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

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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

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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