Interaction of antimalarial drugs with hemin

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Hemin (ferriprotoporphyrin IX) is shown to form complexes with the chloroquine class of antimalarial drugs. The Soret band of hemin becomes optically active upon the addition of chiral drugs. Results on the hemin-induced quenching of the fluorescence of chloroquine are consistent with the formation of a 2:1 hemin:drug complex with a formation constant of $1.4 \times 10^7$ at 298 K. Also a direct comparison of the drug-treated and drug-free parasites themselves, by the noninvasive photoacoustic spectroscopic method, reveals an in vivo interaction between endogenous hemin and the added drug.

Drugs of the chloroquine class are effective against the malaria parasite *Plasmodium* in erythrocytes. Some ideas about the mode of action of these drugs have been forthcoming in recent years. Fitch and coworkers (1-3) etc. have proposed that chloroquine complexes with endogenous hemin (ferriprotoporphyrin IX) in the parasite and that this complex effectively lyases the parasite cells. We have concurrently obtained evidence for the binding of these drugs to the malaria pigment and to hemin and its derivatives (4,5). There have also been other reports (6-8) on the possibility of such a complexation in aqueous solutions. It is therefore of interest to investigate the stoichiometry and the strength of the interaction between this class of drugs and hemin in solution, and to monitor this interaction directly in the parasite itself. We present evidence here for the complex in the solution state, and in the parasites themselves. The stoichiometry and the stability constant that we have found for the hemin:chloroquine complex compares favourably with those estimated for the binding of chloroquine to infected erythrocytes and to hemin (1).

Materials and Methods

Samples of hemin, quinine sulfate, quinidine sulfate, and chloroquine diphosphate of high purity were obtained from commercial sources. The parasite *P. berghei* was grown in mouse blood in the presence

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and absence of drug, as per standard procedures (2,9,10). Circular dichroism (CD) spectra were measured with a JASCO J-20 spectropolarimeter at ambient temperature (295 K). Fluorescence spectra were recorded on a Hitachi model 650-10S instrument using a thermoregulated cell assembly. All CD and fluorescence measurements were made on freshly made solutions and within 30 min of mixing them, in order to avoid problems of time-dependent aggregation and related complications. Photoacoustic (PA) spectra of lyophilized samples of the parasite, diluted with inert compounds such as alumina or glucose, were recorded using an EG & G model 6001 spectrometer.

Results and Discussion

Drug-induced optical activity of hemin

Fig. 1 reveals that a large negative cotton effect is induced in the Soret band of the inherently achiral hemin molecule upon the addition of levorotatory quinine. The diastereoisomer quinidine produces essentially the same effect except that the induced cotton effect is opposite in sign, as expected. The induced optical activity might arise either because of a perturbation of the hemin transitions by those of the drug, or by an exciton coupling between the bands of the hemin placed in the chiral environment provided by the drug; the latter appears likely in light of the large ellipticites seen. The data shown in Fig. 1 are at concentrations and pH that favour the occurrence of hemin in the monomeric form and were recorded shortly after mixing.

![CD spectra of hemin (H), 5 x 10^{-5} M, in the presence of 4 x 10^{-5} M quinine (Q) or 4 x 10^{-5} M quinidine (QD); pH 7.4, 0.02 M phosphate buffer; ambient temperature (295 K). [θ]M is molar ellipticity in deg.cm^2/dmol based on total hemin concentration.](image)

Fig. 1. CD spectra of hemin (H), 5 x 10^{-5} M, in the presence of 4 x 10^{-5} M quinine (Q) or 4 x 10^{-5} M quinidine (QD); pH 7.4, 0.02 M phosphate buffer; ambient temperature (295 K). [θ]M is molar ellipticity in deg.cm^2/dmol based on total hemin concentration.
the two components, in order to avoid any artifacts due to light scattering when large aggregates form. Since the induced CD of hemin required intimate contact between hemin and the drug, the results are consistent with the postulation of a complex in solution. We did not study the effects of the drug chloroquine by this method since it was available only in the racemic form.

Quenching of chloroquine fluorescence by hemin

We turn to a study of the stoichiometry and the stability of the suggested complex. Fig. 2 shows the effect of added hemin on the fluorescence (excitation at 290 nm, emission at 386 nm) of chloroquine. Several observations are noteworthy in this connection: (i) hemin by itself does not fluoresce but quenches the chloroquine emission; (ii) the fluorescence polarization value of chloroquine \( P = 0.08 \pm 0.01 \) was found to be invariant upon hemin addition; (iii) the

![Fig. 2. Fluorescence spectra of chloroquine in presence of hemin.](image)

- **Curve A**: Quenching by increasing concentrations of hemin as: 1 = 0.00 M; 2 = 7.45 x 10^{-6} M; 3 = 1.49 x 10^{-5} M; 4 = 2.23 x 10^{-5} M; 5 = 2.98 x 10^{-5} M; 6 = 7.45 x 10^{-5} M. Chloroquine concentration was kept constant at 2.94 x 10^{-4} M. Temperature 295 K.
- **Curve B**: Stern-Volmer plot of hemin-induced quenching of chloroquine (2.94 x 10^{-4} M) at 295 K.
- **Curve C**: Temperature dependence of the fluorescence intensity of chloroquine (2.94 x 10^{-4} M) in the presence of hemin (7.45 x 10^{-5} M). Solvent in all cases: pH 7.4, 0.02 M phosphate buffer.
Fig. 3. Photoacoustic spectra of *Plasmodium berghei*, lyophilized powder, diluted with alumina. Parasites isolated from the blood of control and drug-treated mice. Modulation frequency 912 Hz. Ambient temperature. The signal strengths in the 630-nm region in both cases were normalized to the same value in order to avoid sample-to-sample variations.

emission intensity of the binary solution was found to increase with temperature; and (iv) when the hemin-dependent quenching was analyzed by the Stern-Volmer equation, the resultant plot was found to exhibit a positive deviation from linearity. Thus dynamic quenching by hemin appears unlikely since in that case one would have expected (i) a change in the polarization value due to alterations in the fluorescence lifetime of chloroquine, and (ii) a drop in the emission intensity with increasing temperature. Further, a simple collisional interaction between the quencher and the fluorophore, or a non-fluorescent 1:1 complex, does not appear likely since the Stern-Volmer plot is not linear. When we analyzed the fluorescence data on the basis of the formation of a 2:1 hemin:chloroquine complex in solution, characterized by the formation constant $K_f = [\text{complex}]/[\text{drug}] \cdot [\text{hemin}]^2$, we obtained a value of $K_f = 1.4 \times 10^7$ at 298 K. This lends credence to earlier suggestions (1,5) and the value of $K_f$ is to be compared with the $K_d$ values reported for the binding of chloroquine to infected erythrocytes ($1 \times 10^{-8}$ M), and to hemin ($3.5 \times 10^{-9}$ M) obtained by dialysis measurements (1).

**Monitoring complex formation in the parasite**

With the suggestion of a hemin:drug complex, and the reported lytic ability of this complex, it becomes of interest to investigate whether such complexation occurs between hemin and the added
chloroquine class of drugs in the parasite. Conventional optical spectroscopy on the parasites themselves is beset with problems of optical opacity and light scattering inherent in such samples in the condensed phase. However, the technique of photoacoustic spectroscopy is useful here. The principles and the application of the PA method in biological systems have been reviewed recently (11-15). Fig. 3 compares the PA spectra of *P. berghei* grown in the presence and in the absence of chloroquine. It is seen that the added drug blueshifts the Soret-band maximum by as much as 25-30 nm, besides increasing the relative intensity ratio of the Soret/630-nm bands. A detailed report of the PA studies will appear elsewhere, and it suffices to note here that the spectral perturbations seen in the PA spectra, upon the addition of the drug, are consistent with the idea of an in situ interaction between the endogenous hemin and the antimalarial drug.

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