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On the kinetics of formation of hemozoin, the malaria pigment

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ABSTRACT

We report on the kinetics of formation of hemozoin as a function of hemin concentration and reaction medium. Evidence is presented for the critical role played by interfacial regions in the efficient conversion of hemin to the malaria pigment.

1. Introduction

Despite years of intensive study malaria remains one of mankind's most dreaded scourges with annual infections and deaths counted in the hundreds of millions [1]. A critical factor in the expression of the disease is a pathway by which the *Plasmodium* parasite protects itself from the presence of extra-protein heme. As the parasite feeds on the protein portion of hemoglobin, large amounts of toxic heme are released. The heme is rapidly oxidized to hemin (iron in the +3 oxidation state) which, in a series of steps, is converted to and stored as an insoluble, non-toxic, largely unreactive assembly called *hemozoin* ("malaria pigment") [2,3]. It is generally believed that quinine and similar drugs owe their efficacy to interference with hemozoin formation [4], but some considerable uncertainty remains as to the molecular details of this inhibition [5].

Although studied for nearly three centuries, the composition and structure of hemozoin were elucidated only within the last twenty years [6,7]; and the mechanism of its formation remains an open question. The basic unit of the assembly is a *reciprocal dimer* in which a propionate group of one hemin occupies an axial position on the iron of a neighboring hemin, and *vice versa* [6]. The iron(III) centers have been shown to be high-spin [8]. Whereas hemin is known to aggregate extensively in aqueous solution, the "normal" dimer involves either stacking interactions or the formation of a μ-oxo linkage between iron centers [9–11]. Some controversy exists as to which of these forms is prevalent under the conditions of interest here (pH ~ 5, *vide infra*); recent evidence indicates that the dimer structure is highly dependent on pH and solvent composition [9,10,12,13]. What is unequivocal is that under these pH conditions, hemin is sparingly soluble in aqueous media and precipitates as *α-hematin* aggregates. This material is readily converted back to a soluble form by modifying the pH or by treatment with any of a number of reagents including DMSO, bicarbonate or pyridine. In contrast, the insoluble aggregate formed by *Plasmodium* based on a reciprocal dimer is resistant to attack by most reagents (including those listed above) and to changes in pH over an extended range. This so-called *β-hematin* aggregate or hemozoin involves hydrogen bonding between adjacent dimer units for which the non-liganded propionate groupings play a key role [7]. It is in this form that *Plasmodium* stores released heme [14].

The formation of hemozoin occurs in a food vacuole produced by the parasite for protease "digestion" of hemoglobin [15]. The optimum pH for this protein degradation activity – the pH at which hemozoin is formed – is about five, and the half-life for the conversion of free hemin into hemozoin has been estimated as in the order of minutes [16]. To simulate *in vivo* conditions, most laboratory studies on the kinetics of hemozoin formation are carried out in the presence of acetate or citrate buffer to maintain the physiologically relevant pH. In one such protocol [17], hemin in DMSO was added to an aqueous solution containing acetate and a water-soluble alcohol. After 16 h the reaction mixture was tested for the presence of hemozoin. A correlation was found between the yield of the malaria pigment and the lipophilicity of the dissolved alcohol.

A second protocol to study hemozoin formation, developed by Egan and coworkers [4,18], uses a closed system reactor in which a large amount of hemin (75 mg) is placed in about 25 mL of 4.5 M

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acetate buffer and maintained at 60 °C (the reaction is extremely slow at physiological temperature). Under these conditions hemin rapidly aggregates and precipitates in the α-hematin form. Samples are withdrawn at intervals over the course of many hours and are cooled on ice, dried for 48 h and eventually studied *via* infrared spectroscopy to determine the extent of conversion to β-hematin. This protocol has been critiqued by its originators who pointed out the non-correspondence of the conditions (especially temperature and method of hemin delivery) to those found *in vivo* [16]. In still another protocol developed by this group a small sample of a hemin stock in aqueous acetone is injected at the interface of a citrate containing aqueous/organic mixture [16]. Using this approach, efficient conversion to hemozoin was observed in the presence of several lipids and two alcohols (1-pentanol and 1-octanol).

We describe below an alternative, complementary method for studying the kinetics of hemozoin formation, which we believe has several advantages; it is more flexible than previous protocols and lends itself to systematic kinetic studies. We compare the results obtained using this new protocol to those already reported, and discuss factors that appear vital for rapid, efficient conversion of hemin into hemozoin.

2. Experimental

2.1. Materials and methods

Fluka and Frontier Scientific hemin were used for these experiments. Chloroquine (ChlQ) diphosphate was a MP Biomedicals product, and L-α-Dioleoylphosphatidyl-ethanolamine (DOPE) (>99% purity) was supplied by Avanti Polar Lipids, Inc. (Alabama). Other more standard chemicals were obtained from Fisher Scientific and Aldrich Chemicals and met ACS standards for high purity.

Reaction vials (8 mL) fitted with teflon caps were used as reaction vessels. These were maintained at a constant temperature using an Isotemp 202 S bath supplied by Fisher Scientific. The vortex devices employed were a Thermolyne MaxiMix II and a Scientific Industry Vortex-2 Genie. Centrifugations were performed on a Sorvall Instruments RC5C fitted with a SS-34 head. Spectroscopic measurements were conducted on a JASCO V-560 spectrophotometer.

2.2. Protocol

A new protocol used for studying the kinetics of hemozoin formation involves several steps:

(i) Prepare a concentrated solution (~10–20 mM) of hemin in DMSO (Stock A). Hemin is very soluble in this solvent and exists as a monomer [19]. To keep the DMSO concentration below 0.5% in the final reaction mixture, it proves convenient to prepare Stock B in aqueous 1 mM phosphate buffer (final pH ~ 10) by a dilution of Stock A by 20 fold. The concentration of Stock B is determined spectrophotometrically using a sample diluted by a factor of one hundred with a 0.1 M NaOH solution. The molar absorptivity of hemin in this medium has been reported as $\varepsilon = 5.84 \times 10^4\ M^{-1}\ cm^{-1}$ [20]. Finally, a large volume (~20 mL) of a hemin reaction solution (Stock C) in 1 mM phosphate buffer (pH ~ 10) is prepared from Stock B such that hemin in the reaction vials will have the desired *final* concentration (50 μM for most of these experiments). Stock C, which is monophasic, is equilibrated at 37° in a constant temperature bath. When diluted by a factor of one-third (*vide infra*), DMSO is less than 0.5% by volume in the reaction mixture.

(ii) Another stock solution (Stock D) is prepared which typically contains 9 mL methanol, 1 mL of co-solvent, and 2.5 mL of 1 M acetate buffer, pH ~ 5. For lipid experiments, a weighed quantity of the lipid is added to this stock. This mixture is monophasic for all but one of the co-solvents we have tested and report on below (heptane). In the heptane case, the organic mixture and acetate solutions were added separately to the reaction vials. In the standard protocol, 250 μL of Stock D is added to each of a selected number of screw-top reaction vials (usually 15–20) that are then equilibrated to 37 °C.

(iii) To initiate the reaction, 750 μL of the thermally equilibrated hemin (Stock C) is added to each vial. The reaction mixture is inverted twice and immediately returned to the constant temperature bath. In an earlier study XRD, FTIR and Resonance Raman spectroscopies were used to show that hemin produces hemozoin in such media [16,21].
Under these conditions, depending on the co-solvent (or presence of a lipid), the hemin either rapidly precipitates (as α-hematin) and/or is selectively taken up in the co-solvent that has formed a separate phase upon the addition of the aqueous hemin stock. The co-solvent represents about 2% of the total reaction volume, and the recorded pH* of the reaction mixture was 5.3.

(iv) At selected times, reaction vials are removed from the bath and plunged into ice. As quickly as possible, 5 mL of a solvent system containing 50% acetone/aqueous pyridine (5%) buffered with 26 mM HEPES is added. The resulting mixture is vortexed for 60 s to ensure that the pyridine has encountered all the hemin regardless of its state. At this point, all hemin that is not part of a hemozoin assembly is converted to soluble hemin $(py)_2$. This assay solution was shown – through application of X-ray diffraction and infrared spectroscopy – not to react with hemozoin but to readily dissolve hemin and α-hematin and convert them to the low-spin dipyridine hemin complex [21].

(v) The mixture is centrifuged for 30 min at 5000 rpm to separate the insoluble hemozoin which settles to the bottom of the vial. The supernatant is then tested spectrophotometrically (λ_{max} at 406 nm) for the presence of hemin as the dipyridine adduct. The data is plotted (Figs. 1–5) as the percentage of hemin converted to hemozoin (% conversion) *vs.* time.

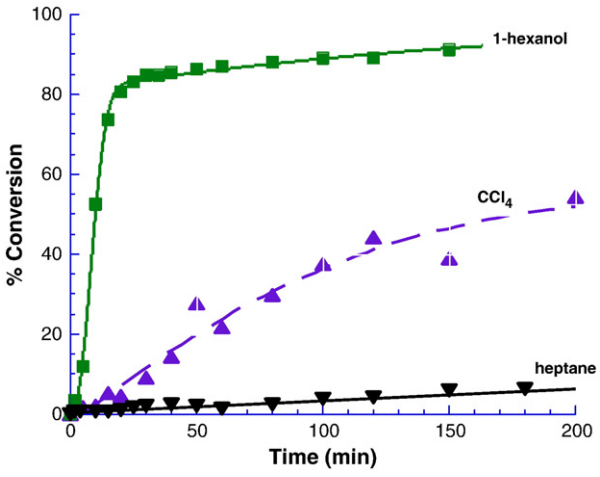

Fig. 1. Kinetic profiles for the conversion of hemin into hemozoin for several co-solvents listed in Table 1. Although the co-solvents represent only about 2% of the reaction volume, they can have a profound impact on the kinetics of conversion. The curves shown represent the best fit of the data obtained by applying Eq. (1). For 1-hexanol, $k = 0.14\ min^{-1}$, $n = 1.3$, $k_s = 0.0054\ min^{-1}$; while for CCl_4, $k = 0.012\ min^{-1}$ and $n = 0.20$. The formation of hemozoin in a heptane/methanol/aqueous medium represents less than 10% conversion over the time window studied (~200 min).