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Quinine and Chloroquine Differentially Perturb Heme Monomer–Dimer Equilibrium

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Abstract

Nuclear magnetic resonance (NMR) measurements of magnetic susceptibility have been utilized to study the equilibrium between two forms (high-spin monomer vs the antiferromagnetically coupled μ -oxo dimer) of ferriprotoporphyrin(IX) as a function of pH. The pH dependence of this equilibrium is significantly altered by the addition of either chloroquine or quinine. Chloroquine promotes the μ -oxo dimer whereas quinine promotes the monomer.

Introduction

Ferriprotoporphyrin IX (FPIX) is a toxic byproduct of the proteolytic degradation of hemoglobin that occurs within the malarial parasite digestive vacuole (DV). Because malaria parasites lack the heme oxygenase pathway, FPIX is detoxified by sequestration into nontoxic crystalline hemozoin¹ within the parasite DV. Antimalarial drugs such as chloroquine (CQ) and quinine (QN) are believed to inhibit the conversion of FPIX into crystalline hemozoin. Although the crystalline structure of hemozoin is known,¹ precisely how hemozoin is formed from FPIX, and precisely how CQ, QN, and other drugs inhibit this process, are still not fully understood. The release of heme upon digestion of hemoglobin by the malarial parasite presumably leads to aqueous soluble heme species. What forms of FPIX interact with antimalarial drugs and what forms lead to the crystallization of hemozoin are among the important questions that remain to be answered before a full understanding of how quinoline antimalarial drugs function can be reached. One of the main difficulties faced by early studies on heme model compounds is the rapid, irreversible oxidation of iron(II) to iron(III) by oxygen and formation of a μ -oxo dimer.² Thus, it is only reasonable to suggest that the μ -oxo dimer is likewise relevant to antimalarial studies. There are several other forms of FPIX that may exist in aqueous solution. For example, a similar water-soluble complex, iron(III) meso-tetrakis (4-*N*-methylpyridiniumyl)porphyrin can exist as a monomeric monoaqua five-coordinated complex, a monomeric diaqua six-coordinated complex, a monomeric monoaqua-hydroxo six-coordinated complex, a μ -oxo dimer, or a bis-hydroxo complex.³ The relative proportions of these species in an aqueous solution have been demonstrated to depend on pH. With a tetrasulfonated tetraphenylporphine (TPPS) system, an equilibrium between monomeric and dimeric species has likewise been established.⁴ In addition to the above, non-covalent dimers as well as larger aggregates of FPIX are also possible forms of FPIX in aqueous solution.

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Of all the possible forms of FPIX that can exist in an aqueous medium, the μ -oxo dimer stands out because of its unique magnetic susceptibility. The oxide bridge affords a strong antiferromagnetic coupling between the two high-spin ($S = 5/2$) ferric ions such that the observed effective magnetic moment per iron is closer to $1.7 \mu_B$ (the theoretical value for $S = 1/2$) than to $5.9 \mu_B$ (the theoretical value for $S = 5/2$). It should be noted that an antiferromagnetic coupling of similar magnitude has also been found in a hydroxo-bridged Fe(III) species, although this form has only been isolated from an organic solvent, CH_2Cl_2 .⁵

In relation to antimalarial drugs, Leed et al. have used nuclear magnetic resonance (NMR) spectroscopy to elucidate the structure of a CQ-FPIX μ -oxo dimer complex.⁶ These studies have derived distances between protons of noncovalently bound antimalarial drugs and the FPIX iron center from the relaxation rates of the protons of the drug. Leed et al.⁶ have also measured the effective magnetic moment of the ferric iron in FPIX using the Evans method.⁷ A magnetic moment, much lower than what would be expected from a high-spin species, was observed, therefore, an antiferromagnetic species (presumably the μ -oxo dimer) was assumed to be dominant in an aqueous solution at pH 6.5, both in the presence and absence of CQ.⁶ A recent article also supports the existence of FPIX mainly as dimers in aqueous solutions. However, in this recent article,⁸ FPIX is concluded to form only a noncovalent back-to-back (π - π) dimer and not a μ -oxo species. Obviously, a consideration of how FPIX behaves in aqueous solution is crucial in understanding how hemozoin is formed and how antimalarial drugs inhibit its crystallization.

In this article, we have reexamined the nature of FPIX in aqueous solutions. Magnetic susceptibility measurements by NMR spectroscopy at different pH values are performed to study in detail the equilibrium between monomer and dimer FPIX species. Because of the difficulty in dissolving heme at low pH, a 40% (v/v) DMSO/ H_2O solution is employed in these studies. Proton spectra are obtained to help determine the identity of the FPIX species that are present. These NMR measurements are repeated in the presence of the antimalarial drugs CQ and QN to determine what effects these drugs have on the speciation of FPIX in aqueous solutions.

Materials and Methods

Chloroquine (CQ) diphosphate, quinine (QN) hydrochloride dehydrate, (d_6)-dimethyl sulfoxide (DMSO), and hemin (FPIX) chloride (Fluka, Buchs, Switzerland) were purchased from Sigma-Aldrich (St. Louis, MO). The Fluka brand of Hemin chloride (guaranteed to be 98% pure) was chosen for this study. This is different from the Sigma-brand bovine hematin and bovine hemin, which according to recent work⁹ is less than 65% pure. Magnetic susceptibility measurements of the Sigma-brand bovine hematin also yielded higher magnetic moments per iron than those of the Fluka brand, suggesting that some of the impurities are paramagnetic. Deuterium oxide (D_2O) was purchased from Cambridge Isotope Laboratory (Andover, MA). Sodium phosphate monobasic and sodium bicarbonate were obtained from Fisher Chemicals (Fair Lawn, NJ). Sodium phosphate dibasic was purchased from EMD Chemicals (Gibbstown, NJ).

For samples in aqueous solutions, phosphate buffer was used. The total salt concentration is kept at 100 mM. The actual pH of the buffer was measured using a Beckman Φ 11 pH meter. For magnetic susceptibility measurements of heme in 40% (v/v) DMSO/ H_2O , hemin stock solutions (10 mM) were made in DMSO- d_6 , whereas CQ stock solutions were made in D_2O . Samples (40% v/v DMSO/ H_2O) were prepared by adding the required amount of heme and then diluting with 100 mM phosphate buffer to give a final FPIX concentration 4 mM. Samples contained in coaxial inserts for magnetic susceptibility measurements were prepared in the same way as the samples containing heme but with the heme solution replaced by pure DMSO-

d_6 . The pH of each sample was taken to be the pH of the aqueous buffer measured using a Beckman Φ 11 pH meter. 1D proton NMR spectra were recorded on a Varian Unity INOVA 500 MHz NMR spectrometer with a proton frequency of 499.789 MHz using Varian VNMR version 5.1 software. Spectra were recorded at 298 K. Chemical shifts are referenced to external tetramethylsilane (TMS). Magnetic susceptibility measurements were made using the Evans method,⁷ using the equation appropriate for a superconducting magnet;

$$\chi_M = \frac{-3\Delta\nu}{4\pi c} + \chi_D \quad (1)$$

where χ_M is the molar susceptibility of the paramagnetic substance in cm^3/mol , $\Delta\nu$ is the chemical shift difference (in ppm) between a reference proton in the sample and that in a solution lacking the paramagnetic compound, c is the concentration of FPIX in mol/mL, and χ_D is the diamagnetic susceptibility of heme (6.9×10^{-4} cgs units). The corrections for the susceptibility of the solvent and the difference in densities of the solvent and the solution are ignored. The water resonance frequency was used as the reporter for the chemical shift difference. Results using water are found to be identical to those obtained using DMSO or an additional reporter molecule such as *tert*-butyl alcohol. The molar susceptibility of FPIX is converted to magnetic moment using the following equation:

$$\mu = 2.8 \sqrt{\chi_M T} \quad (2)$$

Results and Discussion

Figure 1 presents the measured magnetic moment per iron in FPIX in 40% (v/v) DMSO/ H_2O solutions at various pH values. A limiting value of $5.6 \mu_B$ for the magnetic moment per iron is observed at low pH, whereas a value of $2.4 \mu_B$ is seen at high pH.

Proton NMR spectra at acidic (pH 3) and basic (pH 9) 95% (v/v) DMSO- d_6 solutions have been obtained to elucidate further what the above limiting values of the magnetic moment mean in terms of which FPIX species are present. These proton NMR spectra are shown in Figure 2. The use of a larger percentage of DMSO in these solutions allows for a better dynamic range of the NMR spectrum, which greatly improves signal-to-noise ratio. The lines also appear narrower in the presence of DMSO. A corresponding spectrum at pH 9 of an aqueous solution (no DMSO) is shown in Figure 3.

The peaks obtained from an aqueous solution are broader and less well resolved. However, the substantial resonance (at about 5–7 ppm) near the sharp solvent peak clearly points to an FPIX species that has much smaller contact shifts. On the basis of the proton NMR spectra observed, the substantially reduced magnetic moment corresponds to a solution that contains FPIX proton peaks with smaller contact shifts (less than 10 ppm). These magnetic properties are clearly associated with an antiferromagnetically coupled oxo-bridged dimer.¹⁰ The higher observed magnetic moment (at low pH), on the other hand, matches FPIX proton peaks (the $-\text{CH}_3$ substituents on porphyrin, for example) that exhibit much larger contact shifts (60–70 ppm from tetramethylsilane (TMS)). These highly deshielded resonances have been previously assigned to the methyl protons of high spin monomeric FPIX species.¹¹ Therefore, Figure 1 essentially displays the equilibrium relationship between the μ -oxo dimer of FPIX and its monomer as a function of pH. The inflection point of the curve in Figure 1 is at about pH 6.5.

It is not possible to construct a similar curve without adding DMSO to the solutions because the poor solubility of FPIX in water alone at low pH precludes NMR measurements. However, it should be noted that in the absence of DMSO, even at pH as low as 5 (the lowest pH we could reach without precipitation and harboring minimal observable [heme]), the measured magnetic moment is only about $3 \mu_B$ per iron, indicating that in strictly aqueous solutions, the

inflection point for the dimer–monomer equilibrium occurs at a much lower pH (<5). This is reasonable since the formation of the monomer proceeds with protonation of the oxo-bridge of the dimer, and in the presence of DMSO, protonation occurs more readily than when water is the only solvent. Hence, the transition from μ -oxo dimer to monomer is expected to occur at a higher pH in DMSO/water solutions. Another factor that determines the pH at which the transition occurs is the relative stability of the μ -oxo dimer, which can be modified by the sidechains on the porphyrin ring. It is expected that the μ -oxo dimer of FPIX would be more stable than that of a tetrasulfonated tetraphenylporphine⁴ because of the repulsive interactions between the negatively charged sulfonate groups. Thus, unlike tetrasulfonated tetraphenylporphine, the μ -oxo dimer of FPIX requires a lower pH before the monomer becomes the dominant species.

It is important to note that the present work can only discriminate between FPIX species that have different effective magnetic moments per iron. Because the μ -oxo dimer form of FPIX is the only species that provides antiferromagnetic coupling, this form can be safely assigned to the FPIX species that has a lower effective magnetic moment per iron. Although the term monomer is often used to refer to the FPIX species that has a higher effective magnetic moment per iron, it is still possible to have similarly high magnetic moments for an FPIX dimer that does not have the two iron(III) centers bridged by an oxygen atom. Thus, the species with a higher magnetic moment per iron could either be monomeric or a noncovalent dimer.

At low pH, only the high-spin species are present. On the other hand, the spectrum at high pH still contains resonances (although much reduced in intensity) in the 45–70 ppm region, indicating that even in basic solution, there is still a low amount of monomeric species. These observations are likewise in general agreement with those made recently by Gandini et al.³

The measured magnetic susceptibility is related to the rootmean-square of the magnetic moment (eq 2), which makes the averaging of the effective magnetic moment per iron favor the higher spin species. Therefore, the shape of Figure 1 does not translate directly to the shape of a graph that plots fraction of monomer or dimer at equilibrium against pH. Using the observed limiting values at low and high pH, and a squared averaging, the measured magnetic moment at a given pH value can be roughly converted to fraction of FPIX existing as monomer in solution. This is presented in Figure 4. Additional sets of points have been added in this plot, showing the results obtained by repeating the experiment with various amounts of CQ. Uncertainty in the measurements has also been included in Figure 4. The error bars are larger at lower pH, where the observed magnetic moment is higher. The higher magnetic moment leads to greater inhomogeneity, which causes broadening of the NMR lines and a larger uncertainty in specifying the center of these resonance lines. It can be deduced from these results that, at a given pH, addition of CQ shifts the monomer- μ -oxo dimer equilibrium toward dimer formation. The shift in equilibrium is clearly dependent on the concentration of CQ. On the basis of T_1 measurements of the CQ protons, CQ does not interact appreciably with the high magnetic moment form of heme at low pH. Although the CQ lines are broadened in a solution containing heme at pH 5 in 40% (v/v) DMSO, the measured T_1 's are still on the order of a second (similar in magnitude to the proton T_1 's of CQ in the absence of heme), indicating only weak paramagnetic relaxation. In contrast, CQs interaction with the μ -oxo dimer is definitely more evident. With the μ -oxo dimer, the T_1 's of the CQ protons are reduced to milliseconds.⁶ Thus, the preference of CQ for the dimer over the monomer is responsible for the observed change in the FPIX monomer–dimer equilibrium.

Addition of the related quinoline antimalarial QN to heme solution leads to something entirely different. The observed magnetic moment per iron for FPIX dissolved in 40% (v/v) DMSO at pH values of 7 and 10, in the presence of an equimolar amount of QN, are both $5.3 \mu_B$. Because the observed magnetic moment is already high at acidic pH even in the absence of QN, it is

assumed that QN would no longer have an effect on the observed magnetic moment of heme at pH lower than 6. The magnetic moment observed in the presence of QN is very close to the upper limiting value of $5.6 \mu_B$. Unlike CQ, the effect of QN on the magnetic moment seems to result from the formation of a stoichiometric adduct, suggesting that the complex QN forms with FPIX is not in fast exchange with either free FPIX or free QN. With observation of this relatively high magnetic moment, it can be concluded that QN promotes a high-spin monomeric form of FPIX even under basic conditions. The proton spectra of these solutions (data not shown) do not provide additional peaks other than those from monomeric FPIX and quinine. From the NMR spectra, there is no direct evidence that quinine is forming a complex with monomeric heme although the proton resonances of quinine have been significantly broadened. Nevertheless, this newly discovered difference between how CQ and QN affect the equilibrium between FPIX monomers and dimers has significant implications on how these two related drugs function as antimalarial agents. This difference between CQ and QN was not observed in the previous study by Leed et al.⁶ because the samples employed in that work always had the drug in large excess. The lowest QN/FPIX ratio used by Leed et al.⁶ was 10:1, thus, the proton NMR spectrum of quinine would be dominated by excess QN molecules, which are then expected to interact in a similar fashion as CQ with heme, that is, forming noncovalent $\pi-\pi$ complexes.

Both CQ and QN are known to inhibit hemozoin crystallization.¹² Stabilization of the FPIX monomer by QN could be a possible mechanism for preventing the aggregation and crystallization of hemozoin. If this is correct, then how CQ achieves the same by promoting μ -oxo FPIX dimer formation remains a puzzle. One reasonable explanation is that the main precursor of hemozoin is the proposed noncovalent back-to-back dimer.⁸ As mentioned earlier, the magnetic susceptibility measurements can only distinguish between the μ -oxo species and non-oxo-bridged species (noncovalent dimers and monomers). The recent work of de Villiers et al.⁸ showing that even at low pH, diffusion measurements, as well as observation of a broad and weakened Soret band, point to FPIX still existing mainly as dimers (noncovalent, weak or no antiferromagnetic coupling, high-spin) even in acidic solution. With a noncovalent FPIX dimer as a possible precursor for hemozoin formation, one can explain the differences that have been revealed by this study with regard to how CQ and QN affect the FPIX monomer–dimer equilibrium. QN accomplishes hemozoin inhibition by promoting the monomeric heme species. CQ, on the other hand, steers away heme from forming noncovalent dimers by promoting the covalently linked μ -oxo dimer.

However, it is still possible that the μ -oxo dimer is the precursor of hemozoin because this work shows that in aqueous solution, even at pH < 5, the μ -oxo dimer is still the dominant form. Hence, it is likely that it is the μ -oxo dimer form that initially aggregates and precipitates inside the acidic DV of the parasite. Formation of the Fe–O41 head-to-tail dimer from the μ -oxo dimer is postulated to require a substantial energy of activation to break the μ -oxo bridge.⁸ The unfavorable kinetics of this reaction could be relieved by a low dielectric and hydrophobic environment that lipids provide, possibly explaining why hemozoin formation is observed to be catalyzed by lipids.¹³ In this scenario, we could suggest that CQ inhibits hemozoin formation by preventing the μ -oxo dimer from entering lipids. Future work will address these remaining questions.

Conclusions

The magnetic susceptibility measurements presented in this article provide the first detailed dependence of the FPIX monomer–dimer equilibrium on pH. This article reiterates the relevance of the μ -oxo dimer in the study of aqueous solutions of FPIX. The opposite effects that CQ and QN exert on this equilibrium suggest that the mechanism by which CQ interacts with FPIX is quite distinct from that of QN. CQ promotes μ -oxo dimer formation, whereas QN

favors the monomer. One important implication is that pH and other ionic perturbations that have been measured for the DV of CQ – resistant parasites^{14,15} can now be understood to confer resistance to CQ and QN in different ways. For example, even though lower pH would be predicted to promote heme monomer over dimer (Figure 1), in the presence of CQ, lower DV pH for CQ resistant parasites¹⁴ will actually exacerbate CQ's effect on promoting dimer by concentrating more CQ within the DV (Figure 4). However, this is not relevant for QN, in part because of different weak base partitioning (quinolyl N pK_a differ for CQ and QN such that under physiologic conditions CQ is effectively diprotic, whereas QN is monoprotic) and in part because QN stabilizes monomer over dimer. Although it is still not known whether monomeric or μ -oxo dimeric heme is more relevant for formation of hemozoin, it is becoming clear that steps in the CQ versus QN resistance mechanisms that involve heme are distinct.

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Figure 1. Magnetic moment per iron vs pH for 4 mM hemin chloride dissolved in 40% (v/v) DMSO- d_6 /D $_2$ O.

Figure 2. Proton NMR spectra of FPIX in 95% (v/v) DMSO-*d*₆/D₂O at pH 3 (top), and at pH 9 (bottom).

Figure 3.
Proton NMR spectrum of aqueous FPIX at pH 9.

Figure 4. Fraction of FPIX as monomer (based on measured magnetic moment per iron) vs pH: (■) no CQ, (□) 100 μ M CQ, (Δ) 500 μ M CQ, ($\times$) 1 mM CQ, ($\diamond$) 2 mM CQ, and ($\blacklozenge$) 10 mM CQ.