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The reaction of artemisinins with hemoglobin: A unified picture

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Abstract—The reactions with hemoglobin of artemisinin and of its parent compounds, sodium artesunate and dihydroartemisinin, were investigated by visible absorption spectroscopy under standard solution conditions (50 mM phosphate buffer, pH 7, 37 °C). Notably, these antimalarial drugs were found to react with hemoglobin (i.e., ferrous heme), but not with methemoglobin (i.e., ferric heme). The reaction selectively occurs at the heme sites and consists of the progressive, slow decay of the Soret band, as a consequence of heme alkylation and subsequent loss of π electron delocalization. For the various tested compounds the process reaches completion within ~30–70 h. Additional experiments were carried out upon adopting the solution conditions described by Meunier et al. and by Kannan et al. in their recent studies. Some reactivity of artemisinin with methemoglobin was indeed detected after addition of 50% v/v acetonitrile, most likely as a consequence of extensive protein unfolding. A unified description for the reactions of artemisinins with hemoglobin is given.

1. Introduction

Artemisinin and its derivatives are increasingly used in the treatment of multidrug-resistant *Plasmodium falciparum* malaria.[1–4] In fact, the association of artemisinin derivatives with conventional antimalarial agents represents today an effective treatment for malaria while minimizing the risk of developing drug resistance.[5,6]

The mechanism of action of artemisinin and of its analogues is still a matter of intense debate.[2] In agreement with an interpretation previously formulated by Posner,[7] Meunier et al.[8–10] have recently proposed that reductive homolysis of the endoperoxide function, promoted by iron(II) heme, and subsequent formation of C4 centered alkylating radicals, are crucial events for the antimalarial activity of these drugs. Moreover, the same research group demonstrated that heme may be alkylated by artemisinin in *meso* position; an extensive characterization of the resulting heme-artemisinin adducts was carried out.[8–10] On the ground of their results, Meunier et al.[11] suggested that heme is both the *trigger* and the *target* for artemisinin antimalarials. However, alternative mechanisms to explain the biological activity of artemisinins have been suggested as well. In particular, Eckstein-Ludwig and colleagues recently provided solid experimental evidence that PfATP6, a key metabolic enzyme of plasmodium, is potently inhibited by artemisinin and its derivatives; thus, they proposed a theory according to which artemisinins would produce their biological effects by mobilizing intracellular Ca^{2+} stores in analogy to the mechanism of thapsigargin.[12]

In any case, the reactivity of the endoperoxide function of artemisinins with heme iron is considered to be a key factor for the pharmacological activity of antimalarial endoperoxide drugs and, specifically, for their activation mechanism.[13–15] Based on the above arguments, various authors, during the last 15 years, suggested that the reaction of artemisinins with heme containing proteins, in particular hemoglobin, might be of particular biological and pharmacological interest.

We realized, however, that the scientific literature on this topic is quite fragmentary and, also, largely controversial. Already in the early 1990s Meshnick et al. had shown that artemisinin tightly associates to hemoglobin.[16] It was subsequently reported that artemisinin binds weakly to normal hemoglobin but, more strongly, to hemoglobin H.[17] Pandey et al.[18] observed that artemisinin disrupts the hemoglobin catabolism and the

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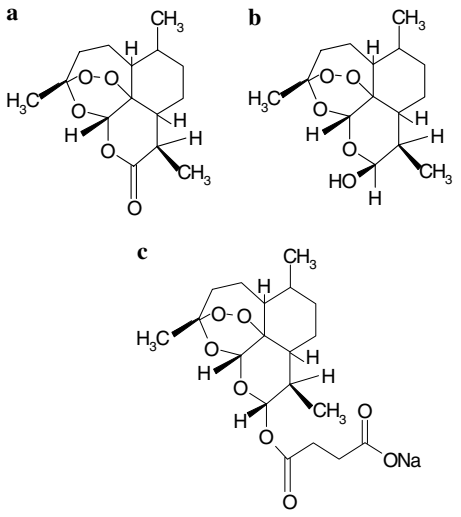


Scheme 1. Schematic drawing of artemisinin (a), dihydroartemisinin (b), and sodium artesunate (c).

heme detoxification systems in the malarial parasite. Among the recent contributions, Meunier and coworkers suggested that artemisinin reacts with either hemoglobin or myoglobin, only in their ferrous state, producing direct heme alkylation.[19] In contrast, Kannan et al.[20] reported that artemisinin reacts with methemoglobin—*but not with metmyoglobin*—causing characteristic changes of the visible absorption spectra and producing either mono- or dialkylation on the porphyrin ring. These latter results, however, were obtained in the presence of high amounts of organic solvents that are known to affect deeply the native solution structure of the protein.

To shed further light on these controversial issues, we have carried out a systematic spectroscopic investigation of the reaction of artemisinin and of its analogues, artesunate and dihydroartemisinin, with hemoglobin, under well-defined solution conditions. Also, in a few additional experiments, the solution conditions described by Meunier et al.[19] and by Kannan et al.,[20] respectively, in their recent works, were reproduced. For comparison purposes, the reactions of artemisinin with both ferric and ferrous protoporphyrin IX were, again, analyzed (Scheme 1).

2. Results

2.1. The reactions of artemisinin with iron(III) and iron(II) protoporphyrin IX

The reactions of artemisinins with either iron(III) protoporphyrin IX (hemin) or iron(II) protoporphyrin IX ('reduced' hemin) were investigated, in detail, in previous studies, under different experimental conditions.[20–24] Remarkably, artemisinins were reported to react both with iron(III) and iron(II) protoporphyrin IX, although with different kinetic profiles, giving rise to characteristic adducts.[21–25] For comparison purposes, we have repeated here, under well-defined and homogeneous solution conditions, the reactions of artemisinin with iron protoporphyrin IX, in both oxidation states. The solution conditions employed for these experiments were the following: 50:50 v/v DMSO/100 mM phosphate buffer, pH 7, 37 °C.

The obtained spectral patterns are shown in Figure 1. Notably, the reaction of artemisinin with iron(II) protoporphyrin IX leads to the progressive decrease and to eventual disappearance of the Soret band as a consequence of disruption of π electron delocalization on the tetrapyrrole ring. The profile of the reaction is, at least, biphasic. At mixing, the addition of artemisinin (given at 2:1 molar ratio) causes drastic decreases of the bands at 520 and 555 nm, a large decrease of the Soret band and the appearance of a new band around 480 nm. Afterward, the latter band and the Soret band, manifest a progressive decrease that is complete within the following 6 h.

At variance, the reaction of artemisinin with iron(III) protoporphyrin IX (*hemin*), performed under identical solution conditions, is characterized by less marked spectral changes; after 24 h, these changes consist of a modest red shift (from 400 to 410 nm) and of a significant decrease in intensity of the Soret band (about 15%). Notably, the latter reaction is very sensitive to the solution conditions. Indeed, complete disappearance of the Soret band is observed, within 24 h, if the reaction is carried out in pure DMSO (data not shown) in agreement with previous results from our laboratory.[24]

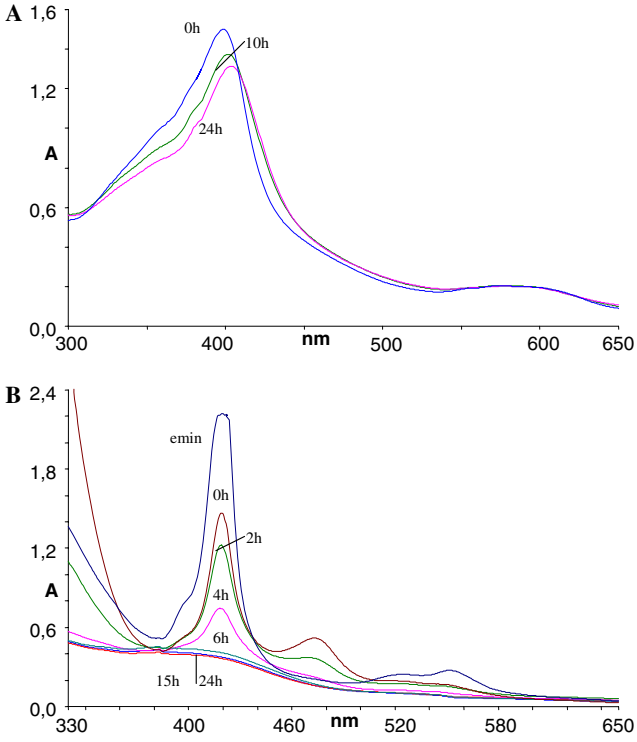
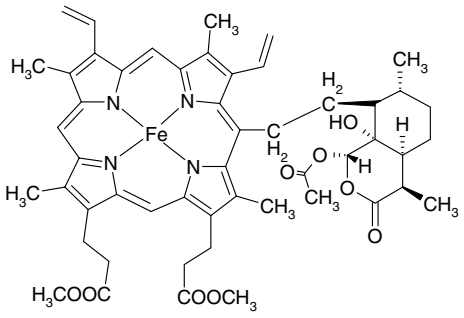


Figure 1. Time dependent spectrophotometric profiles, in the visible, of the reaction of artemisinin with iron(III) protoporphyrin IX (A) and iron(II) protoporphyrin IX (B). The reactions were monitored over 24 h. Solution conditions: iron(III) or iron(II) protoporphyrin concentration was 1×10^{-5} M; the artemisinin/iron ratio was 2:1; 50:50 v/v DMSO/100 mM phosphate buffer, pH 7, 37 °C.



Scheme 2. Schematic drawing of the hemin-artemisinin adduct.

To gain further insight into the mechanism of the above reactions, ESI-MS measurements were carried out on the final solutions, following the reported procedure.[24] In both cases, the ESI-MS spectra revealed the presence of an intense peak at 898 *m/z* and of a parent peak at 838 *m/z* that are diagnostic of the presence of a 1:1 iron protoporphyrin IX/artemisinin adduct according to Scheme 2.[24]

2.2. The reaction of artemisinins with hemoglobin in phosphate buffer, pH 7

The absorption spectrum of hemoglobin, in its ferrous state, is characterized by the Soret band, at 407 nm, plus two weaker bands at 540 and 576 nm (Fig. 2); at variance, the absorption spectrum of methemoglobin shows the Soret band at 400 nm and four broad bands at 500, 540, 580, and 630 nm (Fig. 3).

Stabilization of hemoglobin in its ferrous state was obtained by addition of a twofold stoichiometric amount of ascorbic acid. Then, hemoglobin was reacted, at 37 °C, with a 10:1 molar excess of artemisinin in 50 mM phosphate buffer, pH 7. The time dependent profile of the reaction is shown in Figure 2A: characteristic spectral changes are clearly detected.

Apparently, the reaction is at least biphasic. During the first period, covering ~2 h, a very slight blue shift of the Soret band is observed as well as a pronounced broadening of the small bands at 540 and 576 nm; afterwards, a progressive, continuous decrease in intensity of the Soret band takes place. After 20 h, the intensity of the Soret band is reduced to about 35% of its initial value. Similar profiles were obtained when hemoglobin was reacted with either artesunate or dihydroartemisinin (Figs. 2B and C). The residual intensities of the Soret band, after 20 h, were ~20% in the case of artesunate and ~18% in the case of dihydroartemisinin, respectively.

Upon extending the spectrophotometric monitoring of these reactions over larger time periods (up to 70 h), nearly complete disappearance of the Soret band was observed in all cases.

In contrast, the reaction of methemoglobin with the same amount of artemisinin, under identical solution conditions, did not produce any significant spectral change, over 20 h observation. Only a very marginal

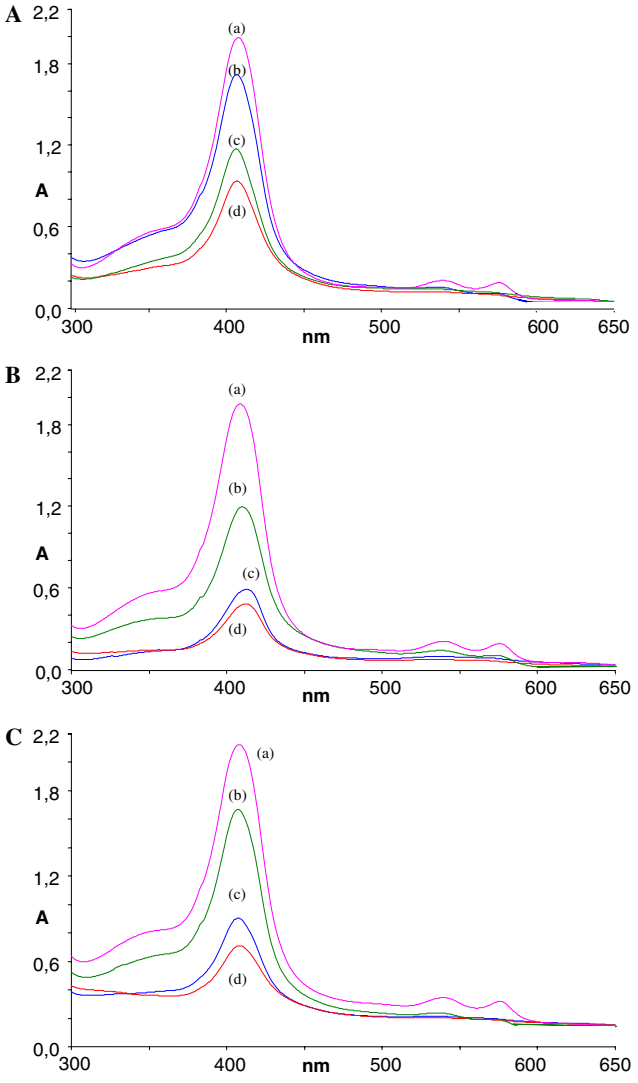


Figure 2. Time dependent spectrophotometric profiles of the reaction of hemoglobin in its ferrous state at 37 °C, with a 10:1 molar excess of artemisinin (A), artesunate (B), and dihydroartemisinin (C) in 50 mM phosphate buffer, pH 7, at *t*: 0 h (a); *t*: 3 h (b); *t*: 14 h (c); *t*: 20 h (d). Hemoglobin concentration is 1×10^{-5} M.

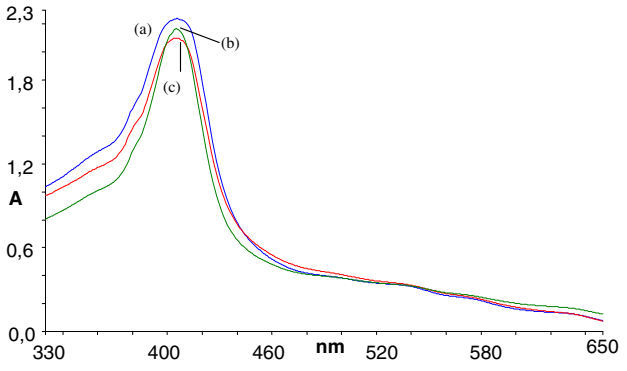


Figure 3. UV–visibile spectra of methemoglobin/artemisinin (1:10) in 50 mM phosphate buffer, pH 7, at *t*: 0 h (a); *t*: 10 h (b); *t*: 20 h (c). Protein concentration was 1×10^{-5} M.

decrease of the Soret band was noted after 20 h (see Fig. 3). Similar results were obtained in the case of sodium artesunate and dihydroartemisinin (data not shown).

2.3. The reaction of hemoglobin with artemisinin in ammonium acetate buffer, pH 8

Afterwards, the reaction of hemoglobin with artemisinin was investigated under the experimental conditions reported by Meunier and colleagues. in a recent paper.[19] Thus, a 1.2/1 artemisinin/heme ratio was used in these experiments, with 10 mM ammonium acetate, pH 8, as buffer. 4% v/v DMSO was added. Again, a spectral pattern very similar to that previously observed when working in the phosphate buffer, pH 7, was obtained with a clear biphasic behavior. In the first phase, a modest blue shift is noted accompanied by a dramatic broadening of the bands at 540 and 576 nm. Later on, the progressive decrease of the Soret band takes place. After 24 h, the intensity of the Soret band has reduced to only 25% of its initial value (Fig. 4A).

Then, we performed the reaction of methemoglobin with artemisinin, under the solution conditions described by Meunier.[19] As shown in Figure 4B, only a very modest decrease of the Soret band was detected over 20 h, in good agreement with Meunier's results.

In order to better understand the specific role of DMSO on the reaction between hemoglobin and artemisinin, additional experiments were carried out in the absence of DMSO. A marked reduction in the rate of the reaction was noted without significant modifications of the overall spectral profile. After 20 h, the observed decrease of the Soret band is only 20%.

2.4. The reaction of hemoglobin with artemisinin in the presence of 50% acetonitrile

Finally, the reaction of hemoglobin with artemisinin was carried out and analyzed under the experimental conditions reported by Kannan et al.[20] that is, in mixed 50:50 v/v acetonitrile/100 mM phosphate buffer, pH 7. First of all, it must be noticed that addition of 50% v/v acetonitrile induces characteristic changes in the absorption spectra of both hemoglobin and methemoglobin. It is well known that organic solvents, in such a high concentration, greatly perturb the solution structure of globular proteins. For instance, a detailed study had been previously reported on the effects of high concentrations of DMSO on the solution structure of hemoglobin.[26] Thus, we propose that the visible spectral changes of hemoglobin and methemoglobin, observed after addition of 50:50 v/v acetonitrile, are the direct consequence of the conformational changes that acetonitrile produces on the overall solution conformation of these proteins and, specifically, on the local structure and the electronic properties of the heme centers.

In the case of methemoglobin, the spectral changes consist of a modest shift of the Soret band and of some marked changes of the spectral features in the 480/650 nm region. On the other hand, addition of 50% acetonitrile greatly perturbs the characteristic visible bands of ferrous hemoglobin at 540 and 576 nm that are replaced by some broader features; these latter changes are indicative of the presence of a iron(III) heme. Thus, these observations strongly suggest that addition of high

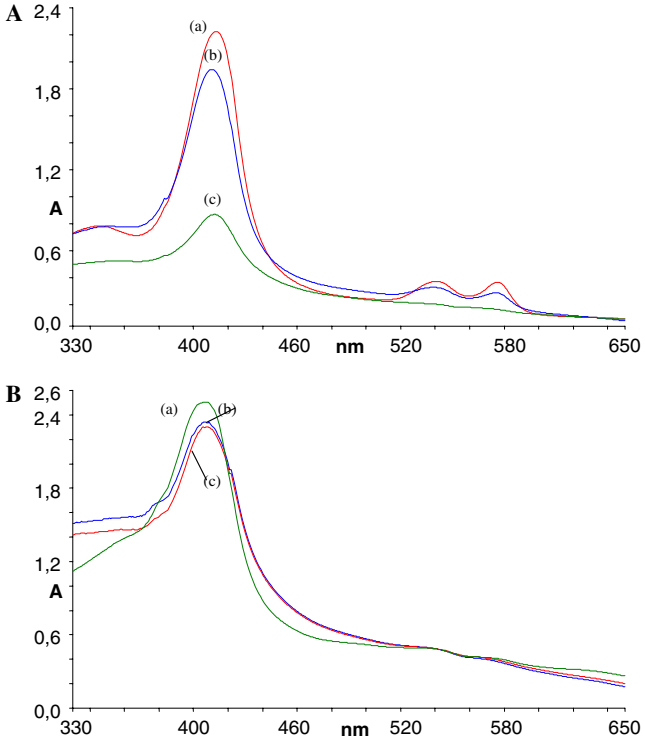


Figure 4. UV–visible spectra of (A) artemisinin/hemoglobin and (B) artemisinin/methemoglobin, 1.2/1 ratio, with 10 mM ammonium acetate buffer, pH 8, *t*: 0 h (a), *t*: 4 h (b), *t*: 20 h (c) (96% buffer, 4% DMSO).

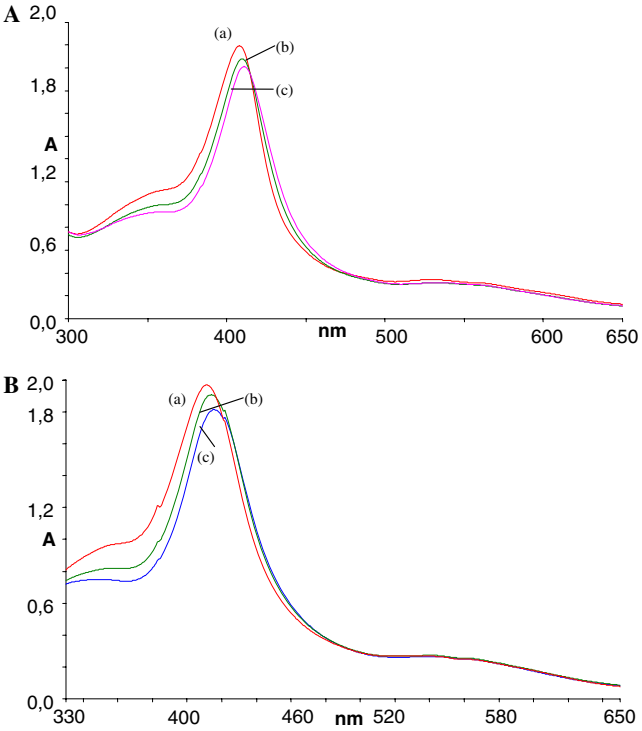


Figure 5. UV–visible spectra of the reactions of methemoglobin (A) and hemoglobin (B) with artemisinin (1:10), in 50:50 v/v acetonitrile/phosphate buffer, pH 7, 37 °C, at *t*: 0 h, *t*: 10 h, *t*: 20 h.

amounts of acetonitrile, under aerobic conditions, promotes quick oxidation of hemoglobin to methemoglobin. Remarkably, the final spectra of hemoglobin and methemoglobin, in the presence of 50% acetonitrile, are nearly identical and diagnostic of the presence of iron(III).

After analysis of the spectral effects produced by acetonitrile, either methemoglobin or hemoglobin, dissolved in the above mixed buffer, was reacted with artemisinin, at a 1:10 molar ratio. The corresponding spectral profiles, collected over 24 h, are shown in Figure 5. Notably both samples exhibit a similar reactivity consisting of a modest decrease of the Soret band. After 24 h, the decrease of the Soret band, in both cases, is only ~15%; this decrease is accompanied by a red shift of about 5 nm. In a way these spectral changes resemble very closely those obtained in the case of the reaction of hemin plus artemisinin in the DMSO/phosphate mixed buffer, as shown in Figure 1A.

3. Discussion

Surprisingly, only a few studies based on visible absorption spectroscopy have been carried out on the reactions of artemisinins with heme proteins in spite of the large current interest on this topic. This prompted us to reconsider, in a systematic manner, the reaction of artemisinin and its analogues with hemoglobin, either in the ferric or ferrous state, mainly relying on the application of visible absorption spectroscopy. Indeed, the latter technique represents an easy, direct and, sensitive method to monitor the iron sites of hemoproteins.

Particular attention was devoted to the standardization of experimental conditions. The solution conditions for this study were settled as follows. All reactions involving proteins were carried out in 50 mM phosphate buffer, at pH 7. Artemisinin drugs were added as concentrated ethanolic solutions, at ratios ranging from 10:1 to 1.2:1 (referred to the concentration of the iron centers). In the final samples, ethanol concentrations never exceeded 1%. Reactions were monitored continuously by absorption spectroscopy in the visible, over ca 24 h at 37 °C. A few additional experiments were carried out in which the solution conditions employed by Meunier et al. and by Kannan et al., in their recent studies were carefully reproduced.

The reactivity of the heme centers with artemisinin and its analogues was judged on the basis of the decrease of the Soret band, a condition that is achieved as a consequence of heme alkylation and subsequent disruption of the π electron delocalized system, as previously described.[24]

Some clear trends could be identified from the spectroscopic analysis of the reaction. Indeed, our data provide unambiguous evidence that artemisinin and its analogues, namely sodium artesunate and dihydroartemisinin, do react with hemoglobin in the ferrous state, giving rise to similar spectral patterns. The reaction, in all cases (i.e., with the three different artemisinin based drugs), leads to the progressive, slow decay and to eventual loss of the Soret band, as a consequence of direct alkylation of the porphyrin ring and of its subsequent degradation. Specific degradation of the porphpyrin ring by peroxides, alkylperoxides, and artemisinin had been previously observed[27–30] Notably, from our results, it emerges that all 4 heme groups bound to hemoglobin are attacked and degraded by artemisinins. In contrast, no decay of the Soret band was detected when artemisinins were reacted with methemoglobin implying a crucial role for the oxidation state of the iron center in governing this type of reactivity.

Similar results were obtained when the experiments were carried out within the similar (mild) solution conditions described by Meunier (i.e., ammonium acetate buffer, at pH 8, in the presence of DMSO 4%; artemisinin/iron molar ratio: 1.2/1).[19] Again, artemisinin was found to react with ferrous hemoglobin and to produce a spectral pattern rather similar to that, previously observed, in phosphate buffer. Conversely, no reaction was observed with methemoglobin. The role played by DMSO in the reaction was specifically addressed; it appears that DMSO, even at this relatively low concentration (4%), greatly accelerates the reaction, possibly by rendering the heme group more exposed to the alkylating attack.

In a final series of experiments, the more drastic solution conditions described by Kannan et al.[20] were adopted. We found that addition of 50% acetonitrile causes a large perturbation of the protein conformation that is reflected in marked changes of the visible spectrum. The structural modifications induced by acetonitrile greatly facilitate quick oxidation of ferrous heme. Notably, under the solution conditions reported by Kannan, in the presence of oxygen, ferrous hemoglobin is rapidly and completely converted into methemoglobin. The reaction of methemoglobin with artemisinin, under the latter conditions, results in a partial decrease of the Soret band over 24 h, accompanied by a modest red shift. These findings suggest that ferric heme, within the unfolded protein conformation induced by acetonitrile, is more prone to attack by artemisinin so that alkylation of the heme group may slowly occur.

The results obtained so far, under homogeneous solution conditions, allow us to propose the following unified picture for the reaction of artemisinins with hemoglobin. Artemisinin and its analogues, under physiological-like conditions, do react with ferrous hemoglobin *but not with methemoglobin*, giving further support to the idea that the iron(II) centers play a relevant role in the activation of artemisinin drugs. All four heme ferrous centers of native hemoglobin are targeted; eventual oxidative degradation of the iron porphyrin chromophore takes place with progressive loss and final disappearance of the Soret band. In contrast, methemoglobin has been shown to be relatively inert toward artemisinins; some reactivity is observed only after addition of substantial amounts of organic solvents. It is argued that the extensive protein unfolding prompted by addition of acetonitrile may render the iron(III) heme

center exposed to attack by the endoperoxide group allowing occurrence of a reaction analogous to that of hemin with artemisinin.

4. Experimental

4.1. Materials

Hemin was purchased from FLUKA Riedel-de Haën. Artemisinin was obtained from SIGMA Chemical Company. Artesunate and dihydroartemisinin were from Dafra Pharma N.V. Fresh ethanolic solutions of the three artemisinins were prepared, at 1×10^{-2} M concentration. Human hemoglobin and human methemoglobin were purchased from Sigma Aldrich. The concentration and the oxidation state of the hemoglobin samples were determined on the basis of their characteristic visible spectra.

4.2. UV–visible spectra

UV–visible absorption spectra were carried out with a Perkin-Elmer Lambda Bio 20 instrument equipped with a thermostatic cell. The kinetic experiments were conducted at 37 °C. Generally, spectra were recorded at 1 h intervals over 24 h. In the case of hemin and reduced hemin, the absorption spectra were recorded in 50:50 v/v DMSO/phosphate buffer ($c = 100$ mmol/l) pH 7. The standard condition for hemoglobin samples was 50 mM phosphate buffer, pH 7. In some cases, the absorption spectra were carried out in the following media: 50 mM buffer phosphate, pH 7, plus 4% DMSO ('Meunier's conditions'); 50:50 v/v acetonitrile/phosphate buffer, pH 7 ('Kannan's conditions').

4.3. ESI MS

MS spectra were registered in positive ion mode, on a HP 1100MSD API-electrospray instrument (Hewlett & Packard, Palo Alto, CA, USA). Capillary temperature was 220 °C, capillary voltage 3.0 V, source voltage 4.2 kV, tube lens voltage 30 V, and collision energy 35%.

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