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Purification and Characterization of Dihydroorotate Dehydrogenase from the Rodent Malaria Parasite *Plasmodium berghei*†

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ABSTRACT: Dihydroorotate dehydrogenase (DHODase) has been purified 400-fold from the rodent malaria parasite *Plasmodium berghei* to apparent homogeneity by Triton X-100 solubilization followed by anion-exchange, Cibacron Blue F3GA-agarose affinity, and gel filtration chromatography. The purified enzyme has a molecular mass of 52 ± 2 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and of 55 ± 6 kDa by gel filtration chromatography, and it has a pI of 8.2. It is active in monomeric form, contains 2.022 mol of iron and 1.602 acid-labile sulfurs per mole of enzyme, and does not contain a flavin cofactor. The purified DHODase exhibits optimal activity at pH 8.0 in the presence of the ubiquinone coenzyme CoQ_6, CoQ_7, CoQ_9, or CoQ_{10}. The K_m values for L-DHO and CoQ_6 are 7.9 ± 2.5 μM and 21.6 ± 5.5 μM, respectively. The k_{cat} values for both substrates are 11.44 min^{-1} and 11.70 min^{-1}, respectively. The reaction product orotate and an orotate analogue, 5-fluoroorotate, are competitive inhibitors of the enzyme-catalyzed reaction with K_i values of 30.5 μM and 34.9 μM, respectively. The requirement of the long-chain ubiquinones for activity supports the hypothesis of the linkage of pyrimidine biosynthesis to the electron transport system and oxygen utilization in malaria by DHODase via ubiquinones [Gutteridge, W. E., Dave, D., & Richards, W. H. G. (1979) *Biochim. Biophys. Acta 582*, 390-401].

Dihydroorotate dehydrogenase (DHODase,[1] L-5,6-dihydroorotate:oxygen oxidoreductase, EC 1.3.3.1), the fourth sequential enzyme in the de novo biosynthesis of pyrimidines, catalyzes the oxidation of dihydroorotate (DHO) to orotate (OA). In many bacterial systems, the DHODase is membrane bound, and its action is intimately linked to the cell's respiratory systems (Karibian & Couchoud, 1974; Larsen & Jensen, 1985). Similarly, in a variety of eukaryotic cells, the enzyme is physically associated with the membrane of the mitochondrion, and again, its action appears to be linked to respiratory electron transport (Jones, 1980; Chen & Jones, 1976; Forman & Kennedy, 1978; Hines et al., 1986; Gero & O'Sullivan, 1985). In contrast, a cytosolic form of DHODase has been isolated from the trypanosomatid protozoans *Crithidia fasciculata* and *Trypanosoma brucei* (Pascal et al., 1983; Pascal & Walsh, 1984), and a similar enzyme has been found in *Escherichia coli* (Larsen & Jensen, 1985).

The parasitic protozoa responsible for human malaria are totally dependent on de novo biosynthesis for their pyrimidine

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[1] Abbreviations: DHODase, dihydroorotate dehydrogenase; L-DHO, dihydroorotate; L-OA, orotate; 5-F-OA, 5-fluoroorotate; DCIP, dichlorophenolindophenol; FPLC, fast protein liquid chromatography.

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requirements [Krungkrai et al. (1990) and references cited therein]. For this reason pyrimidine biosynthesis by malaria parasites, and the enzyme DHODase in particular, has long been considered to be a promising target for chemotherapy. Some progress to this end has already been made with the discovery that 2-hydroxy-1,4-naphthoquinone derivatives possess potent antimalarial activity through inhibition of pyrimidine biosynthesis (Hammond et al., 1985; Hudson et al., 1985). One particularly promising 2-hydroxy-1,4-naphthoquinone derivative (A566C, Wellcome) is currently being tested for prophylaxis and treatment of malaria in human volunteers (Gutteridge, 1989). However, while the parasite DHODase is the presumed site of action of these experimental antimalarials, this parasite enzyme has not been characterized. To facilitate further work in this area and as part of a detailed analysis of pyrimidine metabolism and mitochondrial function in malaria parasites (Krungkrai et al., 1990), we have purified DHODase to apparent homogeneity from the mitochondrial pellet of the rodent malaria parasite *Plasmodium berghei*. We now report the molecular and kinetic properties of the enzyme and its cofactor requirements. In particular, the malarial DHODase, in marked contrast to its mammalian or trypanosomatid counterparts, is shown to be a metalloprotein which does not contain flavin cofactors but which does contain iron in the form of an iron–sulfur cluster.

Materials and Methods

Materials. [*carboxy*-^{14}C]Orotate (52.5 Ci mol^{-1}) was purchased from Du Pont-New England Nuclear. Cibacron Blue F3GA–agarose affinity gel was obtained from Pierce. Fast protein liquid chromatography (FPLC) was carried out on columns from Pharmacia LKB Biotechnology Inc. All other chemicals, reagents, and materials were of the highest quality commercially available and were used without further purification. The quinones were dissolved in 1% Triton X-100 prior to use in the assays.

[*carboxy*-^{14}C]Dihydroorotate (L-[^{14}C]DHO) was synthesized from [^{14}C]orotate as described (Smithers et al., 1978) and purified by FPLC on a Mono Q10/10 anion-exchange column with 0.19 M LiCl as eluent. The radiochemical purity of [^{14}C]DHO was >99%, as determined by thin-layer chromatography (TLC) on poly(ethylenimine)–cellulose (PEI–cellulose) (Christopherson et al., 1978, 1981), and had specific radioactivity of 52.5 Ci mol^{-1}.

Enzyme Assays. The spectrophotometric method, based on 2,6-dichlorophenolindophenol (DCIP) reduction, was used according to Miller et al. (1968). In a typical assay, CoQ_6 was used as the proximal electron acceptor, and DCIP was used as the final electron acceptor. The reaction was monitored by the loss of DCIP absorbance at 600 nm (ϵ = 21 500 M^{-1} cm^{-1}). The reaction (1.0 mL) contained 0.25 mM L-DHO, 0.07 mM CoQ_6, 0.045 mM DCIP, 0.15% Triton X-100, and enzyme (0.25–0.50 nmol min^{-1}) in 100 mM Tris-HCl (pH 8.0). This method was used for routine activity assays during enzyme purification; the addition of 1 mM KCN was included to ensure electron transfer from CoQ_6 to DCIP and not to the electron transport system when the enzyme was still in the membrane-bound form.

In an alternative assay procedure, [^{14}C]orotate (OA) production from [^{14}C]DHO was directly monitored by chromatography using a Mono Q anion-exchange column on FPLC with isocratic elution of 0.2 M ammonium formate (pH 7.0). A standard assay (0.5 mL) contained 0.1 mM [^{14}C]DHO, 0.07 mM CoQ_6, enzyme (0.5 nmol min^{-1}), 0.15% Triton X-100, and 100 mM Tris-HCl (pH 8.0). This method was used to confirm the identity of the enzyme DHODase after purification from each step and to evaluate the backward reaction catalyzed by DHODase from L-OA to L-DHO.

Parasites. P. berghei parasites were cultivated in Balb/c mice. Cell-free extracts of the parasites were prepared as described previously (Krungkrai et al., 1985) in the presence of the following protease inhibitors: 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.03 mg mL^{-1} leupeptin, pepstatin, and *N*-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), and 1 mM ethylenediaminetetraacetic acid (EDTA).

Enzyme Purification. Each step was carried out at 4 °C unless otherwise stated. All buffer solutions contained 2.5 mM L-DHO, which stabilized the enzyme activity during purification. The isolated parasites could be stored for 3 months at −80 °C without loss of DHODase activity. DHODase from *P. berghei* was purified to apparent homogeneity as follows.

Membrane Extraction. The isolated parasites were mixed with 10 mM Tris-HCl (pH 8.0) and protease inhibitors as described above. After three cycles of freezing (dry ice–acetone) and thawing (37 °C), the homogenate was centrifuged (39000*g* for 30 min). The pellet fraction was resuspended with 10 mM Tris-HCl (pH 8.0) containing 1.0 mM EDTA, 1.0 mM PMSF, and 2.5 mM L-DHO and designated the crude membrane extract.

Detergent Solubilization. Triton X-100 was added to the crude extracts to give a final concentration of 0.15%. The mixture was then stirred for 30 min at 4 °C. The supernatant fluid collected after centrifugation (39000*g* for 30 min) contained solubilized DHODase.

FPLC on Mono Q. The Triton X-100 solubilized DHODase was directly applied to a Mono Q 10/10 FPLC column at a flow rate of 1.0 mL min^{-1}. The column was washed with 10 mM Tris-HCl (pH 8.0) containing 0.15% Triton X-100, 1.0 mM EDTA, 1.0 mM PMSF, and 2.5 mM L-DHO (buffer A) and eluted with a linear gradient of 0–0.6 M $(NH_4)_2SO_4$ (70 mL). Active fractions, which eluted at 0.05 M $(NH_4)_2SO_4$, were pooled and desalted by PD-10 columns (Pharmacia) which had been equilibrated with buffer A (Figure 1A).

Cibacron Blue F3GA–Agarose Affinity Column Chromatography. Active fractions from Mono Q chromatography were applied to a Cibacron Blue F3GA–agarose affinity column which had previously been equilibrated with buffer A. The column was washed with 12 mL of buffer A and then eluted with buffer A containing 0.6 M $(NH_4)_2SO_4$ (12 mL) and finally eluted with buffer A containing 2.0 M $(NH_4)_2SO_4$ (Figure 1B). The active 1.0-mL fractions were pooled and concentrated on a Centricon-10 (Amicon).

Gel Filtration on Superose 12 FPLC Column. Final purification was carried out by gel filtration on a Superose 12 column by elution with buffer A containing 0.6 M $(NH_4)_2SO_4$ at a flow rate of 0.5 mL min^{-1}. The column was calibrated with the following proteins: thyroglobulin, 670 kDa; IgG, 158 kDa; ovalbumin, 44 kDa; myoglobin, 17 kDa; vitamin B_{12}, 1350 Da (Bio-Rad). The enzyme eluted at a position which corresponds to 55 kDa (Figure 1C). The purified enzyme was stored at −80 °C.

Electrophoresis. SDS–polyacrylamide gel electrophoresis was performed on a Bio-Rad minislab gel apparatus with a 5% acrylamide stacking gel and a 10% acrylamide running gel in the discontinuous buffer system of Laemmli (1970). The gels were stained with both Coomassie blue R and silver (Wray et al., 1981).

Mono P Chromatofocusing FPLC. The p*I* of the purified DHODase was determined on a Mono P (Pharmacia) chromatofocusing FPLC column. The starting buffer was 25 mM Tris-HCl, pH 9.4; the elution buffer was polybuffer 96

Table I: Purification of DHODase from *P. berghei*

step	total protein (mg)	total act. (milliunits/min)	specific act. [milliunits min^{-1} (mg of protein)$^{-1}$]	yield %	purification (x-fold)
(1) crude membrane extract	146.385	62.070	0.424	100	1
(2) detergent solubilization	20.114	58.863	2.777	90	7
(3) Mono Q FPLC	2.056	45.936	22.340	74	53
(4) Cibacron Blue affinity	0.176	20.465	116.418	33	275
(5) Superose 12 FPLC	0.043	7.229	168.109	12	396

(Pharmacia), pH 6.5. The column was run at a flow rate of 1 mL min^{-1} for 40 min. Fractions (2 mL) were collected and measured immediately for pH and DHODase activity.

Fe, Acid-Labile Sulfur, and Flavin Analysis. The non-heme iron content of pure DHODase (19.608 μg) was determined with a sensitivity as low as 0.1 nmol by use of diphenylphenanthroline following dithionite reduction (Brumby & Massey, 1967). Fe wire was used as standard. Determination of acid-labile sulfur was carried out according to Rabinowitz (1978) with Na_2S as standard. The flavin (FMN) content was measured from the spectrum of the purified DHODase with ϵ = 12 200 M^{-1} cm^{-1} at 450 nm (ca. 0.82 nmol of flavin; A_{450} = 0.010 in 1 mL of solution).

Antibody Production and Immunoblotting. Antiserum directed against purified DHODase was raised in Balb/C mice. Each mouse was injected with 7 μg of antigen in Freund's complete adjuvant, followed by 5 μg in Freund's incomplete adjuvant at 2-week intervals after the first dose. Antiserum was collected after 6 weeks and assessed by immunoblot analysis. When diluted 1:200, the serum could easily detect 70 ng of purified DHODase.

Other Methods. Protein concentrations were determined by the method of Bradford (1976) with bovine serum albumin as standard. Kinetic parameters were determined on homogeneous preparations of DHODase and were fitted with a linear regression analysis. The effect of pH on initial velocity was studied at 37 °C with 100 mM Tris-HCl buffer at various pH (7.0–11.0). Spontaneous reduction of DCIP at pH <7.0 precluded measurement at lower pH. Enzyme solutions containing 2.5 mM DHO were desalted by gel filtration (ExCellulose, Pierce) before kinetic analysis.

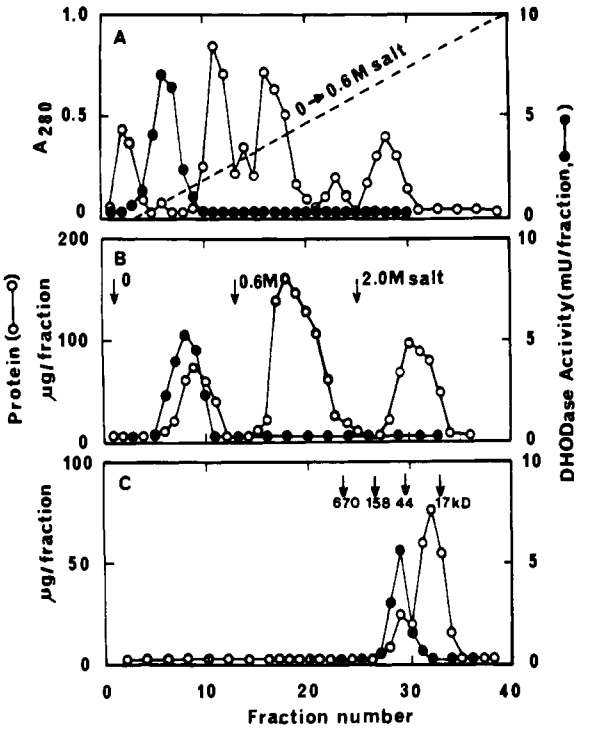

FIGURE 1: Chromatographic profiles of *P. berghei* DHODase purification. Open circles indicate protein. Closed circles indicate DHODase activity. (A) FPLC of Triton X-100 solubilized DHODase on a Mono Q 10/10 column with a linear salt gradient. (B) Cibacron Blue F3GA–agarose affinity column of pooled Mono Q activity with stepwise application of salt. (C) Gel filtration using a Superose 12 column. Arrows show elution position of calibration proteins.

RESULTS

Enzyme Purification from P. berghei. Cell-free extracts of the malarial DHODase were found to be extemely labile. Even in the presence of protease inhibitors at 4 °C, the activity decreased by more than 50% overnight. We first tried to stabilize the DHODase activity with known stabilizers reported for this enzyme and for general use. In separate experiments, the following reagents were included: 2.5 mM L-OA, 1.0 mM DTT, 1.0 mM EDTA, 0.6 M $(NH_4)_2SO_4$, 20% dimethyl sulfoxide, and 50% glycerol. These compounds had no stabilizing effect when the enzyme was stored at 4 °C or even at −20 °C. However, by using 2.5 mM L-DHO in all buffers, we found that the enzyme was stable through overnight storage at −80 °C. This approach facilitated stockpiling of large amounts of parasite extract and the homogeneous purification of enzyme.

Following Triton X-100 solubilization of the parasite pellet, chromatography on Mono Q anion-exchange, Cibacron Blue F3GA–agarose affinity, and finally gel filtration (Superose 12 column) chromatography, the *P. berghei* DHODase was purified ca. 400-fold to apparent homogeneity in 12% yield (Table I and Figure 1). Repeated applications of the purification scheme of Table I gave consistently homogeneous enzyme preparations. The purified DHODase was stable in 100 mM Tris-HCl (pH 8.0), 0.15% Triton X-100, and 2.5 mM L-DHO, at −80 °C, for 3 weeks. Storage at 4 and −20 °C in the same buffer resulted in ca. 40% loss of activity per day.

Physical Characterization of P. berghei DHODase. The molecular weight of the purified enzyme was found to be 51 600 ± 2050 (n = 4) by SDS–PAGE analysis (Figure 2). The native enzyme was estimated to have a molecular weight of 55 000 ± 6000 (n = 3) as determined by gel permeation chromatography on a Superose 12 FPLC column. These data suggest that the malarial DHODase is active in monomeric form. In addition, following the production of antisera to the purified DHODase, Western immunoblot analysis of a crude *P. berghei* cell lysate (run under reducing and nonreducing conditions) revealed a single protein species with M_r 52 000 (Figure 3). The pI of the purified enzyme was determined to be 8.2 (two determinations).

By use of the extinction coefficient for flavin (FMN) at 450 nm (ϵ = 12 200 M^{-1} cm^{-1}), the flavin content of the enzyme at pH 8.0 was calculated to be ≤0.065 ± 0.014 mol/mol of enzyme. Microanalytical characterization of the purified enzyme indicated that 1 mol of enzyme contains 2.022 mol of Fe and 1.602 mol of acid-labile sulfur. These results suggest that the malarial DHODase is a metalloprotein with ca. 2 mol of Fe and 2 mol of acid-labile sulfur per mole of enzyme.

The UV/vis spectrum of the pure DHODase was not consistent with the presence of a flavin cofactor. Neither was the

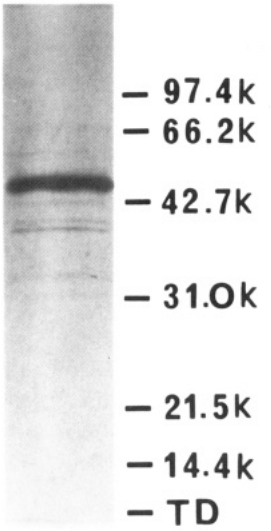

FIGURE 2: SDS–PAGE analysis of *P. berghei* DHODase. A 10% polyacrylamide gel was run in 0.1% SDS. Marker proteins were phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (31.0 kDa), and soybean inhibitor (21.5 kDa). The proteins were first stained with Coomassie blue and then with silver.

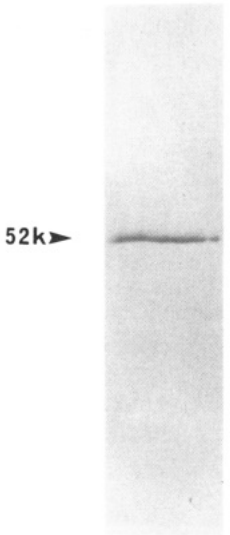

FIGURE 3: Western analysis of a Triton-solubilized extract of *P. berghei* probed with mouse anti-DHODase antiserum.

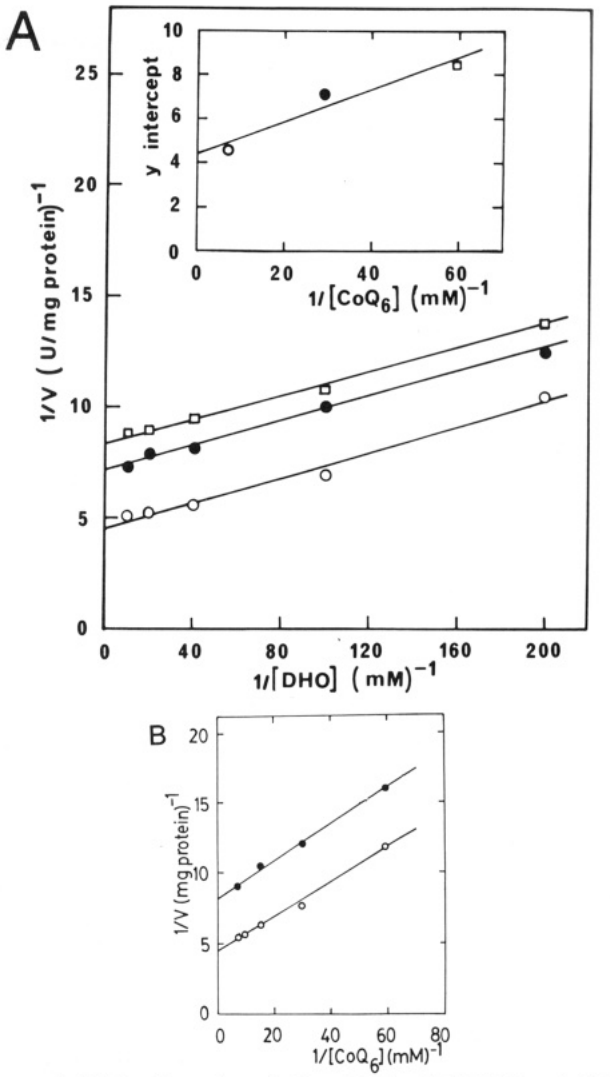

FIGURE 4: (A) Double-reciprocal plots of *P. berghei* DHODase initial velocity kinetics obtained by varying [DHO] at three different fixed [CoQ_6]. Concentrations of CoQ_6 were 16.9 (□), 33.8 (●), and 135.2 μM (○), in a total volume of 1.0 mL at pH 8.0. The slope of each [CoQ_6] line is 0.024 and equal to K_{DHO}/V_{max}. Inset is the secondary plot between the *y* intercept and the concentration of CoQ_6; the slope of this plot is 0.0727 and equal to K_{CoQ}/V_{max}. (B) [CoQ_6] at 0.1 mM DHO (○) and 0.1 mM DHO and OA (●).

UV/vis spectrum indicative of a [2Fe-2S] cluster which, when in the +2 oxidation state, has been reported to display absorbance maxima near 330, 420, and 465 nm (Orme-Johnstone & Orme-Johnstone, 1978). At a protein concentration of 98 μg/mL, A_{415} and A_{450} were determined to be 0.005 and 0.0015, respectively.

Kinetic Analysis of P. berghei DHODase. The purified DHODase had optimal activity at pH 8.0. The reversibility of the DHODase reaction was tested with purified DHODase with [^{14}C]OA as substrate. It was shown that there was no [^{14}C]DHO formation, even in the presence of 100 μM NAD(P)H. These results suggest the malarial DHODase does not function as an orotate reductase. Cosubstrate electron acceptors for the purified DHODase were examined. Maximal rates were obtained when CoQ_6, CoQ_7, CoQ_9, and CoQ_{10} were used as electron acceptors. The simple quinones 2-hydroxy-1,4-naphthoquinone (lawsone) and 2-methyl-1,4-naphthoquinone (menadione) and CoQ_0 did not function as substrates but were found to be inhibitory (50% inhibition at 0.2 mM). When used alone, DCIP itself was a poor electron acceptor (cosubstrate). Similarly, the enzyme does not transfer electrons directly to molecular oxygen. The latter result was obtained by monitoring OA production at 278 nm from the substrate DHO at pH 8.0 in air-saturated buffer; no significant conversion of DHO to OA could be detected. These results suggest that the malarial DHODase possesses a strict requirement for a ubiquinone (CoQ) cofactor. In additional experiments, both FAD and FMN were added to incubation mixtures (in the presence and absence of ubiquinone); neither compound altered the reaction rate.

The apparent Michaelis constants (K_m) for L-DHO and CoQ_6 were estimated from double-reciprocal plots of the initial rates at various substrate concentrations. The K_m values for L-DHO and CoQ_6 were 7.90 ± 2.45 μM (n = 3) and 21.58 ± 5.45 μM (n = 3), whereas the k_{cat} values were 11.44 ± 3.41 min^{-1} and 11.70 ± 2.86 min^{-1} for L-DHO and CoQ_6, respectively. The mechanism of catalysis with two substrates of the malarial DHODase was analyzed by using the initial velocity patterns obtained by varying [DHO] at three different fixed [CoQ_6] and by varying [CoQ_6] at fixed concentrations of [DHO] and [OA]. Double-reciprocal plots of the results (Figure 4) gave parallel straight-line patterns. These results

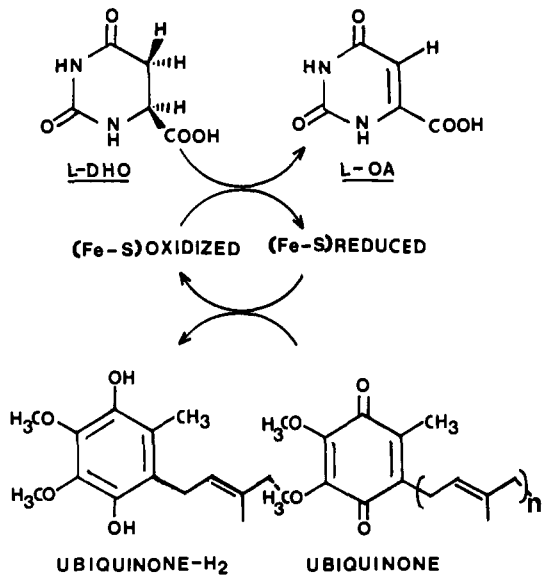

FIGURE 5: Proposed enzymatic catalysis by *P. berghei* DHODase.

suggest that the malarial DHODase displays a ping-pong mechanism for L-DHO and CoQ_6.

L-OA, the product of the enzymatic reaction, was a competitive inhibitor of DHO reduction with a K_i of 30.54 μM. An orotate analogue, 5-fluoroorotate (5-F-OA), also showed competitive inhibition with a K_i of 34.92 μM.

Discussion

Dihydroorotate dehydrogenases have been characterized from a variety of prokaryote and eukaryote systems. While these enzymes all catalyze two-electron oxidation of DHO to OA, considerable differences exist with regard to their physical properties, cellular location, cofactor requirement, kinetic behavior, and the species to which they transfer the reducing equivalents which result from turnover. For example, DHODase from higher eukaryotic cells are located in the mitochondria and are metalloenzymes (Forman & Kennedy, 1978) or metalloflavoenzymes which have both flavin mononucleotide (FMN) and iron–sulfur centers (Hines et al., 1986; Hines & Johnston, 1989). By contrast, the cytosolic DHODases of the trypanosomatid protozoa have been shown to be simple flavoprotein oxidases which deliver electrons directly to molecular oxygen (Pascal et al., 1983). Our interest in the *Plasmodium* DHODase was stimulated both by the supposed sensitivity of this enzyme toward antimalarial drugs of the 2-hydroxy-1,4-naphthoquinone class and also by the relationship of this enzyme to electron transfer and oxygen utilization in the rudimentary mitochondria of the intraerythrocytic forms of malaria parasites. The rodent malarial parasite *P. berghei* was chosen as a suitable source for DHODase characterization because of its relative ease of culture and because it has proved a reliable model of the human pathogen in related studies of malaria biochemistry.

On purification to apparent homogeneity, the *Plasmodium* DHODase was found to be membrane bound and active in monomeric form with an estimated molecular mass of 55 kDa. Cofactor and kinetic analyses of the enzyme-catalyzed reaction suggest that the enzyme catalyzes DHO oxidation through the intermediacy of a single [2Fe-2S] iron–sulfur cluster with ubiquinone acting as an obligatory electron acceptor (Figure 5), although confirmation of this scheme can only come with direct detection of an iron–sulfur cluster by either UV/vis or electron spin resonance spectroscopy. Kinetic analyses (Figure 4), indicate a two-site ping-pong reaction mechanism. The product OA as well as the 5-fluoroorotate derivative was found to be inhibitory. This latter result is very interesting, as 5-fluoroorotate has recently been shown to possess marked antimalarial properties in vitro (Rathod et al., 1989).

This *Plasmodium* DHODase can be classified as a dihydroorotate:ubiquinone oxidoreductase. In marked contrast to the counterpart enzyme in trypanosomatid protozoans, it is physically associated with the particulate fraction of the parasite, and by analogy to other eukaryotic systems, it is likely to be mitochondrial. There is scant information on the functional role of the mitochondrion in the intraerythrocytic malaria parasite. The organism is defined as being microaerophilic (Scheibel et al., 1979); low oxygen tension is required for growth, but it is not involved in energy production (Scheibel, 1988). However, the parasite has recently been shown to possess mitochondrial genes encoding components of a rudimentary electron transport chain, notably cytochrome *b* and cytochrome oxidase subunit I (Vaidya et al., 1989). The molecular properties of the pure malarial DHODase allow for a rationalization of this apparent contradiction. It seems likely, as hypothesized some years ago (Gutteridge et al., 1979), that pyrimidine biosynthesis is linked to electron transport and oxygen utilization in malaria. This simplified mitochondrial electron transport chain would then involve DHODase, ubiquinone, cytochromes *b* and c_1, and cytochrome *c* oxidase and would function to couple DHO oxidation to the reduction of O_2.

In conclusion, with the purification of this malarial DHODase it is now possible to investigate the long-suspected susceptibility of this enzyme toward existing (and novel) antimalarial compounds. It is also possible to undertake (for the first time) detailed analysis of the functional and morphological status of the mitochondria throughout the various stages of the life cycle of the malaria parasite. Toward this end, polyclonal antisera against the *P. berghei* enzyme has been raised which recognize the corresponding protein in the human pathogen, *P. falciparum* (Krungkrai et al., unpublished results), and we are currently using this in attempts to localize and isolate the organelle.

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Purification and Characterization of Recombinant Mouse and Herpes Simplex Virus Ribonucleotide Reductase R2 Subunit†

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ABSTRACT: Overexpression of recombinant mouse and herpes simplex virus ribonucleotide reductase small subunit (protein R2) has been obtained by using the T7 RNA polymerase expression system. Both proteins, which constitute about 30% of the soluble *Escherichia coli* proteins, have been purified to homogeneity by a rapid and simple procedure. At this stage, few of the molecules contain the iron–tyrosyl free-radical center necessary for activity; however, addition of ferrous iron and oxygen under controlled conditions resulted in a mouse R2 protein containing 0.8 radical and 2 irons per polypeptide chain. In this reaction, one oxygen molecule was needed to generate each tyrosyl radical. Both proteins had full enzymatic activity. EPR spectroscopy showed that iron-center/radical interactions are considerably stronger in both mouse and viral proteins than in *E. coli* protein R2. CD spectra showed that the bacterial protein contains 70% α-helical structure compared to only about 50% in the mouse and viral proteins. Light absorption spectra between 310 and 600 nm indicate close similarity of the μ-oxo-bridged binuclear iron centers in all three R2 proteins. Furthermore, the paramagnetically shifted iron ligand proton NMR resonances show that the antiferromagnetic coupling and ligand arrangement in the iron center are nearly identical in all three species.

Ribonucleotide reductase (EC 1.17.4.1) catalyzes the reduction of ribonucleotides to deoxyribonucleotides, the first unique step in the biochemical pathway leading to DNA synthesis (Thelander & Reichard, 1979; Lammers & Follmann, 1983; Reichard, 1988; Stubbe, 1990). Mammalian cells, DNA viruses of the herpes virus group, and some prokaryotes, notably *Escherichia coli*, have a heterodimeric, iron-containing enzyme of $\alpha_2\beta_2$ type. The larger (R1)[1] subunit binds substrates and allosteric effectors and provides redox-active sulfhydryl groups. The smaller (R2)[1] subunit contains binuclear ferric iron centers and a stable tyrosyl free radical essential for activity.

Extensive studies of the *E. coli* R2 protein and its iron/free-radical center, culminating in the elucidation of its three-dimensional structure by X-ray crystallography (Nordlund et al., 1990), have been made possible by development of systems for its overexpression. The crystallographic studies together with data from Lynch et al. (1989) clearly demonstrate that each R2 polypeptide chain contains one binuclear iron center. The tyrosyl radical content as determined by EPR spectroscopy seems to vary and has usually been found to be around 0.5 per polypeptide chain (Sahlin et al., 1989; Lynch et al., 1989).

Mammalian R2 has been purified to homogeneity from hydroxyurea-resistant, R2-overproducing mouse cells (The-

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[1] The terms R1 and R2 for the large and small subunits, respectively, replace the earlier species-based abbreviations, viz., B1 and B2 for the *E. coli* and M1 and M2 for the mammalian ribonucleotide reductase proteins.

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