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Ferryl–oxo heme intermediate in the antimalarial mode of action of artemisinin

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Abstract Fourier transform infrared (FTIR) and resonance Raman (RR) spectroscopies have been employed to investigate the reductive cleavage of the O–O bond of the endoperoxide moiety of the antimalarial drug artemisinin and its analog trioxane alcohol by hemin dimer. We have recorded FTIR spectra in the ν(O–O) and ν_{as}(Fe–O–Fe) regions of artemisinin and of the hemin dimer that show the cleavage of the endoperoxide and that of the hemin dimer, respectively. We observed similar results in the trioxane alcohol/hemin dimer reaction. The RR spectrum of the artemisinin/hemin dimer reaction displays a vibrational mode at 850 cm^{-1} that shifts to 818 cm^{-1} when the experiment is repeated with ^{18}O–O^{18} endoperoxide enriched trioxane alcohol. The frequency of this vibration and the magnitude of the ^{18}O–O^{18} isotopic shift led us to assign the 850 cm^{-1} mode to the $Fe^{IV}=O$ stretching vibration of a ferryl–xoxo heme intermediate that occurs in the artemisinin/hemin dimer and trioxane alcohol/hemin reactions. These results provide the first direct characterization of the antimalarial mode of action of artemisinin and its trioxane analog, and suggest that artemisinin appears to react with heme molecules that have been incorporated into hemozoin and subsequently the heme performs cytochrome P450-type chemistry.

Key words: Artemisinin; Ferryl–oxo; Antimalarial; Resonance Raman; Fourier transform infrared

1. Introduction

Artemisinin is a novel sesquiterpene lactone with an endoperoxide function essential in the chemotherapy of chloroquine-resistant strains of *Plasmodium falciparum* malaria [1–12]. The spread of *P. falciparrum* has initiated a tremendous interest in the mechanism of action, drug development, and biochemistry behind the antimalarial activity of artemisinin and other trioxane analogs [1–4,9–10]. Although the crystal structure of the malaria pigment or hemozoin which accumulates as a crystalline pellet in the cytosol of the erythrocytes has yet to be described, the current consensus based on spectroscopic data is that the pigment consists of a dense lattice of polymerized hemes [13,14]. The heme component of intact malaria pigment obtained from in vitro cultures of *P. falciparum*, has been shown by a variety of spectroscopic techniques to be similar to those of β-hematin and of hemin dimer [15]. Proposed reaction pathways for the endoperoxide activity of artemisinin are difficult to verify without knowing the structure of the intermediates involved in the reductive cleavage of the O–O bond of the endoperoxide by hemozoin.

In the experiments reported here, we have studied the artemisinin and trioxane alcohol/hemin dimer reactions by Fourier transform infrared (FTIR) and resonance Raman (RR) spectroscopies. The application of these vibrational techniques has allowed us to monitor the cleavage of both the endoperoxide and that of the hemin dimer and, to identify the heme iron–ligand stretching modes. We monitored the cleavage of both the O–O and Fe–O–Fe bonds by FTIR spectroscopy and identified the ferryl intermediate by its characteristic iron oxygen stretching frequency (850 cm^{-1} for heme $Fe^{IV}=O$) using RR. Moreover, we have studied the simplified analog of artemisinin, alcohol trioxane, and obtained identical results with those of the artemisinin/hemin dimer reaction. The use of this analog has allowed us to obtain further insight into the artemisinin/hemin dimer reaction; the interaction of this species with hemin dimer also serves as a benchmark for studies in which artemisinin analogues without preserving the lactone can have potent in vivo antimalarial activity. Finally, it allows us to use labeled ^{18}O-endoperoxide and thus identify the ferryl intermediate by its isotope shift. With the identification of the ferryl intermediate, the predominant structure of the final intermediate is known and the reductive cleavage of the O–O bond of the endoperoxide moiety of artemisinin, leading to the biologically relevant damage of the malarial parasite can be described with more certainty.

2. Materials and methods

Artemisinin and protoporphyrin IX were obtained from Aldrich and used as purchased. Trioxane alcohol was synthesized according to Posner [1] and the ^{18}O-labeled trioxane alcohol was synthesized by introducing $^{18}O_2$ during the photo-oxygenation step. The FTIR spectra were recorded from dry film samples at 4 cm^{-1} resolution with a Bruker Equinox 55 FTIR spectrometer equipped with a liquid nitrogen cooled mercury cadmium telluride detector. The final concentration of hemin dimer and artemisinin were 5 mM and 5.5 mM, respectively. All spectra were recorded at 25°C. The RR spectra were obtained from 80–100 μM samples, pH 8.9, in a cylindrical quartz spinning cell maintained at 3–5°C by a stream of cold nitrogen gas. The Raman spectra were acquired by using a SPEX 1877 triplemate with an EG and G (model 1530-CUV-1024S) CCD detector. A Coherent Innova K-90 krypton ion laser was used to provide the excitation wavelength of 413.1 nm. The power incident on the oxidase samples was typically 8–10 mW. Total accumulation time for each spectrum was 30 min. Optical absorption spectra were recorded before and after Raman measurements in order to assess sample stability with a Perkin-Elmer Lambda 20 UV–vis spectrophotometer.

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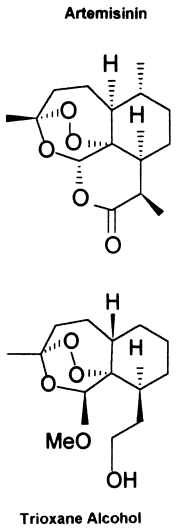

Fig. 1. Structures of artemisinin and trioxane alcohol.

3. Results and discussion

To monitor the endoperoxide moiety of artemisinin and that of the Fe–O–Fe antisymmetric mode of the hemin dimer, the FTIR spectra of the intermediates in the reaction of the drug with the hemin dimer were followed from 30 min to 3 h (Fig. 2). At the earliest times in the reaction the ν(Fe–O–Fe) mode of the hemin dimer located at 897 cm^{-1} (Fig. 2, IA), and the ν(O–O) mode of artemisinin observed at 883 cm^{-1} (Fig. 2, IIA) have lost intensity. As the reaction proceeds, however, both lines have diminished significantly, and show minimum intensity within 3 h. This indicates that the O–O cleavage is oxidizing the hemin dimer and subsequently iron heme–oxygen intermediates are produced. Additional information comes from the high frequency FTIR spectra of the reaction (Fig. 1, III). The lactone mode observed at 1740 cm^{-1} in the spectrum of artemisinin has lost most of its intensity in both the 30 and 180 min spectra of the reaction. This observation suggest that in addition to the endoperoxide moiety the lactone is involved in the reductive decomposition of artemisinin [16].

Establishing the sequence and temporal behavior of intermediates in the endoperoxide reductive cleavage is essential in understanding the linkage of these events with antimalarial activity. The inset of Fig. 3 shows difference spectra of the artemisinin (spectrum A) and alcohol trioxane (spectrum B) induced absorption changes of hemin dimer. Although the formation of the 428 nm peak/361 nm trough is much faster in the artemisinin/hemin dimer reaction, there is little difference between the artemisinin/hemin dimer and trioxane alcohol/hemin dimer spectra. This indicates that the newly formed hemin complex is independent of the structural difference between artemisinin and trioxane alcohol. Direct observation of iron/bound oxygen ligand vibrations is necessary to provide detailed information on iron heme–oxygen intermediates in the artemisinin reduction reaction.

The mid-frequency spectra (Fig. 3A) show the RR spectra of the hemin dimer. Spectrum 3B is that of the artemisinin/hemin dimer and is similar to the hemin dimer with the exception that a new mode appears at 850 cm^{-1}. The most reasonable assignment of the 850 cm^{-1} mode is that it arises from a heme $Fe^{IV}=O$ species. Such an assignment is in perfect agreement with Ó($Fe^{IV}=O$) frequencies observed in other five-coordinate heme $Fe^{IV}=O$ complexes (Table 1). Direct confirmation of this hypothesis requires detection of the $Fe^{IV}=O^{18}$ vibration. Spectrum 3C shows that the 850 cm^{-1} mode is downshifted to 818 cm^{-1} when the experiment is repeated with the ^{18}O–O^{18} enriched endoperoxide. The frequency of this mode and the 32 cm^{-1} isotope shift, which is in agreement with that predicted by a two-body FeO harmonic oscillator approximation, allows us to assign it as the heme

Table 1
Iron–oxygen stretching frequencies (cm^{-1}) of ferryl–oxo porphyrins

Model	ν($Fe^{IV}=O$)	Ref.
$Fe^{IV}=O$ (TPP)	852	[17,18]
$Fe^{IV}=O$ (OEP)	852	[17,18]
$Fe^{IV}=O$ (Protoheme)	850	this work
(NMI) $Fe^{IV}=O$ (Protoheme)	820	[19]
(NMI) $Fe^{IV}=O$ (TPP)	820	[20]
(NMI) $Fe^{IV}=O$ (OEP)	820	[20]

Abbreviations: TPP, tetraphenylporphyrin; OEP, octaethylporphyrin; NMI, 1-methylimidazole.

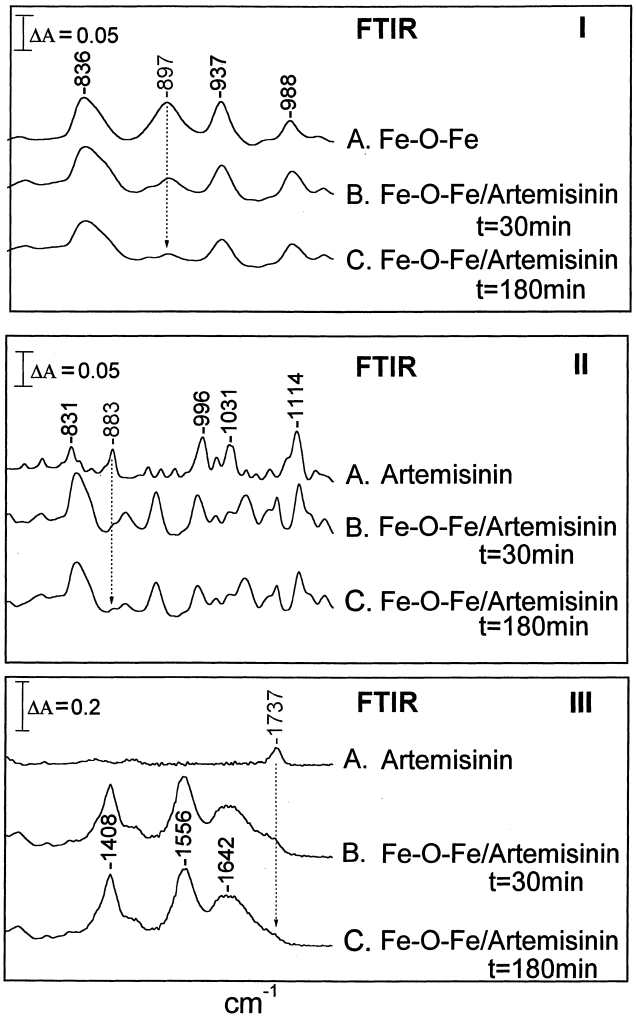

Fig. 2. FTIR spectra of the Fe–O–Fe hemin dimer (IA), artemisinin (IIA) and of artemisinin/hemin dimer reaction (IA, IC, IIB, IIC, IIIB, IIIC) at the indicated times. Total accumulation time for each spectrum was 15 min.

$Fe^{IV}=O$ stretching vibration. The difference spectra B−A and C−A are obtained by subtracting the Raman spectrum of the hemin dimer from that of the hemin dimer reacted with artemisinin and ^{18}O–O^{18} enriched trioxane alcohol, respectively. The fact that such spectra were obtained from several independent preparations makes the presence of the oxygen isotope sensitive-band reliable. The modes located at 850 cm^{-1}, 818 cm^{-1} and the 850 cm^{-1} peak/818 cm^{-1} trough pattern shown in the difference spectra B−C further support our assignment that the 850 cm^{-1} mode arises from a ferryl–oxo ($Fe^{IV}=O$).

A ferryl, non-heme intermediate was previously proposed to exist in the reductive decomposition of artemisinin [9,11]. Our data offer for the first time direct evidence for a heme–ferryl intermediate during ferric heme dimer activation of 1,2,4-trioxanes, and provide an isolated marker line which should be useful for kinetic studies. The characterization of the biologically active, toxic, ferryl intermediate, obtained by the cleaved endoperoxide of artemisinin, is the key step in understanding of the mode of action of artemisinin and its synthetic antimalarial analogs (Fig. 4). Ferryl intermediates are characteristic of monooxygenase metalloenzymes and known to cause oxidative damage to biological macromolecules. The direct observation of a ferryl intermediate supports the mechanism proposed by Posner [9], and has implications for understanding the action of current antimalarial drugs with hemozoin [21]. Confirmation of the ferryl intermediate is particularly important in view of its role in killing the parasite and in the formation of artemisinin–protein adducts that were previously detected when radiolabeled artemisinin was incubated in vitro with *P. falciparum* infected erythrocytes [5].

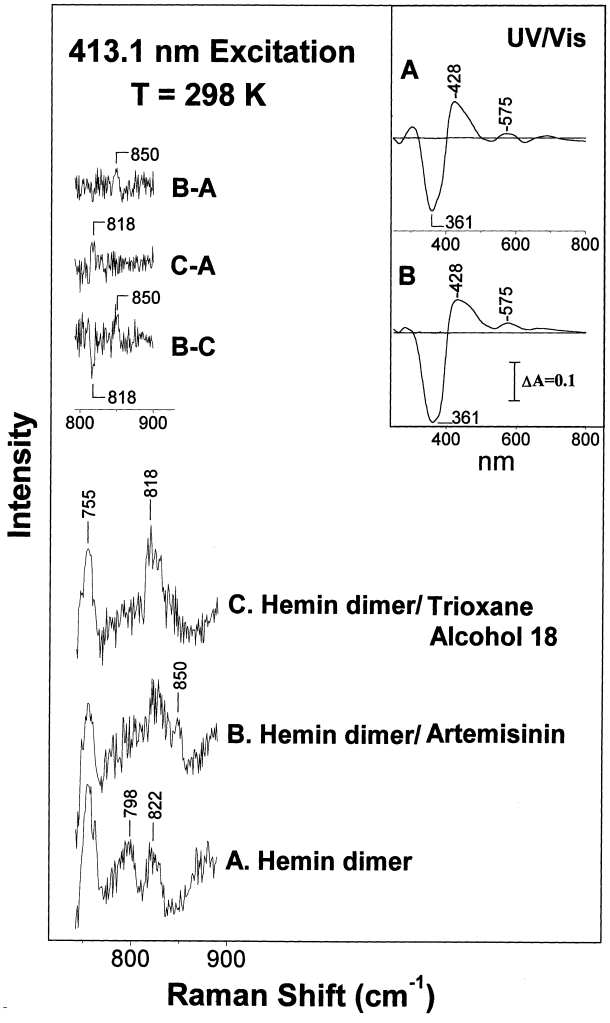

Fig. 3. RR spectra of (A) hemin dimer; (B) hemin dimer/artemisinin; (C) hemin dimer/trioxane alcohol 18 and their difference spectra B−C, C−A and B−A. The isotope scattering runs were repeated three times. Inset: (A) difference optical spectra of artemisinin and (B) trioxane alcohol hemin dimer reactions. The UV–vis spectra were recorded from samples containing 14.4 μM hemin dimer and 25 μM artemisinin. The final trioxane alcohol concentration in the UV–vis spectra was 50 μM.

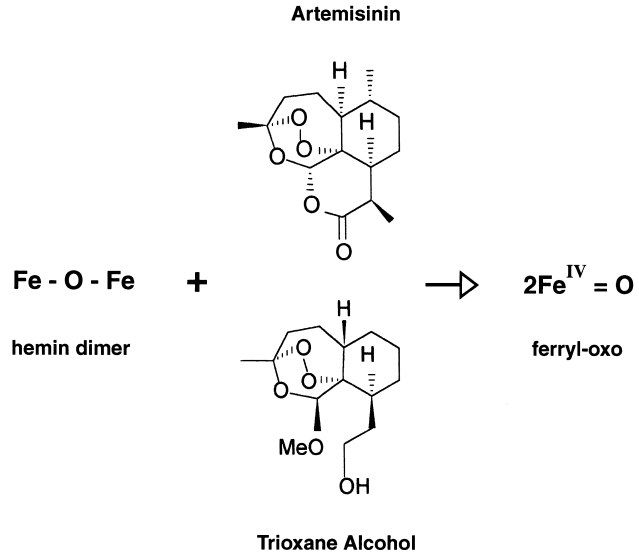

Fig. 4. The reaction of hemin dimer with artemisinin and trioxane alcohol.

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