Determinants of *Ascaris* Hemoglobin Octamer Formation*

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The oxygen-avid, homo-octameric hemoglobin of *Ascaris* (AH) has an unusual structure. Each polypeptide consists of two tandem globin folds followed by a highly charged COOH-terminal tail that contains four direct repeats of His-Lys-Glu-Glu (HKEE). Deletion analysis of the AH tail determined that at least two of the four HKEE repeats are required for efficient octamer formation. Surprisingly, the first two residues of the tail (Glu-His-His-Glu) alone were moderately effective in promoting multimerization. The hemoglobin from *Pseudoterranova decipiens* (PH) also consists of two globin domains followed by a shorter COOH-terminal extension containing only one HKEE repeat. Interchanging the tails of AH and PH revealed that the PH tail is moderately effective in promoting octamer formation. Dissociation analysis of wild-type and mutant AH and PH revealed that the intact octamers are stabilized by interactions between residues within the globin folds, not the tail. Mutational and biochemical studies revealed that one key interaction is contributed by isolucine 15, which lies in the unusually long AB loop of AH. We propose that the AH tail plays no role in stabilization of the quaternary structure once formed but rather functions as an intramolecular chaperone, aiding assembly of the nascent AH octamer.

The perienteric hemoglobin of the parasitic nematode *Ascaris lumbricoides* has long fascinated scientists because of its exceptional oxygen avidity and unique quaternary structure (1). *Ascaris* hemoglobin is a homooctamer comprised of identical 43-kDa polypeptides (2, 3). Each subunit contains two tandem globin domains followed by a highly charged COOH-terminal tail (see Fig. 1) (4, 5). Recombinant domain one (D1) is monomeric in solution, whereas domain two (D2), containing the highly charged COOH-terminal tail, is capable of forming an octamer (6). Excluding the tail, the two globin folds are highly homologous with 63% amino acid identity (4, 5). The 22-amino acid COOH-terminal tail is highly charged (see Fig. 1) and contains four direct repeats of His-Lys-Glu-Glu (HKEE). Molecular modeling suggests that the tails may form an eight-stranded antiparallel β-barrel stabilized by interstrand salt bridges between side chains (5, 7).

The crystal structure of D1 has been determined. It demonstrates remarkable conservation of the tertiary structure compared with globins of diverse species (8). Along with mutagenesis and spectroscopic analyses, elucidation of the crystal structure of D1 has successfully unraveled the molecular basis for the high oxygen avidity (8–12), yet little is known about the motifs responsible for the formation and stabilization of the intact octamer. Numerous attempts by our laboratory and others to crystallize native or recombinant full-length *Ascaris* hemoglobin have failed.

Interestingly, another parasitic nematode, *Pseudoterranova decipiens*, contains a hemoglobin with marked similarity to *Ascaris* hemoglobin. The hemoglobin of *Pseudoterranova* (PH) is extremely oxygen-avid, with kinetic properties similar to those of *Ascaris* hemoglobin (AH) (13). The polypeptide of PH likewise contains two tandemly repeated globin folds (P1 and P2) followed by a COOH-terminal tail (see Fig. 1) (14). The quaternary structure of PH has not been reported in the literature, but the molecule is believed to be octameric. The globin folds of AH and PH share extensive homology (4). The first domains (D1 and P1) are 73% identical, and the second domains (D2 and P2) are 58% identical. However, there is much less homology in their COOH-terminal tails. The tail of PH is 7 amino acids shorter than that of AH, contains only 1 HKEE repeat, and is substantially diminished in the number of consecutive side chains with alternating charges predicted by a β-strand conformation (see Fig. 1). The importance of the PH tail in subunit assembly is unknown.

Using molecular and biochemical approaches, we previously initiated studies to delineate the role of the tail in *Ascaris* hemoglobin octamer formation (15). The addition of the tail to monomeric D1 or myoglobin did not result in multimer formation. Furthermore, removal of the tail from full-length *Ascaris* hemoglobin substantially decreased but did not eliminate the ability to multimerize. These data revealed that the tail is necessary, but not sufficient, for *Ascaris* hemoglobin octamer formation. In this study, we have defined the contribution of the tail to AH octamer assembly and stability and have identified an additional region of AH important in subunit interactions.

**EXPERIMENTAL PROCEDURES**

*Pseudoterranova* Hemoglobin Construct—cDNA encoding the hemoglobin gene of PH was obtained as two partial EcoRI fragments (a gift from Dr. Bill Pohajdak, Dalhousie University). A two-step polymerase chain reaction (PCR)-based strategy was used to assemble a full-length PH cDNA (15, 16). In Reaction I of the first PCR step, amplification of the 5′ EcoRI fragment (18.3) was performed with a forward primer (5′-agggctatggctcaaaaagcggagagatgtc-3′) complementary to the extreme 5′ end of the coding sequence of PH that also provided an NcoI restriction site along with an additional alanine at the amino terminus. The reverse primer (5′-gtggtctagatgtaggtacctattttctgca-3′) contained 21 nucleotides complementary to the extreme 3′ end of the...
coding region of 18.3 plus 21 nucleotides complimentary to the extreme 5’-coding region of the 3’ EcoRI fragment (10.2). In Reaction II, amplification of the 3’ fragment (10.2) was conducted with a forward primer complementary to the reverse primer used in Reaction I. The reverse primer in Reaction II (5’-gcacctagttggtcgtggctttcggc-3’) was complemented with the extreme 3’ end of AH and also provided a stop codon and an NcoI restriction site. The overlapping products of Reactions I and II were diluted 10-fold and amplified in an additional PCR step with the extreme 5’ and 3’ end-specific oligonucleotides, generating a full-length cDNA encoding PH.

PCR was performed in a Robocycler Gradient 40 (Stratagene). Final reaction components were as described (15). Reactions were cycled 30 cycles, generating a full-length cDNA encoding PH. The coding region of 18.3 plus 21 nucleotides complimentary to the extreme terminator Cycle Sequencing Ready reaction kit (Perkin-Elmer). Positive pET-8C as described (15). Positives were verified by cycle sequencing in M13 derivative 11 l. Controls using 10-fold concentrated hemoglobin in the same volume were performed. In controls for 10-fold dilution, incubations were performed as described above, then the reaction was diluted 10-fold with 2 mM NaSCN in 20 mM Tris, pH 7.5, just before injection onto a Superose 12 column. Variation of the concentration of hemoglobin had no effect on elution profiles. In additional control experiments, fractions corresponding to octamer and aggregate species were isolated. Repeat gel filtration of separated octamer and aggregate revealed that these species are stable and are not in equilibrium. Data were collected and analyzed using Dynamax high performance liquid chromatography Method Manager (Rainin).

**Quaternary Structure Determination—**Hemoglobins were analyzed using a Superose 12 gel filtration column (Amersham Pharmacia Biotech) as described previously (15). Except where stated, 50 μl of hemoglobin at 4.1 mg/ml were injected onto the column. Samples with 5 to 20 mg/ml were 10-fold diluted before injection. For the position 15 mutants, slowing of the elution rate by 10-fold to 0.05 ml/min was also performed as an additional control. Variation of the concentration of hemoglobin or the flow rate of the column had no effect on elution profiles. In additional control experiments, fractions corresponding to octamer and aggregate species were isolated. Repeat gel filtration of separated octamer and aggregate revealed that these species are stable and are not in equilibrium. Data were collected and analyzed using Dynamax high performance liquid chromatography Method Manager (Rainin).

**RESULTS**

**Minimal Length of the Ascaris Hemoglobin Tail Required for Function—**In these experiments, successive truncations of the four HKEE repeats of the AH tail were created (Fig. 1). Mutant hemoglobins purified from E. coli extracts migrated on SDS-polyacrylamide gels as predicted (data not shown). To assess quaternary structure, identical amounts of purified truncated hemoglobins were injected onto a Superose 12 gel filtration column. Elution profiles are shown in Fig. 2. The initial narrow elution peak (~15 min) seen in all panels represents aggregated protein eluting in the void volume. Truncation of one or two HKEE repeats, forming AH-t.1 and AH-t.2 respectively, resulted in hemoglobins with elution profiles indistinguishable from that of AH. However, when three or all four of the HKEE repeats were removed (AH-t.3 and AH-t.4, respectively), the quaternary structures were substantially altered. Several intermediate peaks were visible, including a peak at the position expected for monomer (~45 kDa). Complete removal of the tail (AH-t) caused an even more profound disruption of octamer formation. Intermediate oligomers were detected as for AH-t.3 and AH-t.4. Furthermore, very little properly folded and assembled AH-t was formed when treated with other truncated globins, with the majority of AH-t forming aggregates that were not purified.

**Quaternary Structure of Pseudoterranova Hemoglobin—**To determine the assembly state of the two-domain hemoglobin from PH, we initially pieced together a full-length cDNA from two partial cdNAS. After DEAE purification, gel filtration analysis of recombinant PH (Fig. 3) showed an elution profile similar to that of AH, with a peak at the position expected for
octamer (~20 min), confirming unpublished reports. We also cloned and expressed the separated globin domains, P1 and P2. In solution, P1 was monomeric, and P2 was multimeric (data not shown).

**Analysis of AH and PH Tails**—These experiments were performed to determine whether the tails of PH and AH could promote efficient octamer formation of the other hemoglobin. The tails of AH and PH were interchanged, creating AH with the PH tail (AHPt) and PH with the AH tail (PHAt). Gel filtration analysis was performed to determine the quaternary structure of AHPt and PHAt. Both AHPt and PHAt eluted at positions expected for octamer (Fig. 3, ~20 min), demonstrating that the tails are functional on either hemoglobin.

To assess the contribution of the different tails to octamer stability, wild-type and mutant hemoglobins were disrupted with NaSCN. In these experiments, DEAE-purified recombinant hemoglobins were incubated in the presence of 2 M NaSCN for 5 min. The quaternary structure of disrupted hemoglobins was assayed by gel filtration chromatography (Fig. 4). The dissociation of both AH and AHPt resulted in the production of several intermediates; peaks corresponded to the expected migration of tetramer and dimer with little monomer. Disruption of AH-t resulted in similar disruption of the octamer. The elution profiles for both PH and PHAt drastically differed from those of AH and AHPt. PH and PHAt octamers nearly completely dissociated after treatment with NaSCN, resulting in a predominant peak at the elution time expected for monomer (~43 kDa), with small amounts of higher oligomers present.

**Role of Isoleucine 15 in AH Octamer Formation**—In these

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**Fig. 1. Comparison of Ascaris hemoglobin with Pseudoterranova hemoglobin.** The two tandem globin folds (D1 and D2 for AH, P1 and P2 for PH) are represented by rectangles. The amino acid sequences of the tails are shown. Charges of amino acid side chains are also depicted. Arrows point to the sites of newly generated COOH termini in deletion constructs of the AH tail.

**Fig. 2. Deletion analysis of the Ascaris hemoglobin tail.** Successive deletions of HKEE repeats of the AH tail were constructed. Mutant hemoglobins were purified and injected onto a Superose 12 gel filtration column as described under "Experimental Procedures." Hemoglobin elution from the column was monitored by absorbance at 410 nm. Elution times (in min) were compared with those of molecular weight standards (depicted by arrows). The globin being analyzed is shown in the upper left corner of each panel. The initial peak at approximately 15 min represents protein eluted in the void volume. Octamer elutes at approximately 20 min.
studies, the residue at position 15 was interchanged between domain 1 plus the tail (D1-t) and domain 2 (D2) of AH. Using a two-step PCR mutagenesis strategy, isoleucine was substituted for valine in D1-t, resulting in D1-t V15I, and valine was substituted for isoleucine in D2 (containing the tail), creating D2 I15V. Gel filtration analysis of purified, recombinant globins is shown in Fig. 5. D1-t V15I eluted at the position expected for a monomer, just ahead of myoglobin (17.5 kDa), demonstrating no change compared with monomeric D1-t (15). The minor lower molecular weight peak corresponds to a stable degradation product visible by SDS-polyacrylamide electrophoresis (data not shown). D2 and D2 I15V were expressed and purified in parallel. Identical amounts at equal concentrations of the two proteins were injected onto a gel filtration column to assess quaternary structure. The elution profile for D2 demonstrated peaks corresponding the migration of predominantly tetramer and dimer, with some monomer and octamer present. D2 I15V, however, eluted predominantly at the position expected for monomer, with little dimer and other higher multimers present.

**DISCUSSION**

Previously, our laboratory reported that the primary determinants of AH octamer formation lie within the second globin domain (6). Thus, we targeted the tail as a potential multimerization motif and found that the tail was necessary but not sufficient for octamer formation (15). Furthermore, dissociation studies demonstrated that multiple types of interactions stabilize the AH octamer (15). The goals of this study were to further define the role of the tail in AH assembly and stability and to

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**FIG. 3. Analysis of *Ascaris* and *Pseudoterranova* hemoglobin tails.** The tails were interchanged between AH and PH, generating AHPt and PHAt, respectively. DEAE-purified wild-type and mutant hemoglobins were injected onto a Superose 12 gel filtration column as described under “Experimental Procedures.” Hemoglobin elution from the column was monitored by absorbance at 410 nm. Elution times (in min) were compared with those of molecular weight standards (depicted by arrows). The globin being analyzed is shown in the upper left corner of each panel. The initial peak at approximately 15 min represents protein eluted in the void volume. Octamer elutes at approximately 20 min.

**FIG. 4. Dissociation of hemoglobins.** Hemoglobins were incubated for 5 min in 2 M NaSCN (final concentration) as described under “Experimental Procedures.” Samples were then immediately injected onto a Superose 12 gel filtration column as described under “Experimental Procedures.” Hemoglobin elution from the column was monitored by absorbance at 410 nm. Elution times (in min) were compared with those of molecular weight standards (depicted by arrows). The globin being analyzed is shown in the upper left corner of each panel. The initial peak at approximately 15 min represents protein eluted in the void volume. The migration of intact octamer is represented by the dashed line. The elution profile for AH-t was amplified three-fold to visualize peaks.
Dimer (dimer) are labeled. The residues at position 15 were interchanged between D1 compared with those of molecular weight standards (depicted by arrows). The globin being analyzed is shown in the upper left corner of each panel. The initial peak at approximately 15 min represents protein eluted in the void volume. The additional small molecular weight peak seen for D1+t V15I represents a stable degradation product. Peaks corresponding to the expected migration of octamer (o), tetramer (t), dimer (d), and monomer (m) are labeled.

Identify additional regions of the molecule contributing to octamer stability.

Initially, we determined the minimal sequence of the AH tail required for octamer formation. A series of AH mutants with successive deletions of HKEE repeats was constructed. Removal of up to two of the four HKEE repeats had no effect on oligomerization. However, deletion of three or all four HKEE repeats, or the entire tail, substantially altered subunit assembly. Lower order oligomers, including a significant amount of monomer, were apparent for AH-t.3 and AH-t.4. Surprisingly, further deletion of the first four residues of the tail (EHHE), creating AH-t, had a dramatic effect on octamer assembly. In addition to the presence of smaller multimers, the majority of the protein was aggregated, compared with other AH globins. These experiments demonstrated that 12 amino acids containing two HKEE repeats are required for efficient octamer formation. However, a very short, four-amino acid COOH-terminal extension (EHHE) can partially support AH octamer formation.

P. decipiens contains a hemoglobin with striking similarity to AH (4, 14). The polypeptide of PH is also comprised of two tandem globin repeats followed by a COOH-terminal extension. However, the tail of PH is only 7 amino acids shorter than the equivalent HKEE repeat. Similar to AH, recombinant PH forms an octamer, P1 forms a monomer, and P2 is multimeric (data not shown). We hypothesized that the PH tail may function in promoting efficient octamer formation, as does the AH tail. To test this hypothesis, we interchanged the tails between AH and PH, creating AHPt and PHAt. Not surprisingly, both mutant hemoglobins with heterologous tails retained the ability to form an octamer. However, the PH tail was less effective in promoting octamer formation than the AH tail. Both AH and PHAt had more octamer compared with aggregate than their counterparts with the PH tail, AHPt and PH, respectively (Fig. 3). The reduced length of the PH tail is likely not the cause of this reduced efficiency, as the PH tail is 3 amino acids shorter than the completely functional 12 amino-acid tail of AH-t.2. This suggests that the increased number of HKEE repeats and perhaps the number of consecutive alternating charges in a predicted β-strand conformation (5, 7) enhance the ability of the AH tail to promote octamer formation.

Previous work demonstrated that several types of interactions exist between subunits within the intact octamer (15, 17). We hypothesized that the tail participated in one of these types of interactions, stabilizing the octamer. To test this, we dissociated wild-type and mutant globins with NaSCN, previously demonstrated by our laboratory to selectively disrupt AH into intermediate tetramer, dimer, and monomer intermediates (15). Interestingly, the presence of either tail had no effect on the pattern of intermediates seen. AH and AHPt had similar dissociation patterns, with the expected appearance of tetramer and dimer intermediates with little monomer present. AH-t also displayed a similar profile, albeit with peaks of much lower magnitude because of the decreased amount of octamer in the starting material. Much stronger interactions occur between AH subunits than between those of PH, both PH and PHAt rapidly dissociated into monomer. Because changing or deleting the tail had no effect on the pattern of dissociation intermediates detected, we conclude that the major interactions stabilizing the intact octamer occur between residues within the globin folds. However, the AH tail is absolutely essential for efficient de novo octamer formation. Deletion of the AH tail had a drastic effect, resulting in deficient octamer assembly. Furthermore, deletion of more than two HKEE repeats or replacement with the PH tail had a partial effect on proper folding and assembly. Based on these data, we propose that the tail of AH may function as an endochaperone, aiding the de novo assembly of the AH octamer.

Endo- or intramolecular chaperones are regions of proteins required for proper folding. They were first reported for subtilisin, which was found to fold into an inactive conformation in the absence of its propeptide (18, 19). Examples of other well-characterized proteins containing intramolecular chaperones required for folding include α-lytic protease (20), aqulasin (21), and carboxypeptidase Y (22). Additionally, certain oligomeric proteins contain endochaperones required for assembly, such as von Willebrand factor (23), activin A (24), transforming growth factor β-1 (24), yeast 20 S proteasome (25), and collagen (for review see Ref. 26). In all of these cases, the endochaperone is found within propeptide regions and is then cleaved from the mature protein after aiding folding or assembly of the covalently attached polypeptide. The tail of Ascaris hemoglobin represents a novel example of a potential intramolecular chaperone. The tail is not found within the propeptide region of the molecule, and more importantly, is not cleaved from the properly folded and assembled octamer. The unique primary sequence of the Ascaris tail seems to be critical for maximal function. Perturbations of the tail, either by deletion or by replacement with the related tail of PH, altered its ability to promote octamer formation. It is likely that the PH tail functions as an endochaperone, albeit with reduced efficiency.
Our next goal was to determine regions within the globin fold that contribute to octamer stability. Although D1 is monomeric in solution, it formed a weak, symmetric dimer within the crystalline lattice (6, 8). Previously, we determined that the interface of the D1 crystalline dimer is formed by the E- and F-helices, along with the AB loop. To determine whether any of these residues contribute to stabilizing the intact octamer, we made an array of mutations along the proposed interface, interchanging residues between D1 and D2. The goal of these experiments was to either promote multimerization of monomeric D1 and D1+t or to disrupt D2 multimer formation. Most of the mutations disrupted recombinant protein expression. However, alteration of amino acid 15 was revealing. The residue at position 15 is a valine in D1 and an isoleucine in D2 (residue 165 in the full-length polypeptide). Changing the valine to isoleucine in D1+t had no effect on multimer formation, as the mutant molecule remained monomeric. However, when the residue at position 15 was decreased by only one carbon unit from isoleucine to valine, D2 I15V was deficient in multimerization, forming mostly tetramer and dimer with little monomer. These experiments demonstrate that isoleucine 15 in D2 contributes a key interaction stabilizing the multimer. Isoleucine 15 lies in the unusually long AB loop of AH (4, 5). Based on our analysis of the D1 crystalline dimer, valine 15 of D1 interacted with alanine 86, which is a much bulkier hydrophobic methionine in D2. Residues within the AB loop of AH were initially hypothesized to contribute to the high oxygen avidity (5). However, a role for the AB loop in this capacity has yet to be demonstrated. We conclude that the AB loop, specifically isoleucine 15 in the second globin domain, contributes to stabilizing the unique quaternary structure of AH.

These studies have analyzed the contribution of the Ascaris hemoglobin tail to octamer assembly and stability, using deletion analysis, as well as comparison of AH with PH. We determined that the AH tail is essential for efficient octamer formation, despite the fact that it does not stabilize the intact octamer. Based on our data, we propose that the tail of Ascaris hemoglobin may serve as a novel intramolecular chaperone, which is not cleaved from the mature octamer. Future biophysical and cochemical studies will elucidate the precise role of the Ascaris hemoglobin tail in folding and assembly of the octamer.

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