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Deferoxamine: Stimulation of Hematin Polymerization and Antagonism of Its Inhibition by Chloroquine

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ABSTRACT. The iron chelator deferoxamine enhances the clearance of *Plasmodium falciparum* parasitemia and may be useful in drug combinations for the treatment of cerebral malaria. However, the deferoxamine–chloroquine drug combination is antagonistic, or at best additive, against *P. falciparum in vitro*. As chloroquine is thought to exert its antimalarial activity by interacting with hematin released from the proteolytic degradation of hemoglobin in the parasite food vacuole, we hypothesized that deferoxamine might interfere with the ability of chloroquine to inhibit hematin polymerization, since it was reported that deferoxamine interacts with hematin. Therefore, we assessed deferoxamine–hematin binding in more detail and investigated the effect of deferoxamine on hematin polymerization in the presence and absence of chloroquine. Isothermal titration calorimetry (ITC) experiments demonstrated an enthalpy-driven deferoxamine:hematin μ-oxo dimer binding with an association constant of 2.8×10^4 M^{-1} at pH 6.5, a binding affinity 14-fold lower than that measured for chloroquine. At least two of the three hydroxamic acid functional groups of deferoxamine must be unionized for effective binding. We also discovered that deferoxamine antagonized chloroquine-mediated inhibition of hematin polymerization. Unexpectedly, deferoxamine increased the concentration of soluble forms of hematin and enhanced the rate of hematin polymerization. Deferoxamine also could initiate hematin polymerization. In contrast, chloroquine decreased the concentration of soluble forms of hematin and inhibited hematin polymerization. This work supports the postulate that initiation of hematin polymerization requires a higher concentration of soluble hematin monomer than does the elongation phase of polymerization and provides one possible explanation for the observed antagonism between deferoxamine and chloroquine against parasites in culture. BIOCHEM PHARMACOL **58**;5:817–824, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. deferoxamine; hematin polymerization; chloroquine; malaria; association constant; hematin μ-oxo dimer

Deferoxamine enhances the clearance of *Plasmodium falciparum* parasitemia [1, 2], and may be useful in drug combinations for the treatment of cerebral malaria [3, 4]. However, a recent study [5] suggests that the addition of deferoxamine to standard treatment regimens may not necessarily benefit all patients with cerebral malaria. Moreover, the modest antimalarial potency [6] of deferoxamine is a significant drawback, and the deferoxamine–chloroquine drug combination is antagonistic [7], or at best additive [8, 9], against *P. falciparum in vitro*.

It is widely accepted that chloroquine exerts its antimalarial activity by interacting with hematin released from the proteolytic degradation of hemoglobin in the parasite food vacuole [10–13]. It is believed that free hematin, or the chloroquine–hematin complex, is toxic to the parasite and that the interaction of chloroquine with hematin prevents its incorporation into hemozoin. Hemozoin, or malaria pigment [14], is an insoluble polymer, very similar [15] if not identical to β-hematin [16, 17], formed from hematin [hydroxoiron(III)protoporphyrin IX] monomers.

This polymerization reaction can be reproduced *in vitro*, allowing more detailed evaluation of the process [18, 19]. We hypothesized that deferoxamine might interfere with the ability of chloroquine to inhibit hematin polymerization [18, 20], since it has been reported that deferoxamine binds to hematin [21] and protects erythrocytes from hematin-induced lysis [21, 22]. Therefore, we assessed deferoxamine-hematin binding in more detail and investigated the effect of deferoxamine on hematin polymerization in the presence and absence of chloroquine.

MATERIALS AND METHODS

Reagents

Deferoxamine mesylate, hemin chloride, protoporphyrin IX, and chloroquine sulfate were obtained from the Sigma Chemical Co.

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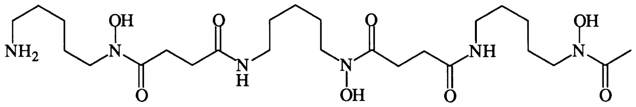
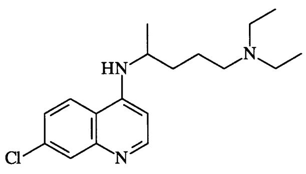


Deferoxamine



Chloroquine

STRUCTURE 1.

Hematin Polymerization

Hematin polymerization experiments were performed as described by Dorn *et al.* [19, 20] either with or without *P. falciparum* K1 trophozoite acetonitrile extract to initiate the reaction. Stock solutions of chloroquine and deferoxamine were prepared in 500 mM acetate buffer, pH 4.8, and added to the reaction mixtures. For the drug combination experiment, the dpm values (incorporation of [^{14}C]hematin into hemozoin) obtained from the assay were expressed as percent inhibition relative to hemozoin formation in a drug-free control. The values of triplicate assays were plotted semilogarithmically (CA-Cricket Graph III 1.5.2), and the IC_{50} values (μM) were calculated graphically ± SD by probit analysis.

Determination of Soluble Hematin Concentration at pH 4.8 in the Presence and Absence of Chloroquine and Deferoxamine

Saturated hematin solutions ($N = 6$) were prepared by adding 1 mL of a 1 mM stock hematin solution (0.01 N NaOH) to 9 mL of 500 mM acetate buffer, pH 4.8, and the resulting solutions/suspensions were sonicated for 2 min and allowed to stand for 10 min. Similar saturated hematin buffer solutions ($N = 6$) also were prepared in the presence of deferoxamine or chloroquine in concentrations ranging from 1 μM to 1 mM. Centrifugation of each sample for 20 min afforded the complete sedimentation of insoluble hematin material. Samples (2–3 mL) of the supernatant were removed and lyophilized to afford a dry hematin solid, which was dissolved in 0.01 N NaOH and quantified by reading the absorbance at 386 nm using a Shimadzu UV-1601PC UV-VIS spectrophotometer and converting absorbance to concentration by a standard curve. This new direct method of assessing saturating hematin solubility provides a similar value (1.2 μM) to that determined earlier (1.4 μM) via an indirect filtration method [13].

*ITC**

ITC experiments were carried out at 37° on an MCS titration calorimeter from Microcal Inc. [23]. Deferoxamine and hematin were dissolved in water because of an observed endothermic interaction between phosphate and acetate buffers and deferoxamine. An aliquot of a 5 mM stock solution of hematin in 0.01 N NaOH was diluted to the required concentrations (0.5 mM) in water, and the pH was adjusted to 6.5. This hematin solution was titrated with a 5 mM solution of deferoxamine using a 100-μL syringe rotating at 400 rpm. Titration experiments ($N = 4$) consisted of 15 injections of 6 μL each. Heats of dilution and mixing were obtained by 15 injections of deferoxamine into water, pH 6.5. After having first subtracted heats of dilution and mixing from each injection heat pulse, association constants (K_a values), enthalpy change (ΔH), and stoichiometry (n) were obtained by construction of binding isotherms by means of nonlinear least squares fitting of the titration data using Origin® software.

Removal of Hematin Iron by Deferoxamine

It has been reported previously [24] that deferoxamine can remove iron from hemozoin. To determine if deferoxamine could remove the iron atom of hematin, solutions of 5 mM deferoxamine and 0.5 mM hematin in 500 mM phosphate buffer, pH 6.9, and 100 μM deferoxamine and 20 μM hematin in 500 mM acetate buffer, pH 4.8, were prepared. The pH 6.9 experiment was conducted because this is the pH of the red cell cytosol [25]. The pH 4.8 experiment was done because this pH falls within the pH range (4.8 to 5.4) of the lysosomal food vacuole [25, 26]. Aliquots of these mixtures were spotted on analytical silica gel TLC plates previously spotted with deferoxamine, hematin, feroxamine, and protoporphyrin IX standards. Two independent developing solvent mixtures of chloroform:methanol:acetic acid were used: 8:1:1 and 9:0.5:0.5. As a confirmatory test, protoporphyrin IX was measured by fluorescence spectroscopy (excitation 395.6 nm, emission 791.6 nm) using a Shimadzu RF5000U spectrofluorophotometer.

FTIR Spectroscopy

As previously described [13], free hematin was separated from hemozoin by filtration and washing. After drying, FTIR spectra were measured with a Nicolet model Magna 550 FT-IR spectrometer (KBr beamsplitter and an MCT detector) and a Nic-plan IR microscope equipped with an attenuated total reflection objective made of ZnSe.

RESULTS

Deferoxamine–Hematin Binding

A representative deferoxamine–hematin titration curve and corresponding binding isotherm from a single ITC experiment are shown in Fig. 1. ITC titration experiments (Table 1) showed that deferoxamine–hematin binding was modest, with a K_a of 2.8×10^4 M^{-1} compared with a value of 40×10^4 M^{-1} for chloroquine. As for chloroquine, deferoxamine–hematin μ-oxo dimer binding was enthalpy-

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* *Abbreviations*: ITC, isothermal titration calorimetry; K_a, association constant; and FTIR, Fourier tranformed infrared.

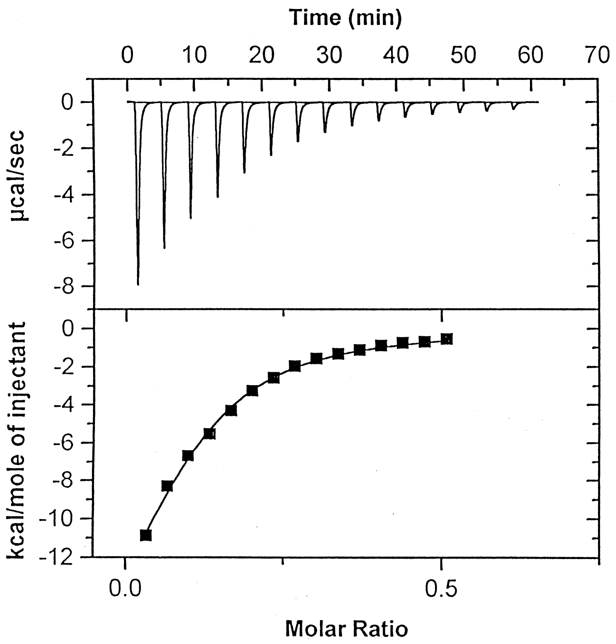

FIG. 1. Heats of binding and binding isotherm for the calorimetric titration of 0.5 mM hematin in water with 6-μL injections of 5 mM deferoxamine in water at pH 6.5 and 37°.

driven. Even though the enthalpy (ΔH) of drug-hematin binding for deferoxamine was substantially greater than that for chloroquine, the entropic penalty (TΔS) for this binding was more than 6-fold that of chloroquine, a result that probably derived from a substantial loss of conformational mobility for the very flexible deferoxamine molecule upon hematin μ-oxo dimer binding and from the increased order of the hydrophobic hydration shell of the deferoxamine–hematin μ-oxo dimer complex compared with free deferoxamine and free hematin μ-oxo dimer. Consequently, the free energy (ΔG) of binding for deferoxamine was substantially lower than that for chloroquine [13]. As hematin monomer [hydroxoiron(III)protoporphyrin IX] exists almost exclusively as its μ-oxo dimer in solution [27, 28], the stoichiometry data indicated formation of a 1:5 deferoxamine:hematin μ-oxo dimer complex.

Deferoxamine–Hematin Demetalation

Using both TLC and fluorescence spectroscopy, we determined that a 5- to 10-fold molar excess of deferoxamine did not remove the iron atom from hematin μ-oxo dimer to form feroxamine and protoporphyrin IX (data not shown) at either pH 4.8 or pH 6.9.

Stimulation of Hematin Polymerization by Deferoxamine

Before looking at the effect of deferoxamine on chloroquine-mediated inhibition of hematin polymerization, we examined the effect of deferoxamine alone on hematin polymerization. Unexpectedly, we found that deferoxamine stimulated rather than inhibited hematin polymerization. When added to an acetonitrile extract of *P. falciparum* trophozoite lysate, which is capable of initiating hematin polymerization, a 2.8-fold increase in the rate of hematin polymerization was evident at a deferoxamine concentration of 1 mM (Fig. 2a). This stimulation also occurred without the addition of the acetonitrile extract of the *P. falciparum* K1 trophozoite lysate, a condition under which hematin polymerization does not normally occur (Fig. 2b). This suggests that deferoxamine itself may serve as an initiator for hematin polymerization. The FTIR spectrum of the product β-hematin formed in both experiments was identical to a spectrum of authentic hemozoin (Fig. 3).

Deferoxamine Antagonism of Chloroquine-Mediated Inhibition of Hematin Polymerization

As we had predicted, deferoxamine antagonized chloroquine-mediated inhibition of hematin polymerization (Fig. 4) at concentrations as low as 100 μM. This concentration is only 10-fold greater than its IC_{50} range of 6–18 μM against *P. falciparum in vitro* [7–9, 29], and it could be postulated that higher concentrations of the order of 100 μM might be attained by deferoxamine in the food vacuole of the parasite where the interaction of chloroquine and hematin μ-oxo dimer presumably occurs [30–32].

Deferoxamine-Mediated Increase in Hematin Solubility at pH 4.8

In addition to the potential role of deferoxamine as an initiator of hematin polymerization, we wondered if deferoxamine enhancement of hematin polymerization was due to a deferoxamine-mediated increase in saturating hematin solubility at pH 4.8, since hematin is rather insoluble at pH < 6.5 [13]. Our data (Fig. 5, left panel) support this hypothesis, as deferoxamine at concentrations from 1 to 1000 μM mediated a 44–58% increase ($0.0001 < P <$

TABLE 1. Stoichiometry and thermodynamics of deferoxamine-hematin binding

Drug	n*	1/n	K_a (10^4 M^{-1})	ΔG (kcal/mol)	ΔH (kcal/mol)	TΔS (kcal/mol)
Deferoxamine†	0.10 ± 0.01	10	2.8 ± 0.1	−6.30 ± 0.11	−20.1 ± 2.0	−13.8 ± 2.1
Chloroquine‡	0.24 ± 0.03§	4.2	40 ± 17§	−7.87 ± 0.37	−10.1 ± 1.0	−2.23 ± 1.20

*Binding stoichiometry.

†Deferoxamine and hematin were dissolved in water, pH 6.5.

‡Chloroquine and hematin were dissolved in 250 mM phosphate buffer, pH 6.5. Data from Dorn *et al.* [13].

§In unpublished work, we found that the K_a and n for chloroquine-hematin μ-oxo dimer binding are independent of ionic strength.

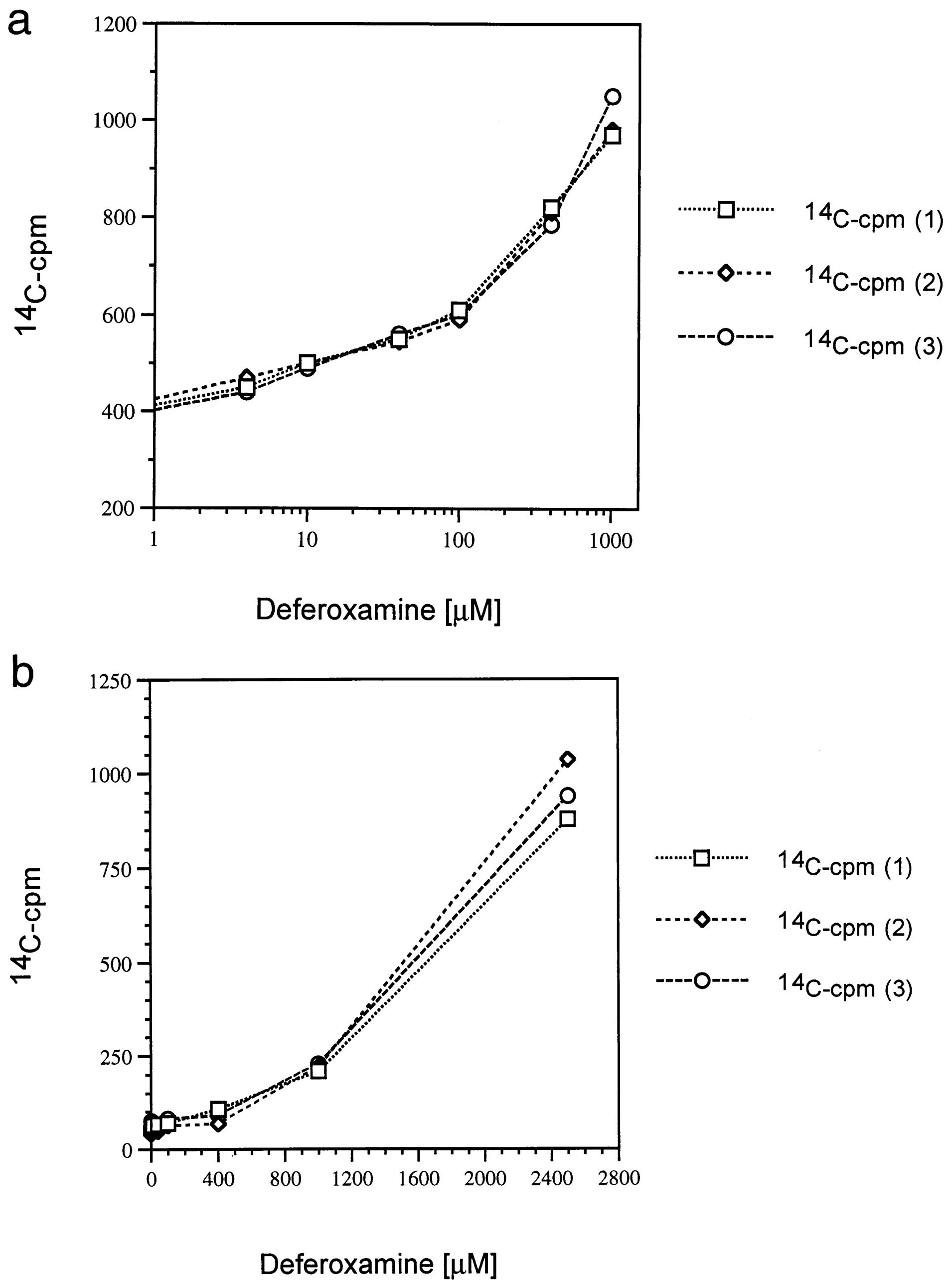

FIG. 2. Effect of deferoxamine on hematin polymerization activity in 500 mM acetate, pH 4.8, with (a) and without (b) acetonitrile extract of *P. falciparum* K1 trophozoite lysate. Reactions were allowed to proceed overnight, and values for three separate experiments are shown for each panel.

0.003) in saturating hematin solubility. However, this deferoxamine-dependent increase in hematin solubility was biphasic; hematin solubility was significantly lower at 100 μM compared with either 10 μM ($P = 0.002$) or 1000 μM ($P = 0.0003$) deferoxamine.

Chloroquine-Mediated Decrease in Hematin Solubility at pH 4.8

We had suggested earlier [13] that other properties of chloroquine–hematin μ-oxo dimer complexes beyond association constants alone may contribute to inhibition of hematin polymerization by chloroquine. One such parameter may be a chloroquine-mediated decrease in saturating hematin solubility, as illustrated in the right panel of Fig. 5. At 1 μM, chloroquine had no significant effect on hematin solubility. In contrast, 1 μM deferoxamine increased hematin solubility significantly. However, in the concentration range of 10–1000 μM, chloroquine significantly ($0.0001 < P < 0.015$) decreased hematin solubility. The maximum solubility decrease was produced with 10 μM chloroquine. At chloroquine concentrations of 100 μM ($P < 0.0001$) and 1000 μM ($P = 0.017$), the decline in hematin solubility was less pronounced than that seen with 10 μM chloroquine.

DISCUSSION

This work demonstrated that deferoxamine binds to hematin μ-oxo dimer and promotes hematin polymerization *in*

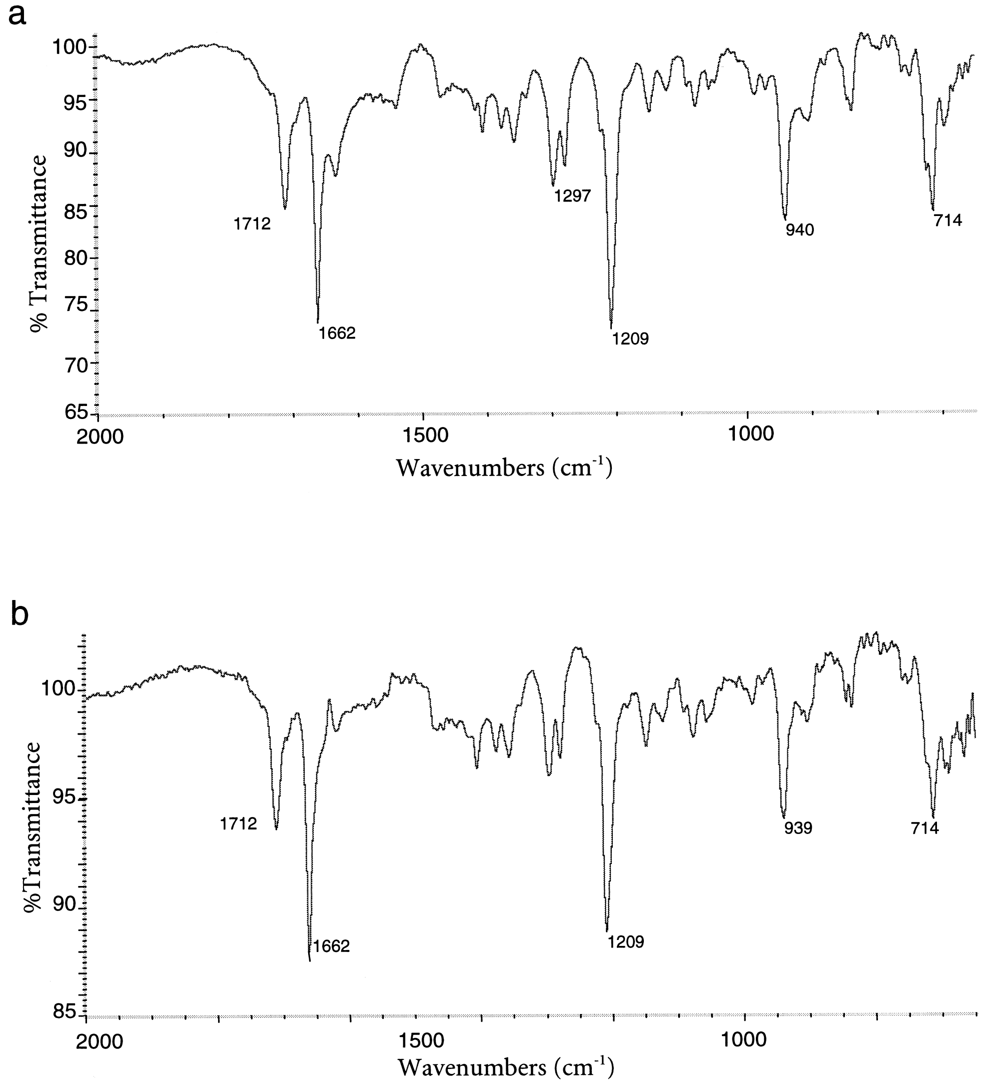

FIG. 3. FTIR spectra of β-hematin initiated by deferoxamine in 500 mM acetate, pH 4.8, with (a) and without (b) acetonitrile extract of *P. falciparum* K1 trophozoite lysate, N = 2.

vitro. It also antagonizes chloroquine-mediated inhibition of hematin polymerization *in vitro*. Unlike chloroquine, which reduces the solubility of hematin, deferoxamine enhanced the solubility of hematin. In this section we will concentrate first on the nature of deferoxamine binding to hematin and then discuss the relevance of this work to our understanding of hematin polymerization.

Deferoxamine–Hematin Binding

Although Baysal *et al.* [21] describe saturable deferoxamine-induced shifts in the Soret band of hematin solutions in the pH range of 6.0 to 8.4, these ITC data represent the first direct determination of a K_a for deferoxamine–hematin binding. These data confirm that significant deferoxamine–hematin μ-oxo dimer binding occurred at a pH (6.0 to 6.5) where none of the three hydroxamic acid functional groups are ionized [33]. Interestingly, Baysal *et al.* [21] observed no spectral shifts characteristic of deferoxamine–hematin binding at pH 9.3, a pH in the midrange of the three ionization constants (8.32, 9.16, 9.94) of the three hydroxamic acid functional groups of deferoxamine [33]. We can conclude that the hydroxamic acid functional groups play a key role in deferoxamine–hematin μ-oxo dimer binding, possibly by forming favorable hydrogen bonding, dipole–dipole, and ion–dipole interactions, and that at least two of the three hydroxamic acids must be unionized for effective binding. The absence of deferoxamine–hematin μ-oxo dimer binding at pH 9.3 [21] might derive from the electrostatic repulsion created when hematin μ-oxo dimer with its four negatively charged carboxylates, and deferoxamine with its two negatively charged hydroxamates, approach each other.

Deferoxamine hydroxamate–iron coordination [21] in the hematin μ-oxo dimer is unlikely to occur, as the two iron atoms in the hematin μ-oxo dimer are displaced some 0.5 Å out of the porphyrin plane toward the bridging oxygen atom [34]. We found that deferoxamine was unable

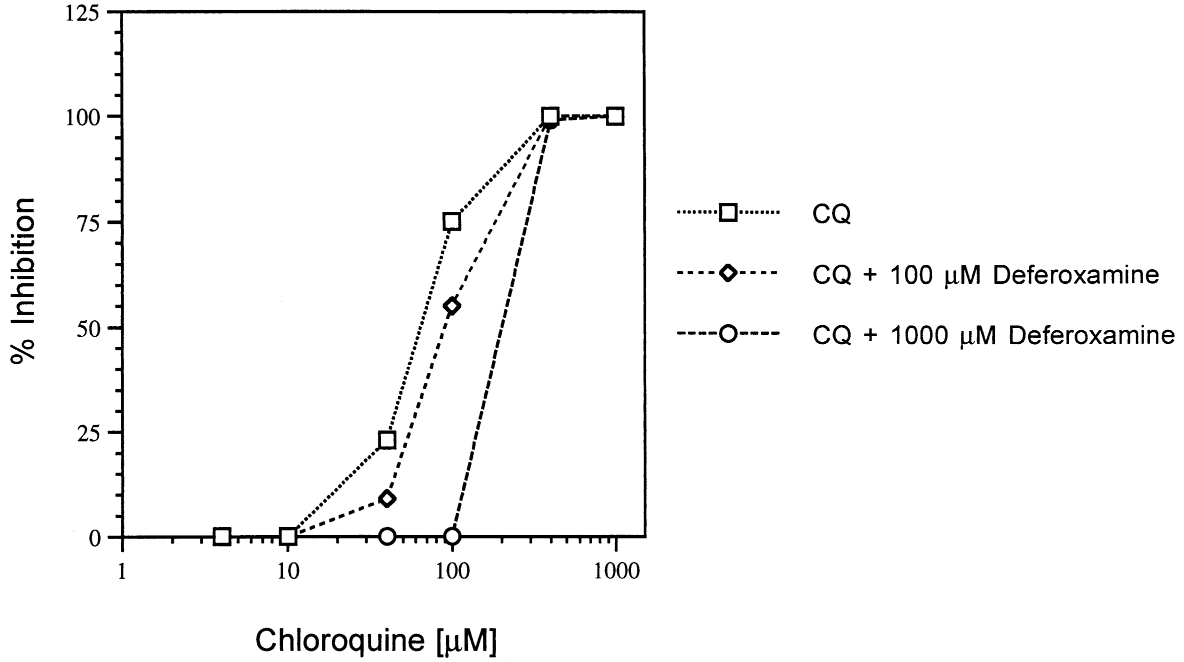

FIG. 4. Deferoxamine antagonism of chloroquine-mediated inhibition of hematin polymerization initiated by an acetonitrile extract of erythrocytes. Individual data points represent the means for three separate experiments; in each case, SD < 1%. Hematin polymerization control values correspond to 18 μM radiolabeled hematin.

to remove iron from hematin. This result was not unexpected, due to the strong iron coordination and the poor accessibility [34] of the iron atoms in the hematin μ-oxo dimer. However, it contrasts with the results of van Zyl *et al.* [24], who claimed that deferoxamine removes non-hematin iron (^{59}Fe) associated with radiolabeled hemozoin. This is a puzzling observation, as pure hemozoin [14, 15] should contain no non-hematin iron.

Effect of Deferoxamine on Hematin Polymerization

The precise antimalarial mode of action of deferoxamine and its derivatives is unknown. It has been suggested that the antimalarial action of deferoxamine is related directly to its ability to permeate into the parasite and deprive the parasite of iron required for its own biosynthesis, rather than chelation of extra- or intraerythrocytic iron [29, 35, 36]. If this is the case, the parasite target(s) for deferoxamine remains unidentified, but from our data it is unlikely to be hematin demetalation, inhibition of hematin polymerization, or initiation of hemozoin depolymerization [37]. Whatever its mechanism of action in the parasite, the ability of deferoxamine to enhance hematin polymerization in the test tube warrants further discussion. On the one hand, deferoxamine may serve as a structural template to initiate hematin polymerization. However, a second potential mechanism to account for both its initiation and enhancement of hematin polymerization may be an in-

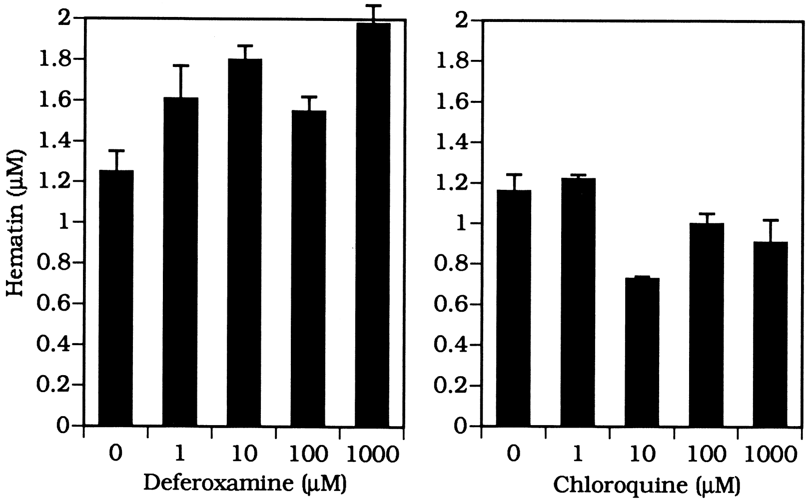

FIG. 5. Saturating hematin solubility in 500 mM acetate, pH 4.8, as a function of deferoxamine or chloroquine concentration. Bars represent the mean ± SD for six experiments.

creased concentration of soluble forms of hematin at low pH in the presence of deferoxamine. This may be particularly significant, as the rate, and most especially, the initiation [19] of hematin polymerization probably is controlled and limited in part by soluble hematin monomer concentration. In contrast, when chloroquine binds to the μ-oxo dimer, the hematin monomer–hematin μ-oxo dimer equilibrium [27] shifts to the right [13, 38] and the concentration of soluble forms of hematin, most notably hematin monomer, decreases.

This work suggests that factors affecting hematin solubility may play a role in perturbing and/or inhibiting hematin polymerization. Chloroquine decreases the concentration of soluble forms of hematin and inhibits hematin polymerization, whereas deferoxamine increases the concentration of soluble forms of hematin and enhances hematin polymerization. Our data provide one possible explanation for the observation that deferoxamine antagonizes the activity of chloroquine against parasites in culture [7], as micromolar levels could antagonize inhibition of hematin polymerization by chloroquine. However, this does not rule out a potential clinical value of combining deferoxamine with chloroquine. For example, proposed advantages of deferoxamine drug combinations such as an enhanced immune response [39], a protection of cell membranes from hematin-induced lysis [21, 22], or a removal of the excess systemic iron [40] liberated from parasite digestion all could be of value regardless of its effect on direct inhibition of parasite growth.

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