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Thermoglobin, Oxygen-avid Hemoglobin in a Bacterial Hyperthermophile*

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The hemoglobin family of proteins, ubiquitous in all domains of life, evolved from an ancestral protein of primordial function to extant hemoglobins that perform a myriad of functions with diverged biochemical properties. Study of homologs in bacterial hyperthermophiles may shed light on both mechanisms of adaptation to extreme conditions and the nature of the ancestral protein. A hemoglobin was identified in *Aquifex aeolicus*, cloned, recombinantly expressed, purified, and characterized. This hemoglobin is monomeric, resistant to thermal and chemical denaturation, pentacoordinate in the ferrous deoxygenated state, and oxygen-avid. The oxygen equilibrium dissociation constant is approximately 1 nM at room temperature, due in part to a hydrogen bond between the bound ligand and a tyrosine residue in the distal pocket. These biochemical properties of *A. aeolicus* thermoglobin, AaTgb, may have been shared by the ancestral hemoglobin, thus suggesting possible primordial functions and providing a starting point for consequent evolution of the hemoglobin family.

Proteins of the hemoglobin (Hb)[3] family, also referred to as the myoglobin (Mb) or globin family, are gas-binding heme proteins found in all domains of life. Hbs have evolved slightly different structures and functions, but both the predominantly helical structure and certain amino acids are well conserved (1). Distinct, but related, classes of Hbs are widespread in Bacteria, Archaea, and Eucarya (2). Classical examples are human Hb and Mb, found in blood and muscle, respectively, which use pentacoordinated heme for ligand binding. Monomeric Mb binds O_2 for intracellular storage; tetrameric Hb binds O_2 cooperatively for intercellular transport. The oxygenated forms of these proteins also dioxygenate nitric oxide, detoxifying it to nitrate as part of a protective function to prevent inhibition of respiration in muscle and brain tissue (3). The first Hb identified in Bacteria was found in *Vitreoscilla stercoraria* (4). Similar single-domain Hbs (SDHbs) in Bacteria possess pentacordinated heme and helical structures similar to that of eukaryotic Hbs except for a deletion in the CE region (5). Protoglobins (Pgbs) in Archaea contain an additional Z helix at the N terminus and bind O_2 weakly (6). Originally found in a unicellular eukaryote (7), truncated Hbs (TrHbs), which are also found in plants and Bacteria, have deletions in the A helix and internal sites, contain hexacoordinated heme in the unliganded state, and have a high affinity for O_2 (8, 9). Full-length hexacoordinate Hbs in vertebrates have moderate O_2 affinity and may be found in all tissue (10, 11) or exclusively expressed in one organ such as the brain (12). Similar hexacoordinate Hbs are also found in plants, which in some cases also contain pentacoordinate forms that allow O_2 transport to symbiotic nitrogen-fixing bacteria in legumes and related species (13).

Hb is also found fused to other domains in Bacteria, Archaea, and eukaryotic microorganisms including pathogenic fungi. The flavohemoglobins (Fhbs), which contain a SDHb and a flavin binding reductase domain (14), serve to detoxify nitric oxide (15). Pgbs fused to sensor modules, globin-coupled sensors (GCSs), act as signal tranducers (16). Some invertebrates contain proteins consisting of multiple fused copies of Hb domains, and certain large extracellular Hbs consist of both Hbs and scaffold proteins that associate to form discrete units for O_2 transport in coelemic fluid (17).

To further characterize the diversity of Hbs and identify properties of the ancestral protein, a search was conducted for homologs in autotrophic hyperthermophiles from Bacteria, organisms that are basal on the phylogenetic tree of life and may therefore resemble the last universal common ancestor (18–20). A gene encoding a protein homologous to Hb was identified in *Aquifex aeolicus*, a hydrogen-oxidizing obligate chemolithautotroph that grows at temperatures of ~95 °C under microaerobic conditions (21). *A. aeolicus* thermoglobin, AaTgb, is monomeric, resistant to thermal and chemical denaturation, pentacoordinate in the ferrous deoxygenated state, and oxygen-avid. A tyrosine residue donates a hydrogen bond and stabilizes bound O_2. Such properties may also belong to the ancestral protein from which extant Hbs evolved.

MATERIALS AND METHODS

Computational Search and Cloning—V. stercoraria Hb (4) was used as the query sequence for BLASTP searches limited to Bacteria using the nr database (22). An initial hit was required to have fewer than 300 residues and an *E* value of less than 0.01. Organisms from which hits emerged were screened for hyperthermophiles that grow optimally at 80 °C or greater. Multiple sequence alignments for comparison with other Hbs were performed with ClustalX (23) and manually adjusted based on known crystal structures. Open reading frame *Aq211*, annotated from the genome sequence of *A. aeolicus* (21), was amplified from genomic DNA and cloned into pET17b with flanking NdeI and BamHI sites. The Y29F mutant was generated with the QuikChange site-directed mutagenesis kit (Stratagene).

Expression and Purification—Expression plasmids were transformed into *Escherichia coli* BL21(DE3) (Novagen). Cells were grown in LB with

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[3] The abbreviations used are: Hb, hemoglobin; Mb, myoglobin; SDHb, single-domain hemoglobin; Pgb, protoglobin; TrHb, truncated hemoglobin; Fhb, flavohemoglobin; AaTgb, *Aquifex aeolicus* thermoglobin; β-ME, β-mercaptoethanol; SWMb, sperm whale myoglobin; CD, circular dichroism; GdmCl, guanidinium chloride.

200 μg/ml ampicillin at 37 °C for 16–24 h after reaching an A_{600} of 0.5. Overexpression was achieved by autoinduction of the *lacUV5* promoter in stationary phase. Frozen cell pellets were thawed, lysed by sonication in 50 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol (β-ME), 1 mM phenylmethylsulfonyl fluoride, pH 6.8, and treated with 60% ammonium sulfate at 4 °C. The supernatant was treated with 95% ammonium sulfate, and the pellet was dialyzed against 50 mM Tris, 1 mM EDTA, 1 mM β-ME, pH 8.7. The dialysate was concentrated, brought to room temperature, and applied to a 30-ml Q-Sepharose Fast Flow (Amersham Biosciences) column. The protein was washed with 1–2 column volumes of 50 mM Tris, 50 mM NaCl, 1 mM EDTA, 1 mM β-ME, pH 8.2, and eluted with 1–2 column volumes of the same buffer with 100 mM NaCl. The red band was collected, concentrated, and loaded onto a Superdex 200 HiLoad 16/60 column (Amersham Biosciences) equilibrated in 25 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA, 1 mM β-ME, pH 6.8.

Spectrophotometry—Visible absorption spectra were acquired on an 8453 UV-visible spectrophotometer (Agilent) at room temperature. To obtain oxygenated spectra, purified protein was reduced with dithionite, and reaction products and excess reducing agent were removed on a Sephadex G-25 column equilibrated in 50 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA, 1 mM β-ME, pH 7.5. To obtain deoxygenated spectra, excess solid dithionite was added directly to an oxygenated sample. Protein concentration was estimated using the ferrous oxygenated sperm whale Mb (SWMb) extinction coefficient of 128 mM^{-1} cm^{-1} at the Soret peak maximum (24).

Hydrodynamic Radius Measurements—Analytical gel filtration was performed by injecting 100 μl of 100 μM protein into a Superdex 75 HR 10/30 column (Amersham Biosciences) equilibrated in 50 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA, 1 mM β-ME, pH 7.5, at 4 °C. Elution times were calibrated with two sets of size exclusion standards: 6.5-kDa aprotinin, 12.4-kDa cytochrome *c*, 29-kDa carbonic anhydrase, and 66-kDa bovine serum albumin (Sigma); and 13.7-kDa ribonuclease, 25-kDa chymotrypsinogen A, 43-kDa ovalbumin, and 67-kDa albumin (Amersham Biosciences). Single-angle dynamic light scattering experiments were performed on a DynaPro-MS/X (Proterion) with 40 μl of 100 μM protein in 25 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA, 1 mM β-ME, pH 6.8. Measurements were obtained at 4, 20, 37, and 55 °C using default acquisition parameters. Data were processed with Dynamics version 5.

Thermal Stability Analysis—Circular dichroism (CD) spectra were acquired on a J-710 spectropolarimeter equipped with a PTC-348W Peltier temperature control device (Jasco). 400 μl of 10 μM protein in 25 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA, 1 mM β-ME, pH 6.8, was analyzed in a 1-mm cuvette (Hellma). After acquisition of an initial spectrum at 20 °C, the sample was heated to 90 °C and a second spectrum obtained. The sample was then cooled back to 20 °C for recording of a final spectrum.

Chemical Unfolding Titrations—AaTgb was oxidized with ferricyanide in the presence of strong light to facilitate dissociation of tightly bound O_2. Apoprotein was prepared by adding cold 100 mM HCl to concentrated ferric AaTgb and SWMb to adjust the pH to 2.2 and then adding 2 volumes of cold methyl ethyl ketone to extract the dissociated hemin (25). The remaining apoprotein was dialyzed against 10 mM potassium phosphate, pH 7.0, overnight at 4 °C, and the concentration determined from the A_{280}. Unfolding of holo- and apoAaTgb and SWMb by guanidinium chloride (GdmCl) was followed at equilibrium by CD changes monitored at 222 nm. The measurements were collected using a combined CD titration protocol in an Aviv spectropolarimeter (26–28). Equilibrium unfolding was obtained by mixing two solutions with the same concentration of protein, one containing only 200 mM potassium phosphate, pH 7.0, and the other also containing 6–7 M GdmCl. Mixing was controlled by a Microlab 500 automatic pipetting system (Hamilton).

Ligand Binding Kinetics—Most AaTgb samples were isolated in the reduced state and used directly. Samples containing significant amounts of ferric AaTgb were reduced with dithionite and passed through a Sephadex G-25 column equilibrated with 1 atm O_2 or air. All experiments were carried out in 100 mM phosphate, 2 mM EDTA, pH 7.0, at 20 °C. The sample solutions were drawn into a gas-tight syringe and inserted into a sealed 1-mm cuvette previously equilibrated with the appropriate O_2 partial pressure. O_2 association time courses were measured after complete laser photolysis of oxygenated AaTgb samples using a 300-ns excitation pulse from a Phase-R 2100B dye laser (29). Bimolecular recombination was measured at 436 nm under pseudo first-order conditions, and simple exponential rebinding time courses were observed for most of the samples. O_2 association rate coefficients, k'_{O_2}, were calculated from the slopes of plots of k_{obs} *versus* $[O_2]$, where k_{obs} is the observed pseudo first-order association rate. O_2 dissociation rate coefficients, k_{O_2}, were determined by analyzing time courses for ligand displacement in a stopped-flow spectrometer. In these experiments, solutions of 5–10 μM AaTgb containing various concentrations of free O_2 were mixed with 100 mM potassium phosphate, pH 7.0, equilibrated with 1 atm CO at 20 °C. The O_2 dissociation rate coefficients were calculated from $k_{O_2} = r_{obs}(1 + k'_{O_2})[O_2]/k'_{CO}[CO])$, where r_{obs} is the observed pseudo first-order replacement rate coefficient, and k'_{CO} and k'_{O_2} are the association rate coefficients for CO and O_2 association, respectively, which were determined independently in the laser photolysis experiments described above (30). CO association time courses were measured either in stopped-flow rapid mixing experiments, in which reduced and deoxygenated AaTgb was mixed with various concentrations of CO, or in laser photolysis experiments where rebinding was followed after complete photodissociation. Rate coefficients for CO dissociation, k_{CO}, were determined by analyzing stopped-flow time courses in which the heme-bound CO was displaced with high concentrations of NO. Equilibrium coefficients for O_2 and CO binding, K_{O_2} and K_{CO}, respectively, were calculated from the ratio of the association and dissociation rate coefficients.

RESULTS

Gene Identification and Sequence Analysis—BLASTP searches using *V. stercoraria* Hb as the query sequence were performed in an attempt to find a Hb gene in a bacterial hyperthermophile. *V. stercoraria* Hb was chosen because it was the first Hb found in Bacteria (4) and is well characterized. The upper limit on amino acid residues in the search criteria was introduced to eliminate fusions with other domains; the ancestral Hb is presumably a single domain protein. Of all the species with potential Hbs, only *A. aeolicus* is a hyperthermophile (21). *Aq211* encodes a protein that aligns well with the Hb fold (Fig. 1) and bears 43% identity to *V. stercoraria* Hb, the query sequence. Among other Hbs, Aq211 bears the most similarity to Fhbs and SDHbs (data not shown). *E. coli* and *Alcalignes eutrophus* Fhb are 40 and 39% identical, respectively, to Aq211. Even SWMb and human Hb β still retain 14 and 17% identity, respectively, with Aq211. Because the protein product is a Hb from a hyperthermophile, the gene will be referred to as *AaTgb*, *A. aeolicus* thermoglobin.

The 139-amino acid sequence of AaTgb conforms well to determinants of the eukaryotic Hb fold. Two strictly conserved residues (1) are indeed present. Histidine occupies the heme-linked, proximal F8 site. Although replaced by other residues in a few truncated Hbs (8, 9), the

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                    ...A1..............B1.......B10.....C2....CD1.......D1
P. catodon SWMb     -VLSEGEWQLVLHVWAKVEADVAGHGQDILIRLFKSHPETLEKFDRFKHLK--TE    52
H. sapiens Hb β     VHLTPEEKSAVTALWGKV--NVDEVGGEALGRLLVVYPWTQRFFESFGDLS--TP    51
A. aeolicus AaTgb   -MLSEETIRVIKSTVPLLKEHGTEITARMYELLFSKYPKTKELFAGASEE-----    49
V. stercoraria Hb   -MLDQQTINIIKATVPVLKEHGVTITTTFYKNLFAKHPEVRPLFDMGRQESLE--    52
E. coli Fhb         -MLDAQTIATVKATIPLLVETGPKLTAHFYDRMFTHNPELKEIFNMSNQRN----    50
A. eutrophus Fhb    -MLTQKTKDIVKATAPVLAEHGYDIIKCFYQRMFEAHPELKNVFNMAHQ------    48

                    .....E1....E7..E11......E20.........F1.F4..F8.....G1...
P. catodon SWMb     AEMKASEDLKKHGVTVLTALGAILKKKGH---HEAELKPLAQSHATKHKIPIKYL   104
H. sapiens Hb β     DAVMGNPKVKAHGKKVLGAFSDGLAHLDN---LKGTFATLSELHCDKLHVDPENF   103
A. aeolicus AaTgb   -----------QPKKLANAIIAYATYIDRLEELDNAISTIARSHVRRN-VKPEHY    92
V. stercoraria Hb   -----------QPKALAMTVLAAAQNIENLPAILPAVKKIAVKHCQAG-VAAAHY    95
E. coli Fhb         ---------GDQREALFNAIAAYASNIENLPALLPAVEKIAQKHTSFQ-IKPEQY    95
A. eutrophus Fhb    -------EQGQQQQALARAVYAYAENIEDPNSLMAVLKNIANKHASLG-VKPEQY    95

                    .............G19...H1.................H21..........
P. catodon SWMb     EFISEAIIHVLHSRHPGDFGADAQGAMNKALELFRKDIAAKYKELGYQG---     153
H. sapiens Hb β     RLLGNVLVCVLAHHFGKEFTPPVQAAYQKVVAGVANALAHKYH---------     146
A. aeolicus AaTgb   PLVKECLLQAIEEVLNP--GEEVLKAWEEAYDFLAKTLITLEKKLYSQP---     139
V. stercoraria Hb   PIVGQELLGAIKEVLGDAATDDILDAWGKAYGVIADVFIQVEADLYAQAVE-     146
E. coli Fhb         NIVGEHLLATLDEMFSP--GQEVLDAWGKAYGVLANVFINREAEIYNENASK     145
A. eutrophus Fhb    PIVGEHLLAAIKEVLGNAATDDIISAWAQAYGNLADVLMGMESELYERSAEQ     147
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FIGURE 1. **Structure-based multiple sequence alignment of AaTgb and other proteins with the Hb fold.** The alignment includes AaTgb, SWMb, human Hb *β*, *V. stercoraria* Hb, *E. coli* Fhb, and *A. eutrophus* Fhb. Only the Hb domains of Fhbs are shown. The helical positions of some residues are denoted, and conserved functional positions are *shaded*.

nearly invariant phenylalanine at the CD1 position is also found in AaTgb and presumably forms van der Waals contacts with bound heme. Key strongly, although not strictly, conserved positions (1) are preserved in the AaTgb sequence. Proline occupies the C2 position, initiating the start of the C helix. Although histidine occupies the distal E7 position in most plant and animal Hbs, this residue is commonly replaced by glutamine in many invertebrate and bacterial Hbs (5) including AaTgb. Leucine occupies the E11 position of the distal ligand binding pocket, in which leucine, isoleucine, or valine are normally found. In most Hbs, the F4 position of the proximal ligand binding pocket contains a leucine residue that serves to prevent heme loss (31). Isoleucine replaces leucine in AaTgb, but likely performs the same function.

The predicted secondary structure of AaTgb resembles that of Fhbs and *V. stercoraria* Hb as opposed to other classes of bacterial Hbs. Unlike truncated Hbs, which lack the N-terminal helix of canonical Hbs (8, 9, 32), AaTgb does contain residues that align well with the A helix of other Hbs. An additional N-terminal Z helix not found in eukaryotic Hbs is found in protoglobins (6) and GCSs (16, 33), but AaTgb does not contain such a segment. All bacterial Hbs seem to lack the D helix of animal Hbs (5), and sequence alignment suggests that the same is true for AaTgb. Similarity with Fhbs and SDHbs, not TrHbs, protoglobins, or GCSs, was presumably selected because *V. stercoraria* Hb was used as the query sequence (data not shown).

Recombinant Expression and Purification—AaTgb was cloned and expressed in *E. coli*. The purified protein migrates as a homogenous band on an SDS-PAGE gel at the expected molecular mass (Fig. 2*A*). Freshly reduced and oxygenated protein yields a Soret peak at 417 nm, and α and β bands at 575 and 544 nm, respectively (Fig. 2*B*). This spectrum of oxygenated AaTgb is very similar to that of oxygenated *V. stercoraria* Hb (34) and *E. coli* Fhb (14), with the β band being larger than the α band. In oxygenated human Hb (35) and SWMb (24), the two peaks are roughly equal in height with the α band being slightly more intense. The spectrum of the deoxygenated AaTgb (Fig. 2*B*) looks similar to pentacoordinate, rather than hexacoordinate Hb. One broad peak at 555 nm replaces the α and β bands, and the Soret peak shifts to 433 nm as expected for a pentacoordinate deoxygenated heme (36, 37). Spectroscopy suggests that recombinant AaTgb is purified predominantly in the ferrous oxygenated state (data not shown).

Oligomeric State—The oligomeric state of AaTgb was determined by measuring the apparent molecular mass of the purified protein based on the hydrodynamic radius. Gel filtration chromatography yields an apparent molecular mass of 21 kDa (Fig. 3). The elution time does not change over the protein concentration range of 100 μM to 10 nM (data not shown), suggesting that the peak represents a single species and not the equilibrium between two oligomeric states at these concentrations. Single-angle dynamic light scattering yields an apparent molecular mass ranging from 13 to 20 kDa; the results at 4, 20, 37, and 55 °C are similar. The polydispersity values range from 10 to 20%, suggesting the presence of a single, monodisperse species. The expected molecular mass of AaTgb with one heme molecule is 16.6 kDa. The hydrodynamic radius measurements are therefore consistent with a monomer in solution; no dimers or larger oligomers are detected.

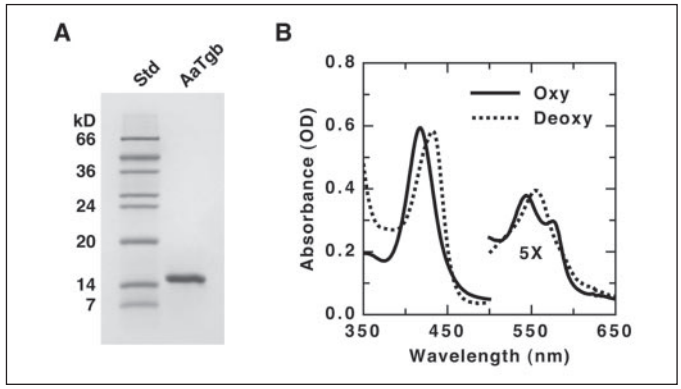

FIGURE 2. **Purification and absorption spectra of AaTgb.** *A*, recombinant AaTgb on a 15% SDS-PAGE gel. *Lane 1*, molecular mass standards. *Lane 2*, purified AaTgb. *B*, visible absorption spectra of oxygenated (*solid line*) and unliganded, deoxygenated (*dotted line*) ferrous AaTgb.

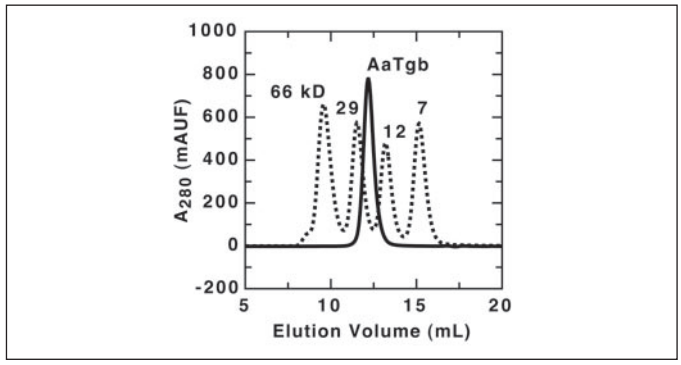

FIGURE 3. **Size exclusion chromatography of AaTgb.** The elution profiles of AaTgb (*solid line*) and molecular weight standards (*dotted line*) are shown.

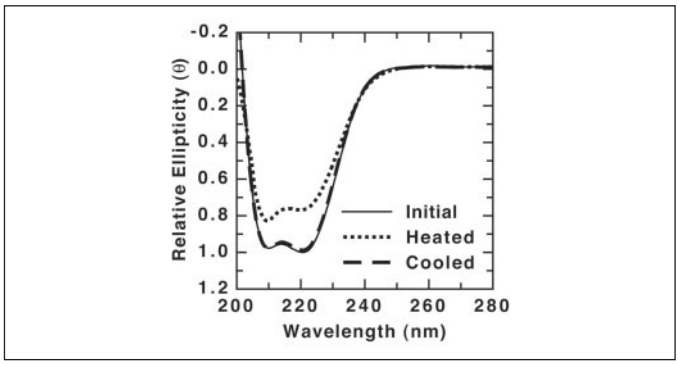

FIGURE 4. **Temperature-dependent CD measurements of AaTgb.** The near UV CD spectra of AaTgb at 20 °C (*solid line*), heated to 90 °C (*dotted line*), and cooled back to 20 °C (*dashed line*) are shown. The amount of circular dichroism is expressed as the measured ellipticity divided by the maximum ellipticity obtained in the initial 20 °C sample.

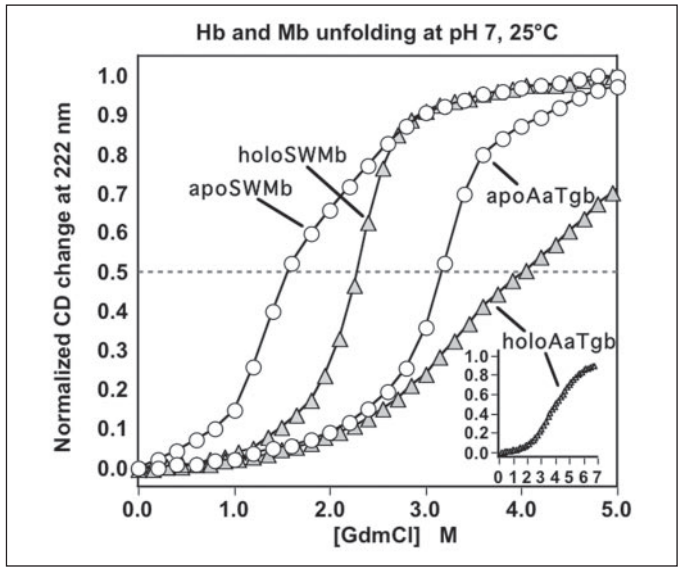

FIGURE 5. **GdmCl-induced unfolding of AaTgb and SWMb at pH 7.0, 25 °C.** The titration profiles of apoAaTgb (*white circles*), ferric holoAaTgb (*gray triangles*), apoSWMb (*white circles*), and ferric holoSWMb (*gray triangles*), are shown as normalized CD changes.

Thermal Stability and Resistance to Chemical Denaturation—The thermal stability of the AaTgb secondary structure was tested by observing the CD spectrum of the protein at different temperatures. AaTgb contains significant α-helical content at 20 °C, and ~75% of the signal at 222 nm is still present even at 90 °C (Fig. 4). Unfolding is reversible as complete refolding of the protein is observed after cooling of the heated sample back to 20 °C. The α-helical signal gradually decreases as the protein is slowly heated from 20 to 90 °C, but no sharp unfolding transition is observed (data not shown). The secondary structure of AaTgb is therefore resistant to thermal denaturation.

The resistance of holo- and apoAaTgb to unfolding by GdmCl was compared with that of holo- and apoSWMb at pH 7.0 and 25 °C (27, 28). A comparison of the unfolding curves shows differences in stabilities (Fig. 5). The midpoint GdmCl concentration for denaturation of apoAaTgb is ~3.5 M and twice as large as that for apoSWMb, ~1.5 M. A similar, ~2-fold increase is observed for ferric holoAaTgb compared with ferric holoSWMb, ~4.2 *versus* ~2.2 M. Estimating unfolding constants is difficult without a complete mechanistic analysis. Fits to a simple two-step scheme or comparisons of unfolding midpoints to SWMb mutants with high stabilities (28), however, indicate that the folding constant for apoAaTgb in these conditions is 100 to 1,000 times greater than that for apoSWMb. A similar large increase in the folding constant is observed for the holoprotein.

Ligand Binding Properties—The reaction rates of reduced AaTgb with O_2 and CO were measured by laser photolysis and rapid mixing methods (TABLE ONE). AaTgb binds O_2 with a k'_{O_2} of 20 μM^{-1} s^{-1} (Fig. 6*A*), which is similar to that for O_2 binding to mammalian Hbs and Mbs (38, 39). k_{O_2}, however, is ~1000-fold slower, 0.019 s^{-1} (Fig. 6*B*). As a result, K_{O_2} for AaTgb is 1100 μM^{-1}, which is ~1,000-fold greater than that for mammalian Hbs and Mbs. The O_2 partial pressure at which ligand binding reaches half-saturation, P_{50}, is calculated to be only 0.0006 mm Hg for AaTgb. k'_{CO}, and k_{CO} for CO binding to AaTgb are 5.1 μM^{-1} s^{-1} and 0.0066 s^{-1}, respectively, and K_{CO} for AaTgb is 780 μM^{-1}, which is only ~30-fold greater than K_{CO} for SWMb. AaTgb binds both O_2 and CO with greater affinity than those of the SDHbs from *Clostridium perfringens* and *Campylobacter jejuni* (40). K_{O_2} and K_{CO} for *C. perfringens* Hb are 120 and 2.0 μM^{-1}, respectively, ~9- and ~400-fold smaller than the same values for AaTgb. Similarly, K_{O_2} and K_{CO} for *C. jejuni* Hb are 140 and 1.1 μM^{-1}, respectively, ~8- and ~700-fold smaller. Because of preferential enhancement of O_2 affinity, AaTgb binds O_2 and CO with similar affinity, yielding a K_{CO}/K_{O_2} of only 0.71. This value is low compared with ratios of ~300 and ~30 for mammalian Hbs and Mbs, respectively. *C. perfringens* and *C. jejuni* Hb show even greater discrimination in favor of O_2 binding than AaTgb, yielding K_{CO}/K_{O_2} values of 0.017 and 0.0083, respectively. The SDHbs from *C. perfringens* and *C. jejuni*, *Ascaris suum* Hb, and AaTgb are members of a small set of Hbs able to preferentially bind O_2 in the presence of CO.

The kinetic parameters for O_2 and CO binding to AaTgb resemble those of the oxygen-avid Hb from the nematode *A. suum* (TABLE ONE), which also has glutamine at the E7 position and tyrosine at the B10 position. Replacement of tyrosine at the B10 position with phenylalanine in the Y29F AaTgb mutant increased k'_{O_2} and k_{O_2} ~10- and ~100-fold to 260 μM^{-1} s^{-1} and 1.9 s^{-1}, respectively. K_{O_2} for the Y29F AaTgb mutant, 140 μM^{-1}, is reduced ~10-fold compared with that of the wild type protein. The Y29F mutation has an opposite effect on CO affinity. k'_{CO} and k_{CO} for CO binding to the Y29F mutant are 83 μM^{-1} s^{-1} and 0.0038 s^{-1}, respectively, resulting in a K_{CO} of 22,000 μM^{-1}. The mutant K_{CO}/K_{O_2}, 150, is ~200-fold greater than that of the wild-type protein. These results suggest that the hydroxyl group of the B10 tyrosine side chain provides a strong hydrogen bonding interaction that allows AaTgb to discriminate in favor of O_2 and against CO binding.

DISCUSSION

Hemoglobin Sequence—AaTgb may be described as a basic Hb. The amino acid sequence is compact, without additional residues or domains at either terminus beyond the A and H helices of the canonical fold (Fig. 1). AaTgb resembles a building block upon which residues may be deleted from or inserted into the termini to yield truncated Hbs (41) or protoglobins (6), respectively. This basic fold may even be fused to other domains to yield Fhbs (14) or GCSs (16), or duplicated and fused onto itself to yield Hbs with multiple copies of the globin domain (17).

Physiological Function—The biological role of AaTgb is not known, although its biochemical properties provide some clues. *A. aeolicus* is cultured in a microaerobic environment consisting of only 1% O_2, but requires O_2 because growth does not occur under anaerobic conditions (21). The high O_2 affinity of AaTgb at 20 °C suggests that significant binding may still occur at high temperatures, but further experiments are required to measure ligand binding properties under conditions physiologically relevant to *A. aeolicus*. Binding with high affinity and a slow dissociation rate, however, seems to preclude functions involving O_2 transport or storage. The sequence

TABLE ONE

Ligand binding parameters for AaTgb in comparison to other heme proteins at pH 7.0, 20 °C

Protein	k'_{O_2}	k_{O_2}	K_{O_2}	P_{50}	k'_{CO}	k_{CO}	K_{CO}	K_{CO}/K_{O_2}
	$\mu M^{-1}s^{-1}$	s^{-1}	μM^{-1}	*mm Hg*	$\mu M^{-1}s^{-1}$	s^{-1}	μM^{-1}	
AaTgb	20	0.019	1100	0.00058	5.1	0.0066	780	0.71
AaTgb Y29F	264	1.9	140	0.0044	83	0.0038	22,000	150
SWMb (38)	17	15	1.1	0.55	0.51	0.019	27	25
SWMb L29Y/H64Q (57)	2.8	1.6	1.8	0.35	0.071	0.014	5.1	2.8
Human Hb α (39)	40	15	2.7	0.23	5.0	0.008	620	230
Human Hb β (39)	90	31	2.9	0.21	7.0	0.007	1,000	340
A. suum Hb (57)	1.5	0.004	370	0.0016	0.21	0.018	12	0.03
C. lacteus Hb (56)	240	180	1.3	0.46	28	0.048	580	450
C. lacteus Hb T48V (56)	30	0.18	170	0.0036	3.0	0.0070	430	2.5
V. stercoraria Hb (54)[a]	200	4.2	48	0.013	ND[b]	ND	NC[c]	NC
		0.15	1300	0.00046				
V. stercoraria Hb[d]	174	0.20	870	0.00070	60	0.037	1,600	1.9
					2.5		68	
C. perfringens Hb (40)	210	1.8	120[e]	0.0052	59	30	2.0[e]	0.017[e]
C. jejuni Hb (40)	150	1.1	140[e]	0.0045	45	40	1.1[e]	0.0083[e]
					1.9		0.048[e]	
E. coli Fhb (53)[a]	38	0.44	86[e]	0.0070	22	0.057	390[e]	4.5[e]
					1.4	0.018	78[e]	
E. coli Fhb Y29F (53)[a]	50	34	1.5[e]	0.41	37	0.12	310[e]	210[e]
		3.3	15[e]	0.040	1.0	0.01	100[e]	
B. subtilis HemAT (52)[a]	19	1,900	0.010	61	0.34	0.067	5.1	510
		87	0.22	2.8				
B. subtilis HemAT (52)[a,f]	19	1,800	0.011	58	0.43	0.070	6.1	550
		50	0.38	1.6				
B. subtilis HemAT Y70F (52)[a,f]	53	19,000	0.003	220	0.47	0.030	16	5300
		1,800	0.029	21				

[a] For proteins with biphasic reaction kinetics, the second phase is described on a separate line.
[b] ND, not determined.
[c] NC, not calculated.
[d] Unpublished data from A. J. Wilkinson, D. A. Webster, D. H. Maillett, and J. S. Olson with recombinant *V. stercoraria* Hb.
[e] Calculated based on reported association and dissociation rate constants.
[f] Sensor domain only.

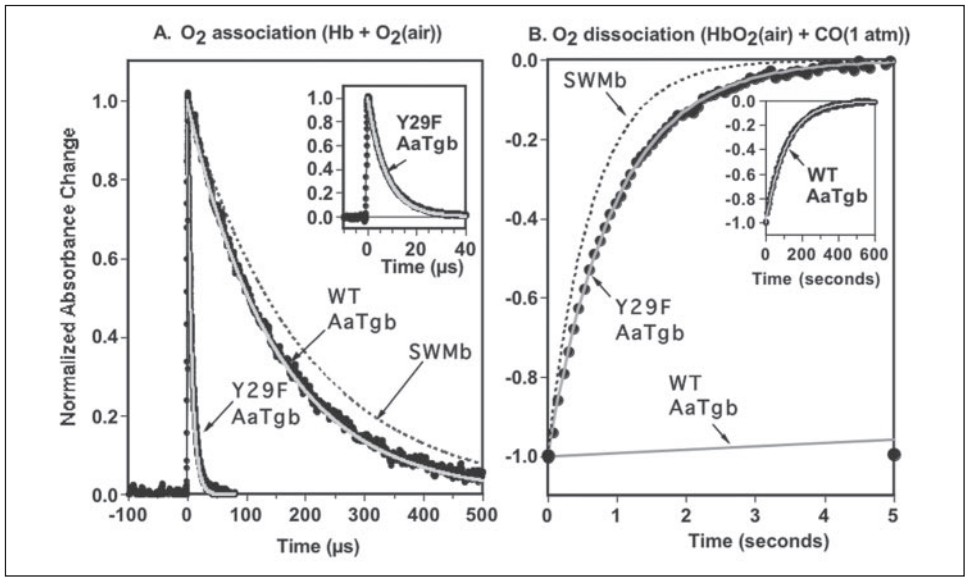

FIGURE 6. **O_2 binding to and dissociation from wild type and Y29F AaTgb at pH 7.0, 20 °C.** *A*, time courses for O_2 association measured by laser photolysis with ~50 μM protein and 250 μM O_2 showing bimolecular rebinding of O_2 from solvent. *B*, time courses for displacement of bound O_2 by CO measured by stopped-flow rapid mixing methods. The concentrations after mixing were ~5 μM protein, 125 μM O_2, and 500 μM CO. The normalized absorbance changes (*black circles* and *black lines*), fits to a single exponential function (*gray lines*), and predicted time courses for SWMb (*dashed lines*) are shown.

of AaTgb is most similar to Fhbs and SDHbs, suggesting a possible common physiological function. As proposed for *V. stercoraria* Hb, perhaps AaTgb presents O_2 to the respiratory system for metabolism (42). FHbs and other Hbs dioxygenate NO (3). Similarly, AaTgb may serve to detoxify excess NO or O_2, which may be detrimental to growth in a microaerobic environment.

Structural Basis of O_2 Avidity—The O_2 affinity of AaTgb, which yields a calculated P_{50} of 0.0006 mm Hg, is among the highest known for

characterized Hbs. The predicted distal heme site contains a B10 tyrosine and an E7 glutamine (Fig. 1). In the structure of the oxygen-avid Hb from *A. suum*, the B10 tyrosine and E7 glutamine preferentially stabilize O_2 by forming two hydrogen bonds with the bound ligand (43). Mutation of glutamine to leucine or alanine in the *A. suum* heme domain results in ~5- and ~60-fold increases, respectively, in k_{O_2} (44). Mutation of tyrosine to leucine or phenylalanine produces an even larger ~200–600-fold increase in k_{O_2} (44, 45), resulting in an ~100-fold decrease in K_{O_2} (45). Thus, in *A. suum* Hb, the B10 tyrosine and E7 glutamine motif enhances O_2 binding, with the B10 tyrosine side chain having a dominant role in O_2 stabilization by electrostatic interactions.

To examine whether the structural basis for high O_2 affinity in AaTgb is similar to that in *A. suum* Hb, the position B10 hydroxyl group was removed by replacing the distal tyrosine with phenylalanine in the Y29F mutant. This mutation increased k_{O_2} ~100-fold, resulting in a net ~10-fold decrease in K_{O_2} (Fig. 6*B*). Furthermore, k_{CO} was relatively unaffected, decreasing less than 2-fold, indicating that this substitution specifically affects bound O_2 (TABLE ONE). In SWMb, dissociation rate constants for O_2, but not CO correlate with the calculated electrostatic field surrounding the bound ligand (46). The high O_2 affinity of AaTgb, shared distal pocket motif with *A. suum* Hb, and the specific effect of the Y29F mutation on k_{O_2} combine to suggest that the B10 tyrosine hydroxyl donates a hydrogen bond directly to bound O_2.

Crystal structures of bacterial Hbs similar to AaTgb, *V. stercoraria* Hb (47, 48), *A. eutrophus* Fhb (49, 50), *E. coli* Fhb (51), and the Hb domain of *Bacillus subtilis* HemAT (33), do not consistently reveal a distal binding site similar to that of *A. suum* Hb. In cases where direct interaction between the B10 tyrosine and bound ligand cannot be observed or deduced, the crystallized biochemical states do not contain the appropriate gas ligands that may order the B10 tyrosine and E7 glutamine side chains. *A. eutrophus* Fhb contained a heme iron of unspecified oxidation state and the alkyl side chains of a complex phospholipid in the ligand binding site (49). *E. coli* Fhb and *V. stercoraria* Hb were both crystallized in the ferric unliganded state (47, 51). Liganded structures of *V. stercoraria* Hb contained large ligands bound to ferric heme (47, 48). The B10 tyrosine does donate a hydrogen bond to cyanide in the ferric state of the *B. subtilis* HemAT Hb domain (33), but this interaction is not observed in the ferrous unliganded state. Mutation of the B10 tyrosine to phenylalanine decreases K_{O_2} ~3-fold in HemAT (52), suggesting that a similar interaction is observed in the ferrous oxygenated state. Although an analogous role for the B10 tyrosine is difficult to infer from the ferric unliganded structure of *E. coli* Fhb, a Y29F mutation does decrease K_{O_2} ~6-fold (53). The structures of the ferrous oxygenated form of these proteins are needed to unambiguously determine the role of the B10 tyrosine in regulating ligand binding. Indeed, the structure of any ferrous oxygenated SDHb or Fhb may provide insights in general mechanisms. AaTgb is a hopeful candidate for crystallization because this Hb is purified in the oxygenated ferrous state after recombinant expression.

Presence of the B10 tyrosine and E7 glutamine is not sufficient to assure O_2 avidity. *C. perfringens* and *C. jenuni* Hb contain both residues in the distal pocket, but have considerably lower affinities for O_2 (40). In contrast, *V. stercoraria* Hb, in which the B10 tyrosine and E7 glutamine have not been observed to directly bind O_2, has been shown to possess high O_2 affinity based on direct kinetic measurements of k'_{O_2} and k_{O_2} (54) (TABLE ONE). In the structure of the truncated neuronal Hb from the worm *Cerebratulus lacteus*, the B10 tyrosine and E7 glutamine participate in a hydrogen bond network with the E11 threonine that prevents stabilization of bound O_2 (55), and high O_2 affinity is generated only after mutation of the E11 threonine to valine (56). Mutation of SWMb to contain both the B10 tyrosine and E7 glutamine only increases K_{O_2} ~2-fold from ~1 to ~2 μM^{-1} (57), still ~300–1000-fold smaller than that of *A. suum* Hb and AaTgb, respectively. The Y29F mutant of AaTgb still has an O_2 affinity ~100-fold greater than wild-type SWMb, and the replacement of the B10 tyrosine with phenylalanine does not affect K_{O_2} in AaTgb as much as the analogous mutation in *A. suum* Hb. Thus, although these studies have shown that the distal tyrosine stabilizes bound O_2, further detailed structural characterization is required to quantitatively understand how AaTgb achieves such high O_2 affinity.

Implications for Hemoglobin Evolution—Although a variety of Hb classes are present in extant organisms, the entire family is likely to have descended from a single ancestral protein. Phylogenetic analysis of Hbs in all three domains of life intimately connects all known Hbs (2), supporting previous suggestions of a common origin (58, 59). Because no single Hb class is widespread in all three domains, the identity of the common ancestor remains unclear. Pgbs have been proposed to be the ancestral form because of presence in both Bacteria and Archaea (6, 60), the two earliest diverging domains of life, even though only a few proteins are known. This argument is not unique, however, because one TrHb was found in Archaea (2), and TrHbs are widespread in the other two domains (8, 9). Characterizing Hbs in organisms at the root of the tree of life is one possible approach to elucidating the nature of the ancestral Hb. Given the widespread distribution of SDHbs and similar proteins, the hypothesis that an extant Hb similar to the ancestral Hb still exists in an organism closely related to the last universal common ancestor may be examined by searching for such proteins in bacterial hyperthermophiles.

A. aeolicus belongs to the family *Aquificaceae*, placed by both ribosomal DNA and combined protein sequences at the deepest branch of bacterial phylogeny (61–63). Rooting of the universal tree of life also places Bacteria as diverging from the last universal common ancestor before Eucarya and Archaea (64, 65). Proteins in *A. aeolicus* may therefore represent living fossils similar to those possessed by the last universal common ancestor to all extant organisms.

Given the frequency of lateral gene transfer in Bacteria and Archaea (66), *AaTgb* may have been acquired from another organism, but no evidence exists directly supporting such a scenario. The *AaTgb* open reading frame GC content is 43.1%, similar to the *A. aeolicus* genomic GC content of 43.4% (21). Lack of a difference fails to provide evidence suggesting recent lateral gene transfer. AaTgb is also resistant to thermal denaturation, suggesting that it functions in a thermophile, not a mesophile. Recent gene transfer would require a gene encoding a thermostable protein from an organism of equivalent GC content; ancient gene transfer could involve any source given that the gene and protein, respectively, evolved the GC content and thermostability, if not already present, to mask such a transfer. Although acquisition of *AaTgb* by lateral gene transfer cannot be formally excluded, both possibilities seem unlikely, and AaTgb may indeed be part of the original *A. aeolicus* proteome.

The ancestral Hb protein may have been monomeric, resistant to thermal and chemical denaturation, pentacoordinate, and oxygen-avid like AaTgb. Because Hb function centers around ligand binding, adaptation for various functions may involve mutations, either local or allosteric, that affect the ligand binding site. Such changes would diverge from the original properties of the ancestral protein. Adaptation from the natural environment of *A. aeolicus* to more aerobic environments may select for lower O_2 affinity, as seen in most other Hbs. In most animal and plant Hbs, such adaptation involves a change in the distal ligand binding site from two hydrogen bond donors, the B10 tyrosine and E7 glutamine, to a single E7 histidine donor. AaTgb is pentacoordinate, suggesting that this heme binding mode preceded hexacoordi-

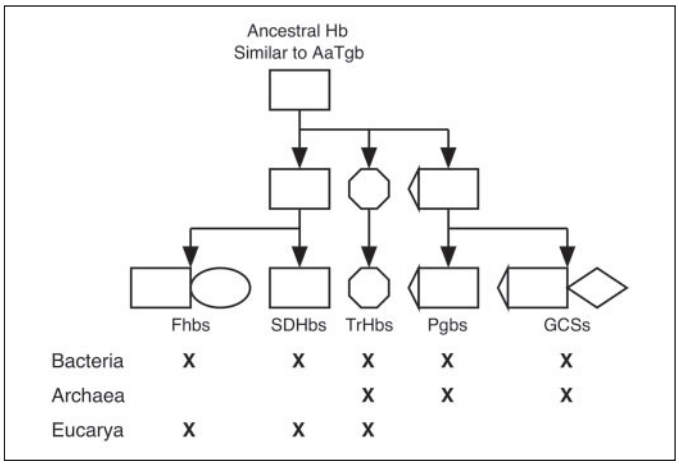

FIGURE 7. **Model of hemoglobin evolution.** Schematic representation based on divergence from a single-domain ancestral protein similar to AaTgb. Combinations of mutations, truncations, insertions, and deletions yield SDHbs, TrHbs, and Pgbs. Gene fusions yield Fhbs and GCSs. Structural elements of the ancestral protein (*squares*), TrHbs (*octagons*), Z helices of Pgbs (*triangles*), flavin reductase domains of Fhbs (*oval*), and sensor domains of GCSs (*diamond*) are depicted as separate elements to illustrate sequence changes. The *table below* the flowchart indicates distribution of extant Hbs among the three domains of life.

nation. Thermostability may also be an ancestral trait lost in the Hb lineage. Oligomerization must be based on a basic monomeric building block, which may have been like AaTgb. Adaptation for various functions may also involve truncation or extension of termini, fusion with other domains, or gene duplication followed by self-fusion.

A parsimonious model for Hb evolution emerges presuming an ancestral protein similar to AaTgb (Fig. 7). SDHbs derive directly from the ancestral protein. From this ancestor, extension or deletion of the N terminus, coupled with other internal mutations, could generate Pgbs and TrHbs, respectively. Fusion of Pgbs with sensor domains would yield GCSs (6) just as fusion of SDHbs with reductase domains would yield Fhbs (14). Although divergence of Pgbs from the ancestral protein must logically precede divergence of GCSs from Pgbs, the temporal sequence of other events is difficult to determine. Different classes of Hbs may then, concurrent with evolution of organisms or through lateral gene transfer, distribute among the three domains of life as currently known (2, 5, 9, 58, 59).

Evolution from the properties of the ancestral Hb could not only vary the range of ligand affinity and specificity, but also generate novel responses such as cooperativity. Hbs require self-association for cooperativity (67), and reduction of O_2 affinity in adaptation may have aided, or even have been necessary, for cooperativity to arise. The O_2 avidity required in microaerobic environments or for certain biochemical functions is not compatible with cooperative binding in aerobic conditions. Mutation of an optimum binding interface resulting in reduced affinity for a ligand, coupled with combinatorial fusion of nonideal binding sites, has been correlated with the presence of cooperativity in the binding of transcription factors to promoter DNA (68). Reducing affinity to generate cooperativity may be a common trend in evolution.

Modification of a building block by mutation, oligomerization, or domain fusion to achieve a myriad of functions is a common trend in evolution. This concept applies to the evolution of Hb from the ancestral protein to the extant diversity of structures and functions. A protein very similar to AaTgb may have served as the basic building block of the Hb lineage.

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