

Figure Legend

Figure 1 :Restriction fragment length polymorphism (RFLP) patterns of 21-Hydroxylase gene mutation.

Lane 1 is DNA size marker (100bp DNA ladder). 21-Hydroxylase gene 's amplicons after digestion with BsiHKAI restriction enzyme; lane 2,3: PCR-RFLP pattern of L homozygotes, 1165bp, 205bp and 88bp length PCR fragments. lane 4,5: PCR-RFLP pattern of L/V heterozygotes,1165bp, 995bp, 205bp, 170bp and 88bp PCR fragments. lane 6,7: PCR-RFLP pattern of V/V homozygotes, 995bp, 205bp, 170bp and 88bp PCR fragments. lane 8: No digestion.

Figure 1. Restriction fragment length polymorphism (RFLP) patterns of 21-Hydroxylase gene mutation.

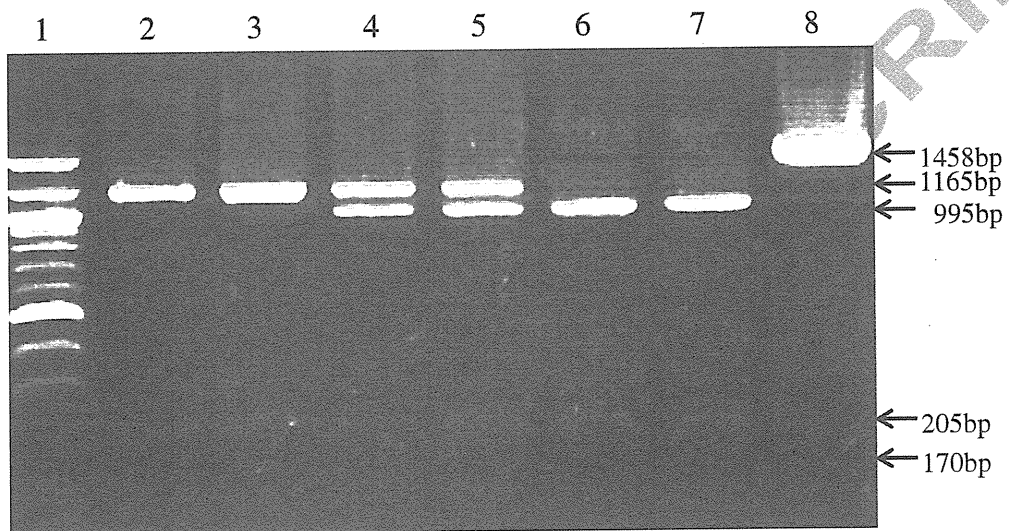


Table 1 Linkage between alleles of HLA-B and HLA-DRB1 with V281L

Allele	obs	exp	diseq	norm_dij	chisq	Degree of Freedom	Pv
<i>B*14:02</i>	14.05729	1.0885	0.01689	0.62017	167.34469	2	<0.0001
<i>B*14:02</i>	3.95052	0.1719	0.00492	0.64835	86.19624	2	<0.0001
<i>DRB1*01:02</i>	10	1.0825	0.01149	0.44772	79.61041	5	<0.0001
<i>DRB1*01:02</i>	6	0.1624	0.00752	1	217.39518	5	<0.0001

Table 2 Allele frequency of the mutant and normal CYP21A2 gene

	Indeterminate n=68(%)	ECG+ &/or Megacolon+ n=156(%)	ECG+ alteration n=75(%)	Megacolon+ n=97(%)	Odds Ratio	P value
L/L & L/V	11(16.2)	6(3.8)	2(2.7)	4(4.1)	0.207 ^a , 0.142 ^b , 0.223 ^c	0.0041 ^a , 0.007 ^b , 0.0118 ^c
V/V	57(83.8)	150(96.1)	73(93.3)	93(95.9)		

^a ECG+ &/or Megacolon+ vs Indeterminate

^b ECG+ alterations vs Indeterminate

^c Megacolon+ vs Indeterminate

Table 3 Allele frequencies of the mutant and normal CYP21A2 gene after exclusion of individuals with Haplotype B*14-DRB1*01

	Indeterminate n=62(%)	ECG+ &/or Megacolon+ n=155(%)	ECG+ alterations n=74(%)	Megacolon+ alterations n=97(%)	Odds Ratio	P value
L/L & L/V	5(8.1)	5(3.2)	1(1.3)	4(4.1)	0.380 ^a , 0.156 ^b , 0.490 ^c	0.153 ^a , 0.092 ^b , 0.313 ^c
V/V	57(91.9)	150(96.8)	73(98.6)	93(95.9)		

^a ECG+ &/or Megacolon+ vs Indeterminate

^b ECG+ vs Indeterminate

^c Megacolon+ vs Indeterminate



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Molecular interaction of the first 3 enzymes of the *de novo* pyrimidine biosynthetic pathway of *Trypanosoma cruzi*

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ABSTRACT

The first 3 reaction steps of the *de novo* pyrimidine biosynthetic pathway are catalyzed by carbamoyl-phosphate synthetase II (CPSII), aspartate transcarbamoylase (ATC), and dihydroorotase (DHO), respectively. In eukaryotes, these enzymes are structurally classified into 2 types: (1) a CPSII-DHO-ATC fusion enzyme (CAD) found in animals, fungi, and amoebozoans, and (2) stand-alone enzymes found in plants and the protist groups. In the present study, we demonstrate direct intermolecular interactions between CPSII, ATC, and DHO of the parasitic protist *Trypanosoma cruzi*, which is the causative agent of Chagas disease. The 3 enzymes were expressed in a bacterial expression system and their interactions were examined. Immunoprecipitation using an antibody specific for each enzyme coupled with Western blotting-based detection using antibodies for the counterpart enzymes showed co-precipitation of all 3 enzymes. From an evolutionary viewpoint, the formation of a functional tri-enzyme complex may have preceded—and led to—gene fusion to produce the CAD protein. This is the first report to demonstrate the structural basis of these 3 enzymes as a model of CAD. Moreover, in conjunction with the essentiality of *de novo* pyrimidine biosynthesis in the parasite, our findings provide a rationale for new strategies for developing drugs for Chagas disease, which target the intermolecular interactions of these 3 enzymes.

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1. Introduction

The *de novo* pyrimidine biosynthetic pathway consists of 6 enzymes required for the production of uridine 5'-monophosphate (UMP). In eukaryotes, the primary structure of the first 3 enzymes in this pathway, carbamoyl-phosphate synthetase II (CPSII; EC 6.3.5.5), aspartate transcarbamoylase (ATC; EC 2.1.3.2), and dihydroorotase (DHO; EC 3.5.2.3), is divided into 2 types [1]. A fusion enzyme of CPSII-DHO-ATC (CAD) is found in animals, fungi, amoebozoans, and also in the red alga *Cyanidioschyzon merolae*

[2,3]. In contrast, the individual, stand-alone enzymes are common among the remaining eukaryotic groups.

Gene fusion results in the formation of multifunctional proteins and is one of the major driving forces in protein evolution. CAD has arisen from an ancient fusion between its monofunctional counterparts, which may result in enhanced channeling of substrates through each catalytic site [4]. From a biochemical viewpoint, it is assumed that formation of a complex of the enzymes preceded gene fusion and contributed to the precise order and topology of the domains within the fused enzyme. Moreover, ATC and DHO constitute an enzyme complex in a subset of bacteria [5,6], suggesting possible complex formation between eukaryotic ATC and DHO. Thus, it is highly likely that a tri-enzyme complex of stand-alone CPSII, ATC, and DHO preceded—and eventually led to—gene fusion to produce the multifunctional CAD protein, which may share tertiary structural similarity.

Abbreviations: CPSII, carbamoyl-phosphate synthetase II; ATC, aspartate transcarbamoylase; DHO, dihydroorotase; CAD, CPSII-DHO-ATC fusion enzyme; UMP, uridine 5'-monophosphate; SBDD, structure-based drug design.

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We have previously showed that CPSII, ATC, and DHO are stand-alone enzymes in the parasitic protist *Trypanosoma cruzi*, which is the causative agent of Chagas disease, an endemic disease in the Latin American countries [7,8]. All 5 genes for the 6 pyrimidine biosynthetic enzymes cluster within trypanosomatid genomes in the following order: CPSII, DHO, OMPDC-OPRT (fused gene comprising the sixth orotidine-5'-monophosphate decarboxylase and the fifth orotate phosphoribosyltransferase enzymes), DHOD (the fourth dihydroorotate dehydrogenase enzyme), and ATC. Thus, the gene order of CPSII, DHO, and ATC in trypanosomatid genomes is consistent with the domain order within CAD [7]. Thus, these findings allowed us to investigate the possibility of complex formation between *T. cruzi* CPSII, ATC, and DHO.

From a clinical viewpoint, *de novo* pyrimidine biosynthesis represents a promising drug target, in particular, against Chagas disease and other trypanosomatid diseases such as sleeping sickness and leishmaniasis caused by *Trypanosoma brucei gambiense/rhodesiense* and *Leishmania* spp., respectively. These trypanosomatid diseases are termed “neglected tropical diseases”, and urgently require effective chemotherapeutics with low toxicity [9,10]. Very recently, we demonstrated the importance of CPSII for the growth of *T. cruzi* in the cytoplasm of the mammalian host cells [11]. Therefore, the 3 enzymes and their complex, if present, represent potential targets of chemotherapy for trypanosomatid diseases.

In the present study, we used recombinant enzymes to examine whether CPSII, ATC, and DHO form a tertiary complex. As a result, we demonstrated that these 3 enzymes interact with each other, suggesting that the multifunctional CAD fusion enzyme was preceded by complex formation between CPSII, ATC, and DHO, possibly mimicking their tertiary structures. Thus, our findings provide important insights into the structural interpretation of evolution of enzymes as well as into the new strategic approaches for the development of drugs against Chagas disease.

2. Materials and methods

2.1. Plasmid construction

The open reading frames (ORFs) for the *T. cruzi* CPSII (GenBank ID: AB005063), ATC (GenBank ID: AB074139), and DHO (GenBank ID: AB010284) genes were subcloned into expression vectors, pET52b (ampicillin-resistant; Novagen, Merck Ltd., Tokyo, Japan), pET28a (kanamycin-resistant; Novagen), and pT-GroE (chloramphenicol-resistant) [12]. Prior to cloning, pET52b was modified using the Gateway[®] Vector Conversion System (Invitrogen, Life Technologies Japan Ltd., Tokyo, Japan) by ligating a reading frame cassette A (RfA) to the *Sma*I site present in pET52b. The resulting plasmid was designated pET52bGW. For cloning of CPSII, the CPSII ORF was amplified using primers (sense, 5'-CACCATGTTGGGG AAAAAGTGAA-3'; antisense, 5'-TCAACTGAACTGCGCTGAAG GAGC-3') and the phage clone carrying CPSII, subcloned into the pENTR vector (Invitrogen), and subsequently cloned into pET52bGW via the Clonase[®] reaction (Invitrogen). For expression of ATC, a pET14b plasmid carrying ATC [13] was digested with *Bam*HI and the resulting DNA fragment was cloned into pET28a. For expression of DHO, the ORF was amplified by PCR using a primer set (sense, 5'-CACCATGACGCGGGTGGAACTGCC-3' and antisense, 5'-CTAAATAGCCTTACCAACAAG-3'), subcloned into pENTR, and finally cloned into pET52bGW. Alternatively, the ORF of DHO was amplified using primers (sense, 5'-GCGAATCCATATGA CGCG-3'; antisense, 5'-GCGGATCCTAAATAGCC-3') and the phage clone carrying DHO [7] and subcloned into the pT7 BlueT vector. The *Nde*I/*Bam*HI fragment was further subcloned into *Nde*I/*Bam*HI-treated pT-GroE, in which the *GroESL* gene was cleaved off.

2.2. Expression of recombinant enzymes

Recombinant plasmids were introduced into *Escherichia coli* BL21 Star[™] (DE3) cells (Invitrogen), either independently or in combination. The *E. coli* transformants were precultured by overnight incubation at 37 °C in Luria–Bertani (LB) medium containing the appropriate antibiotics. Subsequently, a total of 0.8 ml of the bacterial culture was centrifuged for 5 min at 1500g, and the cells were further cultured at 37 °C in 30 ml of antibiotic-supplemented LB medium until the OD₆₀₀ reached 0.8. Expression was induced at 25 °C for 1 h in the presence of 0.4 mM isopropyl-β-D-thiogalactopyranoside. The cells were harvested by centrifugation at 1500g for 10 min and suspended in 2 ml of IP buffer (150 mM NaCl, 10 mM Tris–HCl [pH7.4], 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1.5% NP-40, 0.2 mM sodium orthovanadate, and 0.2 mM PMSF) supplemented with a protease inhibitor cocktail (Complete Mini, Roche Diagnostics K.K., Tokyo, Japan). After disruption of cells by sonication, the lysate was centrifuged at 15,000g, 4 °C for 10 min. The resulting supernatant containing the soluble enzymes was collected and used for immunoprecipitation experiments.

2.3. Antibodies

Rabbit polyclonal IgG was raised against *T. cruzi* CPSII (polypeptide, aa1–17; Sigma–Aldrich, St. Louis, MO) and DHO (polypeptide, aa12–29; BioGate Ltd, Gifu, Japan), and mouse polyclonal antisera was raised against recombinant *T. cruzi* ATC.

2.4. Immunoprecipitation

Immunoprecipitation was carried out using the enzyme-specific antibodies and Protein G Magnetic Beads (New England Biolabs, Beverly, MA) under the conditions recommended by the manufacturer. Briefly, 200 μl of bacterial lysate was incubated at 4 °C for 1 h with 1.5 μg of purified IgG specific for each enzyme, followed by incubation with 25 μl of the beads at 4 °C for 1 h. The resulting precipitates were recovered magnetically, separated by SDS–PAGE, and transferred to PVDF membranes. The membrane was reacted with enzyme-specific antibodies and visualized using alkaline phosphatase-conjugated secondary antibody and colorimetric substrates.

3. Results and discussion

The activities of CPSII, ATC, and DHO enzymes in trypanosomatids have been purified separately by gel chromatography or ammonium sulfate precipitation [14,15], suggesting that the 3 enzymes are not covalently linked, or that weak interactions occur between them. Thus, we aimed to examine the molecular interactions between *T. cruzi* CPSII, ATC, and DHO by using purified enzymes that were independently expressed in a bacterial expression system (Fig. 1). *T. cruzi* ATC was efficiently expressed as a soluble protein and purified to apparent homogeneity, as reported previously [13]. However, individual expression of *T. cruzi* CPSII or DHO was difficult, and these enzymes were mainly expressed as insoluble inclusion bodies. The un-tagged recombinant *T. cruzi* DHO protein appeared to be toxic to *E. coli*, since the transformant colonies were very small (data not shown). This may be due to heterodimerization between bacterial and *T. cruzi* DHO proteins, thereby leading to impaired *de novo* pyrimidine biosynthesis by the bacteria. Furthermore, we also failed to obtain efficient expression of a soluble form of GST-tagged DHO.

Although these results suggest that individually expressed CPSII and DHO were highly unstable in the bacteria, we tried to examine

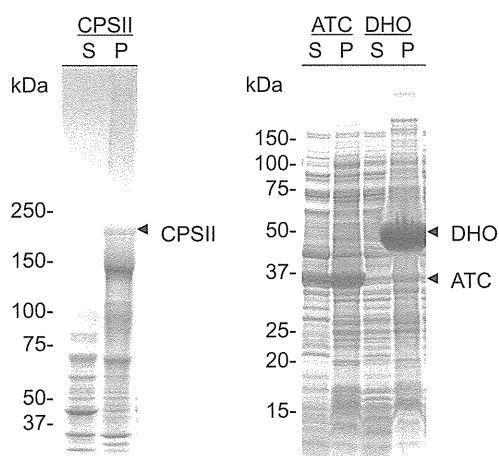


Fig. 1. Expression of the first 3 enzymes of the *Trypanosoma cruzi de novo* pyrimidine biosynthetic pathway in a bacterial expression system carbamoyl-phosphate synthetase II (CPSII), aspartate transcarbamoylase (ATC), and dihydroorotase (DHO) were independently expressed in *E. coli* BL21 (DE3) Star cells, separated by SDS-PAGE, and stained with Coomassie Brilliant Blue. Recombinant CPSII and DHO were expressed mostly in the insoluble fractions (P), whereas ATC was expressed in both soluble (S) and insoluble fractions. The arrowheads indicate the respective protein bands.

whether the individually expressed enzymes formed a complex, which would stabilize these enzymes. The soluble fractions of each bacterial lysate were mixed, immunoprecipitated using the respective antibodies, and probed by Western blot analysis. However, no association was seen between the individually expressed target proteins.

Therefore, we examined the interactions between the 3 proteins by co-expressing CPSII, ATC, and DHO using pET52b (ampicillin-resistant), pET28a (kanamycin-resistant), and pT-GroE (chloramphenicol-resistant) expression vectors, respectively, in order to confer antibiotic selection. Under these conditions, the transformed bacteria grew normally. After induction of expression, the bacterial lysates were immunoprecipitated using either CPSII- or ATC- or DHO-specific antibodies and subjected to Western blot analysis. As shown in Fig. 2, all 3 enzymes were co-precipitated

by a pull-down assay using antibodies specific to either enzyme (Fig. 2). Namely, immunoprecipitation using the CPSII-specific antibody was accompanied by co-precipitation of both ATC and DHO, and the ATC- or DHO-specific antibodies allowed co-precipitation of CPSII plus DHO or CPSII plus ATC, respectively. Thus, these results clearly indicate the existence of a molecular interaction between CPSII, ATC, and DHO.

Co-precipitation of ATC and DHO in the CPSII-specific precipitate was further confirmed by in-gel digestion coupled with liquid chromatography–tandem mass spectrometry (LC–MS/MS) of the corresponding protein bands separated by SDS–PAGE (Supplementary Fig. S1). The sequence coverage scores for ATC and DHO were 52% and 51%, respectively. In addition, the ATC-specific precipitate was found to contain CPSII and DHO, and CPSII and ATC were detected in the DHO-specific precipitate (Supplementary Table S1). Therefore, these findings indicate complex formation by these non-covalently linked enzymes.

However, co-precipitation of CPSII with ATC and DHO does not necessarily indicate a direct interaction of CPSII with both ATC and DHO; if ATC interacts with DHO, the interaction of CPSII with either ATC or DHO would allow co-precipitation of all 3 enzymes. Therefore, we further examined the existence of a direct interaction of CPSII with ATC and DHO. CPSII was co-expressed with either ATC or DHO, and precipitated using the CPSII-specific antibody. Western blot analysis of the precipitates showed direct interactions between both CPSII and ATC and between CPSII and DHO (Fig. 3). Similarly, a direct interaction was also observed between ATC and DHO, which is consistent with results for the bacterial enzymes [5]. These findings strongly suggest that tri-enzyme complex formation between CPSII, ATC, and DHO occurs via direct interactions.

Regarding enzyme stability, native CPSII purified from the kinetoplastid *Crithidia fasciculata*—a close relative of trypanosomatids—was very fragile, and its enzyme activity was rapidly lost during cryopreservation, which may have been accompanied by dissociation of its tertiary structure [16]. In the present study, the molecular interactions between CPSII, ATC, and DHO were stable after repeated freezing and thawing. Therefore, it is possible that complex formation stabilizes enzyme conformation.

Recent advances in drug development have highlighted structure-based drug design (SBDD) as a powerful tool. Moreover, inhibitors of enzymes involved in *de novo* pyrimidine biosynthesis have been extensively studied because of the physiological importance of this pathway, particularly in rapidly growing cells such as cancer cells. However, SBDD has not yet been applied to CAD or its individual counterparts because of difficulties in their expression, purification, and crystallization and the resulting lack of structural information.

In the present study, we demonstrated for the first time the existence of a molecular interaction between *T. cruzi* CPSII, ATC, and DHO enzymes. Furthermore, the efficient expression of these proteins may aid structural analyses and the elucidation of a model of CAD, thereby leading to SBDD. Indeed, phylogenetic analysis has revealed that the trypanosomatid CPSII and ATC enzymes share common evolutionary origins with the corresponding domains in animal CAD [1].

Moreover, we recently identified the crystal structure of *T. cruzi* ATC [13]. This is the first crystallization of any of the 3 enzymes, including CAD proteins in eukaryotes. Therefore, our findings provide insight into SBDD targeted against trypanosomiasis, particularly aimed at inhibiting intermolecular interactions of the tri-enzymes. Although the phylogenetic relationship between *T. cruzi* DHO and the DHO domain of CAD remains unclarified, it is highly likely that the functional tri-enzyme complex of CPSII, ATC, and DHO occurred in the ancestral eukaryotes, thereby leading to the formation of CAD via fusion of the 3 genes.

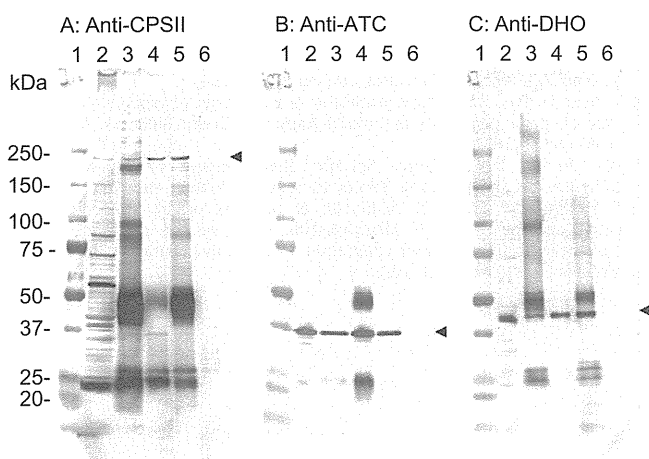


Fig. 2. Immunoprecipitation analysis of *E. coli* lysates co-expressing CPSII, ATC, and DHO. Western blot analysis was performed using antibodies specific to CPSII (panel A), ATC (panel B), and DHO (panel C). The *E. coli* lysates (lane 2) were immunoprecipitated using antibodies specific to CPSII (lane 3), ATC (lane 4), and DHO (lane 5), and examined by Western blotting. Precipitates using protein G beads in the absence of antibodies were loaded as a negative control (lane 6). An arrowhead indicates the respective target band. Lane 1, molecular weight marker.

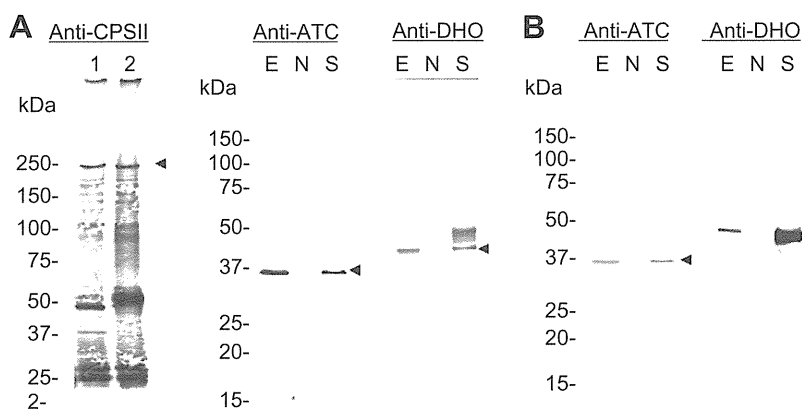


Fig. 3. Detection of the direct interactions between CPSII, ATC, and DHO. In panel A, the bacterial lysates expressing CPSII together with ATC (lane 1) or DHO (lane 2) were immunoprecipitated using antibodies specific to the respective enzyme and probed using the CPSII-specific antibody (left). Alternatively, the bacterial extracts (E) expressing CPSII plus ATC or CPSII plus DHO were immunoprecipitated using the CPSII-specific antibody (S) and reacted with the antibody for ATC (middle) or DHO (right). In panel B, the bacterial extracts expressing ATC and DHO were immunoprecipitated using the antibody (S) for DHO (left) or ATC (right) and probed using the antibody for the counterpart enzymes. The immunoprecipitates in the absence of antibody (N) were loaded as a negative control. An arrowhead indicates the respective target band.

Since the *de novo* pyrimidine biosynthetic pathway is a potential primary target of chemotherapy [17,18], our findings may accelerate strategic approaches against trypanosomatid diseases based on SBDD that target the intermolecular interactions of the first 3 enzymes. Further analyses, including crystallography, are necessary to understand the molecular basis of the tri-enzyme complex.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.12.148.

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Critical importance of the *de novo* pyrimidine biosynthesis pathway for *Trypanosoma cruzi* growth in the mammalian host cell cytoplasm

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ABSTRACT

The intracellular parasitic protist *Trypanosoma cruzi* is the causative agent of Chagas disease in Latin America. In general, pyrimidine nucleotides are supplied by both *de novo* biosynthesis and salvage pathways. While epimastigotes—an insect form—possess both activities, amastigotes—an intracellular replicating form of *T. cruzi*—are unable to mediate the uptake of pyrimidine. However, the requirement of *de novo* pyrimidine biosynthesis for parasite growth and survival has not yet been elucidated. Carbamoyl-phosphate synthetase II (CPSII) is the first and rate-limiting enzyme of the *de novo* biosynthetic pathway, and increased CPSII activity is associated with the rapid proliferation of tumor cells. In the present study, we showed that disruption of the *T. cruzi cpsII* gene significantly reduced parasite growth. In particular, the growth of amastigotes lacking the *cpsII* gene was severely suppressed. Thus, the *de novo* pyrimidine pathway is important for proliferation of *T. cruzi* in the host cell cytoplasm and represents a promising target for chemotherapy against Chagas disease.

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1. Introduction

The parasitic protist *Trypanosoma cruzi* is the causative agent of Chagas disease [1]. The parasitic life cycle comprises 2 phases (the insect and mammalian stages) and includes three developmental forms—epimastigotes, trypomastigotes, and amastigotes [2]. Within the insect vector, the reduviid bug, epimastigotes replicate and transform into metacyclic trypomastigotes by a process termed metacyclogenesis. Non-proliferating metacyclic trypomastigotes invade the mammalian host and subsequently transform into amastigotes in a wide variety of nucleated cells. Intracellular amastigotes multiply by binary fission and then transform back into bloodstream trypomastigotes, which are released into the circulation after host cell disruption.

Pyrimidine is an essential component of nucleic acid structure. Thus, the biosynthesis of pyrimidine is a vital biological process,

which is achieved by both *de novo* synthesis and salvage pathways. Although both pathways have been shown to operate in *T. cruzi*, the parasite has only limited salvage activity. It has been reported that *T. cruzi* preferentially takes up more pyrimidine bases and nucleosides than nucleotides [3]. However, the host cell cytoplasm contains mainly nucleotides [4], which are not efficiently salvaged by *T. cruzi*. In addition, the intracellular amastigote lacks uracil phosphoribosyltransferase and uridine kinase enzymatic activities, the latter of which is not found in the *T. cruzi* genome [5]. Therefore, the *de novo* pyrimidine biosynthesis pathway may be important for *T. cruzi* growth in mammalian hosts.

The *de novo* pyrimidine biosynthetic pathway consists of a 6-enzyme cascade that catalyzes the formation of uridine 5'-monophosphate [6]. The first enzyme in the pathway is carbamoyl-phosphate synthetase II (CPSII, EC 6.3.5.5), which generates carbamoyl-phosphate from L-glutamine, bicarbonate, and two ATP molecules. Moreover, CPSII is a key regulatory enzyme of *de novo* pyrimidine biosynthesis, and increased CPSII activity is associated with the rapid proliferation of tumor cells [7].

In the present study, we established *T. cruzi* lacking the *CPSII* (*TcCPSII*) gene in order to determine the physiological relevance of *de novo* pyrimidine biosynthesis. By analyzing the mutant

Abbreviations: CPSII, carbamoyl-phosphate synthetase II; LIT, liver infusion tryptose; DHOD, dihydroorotate dehydrogenase; MOI, a multiplicity of infection.

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parasites, we found that the *de novo* pyrimidine pathway is important for parasitic growth in the host cell.

2. Materials and methods

2.1. *T. cruzi* culture

T. cruzi epimastigotes (Tulahuen strain) were routinely subcultured on a weekly basis in liver infusion tryptose (LIT) medium (No. 1029, ATCC medium formulations) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 10 μ g/ml hemin (Sigma–Aldrich, Japan) in tightly capped 25-cm² culture flasks at 27 °C. The mammalian stages of *T. cruzi* were maintained in HeLa cells, as previously described [8]. In addition, we used mouse embryonic fibroblast 3T3-Swiss albino cells (Health Science Research Resources Bank, Tokyo, Japan), which were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich Japan) supplemented with 10% FBS. For experiments, 3T3-Swiss cells were infected with metacyclic trypomastigotes at a multiplicity of infection (MOI) of 0.26. The infected cells were washed with PBS 24 h after infection to remove free parasites and cultured for a

further 3 days. Multiple infections of a single host cell by metacyclic trypomastigotes were confirmed as negligible under the experimental conditions employed. Infected *T. cruzi* amastigotes within host cells were stained with Diff-Quik solution (Sysmex, Kobe, Japan) and detected microscopically as previously described [8].

2.2. Metacyclogenesis

Metacyclogenesis was induced as previously described [9]. Epimastigotes from the late logarithmic phase were collected by centrifugation, suspended at a density of 1.0×10^7 cells/ml in 80% (v/v) RPMI-1640 medium and 20% (v/v) Grace's insect medium and incubated at 27 °C. Under these conditions, epimastigotes adhered to the surface of the plastic culture flask, and were released only once they enter metacyclogenesis [10]. For counting of metacyclic trypomastigotes, parasites that appeared in the culture supernatant were stained with Giemsa stain, and their morphology was evaluated by light microscopy according to the relative kinetoplast-nucleus position [10–12]. The efficacy of metacyclogenesis was evaluated by the ratio of epimastigotes to trypomastigotes in the culture supernatant 72 h after induction.

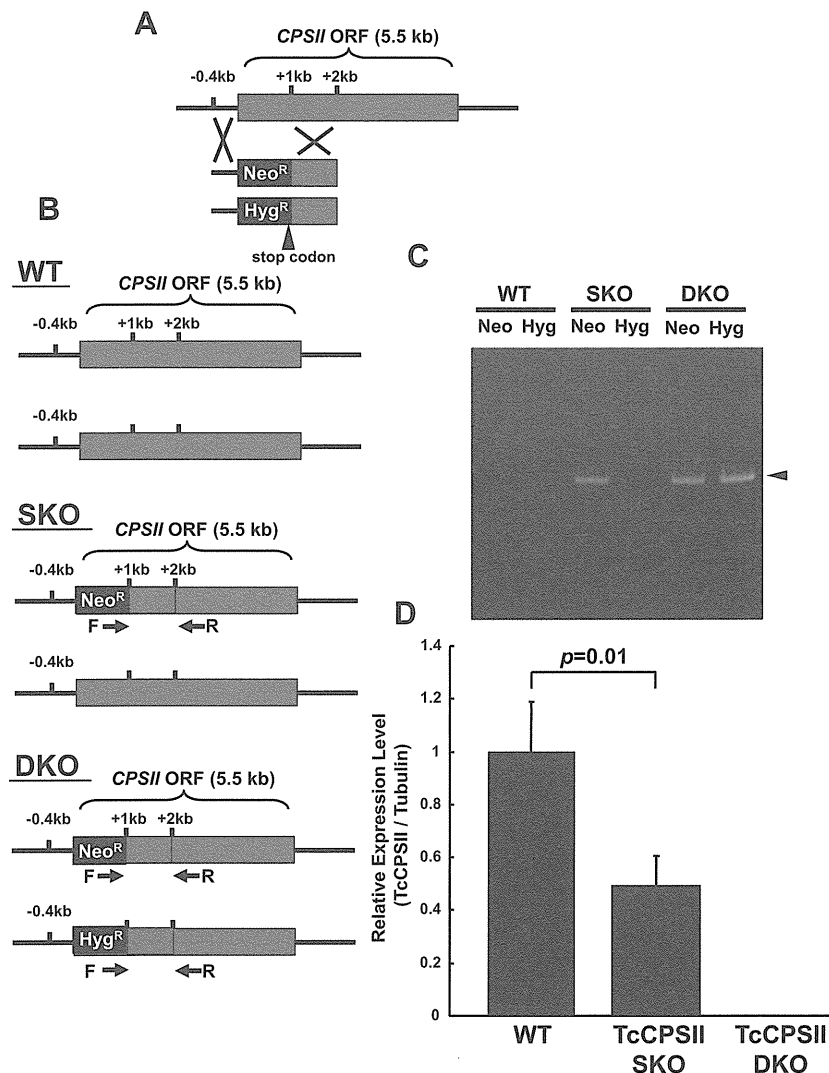


Fig. 1. Production of a *T. cruzi* *CPSII*-null mutant. (A) Diagram of the *TcCPSII* locus and neomycin- and hygromycin-resistance gene cassettes containing 5'-upstream (−0.41 kb) and 3'-downstream (+1.0 kb) sequences corresponding to the downstream region (+1 to +2 kb) of the *TcCPSII* open reading frame (ORF). (B) Schematic representation of *TcCPSII* loci in the WT, single-gene knockout (SKO), and double-gene knockout (DKO) parasites. (C) PCR analysis of genomic DNA from clones of the WT, SKO, and DKO parasites confirmed disruption of 1 locus of the *TcCPSII* gene in SKO parasites and disruption of the 2 loci in DKO parasites, respectively. (D) Real-time PCR analysis of *TcCPSII* mRNA expression levels in WT, SKO, and DKO epimastigotes. The data shown are expressed as fold change and represent the mean \pm SD of three independent experiments.

To examine the infectivity of host cells by metacyclic trypomastigotes, the remaining epimastigotes were killed by adding untreated (not heat-inactivated) FBS, as previously described [13]. The trypomastigotes were collected by centrifugation at 800g for 5 min in 15-ml polypropylene tubes to remove cell debris. The resulting supernatant was centrifuged at 1500g for 10 min at 4 °C, and the pellet containing trypomastigotes was washed three times with 10 ml of DMEM by repeated suspension and centrifugation. The purified trypomastigotes were counted on an improved Neubauer hemocytometer and used for experiments.

2.3. Generation of the knockout DNA cassettes for *TcCPSII*

We constructed two gene-knockout cassettes comprising the 5' flanking region of *TcCPSII*, the neomycin phosphotransferase II gene (*neo^R*) or hygromycin B phosphotransferase gene (*hyg^R*), and an internal sequence from the *TcCPSII* gene (nucleotides +1000 to +2000) to allow disruption of the *TcCPSII* loci by homologous recombination (Fig. 1A). The knockout cassettes were constructed using a MultiSite Gateway® Three-Fragment Vector Construction Kit (Invitrogen) [14]. The pDEST/*TcCPSII*_Neo^R plasmid was constructed by amplifying the 5' flanking sequence of *TcCPSII* (0.41 kb) using a primer set (sense, 5'-GGGGACAACCTTTGTATAGAAAAGTTGGAAATTCGCGTCTTCCTTTTTTCTCCTTTTCTTTT-3' and antisense, 5'-GGGGACTGCTTTTTGTACAACTTGTTTACTTTTTTATGTTTTGTGTACTG-3'; the *EcoRI* site is underlined), KOD-Plus-Neo (TOYOBO), and *T. cruzi* Tulahuen genomic DNA as the template and was subsequently cloned into the entry vector (pDONR P4-P1R) via a BP reaction. Similarly, the coding region of *TcCPSII* (+1000 to +2000 bp) was amplified using a primer set (sense, 5'-GGGGACAGCTTCTTGTAACAAGTGGTTCTCTGTACAGTTTCATCCG-3' and antisense, 5'-GGGGACAACCTTTGTATAATAAAGTTGGAAATTCAGACGTGCATTAACCTCAATT-3') and cloned into pDONR P2R-P3. The *neo^R* gene was amplified using a primer set (sense, 5'-GGGGACAAGTTTGTACAAAAAGCAGGCTATGATTGAACAAGATGGATT-3' and antisense, 5'-GGGGACCACTTTGTACAAGAAAGCTGGTTCAGAAGA-ACTGGTCAAGAA-3') and pTREX DNA as the template and cloned into entry vector pDONR 221. In order to generate the final plasmid, the three recombinant plasmids were subsequently transferred to a destination vector, pDEST R4-R3, according to the manufacturer's instructions. Finally, the knockout DNA cassette was excised from the plasmid backbone with *EcoRI* and used for electroporation.

To construct the pDEST/*TcCPSII*_Hyg^R plasmid, a 0.41-kb 5' flanking sequence from *TcCPSII* was amplified using *T. cruzi* Tulahuen genomic DNA, a primer set (sense, 5'-GGGGACAAC-TTGTATAGAAAAGTTGGAGCTGGCGTCTTCCTTTTTTCTCCTTTTCTTTT-3'; the *PvuII* site for digestion is underlined and antisense, 5'-GGGGACTGCTTTTTGTACAACTTGTTTACTTTTTTATGTTTTGTGTACTG-3') and KOD-Plus-Neo (TOYOBO) and cloned into the entry vector (pDONR P4-P1R) via a BP reaction. Similarly, a region of the *TcCPSII* ORF sequence (+1000 to +2000) was amplified from *T. cruzi* Tulahuen genomic DNA using a primer pair (sense, 5'-GGGGACAGCTTCTTGTAACAAGTGGTTCTCTGTACAGTTTCATCCG-3' and antisense, 5'-GGGGACAACCTTTGTATAATAAAGTTGGAGCTGAGACGTGCATTAACCTCAATT-3'; the *PvuII* site for digestion is underlined) and cloned into pDONR P2R-P3. Using the pTEX-derived plasmid, p72hyg72 [15,16], as a template, the *hyg^R* gene was amplified with a primer set (sense, 5'-GGGGACAAGTTTGTACAAAAAGCAGGCTATGAAAAAGCTGAACCTACC-3' and antisense, 5'-GGGGACCACTTTGTACAAGAAAGCTGGTTCCTTGCCTCGGACGAGT-3') and cloned into entry vector pDONR 221. The three entry clones were subsequently mixed with a destination vector (pDEST R4-R3) to generate the final plasmid by an LR reaction. The knockout DNA cassette was released from the plasmid backbone by *PvuII* digestion.

2.4. Transformation of *T. cruzi* using knockout DNA cassettes

About 1×10^7 early log-phase epimastigotes were suspended in 100 μ l of Amaxa Basic® Parasite Nucleofector Kit 2 solution (Lonza). Transformation of the parasites was carried out using 10 μ g of the knockout DNA cassette and the "U-033" program of an Amaxa Nucleofector Device (Lonza). Stable transformants were selected by incubating cells for 30–45 days in LIT medium containing 0.25 mg/ml G418 (for single-gene knockout [SKO] with *neo^R*) or 0.25 mg/ml G418 plus 0.25 mg/ml hygromycin B with 0.2 mM uracil (for double-gene knockout [DKO] with *neo^R* and *hyg^R*) and cloned by limiting dilution. Integration of the knockout cassette into the precise locus of the *TcCPSII* gene was confirmed by PCR using DNA cassette-specific sense primers (5'-ATCGCCTTGTGACGAGTTCT-3' for *neo^R* and 5'-ACTCGTCCGAGGGCAAAGGAA-3' for *hyg^R*) and the *TcCPSII*-specific antisense primer (5'-CATTGTTGTTCTTGGTGACCCC-3'; +2101 to +2122 bp).

2.5. Real-time PCR

Total RNA was isolated from wildtype (WT), *CPSII* SKO, and *CPSII* DKO epimastigotes using Agilent Total RNA Isolation mini kits (Agilent Technologies, Santa Clara, CA). Subsequently, cDNA was prepared by reverse transcription using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Real-time PCR was performed using an Applied Biosystems 7500 Real-Time PCR System (Applied Bio Systems, Foster City, CA) and the following primers (*TcCPSII*-specific sense, 5'-TGGCCTTTTATTCCAACG-3' and antisense, 5'-CGATGGCCGTACTTCATCTT-3'; *T. cruzi* beta tubulin-specific sense, 5'-TTTGTCCGCAACAACACCTG-3' and antisense, 5'-CTAGTACTGCTCCTCCTCGT-3').

3. Results and discussion

3.1. Construction of *TcCPSII*-knockout *T. cruzi*

T. cruzi is a diploid organism [17]. We previously demonstrated that the *TcCPSII* gene is a single-copy gene in *T. cruzi* Tulahuen and occurs at two gene loci per cell [6]. Thus, we aimed to generate *TcCPSII*-knockout parasites in order to investigate the physiological relevance of the *de novo* pyrimidine biosynthesis pathway in *T. cruzi*.

We constructed two gene knockout cassettes comprising the 5' flanking region of *TcCPSII*, the neomycin phosphotransferase II gene (*neo^R*) or hygromycin B phosphotransferase gene (*hyg^R*), and the coding region of the *TcCPSII* gene (+1000 to +2000 bp) to allow disruption of the *TcCPSII* loci by homologous recombination (Fig. 1A). Integration of the knockout cassettes was confirmed by PCR using a sense primer specific for the resistance marker gene and an antisense primer specific for a region outside the 3' cassette (Fig. 1B and C). For cloning of the DKO parasites, the culture medium was supplemented with uracil in order to complement the defect due to loss of the *CPSII* gene.

We analyzed the expression levels of *TcCPSII* transcripts in WT, SKO, and DKO parasites by real-time RT-PCR (Fig. 1D). A reduction in expression levels of approximately 50% was observed for the SKO parasites, which was directly proportional to the copy number of the functional *TcCPSII* gene. Moreover, transcription of *TcCPSII* in DKO parasites was at trace levels, indicating that DKO parasites are *TcCPSII*-null mutants.

3.2. Physiological importance of *TcCPSII* for the growth of *T. cruzi* epimastigotes

We investigated the physiological importance of *de novo* pyrimidine biosynthesis in epimastigotes by comparing the growth of

WT, SKO, and DKO epimastigotes in LIT medium supplemented with 10% FBS. The parasite growth curves for 9 days of cultivation are shown in Fig. 2. While the growth rates were almost identical for the first 6 days of cultivation, significant suppression of the growth of both SKO and DKO parasites was observed after 9 days of cultivation. Notably, the growth of the DKO parasites was more severely impaired than the growth of SKO parasites.

The addition of uracil (final concentration of 500 μM) rescued the growth defect of DKO epimastigotes to a level comparable to that of SKO parasites between days 7 and 9 (Fig. 2). Therefore, since the LIT medium appeared to contain a sufficient amount of pyrimidine precursors to support the growth of *CPSII*-knockout parasites during the early stages of cultivation, these results suggest that epimastigotes preferentially consume pyrimidine precursors in the medium during the first 6 days of cultivation and subsequently depend on *de novo* pyrimidine biosynthesis due to the absence of precursors after 7 days of cultivation.

To further confirm that the growth defect of DKO parasites was due to the lack of *CPSII* activity, we attempted to rescue parasites by overexpression of *TcCPSII*. The DKO and WT epimastigotes were transformed using a trypanosomal expression plasmid, pTREC [18], which carries the *TcCPSII* gene. However, we were unable to obtain transgenic parasites neither for the DKO nor for the WT background, suggesting that overexpression of *TcCPSII* is highly toxic for the parasites. Furthermore, we found that a high concentration of uracil (>500 μM) impaired the growth of parasites (data not shown). Therefore, as previously reported [3], both *de novo* pyrimidine biosynthesis and salvage pathways were shown to be physiologically active in epimastigotes. Moreover, we found that *CPSII* is required for parasite growth under culture conditions of insufficient salvage substrates.

During the life cycle of *T. cruzi*, epimastigotes transform into metacyclic trypomastigotes, which represent a transmission stage from the insect vector to mammals. Metacyclogenesis is also inducible in conditioned medium lacking pyrimidine precursors [9]. Thus, we examined whether a pyrimidine supply is required for transformation of *CPSII*-SKO and DKO parasites. We compared the efficacy of metacyclogenesis of SKO, DKO, and WT parasites and found no significant difference between the groups (data not shown). Therefore, these results suggest that nucleotide synthesis is not critical for the process of metacyclogenesis.

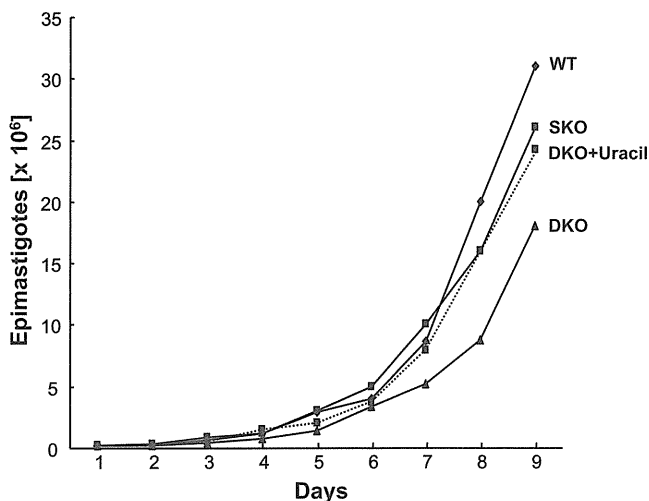


Fig. 2. Physiological role of *CPSII* in the growth of *T. cruzi* epimastigotes. A total of 1×10^6 WT, SKO, or DKO epimastigotes were grown in liver infusion tryptose (LIT) medium, and the average number of parasites from three independent experiments is shown. DKO + uracil represents DKO parasites grown in the presence of 500 μM uracil.

3.3. Physiological importance of *TcCPSII* for *T. cruzi* amastigote growth in the host cell

In general, pyrimidine *de novo* biosynthesis is particularly important in rapidly growing cells [7]. Since *T. cruzi* amastigotes are only proliferative in mammalian hosts, we examined whether *CPSII* and the *de novo* pyrimidine biosynthesis pathway are required for the growth of amastigotes.

We infected 3T3-Swiss fibroblast cells with metacyclic trypomastigotes from WT, SKO, or DKO parasites at an MOI of 0.26, and the average number of intracellular amastigotes per infected cell was compared between the groups. Multiple infections of a single host cell by metacyclic trypomastigotes were negligible under the experimental conditions employed (data not shown). The amastigote counts were significantly reduced for SKO and DKO parasites in a dose-dependent manner (Fig. 3), suggesting that *de novo* pyrimidine biosynthesis is more important for amastigotes than epimastigotes.

Extracellular amastigotes incorporate pyrimidine bases and nucleosides to a significantly lesser extent compared with epimastigotes and trypomastigotes [3]. In addition, the average concentrations of uridine in mammalian tissues and plasma are estimated to be of the micromolar order [4,19]. Therefore, amastigotes are likely to rely on *de novo* pyrimidine biosynthesis. We observed that DKO amastigotes appeared to replicate approximately two times after 4 days of cultivation. These results indicate that the concentrations of intracellular pyrimidine precursors are insufficient to support amastigote replication, resulting in the dependence on *de novo* pyrimidine biosynthesis. Moreover, our observations are in good agreement with a study on another intracellular parasite, *Toxoplasma gondii*, which also has limited pyrimidine salvage ability [20]. Indeed, the *CPSII*-null mutant of this apicomplexan parasite was only able to replicate inside the host cell in culture medium supplemented with >200 μM uracil as a pyrimidine source.

We have previously shown that disruption of 2 of the 3 loci for the dihydroorotate dehydrogenase (*DHOD*) gene, encoding the fourth enzyme of the *de novo* pyrimidine biosynthesis pathway, led to the gradual death of *T. cruzi* epimastigotes, which were no longer viable 2 weeks later [16]. Furthermore, the addition of uridine, cytidine, and thymidine (200 μM each) to the medium did not enable growth of *DHOD* DKO epimastigotes. While these

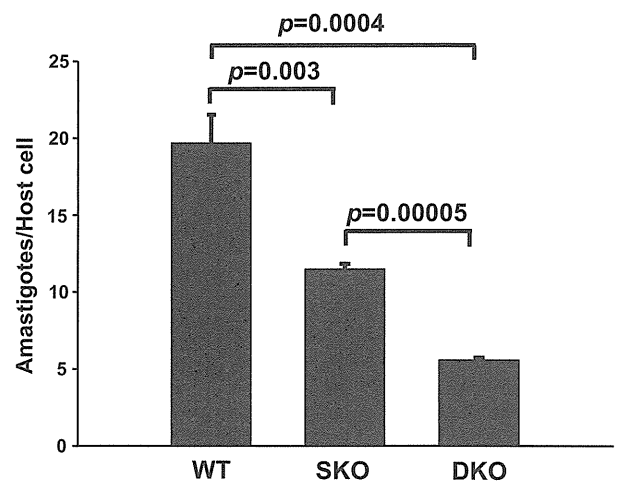


Fig. 3. Physiological role of *CPSII* in the growth of *T. cruzi* amastigotes. A total of 1.5×10^5 3T3-Swiss Albino cells were infected with 4×10^4 WT, SKO, or DKO metacyclic trypomastigotes (MOI, 0.26) and cultured for 4 days. The average number of parasites per infected cell is shown, and data represent the mean \pm SD of three independent experiments.

findings differ from the phenotype of *CPSII* DKO epimastigotes, the phenotypic difference between *CPSII* and *DHOD* KO parasites may be attributed to the coupled reaction of *DHOD*. In *T. cruzi*, catalysis of the oxidation of dihydroorotate by *DHOD* is coupled with the reduction of fumarate to succinate. In addition, this reaction is reversible. Therefore, it is likely that the fumarate-reductase activity of *DHOD*—as well as succinate production by its reverse reaction—contributes to maintenance of the redox balance in epimastigotes. Moreover, *T. cruzi* *DHOD* may use not only dihydroorotate but also other unidentified substrates as electron donors/acceptors for succinate/fumarate metabolism.

Thus, we conclude that the *de novo* pyrimidine biosynthesis pathway is critical for intracellular replication of *T. cruzi*. Our findings provide insight into *CPSII* and the pyrimidine metabolic pathway, which represents a promising target for chemotherapy. The screening of chemical compound libraries by using recombinant enzymes and bioassay systems may facilitate the identification of seed compounds for drug development against Chagas disease.

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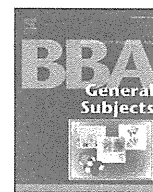
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Review

Mitochondrial fumarate reductase as a target of chemotherapy: From parasites to cancer cells[☆]Chika Sakai^a, Eriko Tomitsuka^{a,b}, Hiroyasu Esumi^b, Shigeharu Harada^c, Kiyoshi Kita^{a,*}^a Department of Biomedical Chemistry, Graduate School of Medicine, The University of Tokyo, Tokyo 113-0033, Japan^b Cancer Physiology Project, Investigative Treatment Division, National Cancer Center Research Institute East, 6-5-1 Kashiwanoha, Kashiwa, Chiba 277-8577, Japan^c Department of Applied Biology, Graduate School of Science and Technology, Kyoto Institute of Technology, Kyoto 606-8585, Japan

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ABSTRACT

Recent research on respiratory chain of the parasitic helminth, *Ascaris suum* has shown that the mitochondrial NADH-fumarate reductase system (fumarate respiration), which is composed of complex I (NADH-rhodoquinone reductase), rhodoquinone and complex II (rhodoquinol-fumarate reductase) plays an important role in the anaerobic energy metabolism of adult parasites inhabiting hosts. The enzymes in these parasite-specific pathways are potential target for chemotherapy. We isolated a novel compound, nafuredin, from *Aspergillus niger*, which inhibits NADH-fumarate reductase in helminth mitochondria at nM order. It competes for the quinone-binding site in complex I and shows high selective toxicity to the helminth enzyme. Moreover, nafuredin exerts anthelmintic activity against *Haemonchus contortus* in *in vivo* trials with sheep indicating that mitochondrial complex I is a promising target for chemotherapy. In addition to complex I, complex II is a good target because its catalytic direction is reverse of succinate-ubiquinone reductase in the host complex II. Furthermore, we found atpenin and flutolanil strongly and specifically inhibit mitochondrial complex II.

Interestingly, fumarate respiration was found not only in the parasites but also in some types of human cancer cells. Analysis of the mitochondria from the cancer cells identified an anthelmintic as a specific inhibitor of the fumarate respiration. Role of isoforms of human complex II in the hypoxic condition of cancer cells and fetal tissues is a challenge. This article is part of a Special Issue entitled Biochemistry of Mitochondria, Life and Intervention 2010.

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1. Introduction

In the general understanding of bioenergetics of higher eukaryotes, oxygen is a most important terminal electron acceptor of mitochondrial respiratory chain (Fig. 1). The major function of the aerobic respiratory chain is the electrogenic translocation of protons out of the mitochondrial or bacterial membrane to generate the proton motive force that drives ATP synthesis by F₀F₁-ATPase. This mechanism of oxidative phosphorylation is conserved basically from aerobic bacteria to human mitochondria. However, recent study on the respiratory chain of the lower eukaryotes which reside

micro-aerophilic environment has shown that the mitochondrial NADH-fumarate reductase system (fumarate respiration) plays an important role in the anaerobic energy metabolism [1]. This system is composed of complex I (NADH-quinone reductase), low potential quinone species and complex II (quinol-fumarate reductase: QFR).

Fumarate respiration is well known electron transport chain in the anaerobic bacteria [2]. Reducing equivalent of NADH is transferred to low potential quinone such as naphthoquinone by complex I and finally is oxidized by fumarate by the fumarate reductase activity of complex II which is a reverse reaction of succinate-ubiquinone reductase (SQR) activity of complex II. By using this respiratory chain, bacteria are able to synthesize ATP even in the absence of oxygen. Recently our study of parasitic nematode, *Ascaris suum*, showed fumarate respiration also plays an important role in the anaerobic energy metabolism of adult worms, which reside in the host small intestine where oxygen tension is low [1]. Although fumarate reductase activities of bacterial and mitochondrial complex IIs are the same reaction, evolutionary positions of each enzyme are quite different. All four subunits of complex II in adult *A. suum* are more closely related to the bacterial and mitochondrial SQR than to bacterial QFR [3–5].

Abbreviations: FRD, fumarate reductase; L3, 3rd stage larvae; LL3, lung stage L3; MK, menaquinone; SDH, succinate dehydrogenase; SQR, succinate-ubiquinone reductase; TCA cycle, tricarboxylic acid cycle; QFR, quinol-fumarate reductase; RQ, rhodoquinone

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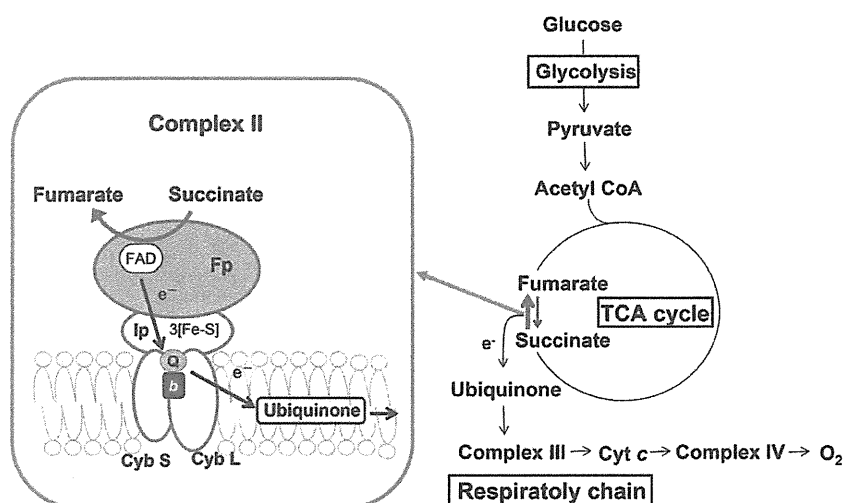


Fig. 1. Complex II is the member of TCA cycle and respiratory chain. Complex II catalyzes the oxidation of succinate to fumarate in the TCA cycle and transports the electron generated by this oxidation to ubiquinone in the respiratory chain. Generally, complex II consists of four subunits. Flavoprotein (Fp) subunit contains a flavin adenine dinucleotide prosthetic group and iron-sulfur protein (Ip) subunit contains three iron-sulfur clusters. There are two hydrophobic cytochromes (Cyb L, Cyb S) subunits. The succinate binding site is located in Fp subunit, while the quinone binding site is formed by three subunits, Ip, Cyb L and Cyb S. Complex II also catalyzes the reduction of fumarate, a reverse-reaction of succinate dehydrogenase, in the respiratory chain of mitochondria from anaerobic animals, such as *Ascaris suum*, as well as anaerobic bacteria.

Thus, mitochondrial QFR is a new enzyme evolved by “reverse evolution” of SQR rather than direct evolution from bacterial QFR [6].

Recently our study has revealed that fumarate respiration functions in some human cancer cells and supports a survival of cancer cells in low nutrition and low oxygen conditions [7,8]. Furthermore, we found complex II with high QFR activity produces reactive oxygen species (ROS) [9]. ROS has been reported to contribute to proliferation and metastasis of cancer cells via the stabilization of hypoxia-inducible factor-1 (HIF-1) [10]. In addition, succinate produced by fumarate respiration also stabilize HIF-1 by the product inhibition of HIF prolyl hydroxylase, which catalyzes the oxygen-dependent hydroxylation of the conserved proline residues in HIF-1 α [11]. Thus, the relationship between accumulation of succinate resulted from functional defect of human complex II by the mutation of the subunits and carcinogenesis has recently become a focus of research [8].

As fumarate respiration is essential for the growth and survival of the parasites and some cancer cells, it should be a promising target of chemotherapy for both parasitic diseases and cancer. In this review, we focus on recent advances in the study of parasite and human mitochondrial fumarate respiration and complex II which is an important component of the system [8].

2. Fumarate respiration of parasite mitochondria

2.1. Life cycle of *A. suum* and changes in respiratory chain

A. suum is the most widely known parasite, and has been studied as a representative of human and livestock parasites [12–14]. Because of its large size, *A. suum* is ideal for the study including biochemical analysis. Adult *A. suum* resides in the small intestine of mammals, and the female produces between 200,000 and 400,000 fertilized eggs per day (Fig. 2). Eggs are excreted with feces and become mature eggs containing infectious 3rd stage larvae (L3) in about 2–3 weeks at normal temperature. The eggs reach the small intestine and hatch, when orally ingested by a host. A hatched larva invades the intestinal wall, and migrates to the liver, lung, trachea, and pharyngeal region, and finally returns to the intestine via the esophagus and stomach, and becomes an adult worm. In humans, the larvae of *A. suum* migrate to several organs including liver and lung and cause a wide variety of nonspecific symptoms such as general malaise, cough, liver

dysfunction, hypereosinophilia with hepatomegaly and/or pneumonia. The oxygen concentration of the small intestine (~5%) is approximately 25% of that outside the body, and provides an environment of low oxygen tension in which the energy metabolism of the adult differs considerably from that of the larvae and the host (Fig. 3). The phosphoenolpyruvate carboxykinase (PEPCK)–succinate pathway, an anaerobic glycolytic pathway, operates in the adult worm, producing ATP under such a hypoxic conditions. This system is used by many other parasites such as *Echinococcus multilocularis* [15], and has also been observed in the adductor muscle of oysters and other bivalves that require energy conversion under anaerobic conditions. It is therefore considered to be a very common pathway for energy metabolism in adaptation to hypoxic environment [16,17].

The first half of the PEPCK–succinate pathway is the same glycolytic pathway found in mammals, in which phosphoenolpyruvate (PEP) is produced. In contrast to aerobic metabolism in mammals involving the conversion of PEP to pyruvate by pyruvate kinase, the *A. suum* adult fixes CO₂ with PEPCK to produce oxaloacetate (OAA). The OAA is converted to malate by the reverse reaction of malate dehydrogenase and transported into the mitochondria to produce pyruvate and fumarate. The NADH formed during production of pyruvate from malate is used in the reduction of fumarate to succinate. The NADH-fumarate reductase system, which is the anaerobic electron transport system characteristic of adult *A. suum* mitochondria, is the final step of this pathway. Unique property of this pathway is discussed in the next section.

In contrast to larvae which require oxygen for their development and possess the respiratory system to be almost the same as that of mammals, cytochrome c oxidase (complex IV) is not found in the respiratory chain of adult *A. suum* mitochondria, and the content of ubiquinol–cytochrome c reductase complex (complex III) is extremely low [18]. In addition to the enzymes, quinone species in the mitochondria also change during the life cycle of *A. suum*. In contrast to adult mitochondria, in which the low-potential rhodoquinone (RQ; $E_m' = -63\text{mV}$) is the major quinone, ubiquinone (UQ; $E_m' = +110\text{mV}$) is the major quinone of larvae (Fig. 4A) [19]. A combination of SQR and UQ, and that of QFR and a low-potential quinone, such as RQ or menaquinone (MK), is also observed in *Escherichia coli* and other bacteria during metabolic adaptation to changes in

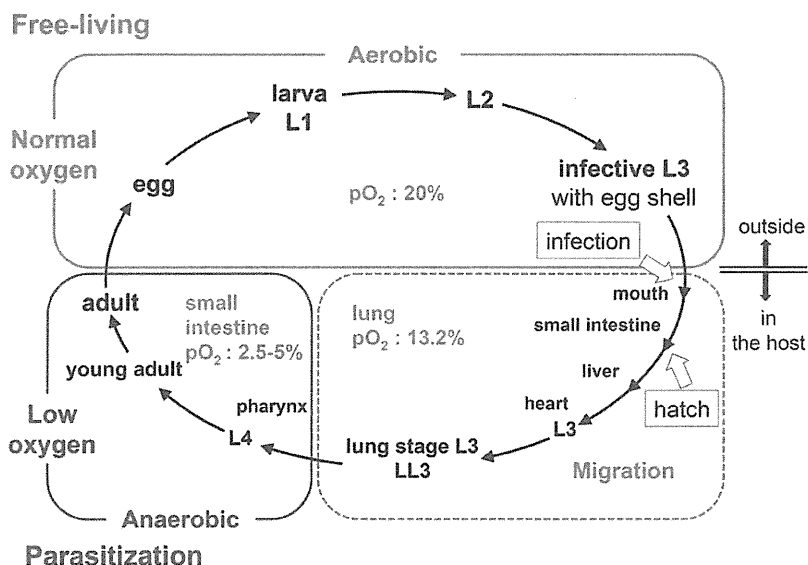


Fig. 2. Life cycle of *Ascaris suum*. Fertilized eggs grow to be infective L3 under aerobic environment. Infective L3 larvae are ingested by the host, reach the small intestine and hatch there. Afterwards, larvae migrate into host body (liver, heart, lung, pharynx), and finally migrate back to the small intestine and become adults. In the host small intestine, the oxygen concentration is low ($pO_2 = 2.5-5\%$) compared with the exogenous environment ($pO_2 = 20\%$). The metabolic pathway of *A. suum* changes dramatically during its life cycle, to adapt to changes in the environmental oxygen concentration [6].

oxygen supply [20,21]. Lower potential of RQ and MK is favorable for the electron transfer from NADH to fumarate (Fig. 4B). In this way, UQ participates in aerobic metabolism in *A. suum* larva, whereas RQ participates in anaerobic metabolism in adult *A. suum*.

Although studies have shown a clear difference in energy metabolism between larval and adult *A. suum* mitochondria, little is known about changes in the properties of mitochondria during migration of *A. suum* larvae in the host. As described later, examination of the changes in enzymatic characteristics and subunit composition of *A. suum* larval complex II from lung stage L3 (LL3) larvae obtained from rabbits showed that properties of LL3 mitochondria differed from those of L3 and adult mitochondria [22]. Protein chemical

analysis revealed that the change in complex II begins with the anchor subunit, and then occurs in the catalytic subunit. Thus, *A. suum* is able to adapt to changes in oxygen concentration in the environment during its life cycle by dynamic change of respiratory chain.

2.2. NADH-fumarate reductase system (fumarate respiration) of *A. suum* adult

The final step of the PEPCK–succinate pathway, which plays such an important role in the anaerobic energy metabolism of the *A. suum* adult, is catalyzed by the NADH-fumarate reductase system as

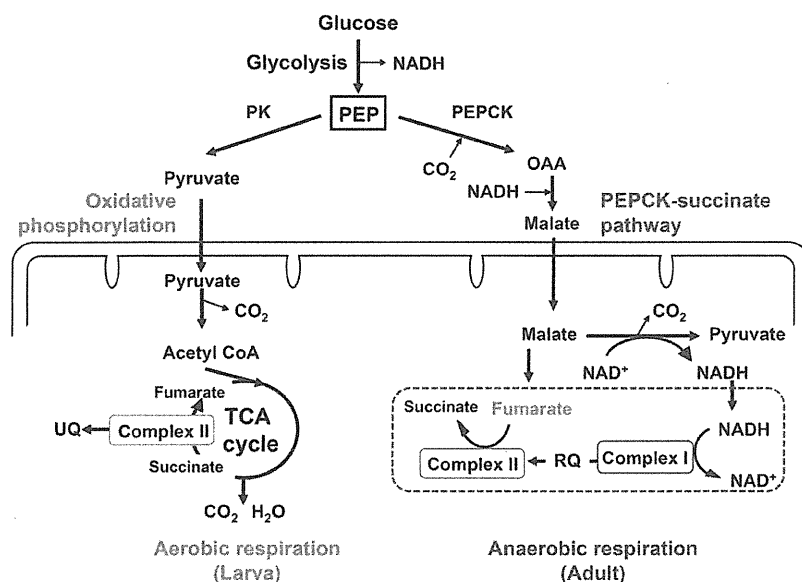


Fig. 3. Glucose metabolism of *A. suum* larval and adult mitochondria. The metabolic pathway of *A. suum* adult has a unique anaerobic electron transport system, NADH-fumarate reductase system. In the phosphoenolpyruvate carboxykinase (PEPCK)–succinate pathway, phosphoenolpyruvate (PEP) produced by a glycolytic process is carboxylated to form oxaloacetate and is then reduced to malate. The cytosolic malate is transported into the mitochondria, where it is first reduced to fumarate, and finally to succinate by the rhoquinol–fumarate reductase activity of complex II. The terminal step is catalyzed by the NADH-fumarate reductase system (boxed in broken lines) comprised of complex I, rhoquinone (RQ), and complex II. PEP, phosphoenolpyruvate; PEPCK, phosphoenolpyruvate carboxykinase; OAA, oxaloacetate [6].

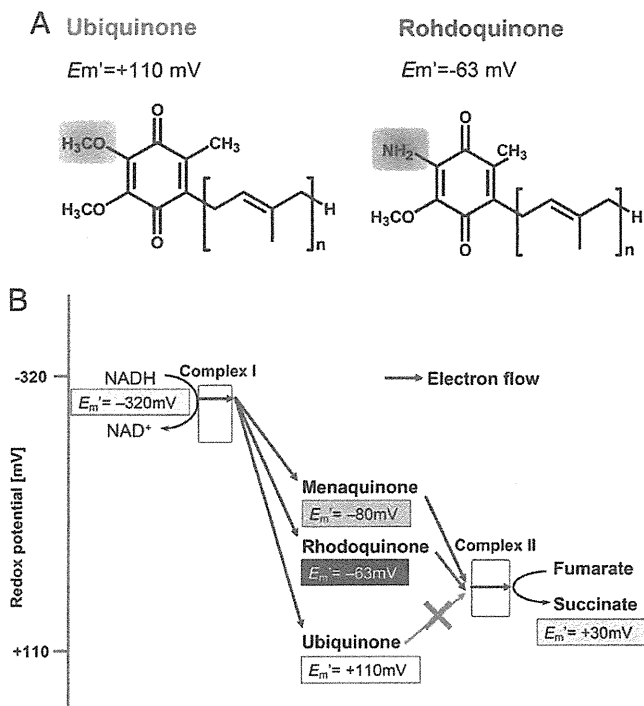


Fig. 4. Chemical structure and redox potentials of the quinones. A. Chemical structures of UQ and RQ. n, numbers of isoprenyl groups in side-chain. B. Redox potentials of quinones and substrates.

described in the previous section. This system is also called “fumarate respiration”. The low-potential rhodoquinone transfers reducing equivalent of NADH via complex I to complex II, and finally succinate is produced by quinol fumarate reductase (QFR) activity of complex II. The merit of this system is to synthesize ATP using the coupling site of complex I even in the absence of oxygen, although its energy efficiency is low (Fig. 5).

A similar anaerobic respiration system exists in the mitochondria of many other parasites, and has also been found in bacteria. Extensive studies of bacteria, including *E. coli*, have revealed the details of this system [23,24]. In *E. coli*, there are two types of complex II, and QFR encoded by the *frd* operon is induced under anaerobic conditions. A low molecular weight mediator between complex I and complex II is menaquinone (MK; $E_m' = -80$ mV), a low-potential naphthoquinone, in the *E. coli* fumarate respiration. In contrast, under aerobic conditions, SQR encoded by *sdh* operon that catalyzes oxidation of succinate is induced [25]. SQR is a dehydrogenase complex in the respiratory system as well as an enzyme in the TCA cycle, and directly connects these systems in aerobic energy metabolism.

Thus, two different enzymes (complex II) are present in *E. coli*, and the bacteria maintain homeostasis of the energy metabolism by controlling the synthesis of these enzymes in response to the environmental oxygen supply. How about the complex IIs of *A. suum*? Biochemical and molecular biological analyses showed *A. suum* also possesses two different complex IIs. However, subunit compositions and expression patterns are more complicated in the parasite complex II.

3. Complex IIs of *A. suum* mitochondria

3.1. Multiple complexes II in *A. suum* mitochondria

The complex II superfamily comprises succinate–quinone reductase (SQR) and quinol–fumarate reductase (QFR), which catalyze the

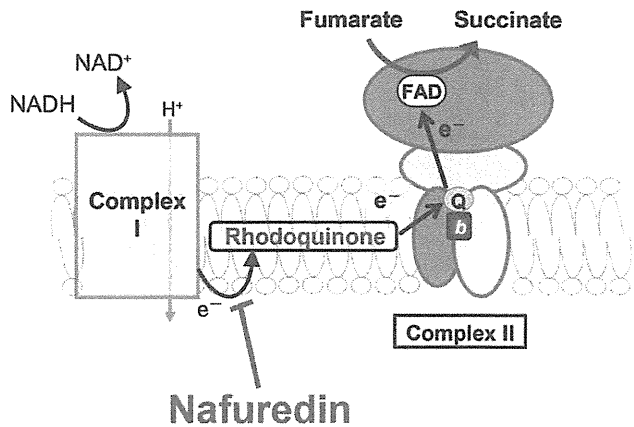


Fig. 5. NADH-fumarate reductase System of *A. suum* as a target of chemotherapy. The differences in energy metabolisms between host and helminths are an attractive therapeutic targets for helminthiasis. NADH-fumarate reductase is a part of a unique respiratory system in parasitic helminths and is the terminal step of the phosphoenolpyruvate carboxykinase–succinate pathway, which is found in many anaerobic organisms. NADH-fumarate reductase system is a potential target for chemotherapy. Nafuredin was found to be competitive inhibitor for rhodoquinone binding site of *A. suum* complex II [1].

interconversion of succinate and fumarate with quinone and quinol. SQR is a component of the aerobic respiratory chain as well as the tricarboxylic acid (TCA) cycle [26]. QFR is a component of the anaerobic respiratory chain in anaerobic and facultative anaerobic bacteria [27] and lower eukaryotes [6,28]. SQR and QFR complexes generally consist of four subunits referred to as the flavoprotein subunit (Fp), iron–sulfur subunit (Ip), cytochrome *b* large subunit (CybL), and cytochrome *b* small subunit (CybS). The Fp and Ip subunits comprise the catalytic domain of the enzyme. The Fp subunit has a FAD as a prosthetic group and contains the dicarboxylate-binding site. The Ip subunit generally contains three iron–sulfur clusters $[2Fe-2S]^{2+,1+}$, $[4Fe-4S]^{2+,1+}$, and $[3Fe-4S]^{1+,0}$. Subunits CybL and CybS, with heme *b* as the prosthetic group, form the anchor domain of the enzyme. This anchors the catalytic domain to the inner mitochondrial membrane and also serves as the quinone oxidation/reduction site [29].

Our previous study showed that *A. suum* mitochondria express stage-specific isoforms of complex II (SQR in larvae/QFR in adult) (Fig. 6). The Fp and CybS in adult complex II differ from those of infective third stage larval (L3) complex II. In contrast, there is no difference in the iron–sulfur cluster (Ip) and CybL between adult and L3 isoforms of complex II. However, recent analysis of the changes that occur in the respiratory chain of *A. suum* larvae during their migration in the host, we found that enzymatic activity, quinone content and complex II subunit composition in mitochondria of lung stage L3 (LL3) *A. suum* larvae is different from those of L3 and adult [22]. Quantitative analysis of quinone content in LL3 mitochondria showed that ubiquinone is more abundant than rhodoquinone. Interestingly, the results of two-dimensional blue-native/sodium dodecyl sulfate polyacrylamide gel electrophoresis analyses showed that LL3 mitochondria contained larval Fp (Fp^L) and adult Fp (Fp^A) at a ratio of 1:0.56, and that most LL3 CybS subunits were of the adult form (CybS^A). This result clearly indicates that the rearrangement of complex II begins with a change in the isoform of the anchor CybS subunit, followed by a similar change in the Fp subunit. At any event, the NADH-fumarate reductase activity of *A. suum* adult worms (~100 nmol/min/mg) are much higher than that of the mammalian host (2–5 nmol/min/mg).

3.2. ROS production from complex II

Mitochondrial respiratory chain is a significant source of cellular ROS. Impairment of the respiratory chain complexes is known to

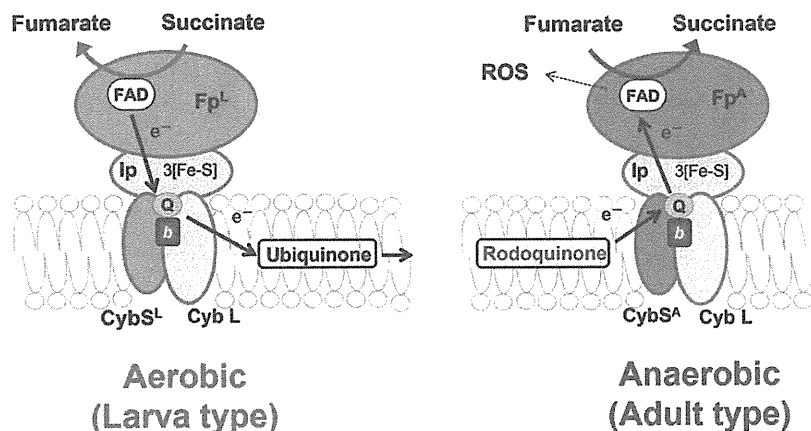


Fig. 6. Schematic representation of *A. suum* complexes II from larva type and adult type. The mitochondrial metabolic pathway of the parasitic nematode *A. suum* changes dramatically during its life cycle, to adapt to changes in the environmental oxygen concentration. *A. suum* mitochondria express stage-specific isoforms of complex II. While there is no difference in the isoforms of the Ip and cybL subunits of complex II between L3 larvae and adult *A. suum*, they have different isoforms of complex II subunits Fp (larval, Fp^L; adult, Fp^A) and cybS (larval, cybS^L; adult, cybS^A) in *A. suum* adult respiratory chain, complex II produces high amount ROS [29].

increase the cellular ROS production [30]. In general, complexes I and III are considered as the two major sites of superoxide and hydrogen peroxide production in the respiratory chain [30–33]. Interestingly, our results show that complex II is the main site of ROS production in *A. suum* adult respiratory chain [9].

Analysis of submitochondrial particles for superoxide (O₂⁻) production using superoxide dismutase inhibitable acetylated cytochrome *c* reduction, and hydrogen peroxide production using catalase inhibitable amplex red oxidation, in the presence and absence of respiratory chain inhibitors, showed the contribution from both the FAD site and quinone-binding site of complex II to produce O₂⁻ and H₂O₂ when succinate is oxidized under aerobic conditions. Considering the conservation of amino acid residues critical for the enzyme reaction between *A. suum* complex II and mitochondrial SQR, our results show the ROS production from more than one site in mitochondrial complex II linked with subtle differences in the amino acid sequences of the enzyme complex.

A. suum adult complex II is a good model to study the mechanism of ROS production from mitochondrial complex II, since amino acid residues conserved among the catalytic domains in mitochondrial SQR enzymes are well conserved in this enzyme and it produces high levels of ROS. Absence of complex III and IV activities in its respiratory chain is an additional advantage of this model. These studies will provide further insight into the possibility of high levels of ROS production from both the FAD site and the Q site in the complex II of *A. suum* adult worm and help to understand the role of mutations in human complex II for carcinogenesis.

3.3. Specific inhibitors of complex II

The differences between parasite and host mitochondria described in this review hold great promise as targets for chemotherapy. For example, the anti-malarial drug Atovaquone, which was recently developed, acts on the mitochondrial respiratory chain [34]. Atovaquone is effective against chloroquine-resistant strains, [35]. The specific target is thought to be complex III, and biochemical analysis has shown that it acts on the ubiquinone oxidation site in the cytochrome *b* of complex III [36,37]. Such a chemotherapeutic approach is also applicable to the helminthes. It has been proposed that the fumarate respiration is the target of such drugs as bithionol and thiabendazole [38,39], but there is no clear biochemical or pharmaceutical evidence to support this idea. However, as described in the previous section, progress in the study of the NADH-fumarate reductase pathway permits screening of new anthelmintic compound.

Nafuredin, selectively inhibits helminth complex I at concentrations in the order of nanomoles [40] (Fig. 7). Kinetic analysis revealed that the inhibition by nafuredin is competitive against RQ (Fig. 5). These findings, coupled with the fact that helminth complex I uses both RQ and UQ as an electron acceptor, suggest that the structural features of the quinone reduction site of helminth complex I may differ from that of mammalian complex I. In fact, the inhibitory mechanism of quinazolines, which effectively kill the *E. multilocularis* protoscoleces, was competitive and partially competitive against RQ and UQ, respectively [41].

The most potent inhibitor of complex II, Atpenin A5, was found during the screening of inhibitors for *A. suum* complex II [42]. To our regret, IC₅₀ of Atpenin A5 for bovine complex II (3.6 nM) was lower than that for *A. suum* complex II (12 nM for QFR and 32 nM for SQR). However, further screening of inhibitors showed that flutolanil, a commercially available fungicide, specifically inhibits *A. suum* SQR [43] (Fig. 7). The IC₅₀ of flutolanil against *A. suum* and bovine SQR was 0.081 and 16 μM, respectively, indicating that flutolanil is a promising lead compound for anthelmintics. To enable rational drug optimization, a crystal of the *A. suum* QFR complexed with

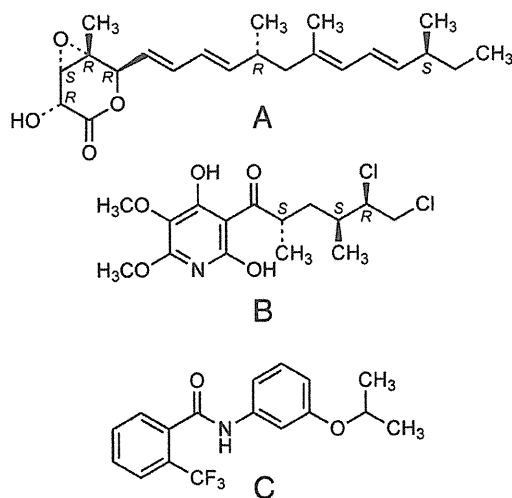


Fig. 7. Chemical structure of inhibitors of complex II. A. Nafuresin, a competitive inhibitor for the rodoquinone binding site of *A. suum* complex II; B. Atpenin A5, a competitive inhibitor for the quinone binding site of complex II of many species; C. Flutolanil, a competitive inhibitor for the quinone binding site of *A. suum* complex II.

flutolanil was prepared by soaking, and X-ray structure analysis has been performed. The current structural model of the flutolanil bound form of the *A. suum* QFR (Harada, unpublished observation) indicates that flutolanil is bound to the same site as those of the quinone binding observed in complex IIs from pig heart mitochondria (pdb code 1ZOY), *E. coli* (1NEK and 1LOV) and avian (1YQ4). The site of the pig enzyme, for example, is composed of ten residues highly conserved across amino acid sequences of these complex IIs; Pro169, Trp173 and Ile218 from the Ip subunit, Ile30, Trp35, Met39, Ser42, Ile43 and Arg46 from the CybL subunit, and Tyr91 from the CybS subunit. However, three residues, Trp35, Met39 and Ile53, are replaced by Pro65, Trp69 and Gly73, respectively, in *A. suum* QFR. The structures of the *A. suum* QFR together with those of QFRs from *Wolinella succinogenes* [24] and *E. coli* [23], and SQRs from *E. coli* [44], pig heart mitochondria [45], and avian heart mitochondria [46] should help clarify the structure–function relationship of complex II and provide useful information for the structure-based design of anthelmintics.

4. Fumarate respiration of human mitochondria

4.1. Human complex II

In human, many cases of diseases caused by mutations in subunits of complex II have been reported. Mutations found in the Ip, Cyb L or Cyb S are associated with the development of pheochromocytoma and paraganglioma [47–51]. It is suggested that the causes of tumorigenesis are ROS production from mutated complex II [52,53] or accumulation of succinate as a result of SQR inhibition [11]. Accumulated succinate inhibits HIF-1 α prolyl hydroxylases in the cytosol, leading to stabilization and activation of HIF-1 α . Thus, succinate can increase expression of genes that facilitate angiogenesis, metastasis, and glycolysis, ultimately leading to tumor progression. On the other hand, no patient with mutation in Fp linked to tumorigenesis has been reported. There are two Fp isoforms in human, which will be discussed later, and this is probably the reason why mutations in Fp are not directly linked to tumorigenesis. Instead, mutations in Fp are linked to severe metabolic disorders resulting from decreased activity of the TCA cycle and impairment of oxidative phosphorylation, although these are rare. These autosome-recessive disorders are manifested as childhood encephalopathy, myopathy, adult optic atrophy, and Leigh syndrome [54–57]. Recently, two new proteins, SDHAF1 (succinate dehydrogenase complex assembly factor 1) and SDHAF2, were found to be the first assembly factors of complex II [53,58]. It was suggested that mutations found in SDHAF1 may result in the reduction of assembled complex II and cause infantile leukoencephalopathy [58]. SDHAF2 is suggested to be required for the

incorporation of the flavin adenine dinucleotide cofactor (flavination) of SDHA (succinate dehydrogenase complex, subunit A, flavoprotein), and it is also necessary for complex II assembly and function [53]. Furthermore, the mutation found in SDHAF2 has been suggested to link to familial paraganglioma [53]

4.2. Isoforms of human complex II

In 2003, we found two isoforms of human Fp, type I and type II [59,60] (Fig. 8). These isoforms differ from each other only in two amino acid residues. Tyr 586 and Val 614 of type I Fp are replaced by Phe 586 and Ile 614 in type II Fp, respectively. Tyr 586 and Val 614 are well conserved among mammals' Fps and type II Fp is found only in human complex II (Fig. 9). Type I Fp gene has an exon–intron structure, while the structure of type II Fp gene has not been determined. The type II Fp gene is not found in the NCBI database and the location has not been clarified yet while type I Fp gene is located on chromosome 5p15 [59,60].

Complex II with type I Fp has isoelectric point (*pI*) of 6–7, whereas complex II with type II Fp shows its *pI* of 5–6. To explain the difference of *pI* values, several reports suggested the phosphorylation of amino acid residues in Fp subunit [7,61]. One of these residues, Tyr 500, is located close to Tyr 586, which is replaced by Phe in type II Fp (Fig. 10). Since the Tyr 586 Phe substitution will certainly destroy a hydrogen bond between Tyr 586 O η and Glu 597 O δ (3.13Å), the local structure around Tyr 586 as well as Tyr 500 phosphorylation status may be different between Fps of types I and II.

The result of biochemical analysis of complex II with each isoform, complex II with each Fp was found to have almost the same SQR specific activities. However, type II Fp has lower optimal pH than type I Fp and at optimal pH of type II Fp, *K_m* value for succinate of type II Fp is lower than type I Fp (Sakai unpublished data). It may be possible that different phosphorylation statuses of complex II with each isoform cause biochemical differences.

4.3. Expression of human complex II containing type II Fp

Our previous study on the expression of isoforms showed that both types were expressed in all the organs tested (liver, heart, skeletal muscle, brain and kidney) and expression of type I Fp was higher than that of type II Fp [59,60]. This tendency was also found in the cultured cells such as Fibroblast, Myoblast, Human Umbilical Vein Endothelial Cells (HUV-EC-C), colon cancer cells (HT-29) and lung cancer cells (A549). However, colorectal adenocarcinoma cells (DLD-1), breast cancer cells (MCF-7) and lymphoma cells (Raji) showed higher expression of type II than that of type I Fp. Type I Fp seems to be essential for the ordinary function of complex II because

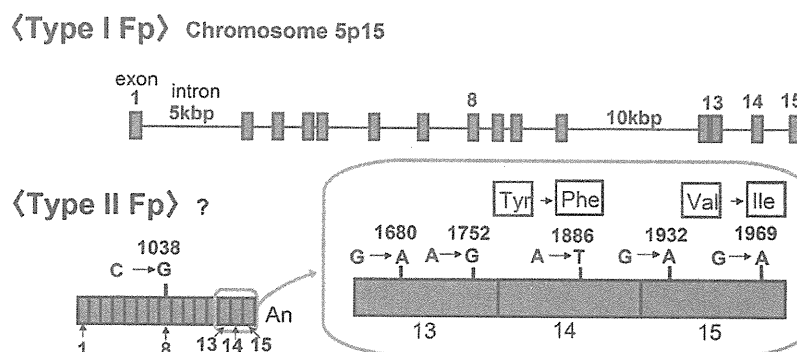


Fig. 8. Fp isoform gene structure. Type I and II Fps differ from each other in six bases in DNA sequences and in two amino acid residues in proteins. Type I Fp gene has an exon–intron structure, while type II Fp gene is suggested to be intron-less. Although type I Fp gene is located on chromosome 5p15, the type II Fp gene is not found in the NCBI database and the location has not been clarified yet [59,60].