Mice infected with the NYU-2 strain of *Plasmodium berghei* were used to study the effect of chloroquine on masking of a lipid that promotes ferriprotoporphyrin IX dimerization. More than 40% of this lipid was masked and unable to promote dimerization in membrane ghosts from erythrocytes of untreated, infected mice. Thus, preparations of membrane ghosts dimerized 57 ± 6 nmol of ferriprotoporphyrin IX during a 2-h incubation, whereas the lipids extracted from these preparations dimerized 101 ± 11 nmol of ferriprotoporphyrin IX (means ± S.D. for four experiments). Exposure of membrane ghosts to sonication or cold significantly increased the extent of masking. In addition, chloroquine treatment of infected mice increased the extent of masking to ~90%. The lipid could be unmasked by extracting it into acetone or by aging erythrocyte membrane ghosts from untreated or chloroquine-treated, infected mice for 24 h at pH 7.4 and 25 °C. These findings indicate that masking and unmasking of a lipid is central to the regulation of ferriprotoporphyrin IX dimerization in malaria parasites. They also indicate that chloroquine impairs the function of this regulatory process.

When mice infected with *Plasmodium berghei* are treated with chloroquine, the parasites lose 80% or more of their ability to produce β-hematin (1), a dimer of ferriprotoporphyrin IX (FP)† (2). Consequently, undimerized, toxic FP accumulates (3) and, presumably, kills the parasites (4, 5). The biochemical basis for the effect of chloroquine on the production of beta-hematin *in vivo* presently is unknown. It is true that chloroquine and other quinoline derivatives, including quinine and mefloquine, bind to FP and inhibit dimerization *in vitro* (3, 6), but something else occurs *in vivo*. In fact, inhibition of FP dimerization by chloroquine is antagonized by quinine and mefloquine *in vivo* (3). These and other observations indicate that chloroquine acts by affecting a regulatory process rather than by directly inhibiting FP dimerization (7). To understand the biochemical basis for the antimalarial action of chloroquine, therefore, it is desirable to know more about the process that regulates FP dimerization.

Current knowledge of FP dimerization in malaria parasites can be briefly summarized as follows. The process is promoted only by the lipid fraction of parasitized erythrocytes (8). Apparently, the lipid serves to concentrate monomeric FP and keep it in a state favorable for dimerization (8). Once formed, FP dimers spontaneously aggregate in an acidic milieu (2) such as occurs in the digestive vacuoles of malaria parasites. These aggregates are insoluble at physiologic pH or lower (5), and, in contrast to undimerized FP, they appear to be nontoxic for malaria parasites.

Since malaria parasites are enriched with linoleic acid, it is likely that linoleate or a linoleate derivative is the lipid that promotes FP dimerization (9). The linoleate probably is released from the inner membranes of hemoglobin-laden endocytic vesicles as they are processed into digestive vacuoles (10). If so, the release of linoleate and of undimerized FP could occur simultaneously and, by immediately co-precipitating with each other, protect the parasite from the toxicities of both (9). Thus, the malaria parasite could regulate FP dimerization and protect itself from FP toxicity by regulating the release and accessibility of linoleate.

In this report, we demonstrate that the lipid that promotes FP dimerization is partially masked in erythrocyte membrane ghosts prepared from mice infected with *P. berghei*. In addition, we show that there is significantly more masking after chloroquine treatment of malarious mice. The term “masking” is used to indicate that some substance is interacting with lipid in such a way as to make it unable to promote FP dimerization.

**EXPERIMENTAL PROCEDURES**

Erythrocytes were obtained from young male Swiss mice infected with chloroquine-susceptible *P. berghei NYU-2* parasites when their parasitemias reached ~1500 parasites per 1000 erythrocytes, usually on day 6 or 7 of infection. In young mice, this parasite infects erythrocytes of all ages, and it produces an asynchronous infection. Male mice weighing ~25 g were purchased from Harlan-Teklad (Madison, WI) for these experiments, and they were infected by intra-peritoneal passage of erythrocytes infected with ~10⁴ parasites. Their care was in accord with St. Louis University policies. When treated with chloroquine, each mouse received 3 μmol of intraperitoneally 6 h before erythrocytes were collected. Parasitemia was determined by counting erythrocytes and parasites in Giemsa stained blood films.

For these experiments, erythrocytes were collected, washed twice, and frozen in liquid nitrogen, all as described previously (1, 3). The frozen erythrocytes were thawed by warming at room temperature when needed for an experiment, and membrane ghosts were collected by centrifugation at 27,000 × g for 10 min and washed twice by resuspension in 10 ml of the standard medium and centrifugation. The initial centrifugation and the washes were at room temperature when the ghosts were scheduled to be aged at 25 °C and at 4 °C when they were scheduled to be aged at 4 °C. The standard medium contained 88 mm sodium chloride, 4.8 mm potassium chloride, and 1.2 mm magnesium sulfate. It was buffered to pH 7.4 with 50 mm sodium phosphate.

To evaluate the effect of aging at 4 and 25 °C on the ability to dimerize FP, aliquots of membrane ghosts equivalent to 50 μl of packed erythrocytes from untreated or chloroquine-treated mice were mixed with standard medium to achieve a total volume of 0.2 ml and preincubated at pH 7.4 and the selected temperatures for various periods of time. No β-hematin was formed during the preincubation, since FP
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Fig. 1. Effect of aging on FP dimerizing activity of membrane ghosts of erythrocytes from mice infected with *P. berghei*. Membrane ghosts were incubated at 4 °C (upper panel) or 25 °C (lower panel) for the periods of time indicated on the abscissa prior to measuring FP dimerizing activity. *Open circles* represent preparations from untreated mice. *Solid circles* represent preparations from mice treated with chloroquine. Means ± S.D. for four experiments at each time interval are shown. FP dimerizing activity is expressed as nmol of FP dimerized per h per ml of packed erythrocytes (normalized to a parasitemia of 1000 parasites per 1000 erythrocytes). The actual parasitemias ranged between 1300 and 1600 parasites per 1000 erythrocytes for untreated mice and between 1000 and 1900 for treated mice.

Related experiments evaluated the effect of preincubation with FP on cold-induced loss of FP dimerizing activity. For these experiments, washed membrane ghosts equivalent to 50 μl of packed, infected erythrocytes were suspended to a total volume of 0.3 ml with standard medium or with 0.2 ml of standard medium plus 0.1 μl of 5 mM NaOH containing 700 nmol of FP. After preincubation at 37 °C for 30 min, both incubation mixtures were incubated for another 2 h at 4 °C. Then 1.5 ml of 0.125 mM sodium acetate (pH 5) and 0.2 ml of 5 mM NaOH containing 700 nmol of FP were added for the assay of FP dimerizing activity as described below. Since one incubation mixture contained more FP than the other when dimerizing activity was assayed, it is important to note that 100 nmol of FP per ml of incubation mixture is sufficient to saturate the process and that additional amounts have no additional effect (1).

In the course of the foregoing experiments, we noticed that sonication had a deleterious effect on the ability of membrane ghosts to dimerize FP. Therefore, we also evaluated the effect of FP added prior to sonication on the loss of FP dimerizing activity. For these experiments, membrane ghosts equivalent to 50 μl of infected erythrocytes were suspended to achieve a total volume of 0.4 ml in 0.125 mM sodium acetate (pH 5) or in a mixture of 0.2 ml of 0.125 mM sodium acetate (pH 5) and 0.2 ml of 5 mM NaOH containing 700 nmol of FP. Immediately upon adding FP to the second tube, both tubes were sonicated (8 × 5-s bursts at a power of 140 watts). For the assay of FP dimerizing activity after sonication, 1.6 ml of 0.125 mM sodium acetate (pH 5) were added to the suspension of membrane ghosts that had received FP prior to sonication, and 1.4 ml of 0.125 mM sodium acetate (pH 5) and 0.2 ml of 5 mM NaOH containing 700 nmol of FP were added to the other suspension for the assay of FP dimerization as described below.

The next experiments were designed to evaluate the extent of masking of the lipid that promotes FP dimerization in membrane ghosts. For these experiments, a protease inhibitor mixture was added immediately after thawing frozen erythrocytes and before membrane ghosts were collected and washed. For each ml of erythrocyte suspension, the protease inhibitor mixture consisted of 100 μl of 40 mM phenylmethylsulfonyl fluoride, 40 μl of 250 μM/ml of pepstatin A, and 20 μl of 1 mM transepoxysuccinyl-l-leucylaminomethyl-cleavable chromogenic substrate. The first two inhibitors were dissolved in absolute ethanol, and the third one was dissolved in 50% ethanol. After adding the protease inhibitor mixture, membrane ghosts were prepared and washed at 4 °C as described above and used for extraction of lipids into acetone.

For extraction of lipids, washed membrane ghosts were suspended in 30 mM n-Octyl glucopyranoside in the standard medium at pH 7.4 (using an equal total volume of the initial erythrocyte suspension) and incubated at room temperature for 30 min with occasional vigorous mixing. Afterward, the supernatant fluid and pellet were separated by centrifugation at 27,000 × g for 10 min at room temperature. Hemolysin FP remained in the pellet. To extract lipids into acetone, the supernatant fluid was lyophilized, acetone at minus 20 °C was added to the resulting powder (10 times the volume of the initial erythrocyte suspension), and the mixture was incubated in an ice bath for 30 min with occasional vigorous mixing. Then the mixture was centrifuged to separate the pellet from the acetone extract.

FP dimerization was measured in erythrocyte ghosts, in acetone extracts, in the pellets that remained after n-Octyl glucopyranoside extraction, and in the pellets that remained after acetone extraction of the lyophilized n-Octyl glucopyranoside extract. To prepare an acetone extract for measurement of FP dimerization, an aliquot was transferred to a polycarbonate tube and thoroughly dried under a stream of nitrogen, after which the dried material was resuspended in 0.1 mM sodium acetate (pH 5) with the aid of sonication. To prepare the pellets for measurement of FP dimerization, they were dried under a stream of nitrogen and suspended in 0.1 mM sodium acetate (pH 5) with the aid of sonication.

**RESULTS**

Chloroquine-induced masking of the lipid that promotes FP dimerization is demonstrated by the data presented in Fig. 1 and Table I. The figure shows the effects of aging at 4 and 25 °C on FP dimerizing activity in erythrocyte membrane ghosts from untreated or treated mice infected with *P. berghei*. As expected, chloroquine treatment caused more than an 80% reduction in FP dimerizing activity. When these ghosts were incubated at 25 °C, however, there was a 22-fold increase in FP dimerizing activity between the 15th and 24th h, from 34 to 748 nmol of FP dimerized per h per ml of packed erythrocytes (normalized to represent a parasitemia of 1000 parasites per 1000 erythrocytes). During the same time period, the FP dimerizing activity in membrane ghosts from infected, untreated mice infected with *P. berghei* was significantly reduced. The data in Fig. 1 and Table I show that chloroquine treatment caused a decrease in FP dimerizing activity, and that this decrease is reversible upon removal of the drug.

**TABLE I**

| Preparation | Treatment | FP dimerized* |
|-------------|-----------|---------------|
| Ghosts      | None      | 57 ± 6        |
| Acetone extract | None   | 101 ± 11      |
| Ghosts      | Chloroquine | 8 ± 2        |
| Acetone extract | Chloroquine | 85 ± 10     |

* Nanomol of FP dimerized per ml of incubation mixture during the 2-h incubation period.
mice nearly doubled. After aging for 24 h at 25 °C, therefore, the FP dimerizing activities of erythrocyte membrane ghosts from untreated and chloroquine-treated, infected mice were approximately equal. Thus, it is apparent that erythrocyte membrane ghosts from infected, chloroquine-treated mice possessed the full complement of the substance that promotes FP dimerization and that most of it was masked. This finding is in agreement with the earlier observation that chloroquine treatment does not reduce the amount of linoleate in erythrocytes from mice infected with *P. berghei* (9) and with the data presented in Table I, which reveal that these erythrocyte membrane ghosts possess the full complement of the substance that promotes FP dimerization and that most of it was masked. This finding is in agreement with previous reports (1, 3), chloroquine from chloroquine-treated, infected mice also are summarized in Table II. The *P.* berghei

| Treatment | FPDA | *p* |
|-----------|------|-----|
| None      | 657 ± 176 (8) | <0.001 |
| 4 °C, 24 h | 47 ± 31 (4) | <0.001 |
| 4 °C, 24 h with FP | 553 ± 57 (4) | <0.01 |
| Sonication | 241 ± 97 (4) | <0.01 |
| Sonication with FP | 622 ± 158 (4) | <0.01 |

*FP dimerizing activity expressed as nM of FP dimerized per h per ml of packed erythrocytes (normalized to a parasitemia of 1000 parasites per 1000 erythrocytes).*

**DISCUSSION**

There are two plausible scenarios to explain chloroquine-induced masking of lipid in malaria parasites. The first one is based on the observation that a large fraction of the lipid exists naturally in a masked state (Fig. 1 and Table I). It assumes that there is a regulated process to unmask lipid as it is needed to promote FP dimerization. This scenario is consistent with the fact that the inner membranes of hemoglobin-laden endocytic vesicles disappear when the vesicles are incorporated into digestive vacuoles (13–16). Since the inner membranes are derived from the parasitophorous vacuolar membrane, this scenario also is consistent with the recent hypothesis that the parasitophorous vacuolar membrane is somehow involved in the formation of hemozoin crystals (17). As the inner membranes disappear, their constituents presumably are released, thus making a lipid, probably linoleate (9), accessible to promote FP dimerization. Since chloroquine halts the incorporation of endocytic vesicles into digestive vacuoles (18–20), it is reasonable to suppose that chloroquine also would halt the unmasking of the lipid that promotes FP dimerization. Indeed, it is possible that chloroquine-induced masking of lipid occurs first and is responsible for halting the processing of endocytic vesicles.

The second scenario is based on evidence that sonication and incubation in the cold inhibits the process of FP dimerization either by affecting the critical micelle concentration or by exposing the process to endogenous inhibitors (Table II). We favor the latter possibility and assume that malaria parasites normally regulate exposure of FP dimerization to endogenous inhibitors. These inhibitors could act either by preventing the unmasking of a lipid or by serving as masking agents. The first possibility can be discarded, however, since FP in the incubation medium protected against inhibition by sonication and cold (Table II). Protection by FP means that a certain amount of lipid was unmasked and accessible to FP before sonication or exposure to cold. Thus, this scenario leads to two conclusions: (a) an endogenous masking agent for the lipid that promotes FP dimerization is present in malaria parasites and (b) an intracellular barrier ordinarily governs exposure of the lipid to this agent. Both conclusions are reasonable, since FP dimerization is compartmentalized either in endocytic vesicles as they are processed into digestive vacuoles or later in the digestive vacuoles. If both conclusions are correct, it would follow that chloroquine acts by impairing the ability of malaria parasites to regulate access of an endogenous masking agent to the lipid that promotes FP dimerization.

It is not yet certain which of the above scenarios best describes the situation in malaria parasites. Possibly, elements of both may be required to completely describe the regulation of FP dimerization. In any case, the observation of chloroquine-induced masking of a lipid provides new insight into the biochemical basis for the antimalarial action of chloroquine.

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