Formation of haemozoin/β-haematin under physiological conditions is not spontaneous

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Abstract Malaria parasite detoxifies free haem, released as a result of haemoglobin digestion, by converting it into an stable, crystalline, black brown pigment known as 'malaria pigment' or 'haemozoin'. Earlier studies have demonstrated the involvement of a parasite-specific enzyme 'haem polymerase' in the formation of haemozoin. However, recently it has been proposed that the polymerization of haem may be a spontaneous process that could take place by incubation of haematin with carboxylic acids (pH 4.2–5.0) even without presence of any parasitic or biological component (*FEBS Letters*, 352, 54–57 (1994)). Here we report that no spontaneous haem polymerization occurs at physiological conditions and the product described in the study mentioned above is not haemozoin/β-haematin (haem polymer) as characterized by us on the basis of solubility characteristics and thin layer chromatography. The infra-red spectroscopic analysis of the product formed though exhibits the bands corresponding to formation of iron–carboxylate bond, similar to that in haemozoin/β-haematin, but was identified as haem–acid adduct. Thus polymerization of haem may not occur spontaneously under the reaction conditions corresponding to food vacuoles of the malarial parasite, the physiological site of haemozoin formation.

Key words: Malaria; Haem; Haemozoin; β-Haematin

1. Introduction

Malaria parasite digests host haemoglobin and utilizes the globin part of the protein for its amino acid requirements, releasing free haem that may be very toxic to the parasite [1–3]. In mammalian system the toxicity of free haem is avoided by its conversion to bilirubin through haem oxygenase-biliverdin reductase pathway [4]. Malaria parasites deal with it by forming a crystalline pigment commonly referred to as 'malaria pigment' or haemozoin [5]. However, the exact molecular mechanisms involved in the formation of this pigment in situ are still under debate [6,7]. Initially the presence of a parasite-specific enzyme termed as 'haem polymerase' was proposed [8,9]. However, the involvement of the enzyme component was questioned later as the reaction was reported to occur even in the presence of preformed β-haematin or malarial haemozoin [10]. A phospholipid component isolated from the non-infected as well as malaria-infected erythrocytes or malarial histidine-rich protein were also found to initiate the formation of haemozoin [11,12].

Haemozoin is a polymer of haem units linked through an iron–carboxylate bond between central iron of one haem and propionate side chain of the other [13]. Egan et al. [14] have recently reported the formation of haemozoin/β-haematin to be a spontaneous process. These workers incubated haematin (ferriprotoporphyrin IX) with different concentrations of acetate (pH 4.5) at 37/60°C and the products were analyzed by infra-red (IR) spectroscopy. In their experiments the product was isolated by filtering the incubation mixture on cellulose acetate/nitrate filters and subsequently washing it with the distilled water. The filters were dried and used for recording the IR spectra. They detected absorbance peaks at 1660 and 1207 cm^{-1} and assigned it to the haemozoin/β-haematin formation.

The conclusion drawn on the basis of these observations that the formation of haemozoin/β-haematin occurs spontaneously was rather premature since the product formed had not been characterized in sufficient detail. Chemical synthesis of β-haematin has already been reported [13]. However, the conditions required for this are highly non-physiological. The most important criterion for the separation of haemozoin/β-haematin from the haematin/haem is their differential solubilities in alkaline bicarbonate buffer (pH 9.0–9.5) and 2.5% sodium dodecyl sulfate (SDS) [13,15,16]. Haem/haematin is readily soluble in these solvents while haemozoin/β-haematin are insoluble. The initial experiments conducted by us showed that the product formed under the conditions described by Egan et al. [14] was readily soluble in bicarbonate buffer as well as 2.5% SDS. Further studies were therefore conducted to characterize the product in detail and also to study the formation of haemozoin.

2. Materials and methods

Haemin and haematin were purchased from Sigma Chemicals Co. (St. Louis, MO). ^{14}C-labelled acetate was from Board of Radiation and Isotope Technology, Bombay, India. All other reagents were of best purity grade available and procured from the local Indian suppliers.

Malarial haemozoin was purified from the erythrocytes highly infected with *Plasmodium yoelii* (parasitemia >60%) or liver from the infected animals, according to the method described earlier [16]. Non-biological/chemical synthesis of β-haematin was carried out by incubating haematin in 4.5 M sodium acetate overnight at 80°C [13]. Purity and polymeric nature of malarial haemozoin and β-haematin were ascertained by the analysis of elemental composition, visible spectra, differential solubility characteristics and also by infrared spectroscopy[16]. To check the formation of β-haematin under physiological conditions non-biologically haematin (5.0 mg/ml) was incubated in solutions with various concentrations of sodium acetate (pH 4.5) as described by Egan et al. [14]. After the incubation, as specified, the mixtures were centrifuged at 10000×*g* and the pellets were washed twice with distilled water. Solubility of the products formed by incubating haematin with various concentrations of acetate under different conditions was checked in different solvents viz., bicarbonate buffer (100 mM, pH 9.5), SDS (2.5% w/v) and methanol. The haem-acetate incubation mixtures were filtered over nitrocellulose filters (0.45 μm, Millipore, USA) as described by Egan et al. [14] and subjected to different kinds of washings. The amount of ferriprotoporphyrin IX

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(haem) retained on the filters after the washing was measured spectrophotometrically [16] after submerging the filters in 0.1 N NaOH for overnight. For recording infrared spectra the solid malarial haemozoin/β-haematin/haematin/haem-acetate incubation products were dried over silica gel and phosphorous pentaoxide and IR spectra were recorded on a Perkin-Elmer 881 (Fig. 1) or Pye Unichem SP3-200 (Fig. 2) IR spectrometer.

A method has been developed for isolation of free haem and β-haematin without extraction/bicarbonate buffer washing by thin layer chromatography (TLC). Haem is highly soluble in methanol. Solubility of haem in methanol was reduced by mixing acetic acid and water and a solvent mixture of methanol/acetic acid/water (8:1.5:0.5, v/v) yielded a distinct spot of haem on TLC over precoated silica gel F_{254} plastic sheets (0.2 mm thickness) (E. Merck, Germany) with R_f value of 0.85 ± 0.05 while the polymerized haem (haemozoin/β-haematin) was insoluble and did not move at all in this solvent and was detected as a distinct spot at the origin only. This provides maximum separation of haem and haemozoin even in short run of the solvent on TLC. For studying the binding of acetate with ferriprotoporphyrin IX, haemin in 0.1 N HCl was incubated with 5 μCi of ^{14}C-labelled sodium acetate at 37°C at least overnight. At the end of incubation the mixture was centrifuged at 10000 × *g* (5 min). The pellets were washed as described and finally dissolved in 100 μl of 0.2 N NaOH and decolourized with 50 μl of hydrogen peroxide. Radioactivity was counted on a Pharmacia-LKB liquid scintillation counter after adding 10 ml of scintillation cocktail (60 g naphthalene; 4 g PPO; and 200 mg POPOP in 1 l of dioxane/methanol; 4:1 v/v).

3. Results and discussion

Solubility of the products obtained under different experimental conditions was studied in bicarbonate buffer, 2.5% (w/v) SDS and methanol. Incubation products of haematin and acetate at 37°C, pH 4.5 (acetate concentration 100 mM, 500 mM, 1 M and 4.5 M) after 6 h of incubation as well as the product formed after incubation at 60°C for 30 min were soluble in bicarbonate and 2.5% SDS (Table 1). All these products had movements similar to that of haemin on TLC (Table 2). However, overnight incubation of haematin at 80°C yielded a product which was not soluble in bicarbonate buffer and 2.5% SDS. The extracts of *Plasmodium yoelii, Plasmodium berghei* and *Plasmodium knowlesi* also contain a bicarbonate buffer/SDS insoluble component [16]. Incubation of haematin in 4.5 M acetate at 80°C for 12 h yielded two components on TLC analysis (Table 2) one corresponding to haem and another corresponding to β-haematin. The component corresponding to β-haematin was insoluble in bicarbonate buffer and SDS solution. It exhibited IR absorbance peaks at 1664 and 1208 cm^{-1} and had similar elemental composition to that of malarial haemozoin. These conditions (high acetate concentration, the temperature of 80°C and overnight incubation time) have already been reported in the literature to result in the formation of β-haematin [13]. Hence it was clear that β-haematin formation occurred only under non-physiological conditions. The products formed under the conditions described by Egan et al. [14] were also characterized by IR spectroscopy (Figs. 1 and 2; Table 2). Incubation of haem with 1 M acetate at 37°C for 6 h or at 60°C for 30 min did yield the product with characteristic peaks at 1660 and 1207 cm^{-1} (Fig. 2B and 1D). Similar peaks were also observed in haemozoin isolated from cell-free *P. yoelii* parasite (Fig. 1A) or liver of *P. yoelii* infected mouse

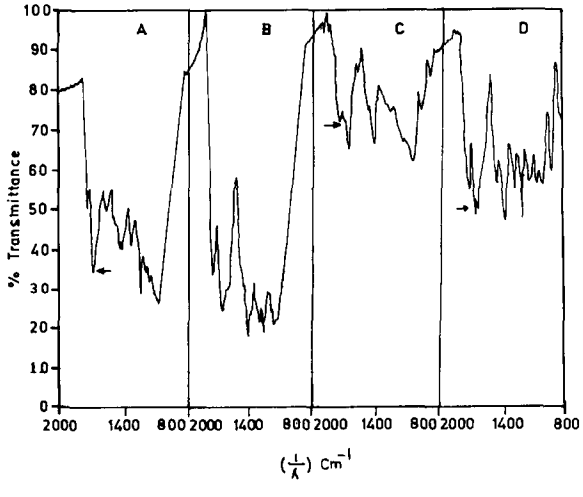

Fig. 1. Infrared spectra of (A) malarial haemozoin, (B) haematin, (C) haematin+phosphoric acid (1 M) incubation (60°C, 30 min) product and (D) haematin+sodium acetate (1 M) incubation (60°C, 30 min) product. The arrow indicates the characteristic peak of iron–carboxylate bond. Other details are described in Section 2.

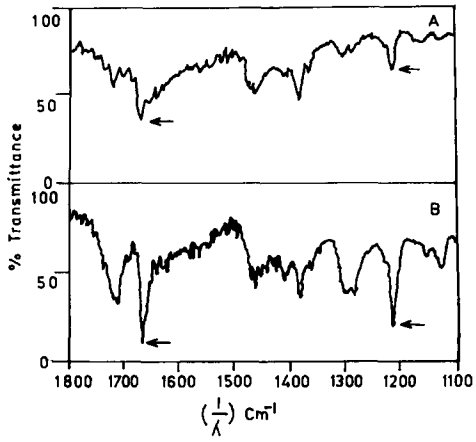

Fig. 2. Characterization of (A) malarial haemozoin and (B) haem–sodium acetate (1 M) incubation (37°C, 6 h) products by IR spectroscopy.

Table 1
Solubility properties of malarial haemozoin, β-haematin and haematin-acetate incubation products

Sample	Solvent		
	Bicarbonate buffer (0.1 mM, pH 10)	SDS (2.5%)	Methanol
Haemin	++++	++	++++
Haematin	++++	+++	++++
Malarial haemozoin	±	−	−
β-Haematin	±	−	−
Haematin-acetate incubation product (s)			
0.1 M 37°C 6 h	++++	+++	++++
0.5 M 37°C 6 h	++++	+++	++++
1.0 M 37°C 6 h	++++	+++	++++
4.5 M 37°C 6 h	++++	+++	++++
4.5 M 60°C 30 min	++++	+++	++++
4.5 M 80°C 12 h	++	++	++

++++, >90% solubility; ++, 40–60% soluble; ±, sparingly soluble; −, insoluble.
Malarial haemozoin was obtained from *Plasmodium yoelii* parasites; β-Haematin was synthesized chemically as described in Section 2.

Table 2
Analysis of haemozoin, β-haematin, haem and haem-acetate incubation mixtures by infra-red spectrometry and thin layer chromatography

Sample			Presence of IR peaks at 1660 and 1207 cm^{-1}	% recovery of FP on TLC	
				Component A (haem polymer)	Component B (free haem)
Malarial haemozoin			present	100	nil
β-Haematin			present	100	nil
Haemin			absent	4±2	87±8
Haem-acetate product					
0.1 M	37°C	6 h	–	3±2	88±9
0.5 M	37°C	6 h	–	5±3	84±11
1.0 M	37°C	6 h	present	4±3	88±7
4.5 M	37°C	6 h	present	5±3	91±7
4.5 M	80°C	12 h	present	43±5	41±7

Values are mean ± SD of triplicate observations. Other details about TLC procedure are described in Section 2.

(Fig. 2A) while haematin did not exhibit these peaks (Fig. 1B). Appearance of the characteristic IR peaks was also observed when haematin was incubated at 60°C for 30 min with 1 M phosphoric acid (Fig. 1C). However, except haemozoin, all other products were soluble in bicarbonate buffer and were characterized as non-polymerized haem on TLC (Table 2).

Iron-carboxylate bond in haemozoin forms due to the linking of ferric iron of one haem molecule to the side chain propionate group of the other haem [13]. Similar type of linkage could be formed between ferric iron of haem and carboxylate group of the acetate [17]. ^{14}C-labelled acetate was used to test this possibility. Distinct binding of ^{14}C-labelled acetate with haematin was observed which was inhibited by chloroquine (Fig. 3). This explained the inhibition of iron–carboxylate bond formation by chloroquine as described by Egan et al. [14]. These results also indicate formation of haem-acetate adduct.

Linkage of organic acids (acetate and phosphate) with haem could not result in polymer formation as further chain elongation is blocked once an acid molecule is attached to haem. This explains the bicarbonate solubility of such a product. Inhibition of this adduct formation by quinoline drugs could be explained by the tendency of these drugs to form complexes with haem, that would inhibit the formation of haem–acid linkage [18,19]. Linkage of acetate to protoporphyrin IX has been reported to give IR absorbance band at 1660 cm^{-1} and the bond formation has been proposed to be similar as in case of haemozoin. Recently reaction of haem polymerization was studied using a HPLC method and no polymerization was found to occur without malarial parasite extract [4]. This is in accordance with the previous studies [10,20,21]. On the basis of all these evidences we propose that the product reported by Egan et al. [14] was not β-haematin but haem-acetate adduct and the polymerization of haem to form haemozoin/β-haematin is not spontaneous but requires the presence of some parasitic or biological material for the initiation and/or continuous formation of haemozoin, whose identity is as yet unestablished. Further studies are needed to unravel the mechanism of this reaction which due to its uniqueness of being found only in the malaria parasite, has the potential of providing a vulnerable target for antimalarial drug design. Considering the present scenario of fast spreading drug resistance and malaria becoming a major global threat, the need to study such promising features of parasite biochemistry cannot be overstressed.

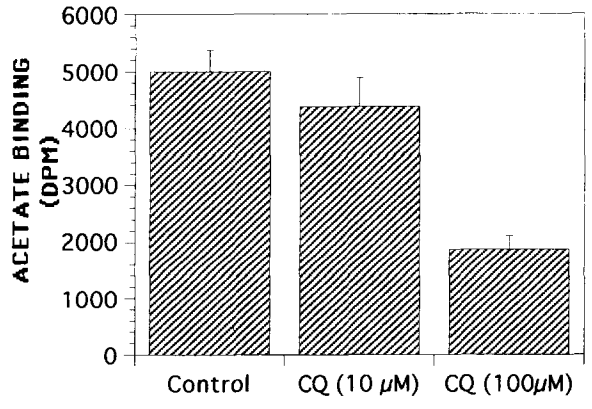

Fig. 3. Analysis of binding of ^{14}C-labelled acetate with haematin. The reaction conditions are described in Section 2.

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