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HEMIN-CATALYZED DECOMPOSITION OF ARTEMISININ (QINGHAOSU)

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Abstract—Artemisinin (qinghaosu) and its derivatives represent an important new class of antimalarial drugs. Previous work suggests that the antimalarial activity of artemisinin may be mediated by a reaction with intraparasitic hemin. Using cyclic voltammetry, artemisinin and dihydroartemisinin were irreversibly reduced at approximately −1 V. In the presence of concentrations of hemin as low as 50 nM, the reduction took place at much lower potentials (−0.435 to −0.460 V). Both reductions took place after adsorption onto the electrode surface. The shift of the reduction potential to more positive values is indicative of a catalytic process similar to that seen with hydrogen peroxide. The catalytic decomposition of artemisinin may play a role in the antimalarial activity of artemisinin.

Malaria infects 270 million people per year and causes 1–2 million deaths [1]. The incidence of malaria world-wide is increasing, in part because of widespread drug resistance. Thus, there is a great need for new antimalarial agents.

Artemisinin (Fig. 1A) is a sesquiterpene endoperoxide which is a potent antimalarial agent. This compound was isolated in 1971 from a Chinese herbal remedy which had been used in the treatment of fevers for over 2000 years. Recently, artemisinin and its derivatives, particularly artemether [the methyl ether of dihydroartemisinin (Fig. 1B)], have been widely used in Asia where over 2 million doses have been given in China alone [1]. Other artemisinin derivatives are currently undergoing Phase I and Phase II clinical testing [1].

A reaction with intraparasitic hemin may mediate the antimalarial activity of artemisinin. Malarial parasites contain large granules of precipitated hemin (known as hemozoin) because they digest host hemoglobin, probably without breaking down hemin [2]. *In vitro*, a reaction between hemin and artemisinin appears to generate organic free radicals, including some which appear to alkylate hemin [3]. When *Plasmodium falciparum*-infected red cells are exposed to radiolabeled drug, this hemin–artemisinin adduct can be isolated [3], suggesting that this same reaction occurs *in situ*. Further evidence for the critical role of hemin/hemozoin in the mechanism of action of artemisinin was obtained by Peters and coworkers [4] who demonstrated that artemisinin was >50 times less effective against a chloroquine-resistant *P. berghei* strain which lacks hemozoin.

In this study, we used cyclic voltammetry to further elucidate the mechanism of the interaction between hemin and artemisinin.

MATERIALS AND METHODS

Chemicals. Hemin (Sigma Chemical Co., St. Louis, MO) stock solutions were prepared by first dissolving the compound in a small volume of 0.25 M NaOH, diluting to a 5 mM concentration with phosphate-buffered saline (PBS) and to a pH between 7.5 and 8.0. This stock solution was prepared daily and stored at 4°. PBS (pH 7.24) was prepared by dissolving 11.5 g of Na_2HPO_4, 2.47 g of $NaH_2PO_4 \cdot 2H_2O$ and 9.0 g NaCl in a liter of deionized-distilled water. Artemisinin (Aldrich Chemical Co., Milwaukee, WI) and dihydroartemisinin (a gift of Dr. Hua-Zhen Pan, Peking Union Medical Center, Beijing, China) were prepared as 10 mM stock solutions in ethanol and stored at 4°. All inorganics were certified A.C.S. grade from the Fisher Scientific Co. (Springfield, NJ). Dehydrated ethanol was from the Quantum Chemical Co. (Tuscola, IL); 30% hydrogen peroxide (reagent grade) with stabilizer was from the Fisher Scientific Co.; and $Fe_2(SO_4)_3$ was from the Baker Chemical Co. (Phillipsburg, NJ). Deionized-distilled water was used to prepare all aqueous solutions.

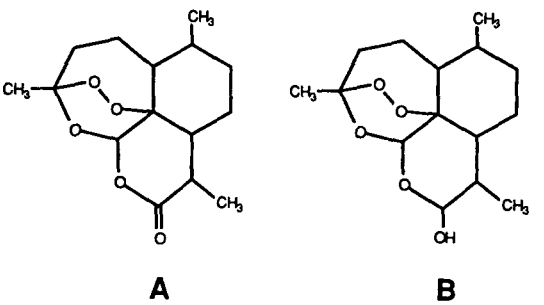

Fig. 1. Structures of artemisinin (A) and dihydroartemisinin (B).

Cyclic voltammetry. Measurements were performed with a BAS-100A electroanalytical analyzer (Bioanalytical Systems, Inc., West Lafayette, IN) at room temperature. The data were stored in a PC-

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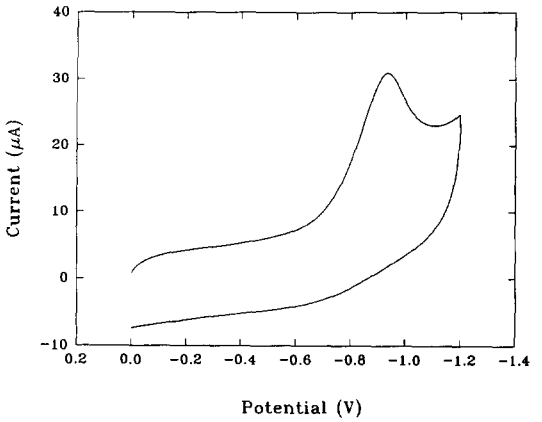

Fig. 2. Cyclic voltammogram of 1 mM artemisinin (scan rate = 0.3 V/sec).

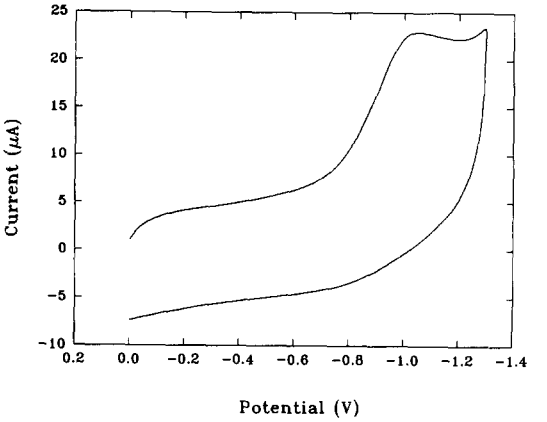

Fig. 3. Cyclic voltammogram of 1 mM dihydroartemisinin (scan rate = 0.3 V/sec).

XT compatible computer. All solutions were degassed with nitrogen during the experiments. The working electrode was a glassy carbon electrode, 3.0 mm in diameter, the reference electrode was Ag/AgCl with saturated KCl, and the auxiliary electrode was Pt wire. The carbon electrode was polished with alumina frequently during the experiments. All cyclic voltammograms were recorded with iR compensation (iR compensation corrects the distortions in cyclic voltammetry caused by solution resistance) [5]. All measurements were made in PBS containing 40% ethanol (v/v).

RESULTS

Reduction of artemisinin and dihydroartemisinin. Artemisinin (1 mM) shows a reduction peak at potentials from −0.873 to −1.054 V at scan rates from 0.03 to 5 V/sec (Fig. 2). No reverse (oxidation) peak was obtained, indicating an irreversible chemical reaction following the reduction that was fast compared to the time scale of the experiment. Dihydroartemisinin showed a similar irreversible reduction, but the peak potential was 50–100 mV more negative than artemisinin (Fig. 3). The cyclic voltammograms of both artemisinin and dihydroartemisinin with background subtraction showed that the reduction peak currents increased linearly with the scan rate, indicative of an electrochemical reduction of adsorbed chemical species on the working electrode surface (Fig. 4) [6]. For comparison, hydrogen peroxide (1 mM) showed a well-defined irreversible diffusion controlled reduction peak from −1.015 to −1.332 V at the same scan rate range as above (data not shown).

Reduction of hemin. Hemin (1 mM) showed a reversible reduction at peak potential from −0.380 to −0.390 V at scan rates from 0.03 to 5 V/sec (Fig. 5). The peak separation was 50 mV at a scan rate of 0.3 V/sec. The reduction and oxidation peaks occurred at nearly the same potential, indicative of reversible reduction of an adsorbed species. As with artemisinin, there was a linear relationship between

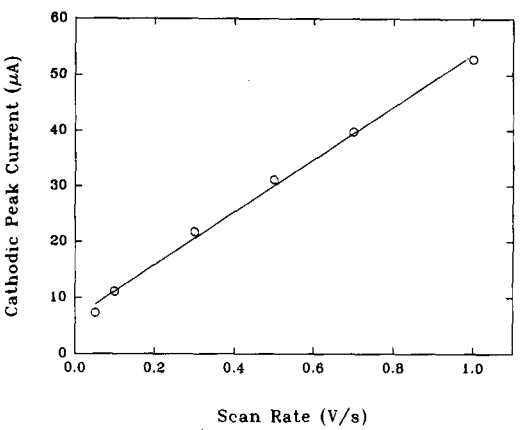

Fig. 4. Linear relationship between the cathodic peak currents and the scan rates in 1 mM artemisinin.

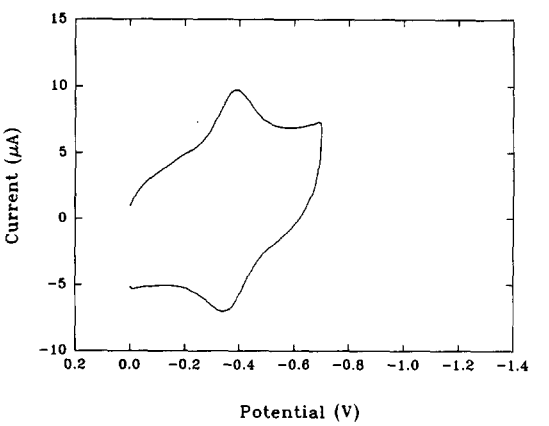

Fig. 5. Cyclic voltammogram of 0.1 mM hemin (scan rate = 0.3 V/sec).

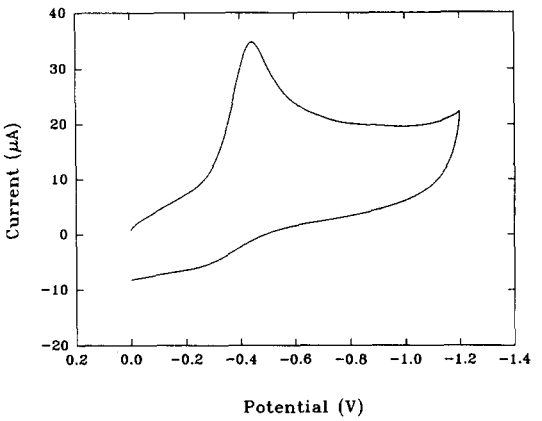

Fig. 6. Cyclic voltammogram of 1 mM artemisinin in the presence of 4.6×10^{-5} M hemin (scan rate = 0.3 V/sec).

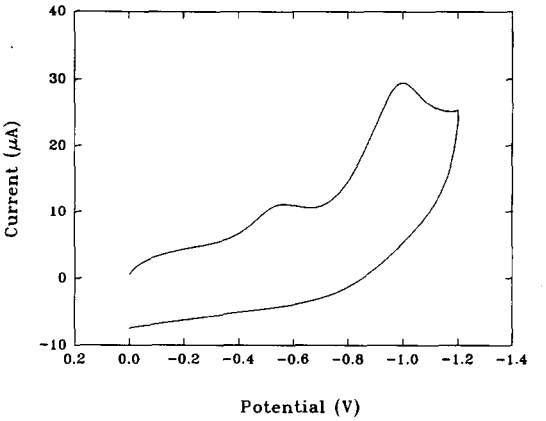

Fig. 7. Cyclic voltammogram of 1 mM artemisinin in the presence of 5×10^{-8} M hemin (scane rate = 0.3 V/sec).

the reduction peak currents and the scan rates (not shown), which is further evidence for the reduction of adsorbed species occurring on the electrode surface.

Catalysis of artemisinin and dihydroartemisinin reduction in the presence of hemin. The reduction peak of artemisinin (1 mM) at around −1.0 V completely disappeared in the presence of hemin at concentrations of 1×10^{-5} M or higher, and a new reduction peak appeared at −0.435 to −0.460 V close to the same peak current as the original peak (Fig. 6). These results suggest that the reduction of artemisinin is catalytic. When the concentration of hemin was between 5×10^{-8} and 1×10^{-5} M, the catalytic peak current was larger at slow scan rates (less than 0.3 V/sec) than at fast scan rates (faster than 0.3 V/sec) and the peak potential shifted to positive potentials with increasing concentrations of hemin. Apparently, at these low hemin concentrations, the rate of the catalysis is on the same order as the time scale of the experiment.

Because the reduction peak potentials of the adsorbed hemin and hemin-catalyzed artemisinin are separated by only 0.1 V, the observed peak results from a combination of the two. The contribution in the catalytic peak from the reduction of the adsorbed hemin could be identified in the cyclic voltammogram from the growing oxidation peak at −0.30 to −0.40 V when the concentration of hemin increased. When the concentration ratio of artemisinin to hemin was relatively large (>10), the interference from hemin was negligible.

The catalytic peak occurred at very low concentrations of hemin. The catalytic peak began to show clearly, especially at slow scan rates, when the concentration of hemin was just 5×10^{-8} M (Fig. 7). A 50 nM concentration is far below the detection limit of cyclic voltammetry, so the new peak cannot be from hemin itself. As the concentration of hemin increased, the catalytic peak current continued to increase (Fig. 8) and the peak potential shifted to the positive potential. The non-catalytic reduction peak of artemisinin decreased as the hemin

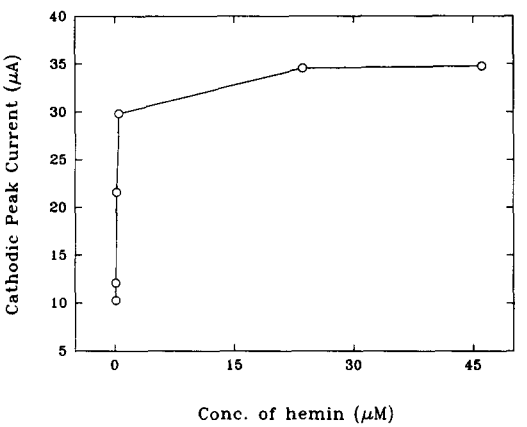

Fig. 8. Cathodic peak currents of 1 mM artemisinin versus different concentrations of hemin added.

concentration increased. The catalytic peak was maximum and the non-catalytic peak disappeared when hemin reached a concentration of 1×10^{-5} M (Fig. 8).

When the concentration of hemin remained at 1×10^{-4} M and the concentration of artemisinin increased from 1.2×10^{-4} M to 1.6×10^{-3} M, the catalytic peak current was linearly proportional to the concentration of artemisinin, providing further evidence that it is indeed the reduction of artemisinin that is catalyzed (data not shown).

Similar results were obtained for the reduction of dihydroartemisinin in the presence of hemin (Fig. 9). As for artemisinin, the catalytic peak current of dihydroartemisinin reached its maximum and the non-catalytic peak disappeared when the concentration of hemin was above 1×10^{-5} M (data not shown).

No catalysis of artemisinin by Fe^{3+}. The reduction of artemisinin (1 mM) was measured in the presence

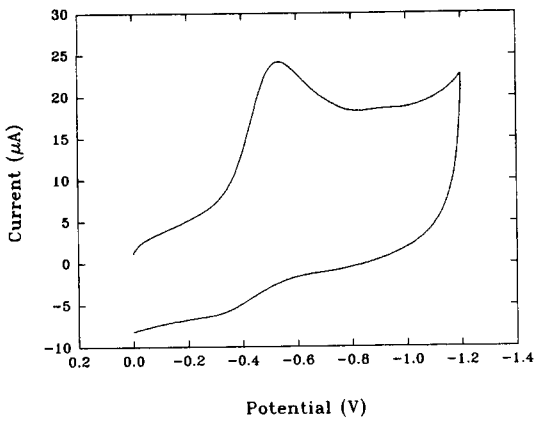

Fig. 9. Cyclic voltammogram of 1 mM dihydroartemisinin in the presence of 5×10^{-5} M hemin (scan rate = 0.3 V/sec).

and absence of ferric sulfate. There was no catalysis shown by concentrations of ferric sulfate ranging from 2×10^{-7} M to 2.2×10^{-4} M.

DISCUSSION

The antimalarial activity of artemisinin may be mediated by a reaction with intraparasitic hemin [3] with the subsequent formation of free radicals [7–9]. The mechanism of the reaction between artemisinin and hemin, however, needs to be elucidated. In this study, we obtained insight into how the reaction might be initiated, since we show that hemin can catalyze the reductive decomposition of artemisinin.

Iron compounds, such as hemin, have long been known to function as catalysts for hydrogen peroxide decomposition [10]. The hemin-catalyzed reductive decomposition of artemisinin and dihydroartemisinin is similar to that of hydrogen peroxide with respect to the appearance of a catalytic reduction peak and the disappearance of the non-catalytic peak [11, 12]. These data suggest that, like hydrogen peroxide, the reduction of artemisinin and dihydroartemisinin is accompanied by the cleavage of the oxygen–oxygen bond.

The mechanism of reduction appears to follow the following scheme:

$$\text{He-Fe(III) (bulk)} \xrightarrow{\text{diffusion}} \text{H-Fe(III) (adsorbed)} \quad (1)$$

$$\text{Q (bulk)} \xrightarrow{\text{diffusion}} \text{Q (adsorbed)} \quad (2)$$

$$\text{H-Fe(III) (ads)} + e^- \longrightarrow \text{H-Fe(II) (ads)} \quad (3)$$

$$n\ \text{H-Fe(II) (ads)} + \text{Q (ads)} + m\ H^+ + m\ e^- \longrightarrow Q_{red} + n\ \text{H-Fe(III) (ads)} + m/2\ H_2O \quad (4)$$

$$Q_{red} \longrightarrow \text{products} \quad (5)$$

where H-Fe(III) represents hemin, and Q represents artemisinin (qinghaosu).

The stoichiometry of reaction (4) has not been determined yet, although it has been shown previously that the hemin-catalyzed reduction of hydrogen peroxide occurs by a two-electron transfer [13].

The hemin-catalyzed reduction of artemisinin could be accounted for in two ways. First, hemin may cause a decrease in the activation energy for reduction. In this case, the increased rate of electron transfer to artemisinin will cause a positive shift in observed reduction peak potential. Second, the peak shift might occur as a consequence of the catalysis of a chemical reaction that follows reduction. This reaction could remove the reduced form of artemisinin and thereby increase the net rate of electron transfer to the drug. In either case, the fact that very small amounts of hemin (50 nM) catalyze the reduction of artemisinin argues against a stoichiometric reaction of hemin with artemisinin taking place on the time scale of the present experiments (seconds).

The endoperoxide moiety of artemisinin appears to be a critical part of the molecule with respect to both its interaction with hemin and its antimalarial activity. Hemin catalyzes the reduction of dihydroartemisinin and artemisinin in an almost identical manner, indicating that the structure of the lactone/lactol ring does not affect the ability of the drug to interact with hemin. Dihydroartemisinin, artemisinin, and many other analogs containing the endoperoxide bridge have comparable antimalarial activities [14, 15]. In contrast, derivatives of artemisinin which lack the endoperoxide bridge lack antimalarial activity [14, 15].

For the hemin-mediated reductive decomposition of artemisinin to occur *in situ*, there must be a physiologically relevant source of electrons. Electrons could potentially be donated to intraparasitic hemin by thiols [3] such as glutathione, which is present in malarial parasites in millimolar concentrations [16, 17]. Alternately, parasite hemin could catalyze the decomposition of artemisinin *in situ* by forming an oxene intermediate as has been shown to occur in reactions between iron(III) porphyrins and various peroxides [18, 19].

The hemin-catalyzed decomposition of artemisinin could lead to the formation of organic free radicals from artemisinin, which would explain the observation that an artemisinin–hemin adduct forms when malaria-infected red cells are treated with radiolabeled artemisinin [3]. In contrast, no such adduct forms when uninfected red cells are incubated with radiolabeled artemisinin [3], suggesting that the drug does not react with hemoglobin-bound hemin. This latter observation could explain why the drug is selectively toxic to the malaria parasite.

Hemozoin plays an important role in the mechanism of action of artemisinin since parasites lacking hemozoin are insensitive to the drug [4]. Hemozoin is composed largely of precipitated hemin [20]. Accordingly, the interaction between hemin and artemisinin observed here, which takes place on the surface of the electrode and not in solution, may be physiologically relevant. Further studies of the

interaction between hemin and artemisinin may aid in the design of new antimalarial agents.

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