RESEARCH COMMUNICATION

The chemical mechanism of β-haematin formation studied by Mössbauer spectroscopy

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Spontaneous formation of β-haematin (malaria pigment) from haematin in acetate solution follows pseudo-zero-order and not autocatalytic kinetics. Acetate appears to facilitate the reaction by solubilizing the haematin and acting as a phase-transfer catalyst, a role which, *in vivo*, could be fulfilled by carboxylic acids or amino acids.

INTRODUCTION

The malaria parasite contains a potential endogenous antimalarial, ferriprotoporphyrin IX (in the form of haematin), released during host haemoglobin degradation [1], the haematin is then removed by conversion into the highly insoluble malaria pigment β-haematin [2]. The polymerization of haematin to β-haematin (haemozoin or malaria pigment) is thus a crucially important chemical reaction in the malaria parasite. It is both essential to the survival of the parasite because it is the process by which it detoxifies haem released during host haemoglobin degradation, and it is the reaction which is blocked [3] by intraerythrocytic antimalarial drugs of the quinoline type (which include chloroquine, quinine and amodiaquin) and so provides a target for the design of new antimalarial drugs. In recent years there has been controversy over the nature of this reaction *in vivo*. Some groups have advocated an enzymic mechanism [4,5] and, subsequently, another has suggested that the reaction is non-enzymic but in fact autocatalytic [6]. More recently it was suggested that the histidine-rich proteins catalyse or initiate the reaction [7]. It has long been known [8] that the polymerization reaction can be carried out in concentrated acetate solutions, for example [2] in 4.5 M acetic acid with heating (70 °C) at low pH (about pH 2). We have previously shown [3] that the reaction is facile at much lower temperatures (down to 6 °C) and acetate concentrations (down to 0.1 M), and at higher pH (4.5). We were also able to show [3] that the reaction proceeds in a mixture of amino acids (0.05 M glutamate, 0.05 M aspartate and 0.4 M glycine, pH 4.5) and that the reaction is inhibited by quinoline antimalarial drugs.

In spite of the obvious importance of this reaction, both in resolving the controversy surrounding the formation of β-haematin *in vivo* and in facilitating the design of novel antimalarial drugs, little is known of the chemical mechanism of the reaction in aqueous solution. Studies of β-haematin synthesis have been performed in strictly non-aqueous media [9], but the kinetics of the process have not been reported. The main reason for the lack of information in aqueous medium is the technical difficulty in studying the rate of this reaction. Since haematin has very low solubility below pH 7 and β-haematin is essentially insoluble, the reaction proceeds as a slurry, making traditional UV–visible spectroscopic techniques useless for monitoring the progress of the reaction. We have found the differential solubility methods used by other groups to be very unreliable for studies of this process. Furthermore, IR spectroscopy, with which haematin and β-haematin can be qualitatively distinguished, is not suitable for quantitative studies because the reproducibility of absolute peak intensities between samples is inadequate. Attempts to use solid-state EPR spectroscopy for this purpose were also unsuccessful as the two compounds are magnetically too similar in terms of Fe–Fe interactions.

We have shown [10] that, using Mössbauer spectroscopy, the starting metalloporphyrin and β-haematin can be clearly distinguished (Figure 1) and we have applied this technique to study the mechanism of β-haematin formation. This seemingly novel application of Mössbauer spectroscopy to monitor a kinetic process in solution provides a direct quantitative measure of the relative amounts of haematin and β-haematin present during the course of the reaction.

MATERIALS AND METHODS

The reaction mixture contained 160 mg of haemin (Sigma) dissolved in 32 ml of 0.1 M NaOH and was neutralized with 3.2 ml 1 M HCl. The appropriate volume of water was added and the mixture was equilibrated at 60 °C. The reaction was initiated by adding the appropriate volume of 12.9 M acetate solution (pH 5), pre-equilibrated at 60 °C, to give the final desired acetate concentration. The final pH of the reaction mixture was 4.3 in the case of 4.5 M acetate (reactions in which 6.0 M and 3.0 M acetate was used were preadjusted to give the same final pH). In the experiment which was seeded with 10% β-haematin at $t = 0$, 16 mg β-haematin (60 min sample) was added immediately after the addition of acetate. All reaction mixtures were stirred continuously with a magnetic stirring apparatus.

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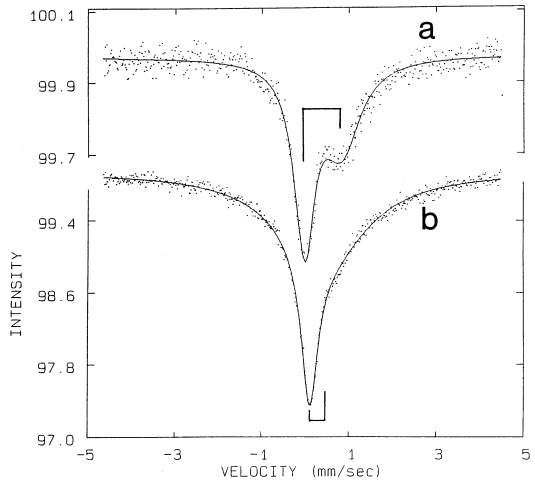

Figure 1 Mössbauer spectra of (a) haematin and (b) β-haematin at 78 K

The positions of the isomer shifts found (see the text for values) for each species are shown on the spectra.

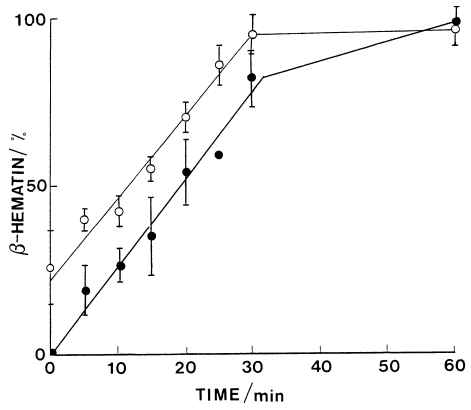

Figure 2 Kinetics of spontaneous formation of β-haematin from haematin at 60 °C in 4.5 M acetate, pH 4.3

Kinetic plots of the formation process in the absence (●) or presence (○) of 10% β-haematin added at t = 0. The infrared spectrum of our 30 min sample was identical in terms of peak positions and relative intensities to those for purified β-haematin [9], confirming that the reaction is essentially complete after this time contrary to a previous report [2]. Error bars are, with the exception of the single point at t = 25 min (unseeded system), one S.E. (3–6 repeats) for the unseeded kinetic trace, and maximum deviation for duplicate repeats in the case of the seeded system.

Samples were removed from the reaction mixtures at various times between 0 and 60 min, were filtered, washed with water and dried, and the finely ground powder mixtures of haematin and β-haematin were mounted in lead sample holders for Mössbauer spectroscopy. Approx. 15 mg of sample was mounted for each time point.

Mössbauer spectra were obtained at 78 K as described elsewhere [10]. For each sample the spectrum was accumulated over approx. 12 h, and varied smoothly with time between the haematin spectrum (Figure 1a) at $t = 0$ and the β-haematin spectrum (Figure 1b) at $t = 60$ min (4.5 M acetate, pH 4.3, 60 °C).

RESULTS AND DISCUSSION

Since any given sample obtained between $t = 0$ and $t = 60$ min (reaction complete in 4.5 M acetate) consists of a mixture of the two species (haematin and β-haematin), the observed spectrum is a composite of two assymetric quadrupole doublets (Figures 1a, 1b). Each doublet consists of two lines that are dependent in shape on a temperature-dependent spin–spin relaxation time [11]. For a given material and temperature the shape of the doublet should be constant provided that the particle size is similar. The data can therefore be analysed in terms of a four-line model, the first two sites having isomer shifts corresponding to those of haematin, and the remaining two those for β-haematin. Thus the curve-fitting procedure can be greatly simplified by using the isomer shifts for haematin and β-haematin as fixed parameters in the optimization process, and their percentage contribution to the observed spectrum can be determined. This allows construction of kinetic curves either for β-haematin formation or for haematin decay, with time. Fixed parameter values were determined as the average of our measurements for haematin and for β-haematin (prepared in 4.5 M acetate, pH 4.3 at 60 °C), the values (±S.D.) used for haematin were -0.063 ± 0.001 mm·s^{-1} and 0.847 ± 0.008 mm·s^{-1} while those used for β-haematin were 0.068 ± 0.001 mm·s^{-1} and 0.371 ± 0.002 mm·s^{-1}.

Figure 2 shows the kinetics of β-haematin formation in 4.5 M acetate, pH 4.3 at 60 °C. Clearly the reaction is zero-order with no evidence of an induction phase (a requirement for an autocatalytic mechanism). Furthermore, in the presence of a 10% seeding of β-haematin the kinetics are still zero-order. The rate obtained for the seeded data is 6.0 μmol·min^{-1}, which compares well with that of the unseeded sample, 6.4 μmol·min^{-1}, indicating that β-haematin is not catalytically involved in the reaction. Furthermore the reaction is essentially complete after 30 min, in agreement with our previous observation [3] that the IR spectrum after 60 min is the same as that obtained at 30 min.

When the reaction was carried out in 3 M and 6 M acetate (results not shown), zero-order kinetics were again observed. However, the zero-order rate for the 3 M system was approx. 20% of that for the 4.5 M system, while for the 6 M system the rate was approx. double that for the 4.5 M system.

These kinetic experiments have clearly demonstrated two important facts about the conversion of haematin to β-haematin, namely that the reaction is an apparent zero-order process and that it is not autocatalytic. The pseudo-zero-order kinetics are consistent with a mechanism in which the reaction proceeds via a small constant concentration of dissolved haematin in a haematin-saturated solution, where polymerization of the dissolved haematin is rate-limiting. This interpretation is supported by the following three observations. First, the overall rate of reaction increases with increasing acetate concentration. Secondly, the concentration of dissolved haematin increases with increasing acetate concentration [3], and finally, the rate of reaction for a given mass of haematin increases with reaction volume [3]. The fact that the zero-order reaction rate is not increased by seeding with β-haematin provides definitive proof that the reaction is not autocatalytic, as claimed by Dorn et al. [6]. *In vitro*, acetate appears to act as a phase-transfer catalyst, a role which could quite realistically be fulfilled *in vivo* by amino acids [3] or proteins, such as those recently suggested by Sullivan et al. [7].

We have previously shown that approx. 2.5 mg of haematin is converted into β-haematin in 1 litre of 0.5 M acetate, pH 4.8 and 37 °C [3] in 7 days. This corresponds to a zero-order rate constant of about 380 pmol/min, which is faster than that

reported by Slater and Cerami [4] under the same conditions, but in the presence of trophozoite extract.

(Zero-order kinetics imply a direct dependence of time taken for complete conversion of substrate into product on total amount of substrate and not on apparent substrate concentration, as is the case for the more familiar first-order kinetics).

Under these conditions, and based on the approximate volume of the food vacuole in the malarial parasite (approx. 5×10^{-16} litre) (estimated from electron micrographs [12]) and the concentration of haemoglobin haem in the erythrocyte cytosol (approx. 22 mM) [13], using the above rate constant all the haematin in the food vacuole would be converted into β-haematin in a maximum time of about 2 μs. Notwithstanding the fact that the conditions of the systems *in vivo* and *in vitro* are not directly comparable, the calculation does indicate that rapid conversion can be brought about easily under acidic conditions similar to those found in the food vacuole, pH 5.2–5.4 [14].

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