INVolvement of Heme in the antimalarial action of chloroquine

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As a world health problem, malaria is among the most important. Over a billion people live in malarious areas, predominantly in the tropics. Approximately 80% of the world’s malaria and 90% of all malaria deaths occur in Africa south of the Sahara. There are over 1.5 million malaria deaths annually, mostly in young children. Four species of malaria parasites commonly infect human beings: Plasmodium falciparum, P. vivax, P. ovale, and P. malariae. Most of the malaria deaths are caused by P. falciparum. This organism is also notorious for its resistance to quinoline antimalarial drugs, such as chloroquine and quinine, which otherwise are highly effective and inexpensive.

One strategy to compensate for quinoline resistance in malaria has been to search for more effective quinoline derivatives. While this search is primarily empirical, it is enlightened to some extent by knowledge of the involvement of heme in the antimalarial action of chloroquine. As a basis for understanding the involvement of heme, I call your attention to the fact that malaria parasites are adapted to live in the hemoglobin-rich cytoplasm of erythrocytes. A simplified flow diagram of hemoglobin catabolism is presented in Fig. 1. At least 3 major processes are involved (1) and each of them may include several steps. The processes are ingestion of erythrocyte cytoplasmic hemoglobin into the parasites’ food vacuoles, digestion of the hemoglobin with the release of amino acids and heme (ferriprotoporphyrin IX, abbreviated FP), and sequestration of FP in the dark brown, refractile malaria pigment known as hemozoin. None of these processes is well understood.

Chloroquine and related quinoline derivatives bind to nonhemozoin FP with high affinity (2). Consequently, these drugs concentrate preferentially in chloroquine-susceptible malaria parasites (2–4). They also interfere with FP sequestration and cause toxic nonhemozoin FP to accumulate (5) to concentrations high enough to kill malaria parasites. Nonhemozoin FP and the complex formed when chloroquine binds to nonhemozoin FP are toxic for malaria parasites (6, 7). Under

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Hemoglobin Catabolism in Malaria Parasites

Erythrocyte Cytoplasmic Hemoglobin

Ingestion

Parasite Vacuolar Hemoglobin

Digestion

FP

Sequestration*

Hemozoin

*Chloroquine interferes with the process of FP sequestration

FIG. 1. Flow diagram of hemoglobin catabolism in malaria parasites.

certain conditions, chloroquine enhances the toxicity of nonhemozoin FP. Just how chloroquine interferes with FP sequestration is the subject of the present paper.

THE STATE OF FP IN HEMOZOIN

Hemozoin is formed and is stored in the parasites’ food vacuoles where the pH is approximately 5. At this pH, hemozoin FP is insoluble (8). In fact, it is insoluble up to a pH at least of 9. It is soluble in dilute NaOH, however, and when solubilized it has all of the characteristics of hematin (8). To explain the remarkable insolubility, FP sequestration was long suspected to be the result of binding of hematin to some other substance in hemozoin, such as a protein or a fatty acid (9). Such a high-affinity binding substance for FP in hemozoin has yet to be discovered. On the other hand, in 1987, we were able to purify an insoluble form of the FP of hemozoin to homogeneity and to show that it consists exclusively of FP aggregates (8). We concluded that the FP in these aggregates was in a special state which rendered it insoluble at the acid pH of the food vacuole (8). Whether or not all of the insoluble FP in hemozoin is in this special state remains to be determined.

We found that the hemozoin FP aggregates, which we had purified, are characterized by insolubility in 2.5% SDS between pH 5 and 8, in 3% aqueous sodium bicarbonate solution, and in chloroform (8). By contrast, hematin is soluble in each of these solvents (8). When suspended in SDS solution, our purified hemozoin FP had a stable, broad Soret band centered at 400 nm and another absorption maximum at
approximately 650 nm (8). Hematin has a narrow Soret band at 400 nm and another absorption maximum at 600 nm. The characteristics of our purified hemozoin FP are identical to those of β-hematin, which can be synthesized by heating hemoglobin in an acetic acid solution (8). We concluded that our purified hemozoin FP probably was identical to β-hematin (8).

Subsequent work by others has more fully characterized β-hematin and confirmed that it is similar if not identical to our purified hemozoin FP (10). The infrared spectrum (10) and x-ray diffraction pattern of β-hematin (11) indicate that it is a polymer in which FP monomers are linked to each other through the coordination of a carboxylate ion of a propionate side chain of one monomer with the central iron atom of an adjacent monomer. The polymer may be further stabilized by hydrogen bonding between the second propionate side chains of adjacent FP monomers (11). The next question, therefore, is: How does the malaria parasite catalyze the formation of a coordination polymer of FP?

**FP SEQUESTRATION**

In 1992, Slater and Cerami (12) and Chou and Fitch (13) reported that crude cell-free preparations of erythrocytes infected with malaria parasites caused exogenous FP to precipitate in a form that is relatively insoluble in SDS and sodium bicarbonate solutions. Both groups concluded that malaria parasites possess an enzyme for FP polymerization. Slater and Cerami named this putative enzyme heme polymerase (12). There has been an extensive but futile search for a protein enzyme with this catalytic function ever since. In the absence of a protein enzyme, various investigators have suggested that FP polymerization may involve autocatalysis by hemozoin FP (14), that the catalyst may be a lipid (15), or that a histidine-rich protein may serve as an enucleation site for FP polymerization (16).

At this point, it should be noted that none of the products of the putative polymerization reactions has been fully characterized. In several studies, they have been considered to be equivalent to our purified hemozoin FP or β-hematin because they are relatively insoluble in SDS and bicarbonate solutions and because they exhibit the correct infrared spectrum (12, 15, 16). Unfortunately, neither the solubility characteristics nor the infrared spectrum of the FP precipitates are sufficient to identify them as pure β-hematin. To establish that insoluble FP aggregates are pure β-hematin, it is also necessary to demonstrate by chemical analysis that the product of the assay is composed exclusively of FP. Such a demonstration has not been re-
ported for any of the "heme polymerase" assays. Therefore, it is preferable to refer to these assays as sequestration assays rather heme polymerase assays.

To illustrate the need for caution in interpreting "heme polymerase" assays, we discovered in one recent set of experiments that the product of a "heme polymerase" assay was contaminated with a form of FP other than β-hematin, based on its visible absorption spectrum (17). These experiments were intended to evaluate whether or not the "heme polymerase" of P. berghei, a malaria parasite of rodents, had the characteristics of a protein. We measured the effect of heat treatment and proteolysis on FP precipitation in vitro by preparations of P. berghei. To our surprise, we found two different "heme polymerase" activities. "Heme polymerase" activity I (HPA I) was destroyed by heat but not by proteolysis. "Heme polymerase" II (HPA II) was destroyed by proteolysis but stimulated by heat. The product of HPA I had the characteristic visible absorption spectrum of β-hematin. The product of HPA II had an anomalous visible absorption spectrum with a narrow Soret band at 400 nm superimposed on the characteristic spectrum of β-hematin, indicating contamination. Moreover, the height of the narrow Soret band increased with increasing exposure of the reaction product to SDS. If only the usual solubility and infrared characteristics had been studied, the significant contamination of this reaction product would not have been detected.

The foregoing results emphasize the possibility that some of the currently used assays of FP sequestration may yield impure products. Therefore, to evaluate for contamination, we repeatedly wash the reaction product with 2.5% SDS until its visible absorption spectrum is stable and typical of authentic hemozoin FP. Using this technique, we have been unable to detect autocatalysis of FP polymerization by purified hemozoin FP from P. berghei. It is also possible that some or all of the FP which precipitates in the presence of lipids and the histidine-rich protein is not β-hematin.

**EFFECT OF CHLOROQUINE TREATMENT IN VIVO ON FP SEQUESTRATION**

For these studies, mice were infected with the chloroquine-susceptible (CS) line of the NYU-2 strain of P. berghei by injecting them intraperitoneally with infected erythrocytes containing 10⁶ parasites. When the parasitemia reached approximately 1000 parasites per 1000 erythrocytes at six days, the mice were treated by intraperitoneal injection of chloroquine, 40 µg per g of body weight. This dose was used
in all the studies reported here but a recent dose response study revealed that as little as 1.6 μg of chloroquine per g of body weight had detectable effects (18).

Fig. 2 shows the effect of chloroquine treatment on the concentration of nonhemozoin FP in parasites in vivo and on FP sequestration in vitro. Nonhemozoin FP is defined as the FP which is soluble in 2.5% aqueous SDS. Chloroquine treatment reduced the ability of parasitized erythrocytes to sequester FP by 85%. Corresponding to the loss of ability to sequester FP, there was a threefold increase in nonhemozoin FP. This chloroquine-induced accumulation of nonhemozoin FP can account for the superior ability of chloroquine-susceptible malaria parasites to concentrate chloroquine, since chloroquine binds with high affinity to nonhemozoin FP (2). Chloroquine-induced accumulation of nonhemozoin FP also is sufficient to account for the antimalarial effect of chloroquine, since both nonhemozoin FP and the chloroquine-FP complex, which is formed by the binding of chloroquine to nonhemozoin FP, are toxic for malaria parasites (6, 19).

**Fig. 2.** Effect of chloroquine treatment on FP sequestration. FP sequestration (“polymerization”) and nonhemozoin FP were measured as previously described (5, 18). FP erythrocytes sequestration is reported as nanomoles of FP “polymerized” per hour per ml of packed erythrocytes normalized to represent a parasitemia of 1000 parasites per 1000 erythrocytes. Means ± SD and numbers of experiments are shown.

Fig. 3 shows the effect of chloroquine treatment in vivo on the ability of infected erythrocytes to catalyze FP sequestration. This study evaluated the time course of the effect of chloroquine. Loss of ability to sequester FP occurred rapidly. Within 2 hours, 50% of the activity had been lost and within 6 hours 80% had been lost. This loss of ability to sequester FP is not due to the binding of chloroquine to FP or to a direct effect of chloroquine on the catalyst (5). Fig. 3 also shows that the effect of chloroquine can be prevented by treating the mice with metabolic inhibitors. The inhibitor used in this case was cycloheximid, but preliminary studies indicate that puromycin and other in-
Fig. 3. Effect of cycloheximide on the chloroquine-induced loss of ability to sequester FP. FP sequestration was measured as previously described (5, 18). FP sequestration is reported as nanomoles of FP sequestered per hour per μmole of preformed hemozoin FP (5). The open circle represents untreated mice, solid circles represent mice treated with 40 μg of chloroquine per g of body weight, open triangles represent mice treated with 0.6 μmole of cycloheximide per g of body weight, and the x symbols represent mice treated with both agents.

Fig. 4. Effect of cycloheximide treatment on chloroquine-induced accumulation of nonhemozoin FP. Nonhemozoin FP was measured as previously described (5, 18) and is reported as nanomoles of FP per ml of packed erythrocytes normalized to represent a parasitemia of 1000 parasites per 1000 erythrocytes. Means ± SD and numbers of experiments are shown.

Inhibitors are similarly effective. Thus, it is probable that new protein synthesis is required for chloroquine to be effective in vivo. Cycloheximide does not reverse the effect of chloroquine.

Fig. 4 shows that cycloheximide not only prevents the chloroquine-
induced loss of ability to sequester FP, it prevents the chloroquine-induced accumulation of nonhemozoin FP. In addition, Fig. 4 shows that quinine treatment prevents the chloroquine-induced accumulation of nonhemozoin FP.

As this result predicts, quinine also prevents the chloroquine-induced loss of ability to sequester FP (Fig. 5). In contrast to cycloheximide, however, quinine treatment reverses the effect of chloroquine in vivo (18). Neither cycloheximide nor quinine in vitro reverses the chloroquine-induced loss of ability to sequester FP. Thus, we conclude that the effects of chloroquine, quinine, and metabolic inhibitors on the catalyst for FP sequestration are indirect, i.e., these agents do not interact directly with the catalyst. Furthermore, we conclude that chloroquine, quinine, and diverse metabolic inhibitors interact with a process which regulates the activity of the catalyst for FP sequestration rather than with the catalyst itself.

The findings summarized in this paper may be taken as evidence that, when chloroquine engages its target in the regulatory process for FP sequestration, it enhances or initiates a chain of events which culminates in increased production, accessibility, or reactivity of a regulator (inactivator) of the catalyst for FP sequestration (18). I propose that this chain of events represents the negative arm of the regulatory process for FP sequestration. I suggest that this negative arm involves protein biosynthesis and that metabolic inhibitors antagonize the chloroquine-induced loss of ability to sequester FP by interfering with protein biosynthesis. How quinine prevents and reverses the effect of chloroquine is more mysterious.
SUMMARY

When malaria parasites digest hemoglobin, they release FP intracellularly. FP is an oxidized form of heme which is toxic for biological membranes. The parasites are not poisoned when they digest hemoglobin, however, because they sequester FP in hemozoin. In fact, the refractile, dark brown substance in hemozoin is sequestered FP. Chloroquine binds tightly to nonhemozoin FP and, under certain circumstances, enhances its toxicity. In addition, chloroquine interferes with FP sequestration and causes toxic nonhemozoin FP to accumulate to lethal levels in erythrocytes parasitized with malaria parasites. Evidently, this is how chloroquine kills malaria parasites. It is desirable, therefore, to know more about FP sequestration and how it is affected by chloroquine. Malaria parasites possess a catalyst for FP sequestration which is modulated by treatment with quinoline antimalarial drugs such as chloroquine and quinine. Chloroquine treatment causes the activity of the catalyst to decrease by 80 to 90 percent. Quinine treatment has no obvious direct effect on the catalyst for FP sequestration. Nevertheless, quinine treatment antagonizes and reverses the chloroquine-induced loss of ability to sequester FP. The effect of chloroquine treatment also is antagonized by various metabolic inhibitors, including inhibitors of protein biosynthesis such as cycloheximide. These findings indicate that chloroquine, like quinine, does not interact directly with the catalyst for FP sequestration. Instead, they are evidence that chloroquine acts by increasing the amount, accessibility, or reactivity of a regulator of the catalyst for FP sequestration. I propose that chloroquine increases the amount of the regulator, which inactivates the catalyst for FP sequestration, which leads to accumulation of nonhemozoin FP, which binds with high-affinity to chloroquine and which ultimately kills the malaria parasite.

REFERENCES

1. Olliaro PL, Goldberg DE. The Plasmodium digestive vacuole: Metabolic headquar
2. Chou AC, Chevli R, Fitch CD. Ferriprotoporphyrin IX fulfills the criteria for iden
3. Macomber PB, O’Brien RL, Hahn FE. Chloroquine: Physiological basis of drug re
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DISCUSSION

Schreiner, George E., VA: Some years ago I visited a hospital in Uganda and there was a whole wing of children with nephrotic syndrome from plasmodium. I wonder if you have ever come across any indication that the altered blood chemistry (like hyperlipidemia, hypoaalbuminemia, etc.) seen in the full nephrotic syndrome has any effect on the action of Chloroquine?

Fitch: No, I haven’t.

Davis, Charlottesville: In the field of rheumatology, we are using more and more chloroquine, usually hydroxychloroquine, for essentially all of the major rheumatic diseases: rheumatoid arthritis, lupus, all of the overlap syndromes. We find this a very safe drug and it is being accepted more and more as a standard. We have very little idea, essentially no idea, of how the drug works. Obviously, it takes months really to be effective in these diseases. I wonder if any spin off of your work on the build up of this product might provide any insight to help us rheumatologists to know what we are really doing in our patients.

Fitch Reply: The effective concentration for Chloroquine for malaria is something on the order of 10 nm. For rheumatic diseases the concentrations need to be higher than that and may be up at micromolar concentrations. I don’t know how it works in rheumatic diseases, but it is more likely to work at the lysosomal level than on the level of hemoglobin metabolism.

Dupont, Houston: How do your studies help us understand the mechanisms of
Chloroquine resistance in P-falsiparim infections? Are there any strategies that you have for circumventing this resistance?

**Fitch:** Well, before I get to P-falsiparim, I'll mention that it is possible to induce Chloroquine resistance in P-bergia, the rodent malaria and various other rodent strains. When you do that, they invariably adapt to living in the red cell without digesting hemoglobin. They become nonpigmented. It is fairly easy in the rodent parasites to understand why they are resistant to Chloroquine. They just don't digest hemoglobin so they don't accumulate that toxic form of FP. In P-falsiparim, even the resistant parasites do digest hemoglobin. They make FP and they sequester it. We have done limited studies with P-falsiparim. In the Chloroquine-susceptible strains of P-falsiparim, Chloroquine interferes with the sequestration as it does in P-bergia, but in the Chloroquine-resistant strain to P-falsiparim, it didn't. We don't know what the change is, but they have adapted to a pathway of sequestration that isn't vulnerable to Chloroquine.

**Griner:** Dr. Fitch, you began your remarks by suggesting that a better understanding of the action of anti-malarials could lead to new drug developments that are more effective. What is the status of interest among drug companies for the development of new drugs?

**Fitch:** Well, most of the people who have malaria are not able to afford expensive medication; so new drug development by pharmaceutical companies is relatively slow. When the army thought it might have to fight in malaria areas, it conducted a big campaign screening hundreds of thousands of drugs and it did produce a new Quinlin, Methliquin, which is on the market. It is called Larium. WHO is also interested in drug development and is supporting to some extent drug development.