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The Malaria Parasite Monitored by Photoacoustic Spectroscopy

Abstract. *Noninvasive photoacoustic spectroscopy was used to study the malaria parasites* Plasmodium chabaudi *and* Plasmodium berghei, *their pigment, and ferriprotoporphyrin IX, which is a by-product of the hemoglobin that the parasite ingests. The results indicate that the pigment consists of ferriprotophorphyrin self-aggregates and a noncovalent complex of ferriprotoporphyrin and protein. Spectra of chloroquine-treated parasites reveal in situ interaction between the drug and ferriprotoporphyrin. Chloroquine-resistant parasites, readily distinguishable by this method, appear to degrade hemoglobin only partially.*

Malaria parasites of the genus *Plasmodium* infect erythrocytes, degrade the hemoglobin there for its metabolism, and store the residual ferriprotoporphyrin IX (FP) in a protein-sequestered form termed malaria pigment or hemozoin (*1*). It has been suggested that free hemin (FP chloride) and a FP-chloroquine complex thought to be formed when this drug is added to the system are toxic to the parasite since they lyse cell membranes (*2*). It is thus of interest to monitor the states in which FP exists in the parasite, observe the changes that it undergoes on drug addition, detect any formation of complexes between antimalarial drugs and FP in the parasite, and investigate any differences between drug-sensitive and -resistant parasite strains.

For a number of reasons, a study in situ is preferable to the usual method of isolating and studying molecular components. First, isolated FP in solution can exist in several states of aggregation depending on the solution conditions (*3, 4*), while the interest here is on its status in vivo. Second, FP appears to complex easily with proteins (*5, 6*), a situation that is more likely to occur in the microorganism. Third, the particulate malaria pigment appears to be a heterogeneous population of molecules and complexes that are not easily studied in solution due to solubility and stability problems. Finally, direct comparisons between drug-treated and untreated parasites and between drug-sensitive and -resistant strains are more meaningfully done in situ. We therefore used photoacoustic spectroscopy, a noninvasive technique particularly suitable for condensed phase samples, in order to establish the nature of the malaria pigment, provide direct evidence for FP-drug interactions in the parasite, and distinguish drug-sensitive strains of the microorganisms from resistant ones—a method of potential diagnostic value.

The principles of photoacoustic spectroscopy and its applications in biology have been described elsewhere (*7–10*). The materials for our study were obtained by standard procedures (*1, 2, 11*). Parasite-infected mouse erythrocytes were incubated in Fitch medium for 30 minutes with or without chloroquine. The parasites were then prepared by saponin lysis and N_2 decompression of the infected erythrocytes (*12*). Samples analyzed were lyophilized powders suitably diluted. Since it is not certain whether all the parasites were intact, the spectra represent conditions in situ rather than in vivo. The spectral parameters of interest are the wavelength maxima of the band in the "red" region (λ_r) and the Soret band (λ_S) of the constituent hemin, their relative intensity ratio I_S/I_r (an internally normalized parameter that characterizes each system studied), band-

Table 1. Photoacoustic spectral data for *Plasmodium* and its components.

Sample	λ_r (nm)*	λ_S (nm)*	I_S/I_r	Remarks
1. Hemin (FP chloride), solid	645	400, broad	2.0	Essentially aggregated; resembles the optical absorption spectrum of the aggregates in solution (*3, 5*)
2. Hemozoin isolated from the parasite	630	400, shoulder at 370	2.5	Monomeric FP indicated
3. Hemin and BSA complex, lyophilized	630	400, shoulder at 370	2.5	Monomeric FP; resembles optical spectrum of the complex in solution (*5, 6*)
4. *P. chabaudi*, isolated and lyophilized; chloroquine-sensitive strain	645, 630	400, minor band ~ 430	2.1	Both monomeric and aggregated heme indicated; 430-nm band suggestive of peptide-coordinated heme (*20, 10*)
5. *P. chabaudi*, chloroquine-resistant strain	645, 630	430	1.3	Only peptide-coordinated heme indicated
6. *P. berghei*, chloroquine-sensitive strain	645, 635	400	2.3	I_S/I_r somewhat higher than in case 4; different parasite strain (also uncloned)
7. *P. berghei*, chloroquine-sensitive and treated with chloroquine	640	370	3.0	Blue-shifted Soret band; FP-drug interaction indicated
8. *P. berghei*, chloroquine-sensitive and treated with mefloquine	640	375	2.5	Blue-shifted Soret band; FP-drug interaction indicated

*Error, ±3 nm.

shifts, occurrence of new bands as peaks or shoulders, and modulation frequency–dependent changes in the signal intensity.

Figure 1 shows the spectra of powdered hemin (FP chloride), of hemozoin isolated from the parasite, and of *P. chabaudi*. The presence of the red band in the 630- to 645-nm region identifies the iron atom in the samples to be in the Fe (III) oxidation state (*4*). Hemin displays a peak at 645 nm and a broad, structureless Soret band at 400 nm. These details, and the overall similarity of the photoacoustic spectrum to the optical absorption spectrum of FP aggregates in solution (*3–5*), allow us to conclude that the hemin sample is essentially in the self-aggregated form. In contrast, in the case of hemozoin [and of FP and bovine serum albumin (BSA) complex (Table 1)], the red band is shifted to 630 nm, the Soret peak is accompanied by a characteristic shoulder near 370 nm, and the I_S/I_r ratio increases to over 2.5. These features are similar to those observed in the solution-state optical spectra of ferrileghemoglobin (*13*) and of complexes of monomeric hemin with human serum albumin (*5*) and with ligandin (*6*), and suggest that FP in hemozoin is in an environment similar to that seen in its complexes with albumin or ligandin. Our results are direct confirmation of the suggestion (*1*) that hemozoin is a noncovalent complex of protein and monomeric FP.

The spectrum of the parasite (Fig. 1 and trace 1 in Fig. 2) shows a split red band at 645 and 630 nm. This, and the I_S/I_r ratio of 2.1, indicates that FP in the parasites occurs both as self-aggregates and as hemozoin. The other peak at 430 nm that is close to the Soret band, being similar to that seen in the photoacoustic spectrum of hemoglobin (*10*), probably arises because of residual hemoglobin (or its degraded products that still contain peptide-coordinated heme) present at the time of isolation.

We suggest that the FP obtained after hemoglobin digestion is processed and stored in the parasite in two ways: as a protein-sequestered complex and as FP self-aggregates, neither of which is toxic to the parasite (*14*). This explains the earlier view (*1, 15*) that the particulate form of the pigment in vivo might contain hemozoin, hematin aggregates, hemichrome, and trapped parasite proteins and also reconciles this view with that of Homewood *et al.* (*16*), who stated that the pigment contains a heterogeneous array of molecules, some of which have surprisingly high weights.

Figure 2 shows spectra of *P. berghei*

Fig. 1. Photoacoustic spectra of solid hemin, isolated hemozoin, and of chloroquine-sensitive (D^+) and -resistant (D^-) clones of *P. chabaudi* isolated by saponin lysis of infected mouse erythrocytes and lyophilized. The samples were diluted with alumina to avoid photoacoustic saturation. Solid-state charge-transfer effects or artifacts were ruled out, since identical spectra were obtained with other diluents, such as glucose. Signal amplitude varied inversely with modulation frequency, and the sample amounts were chosen such that signal intensity varied linearly with concentration. Saturation effects are thus ruled out and the spectra are acceptable (*10*). All spectra have been normalized to the same signal strength of the visible band near 630 nm. This avoids sample-to-sample variations of individual band intensity and affords a characteristic and reproducible set of I_S/I_r values for each of the systems studied. All spectra were obtained at an ambient temperature of 295 K. Carbon black was used as the reference material. The modulation frequency was 40 Hz and the spectral bandwidth in each case was ±3 nm. The EG&G PAR 6001 and EDT OAS 400 photoacoustic spectrometers were used. Photoacoustic intensity is in arbitrary units.

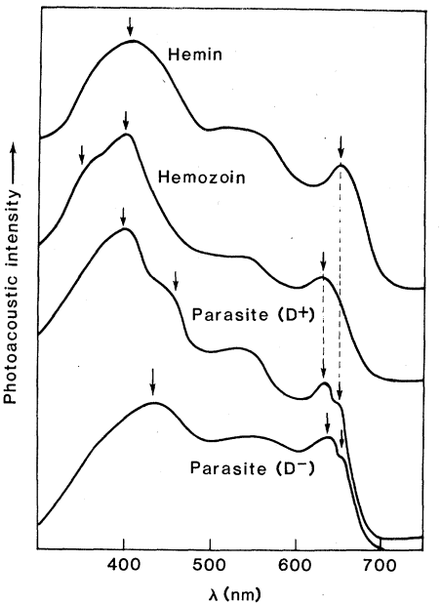


Fig. 2. Photoacoustic spectra of *P. berghei* isolated from infected mouse erythrocytes in the presence and absence of drugs. Note the resemblance of the spectrum of the drug-free parasite to that of *P. chabaudi*. A modulation frequency of 912 Hz was used (*19*). Other experimental details were as described in the legend to Fig. 1.

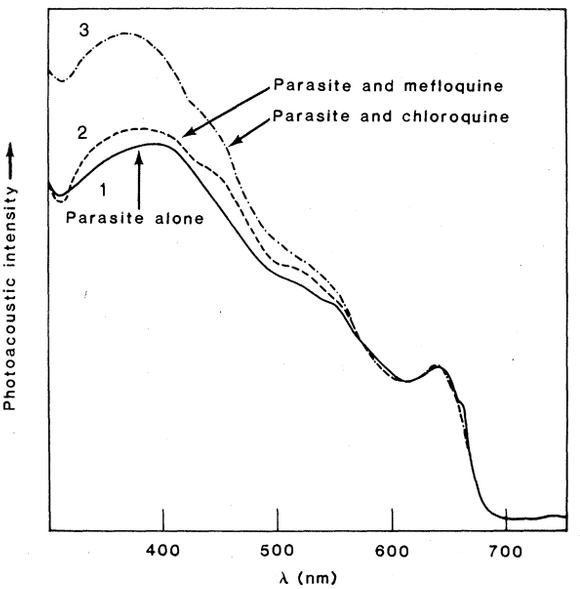
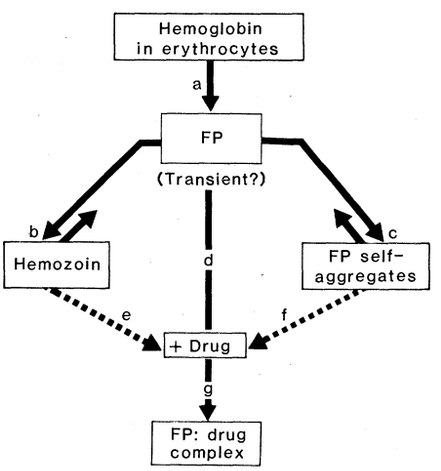


Fig. 3. Model for the processing of FP in the parasite and the mode of action of drug, as suggested by available evidence. Comments: (a) FP is produced after degradation of host globin; (b) hemozoin appears less abundant in drug-resistant parasites (*21*); (c) FP aggregates are indicated by photoacoustic spectra and possibly by the "dense crystals" seen in electron micrographs; (d) interaction with drug is indicated by photoacoustic and other studies (*2, 11, 18*); (e) drug interaction with hemozoin does not appear likely (*11, 15*); (f) this interaction is yet to be established and cannot be disregarded at present; and (g) this complex has membrane-lytic activity (*2*).



grown in mouse erythrocytes in the presence and absence of a few antimalarial drugs. Introduction of a drug causes significant changes in the spectrum of the parasites. The red band is broadened and centered around 640 nm, the Soret band is blue-shifted from 400 to 375 nm or lower, and the I_S/I_r ratio increases significantly. The Soret band shifts indicate FP-drug interaction, while the increase in the intensity ratio points to a reduction in the population of FP self-aggregates on drug addition. In previous studies (*15, 17*), we showed that FP can form a 2:1 molar complex with chloroquine in aqueous solution with a formation constant that appears comparable to the binding of chloroquine to hemin and to parasite suspensions (*18*). We thus believe that our present results provide the first direct evidence for the presence of FP and drug complex in malarial parasites (*19*).

It also appears that the photoacoustic method can readily distinguish between chloroquine-sensitive and -resistant strains of *P. chabaudi* (Fig. 1). Unlike the drug-sensitive strain, the resistant one displays the Soret maximum at about 430 nm and a reduced I_S/I_r ratio of 1.30. The ratio is close to the values of 1.4 and 1.5 seen in the photoacoustic spectrum of hemoglobin (*10*), while the position of the Soret band is similar to that seen in the optical spectra of heme coordinated to a peptide chain, for example, hemoglobin and its derivatives (*20*). Hence, it appears that endogenous FP in the resistant parasite is largely in the form coordinated to a protein chain. It is known that when *P. berghei* becomes resistant to chloroquine, production of the pigment drops (*21*). Fitch and Chevli (*22*) argued that the molecular change responsible for chloroquine resistance could involve either hemoglobin degradation or FP sequestration. In light of our results, it appears likely that drug resistance, at least in *P. chabaudi*, involves the former process in such a way that the degradation of hemoglobin occurs to a stage where the heme group is still held to the peptide chain with the same integrity as in the whole molecule (*23*).

The form of the endogenous FP that complexes with drugs is still not known. Neither the pigment nor aged self-aggregate of FP (*14*) appear to be membranelytic, and free FP, which does lyse membranes, is not detected in the parasite. It is reasonable to conceive of the endogenous FP as being in a state of equilibrium; from this FP the added drug derives its partner to form the lytic complex (Fig. 3). Since the complex can be isolated as a heteromolecular aggregate species (*18, 24*), drug interaction with the FP self-aggregates is a possibility. Drugs do not seem to bind to the pigment (*11*) or to residual hemoglobin (*18*), although they do bind to protease-degraded fragments of hemoglobin (*15*). We propose the scheme shown in Fig. 3 as a model of the mode of action of quinoline drugs on the malaria parasite.

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An Ultrahigh-Speed Analysis of Exocytosis: Nematocyst Discharge

Abstract. *High-speed microcinematography of nematocyst discharge in* Hydra attenuata *Pall. shows that this specialized exocytosis, which appears to result from an increase in the intracapsular pressure, requires a total of about 3 milliseconds. A maximum velocity of 2 meters per second is generated, corresponding to an acceleration of 40,000g. Thus nematocyst discharge is one of the fastest cellular processes in nature.*

The nematocytes (cnidocytes or "stinging cells") of the *Cnidaria* serve various functions such as capture of prey, defense, and locomotion. The essential organelle of these cells, the capsular nematocyst, is an extremely complex secretory product of the Golgi apparatus (*1*). When triggered, it ejects its tubular content by evagination (*2, 3*). Three hypothetical mechanisms have been suggested for this exocytosis (*4*): (i) discharge is caused by a swelling of the capsular matrix due to an influx of water [osmotic hypothesis (*5*)]; (ii) intrinsic tension forces generated during cnidogenesis are released at discharge [tension hypothesis (*6*)]; or (iii) contractile units enveloping the cyst cause the discharge when they increase the cyst's internal pressure by deformation of its wall [contractile hypothesis (*5*)].

We have studied the discharge of stenotele cysts (Fig. 1) in *Hydra attenuata* Pall. (Hydrozoa). This process is normally triggered mechanically by a prey (usually a small crustacean). The cyst everts three stylets (Fig. 1), which join to form an arrowhead that punches a hole