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Oxygen-Avid Hemoglobin of *Ascaris*

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I. Introduction

Ascariasis is one of the world's most prevalent diseases. The causative parasitic nematode, *Ascaris lumbricoides*, infests more than 1 billion people, primarily in the developing world.[1,2] About 1 million people each year manifest severe clinical symptomatology, primarily intestinal blockage and biliary obstruction, and about 20 000 per year die.[3] The most devastating sequelae of this infection result from retarded mental and physical development of countless millions of children with large worm burdens.[4,5] Although the disease is readily cured with available agents, many victims do not have access to treatment and reinfection is a regular occurrence. Ascariasis is a model for studying parasitic nematode diseases in general, many of which are not as easily treated. The *Ascaris* worm is large and is easy to obtain from slaughterhouses, making this organism particularly amenable to biochemical analysis.[6]

This year marks the 50th anniversary of Davenport's discovery of the extraordinary oxygen avidity of *Ascaris* perienteric hemoglobin.[7] Thirty-five years ago the molecule was first proposed to be octameric.[8]

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      A..............     B...............C......         D.....E..
DOM1  NKTRELCMKSLEHAKVDTSNEARQDGIDLYKHMFENYPPLRKYFKNREEYTAEDVQNDPF
DOM2  HAVRHQCMRSLQHIDIGHSETAKQNGIDLYKHMFENYPSMREAFKDRENYTAEDVQKDPF

      .................          F.........    G................
DOM1  FAKQGQKILLACHVLCATYDDRETFNAYTRELLDRHARDHVHMPPEVWTDFWKLFEEYLG
DOM2  FAKQGQRILLACHLLCASYDDEETFHMYVHELMERHERDGVQLPDQHWTDFWKLFEEFLE

      ..   H.................    TAIL...................
DOM1  KKTTLDEPTKQAWHEIGREFAKEINKHGR
DOM2  KKSHLCEHTKHAWAVIGKEFAYEATRHGKEHHEHKEEHKEEHKEEHKEEQH
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Figure 1. Alignment of the tandem globin domains of *Ascaris* hemoglobin. The charged C-terminal tail, the B10 tyrosine, the E7 distal glutamine, and the F8 proximal histidine are in bold letters. Helices are marked above the sequence. (Reprinted with permission from ref 17. Copyright 1995 The American Society for Biochemistry and Molecular Biology.)

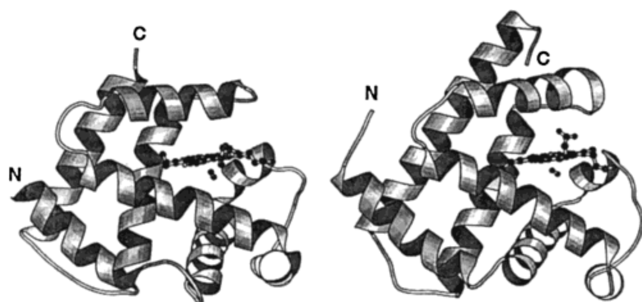

Figure 2. Comparison of *Ascaris* hemoglobin domain 1 (left) and sperm whale myoglobin (right). (Reprinted with permission from ref 13. Copyright 1995.)

A decade ago it was found to possess two oxygen-binding pockets per polypeptide.[9] These studies have raised a series of questions about *Ascaris* hemoglobin: how does it bind oxygen 4 orders of magnitude more tightly than human hemoglobin? How does it form an octamer and why? What is the function of this most unusual hemoglobin (it binds oxygen much too avidly to play a role in delivery)? Only recently have the answers to these questions begun to come to light.

II. Structure

A. Primary Structure and Globin Fold

Ascaris hemoglobin consists of eight identical subunits of about 43 000 molecular weight.[8−10] The hemoglobin gene encodes a polypeptide composed of two globin domains in tandem followed by a C-terminal tail[11,12] (Figure 1). Thus, the Ascaris hemoglobin molecule has a capacity of 16 hemes. The globin domains are 62% identical, and each shares only about 10−15% identity with other globins such as mammalian, plant, and invertebrate species. Nevertheless, the key residues invariant throughout phylogeny, such as the proximal histidine, C2 proline, CD1 phenylalanine (labeling refers to the position in the A−H helices or turns/loops between helices such as AB, CD), and the conserved hydrophobic positions essential for formation of the globin core, are present. This suggested that the basic globin fold shared by a wide variety of animal and plant globins should be present in the *Ascaris* molecule. The crystal structure of the first *Ascaris* hemoglobin domain, which forms a monomer in solution, has subsequently been solved, and the helical arrangement is as expected[13] (Figure 2). There are eight alpha helices as for mammalian globins, and the C alpha positions deviate by a mean of only 1.77 Å when the backbone is superimposed on that of sperm whale myoglobin. The heme moiety is tilted slightly in *Ascaris* hemoglobin (9% compared with mammalian myoglobin), likely due to F helix changes in the proximal pocket (Figure 3). The proximal histidine at F8, which coordinates the heme in its pocket, has a single hydrogen bond to leucine F4, instead of the usual bifurcated hydrogen bond to F4 and F7 in mammalian globins. In mammalian globins, the serine at F7 interacts with the heme propionate via a hydrogen bond. In *Ascaris*, an arginine at this position forms a salt bridge to the propionate.

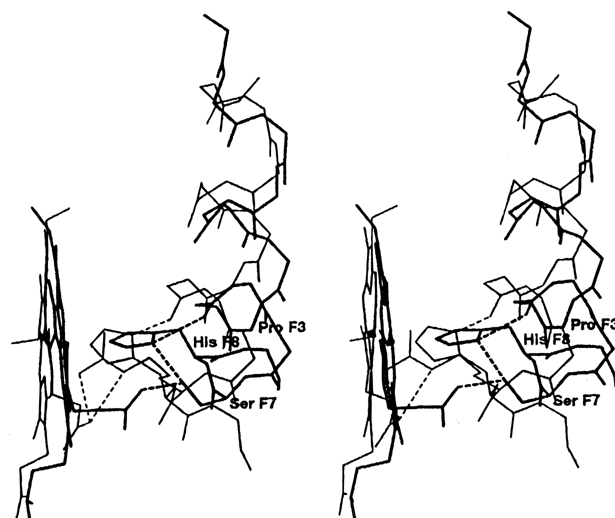

Figure 3. Stereodiagram comparing the F helices and heme groups of *Ascaris* D1 (thin lines) and recombinant sperm whale myoglobin (thick lines). (Reprinted with permission from ref 13. Copyright 1995.)

B. Quaternary Structure

Attempts to obtain crystals of the intact two-domain, homooctameric *Ascaris* hemoglobin have failed in several labs over a number of years. Therefore, our information about subunit assembly has come from microscopy, physicochemical analysis, structural prediction, and mutational analysis. Darawshe and Daniel performed negative stain electron microscopy and found evidence for a two-layer arrangement of tetramers, stacked in eclipsed orientation.[14] The two domains of the globin polypeptide can be expressed separately in *Escherichia coli*.[15] Domain one (D1) is monomeric, while domain two (D2) multimerizes. Since the two domains are highly homologous except for a tail at the C-terminus of the

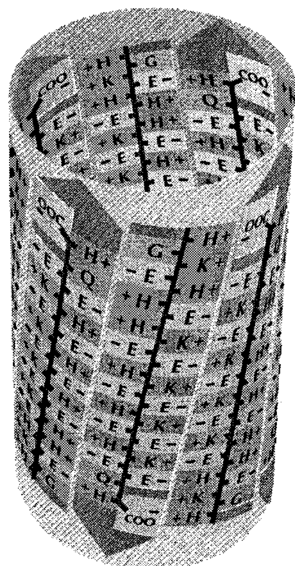

Figure 4. Proposed beta barrel configuration formed by *Ascaris* hemoglobin tails. (Reprinted with permission from ref 16 and Dr. Perutz. Copyright 1993 Current Biology Ltd.)

second domain, suspicion fell upon this region as being important for octamer formation. The C-terminal extension has 22 charged amino acids among the final 23 residues, including four direct repeats of the sequence HKEE.[11] Perutz and co-workers have proposed that this tail may form a beta barrel in which the C-termini from the eight subunits interdigitate, with complimentary charges opposing one another[12,16] (Figure 4). The tail, however, is not sufficient for multimer formation since recombinant D1 with an appended tail remains monomeric, as does mammalian myoglobin with a tail.[17] In fact, the tail is not even strictly required for multimerization, since D2 without a tail can still form various oligomers including octamer, though the efficiency is greatly diminished.[17,18] Furthermore, stability of *Ascaris* hemoglobin with or without a tail is similar when subjected to the chaotropic agent sodium thiocyanate.[18] These data suggest that the tail does not play an important role in holding the octamer together.

That said, the tail does appear to be crucial for efficient octamer assembly.[18] When *Ascaris* hemoglobin is expressed without a tail, large amounts of aggregate form, along with monomeric species. Only a small amount of octamer can be found. With the tail, octamer predominates. One can remove up to two HKEE repeats from the tail and no appreciable effect is seen on octamer formation. When the third repeat is also deleted, efficiency is reduced; when all four repeats are removed, octamer production is further diminished; when the proximal EHHE before the start of the HKEE repeats is also deleted to form the tailless construct, octamer formation is quite low. These results suggest that the C-terminal tail is important for octamer formation, acting as an endochaperone (an endogenous domain that assists in achieving proper assembly) rather than as a determinant of octamer stability in the assembled protein. The other implication is that determinants of subunit interactions must lie within the body of the *Ascaris*

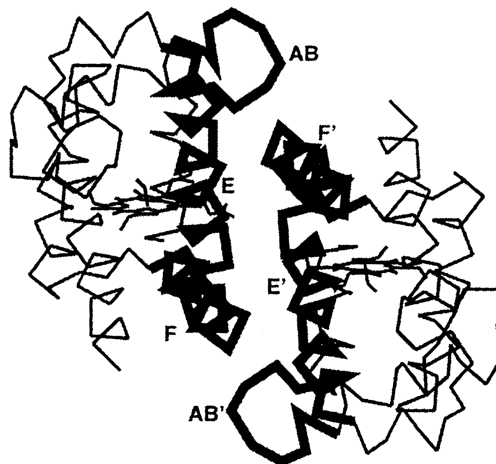

Figure 5. Crystallographic dimer of *Ascaris* D1 suggesting subunit interfaces. The E and F helices as well as the AB loop are bold. (Reprinted with permission from ref 17. Copyright 1995 The American Society for Biochemistry and Molecular Biology.)

hemoglobin domains, especially those of D2, which can octamerize on its own. Intact, two-domain *Ascaris* hemoglobin better forms a octamer than D2 alone, suggesting that there may be some contribution to subunit interactions from D1 as well.[17] In the structural analysis, D1 was found to form a weak crystallographic dimer.[17] Interactions between subunits were seen within the E and F helices and the AB loop (Figure 5). These interactions were predicted to be enhanced in the interface of D2. Mutational studies suggested the importance of isoleucine 15 within the AB loop of D2.[18] Changing this residue in D2 to the valine found in the corresponding position of D1, weakened the multimerization capabilities of D2.

III. Ligand Binding

A. Oxygen

Ascaris hemoglobin has a K_d for oxygen 4 orders of magnitude stronger than that of human hemoglobin under physiological conditions ($P_{50}O_2 = 0.0014$ compared with 25 mmHg)[7,19,20] (Table 1). This extraordinary oxygen avidity derives from a slow off rate. In vacuo, oxygenated *Ascaris* hemoglobin takes several minutes to release its oxygen[7] (compared to milliseconds for mammalian globins[21]) (Table 1). Picosecond spectroscopy after flash photolysis shows a quantum yield after bond breakage of 10^{-4}, i.e., one in 10 000 oxygen molecules manages to escape from the pocket after dissociation from the heme, and thus there is a strong propensity to rebind.[22] The quantum yield is one one-thousandth that of sperm whale myoglobin.

When D1 and D2 are expressed separately in *E. coli*, each is oxygen-avid on its own[15] (Figure 6). This, along with a Hill number of one,[20] suggests that interactions between subunits or domains are not important for oxygen retention. The finding also reduces the puzzle of the molecular basis of oxygen avidity from a repeating, 16 heme, 320 kD octamer down to a single 18 kD globin domain. The basis of oxygen avidity is therefore amenable to study by site-

Table 1. Oxygen Binding Parameters for *Ascaris* Hemoglobin Compared with Mammalian Globins

	k_{on} ($\mu m^{-1}s^{-1}$)	k_{off}	P_{50} (μm Hg)	Hill no.	ref
Ascaris hgb	1.5	0.004	0.001−0.0035	1.0	19,20
SWM	15	14	0.65	1.0	22
human α, T-state	2.9	180			59
human Hgb			25[a]	2.8[a]	60
human β, T-state	11.8	2500			59

[a] Values for human hemoglobin under physiological conditions. SWM: sperm whale myoglobin. hgb: hemoglobin.

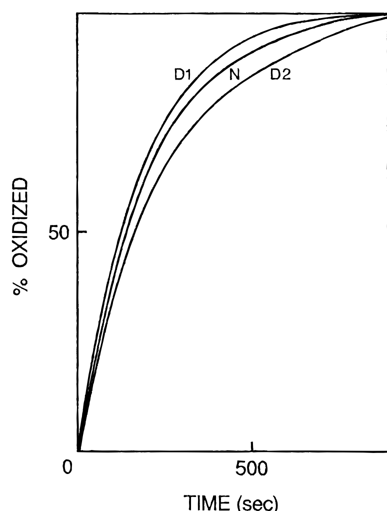

Figure 6. Oxygen dissociation curves for D1 and D2. Oxygen dissociation was monitored by measuring methemoglobin formation (% oxidation) in the presence of potassium ferricyanide. When oxygen dissociates from the heme, ferricyanide converts the heme iron from Fe^{2+} to Fe^{3+}, which is detected spectrally. (Reprinted with permission from ref 15. Copyright 1993 The American Society for Biochemistry and Molecular Biology.)

Table 2. Oxygen Dissociation Rates

globin	k_{off} (s^{-1})	ref
Ascaris D1	0.0059	25
B10 tyr to leu	3.33	25
B10 tyr to phe	1.6	25
E7 gln to leu	0.027	25
SWM	12	22

directed mutagenesis using single point mutations of isolated D1.

Two sites in the globin domain are of particular interest. The first is amino acid B10 tyrosine. This position is a leucine in vertebrate globins. There, the residue juts into the oxygen-binding pocket.[23] Mutation of this leucine to phenylalanine increases the oxygen avidity of sperm whale myoglobin by an order of magnitude for reasons thought to be attributable to the aromatic electron cloud.[24]

Changing the B10 tyrosine to leucine abolishes the extra avidity of *Ascaris* hemoglobin,[25,26] converting the oxygen off rate to nearly that of mammalian myoglobins (or mammalian hemoglobins without the allosteric modulators that decrease oxygen avidity even further) (Table 2). Importantly, crystal diffraction patterns of wild-type domain one and of the leucine mutant are quite comparable, showing that this alteration does not disrupt the overall hemoglobin structure.[25] Even more interestingly, changing the tyrosine to phenylalanine abolishes the oxygen avidity to a similar extent.[25,26] This indicates that the single hydroxyl that differs between phenylalanine and tyrosine is responsible for the extraordinary oxygen avidity of *Ascaris* hemoglobin. It is proposed that this hydroxyl forms a strong hydrogen bond, locking the heme-liganded oxygen in tightly.

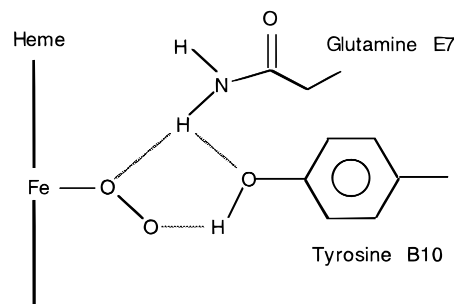

Figure 7. Schematic diagram of the hydrogen bonding network in the active site pocket of *Ascaris* hemoglobin. (Reprinted with permission from ref 13. Copyright 1995.)

Amino acid E7 glutamine is in the position where vertebrate globins have histidine. This residue, known as the distal histidine in vertebrate globins, is important for hydrogen bonding to liganded oxygen.[27] When this amino acid is changed to glutamine in sperm whale myoglobin, the glutamine is still able to hydrogen bond to the molecular oxygen,[28] though avidity decreases slightly.[29] Changing the distal glutamine of *Ascaris* hemoglobin D1 to leucine or alanine accelerates the oxygen off rate by an order of magnitude.[25] This adds support to the expectation that the distal amino acid makes a hydrogen bond to the liganded oxygen. The magnitude of this bond's contribution to avidity is clearly much less than that of the tyrosine. Indeed, the crystal structure[13] confirms that the tyrosine hydroxyl is in perfect position to make a strong hydrogen bond with the distal atom of the liganded oxygen molecule; the glutamine appears to make a weaker hydrogen bond to the oxygen atom coordinated directly to the iron (Figure 7). There is also a hydrogen bond between the tyrosine and glutamine residues. All together, this network of hydrogen bonds serves to grip the liganded oxygen in a molecular vice, resulting in the extreme oxygen avidity first noted 50 years ago.[7]

Direct evidence for these interactions in solution comes from UV resonance Raman spectroscopic measurements.[30] Oxygen binding causes a dramatic change in the tyrosine H-bond signal. Resonance Raman studies of the distal glutamine to leucine mutant show a loosening of the rigidity of the distal pocket, adding further support to the molecular vice concept. Photodissociation experiments suggest the existence of a tight cage with a high probability of ligand rebinding[31] (as proposed by Gibson et al.[22]).

Table 3. Other Ligands (from refs 19, 20, 22)

	k_{on} ($\mu m^{-1} s^{-1}$)	k_{off}	K_d	M ($CO:O_2$)
CO	0.17−0.21	0.018	0.06−0.1	0.04−0.08
NO	4.7−6.5			

This probability decreases with increased temperature due to greater conformational fluctuation allowing ligand escape, thus explaining the substantial temperature factor for oxygen binding,[7,19] which is, in contrast, minimal for mammalian globins.

Further examination of the oxygen-binding pocket has been performed by NMR analysis.[32] The data confirm that the distal E7 and B10 residues exhibit optimal orientations for hydrogen bonding to ligand and to each other. The measurements also provide further evidence for a crowded and constrained distal pocket.

Mutation of sperm whale myoglobin leucine to tyrosine results in a globin that actually has worse oxygen avidity than wild type.[22] The position of this residue in the recombinant myoglobin may not be optimal for interaction with the liganded oxygen. A less sterically hindered triple mutant in which sperm whale myoglobin B10 was changed to tyrosine, E7 to glutamine, and E10 to arginine showed a substantial decrease in oxygen dissociation rate, though not near that of *Ascaris* hemoglobin.[33] NMR studies suggest that the positioning of the distal glutamine in this triple mutant is not optimal for formation of the hydrogen-bonding network of the *Ascaris* molecule.[34]

B. Other Ligands

Carbon monoxide binds to *Ascaris* hemoglobin with rate and equilibrium constants in the same range as those of mammalian globins (Table 3). An unusually low oxygen/CO partition coefficient, M, results from the high oxygen affinity.[19,20] FTIR analysis suggests the existence of a phenolic proton interaction with CO ligand that is absent in the B10 tyr to phe mutant.[31] Nanosecond geminate rebinding (rebinding of ligand that has dissociated but is still in the binding pocket) is decreased in this mutant but actually increases in an E7 glutamine to leucine mutant, probably due to disruption of pocket constraints.[31] The rebinding is only moderate for CO, with a quantum yield of about 0.7 (it is near 1.0 in sperm whale myoglobin).

Nitric oxide binds to *Ascaris* hemoglobin with a rate constant slightly slower than that for mammalian globins.[19,22] Geminate recombination is faster and has a lower yield than that of sperm whale myoglobin.[22] This is consistent with the hypothesis that there is an interaction with the B10 tyrosine (see below). Dissociation constants and affinity of NO binding to the met form have not been measured.

Binding of cyanide and ethylisocyanide has been detected spectrally, as has binding of cyanide and azide to the met form.[35] Rate constants have not been reported.

IV. Body Wall Hemoglobin

In addition to the perienteric hemoglobin that is the subject of this review, *Ascaris* possesses at least one and perhaps several body wall globins.[7,19,36−39] They appear to be disulfide-linked dimers that can be reduced to 15−17 kDa monomers.[40] Thus, the polypeptides are one-half the size of the perienteric hemoglobin. The oxygen affinity of the most abundant form has been measured and is nearly 2 orders of magnitude less than that of the perienteric hemoglobin[7,19] but still an order of magnitude stronger than that of sperm whale myoglobin, again due to a slow dissociation rate. The amino acid sequence has a B10 tyrosine and an E7 glutamine, analogous to the perienteric molecule.[40]

V. Possible Function

There has been much speculation on the function of the enigmatic perienteric hemoglobin. It is widely believed that the molecule binds oxygen much too tightly to ever give it up for cellular delivery on a physiological time scale.[7,38,41]

A. Sink

A role as an oxygen sink has been proposed.[41] *Ascaris* has an anaerobic metabolism, and it is possible that it protects itself against occasional onslaughts of intestinal oxygen by mopping oxygen up with hemoglobin, thereby protecting oxygen-sensitive cells such as sperm and oxygen-sensitive organelles such as mitochondria. The problem with the sink concept is that the hemoglobin supply is limited, so that oxygen can easily saturate the binding capacity (after which the sink is full). Scavenging only makes sense in the sense of a role in oxygen metabolism (see below).

B. Catalase

Another possible protective role comes from the observation that *Ascaris* hemoglobin, like other hemoglobins, has a weak catalase activity.[42] It has thus been proposed that the molecule might function by breaking down any toxic hydrogen peroxide generated by the worm or its host.[43]

C. Carrier

The hemoglobin could serve as a carrier of heme or of iron for gametogenesis.[38] *Ascaris* eggs appear to contain enough heme to account for daily turnover of the iron in hemoglobin-rich perienteric fluid. Whether this heme is free in the eggs or present as intact hemoglobin has not been determined. Simple roles in osmotic balance,[41] similar to that of albumin, or in acid−base buffering[43] have also been suggested.

D. Sterol Biosynthesis

Squalene has been found to co-purify with *Ascaris* hemoglobin, leading to a proposal that the hemoglobin could be involved in sterol biosynthesis.[11] In other organisms, squalene is converted to squalene-2,3-epoxide in a reaction that requires molecular oxygen. An NADPH-dependent ferrihemoprotein reductase also participates in the reaction. The *Ascaris* hemoglobin has an NADPH-dependent reductase activity. Thus, all the components of the squalene epoxide

reaction, squalene, oxygen, NADPH, and a reductase activity, are associated with the hemoglobin. The epoxidation reaction is a critical step in sterol biosynthesis, since the epoxide product cyclizes to form the sterol rings which are then modified to generate cholesterol, ergosterol, vitamin D derivatives, and other compounds. Why would *Ascaris* have a special pathway? These worms lay hundreds of thousands of eggs loaded with sterol each day. The problem that the organisms face is that they live in the folds of the intestines where there is very little oxygen, yet they need substantial oxygen to synthesize sterol for their egg production. It makes perfect sense for them to have evolved an avid hemoglobin that can grab any available oxygen and transfer it directly into sterol biosynthesis. At present this remains an interesting hypothesis. The ability of the *Ascaris* hemoglobin to use NADPH as an electron donor has been confirmed (see below); biochemical attempts to demonstrate squalene epoxidation in worm extracts, perienteric fluid, or with purified hemoglobin have been unsuccessful.

E. Clues from Lower Organisms

Globins have now been found in a wide range of lower organisms, from eubacteria and cyanobacteria, to the single-cell eukaryotes such as yeasts, *Paramecium*, and *Chlamydomonas*, to a variety of other nematodes such as *Pseudoterranova*, *Nippostrongylus*, and *Caenorhabditis*, naming a few of the better characterized ones (see refs 41 and 44−47 for good reviews). Clues to the function of *Ascaris* hemoglobin have come from some of these examples. The denitrifying bacterium *Alcaligenes eutrophus* has a hemoglobin with a globin domain fused to a reductase domain. Gene knockout results in inability of the bacteria to accumulate the transient denitrification intermediate nitrous oxide, though growth as well as production of the pathway endproduct dinitrogen are normal.[48] Nitrogen oxide compounds have been found to induce the expression of globin genes in a variety of bacteria whose globins also have a reductase domain, including *E. coli*, *Bacillus subtilis*, and *Salmonella typhimurium*.[49−54] A knockout of the *Salmonella* hemoglobin gene results in a strain that has increased sensitivity to nitric oxide.[55] Hemoglobin from the related *E. coli* is able to combine oxygen with nitric oxide in an enzymatic reaction to produce nitrate.[50,51] The bacteria are thereby able to detoxify nitric oxide. NADH is required for regeneration of reduced heme, a function presumably mediated through the reductase domain.

F. NO-Dependent Deoxygenase

Ascaris hemoglobin does not have the NADH-dependent reductase domain of the bacterial hemoglobins but instead appears able to use a cysteine sulfhydryl to perform redox chemistry in an NADPH-dependent reaction combining nitric oxide with oxygen.[56] Addition of NO to *Ascaris* oxyhemoglobin results in immediate methemoglobin formation, suggesting direct reaction with the active site oxygen. Interestingly, the B10 tyrosine responsible for the extraordinary oxygen avidity lends superoxide character to the liganded oxygen molecule, dramatically enhancing the reaction with NO. When this reaction is performed with excess NO, NO can be detected bound to cysteine as *S*-nitrosothiol and also to the ferric heme. When NADPH is added to the reaction, NO disappears from the hemoglobin and nitrate accumulates in solution. When *Ascaris* hemoglobin is incubated with excess NO in the presence of oxygen and NADPH, oxygen and NO are consumed and nitrate is formed in amounts indicating multiple rounds of catalysis. More oxygen than NO is consumed, and oxygen is consumed at a slower but significant rate in the absence of NO, suggesting an oxidase reaction that is accelerated by NO.

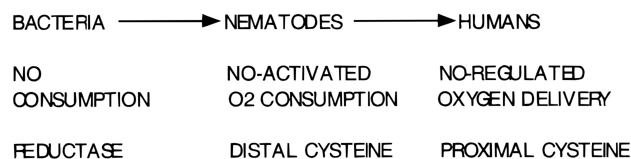

Figure 8. Evolution of the role of NO in hemoglobin function. Proposed function and electron source are listed.

An oxidized mutant *Ascaris* hemoglobin, when incubated with *S*-nitrosocysteine, forms *S*-nitrosohemoglobin with reduction of heme, suggesting that cysteine and heme are redox partners. Mutagenesisexperiments suggest that there is initial capture of NO^+ by a surface cysteine (A7) followed by transfer to the internal E15 cysteine, which lies in the distal ligand binding pocket. It appears, then, that a cysteine sulfhydryl, a heme iron, and nitric oxide participate in a redox triad to yield a deoxygenase activity.

The suggestion from these results[56] is that this hemoglobin serves to protect the *Ascaris* worm against oxygen by catabolizing it enzymatically. Oxygen is thought to be highly toxic to these organisms, which have a predominantly anaerobic metabolism. When drained of the hemoglobin-rich fluid, the pO_2 in the worm's perienteric space goes up dramatically, providing in vivo evidence of the hemoglobin's function.[56] From an evolutionary perspective, NO appears to play an important role in hemoglobin functions, from bacteria that use hemoglobin to control NO levels[49−51] (through reaction with oxygen), to nematodes that use NO to help the hemoglobin control oxygen levels,[56] to mammalian systems in which hemoglobin uses NO to regulate bloodstream oxygen delivery[57,58] (Figure 8).

VI. Conclusions

The perienteric hemoglobin of *Ascaris* is a remarkable molecule because of its oxygen avidity, its multimeric structure, and its hypothesized functions. It comprises a homooctamer of two-domain polypeptides. Its charged C-terminal tail appears to act as an endochaperone, facilitating multimeric assembly. The hemoglobin displays no cooperativity but is extremely oxygen avid, due to a slow off rate. The B10 tyrosine and E7 glutamine form a network of hydrogen bonds to the ligand oxygen and to each other, gripping the oxygen in a molecular vice within

a crowded pocket that makes ligand escape a rare event. The hemoglobin can act as an NO-primed deoxygenase, which is postulated to control oxygen tension, protecting the worm's anaerobic metabolism. Much remains to be learned. We do not have a crystal structure of the intact, octameric molecule. The mechanism of oxygen utilization by this hemoglobin–enzyme has many facets to be established, such as iron spin and oxidation states, NO intermediates, oxygen intermediates, thiyl intermediates, interactions between domains and between subunits, physiological electron donors, and reaction stoichiometry. The role of key amino acids such as the B10 tyrosine, E7 glutamine, and active cysteines needs further pursuit. This hemoglobin has kept scientists intrigued and intermittently preoccupied for 50 years, and there are more good years ahead.

VII. Acknowledgments

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