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FURTHER EVIDENCE FOR THE INTERACTION OF THE ANTIMALARIAL DRUG AMODIAQUINE WITH FERRIPROTOPORPHYRIN IX

GIDEON BLAUER,[*] MUATAZ AKKAWI and ERIKA R. BAUMINGER[†]
Department of Biological Chemistry and [†]Racah Institute of Physics, The Hebrew University of Jerusalem, 91904 Jerusalem, Israel

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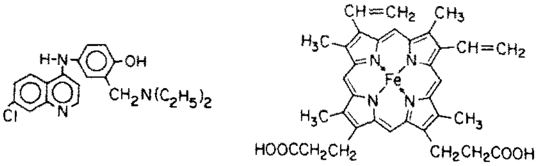
Abstract—Evidence for complex formation of the antimalarial drug amodiaquine (AD) with ferriprotoporphyrin IX (FP) in aqueous medium is presented, in addition to previous preliminary data. A mole ratio of one between the complex components is determined for the insoluble complex at pH 6.7–6.8. Mössbauer data obtained at pH 7–8 and at higher concentrations in the millimolar range confirm the interactions existing between the complex components. These data are considered to aid in removing previous objections to a mechanism of antimalarial action involving complexes of FP with AD and related drugs.



Scheme 1. *Structures of AD (left) and FP (right).*

Complex formation of antimalarial drugs of the quinoline series such as chloroquine, quinidine or amodiaquine (AD[‡]) with ferriprotoporphyrin IX (FP) (Scheme 1), as measured by different spectroscopic methods, has been demonstrated previously (see, e.g. Refs 1–6). In several cases, the formation of specific and soluble aggregates upon complex formation could be shown by hydrodynamic methods, e.g. in the system FP–quinine or FP–quinidine [3, 4]. This complex formation has been considered to be part of a mechanism of action of antimalarial drugs [6]. By prevention of the sequestration of FP into the malarial pigment hemozoin, free FP or its complexes with antimalarial drugs were considered to be toxic to the parasite [7].

Recent publications dealing with alternative modes of action of antimalarial drugs suggest inhibition of a heme polymerase by these drugs [8, 9]. A previous study [10] had failed to find any evidence for the interaction in either aqueous or organic phase between solid FP and AD and this was taken to contradict the FP–drug interaction hypothesis [8, 9]. It therefore seemed of interest to present additional evidence to previous preliminary data on the FP–AD complex [11], using Mössbauer spectroscopy and insolubility of the complex in aqueous medium at pH 6–7.

MATERIALS AND METHODS

Protohemin (chloro-FP). This was obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A., Type I). Stock solutions of about 10 mg hemin in 25 mL of 0.02 N aqueous NaOH were kept for up to 8–10 days at 0–4° in the dark. Their absorption spectra were checked regularly. At pH 6.7–6.8, the maximum absorbance of FP at 366 ± 2 nm (conditions as given for Fig. 1) was found to decrease by about 4% after being kept at room temperature for 1 day, probably due to some precipitation.

AD hydrochloride. This was a Sigma product. Stock solutions in distilled water (10^{-2}–10^{-3} M, pH ≈ 5) were kept at 0–4° and were protected from light. Under the conditions as given for Fig. 1 (pH 6.7–6.8), Beer's Law was found to be obeyed in the concentration range of $(2–8) \times 10^{-4}$ M giving an ε-value of $1.38 \times 10^4 \pm 0.05$ (SD) at 342–344 nm (average of 10 experiments). Solutions of AD (8×10^{-4} M) were stable up to at least 8 days at room temperature, as measured spectrophotometrically. Centrifugation of these solutions for 8 min at 900 *g* or 4 min at 8000 *g* had no measurable effect on the absorption maximum of the solutions at 342–344 nm. However, at concentrations exceeding about 8×10^{-4} M, part of the AD was insoluble. On the other hand, the more concentrated AD stock solutions remained soluble at pH ≈ 5.

Preparation and measurement of the FP–AD complexes. Usually, measured amounts of stock solutions of sodium chloride, phosphate buffer, FP and AD were mixed to give a final volume of 4 mL. The pH was adjusted by 1 N HCl or 0.5 N NaOH. In most experiments reported, the AD stock solution was added as the last component during magnetic stirring. Final solutions were prepared in plastic vessels in order to minimize adsorption of both FP and AD to the wall surface.

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[*] Corresponding author. Tel. (972) 2 585434; FAX (972) 2 666804.

[‡] Abbreviations: FP, ferriprotoporphyrin IX; AD, amodiaquine; DMSO, dimethyl sulfoxide.

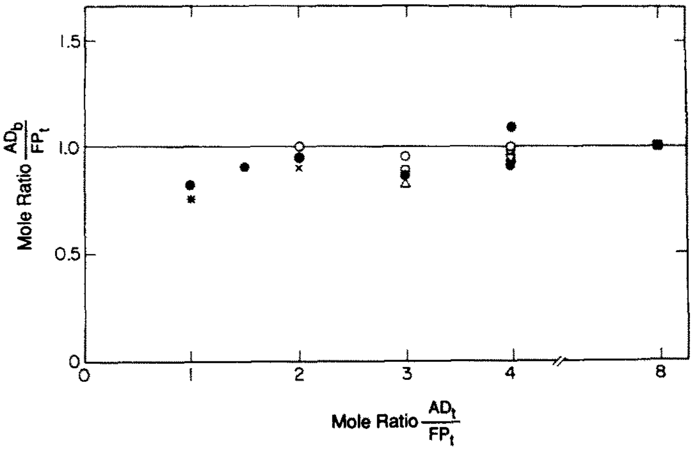

Fig. 1. Interaction of AD with FP. AD bound to FP in the insoluble phase was determined spectrophotometrically at 342–344 nm from the concentration of free AD remaining in the supernatant aqueous solution. FP, 2.0×10^{-4} M; sodium chloride, 0.05 M; phosphate buffer, 0.01 M; pH 6.7–6.8; room temp. (20°) (●) In most cases left to settle for about 1 day; (○) centrifuged at 900 *g* for 8 min; (△) FP dissolved in DMSO prior to addition to the final mixture containing 6 vol. % of DMSO, centrifuged 8 min at 900 *g*; (▽) FP in DMSO, left to settle for 1 day. Exceptions: (■) FP, 1.0×10^{-4} M, left to settle for about 1 day, mole ratio $AD_t/FP_t = 8$; (×) FP, 6.0×10^{-4} M, left to settle for 1 day, (*) Additional centrifugation at 8000 *g* for 4 min. The mole ratio AD_b/FP_t (AD bound to FP total) in the insoluble phase was calculated from the relation:

$$\frac{AD_b}{FP_t} = \frac{AD_{in} - AD_f}{FP_t} = \frac{AD_t}{FP_t} \times \frac{(A_{in} - A_f)}{(A_{in})}$$

where AD_{in} and AD_f are the concentrations of initial (total) and final (free) AD in the solution, respectively; A_{in}, A_f are the measured absorbance values at 342–344 nm of initial (total) AD and final (free) AD, respectively, at constant cell path length. AD_t, FP_t are the total (initial) concentrations of AD and FP, respectively, in the system.

Light absorption spectra. These were measured on a Hewlett–Packard 8452A Diode Array Spectrophotometer, using quartz cells of 0.2 cm path length in most experiments. The reference solvent was water in all cases. For (ultra)centrifugation experiments, a Beckman TLX ultracentrifuge with a 120.2 rotor was used at 18°. It could be shown that under the conditions as given for Fig. 1 and with 6×10^{-4} M AD, the concentration of AD remaining in the supernatant phase was the same if the solution containing the precipitate was centrifuged either at 89,000 *g* for 5 min or at 900 *g* for 8 min. Most systems were therefore centrifuged at 900 *g* for 8 min.

Mössbauer measurements. These were performed using a conventional constant acceleration Mössbauer spectrometer [12] with a 100 mCi ^{57}Co in rhodium source at room temperature and a Harwell proportional counter. Mössbauer spectra were obtained with the samples held in a liquid nitrogen cryostat at 90°K and a liquid helium cryostat at 4.1°K.

RESULTS

Insoluble complex formation of AD with FP determined spectrophotometrically in the aqueous phase

We have observed that under certain conditions (see Fig. 1), excess AD causes complete precipitation of FP. At pH 6.7–6.8, these precipitates settle when kept for several hours or a day at room temperature, leaving clear supernatant solutions which can be analysed spectrophotometrically for AD (absorption maximum at 342–344 nm). Under identical conditions, either free AD or FP remains almost completely soluble (see Materials and Methods). Therefore, insoluble complex formation of the two components AD and FP is inferred. Additional information on this complex is obtained by varying the initial AD concentration at a constant initial concentration of FP, and analysing the remaining AD in the supernatant solution. The absence of FP in this solution was checked in most cases by measurement of the absorbance ratio A_{342}/A_{400} which is about 6 for pure AD. Typical results are summarized in Fig. 1 where the mole ratio AD_{bound}/FP_{total} is plotted as a function of the mole ratio AD_{total}/FP_{total}. Experiments could not be extended beyond concentrations of AD of 8×10^{-4} M because of its low solubility under the given conditions of pH, ionic strength and temperature. However, a single experiment at 1.0×10^{-4} M FP and 8.0×10^{-4} M AD was added. These results clearly indicate a mole ratio of 1 between the complex components. Only at $AD_t/FP_t = 1$, the mole ratio AD_b/FP_t was significantly less than one, requiring considerably longer times or larger centrifugal forces to approach unity. Slow and incomplete complex formation is indicated in this case. In all other cases, the bound FP in the insoluble complex was taken to be essentially equal to the total FP because of the absence of soluble FP in the aqueous phase (see above). Even if relatively small fractions of free FP could precipitate under the given conditions, complex formation with AD appears to be considerably faster. When 6×10^{-4} M AD and 2×10^{-4} M FP were mixed at pH 6.9, and measured spectrophotometrically within about 1 min of preparation of the solution, a small absorption maximum of 580 nm was recorded before the onset of precipitation, indicating complex formation (see Ref. 11 where a peak at 600–610 nm was observed for a 1:1 AD–FP complex at pH 7.4). This peak at 580 nm appeared only in the presence of AD.

It may also be noted that almost the same amount of complex formation is estimated if the system was either left to settle for a day or centrifuged at 900 *g*, usually within a few hours of its preparation (Fig. 1). Therefore, the precipitate should not contain appreciable amounts of free FP sedimenting during centrifugation.

At pH values lower than about 6.5 and at mole ratios of $AD_t/FP_t = 3$, apparently less complex formation was observed. Thus at pH 6.2 and 6.0, the mole ratios AD_b/FP_t were 0.63 and 0.72, respectively, as determined after centrifugation. At pH 5.5, a ratio of 0.69 was obtained, adding initially the AD before the FP, since FP alone precipitated rapidly under these conditions. This is in contrast to pH 6.7–6.8. Adding the AD after precipitation of FP at pH 5.5 under continued stirring of the mixture, the AD_b/FP_t ratio was only 0.36. In all these cases of apparently decreased complex formation, the spectral ratio A_{342}/A_{400} indicated the absence of significant FP in the aqueous phase. Indeed, at pH 5.5 and in the absence of AD, rapid precipitation of 2×10^{-4} M FP occurred. Thus, a mixture of free and bound FP may be present in the solid phase under the above conditions of pH < 6.5 assuming a mole ratio of 1 to prevail also at the lower pH values.

Mössbauer measurements

^{57}Fe Mössbauer spectra obtained for FP and FP–AD at 90°K are shown in Fig. 2. The corresponding Mössbauer parameters, obtained by computer fits to the experimental spectra, are presented in Table 1. The spectra of the same samples measured at 4.1°K were very similar to those observed at 90°K. Each spectrum was analysed by computer fits with one symmetrical quadrupole doublet, though a small asymmetry can be distinguished in all doublets.

All isomer shifts and quadrupole splittings are characteristic for high-spin trivalent iron. As is evident from Table 1, the line width is appreciably broader in pure FP than in the FP–AD system. This broadening, as well as the small asymmetry of the spectra, may be due to slight relaxation effects or to a spread in quadrupole splittings and isomer shifts caused by non-equivalent surroundings of all iron atoms. The parameters obtained for the FP–AD system are very different from those obtained for pure FP at a similar pH. The line width is narrow and the quadrupole splitting is much smaller for FP–AD. This smaller quadrupole splitting shows that the electric field gradient acting on the iron nucleus is smaller and points to a more symmetric distribution of charges around the iron nucleus in FP–AD. The different parameters obtained for FP and FP–AD leave no doubt that AD interacts with FP and causes a drastic change in the charge distribution around the iron. The narrower line width obtained for the FP–AD complex may point to a more unique

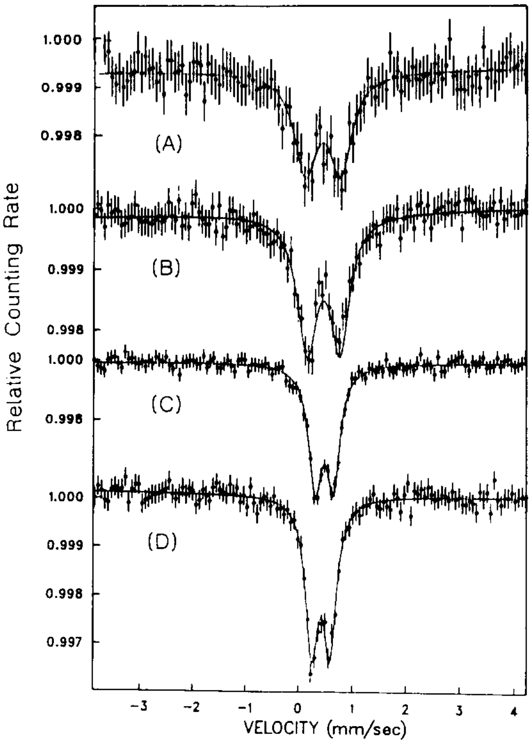

Fig. 2. ^{57}Fe Mössbauer spectra of FP and FP–AD measured at 90°K. (A) FP at pH 7.4; (B) FP at pH 8.5; (C) FP–AD at pH 6.8; (D) FP–AD at pH 8.2. FP, 4×10^{-3} M; AD, 8×10^{-3} M; sodium chloride, 0.05 M. All solutions were left up to 3 days at room temperature before being frozen in liquid nitrogen.

Table 1. Mössbauer parameters of FP and FP–AD at 90°K

System	pH	FWHM (mm/sec)	QS (mm/sec)	IS (mm/sec)
FP	7.4	0.50 (4)	0.66 (1)	0.42 (1)
FP	8.5	0.44 (3)	0.61 (1)	0.42 (1)
FP–AD	6.8	0.29 (1)	0.33 (1)	0.40 (1)
FP–AD	8.2	0.29 (1)	0.33 (1)	0.40 (1)

FWHM, full width at half maximum of each line of the doublets; QS, quadrupole splitting (eqQ/2), equal to the distance between the two lines composing the doublet; IS, isomer shift relative to iron metal at room temperature.

The numbers in parentheses are the errors in the last digit, as obtained from the computer fits.

Other conditions, as given for Fig. 2.

environment around all iron atoms as compared to pure FP at the pH values measured.

DISCUSSION

Previously, spectrophotometric evidence for complex formation between FP and AD was obtained in aqueous solution at pH 7.4 [11]. The present data based on the insolubility of the FP–AD complex and on spectrophotometric determination of unreacted AD confirm the interaction of FP with AD and indicate a mole ratio of unity in the complex under the conditions given. The Mössbauer data obtained at higher concentrations of both FP and AD independently demonstrate the interactions between the complex components.

Considering the rapid establishment of electrochemical equilibria and the reversibility of FP aggregation, the initially alkaline solutions of FP brought to acid pH should act in the same way as FP initially dissolved in a disaggregating medium such as DMSO [13]. Indeed, some relevant experiments included in Fig. 1 give results similar to those obtained with initially alkaline FP.

The stability of the FP–AD complex in a predominantly aqueous medium in a given pH range is considered to be due to the combined effect of different non-covalent interactions such as hydrophobic and electrostatic interactions, the latter involving charged nitrogenous groups on AD and FP carboxylates, as well as possible hydrogen bonding between the aromatic hydroxyl and amino groups and corresponding hydrogen-bonding groups on FP (Scheme 1). In addition, π–π electron interactions between proximate heterocyclic and aromatic moieties of the complex components could add to the stability of the complex. Below about ph 7, FP–AD complexes appear to be formed rapidly but separate slowly from the aqueous phase as shown above.

It has previously been stated that AD does not form a complex with FP in benzene as solvent [10]. This result was considered to present a serious objection to the hypothesis of antimalarial action involving complexes of FP with antimalarial drugs [8–10]. The effective *in vivo* environment of antimalarial drugs is likely to be closer to an aqueous rather than to a strictly non-polar medium such as benzene. Therefore, the present aqueous system may be considered to constitute a preferable model system, despite the relatively low ionic strength used. The complexes formed at lower concentrations may remain soluble for a sufficient period of time to be active even at pH 5.5. This complex formation may effectively inhibit the production of hemozoin by heme polymerase.

In conclusion, the presently confirmed complex formation of FP with AD could play a role in the antimalarial activity of AD and related drugs, although alternative mechanisms have to be considered. In any case, general deductions as to the mechanism of drug action should not be based on the observed absence of complex formation of FP with AD in benzene [8–10].

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