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Chloroquine inhibits heme-dependent protein synthesis in *Plasmodium falciparum*

(malaria parasite/cell-free system/translation/protein synthesis *in situ*/eukaryotic initiation factor 2α kinase)

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ABSTRACT **A cell-free protein-synthesizing system has been reconstituted using the S-30 fraction or ribosomes and the S-100 fraction from *Plasmodium falciparum*. Addition of heme *in vitro* stimulates cell-free protein synthesis strikingly. Chloroquine inhibits the heme-dependent protein synthesis in the parasite lysate. The drug has also been found to inhibit parasite protein synthesis *in situ* at therapeutic concentrations soon after addition to parasite cultures. Ribosomes as well as the S-100 fraction isolated from such chloroquine-treated cultures are defective in protein synthesis. Addition of hemin plus glucose 6-phosphate or high concentrations of GTP, cAMP, and an active preparation of eIF-2 to the parasite cell-free system restores protein synthesis to a significant extent in chloroquine-treated cultures. Under conditions of inhibition of protein synthesis *in situ* by chloroquine in the culture, the parasite eukaryotic initiation factor 2α- (eIF-2α) is phosphorylated in the parasite lysate to a greater extent than that observed in the control culture. Addition of hemin *in vitro* suppresses this phosphorylation. eIF-2α kinase activity is present in the parasite lysate and is not a contaminant derived from the human erythrocytes used to culture the parasite. The heme–chloroquine interactive effects can also be demonstrated with purified eIF-2α kinase from rabbit reticulocyte lysate. It is proposed that chloroquine inhibits heme-dependent protein synthesis in the parasite and this is an early event mediating the growth-inhibitory effects of the drug.**

It is rather surprising that the mechanism of action of chloroquine, a widely used antimalarial drug, is not fully understood. An early suggestion based on intercalation of chloroquine with DNA and inhibition of DNA synthesis (1, 2) has not found favor in view of the millimolar concentrations required for the purpose, whereas nanomolar concentrations in the culture are growth inhibitory. There has, however, been a recent attempt to revive this theory (3, 4). Chloroquine is known to accumulate in the acid vesicles of the parasite and increase the pH and osmolality of the vesicles, leading to swelling and membrane leakiness (5). However, it has also been suggested that alkalinization of the vesicles *per se* is not adequate to explain the toxicity of chloroquine, and substantial alkalinization does not occur within the therapeutic drug concentration range (6). It is known that intraerythrocytic *Plasmodium* digests up to 75% of the host cell hemoglobin in the acid food vacuoles, and the generated heme is present as an inert complex referred to as malaria pigment or hemozoin (7). Chloroquine has been demonstrated to bind to ferriprotoporphyrin IX with high affinity *in vitro* and *in situ*, and it has been suggested that the drug can sequester heme, interfering with the formation of the inert pigment, and the complex can lyse the parasite membrane (7–9). However, there have been arguments against this theory (3, 6).

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Heme is required for protein synthesis in the reticulocyte lysate (10, 11) and a few nonerythroid systems as well (12). We have investigated the possibility whether chloroquine can inhibit the translation process of the parasite by virtue of complex formation with heme, if the parasite protein synthesis were to be heme dependent. Earlier, the protein synthesis rates obtained with *Plasmodia* infecting birds and animals were low, and it was generally concluded that chloroquine is not a potent inhibitor of protein synthesis (13, 14). In the present study we demonstrate that an early event in chloroquine action is an inhibition of heme-dependent protein synthesis in the parasite.

MATERIALS AND METHODS

Maintenance of the Malarial Parasite. *Plasmodium falciparum* FCK 2 strain, a local isolate from Karnataka State, India, was cultured in human O-positive washed erythrocytes *in vitro* using standard techniques (15).

Synchronization of Parasites in Culture. Parasites were synchronized using 5% D-sorbitol (16) and the cultures were pooled at late trophozoite stage with 12–15% parasitaemia. Parasites were released from the erythrocytes with 0.15% saponin (17).

Preparation of the Parasite S-30 Fraction, Isolation of Ribosomes, and Constitution of the Cell-Free System. The free parasites were lysed with 4 vol of ice-cold lysing solution [100 mM KCl/7 mM $Mg(OAc)_2$/380 mM sucrose/6.5 mM 2-mercaptoethanol/50 mM Tris·HCl, pH 7.4/0.14% (vol/vol) Triton X-100] as described for *Plasmodium knowlesi* (18). Leupeptin (15 μM) and placental ribonuclease inhibitor (20 units) were added to the free parasites before adding lysing solution. The suspension was then centrifuged at 12,000 × *g* for 20 min at 4°C. The supernatant was passed through a G-50 spun column and used as the S-30 fraction. The 12,000 × *g* supernatant was centrifuged twice at 10,000 × *g* for 10 min and the second 10,000 × *g* supernatant was centrifuged at 105,000 × *g* for 60 min at 4°C to sediment ribosomes. The ribosomal pellet was rinsed with 200 μl of buffer A [0.25 M sucrose/25 mM KCl/5 mM $Mg(OAc)_2$/50 mM Tris·HCl, pH 7.4] and resuspended in the same buffer. The postribosomal supernatant was passed through a G-50 spun column and used as the S-100 fraction. Cell-free protein synthesis was carried out in 50 μl containing 20 mM Hepes (pH 7.5), 1 mM ATP, 0.1 mM GTP, 5 mM creatine phosphate, 45 units of creatine phosphokinase per ml, 0.5 mM glucose 6-phosphate, 7 mM $Mg(OAc)_2$, 110 mM potassium acetate, 0.375 mM spermidine, 2.5 mM dithiothreitol, 2 μg of wheat germ tRNA, 40 μM (each) of 19 amino acids except methionine (20 μCi, 800 Ci/mmol; 1 Ci = 37 GBq; Amersham), and the S-30

Abbreviations: eIF-2, eukaryotic initiation factor 2; eIF-2α, α subunit (38 kDa) of eIF-2; HRI, heme-regulated inhibitor.

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fraction or S-100 plus ribosomes (10 μg of rRNA). Hemin, when added, was at 15 μM concentration. Chloroquine phosphate and hemin solutions were prepared fresh and were preincubated together on ice for 5 min before the addition of other components. Reactions were initiated with the addition of the S-30 fraction or ribosomes and incubated at 30°C, samples (5 μl) were spotted onto Whatman 1MM filter discs, and radioactivity was measured after washing with trichloroacetic acid (12). All assays were run in triplicate and zero time values were subtracted.

Preparation of Reticulocyte Lysate and Cell-Free Protein Synthesis. Reticulocyte lysate was prepared and cell-free protein synthesis with [^{35}S]methionine was carried out in 25 μl using standard protocols (10, 12).

Phosphorylation of Proteins in Reticulocyte and Parasite Lysate and NaDodSO$_4$/PAGE. Phosphorylation of the parasite eukaryotic initiation factor 2α- (eIF-2α) was assessed in the parasite lysate prepared from *in situ* chloroquine-treated and control cultures. Reaction mixtures were the same as that for protein synthesis except that unlabeled ATP was replaced with [γ-^{32}P]ATP (25 μCi, 3000 Ci/mmol; Amersham) and [^{35}S]methionine was omitted. The protein synthesis mixture (50 μl) was incubated at 30°C for 5 min before [γ-^{32}P]ATP addition. Samples (5 μl) were removed 2 min after ^{32}P addition and mixed with equal volumes of NaDodSO$_4$/protein dissociation buffer. These samples were analyzed on 10% polyacrylamide gels (0.1% NaDodSO$_4$; bisacrylamide:acrylamide ratio, 1:38.5), stained with Coomassie blue, and subjected to autoradiography (19). The rest of the sample was processed for immunoprecipitation. Similar phosphorylation experiments were also carried out with the rabbit reticulocyte lysate.

Immunoprecipitation of Parasite eIF-2α. Samples (2 × 10^5 cpm) were boiled for 3 min in 2% NaDodSO$_4$ sample buffer, diluted 1:10 with buffer containing 1% Triton X-100, 50 mM Tris·HCl (pH 7.4), 150 mM NaCl, and 5 mM EDTA, and mixed with 2 μl of rabbit reticulocyte eIF-2 antiserum. Treatment with *Staphylococcus aureus* cells, washing, and extraction were carried out as described by Maccecchini *et al.* (20). The *S. aureus* cells were removed by centrifugation and the supernatant was subjected to NaDodSO$_4$/10% PAGE and autoradiography.

Effect of Chloroquine *in Situ* on Protein Synthesis by the Metabolic Labeling of Parasites with [^{35}S]Methionine. Parasite cultures (late trophozoites) were incubated at 37°C with or without chloroquine (3 μM) for 30 min and then [^{35}S]methionine (180 μCi/ml of culture) was added to the cultures. After 30 min of labeling, the free parasite pellet was washed three times with excess phosphate-buffered saline, solubilized in 4 vol of buffer containing 50 mM Tris·HCl (pH 8.0), 5 mM EDTA, 5 mM EGTA, 5 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, and 1% (vol/vol) Nonidet P-40, and centrifuged. The parasite proteins in 20 μl of the supernatant were precipitated with 10% trichloroacetic acid. The precipitate was solubilized in 2% NaDodSO$_4$ buffer and subjected to NaDodSO$_4$/PAGE followed by fluorography. The amount of protein loaded in each lane was 140 μg.

Effect of Hemin and Chloroquine *in Vitro* on Heme-Regulated Inhibitor (HRI) Activity. HRI was purified from reticulocytes and assayed for autophosphorylation as well as eIF-2α kinase activities as described by Chen *et al.* (21). Protein kinase assays (20 μl) contained 20 mM Tris·HCl (pH 7.7), 2 mM Mg(OAC)$_2$, 40–60 mM KCl, 25–50 xM [γ-^{32}P]ATP (20–30 Ci/mmol), and HRI preparation (50–100 ng). For the eIF-2α kinase assay, 0.5 μg of rabbit reticulocyte eIF-2 was included (21). Chloroquine and hemin were added at 5 and 10 μM concentrations. The reactions were initiated by the addition of [γ-^{32}P]ATP and incubation was at 30°C for 10 min. The reactions were terminated by the addition of NaDodSO$_4$ denaturing buffer and analyzed by NaDodSO$_4$/PAGE and autoradiography.

RESULTS

Chloroquine Inhibits Heme-Dependent Protein Synthesis in Rabbit Reticulocyte Lysate. The reticulocyte lysate used was stimulated 3-fold with 20 μM hemin during a 30-min incubation period. The response to hemin added 10 min after the start of incubation was found to be decreased by 50%. Chloroquine at a concentration of 10 μM blocked the heme-mediated stimulation by 80% (Fig. 1). Higher concentrations of chloroquine did not lead to any significant increase in the extent of inhibition obtained. Again, chloroquine at these concentrations did not inhibit the basal protein synthesis obtained in the absence of added hemin.

Heme Stimulates Protein Synthesis in Parasite Lysate and Chloroquine Inhibits This Process. Addition of heme (15 μM) to the parasite lysate (S-30) resulted in stimulation of parasite protein synthesis by 3-fold (Fig. 2). The effect of a range of heme concentrations was tested and 15 μM was found to be optimal. Chloroquine at 8 μM was found to strikingly inhibit the heme-dependent increase in protein synthesis. An increase in chloroquine concentration to 16 μM only marginally increases the extent of inhibition. At these chloroquine concentrations, protein synthesis in the absence of added heme is not inhibited by more than 15% (data not presented).

Chloroquine Inhibits Protein Synthesis in *P. falciparum in Situ*. In one set of experiments, [^{35}S]methionine was added to *in situ* chloroquine-treated cultures and incorporation into the parasite proteins was measured. [^{35}S]Methionine incorporation in parasite proteins measured over a 30-min labeling period is inhibited by 50–60% in the drug-treated cultures (control cultures, 47,484 cpm/mg of protein; chloroquine-treated cultures, 17,472 cpm/mg of protein). The autoradiogram (Fig. 3*A*) and the densitometric scan (Fig. 3*B*) indicate that the inhibition is generalized as would be the case if the translation process is interfered with. Similar results were obtained when chloroquine was added *in situ* at a concentration of 0.3 μM to the cultures (data not presented). In the second set, ribosomes and S-100 fraction were prepared from control and chloroquine-treated cultures (3 μM chloroquine

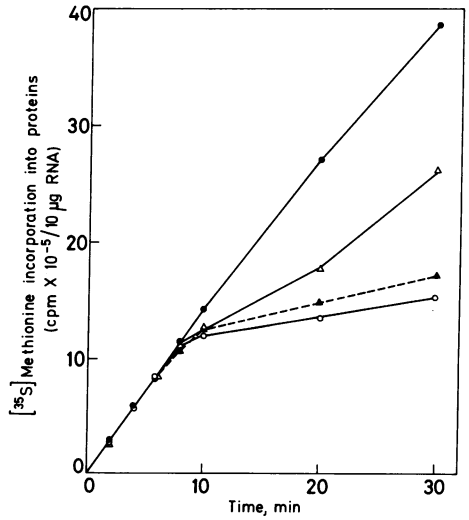

FIG. 1. Inhibition of heme-dependent protein synthesis by chloroquine in rabbit reticulocyte lysate. Protein synthesis was assayed as described in the text. The reaction mixtures (25 μl), which contained the following, were incubated at 30°C and processed: 20 μM hemin added at 0 min (●); 20 μM hemin added at 10 min (△); 20 μM hemin plus 10 μM chloroquine added at 0 min (▲); no additions (○).

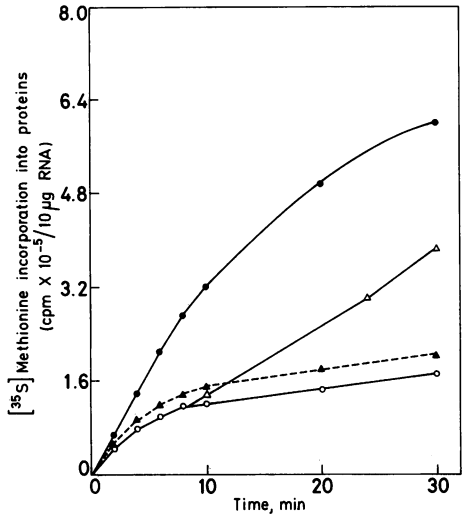

FIG. 2. Inhibition of heme-dependent protein synthesis by chloroquine in parasite lysates. Protein synthesis was assayed as described in the text. The parasite S-30 preparation and other components (50 μl), which contained the following, were incubated at 30°C and processed: 15 μM hemin added at 0 min (●); 15 μM hemin added at 8 min (△); 15 μM hemin plus 8 μM chloroquine added at 0 min (▲); no additions (○).

for 30 min) and cell-free protein synthesis was assessed in mixed preparations. Maximum inhibition in [^{35}S]methionine incorporation is seen when the ribosomes and S-100 fraction are from chloroquine-treated cultures, indicating that these fractions are defective in supporting protein synthesis (Table 1).

Reversal of *in Situ* Chloroquine Effects in Cell-Free Parasite Protein Synthesis. The effects of added hemin plus glucose 6-phosphate or high concentrations of GTP, cAMP, and the active eIF-2 preparation (from reticulocytes) were examined in the cell-free system prepared from control and chloroquine-treated cultures, since it has been reported that these compounds can individually counteract the inhibited protein synthesis in heme-deficient reticulocyte lysates (22–24). Addition of hemin plus glucose 6-phosphate, GTP, cAMP, or the active preparation of eIF-2 to the cell-free system prepared

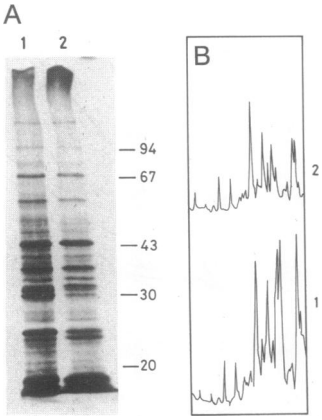

FIG. 3. Effect of chloroquine *in situ* on [^{35}S]methionine incorporation into total proteins of the parasite. The incorporation of [^{35}S]methionine into parasite proteins was determined by analyzing samples from control and chloroquine-treated cultures on NaDodSO$_4$/7.5% PAGE followed by fluorography. (*A*) Autoradiogram of parasite proteins obtained from normal (lane 1) and chloroquine-treated (lane 2) cultures. Molecular mass is indicated in kDa. (*B*) Densitometric scan of lanes 1 and 2 shown in *A*. The autoradiogram was scanned with an LKB 22002 Ultra Scan laser densitometer.

Table 1. Cell-free protein synthesis with control and *in situ* chloroquine-treated *P. falciparum* cultures

Addition	[^{35}S]Methionine incorporation into proteins, cpm × 10^{-3} per 10 μg of rRNA
Ribosome (C)	
+ S100 (C)	163.0
+ S100 (CQ)	111.8
Ribosome (CQ)	
+ S100 (C)	78.6
+ S100 (CQ)	69.6

Cultures were incubated with chloroquine (3 μM) for 30 min. Parasite ribosomes and S-100 fractions from control (C) and chloroquine-treated (CQ) cultures were prepared and cell-free protein synthesis was carried out as described in text.

from chloroquine-treated cultures is able to counteract the inhibited protein synthesis to a significant extent (Table 2).

Chloroquine-Treated Parasite Culture Shows Enhanced eIF-2α Phosphorylation in the Cell-Free System. It is well established that heme deficiency leads to phosphorylation of the initiation factor subunit eIF-2α by HRI, and this results in the inhibition of protein synthesis in reticulocyte lysates (22). Addition of chloroquine *in vitro* to reticulocyte lysate does result in enhanced phosphorylation of a band that comigrates with purified rabbit reticulocyte eIF-2α, overcoming the suppressive effects of added hemin (Fig. 4). Next, the phosphorylation reaction was carried out in parasite lysate (S-30) prepared from normal and chloroquine-treated *P. falciparum* cultures and the products were analyzed by NaDodSO$_4$/PAGE and autoradiography. The band corresponding to a 38-kDa protein and comigrating with the purified rabbit reticulocyte eIF-2α shows a higher level of phosphorylation in the cell-free system prepared from chloroquine-treated cultures than control. Addition of hemin *in vitro* inhibits phosphorylation of this protein (Fig. 5*A*). An additional band with a molecular mass of 33 kDa also shows some increase in phosphorylation in chloroquine-treated cultures, which is inhibited by the addition of hemin *in vitro*. However, the identity of this band is not clear. Quantitative immunoprecipitation analysis also reveals that the parasite eIF-2α in *in situ* chloroquine-treated cultures is phosphorylated to a greater extent than that from control cultures in the cell-free system (Fig. 5 *B* and *C*). This phosphorylation is significantly decreased in the presence of hemin. The eIF-2 polyclonal antibody picks up two other strongly labeled

Table 2. Counteraction of the effects of chloroquine-mediated heme deficiency on cell-free protein synthesis in *P. falciparum* cultures

	[^{35}S]Methionine incorporation into proteins, cpm × 10^{-3} per 10 μg of rRNA			
	Control		CQ treated	
Addition	10 min	20 min	10 min	20 min
Basal (no addition)	124.2	162.0	62.4	78.6
Hemin, 20 μM	163.2	292.4	101.2	146.2
+ Glc-6-*P*	326.4	505.2	247.0	324.2
Hemin, 5 μM				
+ Glc-6-*P*	151.2	186.2	79.2	89.8
+ Glc-6-*P* + cAMP	273.0	452.2	240.6	313.0
+ Glc-6-*P* + GTP	285.0	478.4	231.2	304.2
eIF-2 + Glc-6-*P*	264.2	424.8	180.0	231.2

Glc-6-*P*, glucose 6-phosphate; CQ, chloroquine. The S-30 fraction was used for protein synthesis. Glc-6-*P* was added at a concentration of 500 μM; GTP and cAMP were added at concentrations of 2 mM and 10 mM, respectively. The rabbit reticulocyte eIF-2 (5 μg) used contained α, β, and γ subunits and the 67-kDa protein.

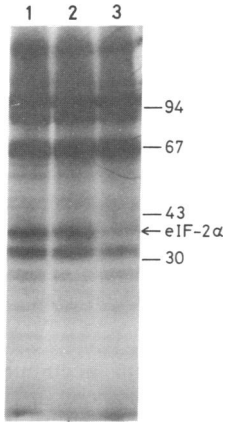

FIG. 4. Effect of chloroquine on eIF-2α phosphorylation in reticulocyte lysate. Lane 1, control (no addition); lane 2, 10 μM chloroquine plus 20 μM hemin; lane 3, 20 μM hemin. Purified eIF-2 and molecular mass markers (given in kDa) were run on separate slots and their migration positions are indicated.

bands, which could be the β and γ subunits, and a few other minor bands. However, none of the bands picked up by the antibody, other than eIF-2α, shows increased phosphorylation in cell-free extracts prepared from chloroquine-treated cultures.

eIF-2α Kinase Activity Is of Parasitic Origin. Attempts to detect HRI in parasite S-30 lysates using a monoclonal antibody against rabbit reticulocyte HRI were not successful. However, this monoclonal antibody is known to be specific to rabbit reticulocyte HRI and does not even react with human HRI (J.-J. Chen and I. M. London, personal communication). But a significant level of phosphorylation of the parasite ribosomal proteins can be detected only with the parasite S-100 preparation (Fig. 6*A*). Significant phosphorylation of any of the parasite ribosomal proteins over endogenous levels (ribosomes in the absence of parasite S-100 or erythrocyte lysate) cannot be detected with the human erythrocyte lysate prepared from the culture except for the phosphorylation of a high molecular mass protein band. The final wash of the parasite pellet also does not have any detectable phosphorylating activity. It is also known that there is little or no detectable HRI activity present in mature erythrocyte (25). In addition, the parasite S-100 preparation, when incubated with purified reticulocyte eIF-2 preparation, is able to phosphorylate a specific band corresponding to eIF-2α (Fig. 6*B*).

Effects of Chloroquine and Hemin *in Vitro* on Purified Rabbit Reticulocyte HRI Activity. It was of interest to examine whether chloroquine would have any effect on purified HRI activity *in vitro*. This cyclic AMP-independent protein kinase specifically phosphorylates eIF-2α and also undergoes autophosphorylation (26). J.-J. Chen and I. M. London (personal communication) carried out the experiments with purified kinase from rabbit reticulocyte lysate and the results

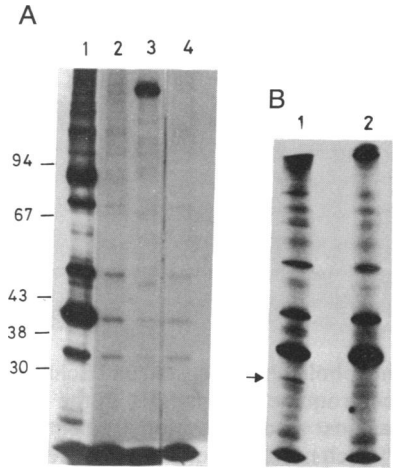

FIG. 6. Phosphorylating activity of parasite S-100 preparation and human erythrocyte lysate. (*A*) Parasite ribosomes, S-100 preparation, the erythrocyte lysate after saponin lysis and release of the parasite, as well as the final wash of the parasite were all checked for their ability to phosphorylate the parasite ribosomal proteins. Lane 1, parasite ribosomes plus S-100 preparation; lane 2, parasite ribosomes plus buffer A; lane 3, parasite ribosomes plus erythrocyte lysate; lane 4, parasite ribosomes plus phosphate-buffered saline wash of the parasite pellet. Incubations were carried out in the absence of hemin. Molecular mass is indicated in kDa. (*B*) Parasite S-100 preparation was incubated in presence (lane 1) and absence (lane 2) of purified reticulocyte eIF-2 preparation (1 μg) and phosphorylation was carried out as described earlier. The arrow indicates the position of eIF-2α.

are presented in Fig. 7 *A* and *B*. It is interesting to note that chloroquine at 5 or 10 μM has very little effect on autophosphorylation of the kinase (Fig. 7*A*). Hemin at 5 or 10 μM inhibits this activity. At equimolar concentrations, chloroquine counteracts the effects of hemin but not completely. Chloroquine at 5 μM and certainly at 10 μM stimulates eIF-2α phosphorylation activity (Fig. 7*B*). Hemin, as expected, inhibits eIF-2α phosphorylation and chloroquine at equimolar concentrations counteracts the effects of hemin significantly.

DISCUSSION

Evidence for formation of the chloroquine–hemozoin complex in the vacuole *in situ* is not unequivocal (3, 6) and the earliest site of lysis due to chloroquine does not appear to be the vacuolar membrane, although the drug has been suggested to act through this mechanism (7–9). Ginsburg and Geary (6) suggest that the drug may be directly inhibiting

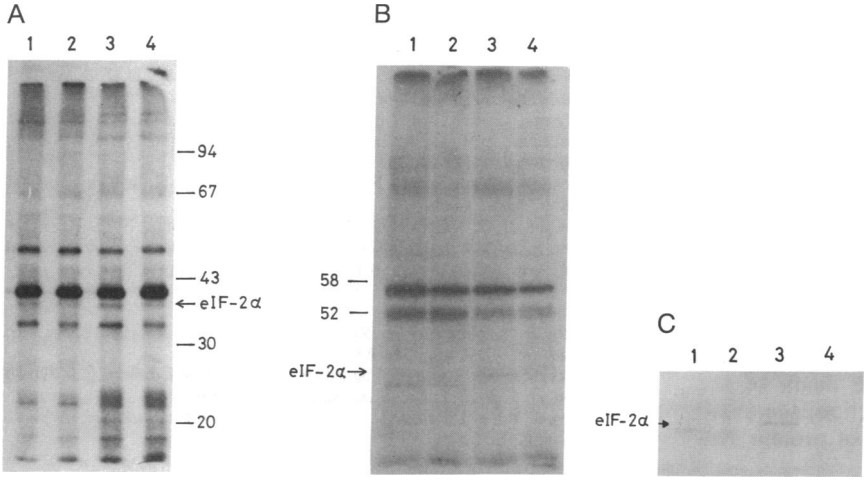

FIG. 5. Effect of chloroquine treatment *in situ* on parasite eIF-2 phosphorylation in parasite lysate. Parasite lysates (S-30) were prepared from control or chloroquine-treated cultures. (*A*) Phosphorylation of total proteins in the parasite lysate. (*B*) Phosphorylation of eIF-2α in the immunoprecipitate from the parasite lysate. (*C*) The eIF-2α region from *B* is presented to provide better clarity. Lanes 1, control lysate (no addition); lanes 2, control lysate (15 μM hemin); lanes 3, lysate from chloroquine-treated cultures (no addition); lanes 4, lysate from chloroquine-treated cultures (15 μM hemin). Blanks run with parasite lysate from control and chloroquine-treated cultures with nonimmune serum did not reveal any phosphorylated band corresponding to eIF-2α. Molecular mass is indicated in kDa.

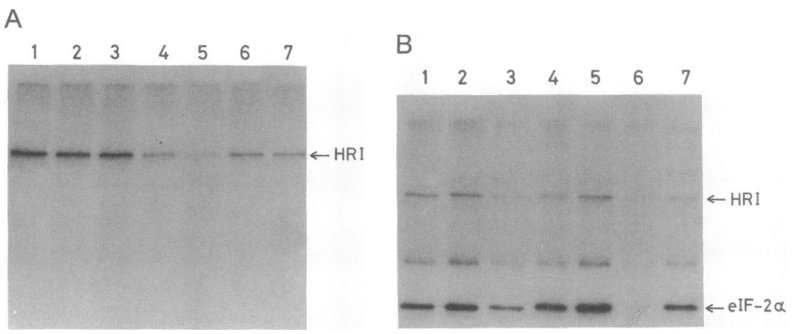

FIG. 7. Effect of hemin and chloroquine on purified HRI activity. Autophosphorylation of HRI (*A*) and phosphorylation of added eIF-2 (0.5 μg) (*B*) were assessed. (*A*) Lane 1, no addition; lane 2, chloroquine (5 μM); lane 3, chloroquine (10 μM); lane 4, hemin (5 μM); lane 5, hemin (10 μM); lane 6, hemin plus chloroquine (5 μM each); lane 7, hemin plus chloroquine (10 μM each). (*B*) Lane 1, no addition; lane 2, chloroquine (5 μM); lane 3, hemin (5 μM); lane 4, hemin plus chloroquine (5 μM each); lane 5, chloroquine (10 μM); lane 6, hemin (10 μM); lane 7, hemin plus chloroquine (10 μM each).

membrane phospholipase. Krogstad and Schlesinger (5) have shown that chloroquine accumulates in the acid vesicles of the parasite against a concentration gradient and alkalinizes the vacuole, which may interfere with a variety of processes, such as receptor-mediated endocytosis, lysosomal enzyme targeting, and lysosomal enzyme-mediated degradation. As already indicated, alkalinization as such does not appear to be sufficient to kill the parasite (6), and in any case the immediate molecular events following the accumulation of chloroquine in the parasite vacuole are not clear.

Meshnick (3) has recently suggested that although the K_d for DNA binding by chloroquine appears very high, it is possible that 0.03–1% of potential intercalation sites might be occupied at therapeutic concentrations. Chloroquine has been found to intercalate more avidly with poly(dG-dC) polynucleotides than other sequences. At this stage, the suggestion of preferential binding of the drug to selective G-C regions of the predominantly (A+T)-rich genome of *P. falciparum* remains to be tested but appears farfetched.

The present studies clearly reveal that the parasite shows heme dependency for protein synthesis just as the reticulocyte lysate. Chloroquine addition *in vitro* inhibits the heme-dependent protein synthesis. The basal level of protein synthesis seen in the absence of added heme may reflect the extent of functional initiation factor available or the endogenous heme that sustains this level of protein synthesis in the cell-free system may not be accessible to chloroquine. This may explain the earlier observation, indicating lack of inhibition of cell-free protein synthesis by chloroquine (14). The parasite lysate from cultures treated with therapeutic concentrations of chloroquine *in situ* for a short period (30 min) manifests enhanced phosphorylation of eIF-2α under conditions of cell-free protein synthesis, which is inhibited by the addition of hemin to the lysate. Addition of hemin, phosphorylated compounds, and eIF-2, known to counteract the inhibited protein synthesis in heme-deficient reticulocyte lysate, has a similar effect with the parasite lysate prepared from *in situ* chloroquine-treated cultures. eIF-2α kinase activity is present in the parasite lysate and is not a contaminant derived from the human erythrocytes used for culturing the parasite. However, further studies are needed to establish whether the heme-sensitive eIF-2α kinase activity detected in the parasite lysate is identical or similar to the HRI characterized in reticulocytes. The results presented in Fig. 7 show that chloroquine prevents inactivation of purified HRI by heme. This is consistent with the notion that chloroquine exerts its effects by complexing with heme.

Finally, the enhanced eIF-2α kinase activity seen in parasite cultures treated with therapeutic concentrations of chloroquine for a short period is associated with a decrease in general protein synthesis. Inhibition of protein synthesis is thus an early event and is possibly responsible for the other defects reported, ultimately leading to the death of the parasite. In this proposed mechanism of action, the availability of chloroquine and heme at the site of protein synthesis would be the deciding factor, and perhaps accumulation of the antimalarial and the hemozoin pigment in the parasite vacuole is basically a detoxication mechanism. We have preliminary evidence to suggest that the parasite synthesizes its own heme, despite the accumulation of the hemozoin pigment derived from the host hemoglobin.

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