

Depolymerization of malarial hemozoin: a novel reaction initiated by blood schizontocidal antimalarials

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Abstract Malaria parasite digests hemoglobin and utilizes the globin part for its nutritional requirements. Heme released as a byproduct of hemoglobin degradation is detoxified by polymerization into a crystalline, insoluble pigment, known as hemozoin. We have identified a novel reaction of depolymerization of hemozoin to heme. This reaction is initiated by the interaction of blood schizontocidal antimalarial drugs with the malarial hemozoin. The reaction has been confirmed, with the purified hemozoin as well as the lysate of the malaria parasite. Pigment breakdown was studied by infrared spectroscopy, thin-layer chromatography and spectrophotometric analysis. It was complete within 2 h of drug exposure, which explains the selective sensitivity of late stages (trophozoites and schizonts) of malarial parasites loaded with the hemozoin pigment to the toxic action of these drugs. It is suggested that the failure of the parasite heme detoxification system due to this reaction results in the accumulation of toxic heme, which alone, or complexed with the antimalarial leads to the death of malaria parasite.

Key words: Malaria; Hemozoin; Heme; Antimalarial; Chloroquine

1. Introduction

Malaria is a disease of global concern with around 300–500 million cases per year and a population of more than 2400 million at risk of infection. The continuous proliferation and cyclic propagation of malaria parasite inside erythrocytes are distinct features of malaria infection and are chiefly responsible for the pathophysiology of the disease. Hemoglobin, the major protein available in erythrocytes, is utilized as a source of nutrition by the parasite. Digestion of hemoglobin by malaria parasite involves a specific sequence of reactions through the action of aspartic as well as cystine proteases and leads to the continuous release of toxic free heme [1–4]. However, simultaneous detoxification and sequestration of heme to an insoluble, crystalline, black-brown pigment named 'hemozoin' prevents the accumulation of free heme [5–7]. Malarial hemozoin has been characterized as a polymer of heme units linked through an iron-carboxylate bridge [8]. The molecular mechanisms of initiation and continuous polymerization of heme to hemozoin in the malaria parasites are still under debate. The involvement of a parasite specific heme polymerase activity in the catalysis of hemozoin formation reported earlier [9–11] has recently been questioned [12,13]. A previous report of heme polymerization activity associated with purified hemozoin/ β -hematin [14] was later attributed to a phospholipid fraction [15]. However, a histidine-rich protein (HRP II) has

recently been localized in the digestive vacuole of malaria parasite which provides a nucleus for the polymerization of heme [16], supporting the theory that the involvement of some parasitic component is necessary for hemozoin formation. Very recently, we have shown that hemozoin formation under physiological conditions cannot occur chemically in the absence of any parasite derived component [17].

The sensitivity of malaria parasites to several antimalarial drugs including the most popular drug chloroquine is confined to the parasite stages that actively degrade hemoglobin and accumulate hemozoin [18]. Blood schizontocidal antimalarial drugs have a selectively fast toxic action on the trophozoite and schizont stages of the parasite which are heavily laden with hemozoin. These stages are subjected to the toxic effect of blood schizontocidal drugs within 2–4 h of exposure. Ring stage parasites have heme polymerization activity, but are relatively insensitive to the toxic effects of these drugs (action time > 12 h) [19]. This indicates that the presence of preformed hemozoin may be necessary for the selective antimalarial action of blood schizontocidal drugs. Thus, inhibition of heme polymerization alone as suggested earlier [5] cannot explain the selective antimalarial action of these drugs.

In this communication, we describe a novel biochemical mode of blood schizontocidal action of the antimalarials through the breakdown of preformed hemozoin.

2. Materials and methods

2.1. Purification and characterization of hemozoin

Hemozoin was purified from mouse blood highly infected (parasitemia 50–70%) with *Plasmodium yoelii* according to a method reported earlier [20]. The purity of hemozoin was ascertained by infrared spectroscopy, and spectrophotometric and elemental analysis [20].

2.2. Depolymerization assay

The hemozoin depolymerization (breakdown) assay was developed on the basis of the differential solubility of hemozoin and heme [6]. Hemozoin was suspended in acetate buffer (pH 5.0, 500 mM) and mildly sonicated to obtain a suspension of fine crystals of the pigment. The pigment was incubated with varying concentrations of chloroquine. The total volume of the incubation mixture was 1.0 ml and incubation was continued for at least 10 h (or as specified) in a shaking water bath at 37°C. The suspensions were centrifuged at 10 000 \times g for 10 min and the pellets were washed twice with distilled water and finally with bicarbonate buffer (100 mM, pH 9.5). Heme is soluble in bicarbonate buffer while hemozoin is not soluble [6]. The pellet of the remaining hemozoin was dissolved in 2.0 N NaOH to convert it into heme (ferriprotoporphyrin IX) and quantitated (as equivalent of heme) by recording the spectra of ferriprotoporphyrin IX (FP IX) in the range of 360–700 nm [21]. In some of the initial experiments the heme released in the bicarbonate wash was also quantitated. However, the quantitation of heme released could not be employed as a routine procedure for the assay of hemozoin breakdown/depolymerization. After incubation the complete separation of hemozoin and heme may only be achieved by repeated washings with 2.5% SDS and bicarbonate buffer. This makes the estimation of heme in the

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pooled washings difficult and leads to low sensitivity of the assay. Moreover, in the experiments conducted with the lysates of malaria infected erythrocytes, the large amount of heme is present as a part of hemoglobin and other proteins make the estimation of heme released due to hemozoin breakdown extremely difficult. Therefore, a reverse assay was standardized based on the estimation of hemozoin in the form of ferriprotoporphyrin IX (FP IX) equivalents after converting it into its constituent heme units by alkali solubilization [8]. The amount of hemozoin depolymerized was calculated by subtracting the hemozoin remaining after incubation with the drug from the amount of hemozoin in control tubes (without any drug) run simultaneously. For depolymerization assay with the *P. yoelii* infected erythrocyte lysate, the blood in acid-citrate-dextrose was washed three times with phosphate-buffered saline and the pellet was finally suspended in 2 vols. of acetate buffer (500 mM, pH 5.0). 100 μ l each of the lysate was dispensed into different tubes and the final volume of the incubation mixture was adjusted to 1 ml. The mixture was incubated at 37°C for 2 h (or as specified) in a shaking water bath with various concentrations of chloroquine. The assay mixture was centrifuged at 10 000 \times g for 10 min and the remaining hemozoin was estimated in the pellets after washing three times with 2.5% (w/v) SDS and once with bicarbonate buffer (pH 9.5, 100 mM). Quinoline drugs are known to accumulate in the food vacuole of the malaria parasite [5]. Hence, the drug concentrations used in these experiments are the effective concentrations at the physiological site of pigment formation. Similar drug concentrations have previously been tested to study the inhibition of heme polymerization [5,9–11,14,16].

3. Results and discussion

Chloroquine (chloroquine) has previously been shown to inhibit the polymerization of heme to hemozoin [5,9–11]. We have been trying to study the molecular and physico-chemical mechanism(s) of this effect. During analysis of the effect of chloroquine on heme polymerization activity in vitro, it was observed that in the control assay mixtures containing the extract of malaria parasite and chloroquine only (without heme substrate) the level of preformed hemozoin decreased significantly as compared to the assay mixtures containing the parasite extracts only (no drug controls). Further, incubation of chloroquine with the purified hemozoin yielded a product with the characteristic spectrum of heme or ferriprotoporphyrin IX on spectrophotometric analysis. This provided us with the clue that chloroquine directly interacts with hemozoin and results in its breakdown. In order to confirm these observations, a series of experiments were performed with the purified malarial hemozoin. Incubation of purified hemozoin with chloroquine in acetate buffer (pH 5.0) resulted in a concentration-dependent decrease in the level of hemozoin with concomitant release of heme from the pigment (Fig. 1A). The breakdown product of this reaction was identified as ferriprotoporphyrin IX by infrared spectroscopy and spectrophotometric analysis as well as by thin-layer chromatography [22]. The infrared spectra of the malarial hemozoin show characteristic absorbance peaks at 1660 and 1207 cm^{-1} . These absorbance peaks have been assigned to iron-carboxylate linkage between heme sub-units and ensure the polymeric nature of the malaria pigment [8]. However, the IR spectra of the breakdown product released due to interaction of chloroquine with malarial hemozoin were similar to that of heme and the absorbance peaks characteristic of hemozoin as mentioned above were absent. These observations clearly establish that interaction of chloroquine with the malarial hemozoin initiates a reverse reaction of conversion of hemozoin to monomeric heme (ferriprotoporphyrin IX). The reaction was therefore termed 'hemozoin depolymerization'. The reaction was

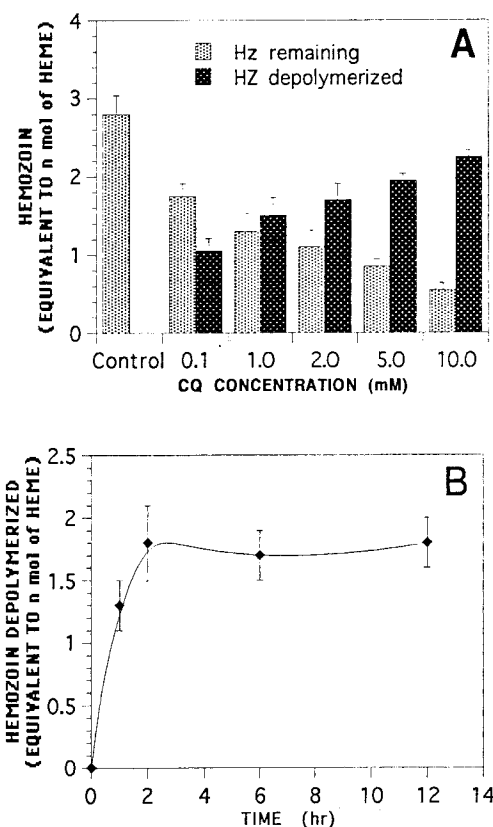


Fig. 1. Effect of chloroquine in vitro on purified hemozoin. (A) At varying concentration of chloroquine and (B) a time course study. Results are presented as mean \pm S.D. of at least three experiments.

complete within 2 h of incubation of hemozoin with chloroquine (Fig. 1 B). A similar effect was observed if, instead of purified hemozoin, lysate of erythrocytes infected with *Plasmodium yoelii* was incubated with chloroquine (Fig. 2A). A time-dependent decrease in the pigment concentration was observed in the samples containing chloroquine. The lysate incubated without the drug did not show any change in the level of hemozoin indicating that during this period there was no detectable formation of de novo hemozoin (Fig. 2B). The decrease in hemozoin level in the presence of chloroquine under these conditions was therefore due to depolymerization of preformed hemozoin rather than the inhibition of hemozoin formation by chloroquine. A similar depletion in the content of hemozoin was also observed when a suspension of intact *P. yoelii* infected erythrocytes was incubated with chloroquine (data not shown). The effect of various factors was also studied on depolymerization of purified hemozoin by chloroquine (Fig. 3). Inclusion of bovine serum albumin in the incubation mixture was found to have no effect on the reaction, but a high concentration of free heme ($> 20 \mu\text{M}$) was inhibitory to the chloroquine-induced hemozoin breakdown (Fig. 3A). The reaction was favored at the pH corresponding to that of the parasite food vacuole, i.e. pH 4.5–5.0 (data not shown). Depolymerization of hemozoin by chloroquine was also dependent on the initial concentration of hemozoin (Fig. 3B). Efficiency of the reaction (in terms of percentage depolymerization) decreased as hemozoin concentration increased. A feed-back effect by the heme released due to the reaction may be responsible for this effect. Other antimalarial drugs

that caused depolymerization of hemozoin include quinine, quinacrine, quinaldine, amodiaquin, mefloquin and halofantrine. Primaquine (a tissue schizonticidal antimalarial), pyrimethamine (a potent inhibitor of malarial dihydrofolate reductase) and desferrioxamine (a ferric iron chelator used in antimalarial chemotherapy) did not show this effect (Fig. 4).

It has been observed for a long time that the blood schizonticidal activity of antimalarial drugs of the 4-aminoquinoline class (e.g. chloroquine, quinine etc.) depends upon active hemoglobin degradation by the parasite and subsequent formation of hemozoin in the parasite food vacuole, the site of hemoglobin degradation [23]. A chloroquine-resistant strain of malarial parasite that produces little or no hemozoin reverts to hemozoin production upon reverting to chloroquine susceptibility [24]. In many other chloroquine-resistant strains where hemozoin is normally produced, the drug resistance has been correlated with less accumulation of the drug inside the parasite digestive vacuole [18]. The malaria parasites treated with chloroquine show clumping of the hemozoin pigment [25]. These indicate that the presence of preformed hemozoin is necessary for selective antimalarial action of these drugs. The reaction we have described herein explains this requirement. 4-Aminoquinoline antimalarial drugs are known to accumulate in the food vacuole of the malaria parasite [26–32] and form complexes with heme [33–36]. Two major mechanisms are suggested for the accumulation of chloroquine and related drugs inside the malarial food vacuole. One is a weak base mechanism; according to it, the chloroquine becomes protonated inside the acidic food vacuole, which makes it

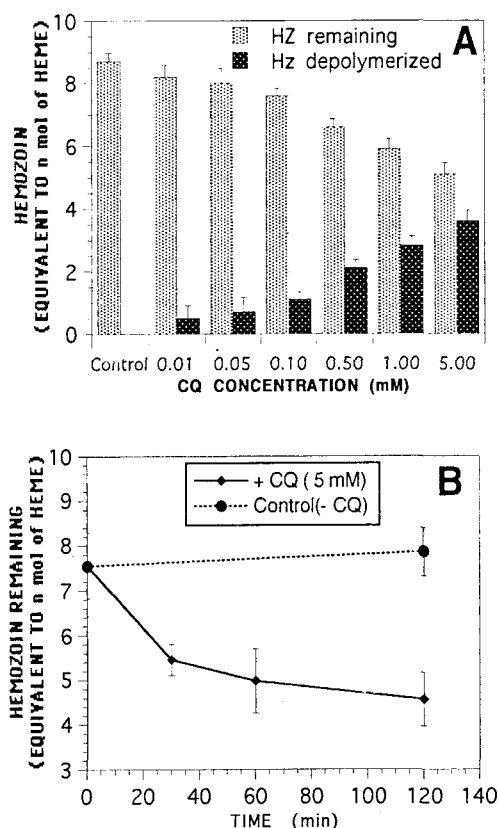


Fig. 2. Effect of chloroquine in vitro on hemozoin content of *Plasmodium yoelii* infected erythrocyte lysate. (A) At varying concentration of chloroquine and (B) a time course study. Results are presented as mean \pm S.D. of at least triplicate observations.

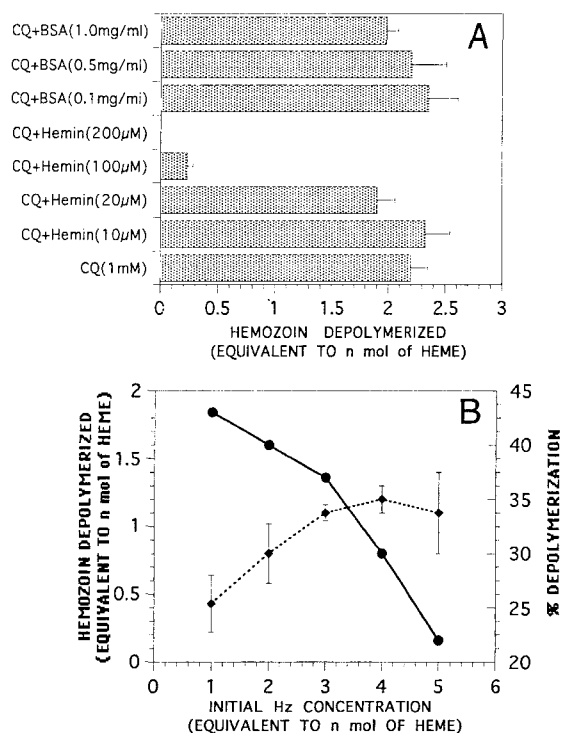


Fig. 3. Effect of heme, bovine serum albumin and hemozoin concentration on depolymerization of hemozoin by chloroquine. (A) Bovine serum albumin and heme and (B) varying concentration of hemozoin (dotted line, nmol of hemozoin (equivalent to heme) depolymerized; solid line, % depolymerization). Each bar/point represents the value mean \pm S.D. of at least triplicate observations.

unable to cross the membrane again and therefore it becomes concentrated as a result of constant influx. Heme has also been identified as a receptor as it forms a complex with chloroquine [23]. A heme-chloroquine complex would not be able to cross the vacuolar membrane and hence leads to drug accumulation [37,38]. Alternatively, a chloroquine-binding protein has also been identified [37]. The efficacy of quinoline drugs may be attributed to their property of accumulation in the malarial food vacuole which results in their effective concentration being several orders of magnitude greater than their therapeutic concentrations. Interaction of these

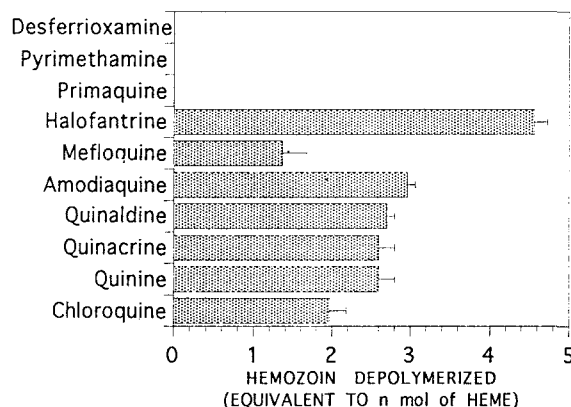


Fig. 4. Depolymerization of purified hemozoin by various antimalarial drugs in vitro. Each bar represents mean \pm S.D. of at least triplicate observations. Concentration of each drug was fixed at 100 μ M level.

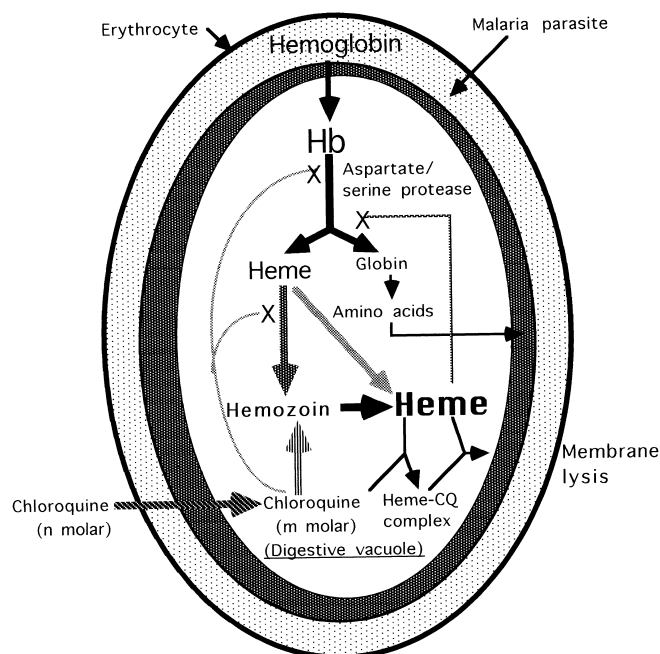


Fig. 5. Biochemical mode of blood schizonticidal action of chloroquine and other related antimalarials through interruption of heme detoxification function of malaria parasite.

drugs with the malaria pigment may start a process of molecular rearrangement responsible for depolymerization of the pigment.

We did not observe hemozoin depolymerization by chloroquine when synthetic β -hematin (prepared by incubating hematin in 5 M acetate at 80°C for 12 h) [8] was used in place of hemozoin isolated from the malaria parasites. Structural differences between chemically synthesized β -hematin and the malarial hemozoin in terms of average number of heme subunits involved in the formation of the polymer (hemozoin/ β -hematin) may be the reason for this anomaly. However, no experimental approach may be designed for determining the average molecular size of malarial hemozoin/ β -hematin.

Formation of hemozoin by the malaria parasite is a slow process which is associated with the degradation of hemoglobin starting at the ring stage and continues up to the late trophozoites. Inhibition of this process alone may not explain the rapid action time (< 2 h) of the blood schizonticides, since the heme accumulated as a result of inhibition of heme polymerization alone may not be sufficient to produce cytotoxic action. Moreover, vacuolar proteases responsible for the degradation of hemoglobin and generation of heme are also inhibited by chloroquine, thereby restricting the availability of heme after the drug treatment. On the basis of the results presented here, we propose that blood schizonticidal antimalarial drugs not only interfere with hemozoin formation as reported earlier [5] but also cause depolymerization of the hemozoin already present in the parasite food vacuoles prior to drug treatment. Their activity may be attributed to the targeting of malaria pigment, converting it back to toxic heme, thus resulting in a total collapse of the parasite heme detoxification system. Release of heme may further initiate the inhibition of vacuolar proteases responsible for hemoglobin degradation by the parasite and exert other toxic effects [39–42]. These results may therefore explain the specific requirement for the presence of the hemozoin pigment for selective

blood schizonticidal action of a wide variety of antimalarial drugs. Based on the information accumulated to date, a scheme has been proposed for the mechanism of the blood schizonticidal action of chloroquine and other related antimalarials (Fig. 5). Further studies on the structural and mechanistic aspects of this reaction may reveal information which could be utilized for the designing of better antimalarial drugs for which the need is pressing due to the rapidly spreading resistance to commonly used antimalarial drugs. Exploitation of this reaction may also help to expedite the clearance of the pigment sequestered in the tissues of the infected host [43], a major factor responsible for the hepatic and splenomegaly which persists for quite some time even after curing of the parasite burden.

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