Characterization of the Products of the Heme Detoxification Pathway in Malarial Late Trophozoites by X-ray Diffraction

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In a process inhibited by the quinoline antimalarial drugs, Plasmodia detoxify heme released during the degradation of hemoglobin by aggregating it into malarial pigment, an insoluble crystalline heme coordination polymer. Synchrotron x-ray powder diffraction patterns for intact desiccated malarial trophozoites and synthetic heme aggregates have been measured; both materials correspond to a single crystalline triclinic lattice with unit cell parameters $a = 12.2176(4), b = 14.7184(5), c = 8.0456(3)$ Å; $\alpha = 90.200(2), \beta = 96.806(3), \gamma = 97.818(3)$ and $Z = 2$. These results unambiguously demonstrate that hemozoin crystallites are identical to synthetic heme aggregates.

Heme is a potent multifunctional regulator whose biochemical levels and distribution are precisely controlled on both intracellular and extracellular levels (1). Efficient regulation of heme is particularly critical for intra-erythrocytic parasites such as Plasmodia which process large quantities of heme in the post-invasion digestion of the erythrocyte’s hemoglobin. Plasmodia, which lack heme oxygenases, detoxify heme by sequestering it into an insoluble heme aggregate termed malarial pigment or hemozoin (2). The quinoline-based family of antimalarials interferes with this process by an as yet unknown mechanism that has recently come under intense scrutiny as part of the effort to combat the spread of chloroquine-resistant strains of Plasmodium. A variety of spectroscopic and bioanalytical techniques indicate that hemozoin is similar to the synthetic aggregated heme phase $\beta$-hematin, which is thought to form strands of hemes linked by propionate oxygen-iron bonds as well as inter-strand propionate hydrogen bonds, Fig. 1 (3-5). Characterization of the carboxylate stretching bands for the propionic acid side chains by IR and Raman spectroscopy provides the best evidence for the presence of iron-oxygen bonds to the propionate side chains (3-5). Unfortunately, crystallographic characterization of these heme aggregates has been hampered by the phase heterogeneity of many synthetic preparations as well as by the small size of the synthetic and natural crystallites isolated from either trophozoites and infected hosts (6). High resolution powder diffraction has been used extensively for the solution of many structural problems (7), and it can solve problems posed by diffraction from microcrystalline phases. In this communication we describe the characterization of $\beta$-hematin derived from both synthetic and natural sources and provide new unambiguous evidence that the heme aggregate present in late stage trophozoites is $\beta$-hematin.

EXPERIMENTAL PROCEDURES

Circa $3 \times 10^8$ chloroquine-susceptible Plasmodium falciparum late trophozoites of the 3D7 clone (NF54 strain) were synchronized with sorbitol and allowed to achieve a 92% level of parasitemia (8). The resulting intact trophozoites were isolated by rapid cooling in liquid nitrogen, and the frozen suspension of cells was lyophilized by freeze-drying in vacuo at $-10^\circ$C. In general this freeze-drying process required 12–14 h and resulted in a fine black free flowing powder. Uninfected erythrocytes from the same source were treated in an identical manner to give a bright red free flowing powder. In their respective experiments, samples of lyophilized trophozoites and erythrocytes were anhydrously loaded onto the diffractometer goniometer which was shrouded by a helium-filled glove bag during both the sample loading and diffraction pattern measurement procedures.

Synthetic $\beta$-hematin was prepared from hemin (Sigma) by treating it with noncoordinating bases in anhydrous conditions, Equation 1 (3, 5).

\[ \text{Fe}^{III}(\text{PP})\text{(NH}_2\text{)}_2\text{Cl} + n \text{HCl} \rightarrow \text{Fe}^{III}(\text{PP})\text{(NH}_2\text{)}_2\text{Cl} \cdot n \text{HCl} \]

(Equation 1)

Briefly, the powder diffraction patterns were measured on beamline X3B1 at the Brookhaven National Laboratories National Synchrotron Light Source using an angular step of 0.01° in 2θ data points. Source x-rays were monochromated by flat Si(111) crystals, and the diffracted beam was analyzed with a Ge(111) crystal. The resulting x-ray flux was continuously monitored for intensity throughout the data collection with an ion counting chamber placed in the x-ray beam immediately before the sample. The wavelength was calibrated by measuring the six most intense peaks of
an aluminum oxide standard, and the system resolution is 0.01° at full width half-maximum (9). For all room temperature measurements a flat plate (either brass or single crystal quartz) geometry was employed. For low temperature measurements a brass rectangular flat plate was used. For the trophozoite measurements the lyophilized sample was packed into a circular well 2 mm deep and 18 mm in diameter (volume ~ 0.51 milliliters). The data reduction was performed with the GUFI software package, and the unit cell was determined by exhaustive trial and error methods with ITO and TREOR (10). The resulting triclinic cells were refined with FULLPROF (7). The final LeBail fits and lattice refinement were performed with FULLPROF (11, 12).

RESULTS AND DISCUSSION

The diffraction patterns for trophozoites and uninfected erythrocytes were measured from 4 to 30°, Fig. 2, traces a and b, Table I. The difference of traces a and b is shown in panel ii of Fig. 2 trace c. Both the desiccated erythrocytes and trophozoites scatter x-rays in two broad bands between 7–10° and 13–20° in 2θ. The origin of these bands is most likely due to scattering from the lipid bilayer membranes in the cells. What is particularly important is that the trophozoites have clear evidence from diffraction spikes from a crystalline species not found in the erythrocytes; moreover, as shown in panel ii of Fig. 2, trace d, these peaks are identical to the sharp diffraction pattern obtained from synthetic samples of β-hematin. The hemoglobin formed within trophozoites thus crystallizes in the same unit cell as β-hematin. The similar intensities of the two patterns in Fig. 2, traces a and d, suggest that the materials are also crystallographically identical on the atomic level as well.

At room temperature high resolution powder diffraction patterns, full width half-maximum < 0.05°, are obtained for β-hematin, [Fe(protoporphyrin-IX)]₇, Fig. 3. The diffraction pattern for this coordination polymer corresponds to a pure single phase which indexes to give a triclinic cell with a high figure of merit, Table I. At lower temperatures, 50 K, the diffraction profile (not shown) is also readily indexed to give a slightly smaller unit cell. The crystallographic parameters for the two temperatures differ principally in contraction along both a and b axes at low temperatures, suggesting that these correspond to the directions of weakest interchain interactions.

The experimental density of β-hematin, 1.45(1) g ml⁻¹, corresponds to the occupation of two heme molecules in the unit cell. For this crystal system there are only two possible space groups: either the two hemes are related by inversion symmetry in space group P-1 or they are crystallographically inequivalent and the space group is P1. In principle, Wilson statistics can be used to determine the presence of an inversion center from a diffraction pattern, but the method is notoriously inaccurate for powder data (13). In the present case the mean of |E·E*| is 0.892 for 266 data out to 2θ = 28° in Fig. 3, a value ambiguously between the limiting values of 0.968 and 0.736 for centro and noncentrosymmetric space groups. In single crystal x-ray diffraction experiments the space group is often confirmed by the complete and successful solution to the crystal structure. In spite of the high resolution present in the diffraction patterns in Figs. 2 and 3, we have been unable to locate the hemes in the unit cells of β-hematin; thus we are unable to unambiguously fit the topological model in Fig. 1 into the experimentally determined lattice with molecular modeling techniques. We attribute this to the severe peak overlap present in the single angular dimension at higher angles in 2θ and the concomitant lack of peak intensity data at higher resolution.

A variety of compositions have been proposed for hemoglobin, and a great deal of uncertainty surrounded early attempts to characterize this seemingly intractable black solid (14, 15). Native isolated hemoglobin is a highly crystalline material and

![Fig. 1. Proposed structure of β-hematin based on spectroscopic data (3-5). Note the formation of strand forming iron-oxygen bonds, with the direction of the chain propagation indicated by the arrows and the hydrogen-bonded propionic dimerization between the chains.](image1)

![Fig. 2. Powder diffraction patterns (λ = 1.1495 Å) for panel I: trace a, lyophilized uninfected erythrocytes, trace b, lyophilized late trophozoites of P. falciparum; panel ii: trace c, difference of two profiles in panel i; and trace d, synthetic β-hematin prepared by the method described in Refs. 3 and 5.](image2)

| Temperature | 2θ range (°) | a (Å) | b (Å) | c (Å) | α (°) | β (°) | γ (°) | V (Å³) | Rwp (%) | Z | R1 (%) | R1 (%) | d₁₀₀ (g cm⁻¹) | d₁₁₀ (g cm⁻¹) |
|-------------|-------------|-------|-------|-------|-------|-------|-------|-------|---------|---|--------|--------|--------------|--------------|
| 293 K       | 4.00–38.00  | 12.2176 (4) | 14.7184 (5) | 8.0456 (3) | 90.200 (2) | 96.806 (3) | 97.818 (3) | 1242.9 (2) | 8.81 | 2 | 2 | 10.2 | 1.45 (1) | 1.436 |
| 50 K        | 4.00–52.07  | 12.1076 (4) | 14.5384 (4) | 7.9808 (2) | 90.868 (2) | 96.705 (2) | 97.133 (2) | 1383.8 (1) | 6.72 | 2 | 2 | 10.2 | 1.477 |

TABLE I

Crystallographic parameters for β-hematin

Data collected with monochromatic x-rays (λ = 1.1495 Å) on a 0–2θ scanning diffractometer with a flatplate geometry on beamline X3B1 at the National Synchrotron Light Source at the Brookhaven National Laboratories.
published transmission electron micrograph images clearly indicate that there is either a regular lattice or d spacing of spacing of 9 ± 2 Å (6). In spite of preliminary suggestions that the heme aggregate present in hemozoin is β-hematin (14), this hypothesis was discounted due to its contamination with proteins from early isolation protocols (16). The development of better isolation procedures, which often employ proteases, has allowed for the separation of hemozoin consisting solely of heme as determined by complete elemental analysis (4). Although the hemozoin isolated by these more rigorous procedures is identical to β-hematin by IR spectroscopy, it has been suggested that the hemozoin may have been modified during the isolation steps (17). The diffraction results presented here provide compelling evidence against this possibility and clearly indicate that the heme aggregate produced in situ is crystalline and has the same lattice as β-hematin.

The central question relating to the structure of hemozoin concerns the mechanism of its biosynthesis and how this is inhibited by the quinoline antimalarials. The biochemical consequences of nonsequestered heme accumulation are profound and include membrane lysis (18) and protease inhibition (19, 20). It was recently recognized that one defense plasmodia have developed against these drugs is efficient drug excretion out of the digestive vacuole (21); however, there may be other adaptations in the chloroquine-resistant strains. Indeed, a host of mechanisms have been proposed for the drug action of the quinolines (15), with the main consensus being the location of their activity in the digestive vacuole. An important step in elucidating the drug action mechanism of these antimalarials is the recent description of the heme aggregating ability of an unusual class of histidine-rich proteins, HRP I–IV, from P. falciparum, which have been cloned and overexpressed (22). These proteins may be the heme polymerase suggested by Slater and Cerami (2) or they may instead initiate heme sequestering (22) which can then be propagated by either enzymatic (2, 23) or nonenzymatic (24–27) heme-sequestering mechanisms. Regardless of these mechanistic details, it is clearly important to have a detailed structural understanding of the metabolic product of heme detoxification.

Several properties of hemozoin make it an ideal excretory product of heme detoxification. First, it is dense and insoluble under physiological conditions and thus represents an irreversible sink for heme released from hemoglobin degradation. Second, hemozoin is a coordination polymer of ferric(protoporphyrin-IX), and thus it will not contribute to the oxygen radical stress from the auto-oxidation that would result from a coordination polymer of ferrous(protoporphyrin-IX). Finally, formation of hemozoin corresponds to an important excretory system for iron, release of which would further exacerbate the oxidative stress of the trophozoites.

The methodology used in this report may be useful in the in situ study of other biominal phases. The study of these materials is most often performed following exhaustive isolation and work up procedures, steps that potentially may alter the crystalline phase. Biominalization and biocrystallization are most often associated with intra or extracellular membranes and their integral scaffold proteins. While many biological processes are facilitated or promoted by biominalization, examples of biocrystallization as a mediator of toxicity or a regulated means of intracellular excretion are rare. Probably the best example of this phenomenon is the crystallization of β-hematin by malarial parasites in the early trophozoite stage. Finally, an important role for hemozoin released into the vasculature during merozoite release may be the suppression of the host’s immunological response by compromising macrophages that phagocitize hemozoin particles (8, 28).

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FIG. 3. Powder diffraction pattern for β-hematin at 293 K between 4 and 38° in 2θ. Trace a, raw data (●) and best fit to all of the peak intensities with lattice and profile parameters adjusted (29); trace b, difference for this fit and observed data.
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