A Novel Enzyme Complex of Orotate Phosphoribosyltransferase and Orotidine 5’-Monophosphate Decarboxylase in Human Malaria Parasite Plasmodium falciparum: Physical Association, Kinetics, and Inhibition Characterization†,‡

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ABSTRACT: Human malaria parasite, Plasmodium falciparum, can only synthesize pyrimidine nucleotides using the de novo pathway, whereas mammalian cells obtain pyrimidine nucleotides from both the de novo and salvage pathways. The parasite’s orotate phosphoribosyltransferase (PfOPRT) and orotidine 5’-monophosphate decarboxylase (PfOMPDC) of the de novo pyrimidine pathway are attractive targets for antimalarial drug development. Previously, we have reported that the two enzymes in P. falciparum exist as a multi-enzyme complex containing two subunits each of 33-kDa PfOPRT and 38-kDa PfOMPDC. In this report, the gene encoding PfOPRT has been cloned and expressed in Escherichia coli. An open reading frame of PfOMPDC gene was identified in the malaria genome database, and PfOMPDC was cloned from P. falciparum cDNA, functionally expressed in E. coli, purified, and characterized. The protein sequence has <20% identity with human OMPDC and four microbial OMPDC for which crystal structures are known. Recombinant PfOMPDC was catalytically active in a dimeric form. Both recombinant PfOPRT and PfOMPDC monofunctional enzymes were kinetically different from the native multi-enzyme complex purified from P. falciparum. Oligomerization of PfOPRT and PfOMPDC cross-linked by dimethyl suberimidate indicated that they were tightly associated as the heterotetrameric 140-kDa complex, (PfOPRT)2(PfOMPDC)2. Kinetic analysis of the PfOPRT-PfOMPDC associated complex was similar to that of the native P. falciparum enzymes and was different from that of the bifunctional human enzymes. Interestingly, a nanomolar inhibitor of the yeast OMPDC, 6-thiocarboxamido-uridine 5’-monophosphate, was about 5 orders of magnitude less effective on the PfOMPDC than on the yeast enzyme. Our results support that the malaria parasite has unique structural and functional properties, sharing characteristics of the monofunctional pyrimidine-metabolizing enzymes in prokaryotes and bifunctional complexes in eukaryotes.

Malaria is a major cause of morbidity and mortality in developing countries. More than a third of the world’s population live in malaria-endemic areas, and 1 billion people are estimated to carry malaria parasites at any one time (1). In Africa alone, there are an estimated 300–500 million cases of malaria and up to 3 million people die from the disease each year (2). Plasmodium falciparum is the causative agent of the most lethal and severe form of human malaria. Chemotherapy of malaria is available but is complicated by both drug toxicity and widespread resistance to most of the currently available antimalarial drugs (3, 4). The need for more efficacious and less toxic agents, particularly rational drugs that exploit metabolic pathways and targets unique to the parasite, is therefore acute (5, 6).

Plasmodia species are dependent on de novo synthesis of pyrimidine nucleotides (Scheme 1) because they lack salvage enzymes (7–10), whereas the mammalian host cells obtain pyrimidines from both de novo and salvage pathways (11, 12). Inhibition of de novo pyrimidine synthesis by specific inhibitors, e.g., orotate analogues (13, 14) and atovaquone (14, 15), thus leads to dramatic reduction in cellular pyrimidine pools and eventual failure of the parasite to survive. Such inhibitors are found to have strong antimalarial activity for P. falciparum grown in vitro and Plasmodium berghei propagated in mice (13, 14).

The final two steps of uridine 5’-monophosphate (UMP) synthesis require the addition of ribose 5-phosphate from...
et al. (23). Solutions of 6-CSNH₂UMP were quantified using the extinction coefficient of ε₂70 = 11 300 M⁻¹ cm⁻¹ (23). 5-Fluoro-UMP (5-FUMP) was prepared from 5-fluoroorotate (5 mmol) by a reaction of yeast OPRT and yeast OMPDC (10 units each) and then purified by HPLC as described (22).

Parasites and Preparation of Parasite Cell-Free Crude Extract. The asexual stage of P. falciparum strain T9 was cultivated by the candle jar method of Trager and Jensen (24) with some modifications as described (19). The parasites were isolated from human red cells and then lysed in the presence of protease inhibitors cocktail to prevent high protease activities in the parasites as described (8, 19). The cell-free crude extract was obtained after centrifugation of the lysate at 27 000 g for 60 min.

Preparation of Native OPRT and OMPDC Enzymes from P. falciparum. The native OPRT and OMPDC enzymes were purified from the freshly prepared cell-free crude extract by three sequential chromatographic steps on Mono Q anion exchange FPLC, UMP-agarose affinity (Sigma), and Superose 12 gel filtration FPLC columns, as described previously (19) with the following buffer: 50 mM Heps, pH 7.4, 5 mM DTT, and 20% glycerol. The purity of the enzymes was assessed by SDS-PAGE. The purified enzymes were stored as aliquots in the presence of 5 mM DTT and 20% glycerol at -80 °C.

Preparation of P. falciparum DNA, RNA, and cDNA. Genomic DNA and total RNA were isolated from P. falciparum as described (21). cDNA was synthesized from total RNA using reverse transcription-polymerase chain reaction (RT-PCR) as described (21).

Cloning, Expression, and Purification of Recombinant PfOPRT from P. falciparum cDNA. The open reading frame (846 bp) encoding OPRT was identified on chromosome 5 in the P. falciparum genome database (25). The PfOPRT was cloned, expressed, and purified as described (21) with some modifications by using a different expression vector and host E. coli. Briefly, the coding region for the PfOPRT was amplified from the cDNA of P. falciparum by PCR and ligated into a pQE30Xa vector (Qiagen). The PfOPRT was expressed in E. coli SG13009 (Qiagen) in the presence of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 18 °C for 18 h. The recombinant protein was purified using Ni²⁺-NTA—agarose affinity chromatography (Qiagen). The His₆-tag was removed from the protein by using factor Xa (Qiagen). The protein was further purified by a Superose 12 gel filtration FPLC column equilibrated with a buffer of 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 5 mM DTT, and 1 mM PMSF. The purified enzyme was eluted with a retention time corresponding to a molecular mass of 66 kDa, which is its dimeric form. SDS-PAGE analysis of the recombinant PfOPRT corresponded to a 33-kDa monomer.

Cloning, Expression, and Purification of Recombinant PfOMPDC from P. falciparum cDNA. The gene homologue encoding PfOMPDC was identified as a single open reading frame (969 bp) on chromosome 10 in the P. falciparum...
genomedatabase (25). RT-PCR wasused to amplify PfOMPDC cDNA using total RNA as the template.

The forward primer was 5′ CGGGATCCATGGGGTTTAAGGTAAAATTA 3′ and the reverse primer was 5′ CCATCGATTTAGTCCATATTGTTTGAATTA 3′ which introduce BamH I and Cla I restriction sites respectively (shown in bold). The PCR conditions were similar to those described for PfOPRT (21). The PCR products were purified and ligated into a pTrcHis-TOPO plasmid (Invitrogen). The PfOMPDC sequence was determined in both directions by the dideoxy chain termination method using an automated Applied Biosystems model 377 sequencer. Competent E. coli TOP10 (Invitrogen) cells were transformed with the recombinant plasmid, induced with 1 mM IPTG at 18 °C, harvested, and stored as cell paste at −80 °C. The recombinant PfOMPDC from the cell paste was purified using a Ni2+-NTA–agarose affinity column. The recombinant protein was then subjected to His6-tag removal using enterokinase (Invitrogen). The protein was further purified by the Superose 12 gel filtration FPLC.

**Enzymatic Assays.** OPRT and OMPDC activities were measured according to previously described spectrophotometric methods (26–28). The OPRT enzymatic reaction was monitored by a decrease in absorbance of orotate, whereas the OMPDC reaction was monitored by a decrease in absorbance of OMP and also by an appearance of UMP in an HPLC chromatogram, as essentially described (19, 22).

**Enzyme Kinetics and Inhibition Studies.** For all kinetic analyses for both OPRT and OMPDC, the purified native and recombinant enzymes, after Superose 12 gel filtration FPLC column chromatography (>97% pure as observed by SDS–PAGE), were used at concentrations of 15–30 nM in 1.0 mL reaction assays, and their kinetics was measured in three to five different preparations. The $K_m$ and $V_{max}$ values were determined by measurement of initial velocities in triplicate with at least five substrate concentrations. The $k_{cat}$ values were calculated as described (29). The catalytic efficiency ($k_{cat}/K_m$) for OPRT and OMPDC and of the 1:1 mixture of OPRT and OMPDC was also calculated.

Where inhibition studies were performed on the mixture of OPRT and OMPDC and on each enzyme separately, three different concentrations of inhibitors were tested for initial velocity measurements. Kinetic data of the initial velocities and inhibitions were fitted to an equation for competitive inhibitors (eq 1) using the method of Cleland (30).

$$v = \frac{V_{max}[S]}{[S] + K_m \left(1 + \frac{[I]}{K_i}\right)}$$

Oligomerization and Chemical Cross-Linking of PfOPRT and PfOMPDC. To demonstrate whether the PfOPRT and the PfOMPDC form an oligomeric multienzyme complex, chemical cross-linking of either PfOPRT or PfOMPDC were performed using the bifunctional reagent 3,3′-dithiobisimidate (DMS) as described (21, 31). The cross-linking reaction was initiated by the addition of DMS (20 µg) to the pure recombinant PfOPRT and/or PfOMPDC (10 µg each), and the reaction incubated at 25 °C for various time intervals. The reaction was then quenched by the addition of 1 M glycine to a concentration of 0.1 M. The cross-linked species were analyzed by SDS–PAGE. The molecular masses of the cross-linked species were also analyzed with a Superose 12 gel filtration FPLC column.

Oligomeric PfOPRT and PfOMPDC enzymes in their native forms were formed by incubating the two enzymes (0.5 mg/mL each) with either 0.25 mM orotate and 0.25 mM PRPP or 0.5 mM UMP in a buffer containing 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 5 mM MgCl₂, and 2.5 mM DTT at 37 °C for 10 min. The oligomeric forms were then analyzed and purified by a Superose 12 gel filtration FPLC column. Both OPRT and OMPDC activities were then measured.

**Miscellaneous Methods.** Protein concentrations were determined by the Bradford assay (32) using bovine serum albumin as standard. Concentrations of pure PfOPRT and PfOMPDC were measured using $ε_{280}$ values of 21 760 and 33 280 M⁻¹ cm⁻¹, respectively. The $ε$ values for both enzymes (monomeric forms) were calculated according to Edelhoch (33).

SDS–PAGE was performed on a Bio-Rad gel apparatus with polyacrylamide gels of 7%–12% in the buffer system of Laemmli (34). The gels were stained with Coomassie brilliant blue R and visualized by Bio-Rad molecular analyst PC software image analysis.

The Superose 12 gel filtration FPLC column was equilibrated with the buffer described before and calibrated with molecular mass markers: blue dextran 2000 (2000 kDa, $V_d$), thyroglobulin (670 kDa), immunoglobulin (158 kDa), bovine serum albumin (66 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B₁₂ (1.35 kDa, $V_i$).

Homology search was performed using the BLAST program (35). Pairwise and multiple sequence alignments of PfOMPDC with other organisms were performed using the CLUSTALW program (36). Hydrophobicity of the PfOMPDC was determined by the DNASIS program (Hitachi) and the Kyte–Doolittle plot as described (37). Western blot analysis was performed to confirm the authenticity of the His$_{6}$-tagged recombinant PfOPRT and PfOMPDC proteins as described (38).

**RESULTS AND DISCUSSION**

OPRT and OMPDC activities in human and multicellular organisms are conferred by 51-kDa bifunctional UMPS protein (11, 16, 27, 39, 40), whereas the enzymes appear to have two forms in lower eukaryotes: monofunctional forms, such as in Saccharomyces cerevisiae (26), or a bifunctional form, as is seen in Trypanosoma cruzi and Leishmania mexicana (41, 42). In bacteria, they are monofunctional enzymes (43). An exception for these enzymes has been found in the malaria parasites, where they exist as a multienzyme complex, (PfOPRT)₂(PfOMPDC)₂ (18,19).

Recent efforts with cDNA microarray analysis of the P. falciparum genes show that the PfOPRT and PfOMPDC genes are actively expressed in the asexual erythrocytic stage (44). The PfOPRT gene and the recombinant protein have been characterized (21). Production of recombinant PfOPRT and PfOMPDC can provide the appropriate quantities of the homogeneous enzymes for characterizations of physical, kinetic, inhibitory effect, and stability properties of individual monofunctional enzyme. The tightly associated complex of
the two enzymes can then be compared to those properties of the multienzyme complex purified from *P. falciparum* grown in vitro.

**Purification and Characterization of Native OPRT and OMPDC Multienzyme Complex from *P. falciparum***

Activities of OPRT and OMPDC in the cell-free crude extracts of *P. falciparum* were ~20–25 nmol min⁻¹ mg⁻¹ protein. The use of Hepes buffer (pH 7.4), 5 mM DTT, 20% glycerol, and the protease inhibitors cocktail stabilized the enzyme activities during purification. Following the crude extracts, the OPRT and OMPDC activities were copurified by Mono Q anion exchange, UMP–agarose affinity, and Superose 12 gel filtration columns with the maximal activity of each enzyme found together. The enzymes were purified with specific activities of ~9–12 µmol min⁻¹ mg⁻¹ protein and ~420–450-fold with overall yield of ~35–40%. This purification strategy provided ~0.25 mg of purified enzymes from 2 L of *P. falciparum* culture having ~2–3 times more active enzymes (kₐₑ values in the range of 10.8–24.6 s⁻¹) than our previous report (19). In vivo concentrations of the enzymes were accurately calculated by using the equation described (27) and the parasite’s intracellular volume of 1.35 mL/g dry weight (45). The cellular concentrations of the enzymes in *P. falciparum* were 60 ± 5 nM (n = 6), about 2.5-fold more than those of the human UMPs (27). The bacteria, such as *E. coli*, and yeast have increased concentrations of the separate monofunctional enzymes by about 10–50-fold, and the stability of both enzymes is decreased (27). The large differences of the in vivo protein concentrations would reflect greater lability of the monofunctional enzymes than the bifunctional human enzyme (27) or *P. falciparum* multienzyme complex in this study.

At the Superose 12 FPLC purification step, the active fractions containing OPRT and OMPDC were eluted in a single peak with a molecular mass of 140 kDa. SDS–PAGE of the purified OPRT and OMPDC resulted in the monomeric forms with the molecular mass of 33 and 38 kDa, respectively. These results indicate the occurrence of the 140-kDa (OPRT)₂(OPMD)₂ complex. The purified complex was stable (>90% activity remaining) for at least 6 months at 20 or −80 °C in 50 mM Hepes buffer, pH 7.4, 300 mM NaCl, 5 mM DTT, and 20% glycerol. Under the same condition but at 4 °C, the enzyme activities were decreased to 50% after 4-week storage. When the enzymes were stored in the absence of DTT and glycerol, their activities were gradually decreased to 50% after 1 week at 4 °C (half-life ~7 days). Repeated applications of the stored enzymes (at 4 °C and 2 weeks) on a Superose 12 FPLC column gave low enzyme activities, eluting with retention times corresponding to their monomeric forms (33 kDa for OPRT and 38 kDa for OMPDC). These labile properties would explain the previous report on the discrete activities of OPRT and OMPDC in *P. falciparum* cytosol observed after one-step purification on a Cibracon blue affinity column (46).

**Purification and Characterization of Monofunctional PfOPRT**

The deduced protein sequence of PfOPRT (281 amino acids) has high similarity (percent similarity indicated in parentheses) to bacterial enzymes, such as *E. coli* (60%) and *Salmonella typhimurium* (56%). It shows low similarity to protozoan *T. cruzi* (30%) and to human OPRT domain of UMPs enzyme (28%). Catalytic residues and the consensus sequences for orotate and PRPP substrate bindings of the two known crystal structures of *S. typhimurium* (47, 48) and *E. coli* (49) OPRTs are well-conserved in the malarial protein sequence (21). Since there is a common metabolic intermediate, OMP, involved in the OPRT and OMPDC reactions (Scheme 1), the protein sequences of both *P. falciparum* enzymes were aligned pairwise. Both enzymes were markedly different in the active site loops and had only 16% identity, which is quite similar to comparisons of both enzymes in yeast and human. These data indicate that OPRT and OMPDC may not share an evolutionary relationship, in contrast to previous suggestions (42, 50). This is consistent with the dissimilarity of the tertiary structures between OPRT and OMPDC.

The cDNA encoding PfOPRT was directly cloned into the vector pQE30Xa and expressed in the *E. coli* SG13009 cells. This strategy yielded ~4 mg of pure PfOPRT from 1 L of cell culture, which was 4-fold higher than that expressed in the expression vector pET-15b and the *E. coli* strain BL21-(DE3) cells containing the RIG plasmid (21, 51). The PfOPRT was a homodimer of two 33-kDa monomers as (PfOPRT)₂, with a 67-kDa molecular mass on a Superose 12 gel filtration FPLC column and a 33-kDa molecular mass by SDS–PAGE (data not shown), which is consistent with the dimeric form in the crystal structures (47–49). The kₐₑ of the recombinant PfOPRT was 4.2 s⁻¹, ~1.5-fold more active than the enzyme obtained in our previous preparation (21). The monofunctional PfOPRT enzyme was stable in the presence of 5 mM DTT and 20% glycerol, i.e., >90% activity remained for at least 3 months at −20 and −80 °C. When the enzyme was stored in the absence of DTT and glycerol, the activity decreased to 50% after overnight storage at 4 °C (half-life ~1 day).

**Cloning and Identification of PfOMPDC Homologue**

The cDNA encoding PfOMPDC was cloned in the pTrcHisTOPO vector and the nucleotide sequence was then verified. The protein sequence of the PfOMPDC consists of 323 amino acids, with 6 Arg (~2 mol %), 17 Asp (~5 mol %), 33 Lys (~10 mol %), and 40 Asn (~12 mol %), a molecular mass of 37 822 Da, and an isoelectric point of 7.6 (Figure 1). The PfOMPDC protein sequence is more similar to the protozoan (*T. cruzi*, *L. mexicana*) (42) and to some bacterial counterparts (*Thermus thermophilus*, *Mycobacterium smegmatis*) (42) than to the OMPDC domain of human UMPs (52). The PfOMPDC sequence has 33, 32, 31, 27 and 14% identity to *T. cruzi*, *L. mexicana*, *T. thermophilus*, *M. smegmatis*, and human enzymes, respectively. However, the sequence identities of the four known crystal structures of OMPDCs, i.e., *Bacillus subtilis* (53), *E. coli* (54), *Methanobacterium thermoautotrophicum* (55), and *S. cerevisiae* (56), are <20% to the malarial enzyme (Figure 1). Similar to PfOPRT (21), PfOMPDC contains an extension of 32 amino acids from its N-terminus (Met1 to Phe32) and a unique insertion of 12 amino acids from Arg72 to Phe83, displaying a hydrophobic index of +1.0. PfOMPDC is one of the longest OMPDC sequences to date, including the *Neurospora crassa* OMPDC (57). The average protein for all 82 species contains 270 amino acids (57). Similar extensions and insertions of different amino acid sequences are found in malarial proteins, but their origin and function remain unknown (21, 58–60).

In comparison with the OMPDC crystal structures from *B. subtilis*, *E. coli*, *M. thermoautotrophicum*, and *S. cerevisiae*
Figure 1: Multiple sequence alignments of *P. falciparum* OMPDC with four known crystal structures. Numbers in parentheses are accession numbers: *B. subtilis* (AA21273), *E. coli* (NP_415797), *M. thermoautotrophicum* (NP_275272), and *S. cerevisiae* (AAA34824.1). Bold letters indicated the most conserved active site residues; italic letters are similar amino acids. Three active site loops (I, II, III) of *Pf* OMPDC are identified and marked by upper lines and underlines.
further characterization of the protein. Our recombinant and a much lower protein yield was obtained. This limits Pf of the than 97%. The purified recombinant enzyme had a specific by 12% gel of SDS of the protein sequence. The purity of the protein, assessed 8) (Figure 2A, inset), corresponding to the predicted value for the bacterial and yeast proteins in the crystal structures (53–56). The active enzyme was stable (>90% activity remaining) in the presence of 5 mM DTT and 20% glycerol for at least 4 months at −20 and −80 °C. When the enzyme was stored in the absence of DTT and glycerol, its activity was decreased to 50% after 3 days at 4 °C (half-life ~ 3 days), and its aggregation was observed at a protein concentration of more than 2 mg/mL.

**Heterotetrameric Formation of Recombinant PfOPRT and PfOMPDC.** Using oligomerization studies of a mixture of the recombinant PfOPRT and PfOMPDC in a stoichiometric ratio of 1:1, the association complex was analyzed by a Superose 12 gel filtration FPLC column after incubating the enzymes with the substrates orotate and PRPP at 300 mM NaCl, Tris-HCl, pH 8.0, for 10 min at 37 °C. Both enzyme activities were coeluted as a single, symmetrical peak at a retention time corresponding to a molecular mass of 140 ± 6 kDa (n = 6) (Figure 2B). There were no enzyme activities remaining at their dimeric positions and also no activities in fractions corresponding to molecular masses higher than 140 kDa, indicating the essentially complete heterotetrameric complex formation between (OPRT)2 and (OMPDC)2. The oligomerization of both enzymes were also performed by incubating the enzymes in the presence or absence of the product UMP; similar results to the enzymes incubated with the substrates were obtained (data not shown). These suggest that the complex would be stabilized by substrate, product, and salt. The fractions containing both PfOPRT and PfOMPDC were pooled and then analyzed by SDS–PAGE, whereupon two bands were observed at their monomeric forms of 33 and 38 kDa for both enzymes (Figure 2B, inset). The purified fractions were subsequently analyzed for their kinetic properties, providing a comparison to their monofunctional forms. Stability of the purified heterotetrameric enzymes was achieved by the addition of 5 mM DTT and 20% glycerol. In the absence of both stabilizers, both enzyme activities were gradually declined ~60% after 1 week at 4 °C (half-life ~ 6 days), similar to their stability properties found in the native complex from *P. falciparum*.

To confirm the complex formation, a second approach using chemical cross-linking of either PfOPRT or PfOMPDC was performed by incubating the enzymes with DMS in a ratio of 1:2 protein to cross-linker at 25 °C for various time intervals, followed by SDS–PAGE analysis. The cross-linked product of PfOMPDC was observed at a greater extent as the dimeric form with a molecular mass of 75 ± 5 kDa (n = 6) within 5-min incubation (data not shown). The cross-linked product of recombinant PfOPRT was a dimer with a molecular mass of 67 kDa, as demonstrated previously (21). Neither recombinant enzyme (10 μg) formed a tetramer when incubating with increasing amounts of DMS up to 100 μg after 60 min and overnight at 25 °C. When the cross-linked dimeric products of PfOPRT and PfOMPDC were mixed and further incubated with DMS for various times, more than 70% of the dimers converted to the tetramers within 5 min and no hexameric form was detected (data not shown).

When a mixture of PfOPRT and PfOMPDC (each 10 μg) was incubated with 40 μg of DMS, there was significant formation of tetramer at 10-min incubation, as shown in Figure 3. At 30 min, more than 70% of the proteins were cross-linked as tetrameric form. There was little hexameric was further purified by a Superose 12 gel filtration FPLC column. The PfOMPDC enzyme activity was eluted in a single peak with a molecular mass of 76 ± 4 kDa (n = 8) (Figure 2A). By SDS–PAGE analysis, the molecular mass of the PfOMPDC had a molecular mass of 38 ± 3 kDa (n = 8) (Figure 2A, inset), corresponding to the predicted value for the protein sequence. The purity of the protein, assessed by 12% gel of SDS–PAGE and image analyses, was more than 97%. The purified recombinant enzyme had a specific activity of ~8–10 μmol min⁻¹ mg⁻¹ protein, 220-fold purification, and 30% yield, and up to 3 mg of pure protein was obtained from 1 L of cell culture. Recently, Menz et al. (62) have used pMICO vector for PfOMPDC expression, and a much lower protein yield was obtained. This limits further characterization of the protein. Our recombinant

<table>
<thead>
<tr>
<th>PfOPRT</th>
<th>PfOMPDC</th>
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<tr>
<td>OPRT</td>
<td>OMPDC</td>
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**Figure 2:** (A) Dimeric form of the recombinant PfOMPDC enzyme. A chromatogram for the Superose 12 gel filtration FPLC column of the purified PfOMPDC is illustrated. The peaks of enzyme activity per 0.5-mL fraction (O) and protein concentration (●) are eluted together at a molecular mass of 76 kDa. Molecular mass markers (Amersham Biosciences) provided in kDa, void volume (Vo), and total eluting volume (Vf) are indicated with arrows. SDS–PAGE on a 12% polyacrylamide gel of the purified PfOMPDC (10 μg) shows a homogeneous preparation at a molecular mass of 38 kDa (inset, lane 2). Low molecular mass markers (Bio-Rad) are given in kDa (inset, lane 1). (B) Heterotetrameric formation of PfOPRT and PfOMPDC enzymes. A chromatogram for the Superose 12 gel filtration FPLC column of the 1:1 mixture of PfOPRT and PfOMPDC dimeric forms is shown. In the 0.5-mL fraction, peaks of both enzyme activities (Δ, PfOPRT; ○, PfOMPDC) and protein concentration (●) are eluted symmetrical at a molecular mass of 140 kDa. Molecular mass markers provided in kDa, void volume (V0) and total eluting volume (Vf) are indicated with arrows. SDS–PAGE on a 10% polyacrylamide gel of the active fractions at 12.5 mL (~140 kDa complex) (30 μg) shows two major bands at molecular masses of 33 and 38 kDa, corresponding to their monomeric sizes (inset, lane 2). High molecular mass markers (Bio-Rad) are given in kDa (inset, lane 1).
The tetramer had a molecular mass of 140 kDa, determined by crossedlinked oligomers were also determined by a Superose 12 gel filtration FPLC column. The tetramer had a molecular mass of the monomer and dimer, and the electrophoretical mobility calculated assuming that these oligomers were all multiples of a linear relationship between the log of molecular mass, particularly due to the major tetrameric form. However, the time-dependent oligomerization of each enzyme corresponded to that expected for sequential cross-linking of monomer.

**Fig. 3**: SDS–PAGE analysis of oligomeric formation between P. falciparum OPRT and P. falciparum OMPDC cross-linked with dimethyl suberimidate (DMS). SDS–PAGE on a 7% polyacrylamide gel shows a time-course of cross-linking between P. falciparum OPRT and P. falciparum OMPDC (each 10 μg) and DMS (40 μg) at 0 min (lane 1), 5 min (lane 2), 10 min (lane 3), 15 min (lane 4), 30 min (lane 5). The letters M, D, T, and H indicate positions of monomeric, dimeric, tetrameric, and hexameric forms. The apparent monomer, dimer, tetramer, and hexamer have log molecular mass of 4.58, 4.85, 5.15, and 5.33, respectively.

**Fig. 4**: Kinetics of cross-linking between the P. falciparum OPRT and P. falciparum OMPDC by dimethyl suberimidate. The experimental details are described in Figure 3. The time dependence of the percentage of each size of oligomers as indicated by the ratio of its band density to the total density of all detected bands is illustrated. The symbols used are as follows: monomer (○), dimer (○), tetramer (●), and hexamer (△).

Kinetic properties of the monofunctional P. falciparum OMPDC and P. falciparum OPRT, their heterotetrameric form, and the native multienzyme complex P. falciparum OPRT and P. falciparum OMPDC, their heterotetrameric form isolated from the 1:1 mixture by a Superose 12 gel filtration FPLC column, and the native OPRT and OMPDC multienzyme complex purified from P. falciparum are shown in Tables 1 and 2. These kinetic values are compared to kinetic parameters reported for human monofunctional OPRT and OMPDC and bifunctional UMPS expressed in the baculovirus system (27, 66). The monofunctional P. falciparum OPRT showed 2-fold lower catalytic efficiency (k_cat/K_m) than the monofunctional human OPRT, but their k_cat values were quite similar. However, the monofunctional P. falciparum OMPDC had 80-fold lower catalytic efficiency than the monofunctional human OMPDC; most of this effect was due to a higher K_m of P. falciparum OMPDC for OMP. In previous studies of mouse UMPS, the K_m of the monofunctional OMPDC expressed in yeast is 3-fold higher than the value obtained for the bifunctional mouse UMPS (39), but these values are quite similar between the recombinant human OMPDC domain and UMPS (27). Comparing the kinetic parameters in three different forms of the two enzymes, the K_m values for both enzymes in the multienzyme complex and in the enzyme mixture are ~3–4-fold lower than these values in the monofunctional forms, and the k_cat values are ~3–4-fold lower than these values in the monofunctional forms.
Table 1: Enzyme Kinetic Constants of OPRT in Monofunctional Recombinant P.falciaparum, a 1:1 Mixture of Recombinant P.falciaparum and P.OMPDC, Yeast Monofunctional OPRT and Bifunctional Human UMPS

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_m$ (µM)</th>
<th>$K_a$ (µM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$k_{cat}/K_m$ (M⁻¹ s⁻¹)</th>
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</thead>
<tbody>
<tr>
<td>P. falciparum monofunctional OPRT</td>
<td>14.4 ± 1.2</td>
<td>1.2</td>
<td>2.5</td>
<td>1.1 x 10⁸</td>
</tr>
<tr>
<td>P. falciparum monofunctional OPRT and P.OMPDC</td>
<td>1.7 ± 0.2</td>
<td>0.8</td>
<td>0.2</td>
<td>2.9 x 10⁸</td>
</tr>
<tr>
<td>P. falciparum multifunctional OPRT and P.OMPDC</td>
<td>3.6 ± 0.8</td>
<td>0.8</td>
<td>0.2</td>
<td>2.9 x 10⁸</td>
</tr>
<tr>
<td>Human OPRT</td>
<td>2.1 ± 0.3</td>
<td>0.7</td>
<td>0.5</td>
<td>2.9 x 10⁸</td>
</tr>
<tr>
<td>Human Bifunctional OPRT</td>
<td>2.1 ± 0.1</td>
<td>1.0</td>
<td>0.9</td>
<td>2.9 x 10⁸</td>
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</table>

Values are from Yablonski et al. (27).

Characterization of Enzyme Inhibition. Lines of evidence for inhibition studies of product UMP and three analogues on two different forms of P.OMPDC are provided and compared to previous observations. Table 3 shows $K_i$ values observed for the inhibitors of monofunctional P.OMPDC, monofunctional yeast enzyme, native P. falciparum OPRT and OMPDC multienzyme complex, and bifunctional human UMPS. In all cases, inhibition was fitted to eq 1 and appeared to be competitive. Previously, we have shown that 5-fluoroorotate is an effective alternative substrate for the P.OMPRT and exhibits strong antimalarial activity with 50% inhibitory effect of approximately 6 nM on P. falciparum grown in vitro and shows potent in vivo antimalarial effect on P. berghei in mice (13, 21). This compound would serve as a subversive substrate for the malarial enzymes. Such type of compounds may be useful in antimalarial drug design targeting malarial OPRT and OMPDC enzyme complex. Moreover, uracil and 5-fluorouracil were not substrates for the P.OMPRT and exhibited weak inhibition (21). The property

values are higher. Catalytic efficiencies of the multienzyme complex and the enzyme mixture are ~6–14-fold greater than those values for the monofunctional forms. However, the $K_m$ values of OMP in these three forms of P.OMPDC are 10-fold less than those reported in the human and yeast enzymes. These results indicate that the tight association between the two enzymes would favor efficient catalysis by lowering $K_m$ and increasing $k_{cat}$.

Characterization of Enzyme Inhibition. Lines of evidence for inhibition studies of product UMP and three analogues on two different forms of the P.OMPDC are provided and compared to previous observations. Table 3 shows $K_i$ values observed for the inhibitors of monofunctional P.OMPDC, monofunctional yeast enzyme, native P. falciparum OPRT and OMPDC multienzyme complex, and bifunctional human UMPS. In all cases, inhibition was fitted to eq 1 and appeared to be competitive. Previously, we have shown that 5-fluoroorotate is an effective alternative substrate for the P.OMPRT and exhibits strong antimalarial activity with 50% inhibitory effect of approximately 6 nM on P. falciparum grown in vitro and shows potent in vivo antimalarial effect on P. berghei in mice (13, 21). This compound would serve as a subversive substrate for the malarial enzymes. Such type of compounds may be useful in antimalarial drug design targeting malarial OPRT and OMPDC enzyme complex. Moreover, uracil and 5-fluorouracil were not substrates for the P.OMPRT and exhibited weak inhibition (21). The property

is similar to S. typhimurium OPRT (47), in contrast to the human OPRT domain of UMPS, in which 5-fluorouracil serves as a substrate and finally converts to 5-fluoro-deoxyUMP, a potent anticancer drug (67). 5-FUMP and 6-aza-UMP were found to be potent inhibitors of P.OMPDC at submicromolar levels, with even lower $K_i$ values in the multienzyme complex. These values are about 15- and 30-fold less than those for the yeast and human enzymes, respectively.

The binding affinities of the UMP analogues to either monofunctional P.OMPDC or multienzyme complex increases in a series: UMP < 6-CSNH2UMP < OMP < 5-FUMP < 6-aza-UMP. The binding order is similar to those of the human OMPDC domain of UMPS (16, 68) and yeast OMPDC (69, 70), except for 6-CSNH2UMP, which is a very potent inhibitor ($K_i = 3.5 \times 10^{-9}$ M) of yeast OMPDC (65, 69) yet only a weak inhibitor of P.OMPDC with $K_i$ values in the range of 1.7–2.4 $\times 10^{-4}$ M. Using commercially available yeast OMPDC with the inhibitor, the obtained $K_i$ was nearly in the nanomolar range, as in the published literature. There have been no reports investigating this inhibitor binding to active site residues of OMPDCs in the known crystals. If 6-CSNH2UMP binds to OMPDC with the uracil ring of the nucleotide situated in the same orientation as the inhibitors in the crystal structures, then the difference in $K_i$ for 6-CSNH2UMP between the yeast and P. falciparum enzymes would be a result of different contacts between the thio-carboxamide group and the respective Lys-Asp-Lys-Asp charged networks at the active sites. If 6-CSNH2UMP binds to OMPDC with the uracil ring of the nucleotide situated in the opposite orientation as the inhibitors in the crystal structures, as has been proposed for the binding of OMP versus these inhibitors (71), then the difference in $K_i$ for 6-CSNH2UMP between the yeast and P. falciparum enzymes would be a result of different contacts between the thio-carboxamide group and the respective portions of the active site near the conserved glutamine—Gln215 of the yeast enzyme—and an unconfirmed residue in the P. falciparum sequence (possibly Asn279). Regardless, the active sites of P. falciparum and human OMPDCs appear quite different and susceptible to variable degrees of inhibition, an inviting prospect for drug intervention.

CONCLUSIONS

The last two enzymes of the de novo pyrimidine biosynthetic pathway in P. falciparum exist as a (OPRT)$_2$-(OMPDC)$_2$ 140-kDa heterotetrameric complex, an organi-
zation that is possibly exploitable for the design of new antimalarial drugs. This proposal, illustrated in Scheme 2, is firmly supported by the lines of evidence as follows: (1) the tightly associated complex of the two recombinant enzymes by native oligomerization induced by substrate, product, and salt, and by dimethyl suberimidate chemical cross-linking; (2) kinetic parameters favoring the multi-enzyme complex prepared from *P. falciparum* and from mixtures of the recombinant enzymes, comparable to the bifunctional human enzymes; (3) inhibition constants of known OMPDC inhibitors confirming stronger affinity to the multienzyme complex than to the monofunctional enzyme, similar to the human enzymes; and (4) less stability of the monofunctional enzymes than the multienzyme complex and the human enzymes.

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