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Dynamics of α-Hb chain binding to its chaperone AHSP depends on heme coordination and redox state

Laurent Kiger [a,*], Corinne Vasseur [a], Elisa Domingues-Hamdi [a], Gilles Truan [b], Michael C. Marden [a], Véronique Baudin-Creuza [a,*]

[a] INSERM, U779, University Paris 11, 78 rue du General Leclerc, 94275 Le Kremlin Bicêtre, France
[b] LISBP, 135 avenue de Rangueil, 31077 Toulouse, France

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ABSTRACT

Background: AHSP is an erythroid molecular chaperone of the α-hemoglobin chains (α-Hb). Upon AHSP binding, native ferric α-Hb undergoes an unprecedented structural rearrangement at the heme site giving rise to a 6th coordination bond with His(E7).
Methods: Recombinant AHSP, WT α-Hb:AHSP and α-Hb^{HE7Q}:AHSP complexes were expressed in *Escherichia coli*. Thermal denaturation curves were measured by circular dichroism for the isolated α-Hb and bound to AHSP. Kinetics of ligand binding and redox reactions of α-Hb bound to AHSP as well as α-Hb release from the α-Hb:AHSP complex were measured by time-resolved absorption spectroscopy.
Results: AHSP binding to α-Hb is kinetically controlled to prevail over direct binding with β-chains and is also thermodynamically controlled by the α-Hb redox state and not the liganded state of the ferrous α-Hb. The dramatic instability of isolated ferric α-Hb is greatly decreased upon AHSP binding. Removing the bis-histidyl hexacoordination in α-HbH58(E7)Q:AHSP complex reduces the stabilizing effect of AHSP binding. Once the ferric α-Hb is bound to AHSP, the globin can be more easily reduced by several chemical and enzymatic systems compared to α-Hb within the Hb-tetramer.
Conclusion: α-Hb reduction could trigger its release from AHSP toward its final Hb β-chain partner producing functional ferrous Hb-tetramers. This work indicates a preferred kinetic pathway for Hb-synthesis.
General significance: The cellular redox balance in Hb-synthesis should be considered as important as the relative proportional synthesis of both Hb-subunits and their heme cofactor. The *in vivo* role of AHSP is discussed in the context of the molecular disorders observed in thalassemia.

1. Introduction

The human adult hemoglobin (HbA) whose main function is the transport of oxygen from the lungs to the tissues is constituted by two α-chains and two β-chains, each associated to a heme molecule. Unlike β-hemoglobin chains (β-Hb), which form homologous tetramers, α-hemoglobin isolated chains (α-Hb) are less stable and may form precipitates acting as active oxidants causing membrane disruption, lipid oxidation, and eventually apoptosis and inefficient erythropoiesis [1]. Recently the alpha hemoglobin-stabilizing protein (AHSP) has been described to stabilize α-Hb in the marrow erythroid cell precursors (basophilic erythroblasts or other late erythroid cells) [2]. The primary function of a chaperone protein is to assist a target protein in reaching a correct intracellular folding mostly by preventing aggregation for oligomeric protein assembly. In contrast to most chaperones, AHSP activity is specific to α-globin (apo or holo) [3–5]. This chaperone does not recognize other globins such as β-Hb, tetrameric Hb or myoglobin [2]. Nevertheless it was not obvious by which mechanism AHSP might stabilize the α-Hb structure since the complex formation is always in competition with the pool of free β-Hb. In fact it seems that there is a slight excess of synthesis of α-globin [6,7] which means that a moderate pool of free α-globin may be present without giving rise to cytotoxic aggregates. Taking into account the very high affinity for αβ dimer formation, coupled with its auto-association to form tetrameric Hb and the high Hb intra-cellular concentration so that the dimer fraction is very low, one can assume that once an α-Hb chain binds to a β-Hb chain it remains bound until the red cell apoptosis. Consequently there are only two ways for AHSP to play its role of chaperone: i) to help stabilize and fold nascent α-Hb and ii) to handle a possible excess of free α-Hb,

Abbreviations: AHSP, alpha hemoglobin-stabilizing protein; AHSP^{WT}, recombinant human wild type AHSP with an N-terminal Gly-Pro-Leu-Gly-Ser peptide; AHSP:α-Hb, complex formed between WT α-Hb chain and recombinant human wild type AHSP; Cyt b5, recombinant soluble domain of human membrane-bound cytochrome b5; GST, glutathione S-transferase; Hb, human adult hemoglobin; MetHb, oxidized Hb; Ngb, neuroglobin; hemin, ferric heme; PBS, phosphate-buffered saline; RBCs, red blood cells; ROS, reactive oxygen species

* Corresponding authors. Tel.: +33 1 49595664, +33 1 49595684.
E-mail addresses: laurent.kiger@inserm.fr (L. Kiger), corinne.vasseur@inserm.fr (C. Vasseur), elisa.domingues@inserm.fr (E. Domingues-Hamdi), gilles.truan@insa-toulouse.fr (G. Truan), michael.marden@inserm.fr (M.C. Marden), veronique.baudin-creuza@inserm.fr (V. Baudin-Creuza).

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such as for β-thalassemia blood disorder. AHSP might exhibit two essential functions: the α chain folding as well as a transient stabilization of the α chain pool until binding to its β chain partner [8].

The binding affinity between a ferrous α-Hb and a β-Hb is several orders of magnitude higher than that for the AHSP:α-Hb complex ($<10^{-12}$ M *versus* 10^{-7} M) [9], as expected for a transient chaperone interaction. Remarkably, there is a redox effect/control on the α-Hb binding to AHSP. The affinity of α-Hb for its chaperone is higher for the ferric state relative to the liganded ferrous state as measured at equilibrium by isothermal titration calorimetry [10] and by fluorescence stopped-flow kinetics [11]. Indeed a large structural rearrangement occurs during the transition from the ferrous to the ferric forms of α-Hb bound to AHSP. During autoxidation, bound O_2 dissociates from the iron atom as the superoxide radical and is replaced in a concerted manner by the distal histidine to form a hemichrome or bis-histidyl ferric iron complex that differs from ferric Hb (metHb) in which a distal water molecule is weakly coordinated at neutral pH [12,13]. To achieve its chaperone function, AHSP binding is favored kinetically since its bimolecular rate of binding to α-Hb is 10 times faster than that for the competitive reaction with β-Hb [14,15]. This kinetic pathway depends also on the *in vivo* relative concentration between free AHSP and free β-Hb. The total AHSP concentration has been estimated to reach 100 μM in erythroid cells [2]. Nevertheless if the monomeric β-Hb concentration does not exceed that of AHSP, a newly synthesized α-Hb should first bind to its chaperone followed by replacement by its β-Hb partner to form the tetrameric Hb $\alpha_2\beta_2$ even though a parallel pathway of direct binding to the β chain cannot be excluded; obviously, the β-Hb chain can be considered as the other natural chaperone of the α-Hb chain.

In this work, we demonstrate the influence of the α-Hb redox state on both its binding to and its release from AHSP based on near UV/visible absorption measurements and confirmed previous measurements based on fluorescence kinetics [11]. Since ferrous heme in aqueous media is instantaneously oxidized upon contact with oxygen, it is assumed that nascent α-Hb will first bind to hemin. It is thus of interest to measure the rate reduction of α-Hb bound to AHSP, compared to that of α-Hb after binding to its β-Hb partner. Furthermore, the influence of the hexacoordination of α-Hb bound to AHSP in its stabilization is investigated by site-directed mutagenesis of the E7 His replaced by a Gln. Our data give new insights on the functional role of the α-Hb binding to AHSP in the first events subsequent to the α-Hb synthesis.

2. Material and methods

2.1. Proteins

HbA was purified as described previously [16]. Oxidized Hb (metHb) was generated by oxidation with a slight excess of potassium ferricyanide. The residual ferricyanide and its reduced form were removed by gel exclusion chromatography on a prepacked Sephadex G25 column (GE Healthcare Life Sciences, Uppsala, Sweden). Human α-Hb and β-Hb chains were split from Hb A by reaction with p-hydroxymercuribenzoate acid following the procedure of Geraci et al. [17] with some modifications [18]. The isolated α-Hb and β-Hb chains were then saturated with CO and stored at −80 °C after dithiothreitol removal.

2.2. Site-directed mutagenesis

The αHis58(E7)→Gln mutation was introduced by site-directed mutagenesis (QuikChange® Lightning Site Directed Mutagenesis kit, Stratagene, Agilent technologies, Santa Clara, CA, USA) using the pGEX6P2-α-AHSP vector as template and the following primers: α^{H58Q} 5′-GGT TAA GGG CCA GGG CAA GAA GGT GG-3′ and 5′-CCA CCT TCT TGC CCT GGC CCT TAA CC-3′. Primers were purchased at Eurofins MWG Operon (Ebersberg, Germany). The pGEX6P2-α-AHSP vector allows the expression of the α-Hb associated with AHSP [19]. The presence of mutation and the integrity of the human α-globin and AHSP cDNA coding regions were checked by DNA sequencing (Eurofins MWG Operon).

2.3. Expression, solubilization and purification of different recombinant proteins

Recombinant wild type AHSP ($AHSP^{WT}$) was expressed as a fusion protein with glutathione S-transferase (GST) in *E. coli* BL21 (DE3) cells containing the pGEX-6P2-AHSP expression plasmid [20]. Recombinant mutated α-Hb was co-expressed with $AHSP^{WT}$ as two fusion proteins with GST in *E. coli* BL21 (DE3) cells containing the pGEX6P2-α-AHSP expression plasmid [19]. The different fusion proteins were solubilized and purified as previously described [15,19,20]. No difference was observed in our functional experiments between the recombinant GST-AHSP: GST-α-Hb complex and the recombinant protein complex after cleavage of the GST moiety [19]. Except when mentioned in the text the cleavage of the GST moiety was achieved directly by addition of PreScission Protease (2 units/100 μg of fusion protein; GE Healthcare Life Sciences, Uppsala, Sweden) on GST proteins bound to glutathione Sepharose 4B (GE Healthcare Life Sciences) in phosphate-buffered saline (PBS) (10 mM Na_2HPO4 and 150 mM NaCl, pH 7.5) containing 1 mM dithiothreitol at 4 °C. The $AHSP^{WT}$ was recovered in the flow-through while the GST moiety and PreScission Protease remained bound to the chromatographic support. $AHSP^{WT}$ was then concentrated by ultracentrifugation (Amicon Ultra, Millipore, Billerica, MA, USA). The $AHSP^{WT}$ concentration was estimated using the coefficient of extinction of 11.46 mM^{-1} cm^{-1} at 280 nm. Hb and isolated Hb chain concentrations were estimated using the coefficient of extinction of 192 mM^{-1} cm^{-1} at 420 nm for the carboxylated form.

The gene coding for the hydrophilic soluble domain and linker segment (108 residues) of the human membrane-bound form of cytochrome b5 (MAEQ…SSSS) was cloned between the *Nde*I and *Bam*HI sites of the vector pET-15b (Novagen, Merck Biosciences, Darmstadt, Germany), giving Hb5-pET15b. Note that the recombinant protein contains a N-terminal poly-His tag sequence. The resulting plasmid was transformed in *E. coli* BL21 (DE3) and protein expression was performed at 20 °C using Terrific Broth supplemented with 1 mM δ-aminolevulinic acid. Protein was purified using a nickel-column (Hi-Trap, GE HealthCare Life Sciences) followed by an ion exchange column (DEAE-Sepharose Fast Flow, GE HealthCare Life Sciences) and finally a gel filtration column (Sephacryl S-200, GE HealthCare Life Sciences). Cytochrome b5 (Cyt b5) concentration and purity were estimated spectrally using a coefficient of extinction of 118 mM^{-1} cm^{-1} at 412 nm and the ratio of the absorbance at 412 nm *versus* 280 nm (ratio above 4.0).

2.4. Absorption spectra and auto-oxidation rate measurement

Absorption spectra were measured with a Cary 50. Kinetics of autoxidation were measured on the GST protein complexes using a thermostated diode-array spectrophotometer (Hewlett Packard 8453, Agilent technologies, Santa Clara, CA, USA). Experimental conditions were 50 mM potassium phosphate and 100 mM NaCl, pH 7.4 at 37 °C in the presence of 10 U of catalase from *Aspergillus niger* (Sigma Aldrich N3515) and superoxide dismutase from bovine erythrocytes (Sigma Aldrich S2515) equilibrated under air.

2.5. Kinetics of CO and O_2 recombination

The CO rebinding kinetics of the AHSP:α-Hb^{WT} and AHSP:α-Hb^{HE7Q} complexes with GST after heme ligand photolysis were measured using a Nd:YAG laser (Big Sky CFR-300, Quantel, Les Ulis, France) generating 8 ns/30 mJ pulses at 532 nm. The laser beam, as well as the monochromatic detection light, were transmitted to the optical cuvette by optical fibers. The detection wavelength was varied from 400 to 440 nm, using

one of two sources: the light from an arc lamp (after the excitation monochromater of a SLM fluorometer, Urbana, IL, USA) or a 430 nm LED (Prizmatix, Israel) equipped with an interference filter (Semrock, Rochester, NY, USA). Samples, 5 μM on a heme basis, were analyzed in 4 × 10 mm quartz cuvettes. Measurements were done at 25 °C in 50 mM potassium phosphate buffer with 100 mM NaCl at pH 7.4. For O_2 binding, an O_2/CO mixture was used in order to study the association of O_2 in competition with CO. After photolysis of CO, a rebinding phase is observed between penta-coordinated hemes and both external ligands in competition, followed by a slow phase of replacement of the oxygenated heme fractions by CO in order for the system to return to the CO-bound steady-state. This phase depends on the bimolecular association rates for both external ligands and their respective dissociation rates. CO dissociation occurs on a slower timescale compared to O_2 and can be treated as an irreversible process.

2.6. Circular dichroism

Circular dichroism (CD) spectra and thermal denaturation curves were measured with a Jasco J810 spectropolarimeter (Jasco, Tokyo, Japan), using a 4 mm optical path length cell. For thermal denaturation curves, the cell temperature was programmed using a Jasco PTC-423S thermoelectric temperature controller. The ellipticity at 222.6 nm was monitored over a temperature range of 0–75 °C, using a bandwidth of 1 nm and a temperature gradient of 1 °C/min. The CD signal was normalized to obtain the unfolded fraction: $f_U = (y_N - y_{obs})/(y_N - y_U)$, where y_{obs} is the observed CD signal and y_N and y_U represent the CD signal of the native and unfolded proteins, respectively. The temperature corresponding to 50% unfolded molecule is the melting temperature, T_m.

2.7. Protein binding kinetics

Kinetics of α-Hb binding in competition with AHSP and β-Hb were recorded with a rapid scanning diode array spectrophotometer (HP8453, Agilent technologies) using only the detection light from a tungsten lamp. The concentration of each protein was 1 to 3 μM (on a heme basis for α-Hb and β-Hb) for experiments with the deoxygenated ferrous or oxidized form. The binding kinetic results were simulated by numerical integration of the equations:

$$d(AHSP:\alpha)/dt = -k_{off,AHSP} \times AHSP:\alpha + k_{on,AHSP} \times \alpha \times AHSP$$

$$d(AHSP)/dt = k_{off,AHSP} \times AHSP:\alpha - k_{on,AHSP} \times \alpha \times AHSP$$

$$d(\alpha\beta)/dt = k_{on,\alpha\beta} \times \alpha \times \beta - k_{off,\alpha\beta} \times \alpha\beta - 2k_{24} \times \alpha\beta \times \alpha\beta + 2k_{42} \times Hb$$

$$d(\alpha)/dt = k_{off,AHSP} \times AHSP:\alpha - k_{on,AHSP} \times \alpha \times AHSP - k_{on,\alpha\beta} \times \alpha \times \beta + k_{off,\alpha\beta} \times \alpha\beta$$

$$d(\beta)/dt = -k_{on,\alpha\beta} \times \alpha \times \beta + k_{off,\alpha\beta} \times \alpha\beta$$

$$d(Hb)/dt = k_{24} \times \alpha\beta \times \alpha\beta - k_{42} \times Hb$$

$$[heme] = AHSP:\alpha + \alpha + \beta + 2\alpha\beta + 4Hb.$$

For the deoxy α-Hb release from AHSP we fit the normalized signal as

$$Signal = (4 \times Hb)/heme.$$

For the ferric α-Hb release from AHSP we fit the normalized signal as:

$$Signal = AHSP:\alpha/heme.$$

where AHSP:α is the complex of AHSP with α-Hb, α is α-Hb, β is β-Hb, αβ is Hb dimer, and Hb is the tetramer which participates in the tetramer to dimer (k_{42}) and dimer to tetramer (k_{24}) reactions.

Nonlinear fittings were performed using Scientist program (MicroMath Scientist, Salt Lake City, UT, USA). The β-Hb pool is in equilibrium between monomer and homotetramer species. Since the β-Hb concentration was no more than a few μM in our experiments, a significant amount of monomeric β-Hb ($K_4 = 12 \times 10^{16}$ M^{-3}) was present available for binding the α-Hb after dissociation from AHSP. Taking into account that a homotetramer β-Hb dissociates into monomers within a few second we finally treated the pool of β-Hb as monomers in our simulations.

2.8. Reduction kinetics of ferric α-Hb bound to AHSP

The kinetics of reduction of ferric α-Hb bound to AHSP were performed under anaerobic conditions, under an atmosphere of 10% CO. The different reducing systems were i) 2 mM sodium ascorbate (Sigma Aldrich 11140) at 36 °C, ii) 0.8 μM of soluble, recombinant Cyt b5 premixed during a few mn with 30 nM spinach ferredoxin $NADP^+$ reductase and 50 μM reduced NADPH in the presence of 5 U of catalase from bovine liver (Sigma Aldrich C40) and SOD from bovine erythrocytes (Sigma Aldrich S2515) at 25 °C iii) 1.5 μM spinach ferredoxin (Sigma Aldrich F5875) in the presence of 30 nM spinach ferredoxin $NADP^+$ reductase (Sigma Aldrich F0628) with 50 μM reduced NADPH at 25 °C and iv) 50 μM reduced NADPH (Sigma Aldrich N5130) mediated by 3 μM methylene blue (Sigma Aldrich M9140) at 25 °C. These kinetics were recorded with a rapid scanning diode array spectrophotometer (HP8453) using only the detection light from a tungsten lamp. The results were compared with those obtained for metHb and for the Hb symmetric valency hybrid (α-$HbFe^{3+}$/β-HbCO)$_2$ in the case of reduction by sodium ascorbate.

3. Results

3.1. Protein purification and absorption spectra

The purity of the different preparations was assessed by analytical gel chromatography and spectroscopy. Usually the protein samples are highly pure after elution from the affinity column. No contaminant was observed on analytical gel chromatography compared to the elution peak arising from AHSP or AHSP:α-Hb complexes. Note that the co-expression of $AHSP^{WT}$ with α-Hb^{WT} or α-Hb^{H58Q} gives after purification an equimolar ratio of both proteins based on SDS-PAGE analysis (data not shown) and the absence of a low molecular weight peak shoulder from analytical gel chromatography. This means that the complex is stable on the μM range of concentration studied in this work. We also compared the absorption at 280 nm, which is sensitive to the aromatic amino-acids of both AHSP and α-Hb but also the heme moiety, with that of the peak in the Soret band essentially due to the heme contribution, as a control of the equimolar ratio of AHSP and α-Hb for a co-expression. For instance the ratio of absorbance of the Soret band *versus* 280 nm is estimated to be 4 (1.5 in the presence of GST fusion proteins) taking for α-Hb CO an extinction coefficient of 192/mM/cm at 420 nm. Unless specified otherwise, all experiments were performed in PBS pH 7.5 at 25 °C.

The absorption spectrum of WT AHSP:α-Hb complex was characteristic of the ferric form with the presence of a stable bis-histidyl coordination which is insensitive to pH as previously shown [12,13]. AHSP:α-Hb^{H58Q} spectra were different for the ferric form since E7 Gln cannot form a 6th coordination bond with the iron (Fig. 1). There was an acid–alkaline transition typical of a ligand replacement from a high spin water molecule to a low spin hydroxyl iron, as for normal Hb or Mb. While the ferric complex displays evidence of a bis-histidyl heme hexacoordination for WT AHSP:α-Hb, the spectrum of the deoxy ferrous iron is characteristic of the usual penta-coordinated form for both WT AHSP:α-Hb and AHSP:α-Hb^{H58Q} complexes (insert Fig. 1); the ligand binding site is thus open for the binding of diatomic ligands such as O_2.

3.2. Unfolding determined by circular dichroism

The redox/ligation state of the α-Hb chain is the key determinant of its stability. Indeed upon oxidation the heme affinity for the globin decreases as shown for Mb [21]; the ferric heme dissociates faster compared to the ferrous heme leading to the globin precipitation. The instability of the ferric native α-Hb was measured by thermal denaturation using the CD secondary structure signature of an α helix in the far UV (Fig. 2A). At 37 °C the α-Hb chains are in near-equilibrium between the unfolded and folded states and thus highly susceptible to lose their heme before undergoing a structural collapse. The ferric form is stabilized by high affinity ligands, such as cyanide which induces a 22 °C increase of Tm similar to that of the oxygenated ferrous form. It is worth noting that the carbon monoxide form induces an increase of 30 °C in the Tm; for this measurement 1 mM sodium dithionite was added to prevent heme oxidation at high temperature and allow reversible binding of heme–CO (a soluble form of the heme in solution at pH 7.4).

As expected the ferric α-Hb^{WT} chain is stabilized upon binding to AHSP leading to a very low fraction of unfolded complex at 37 °C (<0.1%) similar to that for the oxy ferrous form (Fig. 2B). This is explained by the fact that binding to AHSP leads to a large structural change of the ferric α-Hb heme pocket with the formation of a hexacoordinated bis-histidyl conformation of the heme. The mutation of the distal His by a Gln destabilizes the AHSP:α-Hb complex with a 6 °C lower Tm and a decrease of the cooperativity behavior of the thermal unfolding isotherm. At 37 °C the ferric AHSP:α-Hb^{H58Q} complex exhibits about 15% of unfolded fraction which is diminished upon the heme reduction (Fig. 2C). While the secondary structure is not affected by the distal mutation with approximately 80% of alpha-helix content predicted from the CD spectra of the oxy complex (data not shown), the free energy of denaturation is decreased by two fold for the mutated ferric complex compared to the WT (−5 kcal/mol at 37 °C). Note that we did not measure a reverse isotherm after a thermal unfolding cycle to 80 °C. The unfolding process might deviate from a two-state model with some irreversible states of denaturation under kinetic control. As we mentioned previously the isotherms of the complex showed a partial reversibility, mainly due to the AHSP refolding contribution [15].

3.3. Kinetics of the replacement of AHSP by β-Hb chains

Initial binding of AHSP to assist in the proper folding, the newly synthesized α-Hb, is aided by a higher association rate relative to β chains. Unlike the nearly irreversible binding of α to β-Hb chains, the complex of AHSP with α chains has a moderate affinity as expected for a transient species on the Hb folding pathway. We measured the influence of the α-Hb chain redox state on the dissociation kinetics of the AHSP:α-Hb complex and further on its rebinding in competition between free AHSP and β-Hb chains (Fig. 3A & B). These measurements were made for the ferric and deoxygenated ferrous forms of α-Hb, based on the absorption change between the steady-state spectra for the α-Hb bound to its chaperone and to its β-Hb native partner.

In the ferrous state, the formation of a deoxy T-state tetramer leads to an increase of the deoxy spectrum amplitude compared to the contribution of the other deoxy species namely the deoxy α-Hb bound to

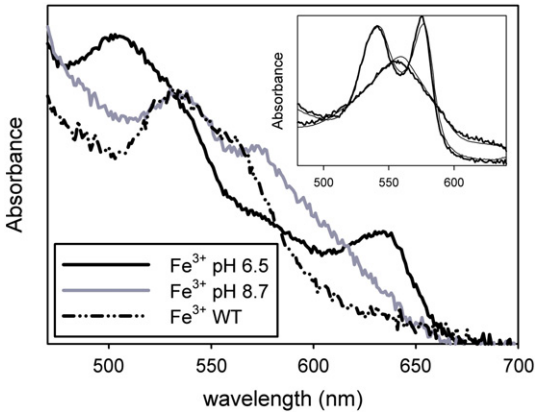

Fig. 1. UV/visible spectra of AHSP:α-Hb (Fe^{3+})^{H58Q} *versus* AHSP:α-Hb (Fe^{3+})WT upon acidic–alkaline pH transition. Ferric α-Hb WT is insensitive to pH and characteristic of a low spin bis-histidyl (E7 and F8) conformation of the heme while E7 Gln α-Hb is mainly in equilibrium with the high spin H_2O molecule and low spin OH^- anion. The insert shows the absorption spectra for the ferrous oxy (α and β bands) and the deoxygenated penta-coordinated ferrous form in the presence of sodium dithionite, which are similar for the H58Q mutant (thick line) and WT AHSP:α-Hb (thin line).

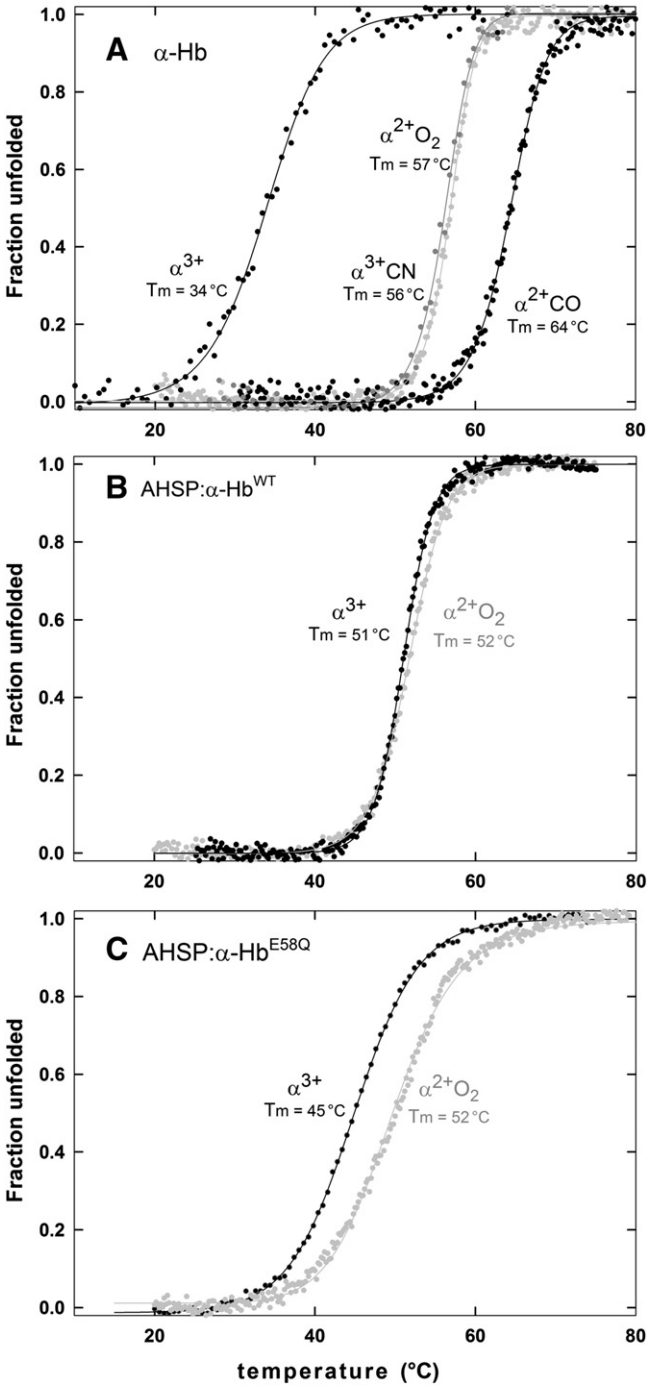

Fig. 2. Thermal unfolding curves for the isolated α-Hb chain (A), AHSP:α-Hb^{WT} complex (B) and AHSP:α-Hb^{H58Q} complex (C) with regard to their redox and ligation states. Measurements were performed for protein concentrations between 2 and 5 μM in 10 mM potassium phosphate buffer with 40 mM NaCl at pH 7.4.

AHSP or within a dimer and the isolated deoxy β-Hb. These last species must be considered as R-like deoxy (R_0) species as opposed to the deoxy (T_0) hetero-tetramer as previously shown (Fig. 3A) [23]. From the deoxy $R_0 \rightarrow T_0$ absorption change one can measure the α-Hb dissociation from AHSP and its subsequent reassociation within a Hb tetramer. The microscopic binding rate constants between AHSP and α-Hb were measured for the first time for a deoxy penta-coordinated state. k_{on} and k_{off} values were similar to those measured for carboxylated ferrous chains using fluorescence emission as detection mode; upon dissociation of the AHSP:α-Hb complex, the tryptophan fluorescence of AHSP quenched by the heme is recovered [20]. Note that such experiments are technically difficult to perform under deoxygenated conditions since the addition of sodium dithionite to keep the sample fully deoxygenated may mask the tryptophan fluorescence emission. As previously

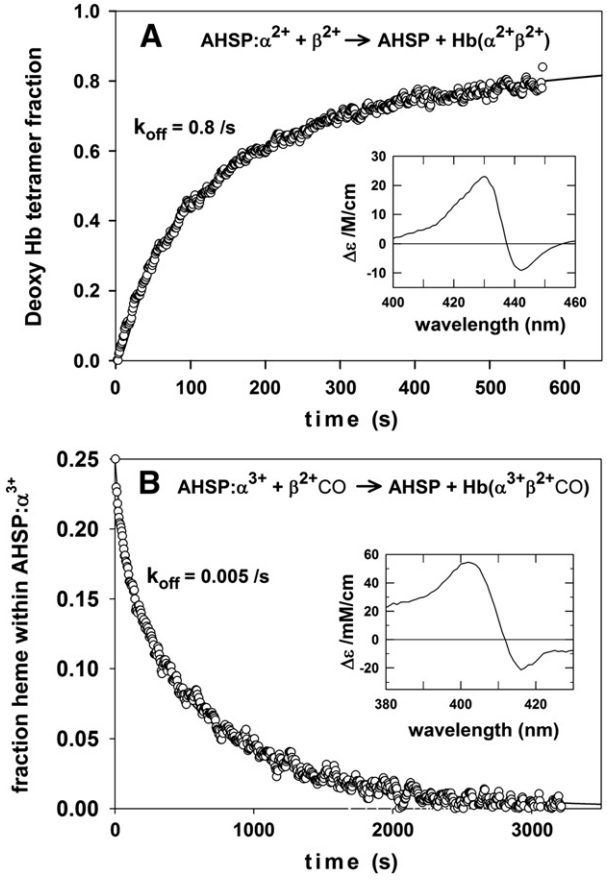

Fig. 3. Replacement reaction. A. Kinetics of ferrous α-Hb release from AHSP complex in competition for rebinding either with AHSP or ferrous β-Hb under deoxygenated conditions. The fraction of Hb tetramer relative to the total of heme species is given *versus* time. The ternary mixture was: 1.2 μM AHSP:α-Hb complex with 1.9 μM free AHSP before the addition of 1 μM β-Hb. The insert shows the variation of absorption monitored in the Soret region due to the formation of (T-state) deoxy Hb tetramer, which displays an increase of amplitude at 430 nm. Simulations indicate a AHSP:α-Hb dissociation rate (k_{off}) of about 1 s and an association rate of 2×10^7 M^{-1} s^{-1}. Best simulations were obtained for the monomer–monomer and dimer–dimer association rates equal to 5×10^5 M^{-1} s^{-1} which are close to those measured for the association between (oxy or CO) liganded α-Hb and β-Hb [11] and for deoxy dimer auto-association [22]. B. Kinetics of ferric α-Hb release from AHSP complex in competition for rebinding either with AHSP or carboxylated β-Hb. Fraction of AHSP:α-Hb complex relative to the total of heme species is given *versus* time. The ternary mixture was: 1.1 μM AHSP:α-Hb complex with 3.3 μM β-Hb. Note that for other protein ratios tested, we obtained similar binding constants after numerical simulations. The insert shows the variation of absorption monitored in the Soret region due essentially to the ferric α-Hb release from AHSP which involves, at this pH, a replacement of the distal His by a water molecule. The best simulations were obtained for the monomer–monomer association rate equal to 1.9×10^6 M^{-1} s^{-1} some 4 times faster than for the above deoxy mixing. This may be due to the charge contribution at the heme site since ferric and ferrous subunits are mixed together. The dissociation rate of the ferric α-Hb is over 100 times slower than for the ferrous form with the same association rate constant of 2×10^7 M^{-1} s^{-1}. The experimental conditions were: PBS at pH 7.5 and 25 °C.

observed for the liganded CO state, best simulations were obtained for a 10 times faster rate of binding of deoxy α-Hb to AHSP compared to that with β-Hb (k_{on} equal to 2×10^7 M^{-1} s^{-1}); the binding rates between α-Hb to AHSP are similar for the CO, deoxy and oxidized forms. The bimolecular rates for Hb monomer–monomer and dimer–dimer association (see figure legends) were allowed to vary as well as the binding parameters for AHSP:α-Hb complex formation. Note that the dissociation rates for Hb dimer and deoxy tetramer are too slow to influence the simulations on the timescale of minutes. Thus, the binding of an external ligand CO or O_2 to the ferrous deoxy α-Hb does not change the overall affinity between α-Hb and AHSP if we compare our data with those for the liganded species reported by the Olson's and Weiss' laboratories [11,24]. The affinity for the complex with ferrous deoxy α-Hb is 40 nM at 25 °C.

Interestingly, in the ferric state the dissociation rate decreases by two orders of magnitude (0.005 s^{-1} at 25 °C; binding affinity of 0.25 nM) compared to the ferrous state as mentioned before based on fluorescence measurements (Fig. 3B) [11]. We are thus in agreement with these latter measurements on the slower dissociation of the ferric state, despite our previous result [15] that did not measure the transition on a slow timescale. In the ferric state α-Hb bound to AHSP is hexacoordinated, but after dissociation from AHSP and binding to β-Hb, the internal E7 distal residue is replaced by a water molecule (or a hydroxyl anion under alkaline conditions). The variation of absorption associated with this ligand transition is shown in Fig. 3B. While the fluorescence technique allows detection of AHSP binding to α-Hb, there is an additional interest of using the absorption detection mode to observe the various heme-based species as pointed out by Mollan et al. [11]. Note that the present kinetics were not strictly mono-exponential but rather extended, since the free [AHSP] increases as the α-Hb release from AHSP complex proceeds toward the formation of the more stable αβ dimeric species.

The higher bimolecular rate of α-Hb for binding AHSP compared to that for binding β-Hb is fully compatible with the AHSP chaperone *in vivo* function to assist the α-Hb folding. However the very slow dissociation rate for the ferric AHSP:α-Hb complex does not support an efficient turnover. Indeed it is reasonable to assume that AHSP should bind first to an oxidized α-Hb if the binding occurs concomitantly or shortly after the α apoglobin synthesis, hypothesis consistent with the results of Komar et al. [25] showing, by the use of a wheat germ cell-free translation, that nascent α-Hb having 86 amino acids can interact with hemin. Even if the heme insertion is a post-AHSP binding event, heme can only acquire its active ferrous form for ligand binding in the apolar heme pocket since a solvent-exposed heme is readily oxidized by H_2O or O_2.

3.4. Kinetics of reduction of the AHSP:α-Hb (Fe^{3+}) complex

We then studied the reduction properties of the oxidized α-Hb bound to AHSP with several enzymatic and chemical redox systems. Kinetics of reduction of the oxidized α-Hb bound to AHSP were made in comparison with α-Hb bound to its native β-subunit within the oxidized Hb tetramer (Fig. 4). To avoid re-oxidation by O_2 after heme reduction, the experiments were performed at low O_2 partial pressure after its replacement by CO gas. Indeed the CO affinity for the heme is two orders of magnitude higher than for O_2 (Table 1) so one can consider in our conditions the reduction as irreversible consecutively to the CO binding. We first tested ascorbate/ascorbic acid chemical redox couple which is part of the natural reducing system found in the RBCs. As shown in Fig. 4A, α-Hb bound to AHSP is reduced about 20 times faster compared to the reduction rate found in the Hb symmetric valency hybrid α-$HbFe^{3+}$/β-$HbFe^{2+}$CO. The reduction rate for metHb is biphasic with a slow component comparable to that measured for the Hb hybrid. Indeed it is well-known that the α-Hb subunits are less susceptible for reduction by ascorbate than their heterologous subunits [26]. Note also that the redox potential is slightly less for the isolated α-Hb *versus*

β-Hb (50 mV *versus* 110 mV) [27]. Thus AHSP binding enhances the rate of reduction of α-Hb by ascorbate relative to the rate for metHb.

We also tested a soluble form of recombinant human Cyt b5 as described in Material and methods section, the natural partner of metHb in red blood cells (RBCs). The reduction rate of AHSP:α-Hb was 2 times faster than that for metHb at pH 7.4 (Fig. 4B). The bimolecular reduction rate at 25 °C was 7×10^3/M/s for AHSP:α-Hb complex at pH 7.4 phosphate buffer with an ionic strength of 0.1 osmol/L (15 mM phosphate and 35 mM NaCl) and 2×10^3/M/s with an ionic strength of 0.3 osmol/L (20 mM phosphate and 100 mM NaCl). We also tested an iron–sulfur electron transport protein from the photosynthetic system, spinach ferredoxin, because of its lower redox potential about −0.4 V but also because the overall charge of this single electron carrier is negative at pH 7.5 so compatible for a bimolecular interaction with the positively charged α-Hb (the AHSP:α-Hb complex interface exhibits essentially hydrophobic contacts) [12,28]. The same feature was observed with 10 times faster reducing rate for the AHSP:α-Hb complex compared to that observed for metHb (Fig. 4C).

Finally we tested methylene blue, which is a potent chemical catalyst for electron transfer reaction and as such facilitates metHb reduction by NADPH; even in this case that allows efficient electron delivery, the reduction rate is about 6 times faster for the AHSP:α-Hb complex compared to metHb (Fig. 4D). For all reducing systems tested with oxidized α-Hb, AHSP binding favors its reduction, thus promoting its release in the ferrous state for binding a β-Hb subunit. Note that the absence of a bis-histidyl hexa-coordination in the AHSP:α-Hb^{H58Q}(Fe^{3+}) complex decreases by three times the rate of ferric heme reduction by ascorbate compared to the WT α-Hb (Fig. 5).

Table 1
O_2 and CO binding and autoxidation properties.

Protein	AHSP:α^{WT}	AHSP:α^{H58Q}	α-HbWT	α-Hb^{H58Q}[a]	α-HbWT[a]
T (°C)	25	25	25	20	20
k_{on}CO (μM^{-1} s^{-1})	1.8	3.5	5.5	6.3	5.2
k_{off}CO (s^{-1})	0.022			0.007	0.016
$k_{on}O_2$ (μM^{-1} s^{-1})	28	31	40	43	40
$k_{off}O_2$ (s^{-1})	120	330	50	42	16
1/KO_2 (μM)	4.2	10.6	1.2	1.0	0.4
$P_{50}O_2$ (Torr)	2.6	6.5	0.75		
KO_2/KCO	350			870	130
Autoxidation (h^{-1}) T (36 °C)	0.9	0.7	0.1		

[a] From [56] 0.1 M phosphate buffer, pH 7.0.

3.5. Kinetics of oxidation

As previously shown α-Hb bound to AHSP becomes oxidized within a few hours [13] much faster than the autoxidation rate of Hb which takes days. In contrast to an isolated α-Hb chain, which readily precipitates at 37 °C after heme oxidation, the AHSP binding avoids the oxidized α-Hb chain precipitation as monitored during the hour timescale absorption change of the reaction in agreement with the above thermal denaturation experiments.

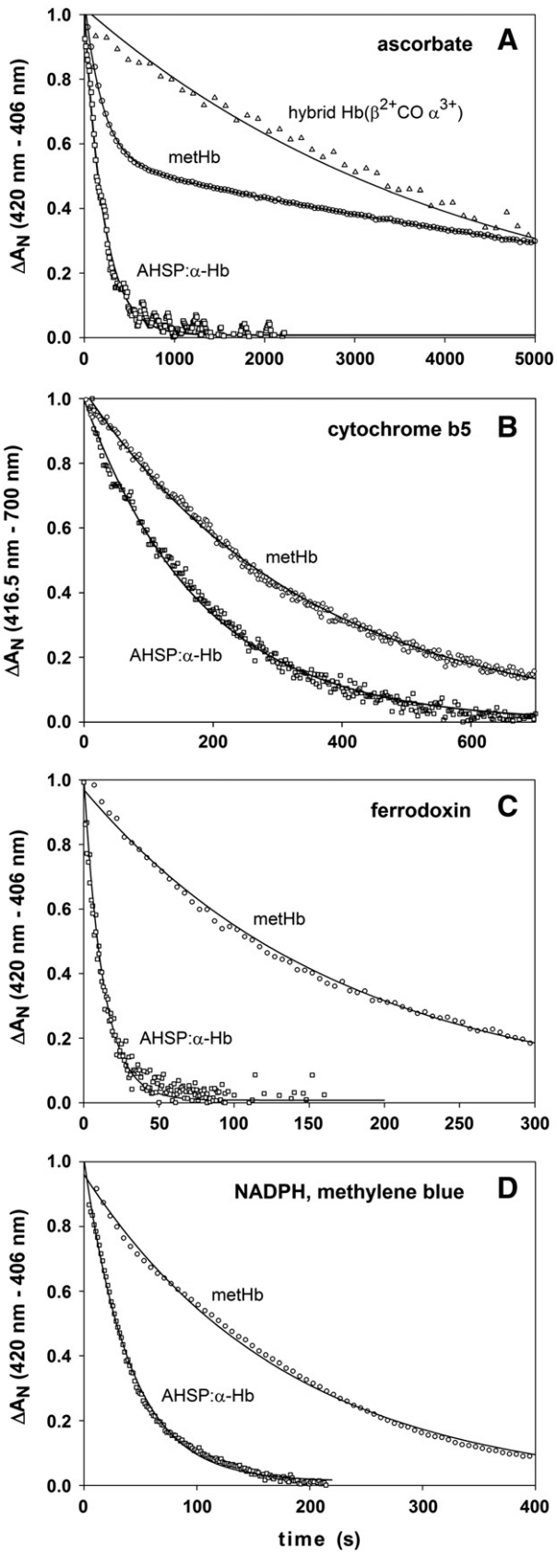

Fig. 4. Reduction kinetics. A. Reduction of the ferric α-Hb bound to AHSP *versus* metHb and the Hb symmetric valency hybrid (αFe^{3+}/β-Hb-CO)$_2$ by ascorbate. The biphasic pattern for the metHb reduction is due to the higher rate for the β-Hb subunits. Experimental conditions were 2 mM ascorbate, 36 °C. B. Reduction of the ferric α-Hb bound to AHSP *versus* metHb by human Cyt b5. Experimental conditions were 0.8 μM recombinant soluble Cyt b5 in the presence of 30 nM spinach ferredoxin reductase with 50 μM NADPH and 5 U catalase and SOD, 25 °C. After the full reduction of Cyt b5 by ferredoxin reductase within a few minutes, 0.6 μM ferric AHSP:α-Hb^{WT} complex or metHb was added. Note that ferredoxin reductase does not reduce the globin samples on this timescale. C. Reduction of the ferric α-Hb bound to AHSP *versus* metHb by ferredoxin. Experimental conditions were 1.5 μM spinach ferredoxin in the presence of 30 nM spinach ferredoxin reductase with 50 μM NADPH, 25 °C. At this substrate concentration, the maximum velocity for reducing the metHb is reached. D. Reduction of the ferric α-Hb bound to AHSP *versus* metHb by NADPH mediated by methylene blue. Experimental conditions were 50 μM NADPH with 3 μM methylene blue. Experimental buffer conditions were PBS at pH 7.5 under 10% CO and [globin] was comprised between 0.5 and 5 μM in these experiments except for reduction by Cyt b5 which were 15 mM phosphate buffer and 35 mM NaCl at pH 7.4.

This faster autoxidation can be explained in part by the lower O_2 affinity of α-Hb bound to AHSP compared to the O_2 affinity of an isolated α-Hb chain or to the R-state affinity of oxy Hb. The O_2 off-rate for α-Hb bound to AHSP is two times faster than that for an isolated α-Hb chain (3–4 times lower O_2 affinity; Table 1). Indeed there is a well-known inverse relationship between O_2 affinity and the autoxidation rate which includes penta-coordinated globins such as Mb or Hb [29]. The E7 His replacement by Gln leads only to a two times lower O_2 affinity for AHSP:α-Hb which indicates that Gln, as a hydrogen bond donor, is able like His to stabilize the Fe–O_2 bond whereas the autoxidation rate is not affected. Thus one can conclude that the distal His is not the main structural determinant of the faster autoxidation of the α-Hb chain bound to AHSP as it has been often proposed, simply because this residue does not bind to the ferrous heme based on the steady-state spectrum of the deoxy complex; the same holds for the E7 Gln which cannot form a 6th coordination bond with the iron. An X-ray structure of the complex AHSP^{P30A}:α-Hb [12] showed a putative O_2 ligand at the proximal side, with the E7 His bound at the opposite distal side of the ferrous heme; the AHSP^{P30A} mutant favors the trans-conformation over the cis–trans isomerization for the WT complex in solution [5,10]. The ferrous complex AHSP:α-Hb^{H58Q} displays spectra typical of pentacoordinated heme (Fig. 1) and bimolecular O_2 and CO rates similar to those of WT α-Hb complex with AHSP (Table 1), suggesting a similar ligand binding pathway and therefore the classical heme structure of native globins with the F8 His coordinated to the iron.

The protective role of the distal histidine binding against reactive oxygen species (ROS) was confirmed by monitoring the oxidation of ferric WT AHSP:α-Hb complex with sub-millimolar H_2O_2 concentrations in comparison with ferric α-Hb^{H58Q}:AHSP complex (Fig. 6). Under these conditions the complex redox chemistry generates first a ferryl species (FeIV═O) and a free radical on the globin moiety followed by other oxidation reactions which finally leads to heme degradation. For instance 20 μM H_2O_2 (10 fold-excess *versus* [heme]) oxidizes irreversibly the ferric α-Hb^{H58Q}:AHSP complex within a few min at 25 °C while on the same timescale only 10% of ferric α-Hb:AHSP complex reacts with 200 μM H_2O (Fig. 6).

4. Discussion

A plausible mechanism has been proposed by which AHSP can achieve its chaperone function by studying the binding dynamics for the formation and the dissociation of the AHSP:α-Hb complex [14,15]. The 10 fold increase in the bimolecular rate of binding of α-Hb to AHSP relative to that for β-Hb, with a consensus value whatever its redox and liganded state of about 10^7 M^{-1} s^{-1} at 25 °C, is a determining kinetic factor for the competition of α-Hb binding to AHSP *versus* β-Hb. The redox state of the α-Hb is also an important factor for driving the dissociation reaction with AHSP; in their ferric state α-Hb chains bind two orders of magnitude more tightly its chaperone due to a very slow dissociation rate [5,11]. We confirm this recent result by a simple absorption measurement and show that the ferrous state in the deoxy penta-coordinated form dissociates 100 times faster as previously shown for the liganded ferrous species [14,15]. Only the bis-histidyl conformation observed after the binding of a ferric α-Hb chain to AHSP stabilizes the complex while in the ferrous α-Hb chain the ligation state does not influence its binding. To obtain this unusual conformation the α-Hb chain undergoes drastic structural rearrangements that further induce long range structural change at the interface with AHSP. The distance between the Cα of the distal and proximal His is shortened by 1.5 Å (length of a covalent carbon–carbon bond) in the ferric AHSP:α-Hb complex by comparison with that in the oxy α-Hb subunit within a Hb tetramer. Structural changes occur at CD corner which are coupled with the position of the E helix and F helix parallel and toward to the heme allowing a 6th coordination bond with the E7 distal His [13,30; Fig. 1 SM]. Note that this hexa-coordinated conformation is also found in numerous globins such as human neuroglobin (Ngb) and cytoglobin [31], reflecting the flexibility of the 3 over 3 α-helical sandwich fold, and more generally in Hb and Mb upon heme oxidation and partial denaturation.

Among possible functional roles of AHSP, fine-tuning of the α-Hb folding is generally expected. Since no change of α-helical content was measured by CD upon binding the α apoglobin to AHSP [5], such a role should rather involve a folding step of the α-Hb shortly after the heminized chain synthesis. Indeed heme binding to an α apoglobin is responsible for a significant increase of its α-helical content which explains the high instability of α apoglobin in solution [32]. To reach its native folding the α apoglobin chain needs a correct heme insertion to further ensure an optimal cooperativity behavior of the Hb tetramer. Even though the first obvious role of AHSP remains the protection of the unstable ferric chain from precipitation in the cytoplasm before its delivery to β-Hb, AHSP might fine-tune the folding of a newly synthesized ferric α-Hb through the formation of the bis-histidyl conformation.

We propose based on this kinetic work the involvement of AHSP in the redox chemistry at the α-Hb heme site. Once the α chain folding is achieved its release to its β-chain partner is triggered by a rapid reduction of the ferric hexa-coordinated heme. Indeed α heme bound to

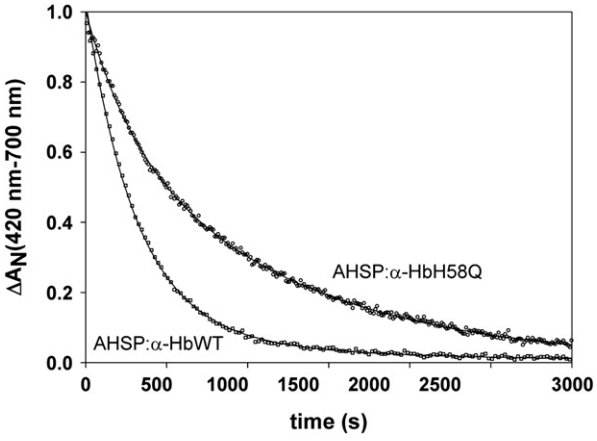

Fig. 5. Reduction of the ferric AHSP:α-Hb $(Fe^{3+})^{H58Q}$ *versus* AHSP:α-Hb $(Fe^{3+})^{WT}$. Experimental conditions were 4 mM ascorbate under 10% CO in 50 mM potassium phosphate, 100 mM NaCl, 10 U catalase and SOD, pH 7.4 at 37 °C. The reduction of WT α-Hb bound to AHSP is monophasic with a rate of 3×10^{-3}/s while for E7 Gln α-Hb the reduction is more biphasic with a main slower rate (65%) of 8×10^{-4}/s.

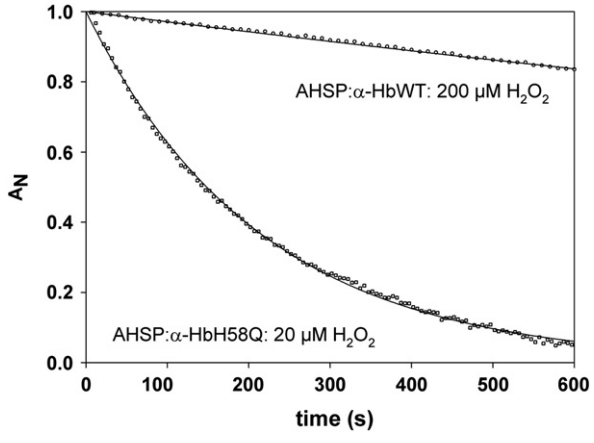

Fig. 6. Oxidation of the ferric AHSP:α-Hb $(Fe^{3+})^{H58Q}$ *versus* AHSP:α-Hb (Fe^{3+})WT by hydrogen peroxide. Experimental conditions were 50 mM potassium phosphate and 100 mM NaCl, pH 7.4 at 25 °C. The WT α-Hb bound to AHSP is less sensitive to H_2O_2 due to the bis-histidyl conformation which disrupts its binding while for E7 Gln α-Hb the oxidation is much faster. The maximum absorption change is shown which corresponds to a drop (about 50%) of the Soret peak as observed for heme proteins upon the formation of irreversible products of heme oxidation. In contrast to MetHb no transient spectrum corresponding to a ferryl species was observed on this time scale.

AHSP is more easily reduced than inside the Hb tetramer and upon its reduction the rate of dissociation from AHSP is one hundred times faster; this higher dissociation rate ensures a better turnover for stabilizing, reducing and delivery of a nascent α-Hb. The reduction properties of ferric α-Hb bound to AHSP seem to be a common property of the hexa-coordinated globin family such as Ngb [33]. The reduction is not limited by the rate constant for the electron transfer like for penta-coordinated globin but by the bimolecular binding rate with the reductant especially if this redox reaction occurs before dissociation of the distal His to avoid a structural rearrangement as a precondition for electron transfer. Amazingly, the reverse reaction of oxidation which is also facilitated for hexa-coordinated globins for instance through the reduction of the molecular O_2 or cytochrome c [34] is probably less favorable for α-Hb bound to AHSP due to the His dissociation in the ferrous state. Indeed the rate of autoxidation is about two orders of magnitude lower than those for strong hexa-coordinated globins [34]. This allows enough time for ferrous α-Hb chains to dissociate from AHSP and then to bind to β-Hb before becoming oxidized and returning to the more stable ferric AHSP:α-Hb complex.

The fact that the reduction of oxidized α-Hb bound to AHSP by the soluble form of Cyt b5 is slightly higher compared to metHb strengthens the role of the cellular redox status as a trigger of the α-Hb delivery to its β-Hb subunits. Indeed Cyt b5 is expected to be the primary electron donor for maintaining a low level of metHb inside the RBCs. The electron transfer between metHb and Cyt b5 is strongly dependent on pH and ionic strength [35,36] which is probably due to the presence of electrostatic contacts between both redox partners. The crystal structure of ferric AHSP:α-Hb complex [13] reveals the presence of predominantly positive charged residues at the globin surface in the vicinity of the heme cavity facing the solvent namely Lys56, Lys60, Lys61, Lys90 and Arg92 which could interact with the negatively charged residues (E44, E43, D60) of Cyt b5 as suggested for the α-Hb chain based on computer simulations [37]. Furthermore Lys 61 ε-amino group can form a hydrogen bond with one propionate carboxylate group in α-Hb within the AHSP complex as well as in metHb [37]. These electrostatic interactions in addition with the α-Hb bis-histidyl heme conformation are certainly important to improve an efficient geometry for electron transfer between AHSP:α-Hb complex and Cyt b5 in order to optimize the electron pathway even though the driving force for electron transfer from Cyt b5 is smaller for AHSP:α-Hb complex than for metHb considering the negative redox potential of −2 mV for Cyt b5 [35] and that of AHSP:α-Hb complex equal to −78 mV [38] compared to those for Hb or the isolated Hb chains lying from +40 to +110 mV [27,38]. Based on a bimolecular rate of 6.2×10^3/M/s for the reduction of metHb by Cyt b5 (50 mM phosphate buffer pH 7.2, 37 °C) and a concentration of Cyt b5 in the RBCs of 0.2 μM, Abe and Sugita [35] calculated the rate for metHb reduction of 1 mM/h assuming 1% of metHb at steady-state and an optimal activity of the Cyt b5 reductase. Since we measured a bimolecular rate of reduction of 7×10^3/M/s (at 25 °C pH 7.4) for the AHSP:α-Hb complex we can also conclude that within a minute a few μM of reduced α-Hb are able to be produced in erythroid cell precursors from its association with AHSP if we assume the presence *in vivo* of a few tenths of μM of reduced Cyt b5 at steady-state and a AHSP concentration of about 0.1 mM. This is consistent, provided that the turnover of the reduction of α-Hb bound to AHSP coupled with its release toward β-Hb chain is stable, with a preferential pathway involving the AHSP binding to α-Hb as a first event during Hb synthesis (the process of the overall α-Hb pool production during erythropoiesis would then require 1–2 days).

Clearly there is evidence that the α-Hb chains are more susceptible to oxidization within the Hb tetramer with regard to the β-chains. For instance the analysis of the relative distribution of both types of ferric subunits can be performed by isoelectric gel focusing since valency hybrids migrate at different positions. Twice the amount of $\alpha^{3+}\beta^{2+}$ hybrids was observed compared to the other symmetrical hybrid containing two ferric β-subunits in a hemolysate from hereditary methemoglobinemia patients [39]. Note that it does not mean that other partially oxidized species are not distributed but due to the dimer exchange in the electric field symmetric hybrids are more stable during the focusing process at 4 °C. The release from AHSP of α-Hb in its ferric form for binding to β-Hb could lead to a partially oxidized Hb tetramer which has a decreased binding capacity and an impaired allosteric transition [40]. Therefore the enhanced rate of reduction for the ferric α-Hb chain within the AHSP complex is also important for delivering a functional Hb tetramer especially due to the lower reduction ability of the α-Hb subunits.

The redox properties and stability of α-Hb are modified by AHSP. One can then consider the relative importance of these roles. The AHSP role could be then more devoted to facilitate the reduction of the α-Hb pool rather than α-Hb folding. In fact a small excess of free α-Hb can cohabitate in RBC with Hb tetramers [6,7,41] provided that the reducing system is able to counterbalance the α-Hb autoxidation. α-Hb chains are not highly unstable in their ferrous state but only and dramatically in their ferric state, where the thermal denaturation studies reveal equal amounts of the unfolded and folded states at 37 °C. Once the complex with AHSP is formed, the Tm increases by almost 20 °C compared to that for the isolated ferric α-Hb. AHSP is thus important for stabilizing the oxidized α-Hb waiting its reduction by the *in vivo* reducing systems. One should also consider that the hexa-coordinated conformation in the oxidized α-Hb bound to AHSP could increase the hemin affinity for α-Hb, which is known to be greatly lower than that of the ferrous heme and as such the main factor of the metHb degradation *in vivo*. Indeed for metmyoglobin the replacement of the low-affinity distal H_2O molecule by cyanide increases the hemin affinity and stabilizes the globin structure [21]. Furthermore hemin is weakly soluble at physiological pH and tends to form dimeric species or non-specific interactions with hydrophobic surfaces and cavities [42,43]. It appears that the binding of the distal E7 His in the AHSP:α-Hb complex does not lead to an increase of the hemin affinity, at least at pH 5.5 and 10 °C [24] so that other additive structural determinants must be responsible for the higher stability of the α-Hb structure after binding to AHSP.

Another putative role of the hexa-coordinated conformation is to protect heme against ROS oxidation because the internal ligand competes with the ROS for heme binding and/or induces steric constraints which prevent its binding. This has been shown for α-Hb bound to AHSP in the presence of a large excess of hydrogen peroxide [13,44] but also for the bis-histidyl hexa-coordinated Ngb [45]. By opposition to an electron transfer reaction involving directly the bis-histidyl bound state, as long as the oxidation–reduction by ROS requires binding to the iron, the distal residue will obviously disrupt such interaction. This is illustrated by the much higher reactivity of the ferric $\alpha\text{-Hb}^{H58Q}$:AHSP toward H_2O_2 compared to the WT complex. In case of α-Hb bound to AHSP this protective role of the hexa-coordination against ROS is only expected for the ferric heme form whereas the oxy ferrous hemes are susceptible to oxidation by ROS [13]. So AHSP exhibits a protective role for the α-Hb chains against ROS oxidations at least after binding the newly synthesized apoglobin and its hemin cofactor.

During normal erythropoiesis AHSP expression seems to follow a pattern similar to that of Hb as revealed from time quantitative PCR on two-phase liquid culture of erythroid cells, with the highest level observed during the production of polychromatic and orthochromatic erythroblasts [46]. It has been also shown that AHSP knockout mice exhibit reticulocytosis indicating a shortened erythrocyte half-life, abnormal erythrocyte morphology with intracellular denatured Hb inclusion with subsequent cellular oxidative damage as observed in β-thalassemia [2]. Thus, the variation of AHSP expression might modulate the phenotype of some hemoglobinopathies. Thereby natural variants of α-Hb with a structural abnormality of the G-H helical region, common to the interface of AHSP:α-Hb and αβ dimer, may be particularly unstable *in vivo* and lead to an α^+-thalassemia phenotype. Several studies have shown that the instability of these α-Hb variants is due to an interaction defect with AHSP [47,48] and/or with β-Hb [49].

Based on this work, mutations located in the distal part of the heme pocket should also be responsible for an alteration of the globin folding through its binding to AHSP if the hexa-coordination with the distal histidine is impaired or if the heme reduction, as a trigger of α-Hb release to its beta chain partner, is less favorable. For all these reasons non-deletional α-thalassemia phenotype might be due to a weak interaction of α-Hb with its chaperone, rather than an intrinsic globin instability, resulting obviously in a cytotoxic accumulation of β-Hb. The same argument holds for β-thalassemia since the relative amount of α-Hb increases compared to that of β-Hb. If the AHSP pool cannot take up and stabilize properly the α-Hb pool then misfolded Hb tetramers might be formed partially oxidized and/or heminized. Obviously AHSP mutations should also be investigated in the future. An AHSP variant V56G has been described recently in the homozygote state to produce a thalassemia phenotype [50]; this variant is less stable than AHSP^{WT} and is significantly unfolded at physiological temperatures (50% loss of alpha helix content) [15]. In β thalassemia pathology, mutation of the AHSP gene or promoter region as well as mRNA instability leading to a decrease AHSP level could be an additional factor of the phenotype expression [51,52].

Finally the expression level of non-deletional α mutation in RBCs can be also affected by the stability of the AHSP:α-Hb complex considering the alpha gene locus. Indeed alpha 2 locus expressed 2–3 more globin than alpha 1 [53]. Consequently the mutated chain fraction can reach 40% which (for a random distribution) leads to 75% of Hb tetramers carrying at least one mutated α-subunit. Without giving a thalassemia phenotype a high proportion of mutated tetramer could impact the RBC oxyphoric functions. The AHSP interaction with a mutated α-Hb chain could affect its level of expression: higher stability compared to that for α-Hb WT leading to a higher expression level and on the other hand a lower expression of a less stable complex. This could be the case for the natural heterozygous variant Hb Boghé α-HbH58(E7)Q for which the mutation is localized on the alpha2 locus [54] whereas the expression level of the mutated α chains reaches only 10% for three heterozygous individuals of the same family. As shown in this work the AHSP:α-Hb^{H58Q} complex exhibits a lower Tm compared to the AHSP:α-Hb^{WT} resulting in a significant unfolded fraction at 37 °C. In addition, the absence of the bis-histidyl hexa-coordination could lead to changes in its susceptibility to oxidation by ROS and to a decrease of the hemin affinity.

It has been recently shown that α-Hb was also present in vessel wall tissues at the junction of endothelial and vascular smooth muscle cells [55] and could play a role in the vessel tone by controlling the NO bioactivity through its NO dioxygenase function. Interestingly the inhibition of the Cyt b5 reductase expression releases the NO suppression via ferrous α-Hb which facilitates its diffusion toward its target, the guanylyl cyclase in vascular smooth muscle cells, inducing finally a vasodilatation response. Since the presence of AHSP was also detected in the endothelial cells, the present results on the regulation of the α-Hb redox state through its binding to AHSP could be transposed to other cell lines.

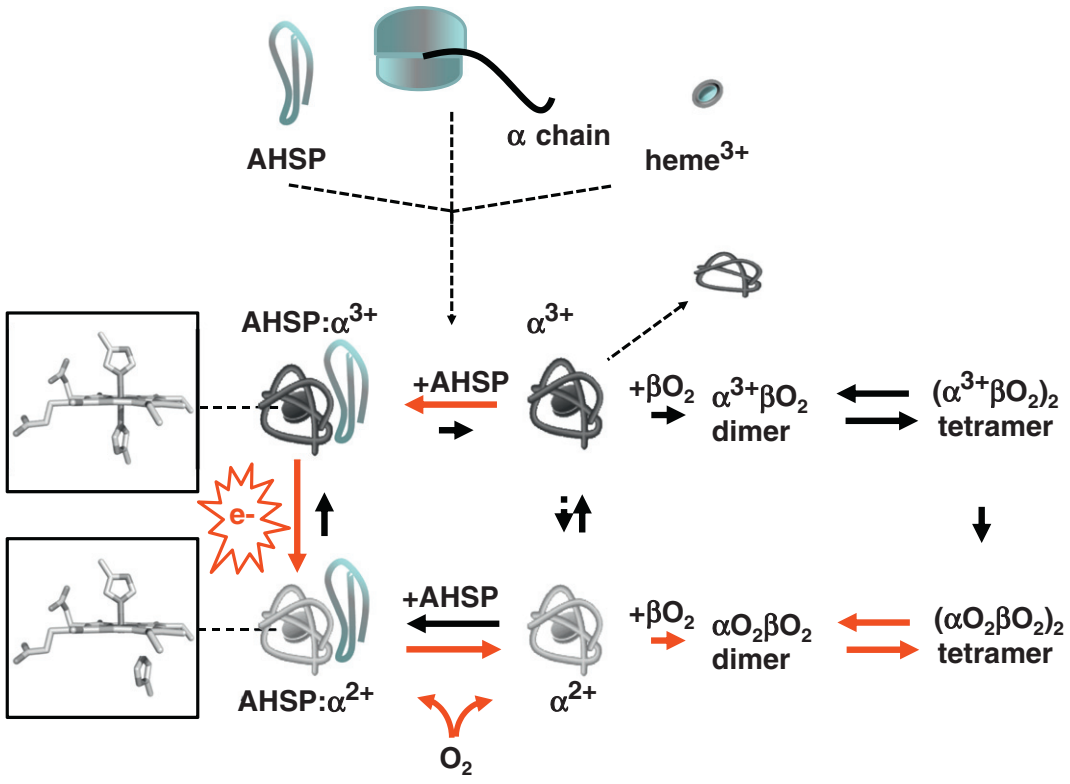

Fig. 7. Putative scheme for AHSP binding to α-Hb. A cofactor ferric heme is incorporated in the α apoglobin concomitantly or shortly before its binding to its chaperone AHSP (thin black dashed lines). Isolated α apoglobin or α-Hb in the ferric state tends to precipitate (thin black dashed lines). The AHSP binding prevails over a direct binding to β chains due to a higher bimolecular rate (and the fact that β chains auto-associate to a homo-tetramer β_4 which dissociate in a few seconds). The correct folding of α-Hb is perhaps enhanced after formation of the bis-histidyl conformation of the heme active site. Then upon reduction of the ferric heme, enhanced within the AHSP complex, the sixth hexa-coordination bond with the distal histidine is removed (allowing O_2 binding) and the α chain is released for binding a beta chain (most likely oxy ferrous due to its high redox potential). The newly formed αβ dimer is delivered in its fully reduced form ready to auto-associate into a functional Hb tetramer. We observe that AHSP accelerates the α-Hb redox reactions and thereby the overall α-Hb/AHSP turnover is facilitated. Indeed the AHSP:α-Hb^{WT} complex after the globin reduction dissociates within a second while in the oxidized ferric state the dissociation is about a hundred times slower. Another pathway for the formation of Hb tetramers might be a direct binding between α-Hb and β-Hb subunits delivering a partially oxidized tetramer with lower O_2 binding capacity and lower binding cooperativity. The pathways depend on the relative AHSP and β chain concentrations in competition for α-Hb binding; for instance, an equivalent steady-state concentration of AHSP and both Hb subunits will lead to 10% of α-Hb binding directly to β-Hb. We therefore consider the dominant pathway for Hb synthesis to be the kinetically favored α-Hb chain binding to its chaperone AHSP, which induces an accelerated reduction and release of the α-Hb chain (solid red lines).

5. Conclusions

AHSP binding to α-Hb is governed by the globin redox state, with the ferric α-Hb exhibiting a higher affinity for AHSP by two orders of magnitude. We show that the ferric AHSP:α-Hb complex can be easily reduced by several chemical and enzymatic systems; the rate is faster than that within the Hb tetramer. Consequently the α-Hb reduction could be the trigger for releasing this globin chain to its final β chain partner in order to produce a fully ferrous functional Hb tetramer as summarized in Fig. 7. Indeed a rapid reduction of the translated α-Hb is crucial since ferric α-Hb is highly unstable. In addition AHSP stabilizes the ferric α-Hb structure by forming a 6th coordination bond with the E7 His giving time for the *in vivo* reducing systems to achieve the ferric α-Hb reduction. This fine-tuning mechanism of AHSP for delivering a reduced functional α-Hb chain also highlights the significant role of the intra-cellular redox balance during erythropoiesis. An additional role of AHSP in RBCs might be protection of ferric α-Hb against ROS binding to prevent the formation of highly reactive ferryl species when the cellular reducing system is overloaded or in the presence of an abnormal excess of free α-Hb during Hb synthesis. Under normoxic conditions the formation of the AHSP:α-Hb(Fe^{3+}) complex should only occur as a transient species; our present results indicate a shorter lifetime of this oxidized form relative to metHbA, thus avoiding excessive ROS production via further redox cycling reactions.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.10.1016/j.bbagen.2013.09.015.

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