Transfer of Human α- to β-Hemoglobin via Its Chaperone Protein

EVIDENCE FOR A NEW STATE*

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The α-hemoglobin-stabilizing protein (AHSP), a small protein of 102 amino acids, is synthesized in red blood cell precursors. It binds specifically to α-hemoglobin (α-Hb) subunits acting as a chaperone protein, preventing the formation of α-hemoglobin-cytotoxic precipitates. We have engineered recombinant AHSP in a pGEX vector to study the functional consequence of interaction between AHSP and α-Hb. By in vitro binding assays, we have isolated the complexes glutathione-S-transferase-AHSP-α-Hb and AHSP-α-Hb. The latter assembles as a heterodimer based on size-exclusion chromatography. These complexes exhibited monophasic CO binding kinetics, as observed for isolated α- and β-subunits of hemoglobin. However, the rate of CO (or oxygen) binding to α-hemoglobin bound to its chaperone is three times slower than that observed for isolated α-hemoglobin, demonstrating a form that is intermediate between R- and T-hemoglobin states. The physiologically relevant replacement of the chaperone by β-hemoglobin chains could be detected by both ligand binding kinetics and tryptophan fluorescence quenching.

The poor solubility of α-chains has remained a puzzle during many decades of hemoglobin research. Special protocols were developed in order to store isolated α-hemoglobin chains (α-Hb), and most genetic engineering studies involved the modification of the β-chains (β-Hb) because of the poor expression yield of the α-chain preparations. The common conclusion was that, in order to avoid unfolding, nascent α-chains must rapidly encounter its partner β-Hb. The discovery of an α-chaperone would change that scenario and possibly the molecular origin of certain thalassemias associated with denatured α-chains (1).

Kihm et al. (2) have described recently a protein induced by GATA-1, an essential erythroid transcription factor that is present at a high level (0.1 μM) in human red blood cell precursors and may act as a chaperone for protecting freshly synthesized α-Hb. This protein, named α-hemoglobin-stabilizing protein (AHSP), also known as being the product of the “erythroid differentiation-related factor gene,” is confined to the erythroid lineage and is down-regulated in transmissible spongiform encephalopathies (3). It binds to α-chains but not to β-chains or to hemoglobin tetramers. AHSP was proposed to maintain the solubility of α-chains, preventing them from forming cytotoxic precipitates as observed in β-thalassemias where they are in large excess or in some unstable hemoglobin variants altering subunit contact areas within the tetramer. More recently, dos Santos et al. (4) have shown that, during maturation of the red blood cell precursor, there is a progressive increase in AHSP gene expression following the expression of the α-globin gene, confirming an important function of AHSP during erythropoiesis.

AHSP is a 102-residue protein whose cDNA and gene have been characterized (GenBank™ AF147435 and AY072612). Some properties of the AHSP-α-Hb complex have already been studied by Gell et al. (5). The AHSP and α-Hb are both monomers in solution and form a heterodimer complex with an association constant of 10^7 M^-1 at 20 °C, compatible with a physiological role (5).

AHSP exhibits some similarities with human tubulin chaperone cofactor A and Rbl2p, its yeast homolog. These chaperone molecules are involved in the tubulin-folding process. α-Tubulin and β-tubulin form a heterodimer that polymerizes to form the microtubule. Free excess β-tubulin is toxic for the cell. It has been shown that Rbl2p and cofactor A bind monomeric β-tubulin to form a heterodimer both in vivo and in vitro (6). This finding suggests that the Rbl2p/cofactor A function was the protected cell from free β-tubulin by binding transiently until it associated with α-tubulin (7). The regions of cofactor A interacting with β-tubulin have been characterized in the three-dimensional structure of the molecule (8).

For a better understanding of the stabilizing role of AHSP on α-Hb, we studied the CO recombination kinetics to determine whether the binding of AHSP modifies the properties of α-Hb. The kinetics, as well as the fluorescence of the single tryptophan of AHSP, allows the observation of the replacement of AHSP by β-Hb.

MATERIALS AND METHODS

Preparation of α-Hb and β-Hb—After reaction of human Hb with p-hydroxymercuribenzoic acid, the α-Hb and β-Hb were purified by cation-exchange chromatography as described previously (9, 10) with slight modifications. They were saturated with carbon monoxide and stored frozen at −80 °C.

Recombinant Plasmid Constructs—Human cDNA was isolated by reverse transcription-PCR from erythroid cells with Superscript II reverse transcriptase (Invitrogen) and TaqDNA polymerase (Qiagen). The primers used for PCR were 5’-GGGGATCCATGGCTCTTCTTA-3’ and 5’-AACCAGCTGACAGGCGGGCTGG-3’. PCR conditions were 94 °C for 1 min, 70 °C for 1 min, and 72 °C for 1 min 30 s (50 cycles). The cDNA band was isolated by agarose gel electrophoresis, cut from the gel, and purified using a Qiagen kit (Qiagen). It was then digested with BamHI and XhoI and desalted on a
microcin HR400 column (Amersham Biosciences). This fragment was ligated in-frame with glutathione S-transferase protein (GST) into pGEX-4T-1 plasmid (Amersham Biosciences), and the AHSP gene-encoding sequence was checked (MWG Biotech, Courtaboeuf, France).

**Expression and Solubilization of GST-AHSP**—The pGEX-AHSP construct was expressed in *Escherichia coli* BL21 cells. The GST fusion protein was purified according to the manufacturer’s instructions. The expression of the GST-AHSP was done in the presence of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside at 37 °C. After cell harvest and centrifugation, the cell pellet was suspended in PBS (150 mM NaCl, 10 mM Na₂HPO₄, pH 7.4) containing 5 mM dithiothreitol and 1 glitler lysozyme. After a 45-min incubation on ice, the cells were disrupted in a Sonifier II disruptor (Branson Ultrasonic, Carouge-Geneva, Switzerland) by a maximum of 3 pulses of 10 s each avoiding frothing. The sonicated solution was incubated in the presence of 1% Triton X-100 for 1 h at 4 °C with slight agitation. The homogenate was then centrifuged at 14,000 rpm for 30 min at 4 °C.

**In Vitro Binding Assays**—After centrifugation, the supernatant containing the GST-AHSP was mixed with glutathione-Sepharose 4B beads (Amersham Biosciences) for 1 h with a volume ratio of 4:1 (supernatant:beads). The beads were washed by 10 (bead) volumes of PBS. CO-bound α-Hb in PBS was added in a previous mixture for 1 h. After incubation, the beads were washed with a 30-bead volume of PBS and then the GST-AHSP-α-Hb complex was eluted by adding a 2-bead-volume of glutathione buffer (10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0). The same preparation was performed with β-Hb.

**Thrombin Cleavage**—1 ml of GST-AHSP or GST-AHSP-α-Hb complex bound to bulk beads was incubated with 120 units of thrombin at 14,000 rpm for 30 min at 4 °C. The size-exclusion chromatography on Superose 12 10/300 GL column was performed in Tris acetate 150 mM, pH 7.5, buffer (A and B) or in PBS buffer (C). Values are the means ± S.D.

**Results**

**Production and Size Characterization of GST-AHSP and AHSP**—After expression in BL21 and solubilization, GST-AHSP was purified on glutathione-Sepharose 4B beads. AHSP was obtained after thrombin cleavage of the bead-bound GST-AHSP. SDS-PAGE indicated that GST-AHSP and AHSP amounted to >95% of the eluted proteins. In this system, GST-AHSP and AHSP showed apparent molecular masses of 26 and 12 kDa, respectively, as predicted from the amino acid sequence of GST-AHSP and AHSP.

Size-exclusion chromatography on Superose 12 10/300 GL column in Tris acetate 150 mM buffer showed that both GST-AHSP and AHSP elute as a single species. Based on a calibration curve, the elution volume of GST-AHSP and AHSP corresponded to 151.3 kDa (Ve = 115.5 ml) and 22.9 kDa (Ve = 14.34 ml), respectively, with typical uncertainty of 0.5 kDa (Fig. 1, A and B). These values are consistent with GST-AHSP-forming tetramers (3.97 subunits) and AHSP-forming dimers (1.9 subunits). The width of the GST-AHSP peak at half-height (0.73 ml) suggested that GST-AHSP had some degree of size heterogeneity. In contrast, the AHSP peak width at half-height was smaller and constant (0.54 ml), indicating that AHSP has a high degree of size homogeneity and stability.

The same experiments were repeated at a higher ionic strength with PBS to decrease ionic interactions (Fig. 1C). GST-AHSP eluted at a higher volume corresponding to a molecular mass of 60 kDa, consistent with a dimer species as reported for recombinant GST (14). However, the peak width at half-height (1.89 ml) was larger, indicating a mixture of forms of a different size. The elution profile of AHSP had little change, displaying a molecular mass of 20 kDa, consistent with a dimer species of narrow peak width at half-height (0.54 ml).

Normally for molecular species that associates reversibly into multimers, the eluted peak profile by size-exclusion chromatography and stability shifts with a decreasing concentration. Irrespective of the applied concentration of AHSP (from 50 μM to 0.8 μM) in Tris acetate buffer, this molecular chaperone eluted at the same volume (Ve = 14.35 ml) corresponding to 22.8 kDa. This result indicates that this elution peak of AHSP is not sensitive to concentration, indicating a stable dimer or possibly that it could reflect a monomeric form with a non-globular geometry of the molecule as reported by Gell et al. (5).

**Complex of GST-AHSP and AHSP with α-Hb**—The GST-AHSP-α-Hb or AHSP-α-Hb complex was studied by in vitro binding assays. GST-AHSP bound to glutathione-Sepharose 4B was incubated with α-Hb. The fixation of α-Hb on GST-AHSP imparted a red color on the Sepharose matrix, demonstrating a specific complex between α-Hb and AHSP as shown in Reaction 1.
AHSP + α-Hb → AHSP-α-Hb

**REACTION 1**

After elution by glutathione buffer, a red eluate was obtained. When the same experiment was done with β-Hb solution, the matrix remained uncolored, indicating no β-Hb binding. A soluble red AHSP-α-Hb complex was generated by thrombin cleavage of GST-AHSP-α-Hb bound to the matrix.

Size-exclusion chromatography on Superose 12 10/300 GL column in Tris acetate 150 mM buffer at pH 7.5 showed that GST-AHSP-α-Hb was eluted as a single species at a volume corresponding to a molecular mass of 179 kDa (Ve = 11.3 ml) (Fig. 1B). This value is slightly lower than that expected for a tetramer of GST-AHSP-α-Hb (~200 kDa); however, the addition of the α-chain may not significantly increase the overall hydrodynamic radius and thus the elution volume.

The AHSP-α-Hb complex also eluted as a single species at a volume corresponding to 33.7 kDa (Ve = 13.75 ml) (Fig. 1, A and B). This value is the result of the formation of an AHSP-α-Hb heterodimer. The dissociation equilibrium of this dimer could be studied by the concentration dependence of the elution profile. Irrespective of applied concentration of this complex (from 25 to 1 μM on a heme basis), the complex was eluted at the same volume corresponding to a heterodimer. The peak width at half-height remained small and constant (0.6 ml), indicating that the heterodimer does not dissociate easily and that the association constant is very high as reported by Gell et al. (5).

**Kinetic Studies**—The recombination traces after photodissociation of CO are shown in Fig. 2. For the GST-AHSP-α-Hb complex, similar for α-Hb controls, the recombination kinetics were monophasic. Generally, isolated α-Hb or β-Hb subunits as well as αβ-dimers exhibit a single rapid phase that is similar in rate to the high affinity R-state Hb tetramer. The rate for CO recombination to the GST-AHSP-α-Hb complex exhibits a rate much slower than that of free α-Hb subunits. The same recombination kinetics was observed with the AHSP-α-Hb complex (Fig. 2), i.e. after cleavage of GST by thrombin. This shows that the influence of AHSP on α-Hb is a 3-fold decrease in the rate of CO rebinding to α-Hb. A similar effect for the kinetics of oxygen binding (data not shown) was observed.

When β-Hb was added to the GST-AHSP-α-Hb or AHSP-α-Hb complexes, the CO recombination kinetics became biphasic as expected for tetrameric Hb, showing the two phases corresponding to the two allosteric states. This result implies a dissociation of the complex GST-AHSP-α-Hb or AHSP-α-Hb, allowing the α-Hb subunits to associate with β-Hb to form the αβ2-tetramers (Reaction 2). This demonstrates the physiological reaction expected for a chaperone.

AHSP-α-Hb + β-Hb → AHSP + αβ2 tetramers

**REACTION 2**

The control reconstitution of α-Hb with β-Hb (without AHSP) also showed kinetics typical of Hb tetramers but with less of the slow phase, characteristic of the deoxy (T-state) conformation. The relative amplitude of slow phase is highly dependent on pH, effectors, and sample history (such as reconstitution). Native Hb will show slightly more of the slow phase relative to reconstituted tetramers. In the present case, one could argue that the yield for reconstitution of allosteric tetramers is higher in the presence of AHSP.

**Fluorescence Studies**—The heme group is an excellent energy acceptor and highly quenches tyrosine or tryptophan fluorescence within Hb subunits, typically by a factor of 50. Because the chaperone has a single Trp and no heme group, the fluorescence intensity can be used to detect the formation of the complexes (Reactions 1 and 2) and to estimate the Tyr to α-heme distance.

The fluorescence of AHSP was typical of an exposed Trp and showed little Tyr contribution (Fig. 3). The AHSP-α-Hb complex (step 1) indicated a decrease by nearly a factor of 3 in intensity; note that the α- or β-chain fluorescence is negligible (Fig. 3). Based on the classical Förster energy transfer theory (15), assuming a random orientation for the donor acceptor pair, the Trp to α-heme distance would be 29 Å. Note that, in the case where the α-Hb chains are added to the cuvette containing AHSP, a significant Tyr fluorescence (with peak near 305 nm) remained.
and the GH corner of the α-Hb, which are involved in the αβ1-packing contact, are exposed toward the exterior and do not participate in the binding with AHSP. This packing contact has been recognized to be essential in avoiding heme oxidation and stabilizing the dimers (18). In this model, there is the possibility of a ternary complex AHSP-αβ with AHSP released only upon Hb (αβ)2-tetramer formation. At a low Hb concentration or with a natural variant that tends to dimerize such as Hb Rothschild, the AHSP would not necessarily dissociate.

An alternate model would be the α1β1-type contact for AHSP binding. In this case, the AHSP must dissociate before the β-chain can bind (Fig. 4, Model 2).

If one treats the AHSP as a pseudo-β-chain, certain comparisons can be made. The single tryptophan residue in AHSP (position 44) would correspond to that in position 37 of the β-subunit. The βp-Trp has a distance to neighboring α-chains of 15 Å for the interdimer (αβp)2 and 29 Å for the intradimer (αβp) pair based on energy transfer quenching (15). The observed fluorescence quenching supports the hypothesis that AHSP occupies the αβp-position. This binding would also be compatible with the observed affinity, since the interdimer complex is relatively weak (1 μM). However, after displacement by the β-chain, the chaperone might occupy the secondary site of weaker affinity. The exact stoichiometry would then depend on the overall β-concentration. At a low concentration, the dimers will be present. Under native conditions of the high Hb concentration, there will be a high percentage of tetramers.

The present results show the expected complex between the chaperone and α-Hb with an intermediate stability not nearly as high as the αβ-complex. Thus, our data demonstrate the physiologically relevant reaction of replacement of AHSP by native β-chains. In addition, a new oxygen affinity was observed for the AHSP-α-Hb complex being intermediate to the R- and T-allostERIC states. For intact Hb tetramers, it is difficult to separate the contribution of the α- and β-chains. It is also difficult to extract an intermediate affinity because of the allosteric nature of Hb. The partially liganded species are never highly populated. The AHSP-α-Hb complex allows the isolation of a new state with an affinity approximately three times lower than the classical R-state.

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