Aggregated Heme Detoxification Byproducts in Malarial Trophozoites: β-Hematin and Malaria Pigment Have a Single $S = {}^5/_2$ Iron Environment in the Bulk Phase as Determined by EPR and Magnetic Mössbauer Spectroscopy

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With the spread of chloroquine-resistant strains of *P. falciparum*[1] and the absence of a suitable replacement for this once very effective antimalarial, there is increasing urgency to understand the biochemistry behind its drug action.[2] The most recent hypothesis, that chloroquine inhibits heme aggregation activity in ring stage, or early, malaria trophozoites,[3] has sustained considerable scrutiny.[4−8] An important recent development is the recognition of a heme-aggregating histidine-rich protein, HRP-II, whose activity is inhibited by low levels of chloroquine.[9] The heme-aggregated byproduct of malarial trophozoites, termed malarial pigment or hemozoin, has been shown by a variety of chemical,[10] spectroscopic,[10,11] and diffraction techniques to be identical to the synthetic phase β-hematin.[10,12] Although a single-crystal X-ray diffraction structure of β-hematin has yet to be described, the current consensus based on spectroscopic data[10,11,13] is that malaria pigment is a dense lattice of propionate-linked hemes.[11] The resulting coordination polymer contains chains of five-coordinate Fe(III) centers which are linked by −(por)FeOC(O)CH_2CH_2(por)Fe− chains and that the second propionic acid is linked to a different chain of propionate-linked hemes through a hydrogen bond (Figure 1). Despite the congruous nature of much of the spectroscopic data for the natural and synthetic phases, it is surprising that there is considerable ambiguity in the EPR data available for this phase. Indeed, there remains considerable confusion about the conflicting interpretations of the available data.[8,10,14,15] In this report we demonstrate by variable-temperature EPR and Mössbauer spectroscopies that much of the confusion concerning the spin state of the iron in malaria pigment stems from the presence of weakly coupled, rapidly relaxing, iron centers which have a net slight rhombic environment. These conclusions are supported by previous magnetic susceptibility results[11] which are in turn only consistent with a high-spin Fe(III) as the predominant paramagnetic species in both the synthetic and natural phases.

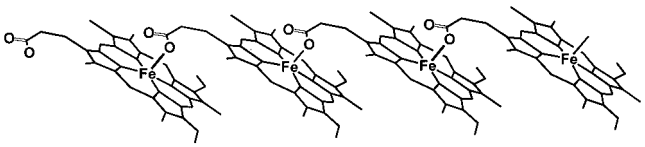

Figure 1. Propionate inter-heme links in β-hematin.

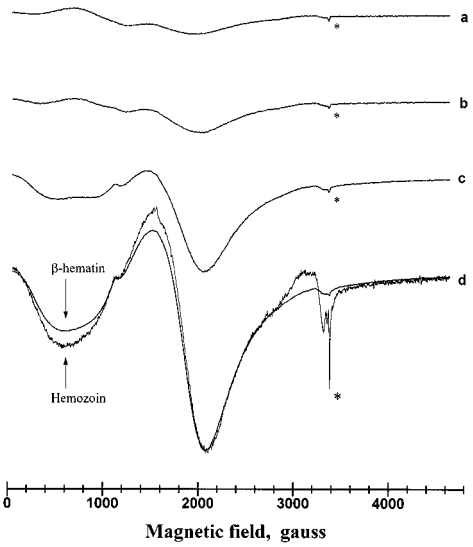

Figure 2. X-band EPR spectra[28] for β-hematin at (a) 21 K, (b) 15.5 K, (c) 9.0 K, and (d) 5.6 K. For comparison the spectrum of hemozoin measured under equivalent conditions at 5.6 K is overlayed in d.

The synthetic method and subsequent sample treatment critically determines the phase homogenity in aggregated heme samples.[16] Phase heterogeneity gives rise to broadened features in the carboxylate stretching bands in the IR[16] and to broad scattering in the X-ray diffraction patterns.[10] To minimize any unexpected effects due to sample treatment, preparation, or isolation of the native malaria pigment from the K1 chloroquine-resistant strain of *P. falciparum*, a nonproteolytic methodology following literature methods was employed.[17] Synthetic β-hematin was prepared by anhydrous dehydrohalogenation of hemin.[19] Both samples were checked by IR spectroscopy before measuring either the EPR or the Mössbauer spectra. Variable-temperature X-band EPR spectra for β-hematin are shown in Figure 2a−d with the spectrum for native malaria pigment shown in Figure 2d. In general these spectra must be acquired for magnetically dilute samples, in this case as a suspension in an ethylene glycol glass, and at low microwave power. While the spectra are necessarily weakened and noisier than for EPR spectra of many heme proteins, the resulting sensitivity and resolution for the spectra of β-hematin and malaria pigment are more than

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adequate. The spectra are strongly temperature dependent, and for temperatures greater than 21 K, rapid relaxation leads to the loss of all absorption intensity. Below 21 K both malaria pigment and synthetic β-hematin have a distinct rhombic pattern with $g = 5.79$, 3.80, and 2.04 at temperatures of 21 K and below. Further cooling below 9 K results in the detection of a broad low-field feature at $g = 12.03$ (Figure 2c). Prior reports have attributed similar features, measured at 10 K and having $g = 3.80$ and 1.95, to be due to low-spin heme.[10] In that case the sample of β-hematin was prepared by acid dehydration of hematin. While one observed peak correlates with our results, the high-field peak may indeed correspond to a low-spin heme, but of a contaminant rather than the bulk phase. Other groups have not described the power dependence of this measurement.[22] Hemozoin-rich fractions isolated from either *P. berghei*, and measured at 23 K[14] or *P. falciparum* at 77 K[15] are dominated by a variety of other bands, in particular an axial high-spin species with $g_\perp = 5.8$ and $g_\parallel = 2.0$ parameters that are similar to the EPR characteristics to monomeric Fe(protoporphyrin-IX)(O_2CCH_3).[23]

The Mössbauer spectra for β-hematin in a weak applied field (Figure 3) support the conclusion about the iron environment drawn from the EPR spectra. The spectra, measured at 4.2, 11.1, and 23.3 K for natural abundance ^{57}Fe, have a relatively narrow spread of 11.5 mm s^{-1}, and the sextet rapidly collapses when the temperature rises above 4.2 K. The isomer shift and quadrupolar splitting values are indicative of a single high-spin ferric site in this lattice. The spectra agree well with the calculated spectra which are based on the spin Hamiltonian, parameters listed in the caption to Figure 3, and using the algorithm described by Schulz, Nyman, and Debrunner.[24] This is indicative of a slightly rhombic high-spin Fe(III) and weak coupling between the Fe(protoporphyrin-IX) centers. Furthermore the Ω and W_0, the spin−spin relaxation and spin−lattice direct process parameters, respectively, correctly model the rapid increase with temperature of the $S = {}^5/_2$ Fe spin fluctuation rate from the nearly slow limit seen in the 4.2 K spectrum. Magnetic Mössbauer spectroscopy allows for these assignments as prior Mössbauer spectroscopic results for β-hematin,[22] malaria pigment,[25] and intact trophozoites[26] have all reported a single broad peak with an isomer shift of 0.2−0.24 mm s^{-1} for all temperatures between 4.2 and 300 K. Given the quality of the fitted spectra and the correlation of the two spectroscopic techniques with each other as well as with magnetic susceptibility measurements,[11] there is no question that both naturally occurring and synthetic malaria pigment has a single high-spin $S = {}^5/_2$ iron environment in the bulk phase. This rules out recent models which have multiple iron environments for aggregates based on a heterotrimeric repeat unit.[27]

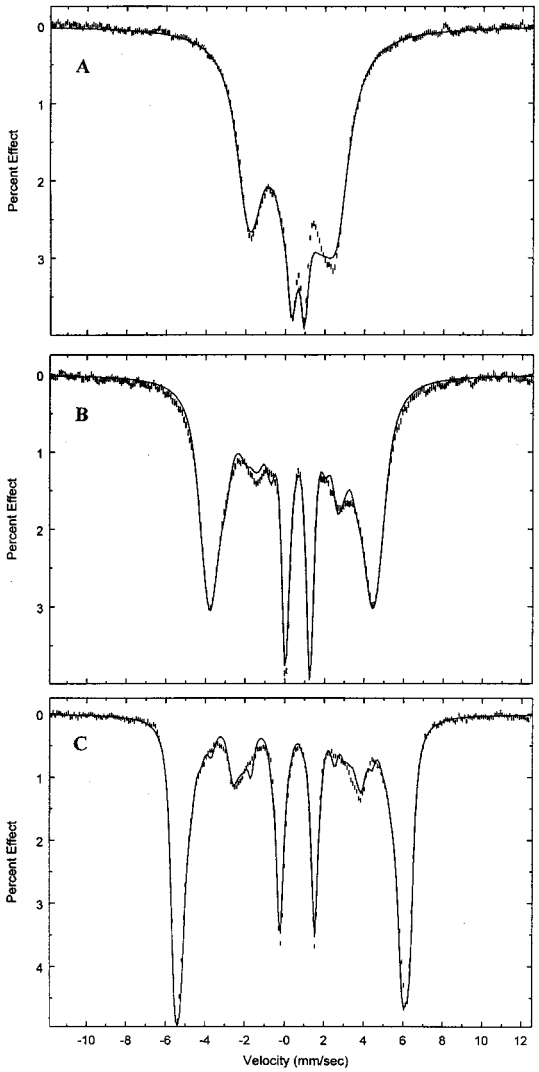

Figure 3. Mössbauer spectrum of β-hematin measured in an applied parallel magnetic field of 4.5 T at (a) 23.3 K, (b) 11.1 K, and (c) 4.2 K. Experimental data are shown as dashs or error bars, and the calculated spectra are shown as solid lines.[29]

These results set the stage for understanding the key surface/protein interactions in malarial pigment. Given the uniformity of the iron environment and the high-quality X-ray diffraction data for both the synthetic and natural phases,[12] it is clear that the major phase of malarial pigment is a highly ordered structure. Numerous early studies gave conflicting results about the protein content of malarial pigment, and it is clear that careful separation of the heme coordination polymer and adsorbates is required to obtain homogeneous samples of malaria pigment. Among these adsorbates are likely to be the proteins responsible for initiating and directing heme polymerization, including HRP-II.[9] Current studies are underway to determine the key interactions between the surface hemes and the adsorbates by exploiting the paramagnetism of the host lattice.

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(20) In homogeneous preparations of β-hematin and malaria pigment there are three sharp bands in the IR at 1712, 1664, and 1211 cm^{-1} with an approximate relative intensity of 1:2.1:1.7.

(21) Samples of EPR were prepared by vortex suspension of 1.1 mg of β-hematin to 0.5 mL of ethylene glycol and syringe transfer of 0.2 mL of this suspension to the bottom of the EPR tube. The glass suspension was formed by plunging this sample into liquid nitrogen, where is was kept before transfer to the precooled EPR cryostat.

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(28) Experimental conditions: sweep width 4600 G, center field 2350 G, tc/ct 164 ms, microwave power 2 mW, modulation amplitude 10 G, receiver gain 6.4 × 10^4 for β-hematin, 5 × 10^5 for malaria pigment, and microwave frequency = 9.464 GHz. All samples were measured as a suspension in ethylene glycol using the same quartz tube. A solvent control was acquired, but a background subtraction was not necessary for data analysis. A small paramagnetic impurity near $g = 2.0$ is marked *.

(29) Fit parameters: $\delta = 0.275$ mm s^{-1}; $\Delta = 0.588$ mm s^{-1}; $\Gamma = 0.35$ mm s^{-1} at fwhm; $\eta = -0.171$; $\Omega = 2.68$ mm s^{-1}; $W_0 = 2.66 \times 10^{-7}$ mm s^{-1} K^{-3}; $\hat{\mathbf{A}}_{xx}/g_n\beta_n = -21.7$ T; $\hat{\mathbf{A}}_{yy}/g_n\beta_n = -20.8$ T; $\hat{\mathbf{A}}_{zz}/g_n\beta_n = -28.8$ T. The parameters E/D set to 0.026 and $D = 18$ K were fixed and not refined for the fitted spectra.