The myoglobin of two trematodes, Paramphistomum epiclitum and Isoparorchis hypselobagri, were isolated to homogeneity. The native molecules are monomeric with $M_r$ 16,000–17,000 and pl 6.5–7.5. In each species, at least four different globin isoforms occur. Primary structure was determined at the protein level. The globin chains contain 147 amino acid residues. Although major determinants of the globin fold are conserved, characteristic substitutions are present. A Tyr residue occurs at the helical positions B10 and E7 (distal position). This is confirmed by NMR measurements (Zhang, W., Rashid, K. A., Haque, M., Siddiqi, A. H., Vinogradov, S. N., Moens, L. & La Mar, G. N. (1997) J. Biol. Chem. 272, 3000–3006). A distal Tyr normally provokes oxidation of the iron atom and the inability to bind oxygen, whereas a Tyr-B10 is indicative for a high oxygen affinity. In contrast, trematode myoglobins are functional molecules with a high oxygen affinity. Molecular modeling predicts two possible positions for the aromatic ring of Tyr-E7: one being outside the heme pocket making it freely accessible to the ligand and one within the heme pocket potentially able to form a second hydrogen bond with the iron-bound oxygen. A hydrogen bond between Tyr-B10 and the bound oxygen as in the Ascaris hemo-globin is predicted as well. The predicted structure may explain the high oxygen affinity of the trematode myoglobins.

The influence of distal amino acid residues on the modulation of ligand binding characteristics to Hb has been the subject of much research (2, 3). Two key residues are His-E7, which stabilizes the iron-bound oxygen by hydrogen bonding and inhibits carbon monoxide by sterical hindrance, and Val-E11, which inhibits sterically the binding of all ligands. This hypothesis is mainly proven by the study of naturally occurring human mutants or engineered sperm whale Mb and by the comparison of the structure-function relationship in other species (4–7). For example, substitution of His-E7 to less polar residues results in reduction of the oxygen affinity due to a large increase of the dissociation rate relative to the association rate (6). Substitution of His-E7 → Tyr in the human Hb M mutants results in a strong decrease in oxygen affinity and an increased autoxidation rate leading to nonfunctional ferri-derivatives (8, 9).

In nonvertebrate globins, His-E7 is often substituted by other residues such as Gln (17.8%), Val (3.4%), Leu (3.4%), and Ile (0.68%) (10). Nevertheless, molecules such as the peritelic fluid Hb and Mb of Ascaris (Gln-E7) and the Mbs of the Aplysiidae (Val-E7) display the highest affinities (6, 11, 12). Therefore, mechanisms for the stabilization of the Fe$^{2+}$–O$_2$ complex, leading to high oxygen affinity, different from that of the vertebrate Hbs and Mbs, must exist.

Site-directed mutagenesis and x-ray analysis of domain I of Ascaris Hb show that the Fe$^{2+}$–O$_2$ complex is stabilized; hence, a high oxygen affinity is created by the formation of two hydrogen bonds: the usual one from the residue at position E7 (Gln) and an additional bond from the phenolate ion of Tyr at position B10 (11, 13, 14). This results in a decreased dissociation rate ($k_{dl}$) (Table I).

X-ray and NMR analysis of the globin of Aplysiia demonstrate that the Fe$^{2+}$–O$_2$ complex is stabilized by the formation of a hydrogen bond between the oxygen and a guanidino-NH proton of Arg-E10 (7, 12, 15). Consequently, the oxygen affinity of Aplysiia Mb is very near to that of vertebrate Mb (Table I).

Hbs/Mbs have been found in the phylum Platyhelminthes, in the representatives of the classes Turbellaria, Trematoda, and Monogenea but are absent from the class Cestoda (16). In Fasciola hepatica, the pigment is intracellular preferentially around the vitellaria and uterine coils. Consequently, it has to be considered as a “tissue Hb” or Mb. The function of the Mbs in these animals is completely speculative as it is assumed that they are facultative aerobes (16). A function in some way in oxygen transport and/or storage is likely. However, the Mb of F. hepatica is extremely antigenic and can be detected in the serum of the host a few days after infection (17). A similar high antigenic character of globins is observed for the Hbs/Mbs of the nematodes where the pigment is found in the excretory/secretory products of Trichostrongylus colubriformis and Nippostrongylus brasiliensis (18–20). The extracellular Hbs of Ascaris suum and Chironomus thummi thummi are also documented as potent allergens (21, 22). The purpose of the
high antigenic character of the Hbs/Mbs of the parasite and their occurrence in the excretory/secretory products is unknown.

Spectral analysis of trematode Mbs and their different derivatives clearly proves the presence of a protoporphyrin IX group in a modified heme environment (23). The major Mb fraction in different trematode species displays a native Mr 15,000–17,000 (24, 25). Minor fractions with Mr 32,000–68,000 composed of monomeric globin chains can also be detected during purification (Table II).

The Mb of Dicrocoelium dendriticum, the small liver fluke, is the best documented trematode globin (Tables I and II). The molecule is monomeric, independent of pH and ligation state (26). Partial sequence data and NMR analysis strongly suggest that the distal His is replaced by Tyr (27, 28). Nevertheless, this monomeric Mb is fully functional, with a high oxygen affinity with a P50 of 0.985 mm Hg, due to a high kcat value, a Hill coefficient of 1.0, and a pronounced acid Bohr effect (26, 29, 30). The latter was proposed to involve the formation and disruption of a salt bridge between a carboxylate side chain and a Lys or Arg residue (26). This is completely in contrast with the human Hb M variants and engineered molecules with a Tyr-E7 which all display a high autoxidation rate very rapidly producing ferri-derivatives unable to bind oxygen (8, 31). Consequently, the Dd Mb must have a specific unknown mechanism to stabilize the Fe(II)-O2 complex. Unfortunately, the primary structure is only partially known, and conflicting data are available for the E-helix (27, 28).

Trematode Mbs are the most primitive animal globins known today; therefore, their Mb must resemble closely the common globin ancestor of the various kingdoms. A phylogenetic analysis based on their primary structure may reveal interesting features.

We here report the primary structure of the Mbs of the trematodes *Paramphistomum epiclitum* and *Isopororchis hypselobagri*.

**Materials and Methods**

**Biological Material—*P. epiclitum* (Platyhelminthes; Trematoda, Par-**

**aphistomatidae) parasitic in the rumen of the common Indian water buffalo *Bubalus bubalis* and *I. hypselobagri* (Platyhelminthes; Tremato-**

**doda: *Isopororchidae* parasitic in the swim bladder of a catfish *Wallago attu* were obtained, respectively, from a local slaughterhouse and fish market in Aligarh, India. The trematodes were washed thoroughly with normal saline and incubated for 1 h at 37°C in 0.15 M NaCl, 8 mM glucose to make them shed their eggs and gut contents. Trematodes were stored at −80°C until use.

**Purification of Trematode Myoglobins and Globin Chains—Tremato-**

**des were homogenized in 3 volumes of 50 mM Tris-HCl, pH 7.0, at 4°C with 1 mM phenylmethylsulfonyl fluoride. The cleared homogenate was differentially precipitated with ammonium sulfate (70% and 90% saturation), and the crude Mb fraction was collected, desalted, and passed through a Sephadex G-75 column equilibrated in 50 mM Tris-HCl,
**Trematode Myoglobins**

### Table III

|        | A-motif | BC-motif | E-motif | Fg-motif | H-motif | Total |
|--------|---------|----------|---------|----------|---------|-------|
|        | NV   | V   | NV   | V   | NV   | V   | NV   | V   | NV   | V   | NV   | V   |
| Phys   | 0.0  | 0.0  | 0.0  | 0.0  | 0.2  | 0.5  | 0.0  | 0.0  | 0.0  | 0.0  | 0.0  | 0.2  | 0.5  |
| Lupi   | 0.0  | 0.0  | 0.0  | 0.0  | 1.0  | 1.2  | 0.0  | 0.0  | 0.0  | 1.0  | 1.0  | 2.2  | 2.0  |
| Asc D1 | 1.0  | 1.0  | 0.0  | 0.2  | 1.0  | 3.9  | 0.0  | 2.4  | 0.0  | 0.7  | 2.0  | 8.2  |
| Asc Mb | 0.0  | 0.0  | 0.0  | 1.2  | 0.0  | 2.9  | 0.7  | 4.1  | 1.0  | 2.4  | 1.7  | 10.6 |
| Pe     | 1.0  | 2.0  | 1.0  | 1.7  | 1.2  | 1.0  | 1.4  | 1.7  | 1.5  | 0.0  | 6.1  | 6.4  |
| Ih     | 0.0  | 2.0  | 0.0  | 0.0  | 0.7  | 3.0  | 2.0  | 1.4  | 1.4  | 0.7  | 0.0  | 5.1  | 7.1  |

**FIG. 1. Purification of Paramphistomum epiclitum myoglobin.** Purification was performed as described under “Materials and Methods.” A, gel filtration chromatography on a Sephadex G-75 column of crude myoglobin fraction. B, SDS-PAGE of fractions representative for the purification. 1, markers; 2, total extract; 3, 0–70% ammonium sulfate precipitate; 4, 70–90% ammonium sulfate precipitate; 5, SG 75 monomeric fraction; 6, globin-3 after focusing (Fig. 2); 7, globin-3 after focusing and RP-HPLC (Fig. 2).

**FIG. 2. Purification of P. epiclitum globins.** Purification was performed as described under “Materials and Methods.” A, separation of globin isoforms by isoelectric focusing. Separation was performed on linear IPG strips with a pH gradient from 4–10. Homogeneity was verified by running on a second dimension SDS-PAGE. Four globin isoforms can be distinguished. B, RP-HPLC of globin-3 after semipreparative one-dimensional isoelectric focusing.

1 The abbreviations used are: CHAPS, 3[(3-cholamidopropyl)dimethylammonium]-1-propanesulfonate; PAGE, polyacrylamide gel electrophoresis; RP-HPLC, reversed-phase high performance liquid chromatography; Pe, Paramphistomum epiclitum; Ih, Isospororchis hypselobagri; Dd, Dicrocoelium dendriticum.
The flow rate of the carrier solvent was 40 µl/min. The capillary voltage was set at 13.81 kV. The source temperature was 80°C. The flow rate of the nebulizing gas and the drying gas were 200 liters/h and 350 liters/h, respectively. The cone voltage was 25 V. The mass spectrometer was scanned from mass to charge 500-1500. Spectra were recorded in the multichannel acquisition mode; results are the averaging of 6 scans. The relative molecular mass (M_r) was calculated using Masslynx software.

Equilibrium Centrifugation—Equilibrium centrifugation was performed in a Beckman Optima XL-A analytical ultracentrifuge according to Yphantis (35). M_r was calculated assuming a partial specific volume, \( \bar{\nu} = 0.725 \text{ ml/g}. \)

Absorption Spectra—Absorption spectra were measured using a SLM-Aminco (DW2000) or HP 8354 spectrophotometer. The oxidized form was obtained by addition of a 10-fold excess of ferricyanide to a 5 mM protein sample, followed by incubation at 37°C for at least 4 h. The spectra of the oxidized form versus pH were made with phosphate buffer (pH <8) or in Tris buffer (pH >8). Kinetics of ligand rebinding were measured after photolysis with 10-ns pulses at 532 nm. Samples were 10 mM on a heme basis, equilibrated under 1 atm of CO or air for the oxygen binding studies.

The ligand replacement reaction (O_2 by CO) is not appropriate in this case involving a very high oxygen affinity. The replacement by CO is very slow and involves an increasing oxygen concentration. The oxygen off-rate was therefore measured by injecting an oxyHb sample into a cuvette containing about 2 mM sodium dithionite equilibrated under nitrogen. The transition from the oxy to deoxy form was followed spectrophotometrically using the HP 8354 with diode array detection. Spectra were recorded every 5 s.

Alignment—The obtained sequences were aligned with relevant non-vertebrate globin sequences using the published templates as a guideline (10, 36, 37). Phylogenetic trees were constructed by the TREECON program (38).

Computer Modeling—A model of the Mb structure of Pe was built using A. suum Hb domain 1 as a template. Insertion/deletions were modeled with the loop modeling algorithm of Ref. 39 which is part of the Brugel modeling package (40). Side chains were modeled using the Dead-End Elimination algorithm (41–43). All modeling work was carried out on a SGI R4000 computer.

RESULTS

Purification and General Characteristics—The Mb of Pe and Ih were purified as described under “Materials and Methods.” As an example, the purification progress for the Pe Mb is documented in Figs. 1 and 2. Similar results were obtained for Ih Mb. The physicochemical characteristics of both Mb molecules are summarized together with data available from literature in Table II.

Absorption Spectra—Absorption spectra of both Mbs in their oxy, deoxy, and carbonmonoxy forms are presented in Fig. 3, A and B. In contrast to the Pe Mb, the Ih Mb is oxidized to its ferric form during purification. Only the deoxy ferrous form was obtained, by addition of dithionite; subsequent addition of oxygen led to the ferric form.

The spectra of both Mbs in function of pH are presented in Fig. 3, C and D. The kinetics after photodissociation of Pe showed a high on-rate, for both oxygen and CO, as for the leghemoglobins. The CO kinetics were monoexponential to within 5% of the total amplitude and were independent of the fraction dissociation, varied by changing the laser intensity. There was thus no evidence for cooperativity within the dimeric forms. Injection of an oxy Pe sample into buffer with dithionite, equilibrated under nitrogen, revealed a slow oxygen dissociation process, requiring 30 s for 2/3 dissociation.

Primary Structure—The primary structure of Pe and Ih globin was reconstructed from N-terminal sequencing of relevant peptides generated by trypsin and protease Asp-N cleavage of the maleylated proteins (Fig. 4).

Alignment—Using the vertebrate and non-vertebrate globin templates, the trematode globin sequences were aligned with globins having known tertiary structures as well as with partial sequence data from D. dendriticum (10, 27, 28, 37) (Fig. 5 and Table III). The presence of Tyr at the distal position (E7)
sequences (10). From this data set, a phylogenetic tree was extended with representative nonvertebrate globin domain I (GLB_ASCSU); among multicellular organisms. The major Mb fractions of lomates, their Mbs represent the most primitive globin type from the Hbs/Mbs of their hosts (16). As trematodes are acoelomates, their Mbs are monomeric (14). Their mass obtained by different techniques clearly confirm each other. The monomeric nature of these Mbs agrees with their intracellular localization (45). Isoelectric focusing under denaturing conditions reveals different isoforms of closely related pI in the range of pH 5–7. Ascaris globin structure is known to directly hydrogen-bond to the heme-bound di-oxygen (as in Fig. 6). The absorption spectra for Pe were typical for most mammalian Hbs, with peaks for the ferrous oxy, deoxy, and CO forms in the range of pH 5–7. Pe, as well as Ih, displays at least four major globin isoforms (Table II; Fig. 2). It is unknown if these isoforms are the products of different genes or if they result from post-translational modifications. Different globin isoforms, in one species, are common in other trematodes as well as in other nonvertebrates (Table II) (46). In the nematodes N. brasilienis, Syngamus trachea, and Ostertachia ostertagi cuticular and body wall globin isoforms have been reported (19, 20). Therefore, it cannot be excluded that the trematode globin isoforms are also tissue-specific.

The oxygen affinity was so high that the deoxy and CO samples still show evidence of a small percentage (~3%) oxy form. This is most obvious for the deoxy sample near 550 nm, where the deoxy absorption peak is flattened, due to a relative minimum of the oxy form. The CO Soret band is lower than for human Hb, also indicating a perturbation by the oxy form.

Injection of an oxyMb sample into a cuvette with sodium dithionite equilibrated under nitrogen allowed a measurement of the oxygen dissociation kinetics; a time constant of 30 s was determined for the oxy to deoxy transition. This is orders of magnitude higher than the millisecond kinetics for most Mb and therefore indicates a very high oxygen affinity as for Hb Ascaris.

The stock samples were in the oxy form. The samples were incubated at 37 °C to study the transition from oxy to the oxidized form. The rate for this process was 0.04/h, a value typical for most Hbs and Mbs. This value is therefore not

**DISCUSSION**

Trematodes contain respiratory proteins clearly different from the Hbs/Mbs of their hosts (16). As trematodes are acoelomates, their Mbs represent the most primitive globin type among multicellular organisms. The major Mb fractions of Pe and Ih are monomeric (M, 16,000–17,000) as are all other trematode globins characterized so far (Table II). Concentration-dependent dimeric forms are observed as well. The molecular mass obtained by different techniques clearly confirm each other. The monomeric nature of these Mbs agrees with their intracellular localization (45). Isoelectric focusing under denaturing conditions reveals different isoforms of closely related pI in the range of pH 5–7. Pe, as well as Ih, displays at least four major globin isoforms (Table II; Fig. 2). It is unknown if these isoforms are the products of different genes or if they result from post-translational modifications. Different globin isoforms, in one species, are common in other trematodes as well as in other nonvertebrates (Table II) (46). In the nematodes N. brasilienis, Syngamus trachea, and Ostertachia ostertagi cuticular and body wall globin isoforms have been reported (19, 20). Therefore, it cannot be excluded that the trematode globin isoforms are also tissue-specific.

The absorption spectra for Pe were typical for most mammalian Hbs, with peaks for the ferrous oxy, deoxy, and CO forms at 415, 432, and 420 nm, respectively (Fig. 3). The oxygen affinity was so high that the deoxy and CO samples still show evidence of a small percentage (~3%) oxy form. This is most obvious for the deoxy sample near 550 nm, where the deoxy absorption peak is flattened, due to a relative minimum of the oxy form. The CO Soret band is lower than for human Hb, also indicating a perturbation by the oxy form.

Injection of an oxyMb sample into a cuvette with sodium dithionite equilibrated under nitrogen allowed a measurement of the oxygen dissociation kinetics; a time constant of 30 s was determined for the oxy to deoxy transition. This is orders of magnitude higher than the millisecond kinetics for most Mb and Hb and therefore indicates a very high oxygen affinity as for Hb Ascaris.

The stock samples were in the oxy form. The samples were incubated at 37 °C to study the transition from oxy to the oxidized form. The rate for this process was 0.04/h, a value typical for most Hbs and Mbs. This value is therefore not
consistent with the correlation of a lower oxidation rate for a higher oxygen affinity (31). The same is true for Hb *Ascaris*, indicating that another mechanism may cause the oxidation in the case of these very high affinity proteins.

From the absorption spectra we have to conclude that the *Pe* Mb is fully functional with a very high oxygen affinity. This confirms previous observations on *Dd* Mb (Table II) but is in contrast with the human Hbs M which have a distal Tyr as well, but are unable to bind oxygen. However, *Ih* Mb shows the same rapid oxidation as Hbs M.

The spectra of the oxidized form were not typical (Fig. 3B). While human Hb shows an acid (Soret at 406 nm with a water molecule as ligand) to alkaline (Soret at 420 nm) transition with a pK value near pH 8, the *Pe* Mb showed only a partial transition. This could indicate a very low pK value (below 4), but the results were hampered by a time dependence in the spectra, especially at the lower pH values. This would indicate a difference in the residues forming the distal side of the heme pocket.

Recombination of CO and oxygen to *Pe* Mb showed high on-rates, as for leghemoglobins. This combined with the very low oxygen dissociation rate, as for Hb *Ascaris*, leads to an extremely high oxygen affinity. As for Hb *Ascaris*, the partition coefficient (ratio of CO to oxygen affinity) is less than 1.

In *Pe* Mb, the iron atom is clearly in the Fe$^{2+}$ (ferrous) form, binding oxygen very strongly. This Mb is very resistant to autoxidation during the purification process, whereas the *Ih* Mb is in the oxy form within the trematode body but as soon as it is exposed to air, autoxidation starts. This suggests a different heme environment in both Mbs. This is confirmed by the difference of the spectra of both Mbs as a function of pH (Fig. 3B). In the hydroxy-met form, the usual 540 and 575 nm maxima are present in both Mbs although in different intensities. However, both spectra are characteristic for the unusual presence of a shoulder at 603 nm. A similar shoulder is observed in the hydroxy-metMb spectrum of *Dd*, and it is proposed, based on analogy with the spectra of human Hb M mutants and hemin in dimethyl sulfoxide, that it substantiates the occurrence of an axially bound tyrosinate (phenoxide) to the central iron atom. Although the sequence of the heme environment of *Dd* Mb is uncertain, a distal Tyr is suggested based on optical spectra and $^3$H NMR analysis (47–49). The similarity in
the hydroxy-metMb spectra of Pe and Ih Mb with the Dd Mb spectra suggest that in the former also a E7 Tyr may occur.

The primary structure of Pe globin-3 was reconstructed from relevant peptides generated by digestion of the maleylated polypeptide with trypsin and endoprotease Asp-N as documented in Fig. 4. All necessary overlaps are presented, and the majority of residues are sequenced at least twice. Moreover, the globin primary structure (C helix to carboxyl terminus) was confirmed by sequencing the coding gene.2 Globin-3 has 147 residues and a mass of 16,643.00 calculated from amino acid sequence. This is in good agreement with the mass determined by mass spectrometry (16,643.2 ± 1.58; Table II) and argues again for the correctness of the sequence. The primary structure of Ih globin-2 is determined in a similar way. It has 147 residues and a mass of 16,499.67 calculated from amino acid sequence. Not all peptide overlaps are presented. However, as the alignment with Pe globin-3 and Dd globin is unambiguous, we are confident that no residues/peptides are missing (Fig. 4).

Using existing templates, the Pe and Ih globin sequences together with the partial sequence of Dd globin are aligned with sperm whale Mb and nonvertebrate globins with known tertiary structure (10, 27, 36, 37) (Fig. 5). The alignment is confirmed (i) by the exclusion of polar residues out of the 33 invariant nonpolar sites listed in (51, 52); (ii) by the alignment of Pro-C2, which determines the folding of the BC corner; (iii) by the heme-linked His-F8; (iv) by the Phe-CD1, which is a heme contact invariably found in globin sequences; and (v) by the alignment of Gly or Ala residues B6 and E8, which are essential for the normal close contact between helices B and E. This results in a low penalty score for the globins against the vertebrate template II (36) and the nonvertebrate template (37) suggesting that the overall globin fold is conserved.

All three trematode Mb sequences show a high degree of homology (~32%) especially in the first half of the molecule. The presence of all helical segments, including a short D-helix, can be accepted. The physicochemical nature of residues at key positions as NA2, B6, B14, C2, CD1, F8, and G5, as well as the residues in the proximity of the heme group, is conserved (Fig. 5). In all three trematode globin sequences, many side chains in the proximity of the heme pocket, especially at the distal side, are aromatic. Deviations of the classical globin pattern also occurs; Leu-A12, Tyr-B10, Tyr-Cα, Tyr-Eγ, Tyr-F4, and Leu-H8 are rather exceptional.

At A12 and H8, the usual Trp is substituted in all three trematode sequences to a Leu, as in nematode globins, which might suggest that the interaction between A and E/H helix is more hydrophobic. The Tyr at position C4 is only precedent in the globin domains of the extracellular Hb of Artemia (53). The occurrence at this position of Ile in the Chironimids Hbs proves that there is space to harbor a bulky side chain (54).

Although an aromatic side chain at F4 is rather common, a Tyr at this position is only shared with T. heteroactus globin I. A Pro residue is present in the middle of the G and H helix (G10, H13) so that a bend of the helices can be expected.

The three trematode globins share the unusual Tyr-B10. This also occurs in legHbs and in the globins of nematodes, protists, and yeasts. All these Hbs share a relative high oxygen affinity (10, 19, 20, 37). This is due to either a high k_{on} (legHbs) or a low k_{off} (Ascaris) value (6, 11). The low k_{off} value, hence the high oxygen affinity, of the Ascaris periericent fluid Hb is due to the formation of a strong hydrogen bond between Tyr-B10 and the distal oxygen of the ligand, combined with a weak hydrogen bond between Gln-E7 and the proximal oxygen whereas the total structure is stabilized by a third hydrogen bond between both residues involved (11, 13, 14). Preliminary results indicate a high oxygen affinity for the Pe Mb, and this confirms the measurements on the Dd Mb (30). In contrast, legHb3 shows a normal oxygen k_{off} rate and a moderately higher oxygen affinity due to a higher k_{on} rate.

Most remarkable, however, is the presence of a Tyr-E7 in all three trematode Mb sequences. This is already suggested by the hydroxy-met spectra described above (Fig. 5B) and confirms previous work on Dd Mb (47–49, 56). As a Hbs is present at the E6 position, confirmation of the nature of the distal residue is essential. 1H NMR measurements on Pe Mb clearly confirm the presence of Tyr-E7, Leu-E11, and Tyr-B10 as proposed by the alignment (1). Therefore, this is the first functional monomeric Mb with confirmation of a Tyr at the distal position. As human Hbs with a distal Tyr always occur as their ferri derivatives (Hb M), unable to bind oxygen, the molecular architecture of the Pe Mb must be so that high oxygen affinity is created and the iron atom protected from oxidation.

Computer modeling of Pe Mb suggests two possible orientations for the Tyr-E7, each of which is at about the same distance (~5 Å) from the nearest atom in Phe-C1D. In one orientation, the Tyr is turned away from the heme (Fig. 6A), thereby making the heme pocket accessible to oxygen. In the other orientation (Fig. 6B), the hydroxyl group of Tyr-E7 forms a hydrogen bond with the iron-bound oxygen atom (distance between oxygen atoms equals 2.5 Å) thereby possibly stabilizing the O2 molecule in the heme pocket. The other oxygen atom of the oxygen binds to Tyr-B10 (distance between oxygen atoms equals 2.7 Å) in the same way as observed in the Ascaris structure (14). Leu-E11 and Phe-G12 are in close contact with each other (2.25 Å between Leu-E11 HD21 and Phe-G12 HE2) and are buried deep into the heme pocket with the closest distance of 4.6 Å between Leu-E11 CD1 and heme-CMB. As Tyr-E7 is close to Leu-E11, this strongly suggests the localization of Tyr-E7 within the heme pocket. This hypothesis is confirmed by NMR data presented in the accompanying paper (1).

Similar reorientation of the distal residue (E7), inside or outside the heme pocket, is observed in the difference Fourier maps of the deoxy- and cyanomet form of Hb III of C. thummi (54). The same is suggested to occur, though to a lesser extent, in the leg Hbs (57). Both globin types display a high oxygen affinity due to a high k_{on} rate created by an accessibility of the heme pocket approaching diffusion limits (6).

Another interesting feature in our model of Pe Mb is that the aromatic Tyr-F4 is not oriented parallel to His-F8. This cannot be due to the presence of an aromatic side chain (Phe) at the nearby G5 position. Indeed, in Aplysia limacina (PDB code 1mba), a Phe is observed at both G5 and F4 while Phe-F4 adopts a parallel orientation to His-F8. We believe that Tyr-F4 is turned away from His-F8 because otherwise its hydroxyl group would be devoided from hydrogen bonding interactions.

In the orientation resulting from the present modeling (Fig. 6), the hydroxyl is positioned in the solvent. Interestingly, the small nearby Ala residue at E14 may favor this well solvated position of the phenol group even more. It is satisfying to observe that, independently from the modeling work, the NMR analysis detects a NOE contact between Tyr-F4 and Ala-E14 which provides additional evidence that Tyr-F4 is turned away from His-F8 (1).

The phylogenetic tree presented in Fig. 7 clearly demonstrates the primitive nature of the trematode Mbs. Both trematode globins have 43.84% identity with each other and share no more than 15–20% with all other globin sequences. Inter-

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2 S. Dewilde, K. A. Rashid, and L. Moens, manuscript in preparation.
3 Q. H. Gibson and L. Parkhurst, unpublished data.
prevention of the tree, however, must be done with caution as the oldest branches are not supported by bootstrapping.

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Trematode Myoglobin

nation of a Hemeprotein from the Small Liver Fluke Dicrocoelium dendriticum.

Ph.D. thesis, University of Zurich

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