Ferriprotoporphyrin IX Fulfills the Criteria for Identification as the Chloroquine Receptor of Malaria Parasites†

Albert C. Chou, Rekha Chevli, and Coy D. Fitch*

ABSTRACT: Mouse erythrocytes treated with a nonspecific protease from *Streptomyces griseus* or infected with chloroquine-susceptible *Plasmodium berghei* possess a saturable process for the accumulation of chloroquine. The process in both cases has high affinity and specificity for drugs in the same chemotherapeutic class as chloroquine. In studies intended to identify the receptor serving protease-treated erythrocytes, hemoglobin was found to be the substrate from which the receptor is created and the receptor was shown to be an aggregated form of ferriprotoporphyrin IX. The affinity and specificity of ferriprotoporphyrin IX for chloroquine also were found to be sufficient to account for chloroquine binding to erythrocytes infected with chloroquine-susceptible *P. berghei*. In equilibrium dialysis experiments, ferriprotoporphyrin IX had a dissociation constant for chloroquine of 3.5×10^{-9} M, and the following values for K_i were obtained in experiments in which various compounds competed with chloroquine for binding to ferriprotoporphyrin IX: amodiaquine, 1.3×10^{-7} M; quinacrine, 1.2×10^{-7} M; quinine, 2.6×10^{-6} M; mefloquine, 1.6×10^{-5} M. When ferriprotoporphyrin IX was adsorbed to the erythrocyte surface, the dissociation constant for chloroquine increased to 2.3×10^{-7} M, indicating that the affinity of the interaction varies appreciably depending on the environment in which it occurs. Erythrocytes infected with chloroquine-susceptible *P. berghei* degrade hemoglobin and accumulate malaria pigment, which contains ferriprotoporphyrin IX, whereas erythrocytes infected with chloroquine-resistant *P. berghei* do not. In addition, when chloroquine-resistant lines of *P. berghei* revert to chloroquine susceptibility, they also revert to the production of malaria pigment. Thus, ferriprotoporphyrin IX fulfills all of the criteria for identification as the receptor responsible for chloroquine accumulation by erythrocytes infected with *P. berghei*.

When mouse erythrocytes infected with *Plasmodium berghei* are exposed to chloroquine [7-chloro-4-[[4-(diethylamino)-1-methylbutyl]amino]quinoline], the first biochemical event is rapid accumulation of the drug (Fitch, 1969). The first morphological event is pigment clumping due to coalescence of digestive vacuoles and formation of autophagic vacuoles (Macomber et al., 1967; Warhurst & Hockley, 1967). Both processes are energy dependent, saturable, and competively inhibited by drugs in the same chemotherapeutic class as chloroquine, including quinine [6-methoxy-α-(5-vinyl-2-quinuclidinyl)-4-quinolinemethanol], quinacrine [6-chloro-9-[[4-(diethylamino)-1-methylbutyl]amino]-2-methoxyacridine], amodiaquine [7-chloro-4-[3-[(diethylamino)-methyl]-4-hydroxyanilino]quinoline], and mefloquine [WR 142 490; α-(2-piperidyl)-2,8-bis(trifluoromethyl)-4-quinolinemethanol] (Fitch, 1972; Fitch et al., 1974c; Warhurst et al., 1972; Warhurst & Thomas, 1975). In addition, the host cell membrane appears to be intimately involved in the process of chloroquine accumulation (Fitch et al., 1978). Receptors for chloroquine have been proposed for the process of accumulation (Fitch, 1969; Fitch et al., 1974c) as well as for the process of pigment clumping (Warhurst et al., 1972) to explain saturability and specificity. In both cases the dissociation constant (K_d) for the interaction of chloroquine with the receptor is estimated to be $\sim 10^{-8}$ M. It is possible that the same receptor serves both processes.

Neither of the groups studying the processes of chloroquine accumulation and pigment clumping have speculated about the chemical nature of the chloroquine receptor. Two candidates have been introduced by others, however. Hahn (1974) considered the possibility that the receptor is DNA because chloroquine can be shown to intercalate between the strands of double-stranded DNA and because he thought that the structural requirements for intercalation are similar to those for binding of antimalarial drugs to the chloroquine receptor. Against this possibility is the low affinity of DNA for chloroquine, with a K_d on the order of 10^{-4} M as measured by equilibrium dialysis or spectrophotometric methods (Stollar & Levine, 1963; Cohen & Yielding, 1965). In addition, it is now known that mefloquine does not intercalate with DNA (Davidson et al., 1977) but does competitively inhibit chloroquine accumulation (Fitch, 1972) and pigment clumping (Warhurst & Thomas, 1975).

Turning to the other candidate for receptor, Macomber et al. (1967) proposed that chloroquine accumulation, pigment clumping, and the pharmacologic effect all are due to binding of chloroquine to ferriprotoporphyrin IX. This compound is a constituent of malaria pigment (Fulton & Rimington, 1953; Homewood et al., 1975; Yamada & Sherman, 1979) and forms complexes with nitrogenous bases such as pyridine, quinine, and chloroquine (Shack & Clark, 1947; Schueler & Cantrell, 1964; Cohen et al., 1964). Unfortunately, Macomber et al. (1967) were unable to detect chloroquine binding to malaria pigment and ferriprotoporphyrin IX has never been found to exist in an uncomplexed state in malaria parasites (Yamada & Sherman, 1979). For these reasons, ferriprotoporphyrin IX has received little serious consideration as a chloroquine receptor until now.

The following observations demonstrate that ferriprotoporphyrin IX deserves further consideration as the chloroquine receptor of malaria parasites. Several years ago we found that treatment with a nonspecific protease from *Streptomyces griseus* can create binding sites for chloroquine on intact, uninfected erythrocytes (Fitch et al., 1974a). These binding

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sites have approximately the same affinity and specificity for chloroquine as the receptor of erythrocytes infected with malaria parasites (Fitch et al., 1978). More recently we have made the new observation that these binding sites are due to the degradation of hemoglobin to produce ferriprotoporphyrin IX, which binds chloroquine with high affinity. In the present report we provide a detailed description of this new observation and of studies of the specificity of chloroquine binding to ferriprotoporphyrin IX.

Materials and Methods

To serve as donors of normal erythrocytes, white male Swiss–Webster mice, weighing 20–25 g, were purchased from Hilltop Laboratories and fed Purina Laboratory Chow and water ad libitum. They were anesthetized with ether, and blood was obtained from an incision which formed a pocket in the axilla and severed the axillary artery. Blood from groups of mice was mixed 1:1 with a buffered medium containing ~1 mg of heparin per mL, and the erythrocytes were washed 3 times with 6 volumes of the buffered medium prior to use in an experiment. With each wash, as much as possible of the buffy coat was removed. Except for studies involving erythrocyte ghosts, which are described in Figure 1, an isotonic medium buffered to pH 7.4 with 50 mM phosphate (Fitch, et al., 1974b) was used.

For studies involving erythrocyte ghosts, blood was collected and washed with ice-cold Tris buffer (310 imosm, pH 7.6). Some of the erythrocytes then were used to study chloroquine accumulation by intact cells, and the rest were used to prepare erythrocyte membrane ghosts by the method of Hanahan & Ekholm (1974) as follows. Washed erythrocytes were resuspended to an hematocrit of 50% in ice-cold isotonic Tris buffer and rapidly mixed with 6 volumes of ice-cold hypotonic Tris buffer (20 imosm, pH 7.6). The lysed suspension was centrifuged at 20000*g* for 20 min at 4 °C, after which the cream-colored, well-packed part of the pellet in the bottom of the tube and the supernatant solution were discarded. The loosely packed part of the pellet was resuspended in 6 volumes of ice-cold isotonic Tris buffer and centrifuged again at 20000*g* for 20 min at 4 °C to obtain a washed pellet. The pellet was washed in this way 4 times. After lysis and each washing, portions of the pellets were removed for study of the effect of proteolytic digestion on chloroquine accumulation as described in the legend for Figure 1.

The binding of chloroquine to hemoglobin, protease-treated hemoglobin, protease alone, and ferriprotoporphyrin IX was studied by equilibrium dialysis. For this purpose Spectropor 3 membrane with a molecular weight cutoff of 3500 was purchased from Spectrum Medical Industries, Inc. It was prepared for use by boiling in deionized, twice distilled water for at least 5 min followed by thorough rinsing. Human hemoglobin, twice crystallized (Type IV), α-chymotrypsin, thrice crystallized from bovine pancreas (Type II), nonspecific protease from *S. griseus* (Type VI), and ferriprotoporphyrin IX (hematin) from bovine blood were obtained from Sigma Chemical Co. The hemoglobin (40 mg/mL) was suspended in the phosphate-buffered medium, pH 7.4, and any insoluble material then was removed by centrifugation at 5000*g* for 10 min to yield a hemoglobin solution of ~30 mg/mL as measured by the cyanomethemoglobin method (Makarem, 1974). This solution was used for treatment with proteases as described in the legend to Figure 2. Ferriprotoporphyrin IX was prepared in the phosphate-buffered medium, pH 7.4, after first dissolving it in a minimal amount of 0.02 N NaOH, and it was allowed to stand at room temperature for ~1 h. Under these conditions the ferriprotoporphyrin IX exists in an aggregated form (Shack & Clark, 1947; Inada & Shibata, 1962). The dialysis was conducted at 4 °C with constant stirring in plastic beakers covered with parafilm. In representative studies the dialysate was monitored serially to ensure that equilibrium was reached. The volume of dialysate was at least 10 times larger than the volume inside the dialysis bag. When inhibitors of chloroquine binding were studied, they were added at the beginning of dialysis to the solution outside of the dialysis bag. The concentration of chloroquine outside of the bag at equilibrium was defined a free chloroquine, and the difference between inside and outside concentrations was used to calculate the amount of chloroquine bound to the material inside the bag. The recovery of [^{14}C]chloroquine in these experiments exceeded 90% of the amount added at the beginning of dialysis.

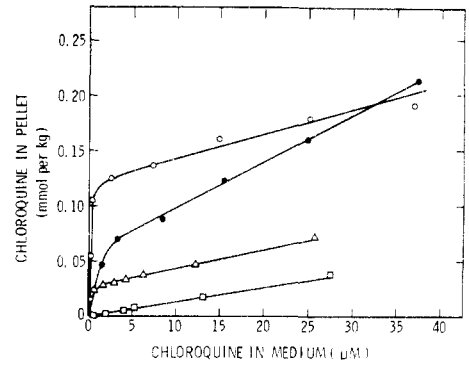

FIGURE 1: Effect of proteolytic digestion on chloroquine binding to erythrocyte membrane ghosts. The preparation of membrane ghosts and the method of washing them are described in the text. 12.5% suspensions of intact erythrocytes or 5% suspensions of membrane ghosts in the phosphate-buffered medium, pH 7.4, containing 1.4 mg/mL (w/v) nonspecific protease from *S. griseus* were preincubated for 60 min at 23 °C. The incubations were terminated by centrifugation at 5000*g* for 15 min, and the pellets were washed twice by resuspending them in 10 volumes of the phosphate-buffered medium followed by centrifugation to recover the pellet. 2% suspensions (v/v) of untreated erythrocytes, of protease-treated erythrocytes, or of protease-treated membrane ghosts in the phosphate-buffered medium were then incubated with [^{14}C]chloroquine for 60 min at 25 °C. The incubation was terminated by centrifugation at 20000*g* for 15 min and separation of medium from pellet for measurement of chloroquine concentration. Intact erythrocytes (●); unwashed membrane ghosts (○); membrane ghosts after one wash (△); membrane ghosts after four washes (□).

[3-^{14}C]Chloroquine (ring-labeled, 1.71 mCi/mmol, New England Nuclear Corp.) was used throughout these experiments to permit radiochemical measurement of chloroquine concentrations (Fitch, 1969). The chloroquine was routinely extracted into heptane (Fitch, 1969) prior to measurement of radioactivity except for the eluate from gel filtration experiments, aliquots of which were added directly to a counting solution containing xylene–dioxane–2-ethoxyethanol, 1:3:3 (Bruno & Christian, 1961), for measurement of radioactivity. A liquid scintillation spectrometer equipped with an external automatic standard for quench correction was used, and the counting error did not exceed 2%.

For the gel filtration experiments, Bio-Gel P-200 was obtained from Bio-Rad Laboratories.

Results

The experiments described in Figure 1 were designed to help localize the precursor of the high-affinity chloroquine binding component. As one of the controls for these experiments, we confirmed the previous observation (Fitch et al., 1974a) that treatment of intact, normal erythrocytes with a nonspecific protease of *S. griseus* causes a large increase in chloroquine binding. Also in agreement with previous work (Fitch et al.,

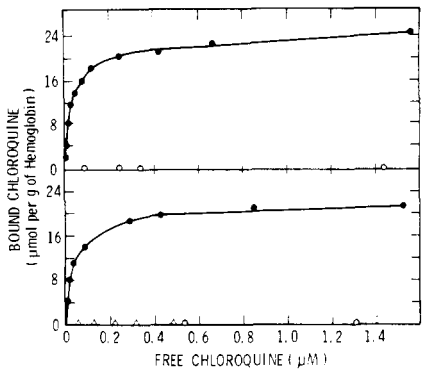

FIGURE 2: Effect of proteolytic digestion on chloroquine binding to hemoglobin as determined by equilibrium dialysis. (Upper panel) Hemoglobin (34 mg/mL) was preincubated with the nonspecific protease of *S. griseus* (0.7 mg/mL) in the phosphate-buffered medium for 60 min at 25 °C. The dialysis bags contained 7.12 mg of degraded hemoglobin and various amounts of [^{14}C]chloroquine in a final volume of 4.5 mL, and they were dialyzed against 50 mL of the phosphate-buffered medium for 65 h as described in the text. In control experiments a similar series of dialysis bags contained [^{14}C]chloroquine and 0.31 mg of protease. Protease-treated hemoglobin (●); protease (○). (Lower panel) Hemoglobin (30.4 mg/mL) was preincubated with α-chymotrypsin (20 mg/mL) in the phosphate-buffered medium for 60 min at 25 °C. The dialysis bags contained 7.12 mg of degraded hemoglobin and various amounts of [^{14}C]chloroquine in a final volume of 4.5 mL, and they were dialyzed against 60 mL of the phosphate-buffered medium for 65 h as described in the text. In control experiments, a similar series of dialysis bags contained either 8 mg of α-chymotrypsin or 7.12 mg of untreated hemoglobin and [^{14}C]-chloroquine. Chymotrypsin-treated hemoglobin (●); chymotrypsin (○); hemoglobin (△).

1974a), the apparent affinity of binding was high ($K_d \approx 5 \times 10^{-8}$ M). After lysis and before washing, there was an increase in the amount of chloroquine binding to the erythrocyte preparation, presumably because an increased amount of precursor was accessible to the protease. After repeated washing, the amount of precursor associated with membranes rapidly decreased and became undetectable. These observations were considered to be consistent with a precursor located in the cytoplasm.

Since hemoglobin is the most abundant protein in the cytoplasm of erythrocytes, it was chosen as the first candidate to be evaluated as a precursor for the chloroquine binding component. The results of these experiments are summarized in Figure 2. There was no binding of chloroquine to the proteases alone or to untreated hemoglobin, which consisted of a 4:1 mixture of methemoglobin and oxyhemoglobin. After treatment either with the nonspecific protease of *S. griseus* or with α-chymotrypsin, high-affinity chloroquine binding dramatically appeared. The maximum binding was 1.5 mol of chloroquine per mol of degraded hemoglobin, and the K_d was 1.4×10^{-8} M. Similar results were obtained by substituting a hemolysate of normal mouse erythrocytes for purified human hemoglobin. In addition to the protease of *S. griseus* and α-chymotrypsin, it was also possible to produce the binding component by treating hemoglobin with trypsin.

Chromatography on a Bio-Gel P-200 column was used to fractionate the proteolytic degradation products of hemoglobin and to help identify the component that binds chloroquine with high affinity. After the digestion mixture was incubated with [^{14}C]chloroquine, there were two radioactive peaks (Figure 3). One of these represented free chloroquine and the other one was bound to a high molecular weight fraction which eluted at the void volume. This fraction had a low protein content and appeared brownish green in color, suggesting the presence of an aggregated form of ferriprotoporphyrin IX. A similar product was obtained by mixing ferriprotoporphyrin IX and chloroquine (Figure 3). Moreover, spectral analysis of the hemoglobin degradation product complexed with chloroquine and of authentic ferriprotoporphyrin IX complexed with chloroquine yielded very similar absorption spectra

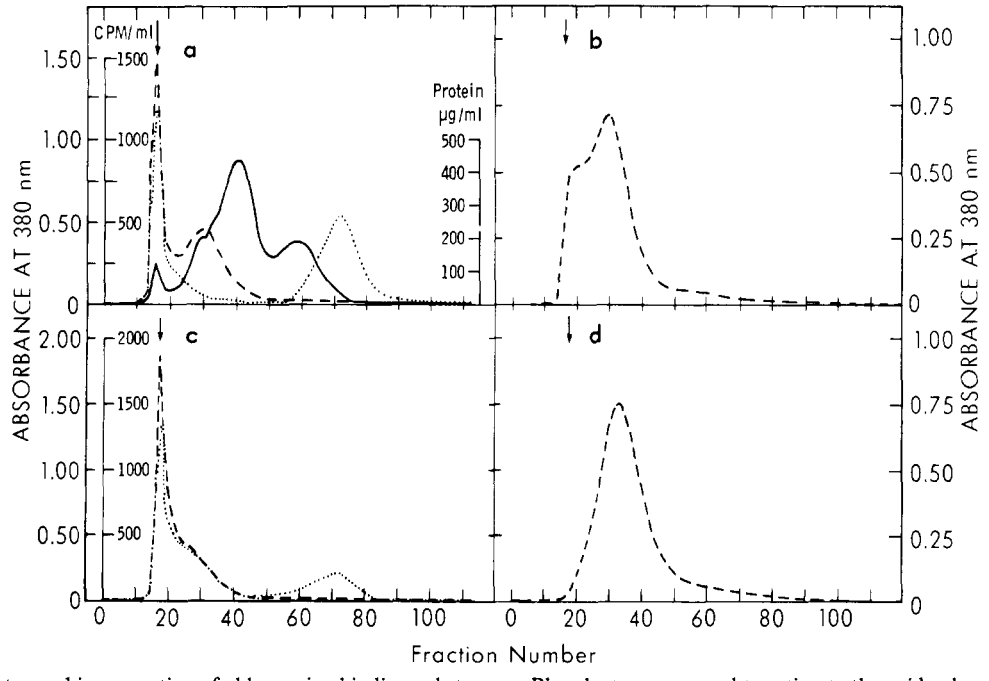

FIGURE 3: Chromatographic separation of chloroquine binding substances. Blue dextran was used to estimate the void volume (arrow). (Panel a) Hemoglobin (28 mg/mL) was incubated with α-chymotrypsin (20 mg/mL) at 25 °C for 1 h, after which [^{14}C]chloroquine (0.93 μmol/mL) was added and the solution was left at 4 °C for 65 h. Then 0.5 mL of the treated hemoglobin solution was loaded onto a Bio-Gel P-200, 50–100 mesh, column (1.5 × 90 cm) equilibrated with the phosphate-buffered medium at 4 °C and eluted with the phosphate-buffered medium. Fractions of 2.5 mL were collected at a flow rate of 12 mL/h and were monitored for radioactivity (dotted line), protein by the Lowry assay (solid line), and ferriprotoporphyrin IX (absorbance at 380 nm, dashed line). (Panel b) Same as panel a but in the absence of [^{14}C]chloroquine. (Panels c and d) Ferriprotoporphyrin IX (0.62 mg/0.5 mL) prepared as described in the text in the presence (panel c) and absence (panel d) of [^{14}C]chloroquine.

Table I: Comparison of Chloroquine Binding Sites[a]

drug (value)	infected erythrocytes[b]	protease-treated erythrocytes[c]	protease-treated hemoglobin	ferriprotoporphyrin IX	pigment clumping[d]
chloroquine (K_d)	1×10^{-8}	5×10^{-8}	1.4×10^{-8}	3.5×10^{-9}	2×10^{-8}
amodiaquine (K_i)	3×10^{-7}	2×10^{-7}	$(4.0 \pm 1.2) \times 10^{-7}$	$(1.3 \pm 0.4) \times 10^{-7}$	NA[e]
quinacrine (K_i)	5×10^{-7}	5×10^{-7}	$(5.9 \pm 2.2) \times 10^{-7}$	$(1.2 \pm 0.4) \times 10^{-7}$	NA[e]
quinine (K_i)	2×10^{-6}	2×10^{-6}	$(3.1 \pm 0.5) \times 10^{-6}$	$(2.6 \pm 0.8) \times 10^{-6}$	4×10^{-7}
mefloquine (K_i)	2×10^{-6}	2×10^{-5}	$(1.4 \pm 0.4) \times 10^{-5}$	$(1.5 \pm 0.02) \times 10^{-5}$	4×10^{-9}
primaquine (K_i)	NA[e]	NA[e]	$(2.2 \pm 1.4) \times 10^{-4}$	$(6.2 \pm 1.6) \times 10^{-5}$	NA[e]

[a] The values for K_d and K_i are given in units of molarity. The experiments were similar to those described in Figures 2 and 5, except that the dialyses were conducted both with and without the addition of a constant amount of inhibitor. The data from six or more separate experiments with six points each were pooled for linear regression analysis to obtain values for K_d for protease-treated hemoglobin and for ferriprotoporphyrin IX. The correlation coefficients exceeded 0.98 in each case. The values for K_i are means ± SD from three or more separate experiments. [b] From Fitch (1972). [c] From Fitch et al. (1974a, 1978). [d] From Warhurst & Thomas (1975). [e] NA, not available.

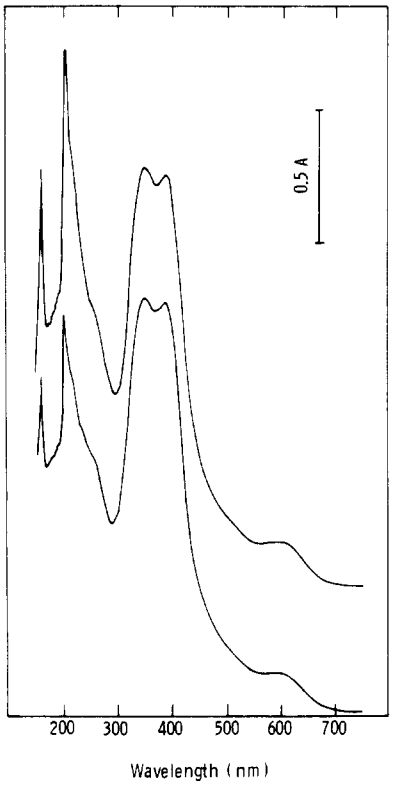

FIGURE 4: Absorption spectra of chloroquine binding fractions. The absorption spectrum of the fraction eluted at the void volume from the proteolysis of hemoglobin (panel a of Figure 3) in the upper tracing is compared with the absorption spectrum of the similar fraction of authentic ferriprotoporphyrin IX (panel c of Figure 3). A Beckman Model 25 spectrophotometer was used to obtain these spectra.

(Figure 4). Under the conditions of these experiments, an aggregated form of ferriprotoporphyrin IX also was produced in the absence of chloroquine, but, in the case of authentic ferriprotoporphyrin IX, the aggregate was not as large as it was in the presence of chloroquine (cf. panels c and d of Figure 3). From the elution patterns shown in Figure 3, the number of ferriprotoporphyrin IX molecules comprising the aggregates was estimated to be on the order of 100 in the absence of chloroquine and in excess of 300 in the presence of chloroquine. It is possible that monomeric and dimeric ferriprotoporphyrin IX and various intermediate-sized aggregates would bind chloroquine with high affinity, but none of these were studied in the present experiments.

Studies of affinity and specificity were performed to further corroborate the identity of the high molecular weight product of proteolysis of hemoglobin. The affinity of authentic ferriprotoporphyrin IX for chloroquine, as evaluated by equilibrium dialysis, was high ($K_d = 3.5 \times 10^{-9}$ M), and ~0.5 mol

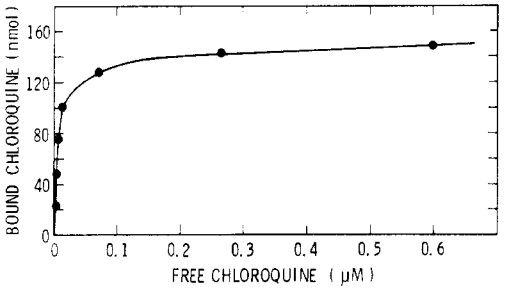

FIGURE 5: Binding of chloroquine to ferriprotoporphyrin IX. Dialysis bags containing 0.223 mg of ferriprotoporphyrin IX, prepared as described in the text, and various amounts of [^{14}C]chloroquine in a final volume of 5 mL were dialyzed against 70 mL of the phosphate-buffered medium at 4 °C for 68 h as described in the text.

of chloroquine was bound per mol of ferriprotoporphyrin IX (Figure 5). The results of studies of specificity are shown in Table I. Five antimalarial drugs were selected to use as inhibitors of [^{14}C]chloroquine binding to evaluate specificity. These same drugs have previously been studied as inhibitors of chloroquine binding in intact erythrocytes infected with malaria parasites (Fitch, 1972) and in uninfected erythrocytes treated with protease (Fitch et al., 1978). Values for K_d and K_i were calculated from double-reciprocal graphs of data obtained in equilibrium dialysis experiments similar to those described in Figures 2 and 5, except that the dialyses were conducted both with and without the addition of a constant amount of inhibitor. Table I compares results from experiments with erythrocytes infected with *P. berghei*, uninfected erythrocytes treated with protease, hemoglobin treated with protease, authentic ferriprotoporphyrin IX, and pigment clumping. The results are in good agreement with each other with the following two exceptions. Firstly, the values for K_i for quinine and mefloquine estimated from pigment clumping experiments are smaller than those from any of the other experiments and the value for mefloquine is particularly noteworthy as it is at least 1000-fold less than is observed in binding studies. Secondly, primaquine [8-[(4-amino-1-methylbutyl)amino]-6-methoxyquinoline] proved to be a competitive inhibitor of chloroquine binding to ferriprotoporphyrin IX. By contrast, primaquine and pamaquine [8-[[4-(diethylamino)-1-methylbutyl]amino]-6-methoxyquinoline] appear to cause a mixed type instead of a competitive type of inhibition of chloroquine binding to erythrocytes infected with chloroquine-susceptible *P. berghei* (Fitch, 1972; Fitch et al., 1974c), and no inhibition of chloroquine binding was observed with 10^{-5} M primaquine in the model system using protease-treated erythrocytes (Fitch et al., 1978). In agreement with the latter observations, 2×10^{-4} M primaquine caused almost no inhibition of chloroquine binding to the hemoglobin degradation product. These findings are consistent

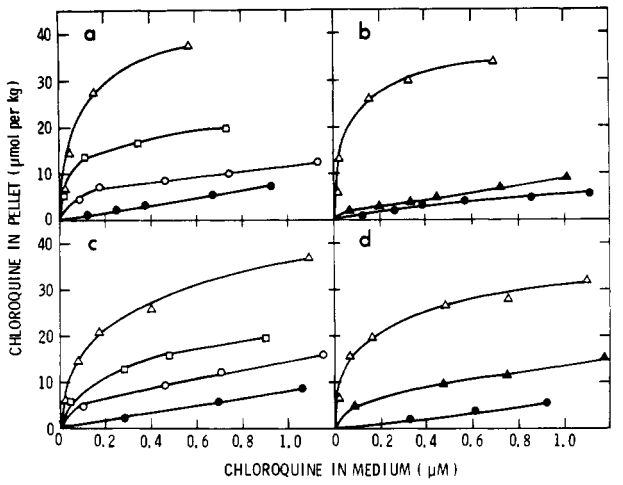

FIGURE 6: Effect of hemoglobin degradation products and ferriprotoporphyrin IX on chloroquine binding to erythrocytes. The hemoglobin degradation products were prepared by incubating 90 mg of hemoglobin with 2.1 mg of the nonspecific protease of *S. griseus* in a final volume of 4.5 mL of the phosphate-buffered medium for 2 h at 25 °C. (Panel a) 5% suspensions of uninfected erythrocytes in the phosphate-buffered medium were preincubated for 60 min at 25 °C with hemoglobin degradation products equivalent to 55 (○), 137 (□), or 275 μg/mL (△) hemoglobin. The control tubes (●) contained erythrocytes and 5.7 μg/mL protease but no hemoglobin degradation products. At the end of the preincubation, the suspensions were centrifuged at 5000*g* for 5 min and the supernatant fluid was removed as completely as possible from the pellet. Then the pellet was resuspended to a hematocrit of 5% in the phosphate-buffered medium containing various concentrations of [^{14}C]chloroquine, and, immediately after mixing, the cells were separated from the medium by centrifugation at 5000*g* for 5 min for measurement of the chloroquine concentration in each. (Panel b) For evaluation of the effect of washing with a solution containing albumin, uninfected erythrocytes were preincubated with hemoglobin degradation products (275 μg/mL) or 5.7 μg/mL protease for 60 min at 25 °C. After preincubation, the pellet was resuspended to a hematocrit of 5% in the phosphate-buffered medium and incubated with or without 1% albumin for 20 min at 25 °C, after which the medium again was removed and the cells were washed once by suspending them in 20 volumes of the phosphate-buffered medium. Finally, the cells were resuspended to a hematocrit of 5% in the phosphate-buffered medium containing various concentrations of [^{14}C]chloroquine and centrifuged immediately after mixing to separate cells from the medium as before for measurement of the chloroquine concentration in each. Hemoglobin degradation products with no albumin (△); hemoglobin degradation products plus albumin (▲); protease alone (●). (Panel c) Uninfected erythrocytes were treated as described for panel a except that ferriprotoporphyrin IX was substituted for the hemoglobin degradation products and no protease was included in the control tubes. The amounts of ferriprotoporphyrin IX were none (●), 2 μg/mL (○), 4 μg/mL (□), and 8 μg/mL (△). (Panel d) Normal erythrocytes were treated as described for panel b except that 8 μg/mL ferriprotoporphyrin IX was substituted for the hemoglobin degradation products and no protease was included in the control tubes. Ferriprotoporphyrin IX with no albumin (△); ferriprotoporphyrin IX with albumin (▲); control tubes without ferriprotoporphyrin IX (●).

with the possibility that various biological products modify the interaction between antimalarial drugs and ferriprotoporphyrin IX.

Further evidence that biological products modify the interaction of ferriprotoporphyrin IX with antimalarial drugs is shown in Figure 6. Panel a of this figure shows that the product of hemoglobin digestion adsorbs to normal, intact erythrocytes and in turn binds chloroquine with a K_d of 1.3×10^{-7} M. Panel c shows that ferriprotoporphyrin IX likewise adsorbs to normal, intact erythrocytes. The amount of ferriprotoporphyrin IX adsorbed and, consequently, the maximal binding capacity for chloroquine were a function of the amount of ferriprotoporphyrin IX to which the cells were exposed (panels a and c of Figure 6). Double-reciprocal graphs of the data in Figure 6 to estimate the K_d of ferriprotoporphyrin IX for chloroquine yielded a value of 2.3×10^{-7} M, which is ~2 orders of magnitude larger than the K_d in the absence of other biological products.

As a final test of the similarity of behavior of the degradation product of hemoglobin and ferriprotoporphyrin IX, the effect of albumin was studied (panels b and d of Figure 6). Washing with a solution of albumin is known to decrease the binding of chloroquine by protease-treated normal erythrocytes (Fitch et al., 1978). Similar treatment also decreased the binding of chloroquine to erythrocytes to which the degradation product of hemoglobin or ferriprotoporphyrin IX had been adsorbed. The decrease in all three cases can be attributed to binding of ferriprotoporphyrin IX to albumin which would keep it in solution instead of adsorbed to the erythrocyte surface.

Discussion

Unequivocal identification of an isolated substance as a drug receptor requires (a) that affinities and specificities of binding of the drug to the substance match those of the receptor, (b) that the drug is relatively ineffective when the putative receptor is absent from the organism, and (c) that drug effectiveness returns when the receptor is reintroduced into the organism. With regard to the chloroquine binding component created by treating the surface of normal, intact erythrocytes with protease, only the first criterion can be applied as no effect of the drug is available to study in this model.

The evidence is compelling that the formation of ferriprotoporphyrin IX accounts for chloroquine accumulation by protease-treated normal erythrocytes. This evidence may be summarized as follows. Ferriprotoporphyrin IX is produced by extensive degradation of hemoglobin by proteases, and, at least in an aggregated form, it binds chloroquine with high affinity and with a specificity closely matching that of normal, intact erythrocytes treated with protease. In addition, washing the erythrocytes in a medium containing albumin reduces the ability to bind chloroquine both by protease-treated erythrocytes and by erythrocytes to which the high molecular weight hemoglobin degradation product or authentic ferriprotoporphyrin IX is adsorbed. With these observations, all of the previously described characteristics of chloroquine binding to protease-treated normal erythrocytes have been duplicated by erythrocytes to which ferriprotoporphyrin IX is adsorbed.

Although visible hemolysis is not usually present after erythrocytes are treated with protease under our conditions (Fitch et al., 1978), only minimal hemolysis would be required to yield enough ferriprotoporphyrin IX to account for our observations, and since hemoglobin would be degraded by the protease, it would not be visible. We conclude, therefore, that protease treatment of normal, intact erythrocytes produces ferriprotoporphyrin IX which adsorbs to the erythrocyte surface and then binds chloroquine with high affinity and with specificity for drugs in the same chemotherapeutic class with chloroquine.

The topography of the chloroquine receptor of *P. berghei* has been inferred from studies of specificity of drug binding (Fitch et al., 1974c). Three features of the receptor were considered essential: "(a) a flat surface large enough to accommodate planar ring systems of 30–40 Å^2"; "(b) a chemical grouping in the surface that favors interaction with compounds having a nitrogen in their ring system such as quinoline derivatives"; "(c) an anionic site located in the proper geometric relationship to the flat surface to attract the protonated terminal nitrogen atom". Ferriprotoporphyrin IX has a flat surface large enough to accommodate a planar ring system

of 30–40 Å^2, it possesses Fe^{3+} to coordinate with the nitrogen of the quinoline ring, and it possesses carboxyl groups that might interact with the terminal protonated nitrogen atom of the side chain of chloroquine and related drugs. It is also possible that the terminal nitrogen atom would coordinate with the Fe^{3+} atom of another nearby ferriprotoporphyrin IX molecule. The latter possibility would account for the stoichiometry of 0.5 mol of chloroquine bound per mol of ferriprotoporphyrin IX. In accord with these topographic features, ferriprotoporphyrin IX in an aggregated form binds chloroquine with high affinity and with specificity corresponding closely to the affinity and specificity of erythrocytes infected with *P. berghei* (Fitch, 1972). Clearly, ferriprotoporphyrin IX fulfills the first criterion to identify it as the drug receptor.

A review of previous knowledge of malaria pigment accumulation indicates that the second and third criteria also are fulfilled by ferriprotoporphyrin IX. Erythrocytes infected with chloroquine-susceptible *P. berghei* possess proteases to degrade hemoglobin (Cook et al., 1961; Levy & Chou, 1973), and they accumulate abundant amounts of malaria pigment (Peters, 1964; Thompson et al., 1965; Ladda & Sprinz, 1969), which contains ferriprotoporphyrin IX (Fulton & Rimington, 1953). Hemoglobin degradation is diminished or absent in erythrocytes infected with chloroquine-resistant *P. berghei* (Eckman et al., 1977) and malaria pigment does not accumulate (Peters, 1964; Thompson et al., 1965; Ladda & Sprinz, 1969). Finally, to satisfy the third criterion, malaria pigment reappears when previously resistant lines of *P. berghei* revert to chloroquine susceptibility (Peters, 1964; Thompson et al., 1965; Ladda & Sprinz, 1969). Therefore, we conclude that ferriprotoporphyrin IX, probably in the form of an aggregate, is the receptor involved in the accumulation of chloroquine with high affinity in erythrocytes infected with *P. berghei*.

Since ferriprotoporphyrin IX fulfills the criteria for identification as the chloroquine receptor of malaria parasites, the relationship between binding to this receptor and the chemotherapeutic action of chloroquine merits consideration. As Macomber et al. (1967) suggested, binding of chloroquine to ferriprotoporphyrin IX may only serve to accumulate the drug and make it available to exert its chemotherapeutic action on the malaria parasite. It is also possible, however, that the chloroquine–ferriprotoporphyrin IX complex would be directly toxic to the malaria parasite. For example, the complex might be involved in pigment clumping, which is the earliest morphological event after exposure of malaria parasites to chloroquine (Macomber et al., 1967; Warhurst & Hockley, 1967). Figure 3 shows that chloroquine increases the size of ferriprotoporphyrin IX aggregates, and in a complex biological mixture such as malaria pigment (Yamada & Sherman, 1979) the presence of chloroquine might cause the formation of aggregates large enough to be visualized as clumping. Macomber et al. (1967) stated that chloroquine does not bind to malaria pigment, but they did not describe their experiments nor did they present any data. Further studies of the interaction of chloroquine with malaria pigment are needed to determine whether or not ferriprotoporphyrin IX is directly involved in pigment clumping.

Identification of ferriprotoporphyrin IX as the chloroquine receptor of *P. berghei* also leaves three other important questions unanswered. Why is there an energy requirement for chloroquine accumulation (Fitch et al., 1974c) and pigment clumping (Warhurst et al., 1972)? Why are the inhibitor constants for quinine and mefloquine so much less for pigment clumping than for the process of chloroquine accumulation (Warhurst & Thomas, 1975)? And does an interaction with ferriprotoporphyrin IX account for the apparent involvement of the host cell membrane in chloroquine accumulation by malaria parasites (Fitch et al., 1978)? Answers to these questions are needed to extend our knowledge of the role of ferriprotoporphyrin IX as a mediator of chloroquine toxicity for malaria parasites.

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Photochemical Studies of 7-*cis*-Rhodopsin at Low Temperatures. Nature and Properties of the Bathointermediate†

Satoru Kawamura,‡ Seiji Miyatani,§ Hiroyuki Matsumoto,‖ Tôru Yoshizawa,* and Robert S. H. Liu

ABSTRACT: The photoreaction of 7-*cis*-rhodopsin derived from 7-*cis*-retinal and cattle opsin was studied by low-temperature spectrophotometry. Upon irradiation of 7-*cis*-rhodopsin at liquid nitrogen temperature (−190 °C) with blue light, its spectrum shifted to the longer wavelengths, indicating the formation of a bathoproduct. The bathoproduct thus formed was found to be identical with bathorhodopsin formed from rhodopsin in their spectroscopic, photochemical, and thermal properties. Therefore, we believe that the bathoproduct is, in fact, bathorhodopsin. The fact that 7-*cis*-rhodopsin can be readily converted to rhodopsin and to 9-cis-rhodopsin shows that the identical retinal binding site of opsin is involved in the three isomeric rhodopsins. These results appear to be consistent with the notion that the chromophore of bathorhodopsin is a twisted all-trans isomer, which is readily obtainable from the 7-cis, 9-cis, and 11-cis isomers.

In 1963 by irradiating rhodopsin at liquid nitrogen temperature (−190 °C) Yoshizawa & Wald (1963) observed the formation of the red-shifted primary photoproduct, bathorhodopsin (earlier known as prelumirhodopsin). The rigid matrix and low temperature used in their experiment apparently did not have a profound effect on the photochemistry of rhodopsin for it has recently been shown by laser flash photolysis of rhodopsin (Busch et al., 1972; Cone, 1972; Rosenfeld et al., 1972) that the same intermediate was generated immediately after illumination.

In the same paper it was also shown by Yoshizawa & Wald (1963) that an intermediate spectrally identical with bathorhodopsin is also formed by irradiation of 9-*cis*-rhodopsin.[1] It appeared natural to suggest (Yoshizawa & Wald, 1963) that an identical intermediate with the strained all-trans polyene geometry is formed from rhodopsin and 9-*cis*-rhodopsin. However, it has also been suggested (Kropf, 1969) that perhaps two spectrally similar bathoproducts are actually formed which may retain something like the original 11-cis and 9-cis geometrical integrities. This suggestion has perhaps found renewed interest after recent postulation of proton transfer as a primary photochemical event of rhodopsin (Fransen et al., 1976; Peters et al., 1977).

The close absorption maxima of rhodopsin (498 nm) and 9-*cis*-rhodopsin (485 nm) hampered the design of a definitive experiment to prove or disprove Kropf's (1969) original suggestion. However, recently DeGrip et al. (1976) reported the formation of 7-*cis*-rhodopsin from highly hindered 7-*cis*-retinal and cattle opsin. The new pigment analogue has a greatly blue-shifted absorption maximum (λ_{max} = 450 nm) from that of rhodopsin (498 nm). It was thought that batho-7-*cis*-rhodopsin (the bathoproduct from 7-*cis*-rhodopsin), if indeed it existed, would also exhibit a similar blue shift from bathorhodopsin and thus be easily distinguishable from the less hindered isomers (bathorhodopsin and batho-9-*cis*-rhodopsin). Furthermore, it was shown that the final photobleaching product of 7-*cis*-rhodopsin is also *all-trans*-retinal and opsin (DeGrip et al., 1976). It will be of interest to determine whether a bleaching sequence identical with that of rhodopsin is also involved in the new pigment analogue. We have therefore studied the photochemistry of 7-*cis*-rhodopsin at low temperatures.

Materials and Methods

Rod outer segments were isolated from cattle retinas as reported previously (Matsumoto et al., 1978). Opsin was extracted with 1% digitonin buffered in 10 mM Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], pH 7.0. The synthesis of 7-*cis*-retinal was reported previously (Ramamurthy & Liu, 1975). The conversion of *all-trans*-retinal to 7-*cis*-retinal has also been reported (Denny & Liu, 1977; Maeda et al., 1978b). To prepare 7-*cis*-rhodopsin, equimolar 7-*cis*-retinal in ethanol was added to opsin and then the mixture was incubated at room temperature for 48 h.

The equipment used for this study and the general procedure have already been described in detail in the literature (Yoshizawa, 1972). Briefly, 7-*cis*-rhodopsin solubilized in digitonin was mixed with glycerol (1:3 v/v). The mixture was placed in an optical cell (path length = 2 mm) which was fixed

† From the Department of Biophysics, Faculty of Science, Kyoto University, Kyoto 606, Japan (S.K., S.M., H.M., and T.Y.), and the Department of Chemistry, University of Hawaii, Honolulu, Hawaii 96822 (R.S.H.L.). *Received August 17, 1979; revised manuscript received December 18, 1979.* This work was supported in part by grants from the Ministry of Education of Japan to T.Y. and from the Japan Society for the Promotion of Science to H.M. and S.K.

‡ Present address: Laboratory of Molecular Biology, University of Wisconsin, Madison, WI 53706.
§ Present address: Faculty of Medicine, University of Tokyo, Tokyo, Japan.
‖ Present address: Department of Biological Sciences, Purdue University, Lafayette, IN 47907.

[1] Since a large number of geometric isomers of rhodopsin are now known to exist, we have decided to abandon the use of common names such as isorhodopsin. In this paper, the term rhodopsin when used carries the implied geometry of 11-cis. A proper prefix will be used in naming an isomer of rhodopsin, e.g., 7-*cis*-rhodopsin or 9-*cis*-rhodopsin, etc.

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