Malarial Dihydroorotate Dehydrogenase

SUBSTRATE AND INHIBITOR SPECIFICITY*

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The malarial parasite relies on de novo pyrimidine biosynthesis to maintain its pyrimidine pools, and unlike the human host cell it is unable to scavenge preformed pyrimidines. Dihydroorotate dehydrogenase (DHODH) catalyzes the oxidation of dihydroorotate (DHO) to produce orotate, a key step in pyrimidine biosynthesis. The enzyme is located in the outer membrane of the mitochondria of the malarial parasite. To characterize the biochemical properties of the malarial enzyme, an N-terminally truncated version of P. falciparum DHODH has been expressed as a soluble, active enzyme in E. coli. The recombinant enzyme binds 0.9 molar equivalents of the cofactor FMN and it has a pH maximum of 8.0 (kcat 8 s−1, Km[H] 0.6 mM DHO (40–80 μM)). The substrate specificity of the ubiquinone cofactor (CoQn) that is required for the oxidation of FMN in the second step of the reaction was also determined. The isoprenoid (n) length of CoQn was a determinant of reaction efficiency; CoQ0, CoQ1, and decylubiquinone (CoQ10) were efficiently utilized in the reaction, however cofactors lacking an isoprenoid tail (CoQ11 and vitamin K1) showed decreased catalytic efficiency resulting from a 4 to 7-fold increase in Kcat/Km. Five potent inhibitors of mammalian DHODH, Redoxal, dichloroallyl lawsone (DCL), and three analogs of A77 1726 were tested as inhibitors of the malarial enzyme. All five compounds were poor inhibitors of the malarial enzyme, with IC50’s ranging from 0.1–1.0 mM. The IC50 values for inhibition of the malarial enzyme are 102–104-fold higher than the values reported for the mammalian enzyme, demonstrating that inhibitor binding to DHODH is species specific. These studies provide direct evidence that the malarial DHODH active site is different from the host enzyme, and that it is an attractive target for the development of new anti-malarial agents.

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Malaria affects between 500 and 900 million people worldwide and causes greater than 2 million deaths per year (1, 2), making this parasitic disease an enormous public health problem throughout the developing world. Currently, several medicinal therapies are available for use as prophylaxis and treatment for at-risk or infected individuals. However, widespread drug resistance against these agents (e.g. chloroquine (3), atovaquone (4), pyrimethamine (5), and sulafadoxine (6)), has compromised the effectiveness of these treatments resulting in the pressing need for the development of new anti-malarial compounds. The identification of targets that exploit the unique biology of the parasite is an essential step in the development of new therapeutics.

Pyrimidines are essential metabolites in all cells. They are required not only for DNA and RNA biosynthesis, but also for the biosynthesis of phospholipids and glycoproteins. The de novo pyrimidine biosynthetic pathway is intact in most organisms, including Plasmodium (7–9). However, unlike mammalian cells, the human malaria parasite, Plasmodium falciparum, cannot salvage preformed pyrimidine bases or nucleosides and utilizes de novo pyrimidine biosynthesis exclusively to meet its metabolic requirements (7, 10–12). The importance of the pyrimidine biosynthetic pathway to the survival of the malarial parasite is demonstrated by the finding that dihydrofolate reductase is a validated target for the treatment of the parasite (13). Furthermore, a number of inhibitors directed at thymidylate synthetase, such as 5-fluoroorotate (5-FO), have nanomolar anti-malarial activity in vitro and cure malaria without toxicity in vivo (14–17). As expected from the inability of the parasite to use nucleosides, the antiproliferative effects of thymidylate synthetase inhibitors on the malarial parasite cannot be reversed by thymidine, whereas mammalian cells cultured in the presence of thymidine were resistant to these inhibitors (16, 17). These studies suggest that additional enzymes in the pyrimidine biosynthetic pathway should be studied for their potential as drug targets in the malarial parasite.

Dihydroorotate dehydrogenase (DHODH) catalyzes the fourth step in the pyrimidine biosynthetic pathway. In human
fumarate or NAD. Although some yeast, eubacteria, and protozoa utilize membrane-bound enzymes. There are, however, no unifying mals, plants, and most Gram-negative bacteria have enzymes, which are mitochondrial in eukaryotes, require respiration, and are activated by respiratory quinones (28). The cytoplasmic enzymes utilize fumarate or NAD, whereas the mitochondrial type utilizes oxidized NAD. The enzyme has also been localized to the mitochondria by electron microscopy. The membrane-bound (28). The cytoplasmic enzymes utilize fumarate or NAD, whereas the mitochondrial type utilizes oxidized NAD.

Dihydroorotate dehydrogenase (DHODH) catalyzes the oxidation of dihydroorotate to orotate utilizing the flavin cofactor FMN in the first of two half reactions (Scheme 1). In the second step, the enzyme catalyzes the re-oxidation of FMNH$_2$ using one of several cofactors. Two forms of DHODH have been described, cytoplasmic and membrane-bound (28). The cytoplasmic enzymes utilize fumarate or NAD$^+$ to oxidize FMNH$_2$, whereas the membrane-bound enzymes, which are mitochondrial in eukaryotes, require respiratory quinones as their physiological oxidant (18, 29). Mammals, plants, and most Gram-negative bacteria have membrane-bound enzymes. There are, however, no unifying rules. Although some yeast, eubacteria, and protozoa utilize fumarate or NAD$^+$, others require respiratory quinones (28). Sequence analysis of the malarial DHODH gene (Fig. 1) demonstrates that it belongs to the mitochondrial-type enzymes (28). The enzyme has also been localized to the mitochondria by studies in parasites (30, 31).

X-ray structures of truncated human DHODH have been solved (32) in complex with orotate and FMN, plus the anti-inflammatory compounds brequinar and A77 1726 (the active metabolite of leflunomide). The enzyme is a $\beta/\alpha$ barrel, and the orotate and FMN stack against each other in the center of the barrel. Brequinar and A77 1726 are thought to bind to the ubiquinone binding site. Consistent with this model, brequinar is a competitive inhibitor of ubiquinone, however, the kinetics of A77 1726 are more complex (33). In the X-ray structure, the inhibitors bind in a channel between two $\alpha$-helices at the N terminus of the molecule that extend away from the $\beta/\alpha$ domain (Fig. 2). This slot forms the entrance to a tunnel that ends at FMN, and presumably extends from the membrane allowing ubiquinone to diffuse into the site during the catalytic cycle. This binding pocket is present only in enzymes of the mitochondrial type.

Biochemical characterization of malarial DHODH has been limited to the study of partially purified enzyme preparations from cultured parasite cells, in which detailed characterization of the enzyme was limited by both purity and quantity of the preparation (30, 31). In this study the truncated soluble form of DHODH from P. falciparum has been overexpressed in E. coli and purified to near homogeneity. The biochemical properties of the recombinant DHODH were examined to determine pH dependence, substrate specificity, and inhibition profiles for a series of compounds that are effective against the human enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**

- L-DHO, 5-FO, FMN, CoQ$_4$, CoQ$_6$ (decyldihydroquinone), CoQ$_{10}$, vitamin K$_3$, DCIP, and all other reagents, unless noted otherwise, were purchased from Sigma Chemical Co. (St. Louis, MO).
- Dichloroallyl lawsone (NCI, National Institutes of Health no. 126771) and Redoxal (NCI/NIH no. 73735) were obtained from the NCI/NIH Developmental Therapeutics Program.
- The "RIG" plasmid was provided by W. G. J. Hol, University of Washington, Seattle, WA.

**Methods**

**Cloning of the P. falciparum pyrD Gene**—The polymerase chain reaction was used to amplify a truncated segment of the pyrD gene from P. falciparum strain C2B genomic DNA encoding the enzyme dihydroorotate dehydrogenase. Primers 1 (CTGGAATTTTTTTCTCATGGATATTATTTTTATAATCC) and 2 (CACCTATAGTGTCGACCGTGTATATTGA- ACTTTTGG), which introduce NcoI and SalI restriction sites, respectively (shown in boldface), were used to generate a 1244-bp DNA fragment. The PCR product was ligated into the pProEX HTa prokaryotic expression vector (Invitrogen, Carlsbad, CA) that produces protein fused to an N terminus His$_6$ sequence. The cloned gene was sequenced to verify that no unintentional mutations were introduced.

The 42-amino acid insert unique to P. falciparum was verified by PCR amplification of a pyrD gene fragment encoding this region using the cDNA library from strain D2 (MB/ATCC, Manassas, VA). Primers 3 (GGAGATACCGCTGATTATAGC) and 2 were used to amplify a fragment of ~750 bp that was directly ligated into ZeroBlunt TOPO vector (Invitrogen, Carlsbad, CA). The cloned pyrD fragment was sequenced with the M13/reverse primer following amplification and purification of plasmid DNA.
H. pylori were taken from GenBank. The sequence of the malarial DHODH gene was previously reported (42). The start sites of the E. coli (Q02127), Pfal
amino acid # 168) are marked in mitochondrial enzymes. Residues consisting of phenylmethylsulfonyl fluoride (200 μM), leupeptin (1 μg/ml), — Cloning and Expression of Human DHODH

Protein Expression and Purification of pfDHODH—Chemically competent E. coli DH5α cells were cotransformed with the His6-DHODH expression vector and the RIG plasmid that encodes the rare tRNAs bound/mitochondrial enzymes, and non-boldface sequences represent cytoplasmic enzymes. Residues in boldface are conserved throughout the mitochondrial enzymes. Residues boxed in grey are within 4.2 Å of either brequinar or A77 1726 based on the structures of human DHODH complexed to these inhibitors (32). The start sites of the E. coli expression constructs for human (human amino acid # 168) are marked in boldface and with arrows. Abbreviations are as follows with accession numbers in parentheses: Human, human (Q02127); Pfal, P. falciparum (Pf3D7/10) (Q82120); Celleg, Caenorhabditis elegans (NP491930); Droso, Drosophila melanogaster (NP116290); E. coli, E. coli (P05201); Hpyl, H. pylori (O25655); Sere, Saccharomyces cerevisiae (P28272); Lacta, Lactococcus lactis (Q96CF9); Tcruz, Trypanosoma cruzi (T03523). Sequences were taken from GenBank™. The sequence of the malarial DHODH gene was previously reported (42). The numbers on the top lines represent the human sequence used for the x-ray structure (Protein Data Bank 1I3d).

Figure 1. Sequence alignment of representative DHODH sequences. Sequences with names displayed in boldface represent membrane-bound/mitochondrial enzymes, and non-boldface sequences represent cytoplasmic enzymes. Residues in boldface are conserved throughout the mitochondrial enzymes. Residues boxed in grey are within 4.2 Å of either brequinar or A77 1726 based on the structures of human DHODH complexed to these inhibitors (32). The start sites of the E. coli expression constructs for human (human amino acid # 168) are marked in boldface and with arrows. Abbreviations are as follows with accession numbers in parentheses: Human, human (Q02127); Pfal, P. falciparum (Pf3D7/10); Celleg, Caenorhabditis elegans (NP491930); Droso, Drosophila melanogaster (NP116290); E. coli, E. coli (P05201); Hpyl, H. pylori (O25655); Sere, Saccharomyces cerevisiae (P28272); Lacta, Lactococcus lactis (Q96CF9); Tcruz, Trypanosoma cruzi (T03523). Sequences were taken from GenBank™. The sequence of the malarial DHODH gene was previously reported (42). The numbers on the top lines represent the human sequence used for the x-ray structure (Protein Data Bank 1I3d).

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Fig. 2. Structure of human DHODH bound to A77 1726 (PDB 1d3h). FMN and A77 1726 are displayed in purple. Residues within 4.2 Å of A77 1726 are displayed; residues that are conserved between human and malarial DHODH are displayed in gray (see sequence alignments), and residues that are variable are displayed in turquoise. Heteroatoms are colored as follows: O, red; N, blue; S, yellow; P, orange. The structure is displayed with the Insight II software (Accelrys Inc., San Diego, CA).

was added followed by 200 μM isopropyl-1-thio-β-D-galactopyranoside. Cells were harvested after 4 h of growth at 30 °C. All other details of the purification are identical to the procedure for isolation of pDHODH outlined above. Approximately 2 mg of purified hDHODH was obtained per liter of medium.

Enzymatic Assays for DHODH Activity—Steady-state measurements to determine the pH dependence of enzyme activity were performed by varying the L-DHO concentration (5−500 μM) and measuring the reduction of 2,6-dichloroindophenol (DCIP) at 600 nm (ε = 18.8 mM−1 cm−1) as described (38). The pH range of the reaction was varied by employing the following buffers (100 mM): MES, pH 6−7; HEPES, pH 7−8; and Tris, pH 8−8.5. DCIP was added to a final concentration of 60 μM in the buffer indicated above at saturating QD (100 μM). Inhibition studies on 5-FQ and IC50 data (Table II) were also performed with this assay. Reactions were initiated by addition of enzyme to a final concentration of 5−50 nM, and the temperature was maintained at 25 °C with a circulating water bath.

For analysis of the CoQ substrate specificity and inhibition patterns by DCL, Redoxal, and CCHNP, formation of L-orotate was measured by circulating water bath. The derivatives of A77 1726 were synthesized in two steps as described (24). In step one the respective para-substituted aniline (Scheme 2, R = NO2, CF3, or CN) was condensed with cyanoacetic acid to form the corresponding cyanoacetamide. In step 2, the product of this reaction was acylated with cyclopropane carbonyl chloride to form the corresponding β-hydroxy enamino. The three resulting compounds (CCHNP, R = NO2, CCHTFP, R = CF3, CCHCIP, R = CN, numbered 49, 17, and 38, respectively, in the original publication (24)) were purified by high-performance liquid chromatography as previously described (24). The identity of each compound was verified by liquid chromatography-MS, MS-MS, IR, and NMR analysis.

RESULTS AND DISCUSSION

Cloning and Expression of Malarial DHODH in E. coli—Malarial DHODH has a significantly longer N-terminal sequence (129 amino acids) than the human enzyme (Fig. 1). The N-terminal sequence contains the membrane-binding domain, which localizes the enzyme to the mitochondria. Soluble overexpression of human DHODH was achieved by truncating 30 amino acids from the N-terminal membrane anchor (40). The truncation was demonstrated not to affect inhibitor binding affinity, nor enzyme activity (41). The x-ray structure of human DHODH (32) also provides evidence that this truncated protein contains the functional catalytic domain (Fig. 2). To obtain soluble malarial DHODH for study, an expression plasmid encoding a truncated enzyme of similar length to the soluble human enzyme was constructed. Genomic DNA from P. falciparum strain C2B served as the template for PCR amplification of a fragment of the pDHODH gene (42), which encodes an enzyme lacking 168 amino acids from the amino terminus. The truncated gene product was ligated into the prokaryotic expression vector pProEXHTa (Invitrogen), which contains an His6-TEV protease cleavage site upstream of the cloning site.

Initial attempts to express malarial DHODH in DH5α cells using the pProEXHTa vector resulted in low protein yields. Expression of P. falciparum proteins in E. coli is often hindered by the high AT content of the genome (82%), which results in the utilization of codons that are rarely used by bacteria (43). To overcome this problem the RIG plasmid was cotransformed with the vector containing the pDHODH gene to provide rare tRNAs encoding Arg, Ile, and Gly (34). In the presence of the
RIG plasmid *P. falciparum* DHODH was expressed in *E. coli* DH5α cells in good yield. The recombinant *pf*DHODH was purified using a combination of Ni2+-agarose and gel filtration chromatography. Typical yields were 1.5–2 mg of purified protein (95% as estimated by SDS-PAGE) per gram of cell paste (Fig. 3A). The UV-visible spectrum of the purified enzyme (Fig. 3B) contains absorption bands at 380 and 454 nm that are characteristic of a flavin cofactor. FMN is tightly bound to the enzyme and copurifies with the protein during all steps. The purified recombinant *pf*DHODH has ~0.9 equivalents of FMN present, based on extinction coefficients of FMN that range from 11 to 12 mm−1 cm−1 at 454 nm.

**pH Dependence of the Steady-state Reaction Catalyzed by *pf*DHODH**—The pH dependence on activity was examined using the coupled DCIP assay. The pH of the reaction buffer was varied from 6.0 to 8.5, and the rates were measured as a function of increasing l-dihydroorotate (at constant decylubiquinone). The data were fitted to a single proton transition (Equation 4) for both the acidic and basic limbs (Fig. 4). Based on this model, maximal catalysis (*k*cat) of 8 s−1 was achieved in the pH range from 7.5 to 8.0. This activity is 90-fold higher than that reported for the partially purified native enzyme from the malarial parasite (30). Activity diminishes above or below the optimal pH range, however, the value of the apparent Michaelis constant, *Km*app (40–80 μM) for DHO remained largely unaffected. The pH dependence was repeated with the direct assay, and identical results were obtained (not shown). Based on these results, HEPES-containing reaction buffer at pH 8.0 was chosen for the standard assay conditions. Subsequent characterization employing either assay gave similar kinetic parameters with the value of *k*cat consistently between 2 and 6 s−1 and *Km*app for DHO ranging from 30 to 60 μM depending on the preparation. The form of the enzyme in which the His8 tag was removed had a *k*cat that was ~1.5-fold lower, however, the *Km*app for l-DHO at saturating CoQD was unchanged.

**Table I**

| Fixed substrate | Varied co-substrate | *k*cat | *Km*app |
|-----------------|---------------------|-------|---------|
|                 |                     | s−1   | μM      |
| l-DHO           | Q0                  | 1.8 ± 0.15 | 114 ± 17 |
| l-DHO           | Vitamin K3          | 2.0 ± 0.28 | 56 ± 18  |
| l-DHO           | Q4                  | 2.0 ± 0.06 | 21 ± 2   |
| l-DHO           | Q6                  | 2.0 ± 0.30 | 15 ± 1   |
| l-DHO           | QD                  | 2.0 ± 0.10 | 16 ± 1   |
| Q4              | l-DHO               | 1.8 ± 0.06 | 35 ± 4   |
| Q6              | l-DHO               | 2.0 ± 0.03 | 62 ± 3   |
| QD              | l-DHO               | 1.8 ± 0.08 | 32 ± 6   |

**Substrate Specificity of *pf*DHODH for the Ubiquinone Cofactor**—The length of the hydrophobic tail of CoQ is a determinant of substrate specificity in the mammalian and bacterial DHODHs. The *H. pylori* enzyme will donate electrons not only to Qo, but also to Q4, whereas the mammalian enzymes are significantly less active with Q0 (26, 39). Furthermore, the *H. pylori* enzyme utilizes menaquinone and menadione (vitamins K2 and K3) for the re-oxidation of FMNH2, but these coenzymes are poor substrates of the human enzyme. To determine the substrate specificity of *pf*DHODH for the ubiquinone cofactor, several quinone cosubstrates were examined to determine the effect of the isoprenoid unit length on enzymatic activity using the direct assay.

The concentration of CoQ was varied (1 to 150 μM) at saturating concentration of l-DHO (500 μM). CoQ4, CoQ6, and CoQD (a soluble synthetic analog of Q10) functioned with similar efficiency as indicated by the calculated specificity constants (*k*cat/*Km*app) of 0.13 μM−1 s−1 (CoQ4 and CoQD) and 0.10 μM−1 s−1 (CoQ6) summarized in Table I. The substrate l-DHO was varied at saturating CoQ concentration (100 μM), and the determined catalytic rates were 1.8 s−1 (CoQ4 and CoQD) and 2.0 s−1 (CoQ6). Cosubstrates that lack the isoprenoid moiety (CoQ4 and vitamin K) had similar catalytic rates to substrates with the hydrophobic chain (*k*cat − 2 s−1), however *Km*app was increased 4- to 7-fold reducing the catalytic efficiency with these substrates (Table I).

**Comparative Inhibitor Analysis for *P. falciparum* and Human DHODH**—To determine if selective inhibitor binding to *P. falciparum* DHODH would be feasible, the inhibition profiles for several of the reported inhibitors of the human enzyme were measured against recombinant *pf*DHODH. Brequinar, AT7 1726, and a number of its derivatives are nanomolar inhibitors of the human enzyme (24, 25). Two structural analogs of brequinar, including Redoxal and DCL, are all also reported to be high affinity inhibitors of the human enzyme (23, 44). These inhibitors are thought to exploit the unique binding site that is found in the membrane-bound type DHODH enzymes, which is the presumed site of CoQ binding. Inhibition studies on *pf*DHODH were conducted with 5-FO, an analog expected to compete directly with the substrate dihydroorotate, with the brequinar analogs available from the NC/NIH compound collection, Redoxal and DCL, and with three AT7 1726 derivatives (CCHNP, CCHTFF, and CCHCP) synthesized for this study.

The substrate analog 5-FO was included in the standard DCIP reduction assay, and the inhibitory effect on enzymatic
activity was measured as a function of increasing L-DHO concentration. A double-reciprocal plot of the data showed a pattern typical of a competitive inhibitor (Fig. 5). The kinetic parameters were calculated from a global fit of the entire data set to Equation 1, describing competitive inhibition. The values of $k_{\text{cat}}$ (2.9 ± 0.05 s$^{-1}$) and $K_{\text{Mapp}}$ (22 ± 2 μM) obtained from the fit were consistent with previous measurements, and the inhibition constant ($K_I$) of 85 ± 10 μM is of similar magnitude to the $K_{\text{Mapp}}$ for L-DHO.

CCHNP, CCHTFP, and CCHCP were synthesized as described under "Experimental Procedures." The $IC_{50}$ values for CCHNP, CCHTFP, and CCHCP were measured as 0.5, 1.1, and 0.7 μM, respectively, against the recombinant malarial DHODH using the DCIP-based assay (Table II). Recombinant truncated human DHODH was prepared as described under “Experimental Procedures” for comparative purposes. Steady-state constants were determined for the purified recombinant human enzyme ($k_{\text{cat}} = 11.0 ± 0.4$ s$^{-1}$ and $K_{\text{Mapp}}$ (L-DHO) = 32 ± 4 μM) using the DCIP assay; these values are similar to previously reported values for this enzyme (40, 41). The $IC_{50}$ values for inhibition of the malarial DHODH by CCHNP, CCHTFP, and CCHCP are 2200-, 5700-, and 2000-fold higher than the values measured for the recombinant truncated human enzyme under the same assay conditions (Table II). Previously, $IC_{50}$ values for these compounds had been reported for the rodent and human enzymes (24, 41). The values for the human enzyme are in good agreement with these previous reports. The $IC_{50}$ values for human DHODH are typically 4- to 10-fold higher than those values measured on the rodent enzymes (41).

The inhibition characteristics of CCHNP against $\rho$/DHODH were further characterized as a function of L-DHO and CoQD concentration by following the formation of orotate using the direct assay. Double-reciprocal plots of the data sets showed that CCHNP is a competitive inhibitor of QD (Fig. 6). Global fitting of each data set was performed using the appropriate equation (“Experimental Procedures”). Global analysis of the inhibition pattern versus QD shows that the data are best fit by the competitive inhibition model and the calculated $K_{\text{Mapp}}$ value for CCHNP is 400 ± 88 μM (Table III). In contrast, global analysis of the inhibition pattern versus L-DHO shows that the data are equally well fitted by either a competitive or non-competitive binding model, and thus the mechanism of inhibition with regard to DHO cannot be conclusively determined by the kinetic analysis. Analysis of the x-ray structure (Fig. 2) demonstrates that the binding sites for DHO and A77

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### Table II

Comparison of inhibitor potency for malarial and human DHODH

| Species           | IC$_{50}$ (μM) | Selectivity (fold) |
|-------------------|----------------|-------------------|
| CCHNP (R = NO$_2$) | 543 ± 77       | 0.25 ± 0.05        | 2200 |
| CCHTFP (R = CF$_3$) | 1080 ± 100    | 0.19 ± 0.02        | 5700 |
| CCHCP (R = CN)    | 712 ± 40       | 0.35 ± 0.07        | 2000 |
| Redoxal           | 71 ± 5         | 0.013 ± 0.0005     | 5500 |
| DCL               | 220 ± 30       | 0.065 ± 0.0001     | 3400 |

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### Table III

Mechanism of inhibition of malarial DHODH

| Inhibitor | Varied substrate | Inhibition type | $K_{\text{Mapp}}$ (μM) |
|-----------|------------------|----------------|-----------------------|
| CCHNP     | L-DHO            | NC             | ND$^a$                |
| Redoxal   | L-DHO$_D$        | C              | $400 ± 88$            |
| QD        | UC               | $59 ± 11$      |
| QD        | UC               | $86 ± 6$       |
| DCL       | L-DHO$_D$        | UC             | $160 ± 40$            |
| QD        | UC               | $250 ± 50$     |

Mammalian DHODH$^b$

| Inhibitor | Varied substrate | Inhibition type | $K_{\text{Mapp}}$ (μM) |
|-----------|------------------|----------------|-----------------------|
| Redoxal   | L-DHO            | NC             | 0.49                  |
| QD        | UC               | 0.40           |
| DCL       | L-DHO$_D$        | UC             | 0.04                  |
| QD        | C                | 0.01           |

$^a$ NC, non-competitive; UC, uncompetitive; C, competitive.

$^b$ Data for human DHODH were taken from Refs. 44 and 45.
Potent inhibition of mammalian DHODH has been achieved with two classes of chemical scaffolds (e.g. A77 1726 and brequinar) that have received significant pharmacological interest for the treatment of human autoimmune disease (19–21). The data we report here demonstrate that analogs of both A77 1726 and of brequinar are poor inhibitors of malarial DHODH. Thus, significant species-selective inhibitor binding can be achieved between the parasite and host DHODH, supporting the hypothesis that selective inhibition of malarial DHODH is feasible.

X-ray structure analysis of brequinar and A77 1726, bound to human DHODH, found a common binding mode for both inhibitors (Fig. 2); this binding site is thought to also bind the cofactor ubiquinone, although direct structural evidence for the ubiquinone binding site has not been obtained (32). For human DHODH, both brequinar and DCL are competitive inhibitors of CoQ analogs consistent with the hypothesis that they bind at the ubiquinone binding site (33, 45). However, both A77 1726 and Redoxal, display non-competitive inhibition patterns with all CoQ analogs tested (33, 44). With the malarial enzyme, CCHNP, which is a structural analog to A77 1726, displays competitive inhibition patterns with QD. These data demonstrate that an A77 1726 analog binds to the same site as CoQ, and they suggest that these analogs bind to the malarial enzyme at the inhibitor binding site that has been defined in the x-ray structure analysis of human DHODH (Fig. 2). For malarial DHODH, Redoxal and DCL are uncompetitive inhibitors of CoQD, suggesting that the binding of inhibitor is dependent on the binding of substrate for the interaction. This analysis is consistent with a binding site in which CoQ and inhibitor bind to separate but adjacent sites.

The brequinar and A77 1726 binding site that is observed in the x-ray structure of human DHODH is composed of amino acids that are highly variable between the human and malarial enzymes (Figs. 1 and 2). Comparison of the structure with the sequence alignment shows that nine of the fifteen residues that are within van der Waals contact (defined as within 4.2 Å) of A77 1726 in the structure are variable between the malarial and human enzymes. These structural differences provide an explanation for the observed differences in the potency of binding of these inhibitors to the two enzymes. The striking differences in inhibitor potency between the malarial and human enzymes strongly suggest that species-selective inhibitors of malarial DHODH can be developed to exploit these structural differences. Finally, recent RNA interference studies, in which malarial parasites were treated with double-stranded RNA-encoding DHODH, correlated a reduction in mRNA levels to reduced parasite growth (46). These studies provide further evidence that DHODH is essential to parasite growth. Thus, the potential to develop selective inhibitors for an essential metabolic enzyme in the malarial parasite suggests that malarial DHODH is an attractive target for the development of new anti-malarial agents.

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