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COMPARISON OF PROTEASES FROM CHLOROQUINE-SENSITIVE AND CHLOROQUINE-RESISTANT STRAINS OF *PLASMODIUM FALCIPARUM*

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Abstract—An aminopeptidase and four hemoglobin-degrading acid proteases have been isolated from cloned strains of chloroquine-sensitive and chloroquine-resistant *Plasmodium falciparum*. Aminopeptidases from both strains showed similar properties including molecular weights of 63,000 and non-competitive inhibition by chloroquine; K_i = 535 and 410 μM for enzymes from the sensitive and resistant strains respectively. The acid proteases from the chloroquine-sensitive strain included a low molecular weight enzyme in the soluble fraction (protease S), an enzyme weakly associated with membrane (protease M_2), and two enzymes strongly associated with membrane (proteases M_3 and M_4). The acid proteases from the chloroquine-resistant strain included protease S, protease M_2, a second enzyme weakly associated with membrane (protease M_1), and protease M_3. All of the acid proteases were inhibited by ferriprotoporphyrin IX and by the chloroquine–ferriprotoporphyrin IX complex, I_{50} = 5–25 μM. The data were consistent with a model for chloroquine action wherein chloroquine acts to divert ferriprotoporphyrin IX from sequestration into malarial pigment, leaving ferriprotoporphyrin IX (or its chloroquine complex) to interfere with digestion of host cytosol by inhibiting hemoglobin-degrading proteases. However, the similarities among the proteases from chloroquine-sensitive and chloroquine-resistant strains of parasites suggest that chloroquine resistance does not result from changes in parasite proteases.

Malarial parasites degrade host cell hemoglobin in order to provide amino acids for growth of the parasites during their intraerythrocytic growth phase [1–3]. Degradation of hemoglobin takes place in a food vacuole within the parasite. This vacuole appears to be similar to a secondary lysosome [4]. A number of studies involving various species of *Plasmodium* have provided evidence for the existence of acid proteases in the parasites [5–8]. These proteases are capable of catalyzing the hydrolysis of hemoglobin and, consequently, are assumed to function within the acidic food vacuoles of the parasites. The degradation of hemoglobin within the food vacuole liberates large quantities of ferriprotoporphyrin IX (hemin) which must be efficiently packaged into non-toxic malarial pigment. Fitch and coworkers [9–14] have provided considerable evidence that ferriprotoporphyrin IX forms a toxic complex with chloroquine that can lyse cells.

In previous studies of proteases from the human pathogen *P. falciparum*, we described an aminopeptidase which exhibits moderate sensitivity to chloroquine [15], and we described a hemoglobin-degrading acid protease which is inhibited by both ferriprotoporphyrin IX and the chloroquine complex of ferriprotoporphyrin IX [8]. Both of these studies utilized a chloroquine-resistant strain of *P. falciparum*. In the present study, we compared five proteases isolated from chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* for their sensitivities to chloroquine, ferriprotoporphyrin IX, and the ferriprotoporphyrin IX–chloroquine complex.

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EXPERIMENTAL

Materials. Ferriprotoporphyrin IX, chloroquine, L-alanine-*p*-nitroanilide, pepstatin and human hemoglobin were purchased from Sigma (St. Louis, MO). [^{3}H]Hypoxanthine was from New England Nuclear (Boston, MA) and [^{3}H]acetic anhydride was from Amersham (Arlington Heights, IL).

Strains of P. falciparum. The chloroquine-resistant strain of *P. falciparum* used in this study, strain FCBI, is a Colombian strain which was cloned by limiting dilution as described by Rosario [16]. The clone used in the present study is denoted as clone NC-1. The chloroquine-sensitive strain of *P. falciparum*, strain Honduras I/CDC (clone HB-3), was cloned by Professor W. Trager, Rockefeller University. This clone was obtained from the Malaria Branch, Center for Disease Control, Atlanta, GA, with permission from Professor Trager.

Cultivation of parasites. P. falciparum was grown in human erythrocytes [17]. Culture dishes contained 2–3% erythrocytes in RPMI 1640 medium supplemented with 5 mM glutamine, 35 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes), 24 mM sodium bicarbonate, and gentamicin, 33 mg/l. Medium contained 10% (v:v) horse serum (Gibco), pH 7.2. When parasitemia reached 12–14%, the parasitized cells were concentrated to 90–95% by flotation on Plasmagel [18]. This method

provides a trophozoite-rich source of parasites. Parasites were isolated from the trophozoite-rich top layer of the Plasmagel by cross-linking of the infected and uninfected erythrocytes with phytohemagglutinin, after which the mixture was passed through a series of sieves [19, 20]. The liberated parasites were purified further by differential centrifugation and were analyzed for purity by use of marker enzymes [21]. Samples of isolated parasites generally exhibited specific activities <0.07 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ for acetylcholine esterase, a sensitive indicator of erythrocyte contamination. Proteins were determined by the method of Bradford [22].

Parasite growth and chloroquine sensitivity. The chloroquine sensitivities of the two cloned strains, HB-3 and NC-1, were determined by monitoring parasite growth through the uptake of [^{3}H]hypoxanthine. Aliquots of medium (250 μl) containing parasitized erythrocytes, 0.5% parasitemia, were added to microtiter wells. Chloroquine, dissolved in serum-free medium, was added to the wells at the appropriate concentrations. Control wells received serum-free medium. After addition of chloroquine, [^{3}H]hypoxanthine (0.5 μCi) was added to each well. Microtiter plates were incubated for 3 days, 37°, in 5% CO_2–95% air, after which the contents of the wells were collected on glass fiber filters, using a cell harvester. The filters were washed with distilled water before being analyzed by liquid scintillation counting. Figure 1 shows the chloroquine inhibition plots for the growth of HB-3 and NC-1, which exhibited IC_{50} values of 27 and 290 nM respectively.

Protein purification. Isolated parasites were disrupted by sonication, five times, 5 sec each, 20 W power, in 0.05 M Tris, pH 7.5, 4°. The sonicate was centrifuged for 30 min at 100,000 *g*. The supernatant fraction and the pellet were used separately for protein purification. Proteases were isolated from the supernatant fraction by high performance liquid chromatography on a TSK-GEL-3000SW column, 60 × 0.75 cm (Toyo Soda, Tokyo) and a Bio-Gel HPHT hydroxylapatite column, 10 × 0.78 cm (Bio-Rad). The membrane pellet was solubilized by sonication two times, 8 sec each, 20 W power, in 0.025 M Tris, pH 7.5, containing 0.5% taurocholate. After centrifugation, the supernatant fraction was used for protein purification by the same HPLC procedures described above.

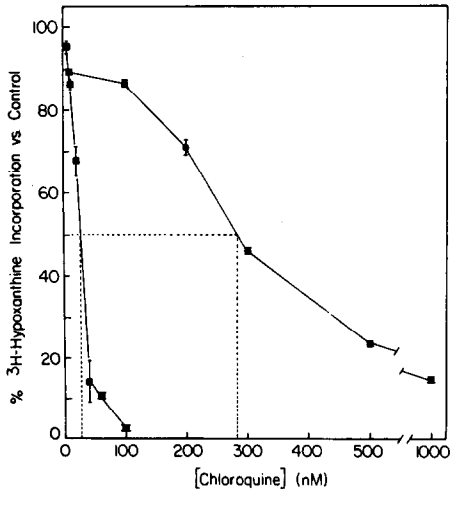

Fig. 1. Inhibition of the growth of *P. falciparum* by chloroquine using the chloroquine-sensitive clone HB-3 (■) and the chloroquine-resistant clone NC-1 (●). I_{50} values are 27 and 290 nM respectively. The error bars indicate the range of values from triplicate determinations. The 100% level represented *ca.* 2000 cpm.

Enzyme assays. Aminopeptidase activity was measured in 0.025 M Tris, pH 7.5, in 1 ml volumes containing 1 mM L-alanine-*p*-nitroanilide, 25°, by following the formation of *p*-nitroaniline at 405 nm, $\Delta\varepsilon = 9600\ M^{-1}\ cm^{-1}$ [23]. Acid protease activity was measured using acid-denatured [^{3}H]hemoglobin, 50 μg/ml, 0.1 μCi/ml, in 0.2 M sodium citrate, pH 4.5, 37°. Activity was determined from the amount of radioactivity in the soluble fraction after precipitation of protein with trichloroacetic acid. [^{3}H]Hemoglobin was prepared by acetylation of hemoglobin with [^{3}H]acetic anhydride [6].

RESULTS

Aminopeptidase from chloroquine-sensitive and chloroquine-resistant strains of P. falciparum. Trophozoite-stage parasites of *P. falciparum* contain a very active aminopeptidase. Previously, we described the properties of this enzyme isolated from a chloroquine-resistant strain, strain FCBI [15]. Aminopeptidase was of interest because of its inhibition by a number of blood schizontocides, including chloroquine.

Trophozoite-stage parasites of chloroquine-sensitive clone HB-3 were disrupted by sonication, and the 100,000 *g* supernatant material was purified by exclusion chromatography on a TSK-G-3000SW HPLC column. The protein and aminopeptidase activity profiles are shown in Fig. 2, left. For comparison, the profile for the purification of aminopeptidases from chloroquine-resistant clone NC-1 is shown in Fig. 2, right. Both cloned strains of *P. falciparum* exhibited high aminopeptidase activity. Specific activities of the 100,000 *g* supernatant fractions from HB-3 and NC-1 were 0.15 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ and 0.41 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, respectively, with 1 mM L-alanine-*p*-nitroanilide as substrate, pH 7.5. The apparent molecular weights of aminopeptidase were determined with a calibrated TSK-G-3000SW column, as shown in Fig. 3. Both aminopeptidases showed molecular weights of 63,000.

The inhibition of the aminopeptidases by chloroquine was determined at pH 7.5, as shown in Fig. 4. Chloroquine was a noncompetitive inhibitor of aminopeptidase from both HB-3 and NC-1; K_i values were 535 and 410 μM respectively. In view of the very similar properties of these aminopeptidases, there is little evidence that chloroquine sensitivity or resistance in strains of *P. falciparum* resulted from altered properties of aminopeptidase. It should be noted, however, that chloroquine-resistant clone NC-1 had significantly higher aminopeptidase specific activity in the crude supernatant.

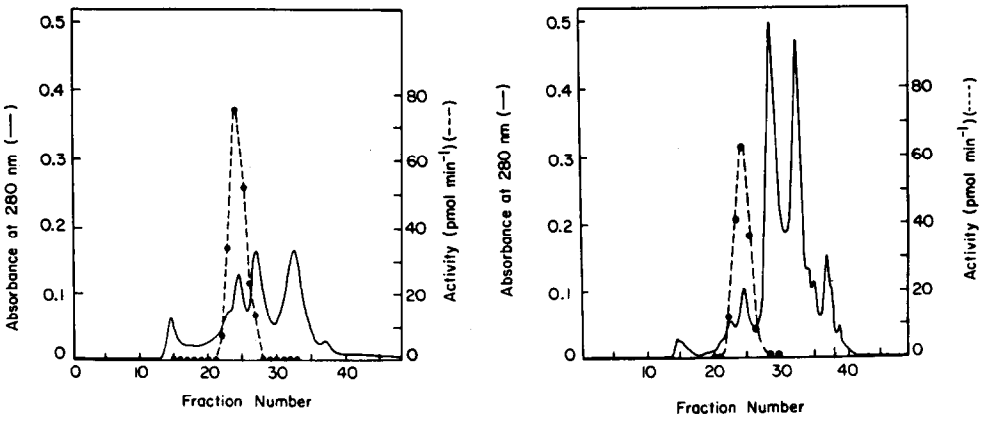

Fig. 2. Activity and protein profiles for the purification of aminopeptidase from chloroquine-sensitive HB-3 (left) and from chloroquine-resistant NC-1 (right). Supernatant material, 250 μl, was applied to a TSK-G-3000SW column, and the column was developed with 0.15 M sodium phosphate buffer, pH 6.0, containing 0.1 M NaCl, at a flow rate of 0.8 ml/min. Aminopeptidase activity was analyzed using L-alanine-*p*-nitroanilide as substrate.

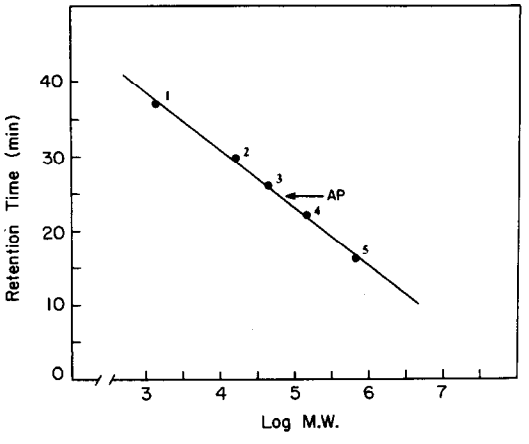

Fig. 3. Molecular weights of aminopeptidase from HB-3 and from NC-1 on a calibrated TSK-G-3000SW column. Aminopeptidase (AP) from both strains migrated with apparent molecular weight of 63,000. Standards (1 to 5) are cyanocobalamin, myoglobin, ovalbumin, IgG and thyroglobulin. Column conditions were the same as Fig. 2.

Hemoglobin-degrading proteases from chloroquine-sensitive and chloroquine-resistant strains of P. falciparum. The 100,000 *g* supernatant material from chloroquine-sensitive HB-3 was added to a TSK-G-3000SW column, and fractions were analyzed for protease activity using [^{3}H]hemoglobin as substrate, pH 4.5. The profile is shown in Fig. 5, left. For comparison, the profile for chloroquine-resistant NC-1 is shown in Fig. 5, right. Clone HB-3 showed a main broad peak of activity with a leading shoulder which migrated as an apparently small protein. Clone NC-1 showed a less pronounced shoulder. The shoulder of activity (Fig. 5, left) for HB-3, fractions 33–36, was concentrated and chromatographed a second time on a TSK-G-3000SW column, and then repeated a third time (Fig. 6). A sharp peak of activity, designated protease S, was observed. Previously, we reported on the properties of a similar low-molecular weight protease from *P. falciparum*, strain FCBI [8]. Protease S can be concentrated on Amicon YM5 membrane but not on a YM10 membrane, consistent with its apparent low molecular weight on the TSK-G-3000SW exclusion column.

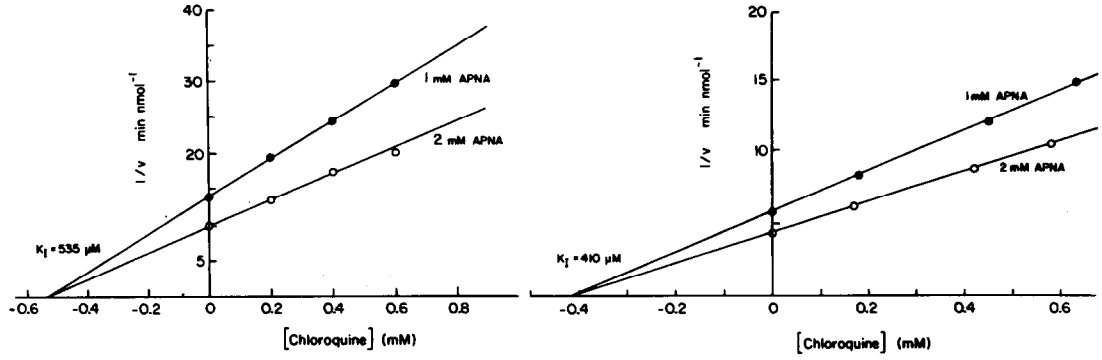

Fig. 4. Dixon plots of the inhibition of aminopeptidase from chloroquine-sensitive HB-3 (left) and from chloroquine-resistant NC-1 (right) by chloroquine. Inhibition was determined at pH 7.5, 25°. Initial rates were measured with 1 mM (●) and 2 mM (○) L-alanine-*p*-nitroanilide.

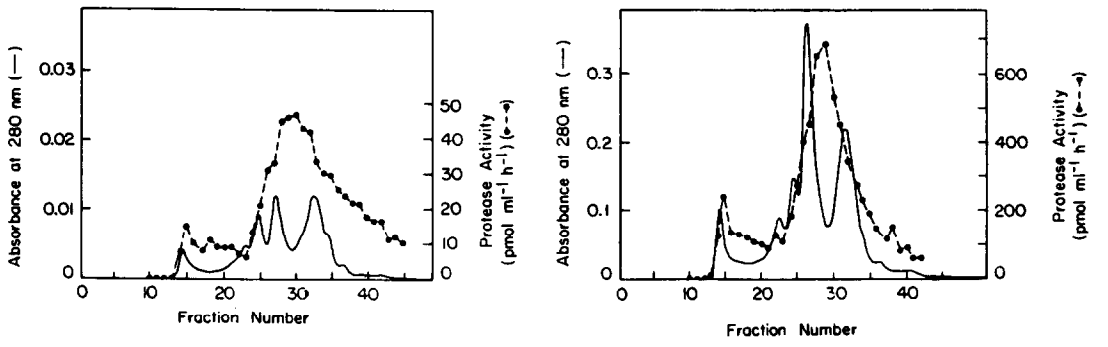

Fig. 5. Acid protease activity of the 100,000 *g* supernatant fraction of *P. falciparum* on TSK-G-3000SW size exclusion chromatography. Left, chloroquine-sensitive HB-3; right, chloroquine-resistant NC-1. Column conditions were the same as in Fig. 2. Activity was measured at pH 4.5 with [^{3}H]hemoglobin.

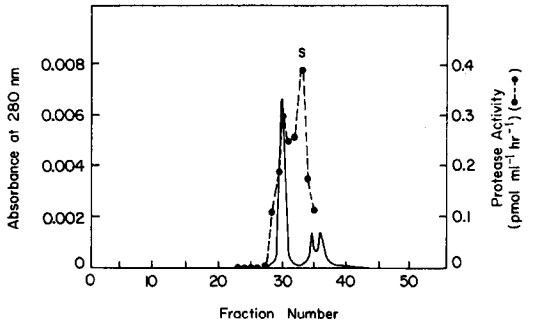

Fig. 6. Chromatography of low molecular weight acid protease activity on a TSK-G-3000SW column. The acid protease activity from chloroquine-sensitive HB-3 (in Fig. 5), fractions 33–36, was concentrated on an Amicon YM5 membrane and then was chromatographed twice on a TSK-G-3000SW column to provide protease S, a hemoglobin-degrading acid protease with an apparent molecular weight of less than 10,000. Protease S from chloroquine-resistant NC-1 behaved similarly.

The major peak of acid protease activity in Fig. 5 (left and right) was concentrated on an Amicon YM10 membrane. The concentrated material was purified further on a Bio-Gel HPHT hydroxylapatite column, as shown in Fig. 7. Chloroquine-resistant clone NC-1 showed two peaks of activity, designated proteases M_1 and M_2. Chloroquine-sensitive HB-3 showed only protease M_2 activity. Proteases S, M_1 and M_2 from chloroquine-resistant NC-1 and proteases S and M_2 from chloroquine-sensitive HB-3 represented 60–65% of the hemoglobin-degrading activity measured in parasite preparations obtained by sonication of trophozoites. A single sonication for 5 sec, 20 W power, appeared sufficient to solubilize all of the protease S, suggesting that this protease is in the soluble fraction of the parasite. Proteases M_1 and M_2 required several repetitions of the sonication procedure to complete their solubilization, suggesting that these proteases are weakly associated with membranes in the parasite. The remaining 35–40% of the hemoglobin-degrading activity could not be solubilized by sonication of the membrane fraction of the parasites.

Hemoglobin-degrading membrane-bound proteases from P. falciparum. Membrane fractions prepared by repeated sonication in 0.025 M Tris, pH 7.5, followed by centrifugation were solubilized in this same buffer containing 0.5% taurocholate. Sonication two times, 8 sec each, solubilized the acid protease activity with 90% recovery of the protease activity. The solubilized material was centrifuged for

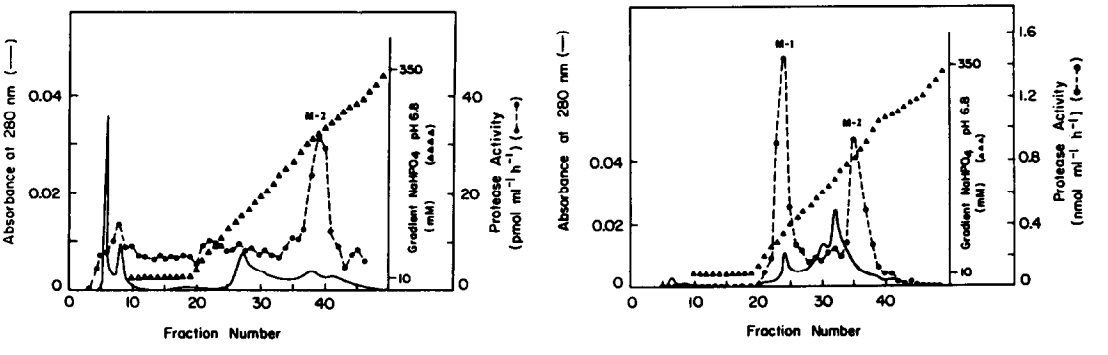

Fig. 7. Purification of acid protease activity on a Bio-Gel HPHT hydroxylapatite HPLC column. Left, chloroquine-sensitive HB-3; right, chloroquine-resistant NC-1. In both cases, fractions 26–31 from Fig. 5 (left, right) were concentrated by pressure filtration on an Amicon YM10 membrane. The concentrated material, 250 μl, was applied to the Bio-Gel column which was developed with a sodium phosphate gradient, 10 to 350 mM, pH 6.8. NC-1 showed two proteases, designated M_1 and M_2. HB-3 showed only protease M_2.

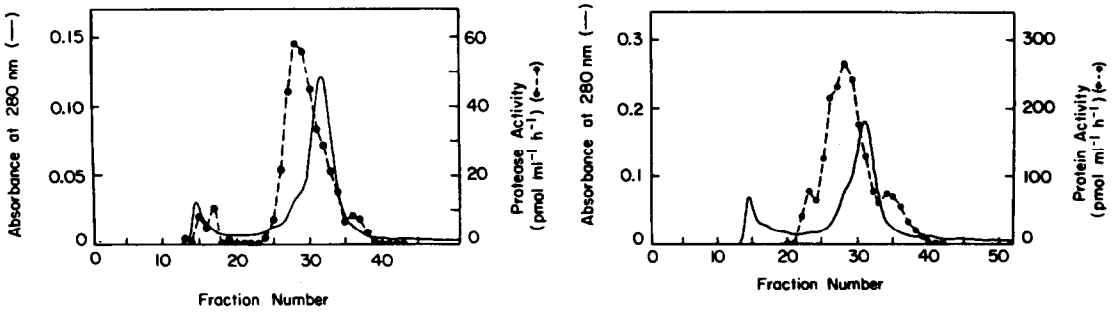

Fig. 8. Purification of detergent-solubilized acid protease activity from the membrane fraction of *P. falciparum* on a TSK-G-3000SW column. Left, chloroquine-sensitive HB-3; right, chloroquine-resistant NC-1. Column conditions were the same as in Fig. 2.

30 min at 100,000 *g*, and the supernatant fraction was added to a TSK-G-3000SW column; profiles are shown in Fig. 8 for chloroquine-sensitive HB-3 (left) and chloroquine-resistant NC-1 (right). In each case the peak of activity was concentrated and was purified further on a Bio-Gel HPHT column, as shown in Fig. 9. Chloroquine-resistant NC-1 showed a single peak of activity, designated protease M_3. Chloroquine-sensitive HB-3 showed a peak corresponding to protease M_3 and an additional peak of activity designated protease M_4.

Inhibition of acid proteases from P. falciparum *by pepstatin.* The hemoglobin-degrading acid proteases from chloroquine-sensitive HB-3 and chloroquine-resistant NC-1 were sensitive to the acid protease inhibitor pepstatin; results are summarized in Table 1. Comparison of the proteases from the two strains shows some differences, however. Protease S from HB-3 was some 350 times more sensitive to pepstatin than was protease S from NC-1. The other acid proteases all showed quite similar sensitivities to pepstatin. Specifically, proteases M_2 and M_3 which appear in both strains exhibited almost identical properties when these two strains were compared. I_{50} values are generally in the nanomolar range.

Inhibition of acid proteases from P. falciparum *by ferriprotoporphyrin IX and by the ferriprotoporphyrin IX–chloroquine complex.* Proteases S, M_1, M_2, M_3, and M_4 were all inhibited by ferriprotoporphyrin IX and by the ferriprotoporphyrin IX–chloroquine complex but not by chloroquine itself. Results are summarized in Table 2. For both inhibitors, I_{50} values were in the 5–25 μM range. There were some modest differences in sensitivities between proteases from chloroquine-sensitive HB-3 and chloroquine-resistant NC-1, especially protease S. In general, however, the differences were small. The values in Table 2 are upper estimates of the inhibition constants. Ferriprotoporphyrin IX aggregates quite rapidly at pH 4.5 [8]. The values in Table 2 represent total concentrations of inhibitors. The concentration of monomeric or dimeric ferriprotoporphyrin IX is probably considerably smaller than the total concentration, due to aggregation. The values in Table 2 were obtained by limiting the assay time to 15 min. The vis-u.v. spectra of fresh solutions of ferriprotoporphyrin IX at pH 4.5 did not show major changes during the first 15 min. At longer times, major changes were seen along with precipitation of aggregates of ferriprotoporphyrin IX. Especially noteworthy is that all of the acid proteases in *P. falciparum* were sensitive to these inhibitors.

DISCUSSION

The identification of the mechanism of action of

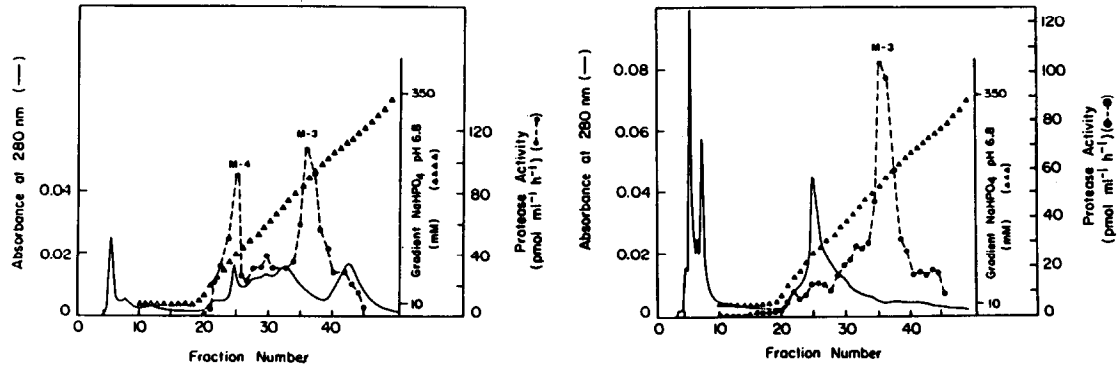

Fig. 9. Purification of membrane-associated acid protease activity from *P. falciparum* on a Bio-Gel HPHT column. Fractions 26–31 from Fig. 8 (left and right) were concentrated on an Amicon YM10 membrane and applied to the hydroxylapatite column. Column conditions were the same as in Fig. 7. For chloroquine-resistant NC-1 (right), a single protease was observed, designated protease M_3. For chloroquine-sensitive HB-3 (left), two proteases were observed, designated M_3 and M_4.

Table 1. Pepstatin inhibition of acid proteases from *P. falciparum*

Protease	I_{50} (nM) HB-3	NC-1
S	0.2	70
M_1		0.7
M_2	0.7	1.0
M_3	0.2	0.2
M_4	0.6	

All assays were performed at pH 4.5, 37°.

chloroquine as an antimalarial drug has been a goal for researchers for many years. This goal has become increasingly important due to the emergence of widespread resistance to chloroquine by *P. falciparum*, the human pathogen which causes the most severe forms of malaria. There are several theories concerning the mechanism of action of chloroquine. These include: (1) chloroquine inhibits nucleic acid synthesis; (2) chloroquine alters the pH of the food vacuole; and (3) chloroquine forms a lytic complex with hemin.

Chloroquine intercalates with DNA, a property which has been used to explain the antimalarial effects of chloroquine [24–28]. However, the antimalarial effects of chloroquine, at least in chloroquine-sensitive strains of *P. falciparum*, are sensitive to nanomolar levels of chloroquine, whereas inhibition of DNA synthesis requires millimolar levels of chloroquine.

Considerable evidence supports the idea that chloroquine accumulates in an acidic food vacuole. Accumulation was first demonstrated by autoradiography [29]. The acidic nature of the vacuole was suggested by the localization of acid phosphatase [30]. Homewood *et al.* [31] first suggested that chloroquine accumulation in the vacuole resulted from the weak base properties of the drug and suggested that accumulation might result in alkalinization of the vacuole so that acid proteases could no longer degrade hemoglobin. Recent studies have provided direct spectral data that the pH of the vacuole is 5.0 to 5.4 [32, 33]. There are conflicting data whether therapeutic levels of chloroquine are sufficient to raise the pH of the vacuole [33, 34]. Nevertheless, accumulation of chloroquine appears to require the acid pH of the vacuole. In addition, chloroquine accumulation appears to exceed the levels expected from its weak base properties [35], reaching millimolar levels in the vacuole [34]. The high concentration of chloroquine in the food vacuole may directly inhibit proteases involved in degradation of hemoglobin [6, 34], rather than raise the pH above the range for protease activity. Another possibility is that the accumulated chloroquine interferes with the proton pump required for maintenance of the pH gradient across the vacuolar membrane [35]. Yet another possibility is that accumulation of chloroquine leads to altered pH in the vacuole which has consequences other than inhibition of proteolysis [33].

Table 2. Inhibition of acid proteases from *P. falciparum* by ferriprotoporphyrin IX and by the ferriprotoporphyrin IX–chloroquine complex

Protease	I_{50} (μM) HB-3 FP-IX*	FP-IX-CQ†	I_{50} (μM) NC-1 FP-IX	FP-IX-CQ
S	25	4	8	5
M_1			8	5
M_2	20	17	5	15
M_3	10	8	8	9
M_4	19	25		

* Inhibition by ferriprotoporphyrin IX (FP-IX).

† Inhibition by the ferriprotoporphyrin IX–chloroquine complex (FP-IX-CQ) when both ferriprotoporphyrin IX and chloroquine were present in a 1:1 ratio. The I_{50} value here represents the total concentration of ferriprotoporphyrin IX, only part of which is in the form of the complex. Conditions: pH 4.5, 37°.

The suggestion that ferriprotoporphyrin IX may aid in the accumulation of chloroquine was first made by Macomber *et al.* [36]. Fitch and coworkers [9–14] have developed this concept extensively. In this model, ferriprotoporphyrin IX is viewed as a receptor for chloroquine, aiding in its accumulation. The chloroquine complex of ferriprotoporphyrin IX as well as ferriprotoporphyrin IX itself have membrane-disrupting properties and can lyse parasites and other cells [10, 11]. Arguments against ferriprotoporphyrin IX as a receptor for chloroquine include the fact that chloroquine accumulates to millimolar levels whereas free ferriprotoporphyrin IX in parasites has not been reported [34]. Certainly free ferriprotoporphyrin IX will not accumulate to millimolar levels since micromolar levels of this compound are sufficient to lyse membranes [10, 11]. It may be that chloroquine accumulation is the result of both the low pH of the vacuole and the availability of ferriprotoporphyrin IX. The question remains whether ferriprotoporphyrin IX and its chloroquine complex are the actual inhibitors of critical processes in the parasite. Ultrastructural studies of the effects of chloroquine on *P. falciparum* did not reveal evidence of deterioration of the vacular membrane. However, ferriprotoporphyrin IX can disrupt cation gradients at concentrations which do not lyse membranes [13].

In our earlier study of protease S, the low molecular weight protease capable of degrading hemoglobin, we suggested a new model for the mode of action of chloroquine [8]. In this model, chloroquine accumulation either by virtue of the low pH of the vacuole or by complexation with ferriprotoporphyrin IX leads to a situation where some ferriprotoporphyrin IX is diverted from its normal sequestration into pigment. The unsequestered ferriprotoporphyrin IX and its chloroquine complex inhibit protease S. The present study extends this model by demonstrating that all four hemoglobin-degrading acid proteases, both in chloroquine-sensitive and in chloroquine-resistant strains of *P. falciparum*, were inhibited by micromolar levels of ferriprotoporphyrin IX and its chloroquine complex. It should be noted, however, that the I_{50} values (Table 2) for inhibition of the various proteases were quite similar

for both strains of parasite. There is no evidence, therefore, that chloroquine resistance resides at the level of these acid proteases. That is, the acid proteases from chloroquine-resistant NC-1 are not less sensitive to ferriprotoporphyrin IX than the proteases from chloroquine-sensitive HB-3. Thus, if the mechanism of action of chloroquine, at least in part, involves the diversion of some ferriprotoporphyrin IX from being incorporated into pigment such that ferriprotoporphyrin IX (or its chloroquine complex) can inhibit the hemoglobin-degrading proteases, we are still left with the question of explaining chloroquine resistance.

The chloroquine-resistant strain NC-1 showed a protease (protease M_1) which was absent in strain HB-3 but lacked one protease (protease M_4) which was present in strain HB-3. Whether proteases M_1 and M_4 are related is not clear. The sensitivities of these two proteases to pepstatin were almost identical (Table 1). Conceivably, protease M_1 in strain NC-1 is similar to protease M_4 in strain HB-3 except that it is more easily released from the membraneous fraction of the parasite. All of the other proteases from strains NC-1 and HB-3 appear to have similar chromatographic properties.

One additional point should be noted. The aminopeptidases from H-3 and NC-1 were sensitive to chloroquine, K_i values *ca.* 0.5 and 0.4 mM respectively. In our previous study of aminopeptidase from *P. falciparum* [15], we showed that the pH optimum is 7.5 and that there is very little activity below pH 6. It appears that aminopeptidase does not have properties that would allow it to function in the acidic food vacuole where chloroquine can accumulate to millimolar levels, levels high enough to inhibit aminopeptidase. However, a recent cytochemical study of an aminopeptidase from *P. chabaudi*, which also is optimally active near neutral pH, demonstrated that this aminopeptidase is associated with digestive vacuoles [37]. Thus, it remains to be demonstrated whether aminopeptidase from *P. falciparum* is located on or in the food vacuole and whether it might be in a location where it would be susceptible to high concentrations of chloroquine.

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