Molecular characterization of the mitochondrial complex II

(succinate-ubiquinone oxidoreductase : fumarate reductase)

from *Plasmodium falciparum*

熱帯熱マラリア原虫ミトコンドリア複合体 II

(コハク酸-ユビキノン酸化還元酵素：フマル酸還元酵素)の解析

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1. Introduction

Malaria causes great suffering to mankind. There are over 2 billion people at risk all over the world [1] and human deaths in subSaharan Africa alone is estimated at 1.5-3 million annually [2]. Malaria is a parasitic disease caused by unicellular parasitic organisms, i.e. protozoa, of the genus *Plasmodium*, and four different forms of malaria occur in man. Among them, the most virulent is that caused by *Plasmodium falciparum*, the agent of lethal tertian malaria. The life cycle of *Plasmodium* spp. is broadly divided into two stages; one is the sexual and subsequent sporogonic stage (production of infective sporozoites) in the female *Anopheles* mosquitoes and the other is the asexual or merogonic stage (multiplication of asexual progeny, the merozoite) in the mammalian host. The latter is subdivided into the exoerythrocytic (in liver parenchymal cell) and erythrocytic stages.

The schedule for the practical use of the malaria vaccine is still rather vague. Chemotherapy using chloroquine was once dominant in treatment especially for *falciparum* malaria but the resistant parasites have spread to almost all endemic areas. The use of alternative compounds has also been followed by resistance. The major reason for this vicious circle is that the modes of action of many antimalarial compounds, including both presently used drugs and seemingly promising novel ones, are poorly understood. This lack of knowledge of organized chemotherapy would be efficiently overcome by exploring the biology of *Plasmodium* in the asexual erythrocytic stage and focusing on preferential targets.

Analyzing the function of mitochondria, the major organelle for metabolism and energy production for a variety of eukaryotic organisms, is critical in attaining this goal. *P. falciparum* is endowed with at least one acristate mitochondrion [3]. Although studies have accumulated on the function of *Plasmodium* mitochondria, detailed biochemical study has been hampered due to difficulties in obtaining workable quantities of functional mitochondria from parasites, and most of our knowledge on the organelle is from indirect evidence. While the antimalarial effects of inhibitors of mitochondrial protein synthesis and of the electron transport system have been detected [4-6], the apparent lack of key enzymes of the Krebs TCA cycle (except for malate dehydrogenase) [7], an acristate mitochondria morphology [8], and the microaerophilic property of parasitized erythrocytes *in vitro* [9] have led to the generally accepted idea that at the intraerythrocytic stage, parasites mitochondria act for pyrimidine biosynthesis [10] (dihydroorotate dehydrogenase; DHODH) [11,12] but the energy requirements are provided exclusively by glycolysis with little or no contribution by the respiratory chain in mitochondria. Recent studies, however, have claimed some modifications to this general idea. The activities of the complex III (ubiquinone-cytochrome *c* oxidoreductase) and complex IV (cytochrome *c* oxidase) were detected [13,14] and cytochrome components were confirmed [15]. At least genes for three components of these enzyme complexes, subunits 1 and 3 of complex IV and apocytochrome *b* of complex III, are found to be encoded on tandem-repeated 6 kb mitochondrial DNAs of various *Plasmodium* spp. ([16] and references therein)[17]. Alterations in their transcripts during erythrocytic

cycle [18] was shown. Moreover, the existence of ADP/ATP translocase in the mitochondrial inner membrane as well as the maintenance of a transmembrane electrical potential of the plasmodial mitochondria [19-21] suggest that plasmodial mitochondria could function in energy metabolism.

Complex II, or succinate-ubiquinone oxidoreductase, is known exclusively as a mitochondrial marker enzyme. It is located in the inner mitochondrial membrane (or cytoplasmic membrane in the case of bacteria) [22,23] and plays a unique role as a component of the TCA cycle as well as the electron transport chain, a direct link between the major systems for energy metabolism. There have been a few reports on complex II in *Plasmodium* spp. but the role of this enzyme in parasite mitochondria is still unknown. Histochemical studies have shown succinate dehydrogenase (SDH) activity at the mosquito or exoerythrocytic (in hepatocytes) stages [24,25] but have failed to detect it in the intraerythrocytic form. On the contrary, preliminary biochemical studies in recent years suggest that complex II can function in intraerythrocytic parasites [13]. However, no reports on complex II at the molecular level have been published yet.

Complex II is generally composed of four polypeptides (Fig. 1) and genes for all the subunits are encoded on the nucleus, not mitochondrial DNA. The largest flavoprotein (Fp) subunit and iron-sulfur (Ip) subunit are hydrophilic and together form a catalytic portion transferring reducing equivalents from succinate to water-soluble dyes such as 2,6-dichlorophenol indophenol (succinate dehydrogenase; SDH), or from reduced methylviologen to fumarate (fumarate reductase; FRD). The Fp/Ip portion is bound to the matrix side of

the inner mitochondrial membrane with two small integral membrane-anchoring proteins containing heme b (cytochrome *b* subunits; CybL and CybS) which seem to be essential for the interaction between the complex and electron carrier quinone species. The mechanism of expression and mitochondrial translocation of these nuclear-encoded subunits has not been clarified in detail, but studies on yeast Fp [26] and *Ascaris suum* Fp [27] revealed the primary structure characteristic of cleavable amino terminal presequences identified in other mitochondrially imported proteins [28-30].

I previously reported (in my master's thesis) the isolation of the genes from *P. falciparum* genome DNA for two catalytic subunits; the flavoprotein (Fp) subunit (Fig. 2) and the iron-sulfur (Ip) subunit (Fig. 4). The deduced amino acid sequences from isolated genome are highly similar to the complex II Fp (Fig. 3) or Ip (Fig. 5) homologues from various species including human hosts. The putative presequence necessary for translocation into the mitochondria [28] and processing motif for the mitochondrial intermediate peptidase [30] were found. The sequences which seem to be essential for the functioning of complex II are well preserved: briefly, histidine and arginine residues in the substrate-binding active site and histidine covalently binding to co-factor FAD (flavin adenine dinucleotide) in Fp; or cysteinyl residues of three distinct ferredoxin-like iron-sulfur clusters in Ip. However, characterized sequences also contain specific sequences like *Plasmodium*-specific insertion or unicellular organisms-specific deletion, which indicates that this is definitely the novel complex II.

For an understanding of the plasmodial complex II and mitochondrial

function in *Plasmodium*, the catalytic direction is the most interesting question on this enzyme. Unlike the mammalian type complex II which functions as SDH, complex II in anaerobic nematodes such as *A. suum* ([31] and references therein)[32,33] show opposite catalytic activity FRD in a unique energy metabolism pathway. It is also noteworthy that *Plasmodium*-infected erythrocytes show microaerophilic property as described above. However, it is still difficult to obtain functional mitochondria for biochemical analysis from *Plasmodium* spp.. Firstly, the preparation method has yet to be established; my repeated attempts to fractionate mitochondria from *P. falciparum in vitro* cultivation have not yet established a protocol. Secondly, electron microscopic analysis shows that in *Plasmodium* mitochondria are not so major organelle as in mammalians. Furthermore, obtaining workable quantities of human malaria parasites for biochemical study is limited by the supply of human blood for the cultivation.

Taking this into account, a molecular approach was planned: closer characterization of previously isolated genes, demonstration of the existence of native proteins by raising antibodies, and eventually, biochemical analysis using recombinant plasmodial complex II: a judicious extension of a previous study. Preparation of the antibodies against this mitochondrial marker enzyme must also be a positive feed back to localize parasite mitochondria which are morphologically aberrant (acristate) at the intraerythrocytic stage.

In the present study, Fp and Ip genes are identified on different chromosomes as single copy genes by genomic Southern hybridization. Southern analysis also identified that no isoforms are present. The expression

of two genes in the asexual erythrocytic stage was demonstrated by RNA blotting and reverse transcriptase - polymerase chain reaction (RT-PCR). Furthermore, recombinant Ip subunit protein was expressed in *Escherichia coli* to analyze the localization of native proteins. Antiserum was raised against this recombinant protein and expression of complex II in the erythrocytic stage was demonstrated by immunofluorescence assay.

2. Materials and methods

2.1. Parasite culture and DNA/RNA isolation

P. falciparum isolates FCR3/Gambia (ATCC#30932, a gift from Dr. Asahi of NIH, Japan), K1 and 3D7 were grown in human red blood cells using an RPMI1640 medium (buffered by 25 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES)) supplemented with 25 mM sodium bicarbonate, 5 mM glutamine, 0.37 mM hypoxanthine, and 10% human plasma [34-37] at 37°C in a candle jar [9] or a multigas incubator (5%-O_2, 5%-CO_2 and 90%-N_2). Human red blood cells and frozen plasma were provided by the Red Cross. Cultures were synchronized by sorbitol lysis [38] for preparation of chromosome-plugs for pulsed field gel electrophoresis. Rodent malaria specie *P. yoelii* 17XL was maintained in ICR mice and used for immunofluorescence assay (a gift from Dr. Torii and Dr. Tsuboi of Ehime university). Free parasites were obtained by saponin lysis. Genomic DNA was isolated using sodium dodecyl sulphate (SDS) and Proteinase K (SIGMA) following standard procedures [39]. Chromosome-plugs were prepared as described in *2.4.*. Total RNA was extracted by the guanidinium thiocyanate - phenol - chloroform (GTC) method described by Chomczynski and Sacchi [40]. Polyadenylated RNA was purified using an oligo(dT) cellulose column (mRNA Purification Kit, Pharmacia).

2.2. Probe preparation for hybridization analysis

P. falciparum flavoprotein (Fp) subunit- or iron-sulfur (Ip) subunit- specific

probes, "pFp" or "pIp", for southern and northern analysis were prepared by polymerase chain reaction (PCR)-labeling for detection with a high sensitivity.

Oligonucleotides primers are:

Fp279:(5'-GCTTTAGGTAATATGACTGAAGATG-3', nt 211-235 in Fig. 2) / Fp280:(5'-AGTTT CCATTATGCCTGGTAATCTT-3', nt 1044-1020) for pFp, or

Ip21:(5'-TGTAGAGAAGGTATATGTGGAAGTTGTGCTATG-3', nt 358-390 in Fig. 4) / Ip22:(5'-ACAACACATAGTACAATTCATAATACCATGGCA-3', nt 840-808) for pIp.

Cloned *P. falciparum* (K1 isolate) Fp or Ip sequences were used as templates and PCR was performed under standard conditions using *Taq* DNA polymerase (GibcoBRL), except for a 2'-deoxynucleotide 5'-triphosphate (dNTP) mixture for labeling and detection without radioisotope: 0.2 mM dATP/dGTP/dCTP, 0.16 mM dTTP and 0.04 mM digoxigenin-labeled dUTP (DIG-dUTP, Boehringer Mannheim).

For Southern blot analysis of chromosomal DNA (*2.4.*), *P. falciparum* chromosome 8-, 9- and 11-specific probes were also prepared according to the results with pFp and pIp. The partial fragments of dihydropteroate synthase (DHPS, on chromosome 8 [41,42]), merozoite surface protein-1 (MSP-1, on chromosome 9 [43]) and exported protein / circumsporozoite protein-related antigen (Exp-1 / CRA, on chromosome 11 [44,45]) were PCR-labeled after cloning.

2.3. Southern blot analysis of the restriction enzyme-digested genomic DNA

1.5 μg of genomic DNA was digested with the various restriction enzymes and run in 0.8% agarose / 1×TAE. The gel was depurinated and denatured under standard procedures, then blotted to a nylon membrane (BIODYNE A 0.2 μm, PALL) using 1 M-NH_4OAc / 0.02 N-NaOH as a transfer buffer. This blot was hybridized with pFp or pIp probes. Hybridization was carried out at 65°C overnight in 5×SSC / 0.02% SDS / 0.1% sarkosyl / 0.5% Blocking Reagent (Boehringer Mannheim). Membranes were washed in 2×SSC / 0.1% SDS at room temperature for 15 min and then in 0.1×SSC / 0.1% SDS at 65°C for 30 min. Detection was performed by alkaline phosphatase-conjugated anti-digoxigenin (DIG-APase) and chemiluminescent substrate for APase (disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo [3.3.1.1]decan}-4-yl)phenyl phosphate; CSPD®, Boehringer Mannheim). The membranes were exposed to X-ray film (X-OMAT AR, Kodak).

2.4. Pulsed field gel electrophoresis (PFGE) of parasite chromosomes

Agarose plugs were made as described by Kemp [46] with slight modifications. *P. falciparum* 14 chromosomes were separated in 0.8% agarose (SeaKemGOLD, FMC BioProducts, Rockland, ME) / 0.5×TBE by PFGE using the clamped homogeneous electric field apparatus (CHEF-DR II, BIO-RAD). Switch time was ramped from 90 s to 300 s for 24 h at 3.5 V/cm, and then from 300 s to 720 s for 24 h at 3.0 V/cm as described by

Hinterberg [47]. Blotting, hybridization, washing and detecting conditions were the same as for the restriction enzyme-digested genomic DNA. *P. falciparum* chromosome 8-, 9- and 11-specific probes were prepared as described above in 2.2..

2.5. Northern blot analysis

For Northern blotting, 0.5 μg of polyadenylated RNA were fractionated by electrophoresis in a 1.1% agarose gel containing 2.2 M formaldehyde in an MOPS (3-(N-morpholino)propanesulfonic acid) buffer and then transferred to BIODYNE A membrane in 20×SSC. Hybridization with pFp or pIp probes was performed at 65°C overnight in 0.1 M Na phosphate (pH7.0) / 7% SDS / 0.1% sarkosyl / 1% Blocking Reagent. Washing and detection were under the same conditions as described above for Southern blotting.

2.6. Reverse transcriptase-polymerase chain reaction (RT-PCR) and DNA sequencing

cDNA was synthesized from 1.25 μg of total RNA and random primers by using Mo-MuLV reverse transcriptase (GibcoBRL) and then PCR was performed under standard conditions without additional dNTP mixtures. The primer sets for RT-PCR were made from the *P. falciparum* gene sequence determined in a previous study (Fig. 2 and Fig. 4):

Fp69:(5'-GTAGCTGCTCAAGGTGGAAT-3', nt 184-203 in Fig. 2) /

Fp70:(5'-ATTCCACCCATATTATAATG-3', nt 1124-1105);

Fp165:(5'-ACACATTTACCATATGAAACATTAAAAGAA-3', nt 991-1020) /

Fp166:(5'-AGTATTTGCATTTATTTTAGGAATATT-3', nt 1371-1345);
Ip21:(5'-TGTAGAGAAGGTATATGTGGAAGTTGTGCTATG-3', nt 358-390 in Fig. 4) /
Ip22:(5'-ACAACACATAGTACAATTCATAATACCATGGCA-3', nt 840-808);
Ip100:(5'-GGAAGTTAATGACACTATGA-3', nt 777-796) /
Ip163:(5'-GTTCTTTAATGGTATCTTCG-3', nt 925-906).

Samples without reverse transcription were used as negative control.

Products from RT-PCR were cloned into a pCR™II vector carrying a single T overhang (TA Cloning Kit, Invitrogen). Sequencing was performed by M13 primers and the dideoxy chain termination method with an automated DNA sequencer (DSQ-1, Shimadzu).

2.7. Expression and purification of recombinant Plasmodium falciparum Ip subunit (PfIp) protein

On many mitochondrial proteins encoded on the nucleus, cumulative data indicate the existence of a processing motif for mitochondrial intermediate peptidase (MIP) [30]. In order to obtain the region of mature PfIp, oligonucleotide primers were designed based on a putative processing motif on a deduced PfIp protein sequence (from Phe-38 to Lys-45, shaded in Fig. 4): a sense 38 mer Ip282:(5'-cgctcg*ccatatg*GAATTTGAAATGAAAAAACAA GTTG-3', nt 136-160 in Fig. 4) and an antisense 37 mer Ip283:(5'-gc*ggatcc*tcaTCATTTTGTTTTTTCCATTTTACTTT-3', nt 966-941). The primers also contain additional nucleotides (shown in the lower case) in order to facilitate cloning into an expression vector in a defined orientation (the

restriction cleavage sites are indicated in *italics*) and to make PfIp codons in the proper reading frame.

These primers were used in PCR amplifications of the PfIp gene using a genomic DNA template. The PCR conditions for amplification (30 cycles) consisted of denaturation at 90°C for 30 sec followed by annealing at 54°C for 30 sec and extension at 72°C for 1 min. The 50 μl reaction mixture contained 0.1 μg of *P. falciparum* FCR3 genomic DNA as a template, 10 nmol of dNTP (0.2 mM each), 40 pmol of each of the primers (0.8 μM), 75 nmol of $MgCl_2$ (1.5 mM), and 4 Units of *Taq* DNA polymerase (GibcoBRL) in the manufacturer's buffer. The amplified product was cloned into a pCR™2.1 plasmid vector (TA cloning kit, Invitrogen) to check sequence. After confirmation, the inserted fragment was digested with *Nde* I and *Bam*H I and subcloned into the *Nde* I / *Bam*H I sites downstream of the $(His)_6$ coding sequence of the pET15b expression plasmid vector (Novagen), and then *E. coli* (BL21(DE3), pLysS) were transformed with the pET15b-PfIp for protein expression. After the transformant was cultured in a phosphate-buffered TB medium (1.2% (w/v) bacto tryptone, 2.4% (w/v) yeast extract, 0.4% (v/v) glycerin, pH7.4) up to ~0.4 OD 550 nm, expression of recombinant PfIp was performed in the presence of an inducing agent, 1 mM isopropyl-1-thio-ß-D-galactoside (IPTG), for 3 h at 37°C. The expressed protein was obtained as inclusion bodies and purified first by sonication / centrifugation. A centrifuged sample was then solubilized by 3M guanidine HCl, affinity-purified by an Ni^{2+}-column using an amino-terminal $(His)_6$ tag and finally eluted with 1M imidazole. The purified protein was dialyzed against 20 mM

Tris-buffered (pH7.9) - 0.1% SDS to remove imidazole and guanidine HCl, and then concentrated by lyophilization. Each step of purification was assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and expression of the intended protein was confirmed by Edman degradation (G1005A-protein sequencer, Hewlett Packard) after removal of the upstream $(His)_6$ tag by thrombin cleavage.

2.8. Anti-PfIp antiserum preparation

Approximately 0.3 mg of purified recombinant PfIp protein, emulsified in Freund's complete adjuvant (FCA), was injected intracutaneaously into a rabbit. The animal was boosted 3 weeks after primary immunization and further booster immunizations were given 4 times at 1 week intervals, each with 0.1 mg of recombinant PfIp in Freund's incomplete adjuvant (FIA). Serum reactivity on the same day of the last two booster immunizations was checked against the recombinant protein by ELISA and blood was taken from the rabbit 7 days after the last immunization.

2.9. Immunoblot analysis

Antisera raised against PfIp (this work), *Bos taurus* (bovine heart) complex II (Fp+Ip subunits) [48] and *Ascaris suum* complex II (adult form) (Ip+CybL+CybS subunits) [49] were used for analyzing cross-reactivity. Purified proteins of *B. taurus* (bovine heart) complex II [48], *A. suum* complex II (adult form) [33] and *E. coli* complex II (SDH isoform) [50] were used as standards of these species. Protein samples were separated by SDS-

PAGE (12.5% single percentage gel) and electroblotted to nitrocellulose membrane (PROTRAN, Schleicher & Schuell) for detection with appropriate dilutions of each antiserum described above. APase-conjugated goat anti-rabbit immunoglobulin G (IgG) (BIORAD) with colorimetric reagents for APase, 5-bromo-4-chloro-3-indolyl phosphate / nitroblue tetrazolium chloride (BCIP/NBT, Boehringer Mannheim), was used for detection of primary antibodies.

2.10. Indirect immunofluorescence assay (IFA)

For IFA, *P. falciparum* FCR3 -, *P. falciparum* 3D7 - and *P. yoelii* 17XL - infected erythrocytes were harvested, washed in phosphate-buffered saline (PBS: pH7.4) and diluted in PBS to a hematocrit of approximately 20%. The erythrocytes were air-dried on multitest slides, fixed with ice-cold acetone and then blocked with PBS containing 5% nonfat dry milk (PBS-Milk) for 30 min at 37°C. The slides were incubated with anti-PfIp antiserum diluted in PBS-Milk for 60 min at 37°C and rinsed with PBS. After incubation with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG serum (mouse and human absorbed; Tago-BioSourceIntl, Camarillo, CA; diluted 1:50 in PBS-Milk) for 30 min at 37°C, the slides were rinsed with PBS and mounted under a coverglass in bicarbonate-buffered (pH9.5) glycerin (including 1,4-diazabicyclo(2,2,2)octane to prevent regression of fluorescence) and observed with a fluorescence microscope (Olympus, Tokyo).

3. Results

3.1. Flavoprotein (Fp) subunit and iron-sulfur (Ip) subunit are present as single genes and located to different chromosomes : chromosome 10 (Fp) and 12 (Ip)

3.1.1. Genome Southern analysis of restriction endonuclease - digested DNA

P. falciparum (K1 isolate) complex II Fp and Ip nucleotide sequences were characterized (previous study) as shown in Fig. 2 (Fp) and Fig. 4 (Ip).

In order to determine the copy number of the genes, *P. falciparum* genome DNA from isolates FCR3 and K1 was digested with restriction endonucleases and analyzed by Southern blotting using pFp and pIp probes (Fig. 6). Probe pFp (fragment flanked by primer set Fp279/Fp280 in Fig. 2) does not contain cleavage sites for *Eco*R I and a single site for *Hin*d III and *Dra* I, whereas pIp (between Ip21 and Ip22 in Fig. 4) has no sites for any of these three enzymes. Two positive hybridizing fragments were detected on *Hin*d III- and *Dra* I-digested lanes hybridized with pFp and a single band for all other lanes. Some of these signals include the presumably less-preserved region out of open reading frame (ORF), but the number of signals was equal to that estimated from the primary structure. All these results strongly suggest that Fp and Ip are present as single genes in the genome of *P. falciparum*. FCR3 genome DNA was further digested with other enzymes (*Ssp* I, *Sph* I, *Pvu* II, *Pst* I, *Sau*3A I, *Hpa* I, *Eco*T22 I, *Nde* I, *Xho* I and *Bam*H I), and the pattern of hybridizing fragments with pFp or pIp probes was consistent with that

expected for single genes (data not shown). The data obtained from these blots also indicate that isolates FCR3 and K1 have the same gene structure for complex II, which was supported by other data: where the clones containing genome DNA from FCR3 and K1 overlapped (between primers Fp69 and Fp166 in Fig. 2, or Ip21 and Ip163 in Fig. 4), the two isolates have identical sequences. Furthermore, hybridization under less stringent conditions (hybridization and washing at 50°C) showed strictly the same signal pattern as under normal conditions (Fig. 6, at 65°C).

3.1.2. Chromosome mapping

The organization of the *P. falciparum* Fp and Ip genes was further examined by pulsed field gel electrophoresis (PFGE) and Southern blot analysis (Fig. 7). Sequential hybridization of a Southern blot of FCR3 and K1 chromosomes with pFp or pIp probes (panel B) and known *P. falciparum* chromosome 8-, 9- and 11- markers (panel C) localized Fp to chromosome 10 and Ip to chromosome 12. This is consistent with the result of genomic Southern analysis showing the presence of single genes.

3.2. Expression of recombinant P. falciparum Ip (PfIp) subunit and preparation of anti-PfIp antibody

3.2.1. Expression of recombinant PfIp subunit

Ip subunit was chosen as the first step to raise antibodies against *P.*

falciparum complex II. A PCR product fragment by Ip282/Ip283 primers, which encodes amino acids 46-321 (Fig. 4) in the *P. falciparum* Ip (PfIp), was subcloned into the pET15b plasmid vector and expressed in *E. coli* as a fusion protein with a $(His)_6$ tag and several other vector-derived amino acid residues, according to the protocol described in Materials and methods *2.7.*. Simple observation on cell pellets demonstrated that an IPTG-induced *E. coli* transformant (*E. coli* BL21(DE3) / pET15b-PfIp, IPTG(+)) were white with brownish-red tinges (Fig. 9, right "PfIp") like those of over-expressed *E. coli* SDH (using *E. coli* sdh^- and frd^- double mutant strain transformed with plasmid carrying *E. coli sdh* [50], data not shown), while those of insert-negative (*E. coli* BL21(DE3) / pET15b, IPTG(+); Fig. 9, left "Control") or uninduced (*E. coli* BL21(DE3) / pET15b-PfIp, IPTG(-)) were white with brownish-green tinges. This difference of color indicates the binding of iron to protein expressed in IPTG-induced transformant, suggesting the expression of recombinant PfIp, i.e. iron-sulfur subunit. Actually, analysis by SDS-PAGE and Coomassie Brilliant Blue (CBB) - staining of cell lysates showed a major band standing out at ~34 kDa (Fig. 10, third lane in panel A, "IPTG+"-marked), which is consistent with the total length of putative mature PfIp and amino acids from the pET15b vector. By contrast, samples that were not red did not express the signal (the left two lanes in panel A). The recombinant PfIp was obtained as inclusion bodies and purification steps (by sonication/centrifugation and Ni^{2+}-column under denatured condition (with 3M guanidine HCl)) gave protein sample showing a single band (Fig. 10, the right lane in A). After dialyzing as described in *2.7.*, purified PfIp was used for

raising anti-PfIp antibody in rabbit (according to *2.8.*).

3.2.2. Antibody raised against PfIp crossreacted with mammalian (bovine heart) Ip

The rabbit anti-PfIp antiserum (diluted 1:10000, *E. coli* lysate added (1:100)) was reacted with recombinant PfIp (Fig. 10, the two lanes on the right in panel B. See also the right lane in A.) but not with insert-negative control (*E. coli* BL21(DE3) / pET15b, IPTG(+); the left "pET"-marked lane). A weak signal was found even without induction (*E. coli* BL21(DE3) / pET15b-PfIp, IPTG(-); second from the left), although protein band was not detected by CBB-staining.

Crossreactivity of this antibody with Ip from two other species was tested using mammalian (*Bos taurus*; bovine heart), multicellular parasite (*A. suum* adult form), and bacterial (*E. coli* SDH isoform) complex II purified from mitochondria (or prokaryotic cytoplasm) of each organism (Fig. 11 (1)). Immuno blot (1:10000, right panel) showed a clear single signal at the location of the bovine heart Ip in addition to the strong one from recombinant PfIp. Almost undetectable signals were observed for Ip homologues of *A. suum* and *E. coli* SDH, even proteins on the nylon membrane are higher than that of PfIp (See the CBB-stain in panel A.). The crossreaction between *P. falciparum* Ip and its bovine heart counterpart was also shown by the pair, PfIp protein / anti-bovine heart complex II (Fp+Ip subunits) antibodies (1:4000) (Fig. 11 (2)). A weak but clear signal was detected at the location of PfIp (second lane from the left), while no such signal was apparent in the *E. coli* lysate of insert-

negative control (the left lane). On the other hand, the same crossreaction test using anti-*A. suum* complex II adult form (Ip+CybS+CybL subunits) antibodies (1:4000) revealed no signals for PfIp (Fig. 11 (3)).

3.3. Expression of Fp and Ip subunits in the asexual, intraerythrocytic cycle

3.3.1. Northern analysis and RT-PCR

To demonstrate the expression of Fp and Ip mRNA transcripts, *P. falciparum* polyadenylated RNA from a non-synchronous culture of intraerythrocytic forms (FCR3 isolate) was analyzed by Northern blotting with pFp or pIp probes (Fig. 8). A single band for each of Fp (left) and Ip (right) was detected and sizes of the transcripts were identified as about 3.3 kb for Fp and 2.4 kb for Ip.

Expression of an identical sequence to genome was shown by RT-PCR. PCR was carried out with primer sets Fp69/Fp70, Fp165/Fp166, Ip21/Ip22, and Ip100/Ip163 using reverse transcription (-) samples as negative control. Sequencing analysis of independent 7 (Fp165/Fp166), 6 (Ip100/Ip163), or 2 (Fp69/Fp70 or Ip21/Ip22) showed that all of these sequences were completely identical to genomic DNA shown in Fig. 2 (Fp) and Fig. 4 (Ip). This result is consistent with the detection of a single transcript by Northern analysis.

3.3.2. Demonstration of expression of Ip peptide using Immunofluorescence

assay (IFA)

In order to examine the expression of native complex II in the intraerythrocytic stage of *Plasmodium*, the rabbit antiserum raised against the recombinant PfIp was used to label acetone-fixed smears of *P. falciparum* - and *P. yoelii* (rodent malaria specie) - infected erythrocytes and reaction was detected by fluorescence (Fig. 12). The antibody (1:100) reacted with *P. falciparum* late asexual stage parasites which have hemozoin, i.e. trophozoite and/or schizont (fluorescence in panels 1-/3-A and strong signals in panels B. See panel 7 and Figure legend.). The fluorescence signals were detected over the whole cell mainly as granular dots. Similar signals were observed with *P. yoelii* (panel 5-A), indicating crossreactivity of this anti *P. falciparum* Ip antiserum. In the negative controls, which were incubated with rabbit preimmune serum (1:100), no fluorescence was detected (panels 2-/4-/6-A).

4. Discussion

The present study reveals that complex II, the mitochondrial marker enzyme, is expressed at the level of protein in *Plasmodium* in the asexual erythrocytic cycle in a mammalian host. This is the first demonstration from *Plasmodium* spp. of nuclear-encoded respiratory chain enzyme components and this report must facilitate the study of plasmodial mitochondria.

In order to know the copy number of these genes and whether these are really functional in parasite mitochondria, enzyme-digested / chromosomal DNA and their messengers were analyzed in the present study. Southern analysis (Fig. 6) gave signal patterns consistent with a single copy gene for both Fp and Ip, with each *P. falciparum* isolate tested. Furthermore, these genes were identified on different chromosomal DNA (Fig. 7) like those of the human [51,52] or yeast [26,53] complex II. Coding Fp and Ip on different chromosomes is quite different from prokaryotic organisms in which the homologues of all four subunits, including Fp and Ip, constitute operon. A cDNA fragment with the same sequence as genome was characterized by RT-PCR and Northern analysis (Fig. 8) using messengers from the erythrocytic stage. Each of the transcripts (3.3 kb for Fp or 2.4 kb for Ip) has untranslated regions (UTR) of more than 1 kb in total, which is calculated from the putative coding region of 1.9 kb for Fp or less than 1 kb for Ip. Although these are unusually longer for mammalian mRNA and sizes of 5'- and 3'-portions remains to be analyzed, long 5'UTRs have been observed in many plasmodial mRNAs such as actin [54] or glucose 6-phosphate

dehydrogenase [55]. Short leader sequences may be optimal for efficient translation. If long 5'UTRs is the case for plasmodial Fp and Ip, they could share unknown biological meaning specific to the translation of plasmodial proteins, such as interaction with ribosomes or with regulatory proteins. These results of genome and messenger analysis and a putative mitochondrial presequence or processing motif on the amino terminal region (previous study) indicate that, as is the case with many mitochondrial proteins encoded on the nucleus [28], each subunit of plasmodial complex II is independently transcribed, then synthesized on cytoplasmic free polyribosomes and post-translationally translocated into mitochondria, and finally processed further and assembled into mature hetelotetramer enzyme complex.

The results of RNA analysis enhanced the preparation of specific antibodies against these two catalytic subunit proteins, which must be useful for the demonstration of native plasmodial complex II and also be positive feed back to identify intraerythrocytic parasites' mitochondria which are morphologically aberrant (acristate) and hard to detect.

The recombinant *P. falciparum* Ip (PfIp) protein was successfully expressed and rabbit antiserum was raised against this recombinant protein (Fig. 10). The expression with high efficiency might be surprising, because PfIp has a high AT content (72% for the expressed fragment) with typical codon usage for *P. falciparum* protein-coding sequences (Coding AT-71%, 1st letter-61%, 2nd letter-69%, 3rd letter-84%; calculated using accumulated data, Codon Usage Tabulated from GeneBank [56]), which is very different from *E. coli* (Coding AT-48%, 1st letter-41%, 2nd letter-59%, 3rd letter-45%). However,

previous studies indicated that this codon bias is not necessarily critical for efficient expression of *P. falciparum* recombinant proteins: it was attained by synthetic genes according to authentic *E. coli* codon usage in the case of dihydroorotate dehydrogenase (DHFR) [57], but equally AT-rich hypoxanthine phosphoribosyl transferase was expressed in large amounts without modification [58]. Other factors, such as stability of mRNA and protein, secondary structure of promoter's ribosome-binding site, and toxicity of product for *E. coli* host, might also be important. The expression as inclusion bodies did not pose any problem for raising antibody in present study, but strategies for correct protein folding will be necessary for next step, the reconstitution of functional *P. falciparum* complex II by expressing recombinant Fp as well as Ip. The electron spin resonance (ESR) analysis of the formation of iron-sulfur clusters, besides preliminary recognition of iron-binding by observation of cell pellets color (Fig. 9), will be required for assessing expression of functional protein.

This anti-PfIp antiserum crossreacted with *B. taurus* (bovine heart) Ip homologue and vice versa (Fig. 11 (1) and (2)), but not with those from *A. suum* (adult form) (Fig. 11 (1) and (3)) and *E. coli* SDH isoform (Fig. 11 (1)). This result indicates that a similar and specific molecular structure is present at epitope(s) on plasmodial and mammalian Ip (Ips of bovine heart [59] and human liver [60] are quite similar. The homology score for the entire sequence is more than 93%.). The section with amino acid residues specifically common to *P. falciparum* and human (but not to *A. suum* or *E. coli*) can also be found, like that upstream of the S-3 iron-sulfur cluster (from

Leu-267 to Arg-269 in Fig. 5). However, the ratio of the signal intensity (*Plasmodium* to bovine heart) of the western blot (Fig. 11 (1) panel B) is far higher than that of the CBB stain (panel A), indicating that the antibody raised here is adequately specific to *Plasmodium* Ip. Furthermore, because host erythrocytes (apart from juvenile ones, i.e. reticulocytes) do not have mitochondria, this weak crossreactivity should not spoil the usefulness of the antibody prepared here for demonstration of the expression of plasmodial complex II in intraerythrocytic form. Negligible cross with *E. coli* also indicates that this anti-PfIp antibody will be useful for assessing the reconstitution of parasite complex II using recombinant proteins of Fp and Ip.

Immunofluorescence assay (IFA) (Fig. 12) confirmed the expression of complex II at the level of protein, providing more convincing data in addition to messenger expression (Fig. 8). It is also noteworthy that crossreaction was observed with rodent malaria parasite *P. yoelii*, which means that mice-infecting parasites can be the model for the human malaria species when analyzing mitochondria. Expression was demonstrated in *P. falciparum* late asexual form parasites, which is in agreement with the messenger level of the three subunits from complex III and complex IV in *P. falciparum* [18]. However, neither identification of the specific stage of the individual parasite nor strict subcellular localization of plasmodial complex II were possible due to low resolution of phase contrast images. The fixation step (by acetone, in this study) could have affected parasite morphology and thus giving a vague image. Although acetone fixation might enable detection of parasite mitochondrial protein even from intact parasitized erythrocytes as shown in

this study, its strong ability to denaturate cell structure could make morphological analysis hard on the other hand. Visualization by cationic fluorescent dye (rhodamine 123) of mitochondria from non-fixed *P. falciparum*-infected erythrocytes showed different fluorescence signals from those in this study [20]. In addition to tests with other fixation methods for IFA (for example, by methanol) to keep cell structure and thus antigenicity, electron microscopic analysis is desirable and will be decisive for subcellular localization of complex II. For strict identification of the expression stage in the erythrocytic cycle, sample preparation from synchronized *in vitro* cultivation is now being tried.

The presence in *Plasmodium* spp. of mitochondrial complex II that constitutes a TCA cycle as well as an electron transport chain is intriguing because the preferable condition for *P. falciparum in vitro* cultivation, the model of intraerythrocytic form in a mammalian host, is microaerobic: neither aerophilic nor obligate-anaerobic [9]. As for the property of plasmodial mitochondria in erythrocytes and the role of complex II, its unique and possible feature can be discussed on the plasmodial electron transport chain (Fig. 13) as described below.

(1) Plasmodial mitochondria are involved, even with limited extent, in aerobic energy metabolism like mammalian and other eukaryotic organisms at the erythrocytic cycle. Complex II functions as succinate dehydrogenase (SDH).

(2) Plasmodial mitochondria have nothing to do with energy metabolism but participate in pyrimidine biosynthesis, and the electron transport chain functions only as an electron disposal sink for dihydroorotate dehydrogenase

(DHODH) [12] via ubiquinone. In this case, plasmodial complex II is fumarate reductase (FRD), the opposite activity of SDH, and fumarate acts as an electron acceptor, second to or instead of oxygen. This theory is consistent with the generally accepted idea that the TCA cycle is absent in the plasmodial intraerythrocytic stage ([7] and references therein).

(3) Plasmodial mitochondria are engaged in anaerobic energy metabolism using complexes I and II, and complex II functions as FRD with fumarate, instead of oxygen, as a terminal electron acceptor. This anaerobic pathway has been clarified in adult *A. suum* ([31] and references therein)[32,33] and other parasitic helminths living under an anaerobic environment.

(4) Plasmodial mitochondria without a complete TCA cycle have a unique, but still mammalian-like electron transport chain. In this case, fumarate is concurrently reduced (by complex II or an unknown mechanism other than complex II) with oxidation of NADH, and then produced succinate is reoxidized by a mammalian-type electron transport chain (including SDH activity by complex II). Preliminary study on a biochemical basis showed a seeming absence of complex I (i.e. rotenone-sensitive NADH-ubiquinone reductase) but also suggested the presence of NADH-fumarate reductase activity (with dehydrogenation of NADH by an unknown mechanism) [13].

The critical question here is whether isozymes are present for plasmodial complex II, and their physiological enzymatic direction: SDH and/or FRD.

Southern analysis gave no evidence of isoforms for plasmodial complex II (Fig. 6). It gave identical signal patterns for both Fp and Ip even under less stringent conditions (at 50°C, data not shown). Another Southern analysis was

also tried using more than 50 clones from PCR products, with pFp or pIp probes. In this experiment, degenerated oligonucleotides corresponding to highly conserved sequence motifs (the same location as primers Fp69/Fp70 or Ip21/Ip22, but degenerated) were employed, with which two Fp isoforms from *A. suum* were distinctively detected by the difference of hybridization intensity (our unpublished data). The result of *P. falciparum* showed without exception a single clear signal with identical intensity (data not shown).

Catalytic direction was examined by homology score using species which have more than one isoforms of complex II with opposite catalytic activities. *A. suum* has two isoforms expressing stage-specifically (SDH in aerobic L2-larva and FRD at anaerobic adult stage) [27,32,33] with corresponding mRNA (our unpublished data). Two stage specific cDNAs were also detected in *Haemonchus contortus* Ip [61]. Furthermore in *E. coli*, SDH is synthesized during aerobic growth, whereas FRD is in an anaerobic culture, and the genes corresponding to each have been well characterized [62-65]. Comparison of the entire amino acid sequence between *P. falciparum* Fp/Ip and each of the homologues from *A. suum* Fp, *E. coli* Fp, *H. contortus* Ip and *E. coli* Ip (by GENETYX computer program) shows homology scores: 59.4% (*A. suum* Fp adult form, FRD) and 61.0% (*A. suum* Fp larval form, SDH); 51.0% (*E. coli sdh* Fp, SDH) and 39.4% (*E. coli frd* Fp, FRD); 58.2% (*H. contortus* Ip-1; expressed throughout the life cycle) and 55.9% (*H. contortus* Ip-2; expressed mainly in an aerobic free-living stage); 49.4% (*E. coli sdh* Ip, SDH) and 33.6% (*E. coli frd* Ip, FRD). No significant difference was observed for *A. suum* Fp - or *H. contortus* Ip - isoforms. For both *E. coli* Fp and Ip,

significantly higher identity to *E. coli sdh* than *frd* was found, but this tendency of *E. coli* is also observed in the *A. suum* Fp adult form whose physiological function has been thoroughly characterized and determined as FRD. In *A. suum* and *E. coli*, stage specific expression of different complex II isoforms was also shown to be coupled with synthesis of different types of quinone species [32,49,66]. In *Plasmodium*, only ubiquinone has so far been detected ([67] and references therein).

In conclusion, two catalytic subunits, flavoprotein (Fp) and iron-sulfur (Ip) subunits, of mitochondrial complex II of *P. falciparum* are detected in uniform with single copy genes. The transcription of these gene sequences was demonstrated at the erythrocytic form in a mammalian host. Furthermore, the expression of native complex II at late intraerythrocytic stages was confirmed. In addition, growth of *P. falciparum* cultivated *in vitro* was inhibited (recent unpublished data) by antisense oligonucleotides against these two subunits, suggesting the physiological importance of this enzyme complex. Demonstration here of the expression of complex II which links the TCA cycle with the electron transport chain could change the generally accepted idea on plasmodial mitochondria, i.e., little or no contribution to energy metabolism. It will also help to understand the mode of action of the electron mediator quinone derivatives (some of which are promising or potential antimalarials). However, the molecular approach at this point and other reports have yet to determine the physiological catalytic property of plasmodial complex II. More detailed analysis is necessary, but a direct approach by measurement of

such a value as the mid-point potential (Em') of the S-3 center of Ip by ESR [68] would again meet the difficulty of obtaining large quantities of functional mitochondria. To attain the overall goal of the present study, construction of functional plasmodial complex II by co-expression of each subunit and biochemical analysis using this reconstituted enzyme are now in progress.

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Figures

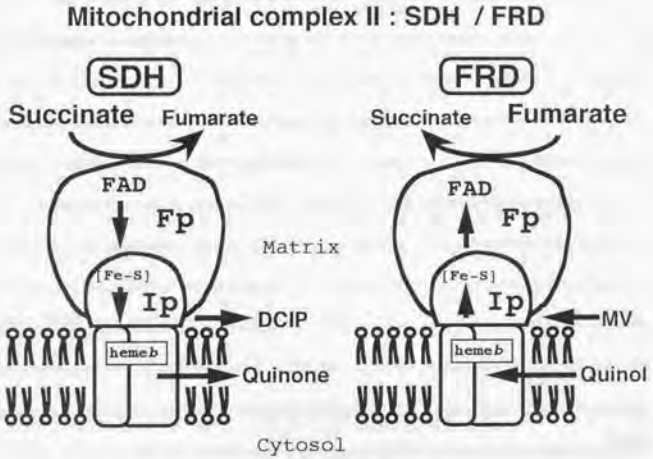


Fig. 1 Molecular organization of complex II in the inner membrane of mitochondria and electron transfer in the complex. SDH, succinate dehydrogenase; FRD, fumarate reductase; Fp, flavoprotein subunit; Ip, iron-sulfur subunit; FAD, flavin adenine dinucleotide; [Fe-S], three iron-sulfur clusters in Ip; DCIP, 2,6-dichlorophenol indophenol; MV, methyl viologen.

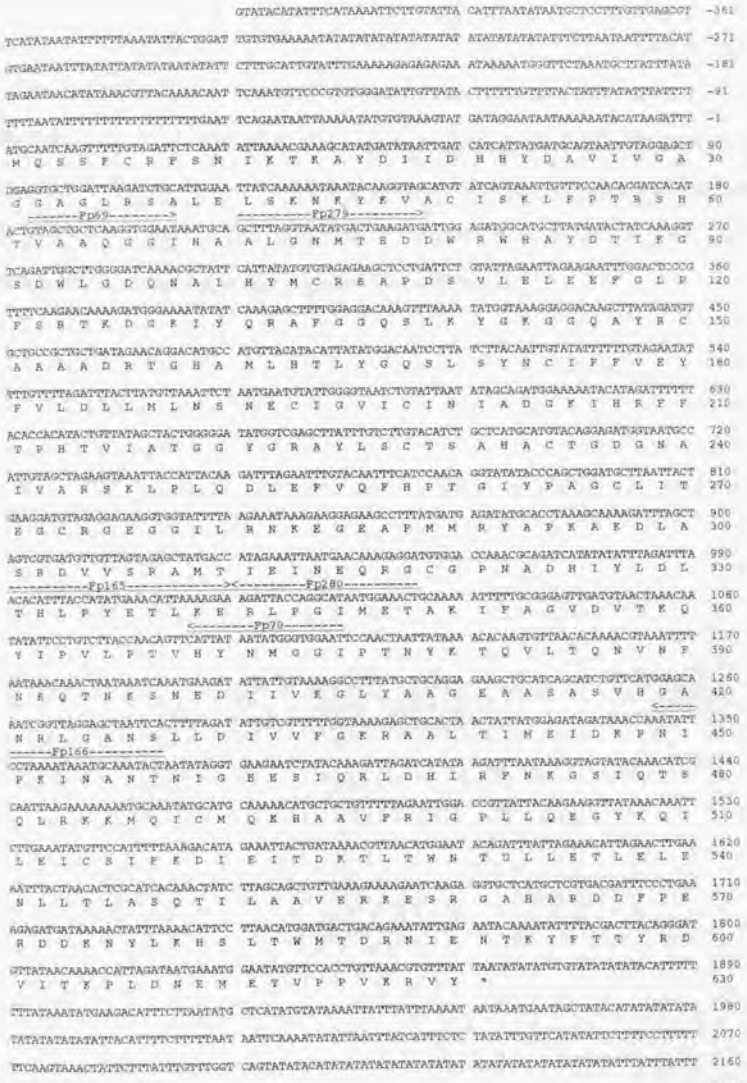


Fig. 2

Fig. 2 Nucleotide sequence of the genomic DNA for the complex II flavoprotein (Fp) subunit from *P. falciparum* K1 isolate. The inferred amino acids are indicated in single letter code below the nucleotide sequence. Nucleotide and amino acid positions are indicated on the right. The stop codon TAA is indicated with *. Fp69, Fp70, Fp165, Fp166, Fp 279 and Fp 280 correspond to the regions for each primer.

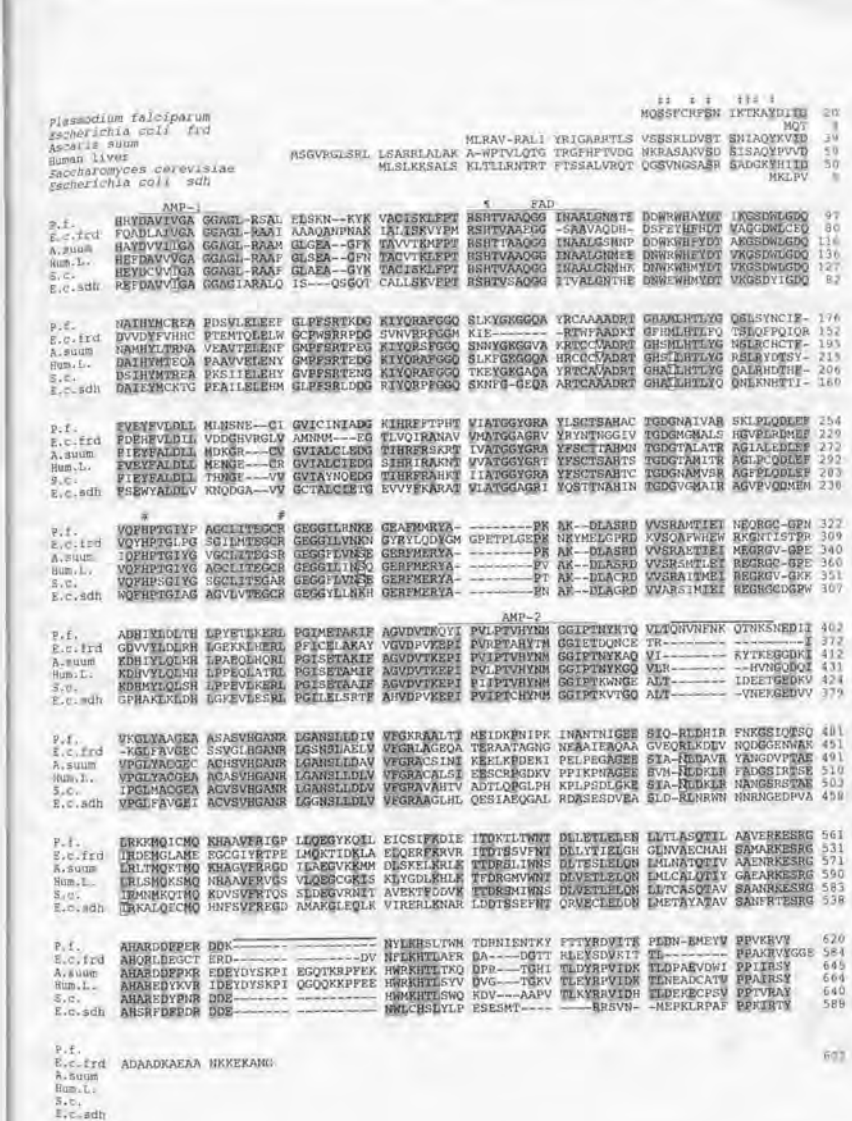

Plasmodium falciparum
Escherichia coli fad
Ascaris suum
Human liver
Saccharomyces cerevisiae
Escherichia coli sdh
AMP-1
FAD
AMP-2

Fig. 3

Fig. 3 Alignment of the amino acid sequence of the complex II Fp subunits from various species. The top line shows that of *P. falciparum*. Dashes indicate gaps introduced to maximize similarity within the alignment. Numbers indicate the position of amino acids from N-terminus (first methionine) of each organisms. The basic and hydroxylated amino acids in N-terminus region are indicated with ‡. # indicate conserved histidine (His-258 in *P. falciparum*) and arginine (Arg-274 in *P. falciparum*) residues in the active site. Three segments interacting with FAD (AMP-1, FAD and AMP-2) are overlined. ¶ indicates FAD-binding histidine (His-60). Specific insertion or deletion sites are overlined twice. Residues that are identical in at least three species are shaded or boxed.

Escherichia coli frd [62]; adult *Ascaris suum* [27]; Human liver [69]; *Saccharomyces cerevisiae* [26]; *Escherichia coli sdh* [64].

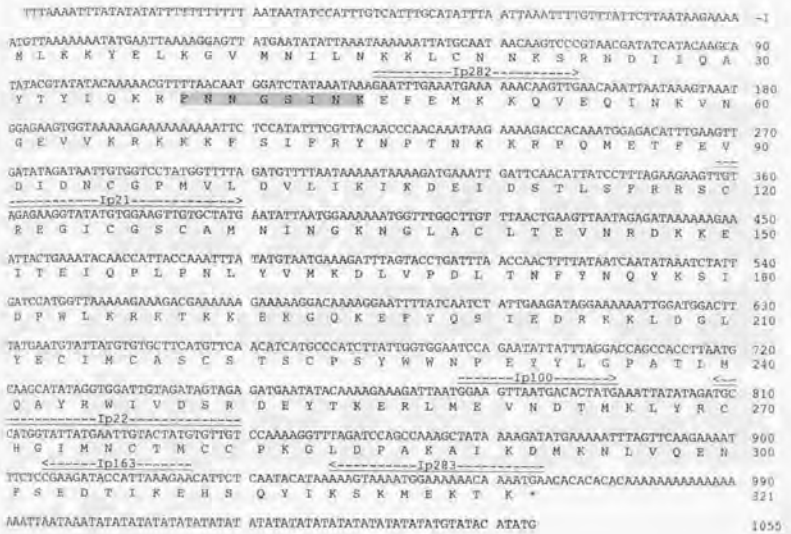


Fig. 4 Nucleotide sequence of the genomic DNA for the complex II iron-sulfur (Ip) subunit from *P. falciparum* K1 isolate. The inferred amino acids are indicated in single letter code below the nucleotide sequence. Nucleotide and amino acid positions are indicated on the right. The stop codon TGA is indicated with *. Putative octapeptides for cleavage by mitochondrial intermediate peptidase are shaded. Ip21, Ip22, Ip100, Ip163, Ip282 and Ip283 correspond to the regions for each primer.

Fig. 5

Fig. 5 Alignment of the amino acid sequence of the complex II Ip subunits from various species. The top line shows that of *P. falciparum*. Dashes indicate gaps introduced to maximize similarity within the alignment. Numbers indicate the positions of amino acids from N-terminus (first methionine except for human liver) of each organisms. Putative octapeptides at plasmodial N-terminus region for cleavage by mitochondrial intermediate peptidase (from Phe-38 to Lys-45) are overlined twice. * indicate conserved cysteine residues in three clusters (S-1, S-2 and S-3; overlined) which comprise the iron-sufur centers (Cys-120, -125, -128 and -140 in S-1; Cys-213, -216, -219 and -223 in S-2; Cys-270, -276 and -280 in S-3 in *P. falciparum*.). Residues that are identical in at least three species are shaded or boxed.

Escherichia coli frd [63]; adult *Ascaris suum* (our unpublished data); Human liver [60]; *Saccharomyces cerevisiae* [53]; *Escherichia coli sdh* [65].

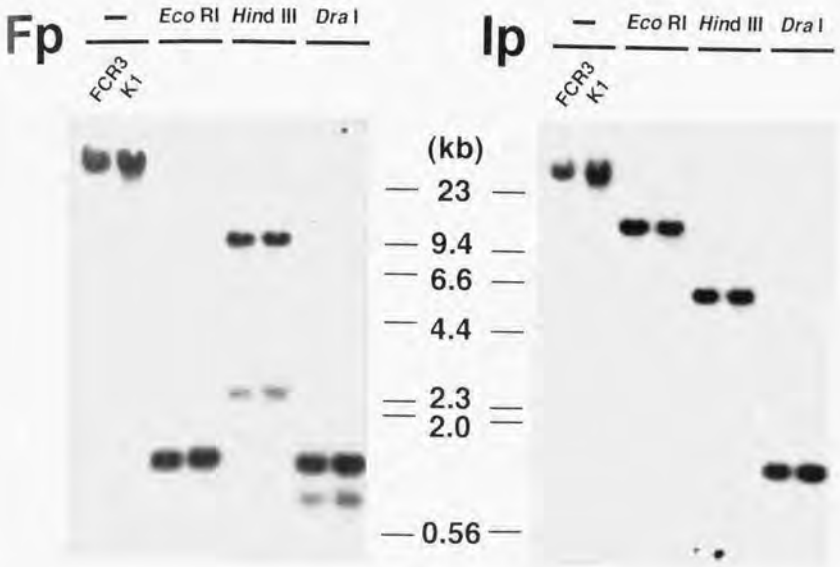


Fig. 6 Southern analysis. *P. falciparum* isolates FCR3 and K1 genomic DNA digested with the indicated enzymes were fractionated and transferred onto nylon membrane. The blots were hybridized with probes consisting of the coding region of the Fp (pFp, left panel) or Ip (pIp, right), respectively. The *Hin*d III digest of λ cI 857 Sam 7 DNA (Takara) was used as size markers. The difference of the intensity between two signals in *Hin*d III- or *Dra* I-digested lanes in left panel is due to the length in which the genome and pFp probe overlap.

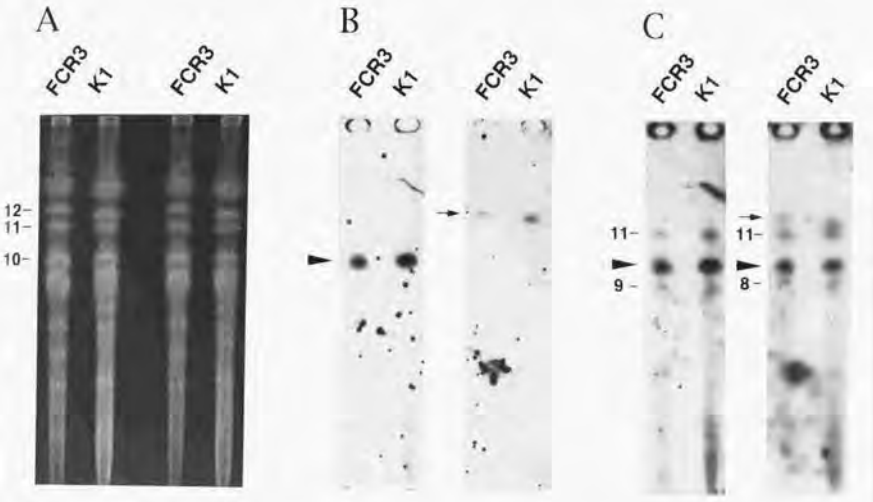


Fig. 7 Chromosome assignment of the *P. falciparum* Fp and Ip genes. (A) Ethidium bromide stained gel of parasite chromosomes from cultures of FCR3 and K1 isolates with chromosome 10, 11 and 12 marked. (B) The chromosomes in panel A were transferred to a membrane and hybridized to pFp (left panel) or pIp (right) probes. The signals were indicated by arrowhead (left panel) or arrow (right). (C) The same filters were rehybridized with the mixture of pFp, chromosome 9- and 11- specific probes (left panel) or pFp, pIp, chromosome 8- and 11- specific probes (right) (See Materials and methods 2.2. on specific probes for each chromosome.). Besides the signals at the same position as panel B, those for chromosome 8, 9 and 11 are also indicated.

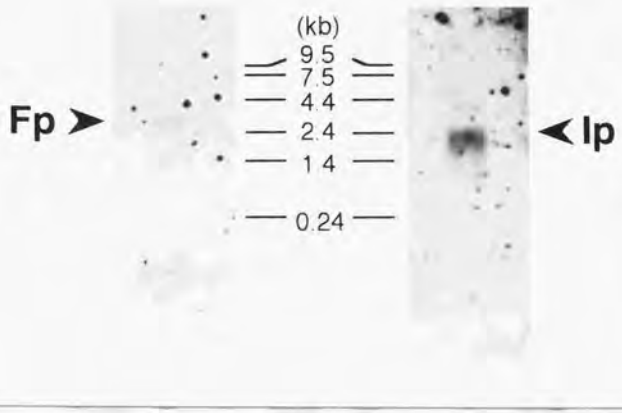


Fig. 8 Northern analysis of isolate FCR3 poly(A)$^+$RNA from asynchronous culture. The blots were probed with DIG-dUTP labeled pFp (left panel) or pIp (right) probes and signals are indicated by arrows. 0.24-9.5 kb RNA ladder (GibcoBRL) was used for estimation of the size of the mRNA.

Fig. 9 Expression of recombinant *P. falciparum* Ip subunit (PfIp) protein inserted into pET expression plasmid vector. After adequate induction of protein expression, *E. coli* cells were harvested by centrifugation. Marked difference of color was observed. Left "Control"; insert-negative control (*E. coli* BL21(DE3) / pET15b, IPTG+). Right "PfIp"; pET vector with PfIp (*E. coli* BL21(DE3) / pET15b-PfIp, IPTG+). Uninduced control (*E. coli* BL21(DE3) / pET15b-PfIp, IPTG-) showed almost the same color as insert-negative.

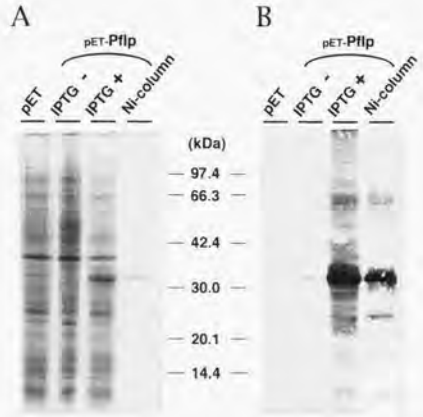
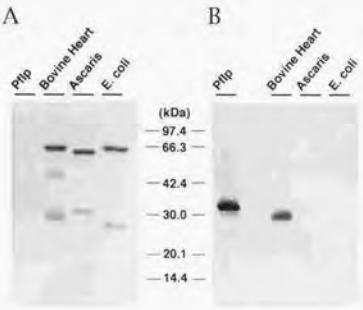
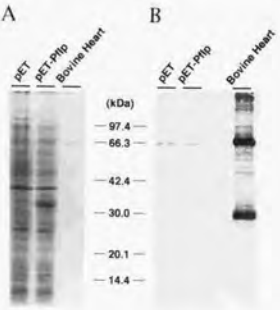


Fig. 10 Expression and purification of recombinant *P. falciparum* Ip subunit (PfIp) protein. (A) Coomassie brilliant blue (CBB)-stained SDS-PAGE. (B) Immunoblot using rabbit antiserum prepared against purified PfIp (1:10000). Samples from left column to the right are: total *E. coli* lysate of insert-negative control (*E. coli* BL21(DE3) / pET15b, IPTG+), pET-PfIp in the absence (*E. coli* BL21(DE3) / pET15b-PfIp, IPTG-) or presence (*E. coli* BL21(DE3) / pET15b-PfIp, IPTG+) of inducing agent IPTG, and induced products which was then purified by sonication/centrifugation, solubilization and affinity-chromatography with Ni^{2+}-column.

(1)


A
B
Pflp
Bovine Heart
Ascaris
E. coli
(kDa)
97.4
66.3
42.4
30.0
20.1
14.4

(2)


A
B
pET
pET-Pflp
Bovine Heart
(kDa)
97.4
66.3
42.4
30.0
20.1
14.4

(3)

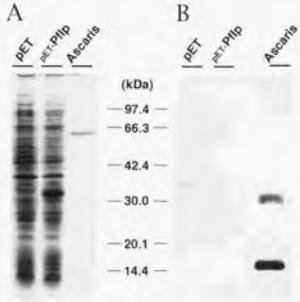

A
B
pET
pET-Pflp
Ascaris
(kDa)
97.4
66.3
42.4
30.0
20.1
14.4

Fig. 11

Fig. 11 Crossreaction of anti-*P. falciparum* Ip subunit (PfIp) antibody with other Ip homologues. (A) CBB-stained SDS-PAGE. (B) Immunoblot using antisera described below. (1) Anti-PfIp antiserum (1:10000). Samples from left column to the right are: purified recombinant PfIp, *B. taurus* (bovine heart) complex II, *A. suum* complex II (adult form) and *E. coli* complex II (SDH isoform). (2) Anti-bovine heart complex II antiserum (1:4000). Samples from left column to the right are: total *E. coli* lysate of insert-negative control (*E. coli* BL21(DE3) / pET15b, IPTG+), recombinant PfIp (*E. coli* BL21(DE3) / pET15b-PfIp, IPTG+), and bovine heart complex II. Because the antiserum was raised against *Bos taurus* (bovine heart) Fp+Ip subunits, strong signal of Fp (~70 kDa) was detected in addition to Ip (~30 kDa) at the lane of bovine heart. (3) Anti-*A. suum* complex II (adult form) antiserum (1:4000). Samples from left column to the right are: total *E. coli* lysate of insert-negative control (*E. coli* BL21(DE3) / pET15b, IPTG+), recombinant PfIp (*E. coli* BL21(DE3) / pET15b-PfIp, IPTG+), and *A. suum* complex II (adult form). Doublet signals at ~15 kDa (the upper one is very weak) at the lane of *Ascaris* are due to antibodies against membrane-anchoring (CybL+CybS) subunits.

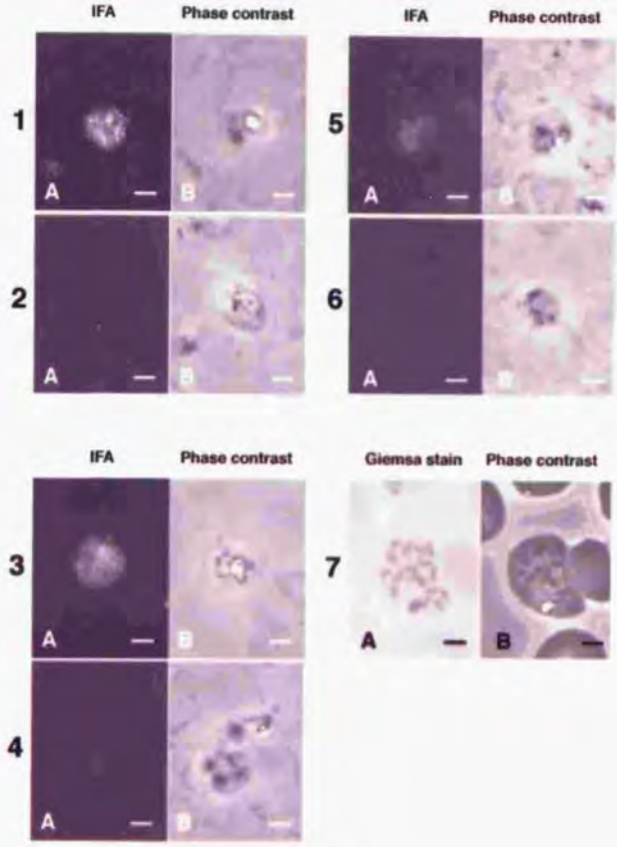

IFA
Phase contrast
IFA
Phase contrast
1
5
A
B
A
B
2
6
A
B
A
B
IFA
Phase contrast
Giemsa stain
Phase contrast
3
7
A
B
A
B
4
A
B

Fig. 12

Fig. 12 Immunofluorescence assay (IFA) on acetone-fixed *Plasmodium*-infected erythrocytes ((A) except for 7-A). The primary antibody was (rabbit) polyclonal antiserum to PfIp (1:100, panels 1/3/5) or pre-immune serum (1:100, panels 2/4/6) and the secondary was FITC-conjugated (goat) anti-rabbit IgG (1:50). (B) Phase-contrast image of the same field as (A). Panels 1/2: *P. falciparum* FCR3, 3/4: *P. falciparum* 3D7, 5/6: rodent malaria specie *P. yoelii* 17XL. Panel 7 shows *P. falciparum* FCR3 schizont stage parasite including typical hemozoin (also known as malaria pigment; polymerized heme derived from digested host hemoglobin.). 7-A: Giemsa stain, 7-B: phase-contrast image. Strong signals in phase contrast image (panels B) were identified as hemozoin. Bar=2 μm

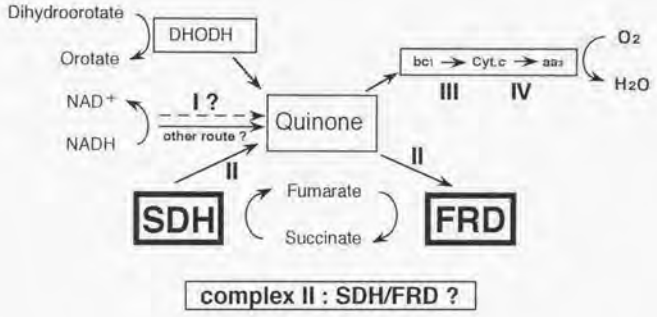
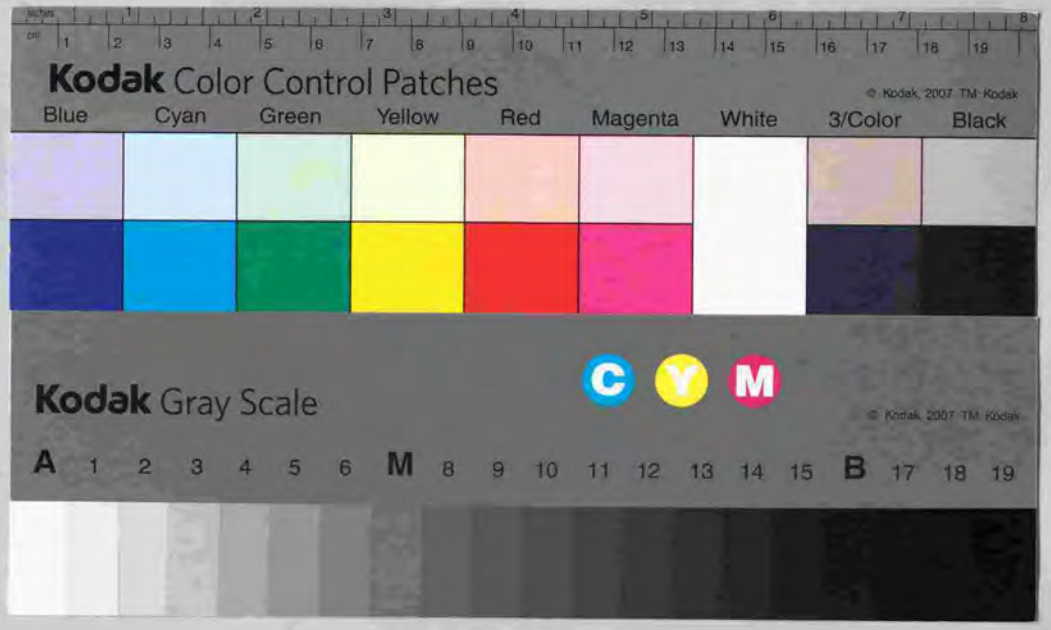


Fig. 13 Proposed pathways of electron transport in plasmodial mitochondria, from the result of this study and the reports so far. The straight lines show electron transfer. See the description on page 25. I, complex I; II, complex II; III, complex III; IV, complex IV; Cyt. *c*, cytochrome *c*; DHODH, dihydroorotate dehydrogenase (in the biosynthetic pathway of the pyrimidine nucleotides); NADH, nicotinamide adenine dinucleotide (reduced form).


Kodak Color Control Patches
© Kodak, 2007 TM: Kodak
Blue
Cyan
Green
Yellow
Red
Magenta
White
3/Color
Black
Kodak Gray Scale
C
Y
M
© Kodak, 2007 TM: Kodak
A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19