyzed by SDS-PAGE.

RESULTS

Absorption Spectra versus Pressure—The absorption spectra of ferrous deoxy α-Hb in the presence of AHSP at pressures from 0.1 MPa to 400 MPa are shown in Fig. 2. The optical spectra at atmospheric pressure (0.1 MPa) of the deoxy ferrous α-Hb or AHSP/α-Hb are similar to those observed for the classical deoxy pentacoordinated form of globin and myoglobin as shown in Fig. 2 (solid blue line). The spectra of ferrous α-Hb and AHSP/α-Hb have the Soret band near 430 nm and a single broad band in the visible region around 555 nm (Fig. 2). Increasing pressure induced a red shift of the Soret band (Fig. 2) and a splitting of visible band into distinct α and β bands at 531 and 561 nm, respectively (Fig. 2). The enhanced amplitude for the α band is similar to that of the ferrous Hx form of the new vertebrate globins Ngb and cytoglobin (13). These spectra are characteristic of the His-Fe-His hexacoordination of the heme iron (9, 13). An overlay of a normalized spectrum of Ngb superimposed the spectra of the AHSP/α-Hb complex at 400 MPa (Fig. 2), indicating a low spin configuration of the heme of the α-Hb chains.

Therefore we can consider an equilibrium between a pentacoordinated and a Hx form as previously reported for tomato hemoglobin (13) and calculate for each spectrum the fraction of Hx form that is enhanced by higher pressure for both isolated α-Hb and the AHSP/α-Hb complex (Figs. 2 and 3), and the thermodynamic parameters characterizing the equilibrium.

After the pressure release from 400 MPa to 0.1 MPa, the AHSP/α-Hb complex exhibits the original pentacoordinated form without any loss of heme, indicating a full reversibility of the hexacoordination. On the other hand, free α-Hb did not recover the deoxy ferrous pentacoordinated form (Fig. 3). The
transition of free $\alpha$-Hb was more abrupt (involving an irreversible protein denaturation) than the transition of the complex (Fig. 3) suggesting that AHSP allowed some degree of flexibility on the structure of $\alpha$-Hb. The pressure that induced the formation of 50% of the Hx form was shifted by 60 MPa in the presence of the $\alpha$-chaperone (120 MPa for $\alpha$-Hb and 180 MPa for the complex) (Fig. 3). Note that the reaction is not reversible for isolated $\alpha$-Hb.

The measured molar reaction volume change ($\Delta V^0 = V^\text{Hexa} - V^\text{Penta}$) associated with iron spin state transition (high to low spin) was obtained from the plot of $\ln(K^\text{eq} = [\text{Hx}]/[\text{pentacoordinated}])$ versus pressure (Fig. 4). The $\Delta V^0$ of the iron spin transition was $-60 \pm 3$ cm$^3$/mol for the AHSP/$\alpha$-Hb complex, which is roughly the equivalent of three water molecules (18 cm$^3$/mol at 1 atm). This value of $\Delta V^0$ is higher than those previously reported for other hemoproteins, which was estimated in general between $-10$ and $-50$ cm$^3$/mol (21–23). This volume change emphasizes the important structural rearrangement of the $\alpha$-Hb heme site when bound to the AHSP as suggested from the recently reported crystallographic data (5).

No dissociation of the $\alpha$-Hb from the AHSP occurred during pressurization. The evidence came from the reversibility of the pressure cycle and the rate of CO recombination at high pressure. A dissociation would likely lead to aggregation of the $\alpha$-Hb subunits as observed for $\alpha$-Hb without AHSP. The products of the pressurized samples were analyzed by size exclusion chromatography; we obtained a single elution peak (data not shown) at a volume corresponding to 33.7 kDa ($V_e = 13.75$ ml) as previously observed for the AHSP/$\alpha$-Hb complex (19).

Kinetic Relaxation of the Low to High Spin Transition and Compressibility of the Heme Pocket—The dynamics of the transition from the low to high spin of the ferrous AHSP/$\alpha$-Hb complex was studied by following the absorbance changes at 536 and 561 nm initiated by a negative P-jump (a rapid decompression) from 300 MPa to 0.1 MPa (Fig. 5). We chose to perform the experiment at 300 MPa because the AHSP/$\alpha$-Hb complex is nearly 100% Hx at this pressure. The absorbance at 561 nm (Fig. 5) as well as at 536 nm (not shown) was normalized by the expected delta absorbance at 561 nm (at 300 MPa), just before the decompression (100% Hx) and after the end of the reaction (100% pentacoordinated). At both wavelengths, the kinetics were similar. The kinetic shape was biphasic with a rapid phase accounting for about two-thirds of the signal, followed by a phase requiring several minutes to obtain a full recovery of the equilibrium form with a rate constant $k = 0.02$ s$^{-1}$. To slow down the rapid phase, we decreased the temperature from 37 to 5 °C. This procedure was still not sufficient to observe the total kinetics. In less than 20 s, we recovered 65% of the pentacoordinated AHSP/$\alpha$-Hb form. The rate constant of the second phase showed a weak temperature dependence with an apparent activation energy of 5.5 kcal/mol$^{-1}$. The Arrhenius plot was linear indicating a simple low to high spin transition.

The spectral shift of the Soret band versus pressure has been associated with the coefficient of compressibility of the heme pocket (24). For both free $\alpha$-Hb-CO and the AHSP/$\alpha$-Hb-CO, we observed a red-shift of the Soret band similar to that for Mb (data not shown); however, unlike myoglobin, the observed red shift was not reversible for the free $\alpha$-chains.

CO Recombination Bimolecular Kinetics versus Pressure—We have investigated the effect of pressure on the CO rebinding kinetics for free $\alpha$-Hb and the AHSP/$\alpha$-Hb complex. The bimolecular association rates at normal pressure are $2 \mu M^{-1} \text{s}^{-1}$ for AHSP/$\alpha$-Hb and $5 \mu M^{-1} \text{s}^{-1}$ for the isolated $\alpha$-Hb chains; the kinetics are monophasic and can be simulated by a single exponential. Increased pressure caused a substantial change in the bimolecular association rates for both; the rates initially increase by about a factor of two, with maximum rates occurring at pressures of 100 MPa for the free $\alpha$-Hb and 150 MPa for the complex. Above these pressures, we observed a decrease of the rates for both protein systems. The increase of pressure was associated with a decrease in the bimolecular amplitude (data not shown).

The plot of $\Delta \ln(k_{\text{CO}})$ versus pressure showed a parabolic shape (Fig. 6) with a peak around 100 MPa for $\alpha$-Hb and 150 MPa for the complex. The activation volume for the bimolecu-
Protection of α-Hb by Its Chaperone AHSP

![Graph](image)

FIGURE 6. Plot of the bimolecular association (on) rate of CO binding versus pressure for the AHSP/α-Hb complex (●) and free α-Hb (○). In the low pressure range, high P accelerates the barrier limited kinetics. Above a certain pressure, the amplitude of the bimolecular phase decreases (more geminate phase), and the slope of the rate versus P reverses sign. High P closes the protein channels leading to a migration limited kinetic rate.

lar CO association rate changes from negative to positive values for both cases. The slope of the first phase (low P region) was \(-4.5 \text{ cm}^3/\text{mol}\) for the complex and \(-7 \text{ cm}^3/\text{mol}\) for the α-chains, whereas the second phase showed a similar slope for both systems with a volume of 5 cm³/mol. It has been reported that negative activation volume is characteristic of the bond formation reaction, and the positive activation volume is described as characteristic of CO diffusion reaction into the protein matrix (15, 25–27). These positive activation volumes for the ligand migration reflect the conformational fluctuations of the proteins for the changes from a “closed” to “open” structure (15, 25–27). Thus AHSP binding to α-Hb does not modify the volume profile for the CO binding reaction, but the peak of the bell shaped curve (transition for the change in sign of the activation volume) was displaced to higher pressures for the AHSP/α-Hb complex (Fig. 6).

Reaction of Ferric α-Hb with H₂O₂—The possibility of a protein–protein radical transfer reaction was investigated by the means of the SDS-PAGE gel. This easy method was extensively used especially to characterize the reactions of sperm whale and horse heart metMb with H₂O₂, which have served as a useful model for the investigation of protein radicals (28–31).

SDS-PAGE analysis of α-Hb after incubation with H₂O₂ shows that the monomeric protein (15 kDa) is partially converted to covalent dimeric products as shown in Fig. 7. Increasing the H₂O₂/protein ratio causes a polymerization of the α-Hb chains, associated with a decrease of initial monomer band. These covalent products could not be attributed to S–S bond formation between the Cys-104 (Cys-104–Cys-104) because the protein samples were treated with dithiothreitol before loading on the SDS-PAGE. At 5 equivalents of H₂O₂ (Fig. 7), a band of high mass molecular appeared, suggesting an aggregation of α-Hb correlating with the disappearance of the α-Hb band near 15 kDa, which became barely detectable at 50 equivalents of H₂O₂ (Fig. 7). Furthermore, these results were correlated with the spectral absorption studies of ferric α-Hb showing a decrease of the Soret band intensity of isolated ferric α-Hb at high H₂O₂ levels (data not shown).

Contrary to that observed for free α-Hb, the α-Hb in the presence of AHSP does not associate into dimers with one equivalent of H₂O₂ (Fig. 7). At high concentrations of H₂O₂, a dimer band appears, but not the band of high molecular mass. This indicates that in the presence of the H₂O₂, AHSP protects the α-Hb from aggregation.

DISCUSSION

The control of protein folding by chaperones is crucial to avoid the problems involving protein precipitation and aggregation. In this work, we demonstrate the protective role of AHSP as a molecular chaperone. High pressure has been used to induce conformational changes in several heme proteins (21, 23), and we have applied such a pressure perturbation to free α-Hb chains and the AHSP/α-Hb complex.

Our results of the spin state changes in the deoxy ferrous proteins versus pressure (Fig. 2) demonstrate the difference in the resistance of isolated α-Hb and the AHSP/α-Hb complex to high pressure denaturation. The low spin or Hx heme iron species are easily differentiated from the high spin forms (pentacoordinated) by the splitting of the α and β visible absorbance bands and the increase of the amplitude of the Soret band. At high pressure, the spectra of the AHSP/α-Hb complex are similar to those of Hx ferrous neuroglobin (Fig. 2) and cytoglobin, indicating that high pressure leads to the formation of the HisE7–Fe–HisF8 heme configuration.

For the pentacoordinated globins such as Mb, the potential sixth ligand is His–E7. From the spectral measurements one cannot distinguish which His is bound to the iron atom. Note that the crystal structure of the ferrous AHSP/α-Hb complex revealed a drastic conformational change in the heme pocket region of the α-Hb (Fig. 1) in which the distal and proximal histidines may reverse roles in terms of being the labile sixth coordination ligand (5).

Reversibility—High pressure induced an irreversible aggregation of the free α-Hb chains. The resistance of the α-Hb chains to precipitation under high pressure was observed only in the presence of its molecular chaperone. This result clearly emphasizes the protective role of the AHSP against denaturation of the isolated α-Hb chains. Based on previous thermal denaturation and pressure studies, it was proposed that the hexacoordination may play the role of a clamp around the
heme, which stabilizes the active site and subsequently the overall protein structure (14, 15). On decompression, the solution of ferrous deoxy AHSP/α-Hb returned to the mainly pentacoordinated form while the free α-Hb remained in a hemichrome state (Fig. 3) known to be cytotoxic for the red blood cells. The irreversibility of the free α-Hb precipitation seems to be directly linked with the irreversibility of the His-E7 coordination to the heme. It is then reasonable to propose that the AHSP may protect the α-Hb chains from precipitation by allowing a flexible heme pocket structure.

The relaxation kinetics of the transitions from high spin to low spin display a rapid restructuring to the pentacoordinated form (Fig. 5), although several minutes are required for a full recovery. This could indicate a multistep process involving the conformation changes of the heme pocket and F-helix as reported in the crystal structure (5). Moreover, it is interesting to note that no dissociation of α-Hb from AHSP occurred even at high pressure. As described by many studies, pressure below 300 MPa lead generally to a reversible dissociation of proteins assemblies into monomers; monomers which undergo to a different conformation from the original (35–37). A gel filtration after decompression gave only one peak corresponding to the peak of the AHSP/α-Hb complex (data not shown). The presence of a unique rate of CO binding to the AHSP/α-Hb complex in the range of 0.1–300 MPa is consistent with the absence of the α-Hb dissociation from the chaperone (Fig. 6), and also indicates that the chaperone activity was still preserved at high pressure.

It is interesting to note that the pressure dependence of the α-Hb bound to the chaperone seems to be different from that of the human tetrameric Hb. A previous study employed high pressure NMR to investigate the structural modification of the tetrameric hemoglobin under high pressure; it was concluded that pressure induces preferentially structural changes at the β heme (32).

Hexacoordination—High pressure favors hexacoordination of the ferrous AHSP/α-Hb complex, with a reaction volume change ΔV° of −60 ml/mol (Fig. 4). Note that a volume change accompanying the heme transition from high to low spin depends on the protein, iron oxidation state, and the presence of an external ligand (15, 23, 33). This reaction volume is much higher than those previously reported for myoglobin or hemo-globin, a volume associated with the compression, which enhances binding of the HisE7 to the heme iron (23). Such a large perturbation could indicate that the AHSP/α-Hb complex undergoes an increase of solvent exposure, which is in agreement with an enhancement of the autooxidation rate of the complex described previously (8, 34). This would indicate that the proximal site of the α-Hb becomes less rigid in the presence of the AHSP and supports the model of a large disorder of the proximal side of the α-Hb heme upon interaction with the AHSP. One direct consequence of these observations is the loss of the key elements that promote the stability of the binding of the di-atomic ligands (O2, CO) in the heme pocket resulting in a decrease of α-Hb affinity for the external ligands in the presence of the AHSP (8). The CO bimolecular rebinding of the α-Hb in the presence of AHSP is slower and thereby shifts the transition from barrier to migration limited kinetics at higher pressures. The binding of the AHSP does not significantly modify the activation volume profile of the CO binding (Fig. 6).

Dimerization—The pathogenesis of the aggregation state of α-Hb is due, at least in part, to the increased level in the red blood cells of reactive oxygen species (ROS) such as superoxide, O2•− hydroxyl radical, OH• or hydrogen peroxide, H2O2. Protein free radicals are considered as intermediates for many enzymes and heme proteins. One example of a typical radical intermediate is that produced by the reaction of the myoglobin with H2O2 (28–31). Based on this example we studied the effect of the H2O2 on the free α-Hb and α-Hb complexed to AHSP.

As shown in Fig. 7, addition of H2O2 can induce a covalent dimerization and/or aggregation of the α-Hb chains. The dimeric species of α-Hb are mainly observed at low concentration of H2O2; further increases in H2O2 lead to higher MW products which reflect an aggregation state of α-Hb. In fact the reaction of ferric α-Hb with H2O2 yields water with the formation of the ferryl species (Fe(IV)=O) heme with a rate of 320 m−1 s−1 (data not shown) close to the value published with the sperm whale Mb (28–31). Reaction of ferric Mb with H2O2 leads to both ferryl and porphyrin radicals. Site-specific mutagenesis and EPR spectroscopy had precisely identified residues implicated in the covalent dimerization of Mb in the presence of H2O2. The tyrosyl radical of the residues Tyr-103 and Tyr-151 have been established to participate in the covalent bond formation between Mb monomers. It is therefore likely that the homodimer of the α-Hb results from the generation of tyrosyl radicals. From the α-Hb crystal structure (Fig. 1), two exposed Tyr residues (Tyr-24 and Tyr-42) may be involved. In the presence of the AHSP, hexacoordination will decrease the reactivity of α-Hb with H2O2. The dimers of α-Hb were observed even in the presence of AHSP, but only at higher concentrations of H2O2. Both Tyr-24 and Tyr-42 are not in the interaction interface between α-Hb and AHSP (Fig. 1). Note that the high molecular weight species were not observed in the presence of the AHSP, again showing the protective role of the chaperone against α-Hb aggregation. Hexacoordination in a flexible heme pocket may thus be the mechanism to provide protection of the α-chains against aggregation. 

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Protection of α-Hb by Its Chaperone AHSP

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