Subunit Interactions in Ascaris Hemoglobin Octamer Formation*

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The oxygen-avid, perienteric hemoglobin of Ascaris is a homoctetamer. Each subunit contains two tandem globin domains that are highly homologous with the exception of a charged COOH-terminal extension. In solution, recombinant domain one (D1) exists as a monomer, whereas recombinant domain two with the COOH-terminal tail (D2) is primarily an octamer. To examine the role of the COOH-terminal extension in Ascaris hemoglobin multimer formation, we attached the tail to the monomeric, heme-containing proteins, myoglobin and D1; neither construct was capable of multimer formation. Additionally, we removed the tail from both full-length Ascaris hemoglobin and D2. This substantially decreased, but did not eliminate, multimerization. We further characterized subunit interactions by disrupting full-length hemoglobin multimers with the chaotropic salt, NaSCN, which yielded intermediate oligomers. In solution, D2 demonstrated a greater propensity to dissociate than full-length hemoglobin, indicating that D1 contributes to octamer stability. D1 formed a weak dimer in its crystal; thus, we analyzed interactions along the subunit interface. Hydrogen bonds as well as hydrophobic and electrostatic forces appeared to contribute to dimer formation. Amino acid substitutions along this interface in D2 are predicted to enhance subunit interactions for that domain. Our studies reveal that the COOH-terminal tail is necessary, but not sufficient, for efficient octamer formation. Other regions, possibly within the E- and F-helices and AB loops of both domains, appear to be important for Ascaris hemoglobin octamer formation.

The perienteric fluid of the parasitic nematode Ascaris contains an abundant hemoglobin with exceptional oxygen avidity (1). This globin is an octamer composed of identical 43-kDa polypeptides (2, 3). Each subunit consists of two tandem globin domains. A highly charged tail is found at the COOH terminus of the second domain (Fig. 1A) (4, 5). The crystal structure of the first globin domain has been solved, revealing striking similarities to the tertiary structures of globins from diverse species (6). The heme pocket contains a network of hydrogen bonds, including one contributed by a critical tyrosine, which locks the liganded oxygen in place. Mutagenesis studies have provided further support for this (7, 8).

Although the molecular basis of the oxygen avidity of Ascaris hemoglobin has now been described in some detail, structural motifs responsible for the formation of this remarkable multimer have not yet been elucidated. However, molecular cloning and expression of the separated domains have provided interesting clues (9). Domain one (D1), which does not have the COOH-terminal extension, exists as a monomer in solution. In contrast, domain two (D2), which contains the tail, is capable of polymerizing to an octamer. Excluding the tail, the primary structures of D1 and D2 are highly homologous with 63% amino acid identity, implicating the tail as a potentially important component in multimerization (4, 5). The 23-residue COOH-terminal extension is highly polar, possessing 22 charged residues, including four His-Lys-Glu-Glu (HKEE) diads. These charged residues contribute to dimer formation. Amino acid substitutions along this interface in D2 are predicted to enhance subunit interactions for that domain. Our studies reveal that the COOH-terminal tail is necessary, but not sufficient, for efficient octamer formation. Other regions, possibly within the E- and F-helices and AB loops of both domains, appear to be important for Ascaris hemoglobin octamer formation.

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The abbreviation used is: PCR, polymerase chain reaction.
The purpose of this experiment was to assess the ability of the highly charged Ascaris hemoglobin tail to promote multimer formation of a structur-
ally related, monomeric protein. Secondary and tertiary structures of mammalian myoglobins and Ascaris hemoglobin domains are very similar, despite low amino acid homology (6, 12). A two-step PCR strategy as described above was utilized to add the Ascaris hemoglobin tail to the COOH terminus of dog myoglobin, resulting in Mgb+t. The mutant protein was expressed in E. coli and migrated on SDS-polyacrylamide gel electrophoresis as predicted, based on molecular mass (data not shown). Recombinant Mgb+t had the visible absorbance spectrum characteristic of a myoglobin, indicating the presence of a properly folded, functional, heme-containing protein. In order to assess quaternary structure, the recombinant protein was analyzed using gel filtration chromatography. Mgb+t eluted at the position expected for a monomer (21 kDa), just ahead of myoglobin without a tail (17.5 kDa), with no detectable multimeric species (Fig. 2). The minor lower molecular weight peak is likely due to degraded recombinant protein.

Analysis of Ascaris Hemoglobin Constructs—To determine the role of the COOH-terminal tail in Ascaris hemoglobin multimerization, constructs of full-length hemoglobin and the separated domains, D1 and D2, were created using PCR (9). D1 plus the COOH-terminal tail (D1+t), D2 lacking its tail (D2−t), and full-length hemoglobin lacking the tail (AH−t) were also constructed (Fig. 1). Mutant proteins were expressed in E. coli, purified, and analyzed by gel filtration chromatography. As expected, D1 was predominantly monomeric (Fig. 3A). Full-length hemoglobin (AH) eluted at the position expected for an octamer (Fig. 3B). At the same concentration, D2 was predominantly octameric, but also formed detectable amounts of dimer and an additional multimeric species, most likely tetramer (Fig. 3C). AH−t was drastically reduced in its ability to form an octamer (Fig. 3B). Elution times indicated the presence of a variety of species, including monomer, dimer, and tetramer with little octamer. D2−t was similarly reduced in its ability to multimerize (Fig. 3C). Major peaks were detected at elution times representing primarily dimer, and to a lesser extent, monomer. Minor peaks at elution times corresponding to tetramer and octamer were also detected. In both tail-less constructs and D2, a substantial amount of high molecular weight aggregate was observed. D1+t remained monomeric with an elution profile almost indistinguishable from that of D1 (Fig. 3A).

Dissociation of Full-length Ascaris Hemoglobin—In order to gain insight into the nature of the interaction(s) involved in octamer formation, NaSCN, a chaotropic salt, was utilized to dissociate Ascaris hemoglobin multimers. For these experiments, full-length recombinant hemoglobin was incubated in the presence of varying concentrations of NaSCN. After 20 min, samples were subjected to gel filtration chromatography to assess quaternary structure (Fig. 4). Hemoglobin peaks were detected by monitoring absorbance at 410 nm in order to selectively detect only properly folded molecules. As expected, Ascaris hemoglobin with no added NaSCN eluted at a position consistent with an octamer. In the presence of 1 M NaSCN, analysis revealed shoulders at tetramer and dimer positions. However, a further increase to 1.5 M NaSCN resulted in a substantial decrease in the elution peak corresponding to octamer with a concomitant rise in peaks representing tetramer and dimer. A small peak corresponding to monomer (Mr = 43,000) was also detected. Dissociation was accentuated in the presence of 2 M NaSCN; elution times suggested a further decrease in octamer along with a marked rise in dimer.

In the next part of this experiment, purified full-length hemoglobin was incubated in the presence of 2 M NaSCN for increasing amounts of time to induce further dissociation of multimers. Quaternary structure was again assessed by gel filtration chromatography (Fig. 5). At 20 min, as in Fig. 4D, substantial dissociation into dimer and tetramer was observed. By 60 min, there was additional loss of octamer and tetramer populations with a striking increase in dimer. Upon incubation...
for 90 min, more substantial dissociation into monomer was observed. Similar results were obtained upon incubation for 135 min (data not shown). After 180 min, no peaks were observed, due to subunit denaturation with subsequent loss of the heme moiety.

**D1 Dimer Interface Analysis—**Although D1 exists as a monomer in solution (9), it forms a weak dimer in the crystalline lattice. The largest intermolecular contact, approximately 1100 Å² in area, occurs across a crystallographic dyad and is three times larger than any other contacts. The interface of the D1 dimer is at the E- and F-helices of each subunit with additional contacts from residues in the A-helix and AB-loop to the E- and F-helices (Fig. 6). Significant amino acid interactions between D1 subunits are summarized in Table I. The arginine residue at position 3 (Arg₃) in the A-helix forms a strong ionic interaction with Asp⁷₃ in the E-helix, whereas Glu⁹₀ and Arg⁹₄ interact more weakly. The main chain amide and carbonyl oxygen atoms of Val¹⁵ form hydrogen bonds with the side chains of Glu⁸₂ and Arg⁹₉, respectively. A number of additional side chains are located within the interface and make contact with residues in the other subunit, including Val⁷₃ with Val⁷₃ and Ala⁸⁶ with Val¹⁵ and Leu⁶⁹.

**DISCUSSION**

Recently, the crystal structure of Ascaris hemoglobin domain one was reported (6). However, because D1 alone does not form multimers in solution (9), this structure has not defined regions of the full-length molecule involved in octamer formation. The two tandem globin folds are highly homologous with the exception of a highly charged, 23 residue COOH-terminal extension on D2, which contains 22 ionizable residues, including four direct HKEE repeats (4, 5). Perutz and colleagues (5) have suggested that this tail may be capable of forming an eight-stranded β-barrel. Thus, we targeted the Ascaris hemoglobin tail as a potentially important structure required for octamer formation.

We first tested the ability of the tail to directly promote multimerization of a structurally related but monomeric protein. Myoglobin served as an appropriate candidate, due to its homologous compact, highly α-helical structure (6, 12). The engineered myoglobin, Mgb-t, was incapable of forming multimers. This raised the possibility that the myoglobin either contained regions that interfered with multimerization or lacked certain motifs necessary for multimer formation. In
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In this study, mutagenesis experiments demonstrated that the COOH-terminal tail is required for stable octamer formation, but is not sufficient to promote multimerization alone. Dissociation experiments and analysis of the D1 crystalline dimer provided further insight into subunit interactions. Unquestionably, solution of the crystal structure of full-length Ascaris hemoglobin or D2 will provide the most direct approach for elucidating the structural basis of octamer formation. This will allow direct visualization of quaternary structure, including relevant contacts between globin domains, as well as interactions between the charged COOH-terminal tails.

To address this, we constructed a mutant of a completely unrelated molecule, glutathione S-transferase, which upon addition of the charged tail was also incapable of forming multimers (data not shown). Taken together, these data suggest that interaction of other regions in the Ascaris hemoglobin molecule are essential for multimerization. Indeed, construction and analysis of D1+t demonstrated that the tail alone is unable to promote multimer formation of the monomeric, Ascaris hemoglobin domain. In contrast, full-length hemoglobin without a tail, as well as D2 without a tail, were still capable of forming the full complement of multimers, albeit in a poorly controlled fashion. The most abundantly observed multimer in these tail-less hemoglobins was a dimer, suggesting that the regions responsible for dimer formation lie within the globin fold of D2. Based on these data, we propose that the ability to multimerize is intrinsic to the globin region of D2 and that the COOH-terminal tail serves to stabilize the Ascaris hemoglobin octamer.

Previously, Darawshe and Daniel (3) proposed that Ascaris hemoglobin adopts a two-layered arrangement with four monomers in each layer. Using cross-linking analysis, the distribution of cross-linked species corresponded to theoretical values predicted from a two-layered arrangement with one type of interaction within a layer and another type between layers. Based on these results, the investigators suggested that a disruptive agent could possibly discriminate between these interactions, yielding tetramer and dimer intermediates. Additionally, the polar zipper model of Perutz and co-workers (5, 10) aligns four monomers on either side of the β-barrel, consistent with the arrangement proposed by Darawshe and Daniel (3). A previous investigation using pH extremes showed dissociation of Ascaris hemoglobin directly into dimers and monomers (3). In our studies, a chaotropic salt, NaSCN, served as a more selective reagent for disruption of the octamer. If only one type of interaction was responsible for octamer formation, then the multimer should dissociate directly into monomers. However, gel filtration analysis yielded elution times corresponding to dimer as well as tetramer, indicating that several types of interactions exist within the octamer. In our NaSCN experiments, it is possible that some subunit reassociation occurred during gel filtration analysis. Regardless, the detection of intermediate species indicates the existence of multiple types of interactions between subunits. These data, combined with our analysis of the tail-less mutants, suggest that interactions between the globin folds, in addition to those between the charged tails, are important for octamer assembly.

Although D2 is certainly capable of multimerization, its ability to form an octamer is reduced in comparison to the full-length molecule, demonstrated by the presence of detectable intermediates. Furthermore, upon dilution, D2 readily dissociated, whereas the full-length molecule remained octameric (data not shown). These results suggest that the first domain in native Ascaris hemoglobin may contribute additional interactions required for the formation of a stable octamer, despite the inability of separated D1 with the tail to multimerize. The fact that D1 forms a weak dimer in the crystal provides further support for this hypothesis. Distinct from vertebrate hemoglobin tetramer interactions (12), the D1 interface is formed by the E- and F-helices along with the AB loop. Examination of surface residues at the D1 dimer interface revealed numerous favorable hydrophobic and electrostatic interactions. This dimer may represent actual molecular interactions that occur between D1 subunits within native Ascaris hemoglobin in solution. Furthermore, an analogous interface may serve as a major site of D2 subunit interactions. Interestingly, Val73 and Ala86 in D1 are replaced with leucine and methionine, respectively, in D2. Both of these substitutions result in side chains with greater hydrophobic surface areas than D1, which could serve as a strong driving force promoting multimer formation.

Table 1

| Distance | Å     |
|----------|-------|
| Arg9 N 1 | 3.05  |
| Arg9 N 2 | 2.88  |
| Val13 O  | 2.98  |
| Val73 C 1 | 3.59 |
| Ala86 C 1 | 3.98 |
| Glu82 O 1 | 3.82 |
| Arg9 N 2 | 3.30  |

Fig. 6. Stereo diagram of the D1 dimer interface. Helix backbone atoms of each D1 subunit are shown with the AB-loop and the E- and F-helices highlighted in bold.
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