A two-dimensional $^1$H NMR study has been carried out on the heme cavity of the extreme oxygen-avid and autoxidation-resistant oxy-myoglobin complex from the trematode Paramphistomum epiclitum, and the residues were identified which potentially provide hydrogen bond stabilization for the bound oxygen. Complete assignment of the heme core resonances allows the identification of 10 key heme pocket residues, 4 Phe, 4 Tyr, and 2 upfield ring current aliphatic side chains. Based solely on the conserved myoglobin folding topology that places the E helix-heme crossover and the completely conserved Phe(CD1)-heme contact at opposing meso positions, the heme orientation in the cavity and the E helix alignment were unambiguously established that place Tyr$^{66}$ at position E7. Moreover, all eight aromatic and the two aliphatic side chains were shown to occupy the positions in the heme cavity predicted by amino acid sequence alignment with globins of known tertiary structure. The dipolar contacts for the Tyr$^{22}(B10)$ and Tyr$^{66}(E7)$ rings indicate that both residues are oriented into the heme cavity, which is unprecedented in globins. The ring hydroxyl protons for both Tyr are close to each other and in a position to provide hydrogen bonds to the coordinated oxygen, as supported by strong retardation of their exchange rate with bulk solvent. A more crowded and compact structure increases the dynamic stability of the distal pocket and may contribute to the autoxidation resistance of this myoglobin.

Myoglobin and hemoglobin are oxygen binding proteins that reflect extraordinary structural homology in spite of often very limited sequence homology (1, 2). Both are composed of ~150-residue globular proteins containing eight helices (labeled A–H) and an iron-protoporphyrin-IX (heme) bound to the completely conserved His(F8); the only other completely conserved residue is Phe(CD1). Among the vertebrate Hbs/Mbs, the key hydrogen bond via Arg(E10) (8), and the parasitic trematode, Ascaris suum, HbI, which provides two hydrogen bonds to the bound O$_2$ via a relatively common Gln(E7) and an uncommon Tyr(B10) (9, 10). Tyr(B10) is present in several other trematode Hbs/Mbs (11, 12), among others (13–15).

The Tyr(B10) hydrogen bond has been identified as the source of the extra stabilization that leads to extreme O$_2$ avidity in A. suum HbI through primarily an extremely slow O$_2$ off-rate (9, 12); the autoxidizability is similar to that of common Mbs/Hbs. The Tyr(B10) is present in Lucina pectinata Hbs (13, 14), A. suum MB(12), and legume Hb(16, 17) but does not result in similarly slow O$_2$ off-rates and likely does not provide a hydrogen bond to the O$_2$. $^1$H NMR studies of the nematode Dicrocoelium dendriticum Hb had identified a Tyr hydrogen-bonded to the bound ligand (18, 19). However, it is clear that Tyr at position B10, and possibly E7, can strongly stabilize the Fe-O$_2$ bond in some cases but not in others. Engineering a Tyr(B10) and Gln(E7) into sperm whale Mb to mimic A. suum HbI failed to reproduce the high oxygen affinity for the latter Hb but demonstrated that Tyr(B10) provides H bond stabilization at the bound O$_2$ (20).

Alignment of the amino acid sequence of the globin from the trematode Paramphistomum epiclitum clearly reveals Tyr at position B10 and places, unprecedented, Tyr$^{66}$ rather than His$^{65}$ at position E7 (21), as shown in Fig. 1. P. epiclitum Mb has a high oxygen affinity created by a high "on-rate" and is rather resistant to autoxidation. In order to shed light on the nature of the active site in this unusual P. epiclitum MbO$_2$ complex, unequivocally establish the alignment of the E helix, and determine the potential role of Tyr(E7) and Tyr(B10) interacting with bound oxygen, we report herein on a solution $^1$H NMR study of its heme cavity. Our interests are to assess the degree to which homologous residues on the various helices make contact with the heme when compared with A. suum HbI (9, 10) or sperm whale Mb crystal structures (22) and as suggested by the molecular modeling (21). The limited availability of the protein dictated that a choice had to be made between sample concentration and sample purity for the desired two-dimensional $^1$H NMR study. It was concluded that the needed ~1.5 mM concentration was possible only for a sample ~60%

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1 The abbreviations used are: Hb, hemoglobin; Mb, myoglobin; NOESY, two-dimensional nuclear Overhauser spectroscopy; TOCSY, two-dimensional total correlation spectroscopy; DSS, 2,2’-dimethyl-2-pentene-5-sulfonate.

2 Q. H. Gibson and L. J. Parkhurst, personal communication.
pure, with the remainder one or more of the minor Mb isomers. This sample heterogeneity limits the scope of the study but not the details to which the positions of the identified heme cavity residues can be placed relative to each other and to the heme.

EXPERIMENTAL PROCEDURES

Protein Purification—P. epiclitum (Platyhelminthes, Trematoda, Paramphistomatidae) Mb was prepared by ammonium sulfate precipitation and gel filtration chromatography as described elsewhere (21). The resulting preparation is homogeneous in M, as evidenced by SDS-polyacrylamide gel electrophoresis and equilibrium centrifugation. Separation by isoelectric focusing under native conditions reveals two major isomers differing in less than 0.1 pH unit which could not be purified further. Isoelectric focusing under denaturing conditions reveals four different globin isomers. It is not known if these are real isomers, coded by different genes, or if they are the result of post-translational modifications or artificial modifications during the isolation process. Due to its high oxygen avidity, the purified protein remains in the oxy-Mb form as shown by spectral analysis (21). The final oxy-Mb sample contained ~1.5 m
total heme concentration in 50 mM NaCl and 50 mM phosphate buffer at pH 7.1. The 1H2O solution was subsequently converted to 2H2O solution using an Amicon ultrafiltration cell. Solution pH was adjusted with NaOH (Na2H) or HCl (2HCl) solution.

NMR Spectra—1H NMR data were collected on a GE Omega-500 spectrometer operating at 500 MHz for protein samples in both 1H2O and 2H2O. The observed chemical shifts are referenced to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) via the solvent signal. Solvent suppression, when necessary, was achieved by direct saturation during the relaxation delay. Phase-sensitive TOCSY (23, 24) and NOESY (25) were used. The 90° pulse width was 8 μs (~25 μs for TOCSY). 128 scans were collected for each block with a repetition time of 1.2 s. Two-dimensional data sets were processed by FELIX software on a Silicon Graphics workstation. Both NOESY and TOCSY spectra were processed by FELIX software on a Silicon Graphics workstation. Two-dimensional data sets were processed by FELIX software on a Silicon Graphics workstation.

RESULTS

Sample Heterogeneity—The resolved portions of the 500 MHz 1H NMR spectrum of P. epiclitum MbO2 in 90% 2H2O, 10% 1H2O at 30°C, pH 7.1, are shown in Fig. 2. Inspection of both the low field and high field resolved portions of the spectrum reveals a molecular heterogeneity indicative of two obvious compounds, in relative ratio ~2:3. In the low field spectrum, the three likely meso-H candidates differ in intensity with the extreme low field peak being broader and significantly more resonant than the other two equal intensity resolved low field peaks. Similarly, the high field portion of the spectrum reveals several ring-current shifted peaks with intensity for methyl groups for both the major and minor components. Contributions from signals from both components in the composite peak centered at ~0.8 ppm are partially resolved at 38°C (Fig. 2B). Our interest is in the peak assignment and structure elucidation of solely the major component whose residue origin in Fig.

2 are labeled by the one-letter amino acid code with the deduced helical positions given in parentheses. Resonances for any minor component are simply labeled Xi; the possible relationship of the minor to the major component will be addressed briefly below. The determination of the alignment of the E helix and nature of the interaction of distal residues with the bound ligand are pursued by identifying contacts to the heme, with emphasis on resolved aliphatic residue peaks in the ring-current upfield spectral window and the aromatic side chains, each of which can be readily identified by highly characteristic TOCSY cross-peak patterns even in an impure sample. To guarantee that all assignments relate solely to the major isoform of interest, we report assignments only when we observe multiple dipolar contacts between pairs of residues or between a residue and two or more heme substituents.

Heme Assignment—The use of the heme as a “template” to place on the upfield aliphatic and low field aromatic side chains first requires unambiguous assignment of the heme resonances. The low field 8–11 ppm portion of the NOESY spectrum in Fig. 3 reveals four nonlabile peaks which fail to exhibit TOCSY peaks and hence must arise from meso-Hs; two are resolved (the heme positions are labeled in Fig. 4). The four meso-Hs exhibit strong NOESY cross-peaks to two, one, one, and no likely heme-methyls in the 2.5–3.8 ppm window (Fig. 3B) which also fail to exhibit TOCSY cross-peaks. These dipolar contacts therefore differentiate the δ-meso-H (nuclear Overhauser effects to 1-CH3 and 8-CH3), γ-meso-H (no heme methyl neighbor), and the pair α-meso-H, β-meso-H (nuclear Overhauser effect to 3-CH3 or 5-CH3). TOCSY identifies two AMX spin systems at low field (Fig. 5A) with chemical shift and spin-coupling patterns diagnostic of two vinyl groups in a diamagnetic protein. NOESY cross-peaks from one vinyl to one
of the 1-CH₃, 8-CH₃ pair identify 1-CH₃ and the 2-vinyl group and, by inference, 8-CH₃ and the 4-vinyl group (Fig. 5B). The NOE spectra of the 2-vinyl group to a meso-H differentiate α-meso-H from β-meso-H and identify both the 3-CH₃ and 5-CH₃ (Fig. 5B) The remaining γ-meso-H exhibits NOE cross-peaks to two sets of spin-coupled protons near 3.5 ppm (Fig. 5B) which exhibit NOE cross-peaks to 3-CH₃ or 8-CH₃. These identify the H₃ for the two propionate groups and complete the heme ring core substituent assignment in a unique and self-consistent manner (26, 27) that guarantees the major component alone is being characterized. The chemical shifts for assigned heme resonances are listed in Table I.

**Table I.**

| Chemical Shift | Protons          | Assignments |
|----------------|------------------|-------------|
| 3.3 ppm        | 3-CH₃, 8-CH₃     | H₃ for propionate groups |
| 3.5 ppm        | 1-CH₃, 2-vinyl   | 1-CH₃, 2-vinyl group |
| 3.8 ppm        | 5-CH₃            | 5-CH₃ |
| 4.0 ppm        | 4-vinyl          | 4-vinyl |
| 4.2 ppm        | 1-CH₂            | 1-CH₂ |

**Heme Pocket Residues**—Assuming the basic Mb fold and a heme orientation about the α-γ-meso-H axis for *P. epiclitum* Mb that is the same as in *A. suum* HbI (9, 28) and sperm whale Mb (22), the sequence alignment and homology model (21) predict the following for aromatic side chain contacts to the heme: Tyr³⁴(C4) with pyrrole A, Phe¹⁰⁸(G5), Phe¹¹⁵(G12) with the pyrrole A/B junction, Tyr⁴²(C4) with pyrrole B, Phe⁴⁶(CD1) with pyrrole C, and, if oriented into the heme pocket, Tyr⁶⁶(E7) with the pyrrole C/D junction. In addition, Tyr⁴⁲(B10) and Phe⁴⁶(B14) are expected to contact Phe⁴⁶(CD1). Lastly, the upfield shifted E helix position residues Leu⁷⁰(E11) and Ala⁷³(E14) are expected to make contact to the pyrrole A/B junction. The expected dispositions relative to the heme for the eight aromatic side chains, Leu⁷⁰(E11), and Ala⁷³(E14) is shown schematically in Fig. 4. It is to be noted that, although the assignments for amino acid residues and their placement near the heme and each other are presented for simplicity as confirmations of aspects of the homology model (21), the conclusion on the E helix alignment and conserved folding topology are, in fact, determined independently by ¹H NMR data.

The TOCSY spectrum (Fig. 6A) for the upfield methyl peak at −1.1 ppm reveals its origin as a Leu, which has dipolar contacts to the heme 1-CH₃ (Fig. 6B), 2-vinyl (Fig. 5D), and δ-meso-H (Figs. 3C). The TOCSY map (Fig. 6A) also identifies a complete Ala spin system with similarly strong NOE cross-peaks to the heme 1-CH₃, 8-CH₃ (Fig. 6B), and δ-meso-H (not shown). The upfield ring current shifts and contacts to the 1-CH₃ and 8-CH₃ are characteristic of Leu⁷⁰(E11) and Ala⁷³(E14) in the normal Mb fold if the heme is oriented like in sperm whale Mb (22) or *A. suum* Hb (9, 28). While the peptide NHs for both residues could be located, the small chemical shift dispersion and the resultant spectral congestion precluded tracing the backbone to uniquely elucidate the sequence origin of the two residues; however, the expected NOE cross-peaks from Ala(E14) C₆H₃ to Leu(E11) C₆H₃ and C₆H₆ (Fig. 6B) confirms their i, i + 3 positions on a helix.

The TOCSY spectrum in Fig. 5A for the aromatic spectral window exhibits all the cross-peaks for four three-spin (rotationally averaged Phe rings) and four two-spin (rotationally averaged Tyr rings), all of which can be demonstrated to arise from the major isomer for which the heme has been assigned. NOE cross-peaks to both 5-CH₃ (Fig. 5C) and β-meso-H (Fig. 3A) for two ring protons identify Phe⁴⁶(CD1), whereas the two ring protons of the other Phe rings yield NOESY cross-peaks to α-meso-H (Fig. 3A) 3-CH₃ (Fig. 5C), and α-meso-H (Fig. 3A) and 2-vinyl (Fig. 5B), thereby uniquely identifying Phe¹¹⁵(G12) and Phe¹⁰⁸(G5). Both protons of two Tyr rings exhibit NOESY cross-peaks to 3-CH₃ (Fig. 5C), 4-vinyl (Fig. 5B), and 1-CH₃ (Fig. 5C), identifying Tyr⁴²(C4) and Tyr⁴²(C4), respectively. Both protons of one of the two remaining Tyr rings fail to exhibit NOESY cross-peaks to any heme substituent but display such cross-peaks to the Phe⁴⁶(CD1) ring (Fig. 5B) and...
Leu$^{70}$(E11) methyls (Fig. 5D) which uniquely label it as Tyr$^{32}$(B10). The remaining Phe ring exhibits NOESY cross-peaks to both the Phe$^{46}$(CD1) and Tyr$^{32}$(B10) rings (Fig. 5B), as is characteristic of Phe$^{36}$(B14). The remaining Tyr ring exhibits NOESY cross-peaks to both the heme 6-H$_{as}$ (not shown) and Leu$^{70}$(E11) methyls (Fig. 5D), and hence must arise from Tyr$^{66}$(E7). Other expected and observed intra residue cross-peaks are between Tyr$^{94}$(F4) and Ala$^{73}$(E14) (not shown) and Tyr$^{32}$(B10) ring and Leu$^{70}$(E11) methyls (Fig. 5D).

Comparison of NOESY maps in $^1$H$_2$O and $^2$H$_2$O reveals the presence of two labile protons with a weak NOESY cross-peak.
between them (Fig. 5E), for which the absence of any TOCSY cross-peaks indicates that they likely arise from two Tyr ring hydroxyls (labeled OH1 and OH2 in Fig. 4). OH1 exhibits moderate intensity NOESY cross-peaks to the rings of Tyr^{32}(B10) and Tyr^{66}(E7), as well as cross-peaks to the ring (C,H) of Phe^{46}(CD1) (Fig. 5B), and to a C,H{3} of Leu^{70}(E11) (Fig. 5D), as shown schematically in Fig. 4. OH2 exhibits moderate intensity NOESY cross-peaks to both the ring of Tyr^{66}(E7) (Fig. 5E) and the C,H{3} of Leu^{70}(E11) (Fig. 5E). These NOESY patterns identify OH1 and OH2 as the side chain hydroxyl protons of Tyr^{32}(B10) and Tyr^{66}(E7), respectively, and places both labile protons well within the heme cavity and in the vicinity of the ligand. The hydroxyl protons of Tyr^{94}(F4) and Tyr^{42}(C4) could not be located, either because of spectral congestion and/or rapid exchange with solvent. The absence of detectable magnetization transfer to the Tyr^{32}(B10) and Tyr^{66}(E7) hydroxyl protons upon saturating the solvent resonance dictates that the labile proton lifetime, \( \tau_{\text{OH}} \), are much longer (by \( > 10 \)) than the expected T_{1} for the resonances (\( \sim 250 \) ms), leading to \( \tau_{\text{OH}} \geq 2 \) s at pH 7.0 (29). The positions of the 10 identified heme pocket residues relative to the heme and to each other and the observed NOESY cross-peak patterns are depicted schematically in Fig. 4, and the chemical shifts are listed in Table II.

The Minor Component—Sufficient assignments are pursued in order to establish whether the minor component (~40%) arises from the heme reoriented 180° about the \( \alpha, \gamma \)-meso axis (30, 31), from another polypeptide chain (isoform), or from damage or modification during isolation. The upfield TOCSY map reveals three minor component methyl peaks (labeled X_{2}, X_{3}, and X_{4} in Fig. 2A) which exhibit spin topology indicative of three CH_{2}-CH_{3} fragments (Fig. 6A) of three different Ile, two of which exhibit NOESY cross-peaks between each other (Fig. 6B), to the low-field resolved meso-H (Fig. 3C) and its adjacent methyl, as well as to an aromatic side chain (not shown). The presence of upfield Leu signals for the major component and Ile signals for the minor component in solution indicates that the heterogeneity is due to the presence of two different polypeptide chains and not due to either heme orientational isomerisms or damage to the major component. Lastly, the significantly broader resolved meso-H signal and strong intensity of NOESY cross-peaks for the minor relative to the major component suggests that the minor component may be oligomeric. Assignments were not pursued further for the minor component.

DISCUSSION

The unique assignment of the heme for the major isoform of \( P. \) epiclitum MbO_{2}, together with the observation of all aromatic ring contacts to the heme and among each other, as expected by amino acid sequence homology with globins of known tertiary structure, provide direct evidence that the heme pocket architecture is very similar to that of other typical Mbs/Hbs. These data also establish both the alignment of the E...
helix as proposed (21), with a Tyr66 at position E7, and the orientation of the heme as found in sperm whale Mb and A. suum Hbl. The residues Tyr32(B10), Phe36(B14), Tyr42(C4), Phe46(CD1), Leu70(E11), Ala73(E14), Tyr48(F4), and Phe106(G5) generally occupy the same positions in the heme pocket as do the homologous residues in other globins. The position of Tyr32(B10) over the heme is supported by both its upfield ring current shifted side chain protons and its NOE5Y pattern to surrounding residues. The Tyr32(B10) ring labile proton also exhibits NOE5Y cross-peaks to a series of peripheral residues (Phe66(CD1), Leu73(E14)) that both confirm its position and exhibit strong NOE5Y cross-peaks to the Phe115(G12) ring over the pyrrole A/B junction (Fig. 5). The Tyr32(B10) ring labile proton also places it in a position where it could provide the second hydrogen bond to the bound oxygen, as well as form a hydrogen bond to the Tyr110 hydroxyl group to stabilize the optimal orientations of both Tyr32(B10) and Tyr66(E7) for hydrogen bonding to O₂. The role of both Tyr32(B10) and Tyr66(E7) hydroxyl protons in forming hydrogen bonds to the ligand is independently supported by the very slow exchange rate with bulk water, with estimated lifetime ~2 s at pH 7.1, some 10⁴ slower than for a free Tyr at this pH (29). This is the first case where a Tyr is demonstrated not only to occupy position E7 but to orient into the heme pocket of a functional globin in a manner common to His(E7) in mammalian Mbs and Hbs.

The present study, however, does not shed much light on the extraordinary resistance of P. epiclitum MbO₂ to autoxidation when compared with Hbs of other nematodes such as Isopararochis hypsolbargi (21) and nematodes such as A. suum (9). Autoxidation of Mb is strongly dependent on hydrogen bonding to the bound ligand by the distal residue and the size and hydrophobicity of the distal side of the heme pocket (5). NMR data on P. epiclitum MbO₂ indicate the formation of two hydrogen bonds by Tyr32(B10) and Tyr66(E7) to the bound oxygen and establish the presence of an unprecedented number of bulky hydrophobic residues in the crowded distal heme pocket (21). A more crowded distal pocket for P. epiclitum Mb than other Mbs, Hbs is supported by the observed line broadening of the Phe66(CD1) ring protons. In spite of its close proximity to the heme, “H NMR has shown that this highly conserved aromatic ring undergoes rapid 180° ring reorientation that completely averages the environments of the two Hs (and two Hs) ring protons in common Mbs and Hbs (27, 33, 34). The significant broadening of the Phe66(CD1) H resonance (Fig. 2A and B) and cross-peaks (Fig. 5B) in P. epiclitum MbO₂ is indicative of slow ring reorientation and suggests a more compact and dynamically more stable distal pocket than in other NMR characterized Hbs and Mbs, which likely contribute to the resistance to autoxidation.

It is clear, however, that the presence of certain residues in the heme pocket (i.e. Tyr110) is insufficient information for predicting oxygen binding and autoxidation resistance. This is illustrated by the A. suum Mb and A. suum Hbl, both with Tyr110 and Glu(E7), but with differences of a factor 50 in kₐₐₜ for oxygen (9, 10, 12), and by the P. epiclitum and I. hypsolbargi Mbs, which exhibit very different autoxidation rates in spite of both possessing Tyr110 and Tyr(E7) (21). Indeed, the complete tertiary structure is involved in fine tuning the orientations of residues to their critical positions. Crystallization experiments with different trematode Mbs are in progress.

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TABLE II

| Residue | Peak | 8° |
|---------|------|----|
| Leu70 (E11) | NH | 8.49 |
| | C₅ | 3.48 |
| | C₅/C₅H₁ | 0.620/0.91 |
| | C₅ | 0.56 |
| | C₅/C₅H₁ | -0.81/1.10 |
| Ala73 (E14) | NH | 7.81 |
| | C₅ | 4.60 |
| | C₅H₁ | 1.70 |
| Tyr32 (B10) | C₅H₅ | 5.87 |
| | C₅ | 5.48 |
| | OH (OH1) | 8.12 |
| Phe36 (B14) | C₅H₅ | 6.99 |
| | C₅ | 6.80 |
| | C₅H₁ | 5.81 |
| Tyr42 (C4) | C₅H₅/C₅H₅ | 6.857/0.4 |
| Phe56 (CD1) | C₅H₅ | 7.09 |
| | C₅ | 6.04 |
| | C₅H₁ | 6.44 |
| Tyr66 (E7) | C₅H₅ | 7.42 |
| | C₅ | 6.75 |
| | OH (OH2) | 8.50 |
| Tyr48 (F4) | C₅H₅/C₅H₅ | 7.71/7.16 |
| Phe106 (G5) | C₅H₅ | 7.50 |
| | C₅ | 7.49 |
| | C₅H₁ | 7.19 |
| Phe115 (G12) | C₅H₅ | 7.10 |
| | C₅ | 6.96 |
| | C₅H₁ | 5.82 |

* Chemical shift, in ppm, referenced to DSS, in 1H2O, pH 7.1 at 30 °C.

NMR Analysis of Trematode Myoglobin Heme Cavity, Hydrogen Bonding

3005
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