Biochemical and Biophysical Research Communications **290,** 595–601 (2002)
doi:10.1006/bbrc.2001.6231, available online at http://www.idealibrary.com on IDEAL®

A Physiochemical Mechanism of Hemozoin (β-Hematin) Synthesis by Malaria Parasite

Abhai K. Tripathi,* Satyendra K. Garg,† and Babu L. Tekwani*,[1]
*Division of Biochemistry, Central Drug Research Institute, Lucknow-226001 (UP), India; and
†Department of Microbiology, Dr. R. M. L. Avadh University, Faizabad (UP), India

Received December 3, 2001

Malaria parasite homogenate, the lipid extracts, and an unsaturated fatty acid, linoleic acid, which have been shown to promote β-hematin formation *in vitro*, were used to investigate the mechanism of hemozoin biosynthesis, a distinct metabolic function of the malaria parasite. *In vitro* β-hematin formation promoted by *Plasmodium yoelii* homogenate, the lipid extracts, and linoleic acid were blocked by ascorbic acid, reduced glutathione, sodium dithionite, β-mercaptoethanol, dithiothreitol, and superoxide dismutase. Oxidized glutathione did not show any effect. Preoxidized preparations of the lipids extracts or the *P. yoelii* homogenate failed to catalyze β-hematin formation. Depletion of oxygen in the reaction mixtures also inhibited the lipid-catalyzed β-hematin formation. Under the reaction conditions similar to those used for the *in vitro* β-hematin formation assay, the antioxidants and reducing agents mentioned above, except the DTT and β-mercaptoethanol, did not cause degradation of heme. β-Hematin formation was also inhibited by *p*-aminophenol, a free radical chain reaction breaker. Hemozoin biosynthesis within the digestive vacuoles of the malaria parasite may be a lipid-catalyzed physiochemical reaction. An oxidative mechanism may be proposed for lipid-mediated β-hematin formation, which may be mediated by generation of some free radical intermediates of heme.

Key Words: **β-hematin; hemozoin; glutathione *Plasmodium yoelii;* plasma; linoleic acid; malaria; superoxide dismutase.**

During intraerythrocytic growth and proliferation hemoglobin is utilized as a major source of nutrition by the malaria parasite (1). Heme (ferriprotoporphyrin IX), released as a toxic byproduct, is sequestered into the black brown crystalline form, commonly referred to as "hemozoin" or the malaria pigment (2, 3). Alternate mechanisms for detoxification of free heme have also been proposed, they include glutathione dependent heme degradation (4) and peroxidative degradation of heme (5). Heme detoxification functions of malaria parasite are potential targets for blood schizonticidal antimalarials (6, 7). Through X-ray diffraction and IR spectroscopy, the structure of hemozoin has been found to be similar to β-hematin, in which heme monomers are linked to each other through co-ordinate bond between iron of one unit to propionate carboxyl group of another ferriprotoporphyrin IX (2.3). The most recent crystallographic studies with β-hematin have indicated that reciprocal iron carboxylate bonds form heme dimers and these dimers are also linked through hydrogen bonding between the remaining propionate groups (8). The mechanism of hemozoin biosynthesis under physiological conditions within the parasite digestive vacuoles, the physiological site for hemoglobin degradation, is still under debate. Initial reports on this aspect described this as an enzymatic reaction (9, 10). However, later reports ruled out the presence of such an enzyme (11–13). In contrast to some of the reports proposing hemozoin biosynthesis to be a spontaneous chemical reaction (14), it is now established that, the formation of β-hematin under physiological conditions is possible only under specific biochemical/physiochemical environment (13, 15, 16). Different parasite factors have been demonstrated to promote β-hematin formation. Some lipids particularly phospholipids (13) or unsaturated fatty acids (16) or even the preformed hemozoin/β-hematin (11) have been demonstrated to initiate or catalyze formation of a product identical to that of hemozoin/β-hematin. The factor(s) catalyzing β-hematin formation in hemozoin or the homogenate of malaria parasite were extracted into acetonitrile (11, 12). Screening of a *P. falciparum* cDNA library with the antibodies developed against partially purified hemozoin, identified the clones of HRPII, which was found to initiate β-hematin formation *in vitro* (17). We have recently reported that the

[1] To whom correspondence and reprint requests should be addressed. Fax: 91-522-223405. E-mail: bltekwani@india.com.

0006-291X/02 $35.00

plasma from mice infected with *P. yoelii* could also promote β-hematin formation *in vitro* (18). We report herein some experimental evidences for explaining mechanism of β-hematin formation mediated by the malaria parasite homogenate and also by the lipids extracts, under the chemical/physiological conditions similar to that in the digestive vacuoles of malaria parasite, the physiological site of hemozoin biosynthesis.

MATERIALS AND METHODS

Materials. Hemin HCl, proteinase K, SDS, reducing agents and antioxidants were purchased from Sigma Chemical Co., U.S.A. Hemozoin was prepared from the erythrocytes infected with *P. yoelii* according to the method described earlier (19). β-Hematin was prepared chemically by incubating hemin in 4.5 M Na–acetate buffer (pH 4.8) overnight at 80°C (2). All other chemicals and reagents were of high purity analytical grade and were procured from the local market.

Parasite and the experimental host. Infection with *Plasmodium yoelii* was maintained in Albino mice (Swiss strain) as described earlier (20). Blood from uninfected or infected mice with parasitemia more then 50% was collected in sterile ACD by cardiac puncture, and centrifuged at 500*g* for 10 min at 4°C to pellet the erythrocytes. Plasma was collected in separate tube, centrifuged again at 10,000*g* for 10 min and passed through nitrocellulose membrane filter (0.22-μm pore size) to remove any membrane contamination or the traces of hemozoin, if present any. The clear plasma was stored in aliquots at −20°C till further use for extraction of lipids or the evaluation of β-hematin formation. The RBC pellet was washed twice with chilled phosphate buffered saline (10 mM, pH 7.4) and parasite homogenate was prepared as described earlier (20).

Preparation of total lipids extracts. Total lipids from the plasma of *P. yoelii*-infected mice, plasma of normal mice and *P. yoelii* homogenate were extracted by 4–5 vol of cold chloroform: methanol (2:1 v/v). After vigorous shaking the tubes were centrifuged for 15 min at 3000*g* at 4°C. Three distinct layers were formed viz., upper aqueous layer, the middle interface which contains the protein precipitate and the lower organic phase containing the total lipids. Upper, middle and the lower layers were collected in separate tubes. The solvent of the organic phase in the lipids extracts was evaporated under nitrogen and the residue was dissolved in DMSO and used for β-hematin formation assay.

In vitro β-hematin formation assay. Formation of β-hematin *in vitro* was assayed by the estimation of formation of the pigment insoluble in SDS (2.5% w/v) and bicarbonate buffer (20). The assay mixture contained, sodium acetate buffer (100 mM, pH 4.8), hemin (100 μM) in a total volume of 1.0 ml. Reaction was initiated by the addition of either of the factors mentioned, followed by an over-night (12–16 h) incubation at 37°C on a rotary shaker. Each assay was run at-least in triplicates. Two controls (each in triplicate) were run simultaneously, one without hemin and another without any initiating factor. The later control determines spontaneous formation of β-hematin, if any. The reaction was terminated by centrifugation at 10,000*g* for 10 min and the pellet was washed twice with Tris buffer (100 mM, pH 7.8) containing SDS (2.5% w/v) and once with bicarbonate buffer (100 mM, pH 9.2). The final β-hematin pellet was solubilized in 50 μl of 2 N NaOH and diluted further to 1.0 ml by 2.5% SDS. The absorbance of the solution was recorded at 400 nm. A milli-molar extinction coefficient of 91 mM^{-1} cm^{-1} was used for the quantification of β-hematin heme (20).

In vitro heme degradation assay. Degradation of heme in the presence of various antioxidants and reducing agents was studied under the conditions similar to those used for *in vitro* β-hematin formation assay, as described above. Under acidic conditions, i.e., pH 4.8, heme tends to aggregate and almost all the free heme as well as β-hematin, if formed any, are recovered in the pellet. The total heme recovered in the pellet is almost equal to the heme added into the reaction mixture. The reaction mixtures, containing acetate buffer (100 mM, pH 4.8), heme (100 mM) and the reducing/antioxidant agent (as specified) in a total volume of 1 ml, were incubated overnight (12–14 h) at 37 C with constant shaking. The reaction mixtures were centrifuged at 10,000*g* for 10 min. The pellets containing total heme (free heme + β-hematin, formed newly if any) were dissolved in 2 N NaOH (50 μl). The volume was made up to 1 ml with 2.5% SDS and total heme content was measured by recording absorbance at 400 nm as described earlier (20). Each assay was run at least in triplicates. Control reaction without any antioxidant or reducing agent was also set up simultaneously. Percent degradation of heme due to antioxidants and reducing agents was calculated by comparing the heme recovered in control reaction without any antioxidants/reducing agents.

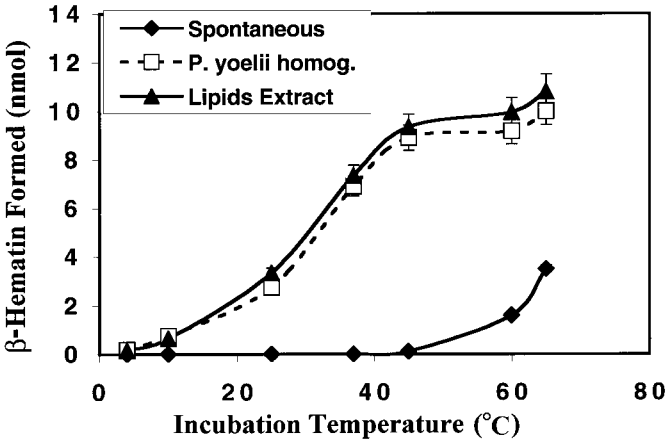

FIG. 1. Spontaneous (chemical) β-hematin formation and β-hematin formation activity of *Plasmodium yoelii* homogenate and the lipids extracts at varying temperatures of incubation. Each point represents the mean ± SD value of at least triplicate observations. Values are given as nanomoles of β-hematin formed in 1 ml of reaction mixture containing 1 nmol of preformed hemozoin heme (in the case of *Plasmodium yoelii* homogenate) or 10 μg of the lipid extract.

RESULTS

Formation of β-hematin was evaluated *in vitro* at different incubation temperatures, in the presence and absence of *P. yoelii* homogenate or the lipids extract (Fig. 1). The reaction mixtures were incubated at the specified temperature for 12–16 h and formation of β-hematin was assayed. In the reaction mixtures with the parasite homogenate or the lipids extracts significant β-hematin formation was observed even at 4 and 10°C, while no β-hematin formation was observed in the control reaction mixtures containing only heme in acetate buffer. At 37°C when the spontaneous β-hematin formation was still undetectable considerable formation of β-hematin occurred in the presence of the parasite homogenate or the lipids extracts. These observations confirmed that at physiological temperatures formation of hemozoin/β-hematin is not sponta-

TABLE 1

Effect of Proteinase k (Pk) Treatment on *in Vitro* β-Hematin Formation Activity of *Plasmodium yoelii* Homogenate and the Lipid Extracts

Experiment	β-Hematin formation (nmol)	β-Hematin formation % of control
P. yoelii homogenate (Control 1)	13.18 ± 0.36	**100**
P. yoelii homogenate (Pk treated)		
a. Total homogenate	1.63 ± 0.17	11.8
b. Pellet	13.05 ± 0.14	94.5
c. Supernatant	0.56 ± 0.09	4.0
P. yoelii homogenate + supernatant of Pk-treated homogenate	2.29 ± 0.13	16.6
Lipid extracts (Control 2)	11.02 ± 1.01	**100**
Lipid extracts + supernatant of Pk-treated homogenate	2.20 ± 0.10	19.9

Note. Values are means ± SD of at least triplicate observations.

neous but requires certain catalytic factors. At higher temperatures, i.e., 45, 60, and 65°C, a greater amount of β-hematin formation was observed in the presence parasite homogenate as well as the lipids extracts compared to that at 25 and 37°C. The results indicate better efficiency of β-hematin formation at higher temperatures. At non-physiological temperatures (60 and 65°C) significant spontaneous formation of β-hematin was also observed.

When the *P. yoelii* homogenate or the lipids extracts were subjected to heat treatment no significant effect was observed on their activity to promote β-hematin formation (data not shown). Treatment of parasite homogenate with proteinase k resulted in significant loss of its activity to promote β-hematin formation (Table 1). When proteinase k treated *P. yoelii* homogenate was further fractionated into the pellet and the soluble fractions, we found that the pellet fraction still promoted significant β-hematin formation, whereas, the soluble fraction did not. When the soluble fraction from the proteinase k treated *P. yoelii* homogenate was added to the assay mix with the original untreated *P. yoelii* homogenate or the lipids extract, this inhibited the β-hematin formation.

The factors in *P. yoelii* homogenate and plasma from infected mice, which promote β-hematin formation, were recovered in the lipids extracts. RBC lysate and plasma from the normal mice as such did not promote β-hematin formation but their lipids extracts promoted this reaction with the efficiency almost comparable to that of the lipids extracts from the parasite homogenate (Fig. 2). Presence of significant amount of the reducing agents and the efficient antioxidant machinery in these preparations might prevent the β-hematin formation. To test this hypothesis effect of antioxidants and reducing agents was tested on the parasite homogenate and the lipids extract mediated reactions of β-hematin formation. Chemical as well as biochemical reducing agents and antioxidants viz., sodium dithionite, DTT, β-mercaptoethanol, reduced glutathione, ascorbic acid and super oxide dismutase, when incorporated in the assay mixture, they all almost completely blocked the formation of β-hematin (Table 2). *p*-Aminophenol, a free radical chain reaction breaker (21) also blocked the reaction almost completely (Table 2). These observations, strongly suggests the involvement of some free radical chain reaction, in the process of β-hematin formation. It is well known that the peroxidation of lipids in the presence of free heme progresses as a chain reaction, leading to the generation of several lipid peroxidation products (21). The reaction mixtures fluxed with nitrogen (which results into depletion of oxygen) showed markedly low β-hematin formation compared to the control reaction mixture either *P. yoelii* homogenate, the lipids extract or linoleic acid as the initiating factors (Fig. 3). The *P. yoelii* homogenate and the lipids extract were preoxidized by incubation with 2 mM H_2O_2 for 1 h at 37°C. The oxidized samples were dialyzed with three changes of PBS to remove any H_2O_2. These oxidized samples were also unable to promote *in vitro* β-hematin formation.

To analyze that the effect of reducing agents and antioxidants mentioned above on β-hematin formation was not due to the degradation of heme, effect of the reducing agents and antioxidants was also tested on recovery of total heme contents during the incubation. No change in the recovery of total heme was noticed in the presence of various antioxidants and reducing agents as shown in Table 3, except DTT and β-mercaptoethanol, which caused 19 and 90% decrease respectively in recovery of heme in the pellet. This indicates that reduced glutathione, which has earlier been shown to cause degradation of heme at pH 7.0 (4) and other reducing agents/antioxidants do not degrade heme under the conditions of β-hematin formation.

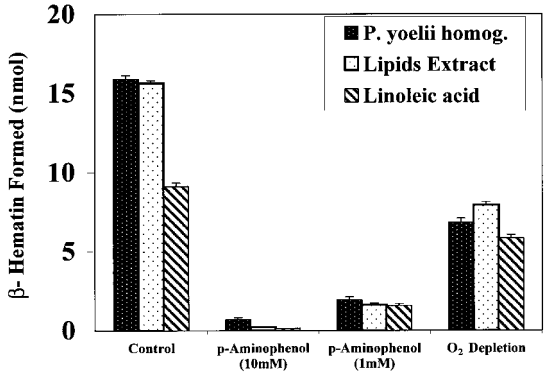

FIG. 2. Analysis of β-hematin formation activity in different preparations and their lipids extracts. Each bar represents the mean ± SD value of at least triplicate observations.

TABLE 2

Effect of Chemical as Well as Biochemical Reducing Agents and Antioxidants on β-Hematin Formation (HF) Activity of *P. yoelii* Homogenate, Lipid Extracts, and an Unsaturated Fatty Acid (Linoleic Acid)

	P. yoelii homogenate		Lipid extracts		Linoleic acid	
Expt.	HF (nmol)	HF (% of control)	HF (nmol)	HF (% of control)	HF (nmol)	HF (% of control)
Control	11.19 ± 0.69	100.00 ± 6.16	12.69 ± 0.46	100.00 ± 6.05	9.17 ± 0.21	100.00 ± 2.29
GSSG	11.81 ± 1.01	105.00 ± 9.02	13.08 ± 0.87	103.00 ± 3.62	9.00 ± 0.51	98.00 ± 5.56
GSH	1.97 ± 0.13	17.60 ± 1.16	1.08 ± 0.06	8.47 ± 0.47	0.87 ± 0.11	9.46 ± 1.19
SOD	3.19 ± 0.20	28.46 ± 1.86	2.01 ± 0.55	15.83 ± 1.65	1.33 ± 0.12	14.48 ± 1.30
A acid	0.70 ± 0.13	15.19 ± 2.05	0.34 ± 0.06	2.65 ± 0.47	0.43 ± 0.09	4.46 ± 0.99
Na dith	0.55 ± 0.10	4.91 ± 0.89	0.98 ± 0.14	7.70 ± 1.10	0.16 ± 0.01	1.08 ± 0.11
DTT	1.21 ± 0.15	10.80 ± 1.34	1.41 ± 0.55	11.11 ± 4.33	0.10 ± 0.01	1.08 ± 0.11
β-ME	0.56 ± 0.06	5.00 ± 0.58	0.00 ± 0.00	Nil	0.00 ± 0.00	Nil

Note. The final concentrations of all the reducing agents and antioxidants used are GSSG, 1 mM; GSH, 1 mM; SOD, 25 U/ml; A acid (ascorbic acid), 5 mM; Na dith (sodium dithionite), 5 mM; DTT, 5 mM; and β-ME, 1%. Values presented are means ± SD of at least triplicate observations.

DISCUSSION

The observations presented in this communication and also some earlier reports (13, 15, 16, 18) indicate that under physiochemical environment similar to that of the malarial digestive vacuoles, the site of hemoglobin degradation and the hemozoin biosynthesis, formation of β-hematin is neither an enzymatic reaction nor a spontaneous chemical process, but may be a physiochemical reaction. Recently hemozoin formation has also been referred to as a biomineralization process (22) The physiological conditions prevailing within the, acidic digestive vacuoles of the malaria parasite provide a suitable physiochemical environment for conversion of heme to β-hematin/hemozoin. Though, both proteins as well as lipids mediated β-hematin formation remain valid hypotheses but later seems to be more relevant, when mechanism of hemozoin synthesis is considered as a simple physiochemical reaction (23). Some reports earlier have shown loss of β-hematin formation activity of the parasite lysate by proteinase k and heat treatment (9, 24). However, contradictory reports have been made when different types of preparations were tested for promoting the β-hematin formation (11, 13, 24). The detailed investigations on this line suggest that the loss in β-hematin formation activity of the parasite homogenate by proteinase k treatment may not be due to the proteinaceous nature of the factors promoting β-hematin formation. But this may be rather be due to the fact that proteinase k treatment generated some peptides, which interact with heme monomers and prevent the formation of iron-carboxylate bond, a necessary reaction for β-hematin formation. The peptides corresponding to a repetitive heme binding motif of HRP II, a major protein produced by *P. falciparum*, have earlier been shown to inhibit the β-hematin formation *in vitro* (25).

The factors responsible for promoting β-hematin formation in parasite homogenate and also the plasma

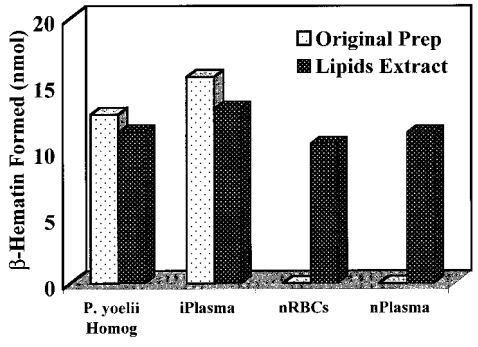

FIG. 3. Evaluation of effect of *p*-aminophenol and oxygen depletion on *in vitro* HF activity of *P. yoelii* lysate, lipid extracts, and linoleic acid. Oxygen was depleted by, flushing nitrogen through the reaction mixtures as described. Values represented by each bar are means ± SD of at least triplicate observations.

TABLE 3

Evaluation of Different Reducing Agents and Antioxidants for Degradation of Heme *in Vitro*

Experiment	% Recovery of heme	% Degradation
Control	97.17 ± 2.9	—
GSH	96.17 ± 6.0	0.47
GSSG	96.19 ± 5.1	1.00
DTT	78.57 ± 1.9	**19.14**
Na dithionite	99.12 ± 2.9	Nil
Ascorbic acid	97.28 ± 3.2	Nil
4-Aminophenol	98.16 ± 4.3	Nil
β-Mercaptoethanol	9.66 ± 1.3	**90.05**
SOD	98.45 ± 5.8	Nil
Oxygen depletion	97.18 ± 2.9	Nil

Note. The concentration of each reducing agent and the antioxidant was the same as mentioned in Table 2. Values presented are means ± SD of at least triplicate observations.

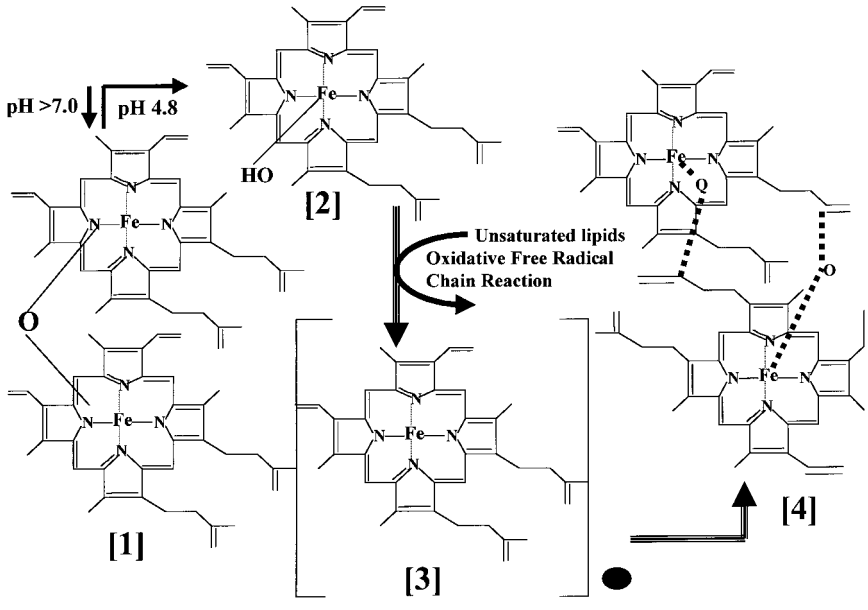

FIG. 4. Scheme depicting the proposed physiochemical mechanism of β-hematin formation. [1] μ-Oxo dimer form of heme at pH. 7.0. [2] Monomer form favored at pH 4.8 and in the macromolecular environment in parasite digestive vacuoles in the presence of lipids. [3] An activated free radical intermediate of heme may be generated due to oxidation of lipids. [4] β-Hematin is formed as a result of formation of reciprocal iron carboxylate bonds.

from *p. yoelii* infected mice were recovered in the chloform:methanol extracts containing the total lipids. Total lipids fraction of the malaria parasite contains 80% or more unsaturated fatty-acids, which is characteristically rich in oleic acid (26). A preliminary GC–MS analysis of the total lipid fractions obtained from *P. yoelii* lysate as well as plasma from infected mice, indicates the presence of mainly unsaturated fatty acids (data not shown). The characteristics of β-hematin formation in the presence of these lipids fractions were almost similar to that reported earlier for different lipid or protein factors (13). The product formed shows molecular and chemical characteristics similar to that of malarial hemozoin and β-hematin. In a recent report it has been shown that approximately 70% activity of cell free preparation of erythrocytes infected with *P. berghei* was recovered in the chloroform extract (16). The normal RBC homogenate or plasma from normal and healthy mice though did not initiate β-hematin formation *in vitro* but their lipids extracts did. This observation is also supported by a recent report which mentions that normal RBC ghost do not support β-hematin formation, but RBC ghosts treated with NaOH, HCl or acetic acid promoted β-hematin formation (27) The normal RBC lysate or the plasma of healthy control mice contain several antioxidants to nullify oxidative stress and prevent the oxidation of its lipid content (28). Malaria infection is accompanied with marked depletion of antioxidants in the blood and reduction in functions of the host to scavenge reactive oxygen intermediates (29–31). Presence of effective antioxidants, in normal erythrocytes and the plasma of normal mice may be preventing β-hematin formation. This hypothesis was verified experimentally by evaluating the effects of chemical as well as biochemical antioxidants and reducing agents on β-hematin promoted by either *P. yoelii* homogenate, lipids extracts or linoleic acid. As was assumed, all antioxidants/reducing agents tested blocked β-hematin formation. Depletion of O_2, by passing N_2 in the reaction mixture, pre-oxidation of lipids extracts as well as *P. yoelii* homogenate by H_2O_2, also inhibited the β-hematin formation. The inhibition of β-hematin formation by p-aminophenol, a free radical chain reaction breaker (21), indicates toward involvement of some free radical chain reaction in this process. Better efficiency of the reaction of β-hematin formation at higher temperature (>45°C) also indicate toward similar mechanism, as oxidation of lipids is more pronounced at higher temperature. The antioxidants and reducing agents did not degrade heme. Glutathione which has earlier been shown to cause degradation of heme at neutral pH (4) could not degrade the same at acidic pH, a necessary condition for β-hematin formation. On the basis of our observations and also some earlier reports, it may be postulated that the lipids which could be easily oxidized in the presence of free heme, in the acidic environment, may promote β-hematin formation. Oxidation of lipids seems to promote the formation of iron carboxylate bond. Based on the results presented in this communication a free radical dependent mechanism may be proposed for biosynthesis of hemozoin by malaria parasite (Fig. 4).

Acidic environment (pH around 4.5 to 5.0), depletion of antioxidants and presence of ferriprotoporphyrin IX in Fe^{3+} state and also a sustained free radical chain reaction are the necessary physiochemical conditions for β-hematin formation. Acidic environment favors the propionic acid moieties of FPIX in partially ionized state as the pK_a value of these has been estimated to be 5.0 (13). The recently proposed model based on X-ray crystallographic analysis of β-hematin indicates that the heme dimers are additionally linked to each other through hydrogen bonding between remaining propionate groups (8). Heme in solution tends to remain in equilibrium between monomeric and μ-oxo-dimeric forms (Fig. 4). The biomolecular environment of malaria digestive vacuoles, as mentioned above, allows heme predominantly in the monomeric form (32). The lipids, which could promote the β-hematin formation, may be postulated to cause shift in the equilibrium between monomeric and μ-oxo-dimeric forms to predominantly toward monomeric form. Subsequently free radical chain reaction initiated due to heme dependent peroxidation of lipids generates some activated intermediates of FPIX, which may facilitate the formation of iron carboxylate bond and synthesis of β-hematin/hemozoin. Based on these observations a scheme postulating an oxidative mechanism of β-hematin formation has been given in given Fig. 4. Understanding of mechanism of hemozoin biosynthesis by malaria parasite may be useful in development of novel inhibitors as potential antimalarials.

ACKNOWLEDGMENTS

The authors are grateful to the Director, CDRI, for providing necessary facilities. A.K.T. is grateful to the Council of Scientific and Industrial Research (CSIR), New Delhi, for the award of a Senior Research Fellowship. This paper bears CDRI Communication 6004.

REFERENCES

1. Francis, S. E., Sullivan, D. J., Jr., and Goldberg, D. E. (1997) Hemoglobin metabolism in the malaria parasite. *Annu. Rev. Microbiol.* **51,** 97–123.
2. Slater, A. F. G., Cerami, A., Swiggard, W. J., Orton, B. R., Flitter, W. D., Goldberg, D. E., Cerami, A., and Henderson, G. B. (1991) An iron carboxylate bond links the heme units of malaria pigment. *Proc. Natl. Acad. Sci. USA* **88,** 325–329.
3. Bohle, D. S., Dinnebier, R. E., Madsen, S. K., and Stephens, P. W. (1997) Characterization of the products of the heme detoxification pathway in malarial late trophozoite by X-ray diffraction. *J. Biol. Chem.* **272,** 713–716.
4. Ginsberg, H., Famin, O., Zhang, J., and Krugliak, M. (1998) Inhibition of glutathione dependent degradation of heme by chloroquine and amodiaquine as a possible basis for their antimalarial mode of action. *Biochem. Pharmacol.* **56,** 1305–1313.
5. Loria, P., Miller, S., Foley, M., and Tilley, L. (1999) Inhibition of the peroxidative degradation of heme as the basis of action of chloroquine and other quinoline antimalarials. *Biochem. J.* **339,** 367–670.
6. Sullivan, D. J., Jr., Gluzman, I. Y., Russe, L. D. G., and Goldberg, D. E. (1996) On the molecular mechanism of chloroquine's antimalarial action. *Proc. Natl. Acad. Sci. USA* **93,** 11865–11870.
7. Slater, A. F. (1993) Chloroquine: Mechanism of drug action and resistance in *Plasmodium falciparum*. *Pharmacol. Ther.* **57,** 203–235.
8. Pagola, S., Stephens, P. W., Bohle, D. S., Kosar, A. D., and Madsen, S. K. (2000) The structure of malaria pigment β-hematin. *Nature* **404,** 307–310.
9. Slater, A. F. G., and Cerami, A. (1992) Inhibition by chloroquine of a novel heme polymerase enzyme activity in malaria trophozoite. *Nature* **355,** 167–169.
10. Chou, A. C., and Fitch, C. D. (1992) Heme polymerase: Modulation by chloroquine treatment of a novel heme polymerase enzyme activity in malaria. *Life Sci.* **51,** 2073–2078.
11. Dorn, A., Stoffel, R., Matile, H., Bubendorf, A., and Ridley, R. (1995). Malarial hemozoin/β-hematin supports heme polymerization in the absence of protein. *Nature* **374,** 269–271.
12. Bendrat, K., Berger, B. J., and Cerami, A. (1995) Heme polymerization in malaria. *Nature* **378,** 138.
13. Dorn, A., Vippagunta, S. R., Matile, H., Bubendorf, A., Vennerstrom, and Ridley, R. G. (1998) A comparison and analysis of several ways to promote hematin (heme) polymerization and an assessment of its initiation *in vitro*. *Biochem. Pharmacol.* **55,** 737–747.
14. Egan, T. J., Ross, D. C., and Adams, P. A. (1996) Quinoline antimalarial drugs inhibit spontaneous formation of β-hematin (malaria pigment). *FEBS Lett.* **352,** 54–57.
15. Pandey, A. V., and Tekwani, B. L. (1996) Formation of malaria pigment/β-hematin under physiological conditions is not spontaneous. *FEBS Lett.* **393,** 189–192.
16. Fitch, C. D., Cai, G. J., Chen, Y. F., and Shoemaker, J. D. (1999) Involvement of lipids in ferriprotoporphyrine IX polymerization in malaria. *Biochim. Biophys. Acta* **1454,** 31–37.
17. Sullivan, D. J., Gluzman, I. V., and Goldberg, D. E. (1996) *Plasmodium* hemozoin formation mediated by histidine rich proteins. *Science* **271,** 219–222.
18. Tripathi, A. K., Gupta, A., Garg, S. K., and Tekwani, B. L. (2001) *In vitro* b-hematin formation assay with plasma of mice infected with *Plasmodium yoelii* and other parasite preparations: Comparative inhibition with quinoline and endoperoxide antimalarials. *Life Sci.* **69,** 2725–2733.
19. Pandey, A. V., Tekwani, B. L., and Pandey, V. C. (1995) Characterization of haemozoin from liver and spleen of mice infected with *Plasmodium yoelii*, a rodent malaria parasite. *Biomed. Res.* **16,** 115–120.
20. Pandey, A. V., Singh, N., Tekwani, B. L., Puri, S. K., and Chauhan, V. S. (1999) Assay of β-hematin formation by malaria parasite. *J. Pharm. Biomed. Anal.* **20,** 203–208.
21. Tappel, A. L. (1953) The mechanism of the oxidation of unsaturated fatty acids catalyzed by hematin compounds. *Arch. Biochem. Biophys.* **44,** 378–395.
22. Zigler, J., Linck, R., and Wright, D. W. (2001) Heme aggregation inhibitors: Antimalarial drugs targeting an essential biomineralization process. *Curr. Med. Chem.* **8,** 171–189.
23. Tripathi, A. K., and Tekwani, B. L. (1999) Formation of β-hematin in malaria parasite: Lipids edge over the proteins as possible mediator. *J. Parasitic Dis.* **23,** 61–70.
24. Fitch, C. D., and Chou, A. C. (1996) Heat labile and heat stimulable heme polymerase activities in *Plasmodium berghei*. *Mol. Biochem. Parasitol.* **82,** 261–264.
25. Pandey, A. V., Joshi, R., Tekwani, B. L., Singh, R. L., and Chauhan, V. S. (1997) Synthetic peptides corresponding to a repetitive sequence of malarial histidine rich protein bind heme and inhibit hemozoin formation *in vitro*. *Mol. Biochem. Parasitol.* **90,** 281–287.

26. Holz, G. G. (1977) Lipids of the malarial parasite. *Bull. WHO* **55,** 237–248.

27. Orjih, A. U. (2001) On the mechanism of hemozoin production in malaria parasite: Activated erythrocyte membranes promote b-hematin synthesis. *Exp. Biol. Med.* **226,** 746–752.

28. Eaton, J. W., Eckman, J. R., Berger, E., and Jacob, H. S. (1976) Suppression of malaria infection by oxidant sensitive host erythrocytes. *Nature* **264,** 758–760.

29. Das, B. S., and Nanda, N. K. (1999) Evidence for erythrocyte lipid peroxidation in acute falciparum malaria. *Trans. Royal Soc. Trop. Med. Hyg.* **93,** 58–62.

30. Simoes, A. P., Van den berg, J. J., Roelfsen, B., and Op den Kamp, J. A. (1992) Lipid peroxidation in *Plasmodium falciparum* parasitized human erythrocytes. *Arch. Biochem. Biophys.* **298,** 651–657.

31. Buffinton, G. D., Hunt, N. H., and Cow den, W. B. (1988) Detection of short chain carbonyl products of lipid peroxidation from malaria parasites (*Plasmodium vinckei*) infected red blood cells exposed to oxidative stress. *Biochem. J.* **249,** 63–68.

32. Moreau, S., Perly, B., Chachaty, C., and Deleuze, C. A. (1985) Nuclear magnetic resonance study of the interaction of antimalarial rugs with porphyrins. *Biochim. Biophys. Acta* **840,** 107–116.