On the Mechanism of Hemozoin Production in Malaria Parasites: Activated Erythrocyte Membranes Promote β-Hematin Synthesis

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The ferriprotoporphyrin IX (FP) molecules released by intraerythrocytic malaria parasites during hemoglobin digestion are converted to β-hematin and are stored in the parasites' food vacuoles. It has been demonstrated in cell-free medium that the incorporation of FP into β-hematin under physiological conditions requires a catalyst from parasite lysates or pre-formed β-hematin. In the present studies, lysates of *Plasmodium falciparum*-infected erythrocytes were suspended in 1 M NaOH and were washed with phosphate buffer, pH 7.6. When the cell extracts were incubated with hematin in 0.5 M sodium acetate buffer, pH 5, for 20 hr at 37°C, a large quantity of β-hematin was formed. To determine whether parasite components were necessary for the β-hematin formation, normal erythrocyte ghosts were similarly treated with 1 M NaOH and then incubated with hematin. In repeated experiments it was found that, on the average, 70% of the hematin was converted to β-hematin. Membranes treated with HCl or CH_3COOH also promoted the formation of β-hematin, while untreated membranes were ineffective. The possibility that metabolic activities in the food vacuoles of malaria parasites may activate membrane fragments, from hemoglobin vesicles, to promote β-hematin formation is discussed in this paper. [Exp Biol Med Vol. 226(8):746–752, 2001]

Key words: hematin; β-hematin; synthesis; activated membranes; catalyst

Heme released from hemoglobin as protein-free ferriprotoporphyrin IX (FP) has been shown to lyse different types of cells, including malaria parasites (1–4). It also inhibits enzyme activities (5), and the growth of bacteria and viruses (6–9). FP is mainly toxic when it is in a solubilized (monomeric) state, but becomes nontoxic when converted to β-hematin. This may explain why malaria parasites seem not to be adversely affected by the large quantities of FP that they generate during hemoglobin catabolism in erythrocytes. They incorporate the released FP into haemozoin (malaria pigment) that is stored in the food vacuoles of the parasites. It has been determined that FP molecules in hemozoin are linked by iron-carboxylate bonds (10), and that the product is identical to chemically synthesized β-hematin (10–12). Pagola *et al*. (13) have recently determined that the FP molecules in β-hematin are linked into dimers through reciprocal iron-carboxylate bonds to one of the propionic side chains of each porphyrin, and the dimers form chains linked by hydrogen bonds in the crystal. Earlier investigators had reported that β-hematin was a polymer (10, 12).

The mechanism of β-hematin synthesis in malaria parasites is currently being studied very intensely because it has been found to be susceptible to inhibition by antimalarial drugs. Therapeutic doses of chloroquine inhibited hemozoin production in human erythrocytes infected with chloroquine-susceptible, but not chloroquine-resistant *Plasmodium falciparum* (14), and it is believed that the inhibition causes monomeric FP to accumulate and kill the parasites. Understanding the process of hemozoin formation may, therefore, contribute to elucidation of the mechanism of drug resistance.

In cell-free conditions, β-hematin is readily synthesized by suspending hematin or hemoglobin in acetic or propionic acid and then heating the suspension at about 80°C (10, 11). Under strictly anhydrous conditions, β-hematin has been prepared at room temperature by dehydrohalogenation of hemin (15). Slater and Cerami (16) produced β-hematin at 37°C by adding crude *P. falciparum* hemozoin to a suspension of hematin in sodium acetate buffer, approximately pH 5. They hypothesized that hemozoin contains heme polymerase enzyme activity that catalyzes FP conversion to β-hematin. Catalysis of β-hematin formation by *P. falciparum* hemozoin, as well as by hemozoin from rodent malaria parasites, has been confirmed by other groups of investigators (17–19). The active material was said to be a compo-

This work was funded by Kuwait University Research Administration Project No. MH 033.

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Received October 10, 2000.
Accepted March 27, 2001.

0037-9727/01/2268-0000$15.00
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nent of hemozoin that is not soluble in physiological solutions. The two main components of unpurified lysates of malaria-infected erythrocytes are β-hematin and the membranes of erythrocytes and parasites.

The heme polymerase hypothesis has been challenged by Dorn *et al*. (17) who showed that treatment of hemozoin with proteinase K and chloroform-methanol to remove associated proteins gave material that was as effective as untreated hemozoin in promoting β-hematin formation. They concluded that β-hematin formation is a chemical process, dependent only on the presence of heme-derived material associated with previously formed β-hematin, and not on protein. However, their experiments did not provide any clue to how biological synthesis of hemozoin is initiated.

A new hypothesis on the source of the catalyst that initiates and promotes β-hematin synthesis in malaria parasites is proposed in the present paper. It is based on the fact that intraerythrocytic plasmodia transport vesicular membranes, together with hemoglobin, into their food vacuoles. There, the membranes are lysed, possibly by hydrolysis, and hemoglobin is digested to FP and amino acids. Data presented here show that under certain conditions, membrane fragments promote conversion of FP to β-hematin at food vacuole pH and physiological temperature.

Materials and Methods

Removal of β-Hematin from Parasite Lysates. *P. falciparum* parasites were grown *in vitro* for 5 days in human erythrocytes, blood group O, rhesus factor positive. The culture was transferred into 15-ml plastic tubes and centrifuged for 10 min at room temperature and 3000 rpm, using a table-top centrifuge (MSE Mistral 2000). The supernatant was discarded and the pellet was washed once with saline solution (0.145 M NaCl). The washed pellet was suspended in saline at approximately 5×10^8 erythrocytes per 1 ml, and 2-ml aliquots of the suspension were prepared in Oak Ridge tubes with screw caps. They were centrifuged for 30 min at 4°C and 19,000 rpm, using a Beckman J2-MI centrifuge equipped with a JA-20 fixed angle rotor (Beckman Instruments, Fullerton, CA). After centrifugation, the supernatants were carefully removed and discarded. To hemolyse the erythrocytes, 10 ml of cold 5 mM phosphate solution, pH 7.6, was added to each of the tubes, which were subsequently covered with their caps and shaken vigorously by hand. They were kept on ice for 10 min before being centrifuged for 30 min at 19,000 rpm. The supernatants were discarded and the pellets (parasite lysates) were washed twice by suspending them each time in 10 ml of cold 20 mM Tris-buffered solution, pH 7.2, and centrifuging them for 60 min at 19,000 rpm. After the final wash, 0.4 ml of 1 M NaOH was added to each of the tubes and they were shaken vigorously by hand and kept at room temperature for 5 min. Four milliliters of 20 mM Tris-buffered solution, pH 7.2, was added to the tubes, followed by vigorous shaking by hand and incubation at room temperature for 30 min. The samples were centrifuged for 60 min at 19,000 rpm, after which the supernatants were discarded. The pellets were resuspended in 5 ml of 5 mM phosphate solution, pH 7.6, and centrifugation was repeated, after which the supernatants were discarded.

Normal Erythrocyte Membranes. Normal human blood samples were collected in tubes that contained the anticoagulant EDTA(Na_2). Most of the experiments were done with blood group O, rhesus factor positive erythrocytes. The blood samples were centrifuged for 10 min at room temperature and 3000 rpm, after which the supernatant plasma and the top layer of the pellet (buffy coat) containing white blood cells and platelets were discarded. The erythrocyte pellet was washed twice with 8 ml of cold saline solution, centrifuging each time at room temperature and 3000 rpm. A suspension of the washed cells was prepared in saline solution to contain 10^7 to 10^9 erythrocytes per 1 ml. Two-milliliter aliquots of the suspension were prepared in high-speed Oak Ridge tubes. The aliquots were centrifuged for 30 min at 19,000 rpm, and the supernatants were discarded. Each tube was shaken briefly by hand to loosen the pellet. Ten milliliters of cold 5 mM sodium phosphate solution, pH 7.6, was added to the tubes. It were shaken vigorously by hand and kept on ice for 10 min before centrifuging for 60 min at 19,000 rpm. The supernatants were discarded and the pellets containing the erythrocyte membrane ghosts were washed twice by resuspending them each time in 10 ml of cold 20 mM Tris-buffered solution, pH 7.2, and centrifuging for 60 min at 19,000 rpm.

Activation of Membranes with NaOH. After lysis and washing of erythrocyte membranes as described above, 0.4 ml of 1 M NaOH was added to the tubes. The tube was shaken vigorously by hand and kept at room temperature for 5 min. Four milliliters of Tris-buffered solution, pH 7.2, was added to the tube and the suspension was shaken vigorously before further incubation at room temperature for 30 min. The suspension was centrifuged for 60 min at 19,000 rpm, the supernatant was discarded, and the pellet was washed twice with 5 ml of 5 mM sodium phosphate solution, pH 7.6.

Hematin. Hemin (16.3 mg, Sigma, St. Louis, MO) was dissolved with 0.4 ml of 1 M NaOH and diluted to 1 mM with distilled water. The solution was passed through a membrane filter, 0.2-μm pore size, to remove undissolved particles. Hematin solution (0.4 ml) was diluted with 2.5% of SDS buffered with 25 mM Tris to pH 7.8 and the absorbance at 400 nm wavelength was used with the millimolar extinction coefficient (90.8) (20) and the total volume (milliliters) to calculate hematin concentration. The stock hematin solution was kept on ice until used, usually within 1 hr. This solution was used as substrate for β-hematin synthesis, and in this paper it is simply called hematin.

Synthesis and Purification of β-hematin. Four milliliters of 0.5 M sodium acetate solution, pH 5, was added to Oak Ridge tubes with or without membranes. The required concentrations (nanomoles) of the hematin pre-

pared above were added to the tubes and mixed thoroughly. The tubes were incubated for 20 hr (overnight) at 37°C. The suspension was then centrifuged for 30 min at 19,000 rpm and the supernatant was discarded. The pellet was suspended in 10 ml of 2.5% SDS buffered with 25 mM Tris to pH 7.8, and kept at room temperature for at least 2 hr to dissolve the hematin aggregates that were not incorporated into β-hematin. It then was centrifuged for 60 min at 19,000 rpm. The supernatant was removed and the pellet was resuspended in 10 ml of 2.5% SDS buffer, pH 7.8, and was kept for 1 hr at room temperature. The suspension was centrifuged for 60 min at 10°C and 19,000 rpm. The supernatant was removed and the SDS-insoluble pellet was the β-hematin (14).

Calculation of β-Hematin Concentration. All β-hematin concentrations in this paper have been given in nanomoles hematin. For this purpose, the SDS-insoluble pellet of β-hematin was completely dissolved with 0.4 ml of 1 M NaOH. The solution was then diluted with 2.5% SDS buffered with 25 mM Tris to pH 7.8, to a volume that did not have spectrophotometric absorbance higher than 0.99 at 400 nm wavelength. The absorbance at 400 nm was then used with the millimolar extinction coefficient (90.8) and the total volume (milliliters) to calculate hematin concentration.

Statistical Analysis. Means and SD were obtained with a computer using Graphpad Instat software. The two-tailed *P* values were used to determine significant differences.

Results

β-Hematin Synthesis with NaOH-Insoluble Extracts from Parasite Lysates. To remove all heme-type products from lysates of *P. falciparum*-infected erythrocytes, the preparation was treated with 0.4 ml of 1 M NaOH and washed twice with 10 ml of phosphate buffer, pH 7.6. The supernatant after the first wash contained 77 nmol of hematin, whereas that of the second wash did not contain a measurable level of hematin. The pellet was mixed with 800 nmol of hematin in sodium acetate buffer, pH 5, and was incubated for 20 hr at 37°C. It was found that 345 nmol of the hematin became converted to β-hematin. This result suggests that at least some of the activity found in lysates of *P. falciparum*-infected erythrocytes was due to a material that was insoluble in NaOH.

Characterization of β-Hematin. When hemin dissolved in NaOH solution was added to sodium acetate buffer, pH 5, with or without cell lysates, large hematin aggregates were formed immediately. After 20 hr of incubation at 37°C, the suspension was centrifuged and the supernatant was clear and colorless, indicating that there was little, if any, monomeric hematin left in the acidic solution. The pellet was suspended in 10 ml of SDS, pH 7.8, and kept at room temperature for 2 hr. No pellet was found in membrane-free samples after centrifugation, indicating that no β-hematin was formed. Spectrophotometric examination of the supernatant showed a characteristic absorption spectrum of monomeric hematin in SDS (Fig. 1) (14, 21). In the sample containing cell lysates and where β-hematin was formed, a dark-brown pellet was recovered after centrifugation of the initial suspension in SDS, and it remained insoluble during repeated washing with SDS solution. When sonicated and mixed vigorously with SDS solution, it formed a suspension that had an absorption spectrum (Fig. 2) similar to that of *P. falciparum* hemozoin (14). The characteristic absorption maximum for β-hematin in slightly alkaline SDS (pH 7–8) is usually found within 640 to 670 nm range (14, 18). In the present work it was automatically identified at 646 nm by the spectrophotometer (Fig. 2). A similar spectrum has also been reported with β-hematin synthesized by dehydrohalogenation of hemin (15). In contrast, monomeric FP solution in SDS has no absorption maximum within 630 to 700 nm (14). Like hemozoin, β-hematin synthesized by activated membranes dissolved readily in NaOH, and when mixed with SDS the solution gave an absorption spectrum similar to that shown in Figure 1.

Promotion of β-Hematin Synthesis by Activated Membranes. Membrane preparations from 1.5 to 3×10^9 normal erythrocytes were suspended in sodium acetate buffer, pH 5, and 800 nmol of hematin was added to the tube. After 20 hr of incubation at 37°C, it was found that on the average, 15 nmol of hematin aggregate had become insoluble in 2.5% of SDS buffered with 25 mM Tris to pH 7.8. This calculation was based on the amount of hematin that apparently did not dissolve in the SDS solution during washing (see Materials and Methods). The quantity was too small to give a clear absorption spectrum of β-hematin. In contrast, measurable quantities of β-hematin were produced when activated membranes were used. In a total of 15 experiments in which NaOH-treated erythrocyte membranes were incubated with 800 nmol of hematin, the quantities of β-hematin produced ranged from 413 to 719 nmol (mean 566 ± 84).

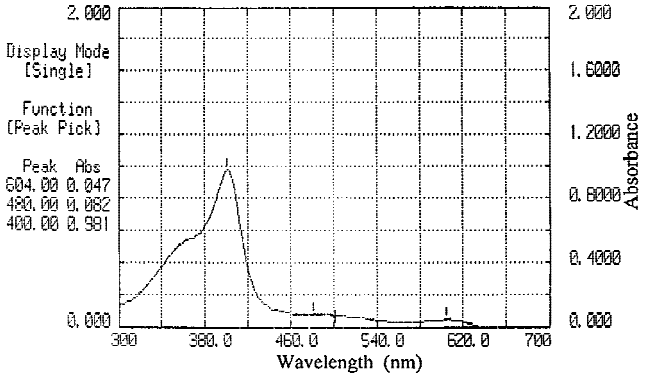

Figure 1. Electronic absorption spectrum of hematin in SDS. Hemin solution in NaOH was added to sodium acetate buffer, pH 5, and incubated at 37°C for 20 hr. After incubation, the suspension was centrifuged and the supernatant was discarded. The pellet was dissolved in 2.5% SDS solution buffered with 25 mM Tris to pH 7.8. The characteristic absorption maximum for monomeric hematin in SDS solution is indicated at 400 nm.

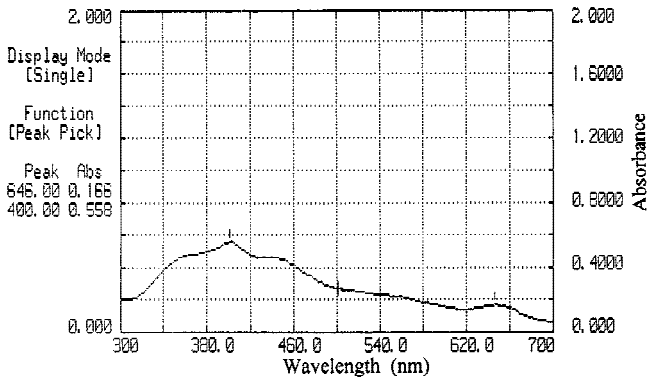

Figure 2. Electronic absorption spectrum of β-hematin suspended in SDS solution, pH 7.8. β-hematin was synthesized using NaOH-treated erythrocyte membranes as catalyst. The characteristic peak for β-hematin is indicated at 646 nm.

Membrane Activation Depends on NaOH Concentration. In a preliminary experiment it was found that erythrocyte membranes treated with 0.001 M NaOH for 30 min converted only 28 out of 800 nmol of hematin to β-hematin. It, therefore, became necessary to examine the effect of NaOH concentration on membrane activation to catalyze β-hematin synthesis. Serial dilutions of 1 M NaOH were prepared with distilled water and were used to activate 1.5 × 10^9 erythrocyte membranes for 60 min at room temperature. The results of β-hematin synthesis carried out with these preparations are summarized in Figure 3. The concentrations of unincorporated hematin in the samples were also measured. Although the mean quantity of the β-hematin synthesized by membranes that were treated with 0.125 M NaOH was less than that synthesized by membranes that were treated with 0.25 M, 0.5 M or 1 M NaOH, statistical analysis indicated that the differences were not significant. However, the difference between the quantities of β-hematin synthesized by membrane that were exposed to 0.063 and 1 M NaOH was statistically significant ($P = 0.0018$). As expected, the mean concentration of unincorporated hematin was significantly higher in samples containing membranes that were hydrolyzed with 0.063 instead of 1 M NaOH ($P = 0.0015$). The conclusion from this experiment is that NaOH concentrations lower than 0.125 M may not produce optimal activation of membranes (within 60 min at room temperature) for promotion of β-hematin synthesis.

Time Necessary for Optimal Activation of Membranes with NaOH. In repeated experiments it was observed that there was little or no quantitative difference in β-hematin formed by membranes activated with 1 M NaOH for 5 or for 60 min. However, when 0.25 M NaOH was used for activation, there was a statistically significant difference ($P = 0.0031$) between activation of membranes for 5 min and for 30 or 60 min. (Fig. 4).

Quantity of Membranes Necessary for Catalysis of β-Hematin Synthesis. The quantity of β-hematin produced depended on the number of erythrocytes from which membranes were prepared, and on the concentration of hematin. In the experiments summarized in Figure 5, 800 nmol of hematin was used and the quantities of membranes were varied. The highest mean amount of β-hematin was produced when activated membranes prepared from 1.5 × 10^9 erythrocyte were used. Increasing the number of erythrocytes to 3 × 10^9 did not increase β-hematin synthesis. On

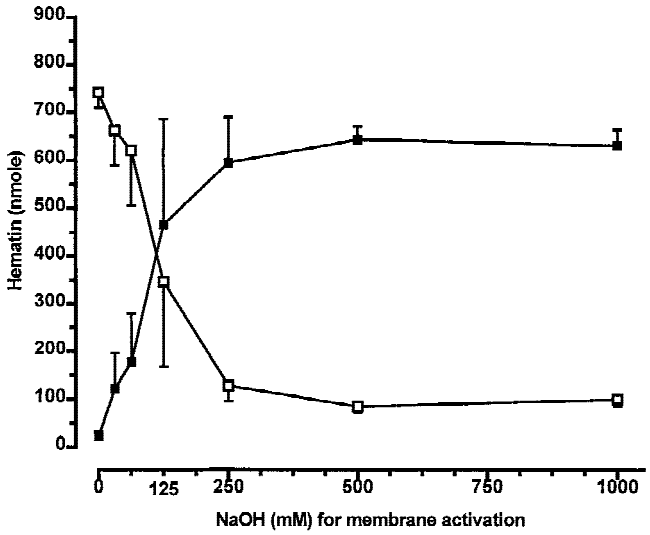

Figure 3. Effect of NaOH concentration on activation of membranes. Erythrocyte membranes (1 × 10^9) were exposed to the indicated mM of NaOH for 60 min at room temperature. After the exposure, the membranes were washed with phosphate solution, pH 7.6. To synthesize β-hematin, 4 ml of sodium acetate buffer, pH 5, and 800 nmol of hematin were added into each tube and incubated for 20 hr at 37°C. The experiments were carried out in triplicates and the mean concentrations ± SD of β-hematin produced (■) and those of unincorporated hematin (□) are shown.

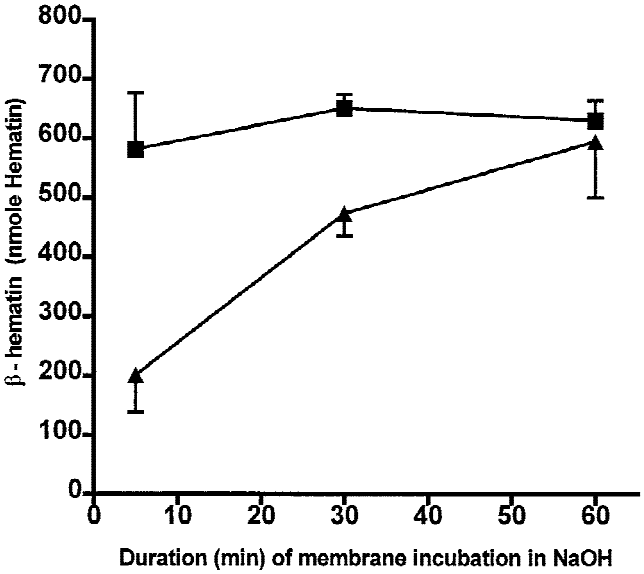

Figure 4. Effect of exposure time on membrane activation by NaOH. Membranes from 1.5 × 10^9 erythrocytes were incubated in 0.25 M (▲) or 1 M (■) for the indicated minutes at room temperature. The samples were then washed with phosphate solution, pH 7.6. β-hematin synthesis was carried out with 800 nmol of hematin. The experiments were done three times and the mean concentrations ± SD of the β-hematin produced are shown.

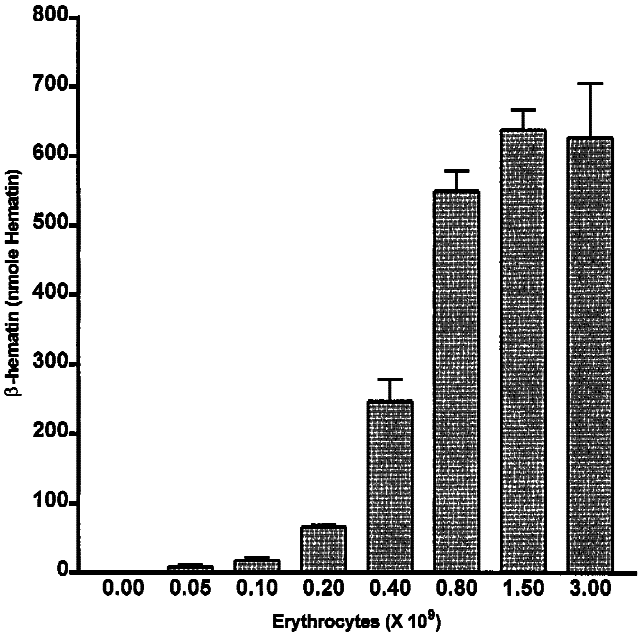

Figure 5. Effect of activated membrane concentration on β-hematin synthesis. The indicated quantities of membranes (number of erythrocytes) were activated through incubation for 5 min in 0.4 ml of 1 M NaOH, after which they were washed with phosphate solution, pH 7.6. To synthesize β-hematin, the activated membranes were mixed with 800 nmol of hematin in 0.5 M sodium acetate solution, pH 5, and the suspension was incubated at 37°C for 20 hr. The experiments were done in triplicates and the mean concentrations ± SD of hematin incorporated into β-hematin are shown.

the other hand, the amount of β-hematin produced decreased by about 90% when 1.5 to 2×10^8 erythrocytes were used for preparation of activated membranes.

Effect of Hematin Concentrations on β-Hematin Synthesis. In a total of 15 experiments, 50% to 90% (mean 70%) of 800 nmol of hematin was converted to β-hematin when incubated with 1 to 3×10^9 NaOH-activated membranes. When 400 or 200 nmol of hematin was used, 71% to 75% (mean 73% ± 2%, three experiments) was incorporated into β-hematin.

Activation of Erythrocyte Membranes with Other Chemicals to Catalyse β-Hematin Synthesis. Acetic acid (CH_3COOH), HCl, and SDS were tested to find out whether chemicals other than NaOH could activate membranes to catalyze β-hematin synthesis. These chemicals are commonly used in experimental production of β-hematin. 1.5×10^9 erythrocyte membranes were suspended in 0.4 ml of 3.5 M acetic acid or 1 M HCl or 4 ml of 2.5% SDS in 25 mM Tris buffer, pH 7.8. The suspensions were incubated at room temperature for 30 to 60 min and were then washed with phosphate buffer, pH 7.6, as was described above for membranes that were activated with NaOH. After centrifugation, there was no pellet in the sample that was treated with SDS, indicating that SDS dissolves membranes. To synthesize β-hematin, 4 ml of sodium acetate buffer, pH 5, and 800 nmol of hematin were added to the tubes and incubated for 20 hr at 37°C. The results are summarized in Table I. The mean quantity of β-hematin produced with NaOH-activated membranes was 30% and 93% higher than those produced with HCl-activated and CH_3COOH-activated membranes, respectively.

Table I. Conversion of Hematin to β-Hematin by Erythrocyte Membranes Treated with Different Chemicals

Membranes treated with	Hematin (nmole) converted to β-hematin
Phosphate buffer[a]	15 ± 4 (8)
SDS (2.5%)	17 ± 7 (4)
CH_3COOH (3.5 M)	42 ± 3 (3)
HCl (1 M)	386 ± 32 (3)
NaOH (1 M)	566 ± 84 (15)

Note. Membranes were incubated at room temperature for 60 min (in CH_3COOH and HCl), 30 min (in SDS pH 7.8), or 5 to 60 min (in NaOH). Eight hundred nanomoles of hematin was used in all samples for β-hematin formation. Results are given in mean ± SD. Number of experiments are indicated in brackets.
[a] Incubation in phosphate buffer served as normal control.

Discussion

It is still uncertain whether hematin molecules spontaneously form β-hematin at conditions that exist in the food vacuoles of malaria parasites, which are approximately pH 5 and 37°C. The initial suggestion that this happens spontaneously was based on detection of β-hematin bands by infrared spectroscopy (22). However, another study showed that FP aggregates formed in the absence of a catalyst or high temperature were soluble in bicarbonate buffer, SDS, or methanol, and therefore were not β-hematin (19). Nevertheless, spontaneous conversion of [^{14}C]-hemin to β-hematin that was resistant to washing with 2% SDS in 0.1 M sodium bicarbonate, pH 9.1, has been reported (23). The preferred method for separation of β-hematin from other FP aggregates involves exposure to SDS solution at pH 7 to 9 (11, 14, 16, 24). Unlike other FP aggregates, β-hematin is resistant to solubilization with aqueous SDS at approximately physiological pH values and below. In experiments carried out in the course of our earlier studies (14), but not published, the use of sodium bicarbonate, pH 9.1, in addition to 2.5% SDS for purification of β-hematin was found to be unnecessary.

In the present study, approximately 2 nmol of hematin was recovered from tubes in which 800 nmol of hematin was incubated alone in sodium acetate buffer, pH 5, for 20 hr at 37°C. It is unclear whether the recovered hematin was incorporated into β-hematin or was simply bound to the tubes, although it could not be washed away with SDS. Equally uncertain was the state of the 15 nmol of hematin that was recovered when 800 nmol of hematin was incubated with normal erythrocyte membranes. It is known that hematin binds tightly to various components of erythrocyte membranes (25, 26) and may not easily be washed away with SDS solution. In any case, there was no doubt that

large quantities of β-hematin were produced when hematin was incubated with activated erythrocyte membranes.

In this paper, the definition of activated membranes is limited to the ability of membranes that have been exposed to alkali or acids to promote β-hematin production. Although the membrane activation reported here was done at room temperature, an experiment carried out at 37°C with 1 M NaOH produced similar results.

Erythrocyte membranes are made up of approximately 52% proteins and 40% lipids. The lipids consist of phospholipid, cholesterol, and a small proportion of glycolipids (27). Chemical analyses of activated membranes were not carried out in this study, but it is known that when lipids are exposed to a strong base or acid, they hydrolyze to form fatty acids, alcohol, and other compounds. Therefore, it is believed that treatment with NaOH or HCl denatured the protein and hydrolyzed the lipid components of the membranes, possibly exposing fatty acids. Other investigators have used acetonitrile or chloroform to extract fatty acids from erythrocyte lysates and have converted hematin to β-hematin with the extracts (28, 29). In the present study, lipids were not specifically separated from other membrane components; therefore, it cannot be ruled out that altered membrane proteins were involved in the observed catalysis of β-hematin production.

The material that converted hematin to β-hematin in this study seemed to be insoluble in aqueous solutions. The activated membranes were suspended in 0.5 M sodium acetate, pH 5, and were passed through a membrane filter with a 0.2-μm pore size. It was found that the filtered solution did not catalyze β-hematin formation, whereas materials retained in the filter retained the catalytic activity. Similarly, when activated membranes were suspended in sodium acetate buffer and centrifuged cold for 60 min at 19,000 rpm, only the pellet converted hematin to β-hematin. On the other hand, when a suspension of activated membranes was passed through a filter with a 0.45-μm pore size, both the filtrate and the material that was retained in the filter were effective in converting hematin to β-hematin. These observations suggest that the minimum size of activated membrane fragments that catalyzed β-hematin synthesis was larger than 0.2 μm.

It is widely believed that the biological function of hemozoin production is to minimize intracellular accumulation of free FP in malaria parasites. Therefore, a mechanism that converts only a small fraction of available FP to β-hematin is unlikely to provide an effective protection. This delicate balance in parasite metabolism has so far received little attention in the experimental attempts to develop cell-free systems for hemozoin synthesis. In the present study, it was found that on the average, only 18% of the hematin incubated in sodium acetate buffer, pH 5, at 37°C for 20 hr was not incorporated into β-hematin. Seventy percent of the hematin was recovered in β-hematin and approximately 10% was lost possibly through technical errors. The question is whether intracellular conversion of FP to β-hematin in the proportion demonstrated in this study would be effective in protecting malaria parasites. Studies have shown that intraerythrocytic parasites incorporate virtually all the FP molecules that they generate from hemoglobin catabolism into β-hematin (30).

A highly effective method for β-hematin production in cell-free medium at 37°C has been identified in this study. Its biological relevance would depend on identification of a more physiological mechanism, including enzymes, for activation of membranes to catalyze β-hematin synthesis. The likely sources of membrane fragments are hemoglobin vesicles. During erythrocyte invasion, the *Plasmodium* merozoite invaginates a small portion of the host cell membrane to form parasitophorous vacuole membrane (PVM) that separates the parasite from the cytosol of the erythrocyte. Feeding is accomplished by invagination of the PVM and the parasite's plasma membrane (PPM) to form a vesicle in which hemoglobin is transported (31). Thus, the vesicle has an inner membrane that originated from PVM and an outer membrane that originated from PPM. Apparently, the first vesicle formed this way is transformed to a food vacuole that is bounded by one membrane. It has been suggested that the inner membrane of the initial vesicle becomes digested (32), leaving only the outer membrane to compartmentalize the metabolic processes. The outer membranes of subsequently formed vesicles are incorporated into the membrane of the food vacuole, while the inner vesicular membrane is transported into the food vacuole and lysed to release hemoglobin for digestion to generate FP (33). It is reasonable to propose that metabolic activities in the food vacuole would activate membrane fragments, possibly through hydrolysis, to catalyze the conversion of FP to β-hematin.

I thank Alex A. and Electricwala Q. Y. for technical assistance.

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