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Molecular and kinetic properties of mitochondrial dihydroorotate dehydrogenase purified from Plasmodium falciparum

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Suriyamonthol N, Bhumiratana A, Krungkrai SR, Krungkrai J. Molecular and kinetic properties of mitochondrial dihydroorotate dehydrogenase purified from Plasmodium falciparum. Chula Med J 1994 June; 38(6): 325-335

Dihydroorotate dehydrogenase (DHODase), which catalyzes the fourth reaction of pyrimidine biosynthesis from L-dihydroorotate (L-DHO) to L-orotate (L-OA), was detected and purified from the human malarial parasite Plasmodium falciparum (a T9/94 mutant line), cultivated in vitro. Purification of the malarial DHODase was achieved using the detergent solubilization of mitochondrial pellet isolated by differential centrifugations and followed by Mono Q anion-exchange, Cibacron Blue dye-affinity and Superose 12 gel-filtration columns on a fast protein liquid chromatographic (FPLC) system. The DHODase activity was stage-dependent and found to be extremely labile.

The apparent relative molecular weight of the enzyme was estimated as 55±4(n=6) kilodaltons. It had N-terminal blocked. The optimal enzymatic reaction required the presence of coenzyme Q (CoQ_9 and CoQ_{10} forms). The apparent Michaelis constants (K_m) for L-DHO and CoQ_9 were found to be 18±2 and 22±5 (n=6) μM, respectively. L-OA, an enzymatic product, was a strong competitive inhibitor of the DHODase with an inhibitory constant (K_i) of 31±4 μM. The L-OA analogs showed inhibitory effect on the isolated enzyme and also had antimalarial activity against P.falciparum in vitro.

Key words: *Dihydroorotate dehydrogenase, Mitochondria, Plasmodium falciparum, Malaria.*

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Received for publication. May 30, 1994.

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ณัฐประภา สุริยมณฑล, อดิศักดิ์ ภูมิรัตน์, สุดารัตน์ กรึงไกร, จิระพันธ์ กรึงไกร. คุณสมบัติทางโมเลกุลและจลนศาสตร์ของเอ็นไซม์ไดไฮโดรออโรเททดีไฮโดรจีเนสที่เตรียมบริสุทธิ์จากไมโตคอนเดรียของเชื้อพลาสโมเดียมฟัลซิปารัม. จุฬาลงกรณ์เวชสาร 2537 มิถุนายน; 38(6) 325-335

ได้ตรวจพบและเตรียมเอ็นไซม์ไดไฮโดรออโรเทท ดีไฮโดรจีเนส ให้บริสุทธิ์ จากเชื้อมาลาเรีย พลาสโมเดียม ฟัลซิปารัม ที่เลี้ยงในจานทดลอง เอ็นไซม์นี้จะเร่งปฏิกิริยาที่ 4 ของขบวนการสังเคราะห์เบสไพริมิดีน จากสารไดไฮโดรออโรเทท ให้ได้เป็น ออโรเทท การเตรียมเอ็นไซม์ให้ได้บริสุทธิ์กระทำโดยนำน้ำสกัดจากการละลายไมโตคอนเดรียที่เตรียมได้จากเชื้อมาลาเรีย มาผ่านลงคอลัมน์แบบ Mono Q anion-exchange แบบ Cibacron Blue dye-affinity และแบบ Superose 12 gel-filtration chromatography บนเครื่องแยกโปรตีนแบบประสิทธิภาพสูง จากนั้นนำเอ็นไซม์ที่เตรียมได้บริสุทธิ์มาตรวจสอบคุณสมบัติทางโมเลกุลและจลนศาสตร์บางประการ และพบว่าการทำงานของเอ็นไซม์นี้จะสูญเสียสภาพธรรมชาติโดยง่าย และขึ้นอยู่กับระยะการเจริญเติบโตของเชื้อมาลาเรียในเม็ดเลือดแดง และจะมีน้ำหนักโมเลกุล เท่ากับ 56±4 กิโลดาลตัน และตรวจพบอีกว่าปลายด้านอะมิโนของเอ็นไซม์ถูกปิดกั้น การทำงานของเอ็นไซม์ต้องการโคเอ็นไซม์คิวเพื่อให้ได้การเร่งปฏิกิริยาเกิดขึ้นดีที่สุด ได้ศึกษาคุณสมบัติทางจลนศาสตร์ต่าง ๆ ของเอ็นไซม์ รวมทั้งได้ทำการทดสอบสารที่มีโครงสร้างคล้ายออโรเททว่ามีผลต่อการทำงานของเอ็นไซม์และมีคุณสมบัติในการต้านเชื้อมาลาเรีย พลาสโมเดียมฟัลซิปารัม ที่เจริญเติบโตในจานทดลอง

Dihydroorotate dehydrogenase (DHODase,L-dihydroorotate: oxygen oxidoreductase, EC 1.3.3.1), the fourth sequential enzyme in the de novo biosynthesis of pyrimidines, catalyzes the oxidation of L-dihydroorotate (L-DHO) to L-orotate (L-OA). The malarial parasites are totally dependent on de novo biosynthesis for their pyrimidine requirements .[1-3] For this reason, pyrimidine biosynthesis in malarial parasites, and the enzyme DHODase in particular, has long been considered to be a promising target for chemotherapy.[4,5] Some progress to this end has already been made with the discovery that 2-hydroxy-1,4-naphthoquinone derivatives and L-OA analogs possess potent antimalarial activity through inhibition the DHODase activity of pyrimidine biosynthesis.[3,5,6] However, while the parasite DHODase is the presumed site of action of these experimental antimalarials, only a monkey parasite Plasmodium knowlesi[7] and a rodent parasite P.berghei[8,9] enzymes have been partially characterized.

In the present study, we detected and purified the enzyme DHODase from mitochondrial pellet of a human malarial parasite, P.falciparum, cultivated in vitro. We also reported some molecular and kinetic properties of the purified DHODase, e.g., stability, developmental stage-dependency, molecular weight, substrate affinity and inhibitory constants values of an enzymatic product, L-OA.

Materials and Methods

Cultivation of malarial parasite

P.falciparum was cultivated by a modification of the method of Trager and Jensen,[10] using a 6%-8% hematocrit of type O human red blood cells suspended in RPMI 1640 medium supplemented with 10% type O fresh human serum. The parasite strain used in this study was a mutant line of T9/94 clone, which has been described before [11] and kindly provided by Professor Sodsri Thaithong of the Faculty of Science, Chulalongkorn University. Synchrony of the parasite was maintained by the sorbitol procedure of Lambros and Vanderberg.[12]

Preparation of host cell-free parasite homogenate and mitochondrial pellet

The parasites were isolated from the host red cells by incubation in an equal volume of 0.15% saponin in phosphate-bufferred saline (PBS), pH 7.4, at 37° C for 20 min.[13] The intact parasites were then washed at least 4 times (8,000 x g,10 min) with ice-cold PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The parasite homogenate was prepared and then processed for obtaining crude mitochondrial pellet using differential centrifugations by the method of Krungkrai et al.[14]

Purification of mitochondrial dihydroorotate dehydrogenase.

Triton X-100 (a nonionic detergent) was added to the mitochondrial pellet to give a final concentration of 0.15%. The mixture was then stirred for 30 min on ice. The supernatant fluid collected after centrifugation at 39,000 xg for 30 min, contained solubilized DHODase. This fraction, called 'detergent solubilization', was used as the starting material for purification.

FPLC on Mono Q anion-exchange chromatography

The Triton X-100 solubilized DHODase was directly applied to a Mono Q HR 5/5 FPLC column at a flow rate of 1.0 ml/min. 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 $(NH_4)_2SO_4$. Active fractions (at 0.03 M salt) were pooled and dialyzed against the buffer A.

Affinity chromatography on Cibacron Blue F3GA-agarose column

The dialyzed sample containing DHODase after the Mono Q FPLC was applied to a Cibacron Blue F3GA-agarose affinity column which had previously equilibrated with the buffer A. The affinity column was washed with 12 ml buffer A and then eluted with 12 ml buffer A containing 0.6 M $(NH_4)_2SO_4$ and finally eluted with buffer A containing 1 M $(NH_4)_2SO_4$. The active enzyme in 1.0-ml fractions were pooled and concentrated in Centricon-10 concentrating tubes (Amicon).

Gel-filtration chromatography on Superose 12 FPLC column.

The active fraction from the affinity step (at 0.6 M salt) was further purified by gel-filtration chromatography on a Superose 12 column by FPLC. The enzyme DHODase was eluted with buffer A containing 0.6 M$(NH_4)_2SO_4$ at a flow rate of 0.5 ml/min. The Superose 12 column was calibrated with known molecular weight proteins (BioRad) and then used to determine the molecular weight of the DHODase purified from P.falciparum.

Enzyme assays

The spectrophotometric method, based on 2,6-dichlorophenolindophenol (DCIP) reduction, was used according to Krungkrai et al.[8] Confirmation of the enzyme identity was also carried out. [8,9]

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a 5% acrylamide stacking gel and a 10% acrylamide running gel in the discontinuous buffer system of Laemmli. [15]

Amino-terminal sequencing.

The purified enzyme was run on a 10% reducing SDS-PAGE gel as described earlier. The protein was then blotted from the gel onto an Immobilon-P (Millipore) membrane electrophoretically.[16] After visualization of the protein with Coomassie Blue R, the band was excised and extensively destained and then used for sequence analysis.

In vitro antimalarial test.

Antimalarial activity against P.falciparum in vitro was monitored by examining % parasitemia at 96 hours in the presence of various concentrations of L-OA analogs according to the method of Krungkrai et al.[6]

Other methods

Protein concentrations were determined by the method of Bradford[17] with bovine serum albumin as the standard. Kinetic parameters were determined with various compounds and were fitted with least-squares analysis of ENZFITTER program.

Results

Stage-dependent dihydroorotate dehydrogenase activity.

The DHODase activity in crude parasite homogenate and Triton X-100 detergent solubilization of mitochondria from P.falciparum in vitro growth were examined. The specific activites of DHODase in the crude homogenate and the detergent solubilized fraction were found to be 8.31±6.26 (n=13) nmol/min/mg protein, and 3.30±2.61 (n=13) nmol/min/mg protein, respectively.

The stage-dependency of DHODase activities was performed from the synchronized P.falciparum culture of rings, trophozoites and schizonts stages. As shown in Table 1, the highest enzyme activity was found in the trophozoites whereas the rings contained the lowest activity. In addition, the amount of proteins in these three stages of the parasite were related to the activities of the enzyme DHODase.

Table 1. Stage-dependent dihydroorotate dehydrogenase activities in P.falciparum

Stages	Activities (nmol/min)[1]	Protein (mg)
Rings	0.18±0.02[2]	11.7±1.2
Trophozoites	4.90±0.15	38.4±3.5
Schizonts	2.70±0.11	32.6±2.3

[1]Enzyme activities were expressed as nmol/min/10^9 parasites.
[2]Results were taken from three preparations of the culture.

Purification of P.falciparum dihydroorotate dehydrogenase.

Purification of DHODase enzyme from P.falciparum in vitro growth was achieved by using the mitochondria pellet isolated from host cell-free parasites. Following Triton X-100 (detergent) solubilization of the mitochondria, chromatography on Mono Q anion-exchange (Figure 1), Cibacron Blue F3GA-agarose affinity (Figure 2), and finally gel-filtration (Superose 12 column) fast protein liquid chromatography (FPLC), the P.falciparum DHODase was purified ca. 10-fold with a 5% yield (Table 2). Repeated applications of the purification scheme of Table 2 gave consistently heterogeneous preparations of the enzyme. The enzyme from each step of the purification was checked for either homogeneous or heterogeneous purity by SDS-PAGE. These results are shown in Figure 3 and Figure 4. The existence of a protein band at molecular weight of 55 kDa in all steps of the purification scheme was noted. However, the final step of purification on Superose 12 gel-filtration FPLC showed two major bands, the upper band (55 kDa) was found to be the DHODase enzyme by using immunoblotting assay against the antibody to the malarial DHODase (Ref.8, data not shown).

Table 2. Purification of dihydroorotate dehydrogenase from P.falciparum

Steps	Protein (mg)	Activity (nmol/min)	Specific activity (nmol/min/mg)	Purification (x-fold)
1. Detergent solubilization	6.14	18.08	2.94	1.00
2. Mono Q FPLC	0.84	3.90	4.64	1.58
3. Cibacron Blue affinity	0.18	3.90	21.67	7.37
4. Superose 12 FPLC	0.03	0.90	30.00	10.20

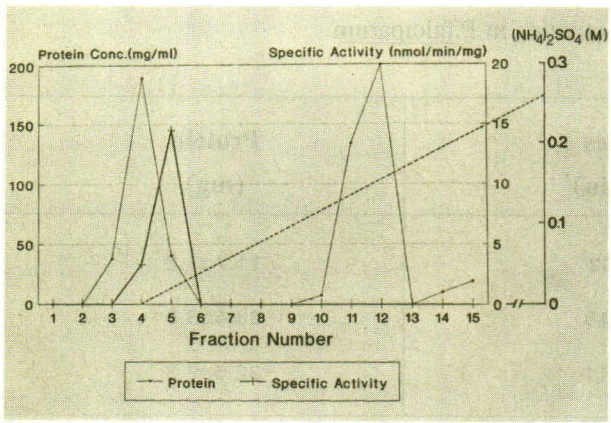

Figure 1. Chromatographic profile of DHODase activity of P.falciparum, eluted from MonoQ anion-exchange column of FPLC system.

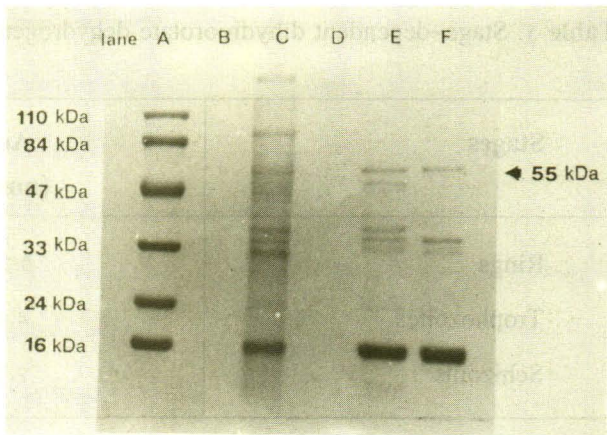

Figure 2. Chromatographic profile of DHODase activity after the Mono Q column of Figure 1,eluted from Cibacron Blue dye- agarose affinity column.

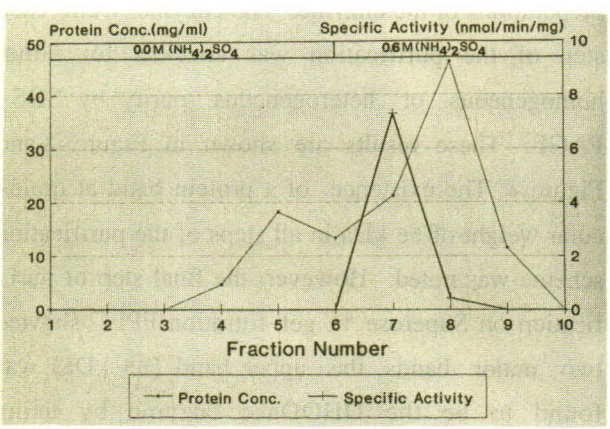

Figure 3. SDS-PAGE pattern of enzyme DHODase in each purification step. The arrow head indicates position of the enzyme.
Lane A,standard molecular weight markers; the molecular weight of each protein band was labelled on the left margin.
Lanes B and D,reagent blank.
Lane C, Triton X-100 detergent solubilizing fraction.
Lane E, pooled active fractions from Mono Q column.
Lane F, pooled active fractions from Cibacron Blue dye-agarise affinity column.

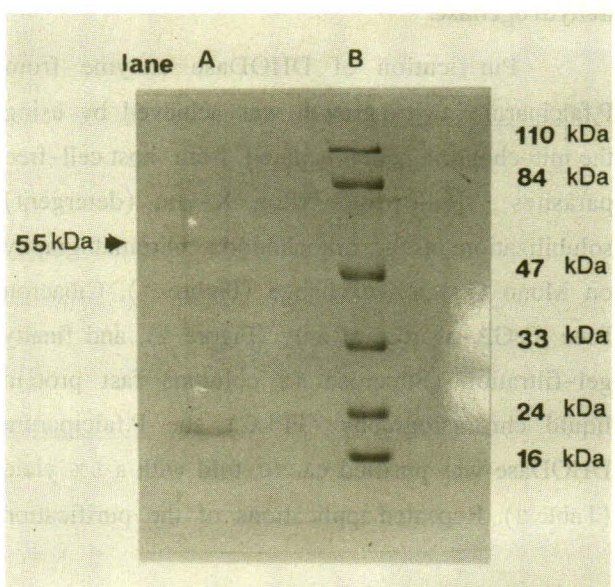

Figure 4. SDS-PAGE analysis of purified DHODase from the last step of purification. The arrow head indicates position of the enzyme.
Lane A, purified DHODase from the Superose 12 FPLC column.
Lane B, standard molecular weight markers; the molecular weight of each protein band was labelled on the right margin.

Molecular and kinetic properties of purified P.falciparum dihydroorotate dehydrogenase.

An attempt has been made to determine NH_2-terminal sequence for aiming at molecular cloning of the malarial DHODase. It was found that the malarial DHODase had NH_2-terminal blocked. Therefore, no amino acid sequence data was obtained.

The molecular weight of the malarial DHODase was determined by using SDS-PAGE analysis (Figure 4) and by constructing the calibration curve as shown in Figure 5. It was found that the molecular weight of the malarial DHODase was 55±4 (n=6) kDa.

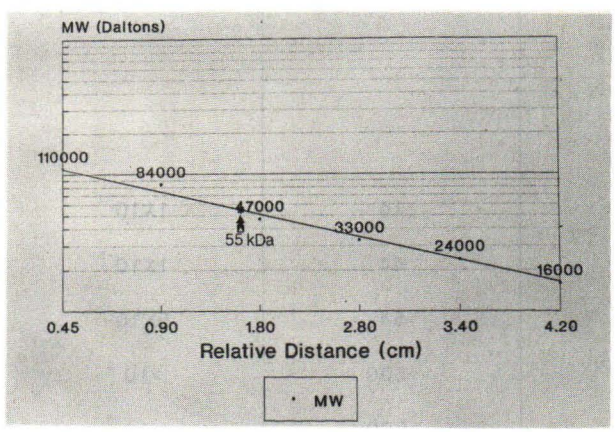

Figure 5. Calibration curve for molecular weight determination on SDS-PAGE analysis. *indicates position of the malarial DHODase purified from P.falciparum

The enzyme DHODase was found to be extremely labile. Even in the presence of protease inhibitors (EDTA, PMSF), the activity was decreased by more than 80% overnight at -20° C. However, the enzyme was more stable at -196° C (liquid nitrogen) than at -20° C (Figure 6).

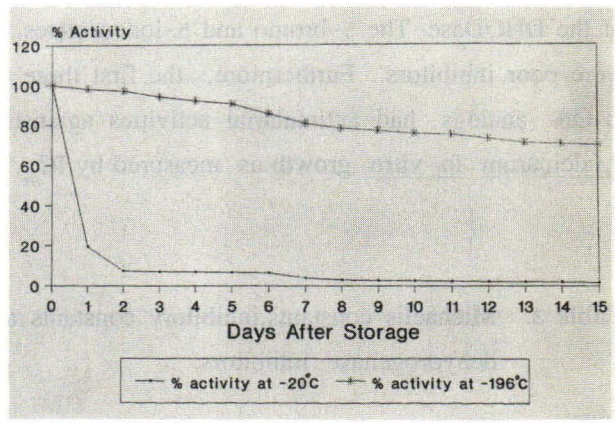

Figure 6. Stability of P.falciparum DHODase tested at -20° C and -196° C. The first day of laboratory experiment is considered as day 'O' and represents 100% activity of the enzyme.

Cosubstrate electron acceptors for the malarial DHODase were examined. Maximal activities were obtained when coenzymes Q_9 and Q_{10} (CoQ_9 and CoQ_{10}) were used as electron acceptors. In the presence of 2,6-dichlorophenolindophenol as proximal electron acceptor, the apparent Michaelis constants (K_m) for L-DHO and CoQ_9 were found to be 18±2 (n=6) μM and 22±5 (n=6) μM, respectively. L-OA, an enzymatic product, was a strong competitive inhibitor of the enzyme with an inhibitory constant (K_i) of 31±4 (n=6) μM.

The L-OA analogs (5-substituted L-OA) were tested for inhibiting the activities of malarial DHODase, and Table 3 shows the inhibitory effect of the L-OA analogs against the isolated malarial enzyme. By using EC_{50} values, effective concentration of the compounds tested resulted 50% inhibition of the enzyme activities. It was found that 5-fluoro, 5-methyl and 5-aminoorotates were strong inhibitors

of the DHODase. The 5-bromo and 5-iodoorotates were poor inhibitors. Furthermore, the first three L-OA analogs had antimalarial activities against P.falciparum in vitro growth as measured by IC_{90}, the concentration of compounds resulting in 90% inhibition of parasite growth after 96-hr treatment. The last two L-OA analogs (5-bromo and 5-iodoorotates) showed little antimalarial activity effect (Table 3).

Table 3. Michaelis constants,inhibitory constants and antimalarial activities of P.falciparum dihydroorotate dehydrogenase inhibitors.

Compounds	K_m (μM)	K_i (μM)	EC_{50} [1] (μM)	IC_{90} [2] (M)
Substrates				
L-DHO	18±2			
CoQ_9	22±5			
Inhibitors				
L-OA		31±4		
5-fluoroorotate			16	$1x10^{-7}$
5-methylorotate			44	$1x10^{-5}$
5-aminoorotate			54	$5x10^{-4}$
5-bromoorotate			>500	$>10^{-3}$
5-iodoorotate			>500	$>10^{-3}$

[1] EC_{50} : concentration of compound giving 50% inhibition of the malarial DHODase activities (average from 2 separate experiments).
[2] IC_{90} : concentration of compound giving 90% inhibition of P.falciparum in vitro growth.

Discussion

The DHODase enzymes have been purified and characterized from a variety of procaryotic and eucaryotic systems, including parasitic protozoans. While these enzymes all catalyze two-electron oxidation from L-DHO to L-OA, some differences exist with regard to their physical properties, subcellular location, cofactor requirement, kinetic behavior, and the compounds to which they transfer the reducing equivalents which result from the enzymatic turnover. For example, the bacterial DHODase is membrane-bound and contains flavin mononucleotide (FMN) binding site, and its action appears to be linked to the cell's respiratiory system.[18,19] The cytosolic DHODase of the parasitic protozoans have been found in Crithidia fasciculata and Trypanosoma brucei,[20] P.berghei and P.falciparum,[21] in addition to the existence of the mitochondrial DHODase form in P.berghei.[8,9] By contrast, the DHODase enzymes from higher eucaryotic cells are located in the mitochondria.[22,23] Our main interest in the P.falciparum DHODase was stimulated by 2 factors: (1) the supposed sensitivity of the enzyme toward a new class of antimalarial

drugs (2-hydroxy-1,4-naphthoquinones), e.g., atovaquone (Wellcome); (2) the relationship of the enzyme to electron transfer and O_2 utilization in the mitochondria of the intraerythrocytic forms of P.falciparum.

With the observation that the late trophozoites and schizonts stages (mature forms) of P.falciparum contained more DHODase activities than the ring stage parasites (young form), the synchronized culture of mature forms were made and used for the enzyme purification. The purification of the enzyme from crude mitochondrial pellet of P.falciparum was achieved by using the following conditions and chromatographic systems: (1) Triton X-100 (0.15%) detergent solubilization; (2) Mono Q anion-exchange FPLC;(3) Cibacron Blue dye-agarose affinity chromatography; and (4) Superose 12 gel-filtration chromatography. The DHODase obtained by these purification steps was apparently near electrophoretic homogeneity, based on SDS-PAGE analysis (Figure 4). The upper intense band on the gel was confirmed by Western blot analysis using antibody to P.berghei DHODase .[8] Based on the native behavior of the enzyme DHODase eluted in the Superose 12 gel-filtration chromatography (Table 2), the molecular weight of the active monomeric native form was estimated to be 55 kDa, which was closely related to the molecular weight of the enzyme (denature form) calculated by SDS-PAGE analysis (Figure 5). These results suggest that the P.falciparum DHODase exists as monomeric form that has been found in the bacterial enzymes.[18,19] It also had blocked in N-terminal of the protein, thus the amino acid sequence of the DHODase has yet to be known. The molecular properties of the P.falciparum DHODase were similar, but not identical, to the characterizied DHODase from P. knowlesi [7] and P.berghei. [8] The enzyme DHODase is presumalbly associated with mitochondrion of P.falciparum but verification of the localization of this enzyme will be necessary.

On kinetic analyses, the DHODase activity of the parasite was found to be extremely labile (Figure 6). The enzyme required CoQ_9 (ubiquinone-45) or CoQ_{10} (ubiquinone-50) for its optimal activity. The K_m values for both L-DHO and CoQ_9 (Table 3) suggest that physiological concentrations of the substrates (at μM levels) can be achieved for the DHODase function during erythrocytic phase maturation in in vitro growth. The product (L-OA) of the enzymatic reaction and a series of 5-substituted orotate analogs were found to inhibit the DHODase activity. Antimalarial activities against both P.falciparum in vitro and P.berghei in vivo of some of these derivatives, e.g., 5-fluoroorotate, has recently been reported by our groups.[5,6] Some orotate analogs were also found to have an antimalarial effect (Table 3). Our results suggest that the enzyme DHODase may be the target site of these orotate analogs.

In general, the DHODase synthesis is regulated at the transcriptional level in yeast Saccharomyces cerevisae through positive control by the L-DHO intracellular pool,[24] and probably at the translational level in bacteria Salmonella typhimurium through the secondary structure of the 5′ end transcript of the DHODase gene. [25] Recently, we have found the existence of degradative orotate reductase, which catalyzes reduction of L-OA to L-DHO, in both P.berghei and P.falciparum.[21] The malarial orotate reductase activity, about 2-5 times higher than the DHODase, [8,9] may play a role in controlling the DHODase synthesis by increasing intracellular level of L-DHO which it will induce more DHODase enzyme which is responsible for the de novo pyrimidine synthesis during intraerythrocytic growth of the parasite. However, the regulatory mechanism of the DHODase synthesis in the malarial parasites needs to be clarified since the DHODase gene homologue of P.falciparum has recently been cloned and sequenced.[26] In this particular case, we plan to do more work on the regulatory system in P. falciparum.

Acknowledgement

We wish to thank Krisna Phakanont and Rachanok Kanjanarithisak for technical assistance in supplying P.falciparum culture and training some experimental work in the laboratory. Thanks are also extended to Chanai Malengloy and Kanjana Kowvasuta for typing and correcting this manuscript. This work was a part of the masters thesis of Nattprapa Suriyamonthol and was supported by the Phra Mahitaladhibet Adulyadejvigrom Phra Bharomrajchanok Fund. This work in the author's laboratory was also supported by the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, and the Faculty of Medicine and Research Affairs Division of Chulalongkorn University.

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