Mechanisms of hematin crystallization and inhibition by the antimalarial drug chloroquine

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Hematin crystallization is the primary mechanism of heme detoxification in malaria parasites and the target of the quinoline class of antimalarials. Despite numerous studies of malaria pathophysiology, fundamental questions regarding hematin growth and inhibition remain. Among them are the identity of the crystallization medium in vivo, aqueous or organic; the mechanism of crystallization, classical or nonclassical; and whether quinoline antimalarials inhibit crystallization by sequestering hematin in the solution, or by blocking surface sites crucial for growth. Here we use time-resolved in situ atomic force microscopy (AFM) and show that the lipid subphase in the parasite may be a preferred growth medium. We provide, to our knowledge, the first evidence of the molecular mechanisms of hematin crystallization and inhibition by chloroquine, a common quinoline antimalarial drug. AFM observations demonstrate that crystallization strictly follows a classical mechanism wherein new crystal layers are generated by 2D nucleation and grow by the attachment of solute molecules. We identify four classes of surface sites available for binding of potential drugs and propose respective mechanisms of drug action. Further studies reveal that chloroquine inhibits hematin crystallization by binding to molecularly flat (100) surfaces. A 2-μM concentration of chloroquine fully arrests layer generation and step advancement, which is ∼10^4× less than hematin’s physiological concentration. Our results suggest that adsorption at specific growth sites may be a preferred mode of hemozoin growth inhibition for the quinoline antimalarials. Because the atomic structures of the identified sites are known, this insight could advance the future design and/or optimization of new antimalarials.

Significance

Approximately 40% of the global population is at risk for malaria infection and 300–600 million clinical episodes of Plasmodium falciparum malaria occur annually. During the malaria parasite lifecycle in human erythrocytes, heme released during hemoglobin catabolism is detoxified by sequestration into crystals. Many of the common antimalarials are believed to suppress the parasite by inhibiting hematin crystallization. We present, to our knowledge, the first evidence of the molecular mechanisms of hematin crystallization and antimalarial drug action as crystal growth inhibitors. These findings enable the identification and optimization of functional moieties that bind to crystal surface sites, thus providing unique guidelines for the discovery of novel antimalarials to combat increased parasite resistance to current drugs.

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Results and Discussion

Aqueous or Organic Medium for Hematin Crystallization. The parasite DV presents a complex environment. The vacuole comprises membrane interfaces (20), an acidic aqueous solution with pH 4.8–5.5 (30), and lipids, mostly mono- and diglycerides, resulting from the degradation of the transport vesicle membranes that carry hemoglobin into the DV (9, 16, 18–20). The location and structure of the lipid subphase in the parasite DV is a subject of debate. Electron microscopy observations have indicated that the lipids self-assemble into nanospheres suspended in the DV (9, 16); however, recent studies indicate the absence of suspended lipid structures and suggest that the lipids line and thicken the DV membrane (18–20). Hemozoin crystals have been observed immersed in the lipid nanospheres (9, 16), or with their basal surfaces attached to the DV membrane (18–20) and other crystal faces apparently exposed to the aqueous subphase. Whereas it has been hypothesized that hemozoin crystals nucleate on the DV membrane (17, 20), the debate on the medium, aqueous or organic, from which soluble hematin reaches the crystals and associates to them has yet to be reconciled (18–20, 31–33).

To address this issue, we tested the feasibility of hematin crystallization from solutions that mimic either the aqueous phase or the lipid structures in the DV. First, we attempted to grow hematin crystals larger than the hemozoin crystals extracted from the DV of P. falciparum, which are less than 1 μm in their longest dimension (16). Our group and others have produced β-hematin crystals in aqueous solution, achieving a marginal increase in crystal size (≤3 μm), but only through the use of nonphysiological conditions (i.e., ionic strength 0.5–5 M) (34). Our attempts to grow β-hematin in aqueous solution with citric and acetate buffers (both at pH 4.8) as a surrogate for the DV produced crystals with a morphology distinctly different from that of hemozoin (34). We then used an analog to the lipid subphase in the DV, a solution of n-octanol saturated with citric buffer at pH 4.8 (details of the preparation are provided in SI Text) and referred to as citric buffer-saturated octanol, CBSO (35). In this solvent, we grew 30-μm β-hematin crystals that possessed the characteristic morphology (Fig. 1B) and powder X-ray diffraction pattern (Fig. S1C) of hemozoin crystals extracted from the DV of P. falciparum (17). Our results revealed that both the organic and aqueous components are critical for crystal growth. For instance, β-hematin crystals failed to grow in anhydrous n-octanol, which seems to suggest that H+ ions are a necessary component of the growth medium, presumably to facilitate the formation of hydrogen bonds in the crystal structure (35). Our analysis of a representative blend of lipids in the DV suggests that there is ~8.5% (by mass) dissolved water (Fig. S2).

As a second test, we used in situ atomic force microscopy (AFM) to monitor the evolution of unfinished layers on large β-hematin crystals in the presence of multiple aqueous solvents (Table S1). The unfinished layers did not grow despite the abundant growth sites presented on the curved steps (Fig. S3). A similar outcome was observed for anhydrous n-octanol (Fig. S4). However, this behavior is in direct contrast with the continuous growth of layers that were observed in CBSO solutions, as discussed below. As a third test, we determined the solubility of hematin in CBSO. Spectroscopic analyses (34) revealed that this solubility is ~10^3× higher than in aqueous buffer at pH 4.8 (Fig. S64), which is not surprising given that hematin is a hydrophobic molecule. Because crystal growth rates roughly scale with the solubility (23), this disparity in the magnitude of hematin solubility indicates that crystallization from an organic phase is a significantly faster method of heme detoxification than from an aqueous phase. Below, we use the data on hematin solubility for quantitative analyses of hematin crystallization in CBSO.

An argument that is presented in the literature in favor of aqueous crystallization of hematin is the putative low solubility of the quinoline antimalarials in organic liquids, which would seemingly reduce their efficacy toward crystals growing in a lipid phase. To this end, we determined the solubility of chloroquine (CQ), a common antimalarial drug, in CBSO and citric buffer at pH 4.8 (Fig. S6B). The solubility of CQ in CBSO is 0.19 mM, which is ~10^3× less than its solubility in the aqueous solvent, but it is still twofold higher than the solubility of hematin in CBSO (~0.1 mM). Collectively, these measurements suggest that the CQ solubility in CBSO is sufficient for growth inhibition by surface binding, a mechanism discussed in greater detail below. These arguments advocate that the lipid structures in the DV may be a preferred environment for hematin crystallization. The difficulty in crystal growth from a physiologically relevant aqueous environment may be attributed to hematin’s low solubility (2 nM) (34) or its propensity to form oligomers (36, 37) that could potentially adhere to the crystal surface and slow or block its growth. Even if one accepts that the crystals are not suspended in lipid nanospheres located in the DV bulk, but are attached to the DV membrane so that only one of the basal faces is exposed to lipids lining the DV membrane (18–20), this contact may be sufficient to ensure growth of the physiological hematin crystals.

The Crystallization Mechanism in CBSO. Guided by the conclusion of preferential β-hematin growth in organic solvents, we used CBSO supersaturated with hematin as a growth medium and focused on the (100) faces of β-hematin. We used large β-hematin crystals prepared in the biomimetic CBSO solutions discussed above and performed, to our knowledge, the first time-resolved in situ AFM study of hematin crystal growth. In situ AFM has proven to be a valuable technique for elucidating structural and dynamic characteristics of classical and nonclassical crystallization mechanisms (38–41). AFM topographical images (Fig. 1C) reveal the presence of unfinished layers on a (100) face with heights h = 1.17 ± 0.07 nm, close to the unit cell dimension in the [100] direction (a = 1.22 nm, Fig. 1D) (7).

**Fig. 1. β-Hematin crystals. (A) Structure of hematin. (B) AFM image of a β-hematin crystal on a glass substrate reveals a morphology similar to hemozoin crystals isolated from P. falciparum. (Scale bar, 2 μm.) (C) A 3D AFM height image of a (100) face reveals the presence of unfinished layers. The step height h = 1.17 ± 0.07 nm was determined by averaging measurements from multiple images. (D) Molecular model of β-hematin using the software package Diamond illustrates an unfinished layer (C atoms in white) on a (100) face (C atoms in blue).**
We observed a classical layer-by-layer mechanism wherein new crystal layers nucleate and grow by the attachment of solute molecules to advancing steps. Analyses of successive snapshots from AFM movies reveal that new layers may either grow (I–III in Fig. 2A–D), dissolve (IV in Fig. 2A–D), or retain a steady size during continuous imaging (V in Fig. 2C and D) depending on their radius $R$ (Movie S1). We observe a reduction in the critical radius $R_{\text{crit}}$ for island growth or dissolution with increasing hematin concentration (Fig. 2E), which is consistent with classical nucleation theory (CNT) applied to 2D crystal islands on a substrate (43). According to CNT, islands form as a result of fluctuations of the concentration of molecules on the surface. The dependence $R_{\text{crit}}(c_{\text{h}})$ is governed by the Gibbs–Thomson relation, according to which $R_{\text{crit}} = \Omega k_B T \ln(c_{\text{h}}/c_e)$ (23) where $\Omega = 0.708$ nm$^3$ is the volume of one molecule in the crystal (7); $\gamma$ is the surface free energy of the layer edge; $k_B$ is the Boltzmann constant; $T$ is temperature; $c_{\text{h}}$ is hematin concentration; and $c_e$ is hematin solubility in CBSO. The correspondence between the experimentally determined $R_{\text{crit}}$ and the a priori CNT prediction in Fig. 2F indicates that the generation of new layers on growing $\beta$-hematin surfaces is governed by the thermodynamics of hematin crystallization.

Analysis of in situ AFM images permits the determination of layer nucleation $J_{2D}$ as the number of islands that exceed $R_{\text{crit}}$ per unit area per time. According to CNT, $J_{2D} \propto \exp(-\Delta G_{2D}/k_B T)$, where the free-energy barrier for layer nucleation, $\Delta G_{2D} = \pi k_B T R_{\text{crit}}^2$, decreases with increasing $c_{\text{h}}$, leading to an exponential increase of $J_{2D}$ with $\ln(c_{\text{h}}/c_e)$ (43, 44). Data in Fig. 2F are qualitatively consistent with this prediction, although the increase in $J_{2D}$ with $\ln(c_{\text{h}}/c_e)$ is weaker than this trend. This is expected because $J_{2D}$ is regulated by surface supersaturations that are lowered from the bulk value during growth at high deviations from equilibrium, whereas $R_{\text{crit}}$ responds to surface supersaturations equilibrated with the bulk, as evidenced by the fluctuations of surface islands around their critical size in Fig. 2A–D.

Upon nucleation, layers advance across the surface, merge with adjacent islands, and eventually cover the entire face (Fig. 3A and B). The island morphology undergoes a temporal shift from an anisotropic to an anisotropic shape that elongates along the $c$ direction. The velocity $v$ of advancing steps was determined from the average displacement $\Delta x$ of steps over time by the comparison of successive AFM images, similar to those in Fig. 3A and B. We observed a faster step velocity along the $c$ direction, consistent with high $c/b$ aspect ratios of islands and bulk crystal habit, which may be attributed to the differences in kink structure (45) or density (46) along each step edge. Herein, we report step velocity in the dominant $c$ direction.

The step velocity $v$ exhibits a linear dependence on hematin concentration $c_{\text{h}}$ (Fig. 3C) if the steps are separated by more than 150 nm. This linearity indicates that hematin molecule addition to $\beta$-hematin crystals is a first-order reversible process where $v(c_{\text{h}})$ reaches zero at $c_e$ and becomes negative at $c_{\text{h}} < c_e$, denoting step retreat due to crystal dissolution. The estimate of crystal solubility $c_e = 0.16$ mM from in situ AFM (Fig. 3C) is in excellent agreement with bulk crystallization data (Fig. S5). The coefficient of proportionality between $v$ and $c_{\text{h}}$ is referred to as the step kinetic coefficient $\beta$ defined from $v = \beta \Omega (c_{\text{h}} - c_e)$. In turn, $\beta$ is proportional to the effective first-order rate constant $k$ for association of molecules to the steps ($\beta = a k$, where $a$ is the molecule size). From the data in Fig. 3C, $\beta = 4.3 \mu$m s$^{-1}$ and $k \geq 10^5$ s$^{-1}$ for steps moving in the $c$ direction.

The observations in Figs. 2 and 3 indicate a strictly classical mechanism of hematin crystallization, observed for numerous other solution-grown crystals (23, 45, 47, 48). Note that in our AFM studies of growing crystal faces, we never detected the association of preassembled species, such as hematin oligomers, which would be indicative of nonclassical growth (24–26, 49).
Concurrent with this observation, characterization of the homogeneity of supersaturated hematin solutions in CBSO by dynamic light scattering revealed the absence of any aggregates of size 1 nm and larger (35). This excludes the possibility of nonclassical crystallization of hematin.

The values of \( J_{2D} \) and \( \nu \) in Figs. 2 and 3, respectively, may provide insight into hematin crystallization in vivo. For instance, electron micrographs of hemozoin crystals in the parasite DV reveal that the crystals can reach thicknesses in the [100] direction of \(-100\) nm within 20 h (9). Our in vitro assays suggest that this approximate rate of crystallization occurs around \( c_{H} = 0.22\) mM. Indeed, at this \( c_{H} \) the step velocity in the \( \bar{c} \) direction is \( v = 0.10\) nm \(s^{-1} \) (Fig. 3C) and the corresponding density of steps (determined by the rate of nucleation of new layers \( J_{2D} \)) is \( \cong 0.008\) nm\(^{-1}\). The crystal growth rate is the product \( r = h(l^{-1}) v \approx 8 \times 10^{-4}\) nm\(^{-1}\); this product is independent of the direction of step motion (50). With this \( r \), a crystal that is 100 nm thick in the [100] direction has grown for \(-18\) h, which is comparable to that of hemozoin in vivo. The closeness of the two time periods suggests that the measured layer nucleation rates and step velocities are physiologically relevant. It also suggests that the hematin concentration in vivo is close to 0.22 mM. This value is only slightly higher than the solubility of 0.16 mM and significantly lower than the potential maximum of 1.6 mM (i.e., the total hematin generated in an average parasite DV) (9). These two considerations imply that hematin is incorporated into hemozoin crystals soon after its release during hemoglobin catabolism. Thus, even a moderate delay in crystallization may induce a significant accumulation of toxic hematin, leading to parasite eradication from its host.

Direct AFM observation of nucleation of new layers and their spreading by incorporation of molecules identified four classes of sites on the surface of growing hematin crystals that are important for growth and constitute potential binding sites for antimalarial drugs. These sites are illustrated in Fig. 4A for the \{100\} faces and their atomic structures are depicted in Fig. 4B–G.

Class 1 consists of molecularly flat surfaces that are typically located between steps (Fig. 4B). Class 2 refers to nuclei of new layers (Fig. 4C), which may comprise several molecules, and can potentially exhibit structures that are distinct from those of larger layers. Class 3 includes the kinks located along the steps (Fig. 4D–F). There are four types of kinks, obtuse and acute, located on steps spreading the positive and negative \( \bar{b} \) and \( \bar{c} \) directions. Finally, class 4 contains groups of closely spaced steps that may host large inhibitor molecules capable of bridging multiple step edges–terraces, as illustrated in Fig. 4G.

**The Mechanism of Action of CQ.** The prevailing hypotheses regarding the suppression of hemozoin formation collectively assume that antimalarials increase \( c_{H} \) in parasite DVs. This process can occur if drug molecules form noncrystallizable complexes with hematin in solution (i.e., hematin complexation) (51) and/or if the molecules bind to hemozoin crystals and impede the addition of solute to growing steps (i.e., crystal growth inhibition) (17). The latter mechanism was proposed by Sullivan for quinoline drugs (52) and by Leiserowitz and coworkers for artemisinin (53). To date, definitive evidence for antimalarial mode(s) of action remains elusive. Here we identify the mechanism of \( \beta \)-hematin crystal growth inhibition by CQ. In situ AFM measurements reveal that the addition of CQ to hematin growth solutions leads to slower step growth, fewer 2D nuclei, and more rugged step edges (Fig. 5A–E and Movie S2). The impact of CQ at concentrations \( c_{CQ} = 2 \mu M \) on layer generation and step propagation is summarized in Fig. 5F–H. There is an exponential decay in \( J_{2D} \) with increasing \( c_{CQ} \) that is accompanied by a monotonic decrease in \( \nu \) (Fig. 5G and H). We observe a complete suppression of layer nucleation and step growth at \( c_{CQ} = 2 \mu M \). These observations are consistent with the hypothesis that CQ impedes crystallization by binding to hematin crystal surfaces, similar to inhibition mechanisms established for many biogenic and synthetic crystals in the literature (23, 54). Furthermore, the data suggest that a complexation mechanism cannot fully account for the reduced growth rates measured by in situ AFM. If, for example, we assume that CQ forms a 1:1 CQ–hematin complex, the free hematin concentration would decrease by 2 \( \mu M \). Based on the \( v(c_{H}) \) dependence in Fig. 3D, this decrease would engender a negligible reduction in \( \nu \). This interpretation, however, does not rule out a potential role of CQ–hematin complexes (51) in hematin growth suppression because AFM imaging cannot identify the adsorbed inhibitor species.

CQ could adsorb to any of the sites listed in Fig. 4. AFM studies reveal a site specificity of CQ for \{100\} terraces. The concomitant suppression in \( J_{2D} \) and \( \nu \) at \( c_{CQ} = 2 \mu M \) and the appearance of protrusions along advancing steps (Fig. 5D and E) are consistent with a step-pinning (stopper) mechanism (55), i.e., CQ molecules preferentially adsorb on the crystal surface and block step propagation. This mechanism assumes that inhibitor surface coverage is governed by the dynamics of adsorption. If the separation between a pair of adsorbed inhibitors...
is less than 2R_{cst}, the adsorbates enforce a curvature at which the advancing step is undersaturated and growth is arrested (55). At intermediate inhibitor surface concentrations, step pinning produces rugged steps (Fig. 5E, arrow) and impedes 2D layer nucleation. The significance of this mechanism is reflected in the sensitivity of crystallization to c_{CQ} (Fig. 5 F–H). When c_{CQ} drops to 0.25 μM, both the generation and the growth of new layers proceed with considerable rate. This high sensitivity may be a critical factor underlying the increased resistance of P. falciparum to CQ (2). Specifically, resistant strains may have developed means to lower c_{CQ} in the DV to levels that permit effective heme detoxification.

**Conclusions**

Here we present, to our knowledge, the first determination of the mechanism of CQ drug action on hematin crystallization. Our results provide definitive evidence resolving several long-standing open questions on hematin crystallization and heme detoxification. The hematin solubility in organic and aqueous solvents and the morphology and dynamics of hematin crystal surfaces held in supersaturated solutions suggest that water-saturated amphiliphic organic solvents are a preferred growth environment. Time-resolved in situ AFM observations demonstrate that hematin crystallization follows a strictly classical mechanism of crystallization wherein new crystal layers are generated by 2D nucleation and grow by the association of molecules from the solution. These mechanistic details identified four distinct classes of crystal surface sites that play crucial roles in growth and could be potentially blocked by crystallization inhibitors to prevent heme detoxification. We provide direct evidence that CQ adsorption on hematin crystal surfaces arrests heme detoxification by suppressing surface growth at concentrations as low as 2 μM. The mechanism of CQ drug action was conclusively identified: CQ adsorbs on (100) terraces between hematin growth steps and blocks step propagation. Collectively, these findings may engender a paradigm shift in the rational design of antimalarial drugs wherein the identification of molecules with site specificity for binding to hematin crystal surfaces provides both a vital criterion and platform for experimental and computational drug screening.

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