Molecular Microbiology (2005) **55**(1), 27–38 doi:10.1111/j.1365-2958.2004.04398.x

The human malaria parasite *Plasmodium falciparum* possesses two distinct dihydrolipoamide dehydrogenases

Paul J. McMillan,[1] Luciana M. Stimmler,[2] Bernardo J. Foth,[2†] Geoffrey I. McFadden[2] and Sylke Müller[1*]

[1]*Institute of Biomedical and Life Sciences, Infection and Immunity, University of Glasgow, Glasgow G12 8QQ, UK.*
[2]*Plant Cell Biology Research Centre, School of Botany, University of Melbourne, Parkville, VIC 3010, Australia.*

Summary

The *Plasmodium falciparum* genome contains genes encoding three α-ketoacid dehydrogenase multienzyme complexes (KADHs) that have central metabolic functions. The parasites possess two distinct genes encoding dihydrolipoamide dehydrogenases (LipDH), which are indispensable subunits of KADHs. This situation is reminiscent of that in plants, where two distinct LipDHs are found in mitochondria and chloroplasts, respectively, that are part of the organelle-specific KADHs. In this study, we show by reverse transcription polymerase chain reaction (RT-PCR) that the genes encoding subunits of all three KADHs, including both LipDHs, are transcribed during the erythrocytic development of *P. falciparum*. Protein expression of mitochondrial LipDH and mitochondrial branched chain α-ketoacid dihydrolipoamide transacylase in these parasite stages was confirmed by Western blotting. The localization of the two LipDHs to the parasite's apicoplast and mitochondrion, respectively, was shown by expressing the LipDH N-terminal presequences fused to green fluorescent protein in erythrocytic stages of *P. falciparum* and by immunofluorescent colocalization with organelle-specific markers. Biochemical characterization of recombinantly expressed mitochondrial LipDH revealed that the protein has kinetic and physicochemical characteristics typical of these flavo disulphide oxidoreductases. We propose that the mitochondrial LipDH is part of the mitochondrial α-ketoglutarate dehydrogenase and branched chain α-ketoacid dehydrogenase complexes and that the apicoplast LipDH is an integral part of the pyruvate dehydrogenase complex which occurs only in the apicoplast in *P. falciparum*.

Accepted 16 September, 2004. *For correspondence. E-mail s.muller@bio.gla.ac.uk; Tel. (+44) 141 330 2383; Fax (+44) 141 330 4600. †Present address: Département de Microbiologie et Médecine Moleculaire, CMU – Faculté de Médecine, Université de Genève, CH-1211 Genève 4, Switzerland.

Introduction

Dihydrolipoamide dehydrogenase (LipDH) is an integral component of the α-ketoacid dehydrogenase multienzyme complexes (KADHs) pyruvate dehydrogenase (PDH; EC: E1: 1.2.4.1; E2: 2.3.1.12; E3: 1.8.1.4), α-ketoglutarate dehydrogenase (KGDH; EC: 1.2.4.2; E2: 2.1.3.61; E3: 1.8.1.4) and branched chain α-ketoacid dehydrogenase (BCKDH; EC: E1: 1.2.4.4; E2: 2.3.1.168, E3: 1.8.1.4) as well as the glycine decarboxylase complex (Perham *et al*., 1996; Douce *et al*., 2001; Mooney *et al*., 2002). KADHs are composed of three subunits, a decarboxylase or E1 subunit, a dihydrolipoamide transacylase or E2 subunit, and the LipDH or E3 subunit. The decarboxylase and transacylase subunits of different KADHs are specific for their substrates but the complexes generally share the same LipDH subunit, although there are reports of LipDH isozymes that are specific for one of the KADHs (Carothers *et al*., 1989). The glycine decarboxylase system comprises a pyridoxal phosphate-containing protein (P-protein), a hydrogen carrier protein (H-protein), a T-protein, which requires tetrahydrofolate as cofactor, and an L-protein, which is equivalent to LipDH. LipDH is responsible for the re-oxidation of dihydrolipoamide that is covalently bound to the transacylase subunit of KADHs or the H-protein of the glycine decarboxylase complex (Koike *et al*., 1963; Mooney *et al*., 2002).

In mammalian cells, KADHs are located solely in the mitochondria where PDH provides acetyl-CoA and NADH for the tricarboxylic acid (TCA) cycle and oxidative phosphorylation, and KGDH is an integral part of the TCA cycle. Plants possess distinct organelle-specific PDHs – one acting as a link between glycolysis and the TCA cycle in mitochondria, the other providing acetyl-CoA and NADH for essential metabolic pathways such as fatty acid biosynthesis in the chloroplast (Mooney *et al*., 2002). Accordingly they require distinct *lipdh* genes encoding proteins that are targeted to their mitochondria and plas-

tids respectively (Lutziger and Oliver, 2001; Mooney *et al*., 2002).

The human malaria parasite *Plasmodium falciparum* is a major health threat in the developing world and the increasing incidence of drug resistance emphasizes that new therapies are urgently required to combat the disease which claims 1–2 million lives annually. *Plasmodium* and other apicomplexan parasites possess a relict plastid, the so-called apicoplast (McFadden *et al*., 1996; Fichera and Roos, 1997). As such an organelle is not present in humans, the elucidation of its roles in parasite survival has attracted considerable interest. It has been found that type II fatty acid biosynthesis and non-mevalonate isoprenoid biosynthesis both occur in the organelle and that as these pathways differ fundamentally from those occurring in mammals they offer excellent potential for therapeutic intervention (Jomaa *et al*., 1999; Surolia and Surolia, 2001; Waller *et al*., 2003).

It has been found that *Plasmodium* is similar to plants in that it possesses a PDH in its plastid, but contrary to plants the parasite apparently lacks a mitochondrial PDH (Gardner *et al*., 2002; Foth *et al*., 2004). However, genes encoding KGDH and BCKDH are present in the parasite genome and these are predicted to be mitochondrial (Gardner *et al*., 2002). In agreement with this distribution of KADHs, it has recently been shown that both *P. falciparum* and *Toxoplasma gondii* contain organelle-specific lipoylation pathways which provide lipoic acid, the essential cofactor for KADHs, to the apicoplast and mitochondrial KADHs (Thomsen-Zieger *et al*., 2003; Wrenger and Müller, 2004). This apparent division of KADHs into the apicoplast and mitochondrion suggested that *P. falciparum* possesses distinct organelle-specific LipDHs that interact with the apicoplast and mitochondrial KADHs respectively. This study was undertaken to determine whether this is the case and whether the complexes all function in the erythrocytic stages of the parasite.

Results

Identification of α-ketoacid dehydrogenase complexes in P. falciparum

Searching the *P. falciparum* genome database revealed that the parasite contains genes encoding the components of three different KADHs and some of the components comprising a glycine decarboxylase complex (Table 1). The components of BCKDH and KGDH as well as the H- and T-proteins of the glycine decarboxylase complex all have predicted mitochondrial targeting peptides at their N-termini. The genome analysis also revealed a single apicoplast-specific PDH, as detailed by Foth *et al*. (2004), but surprisingly the lack of a mitochondrial version of this enzyme complex. Consistent with the potential distribution of *Plasmodium* KADHs in different cellular compartments (apicoplast and mitochondrion), two distinct *lipdh* genes were found – one with a putative apicoplast targeting sequence and one with a potential mitochondrial targeting sequence.

Sequence analyses of lipdh

The predicted mitochondrial *lipdh* gene encodes a protein of 513 amino acids and a theoretical molecular size of 57.2 kDa. The precise cleavage site for the mitochondrial transit peptide is unknown but is predicted to be between

Table 1. Genes encoding the components of α-ketoacid dehydrogenase complexes and the glycine decarboxylase complex in *P. falciparum*.

				Prediction scores[a]			
Gene	Chromosome	Localization	Evidence	PLASMOAP	MITOPROT	TARGETP	Presequence
PDH E1α	11	Apicoplast	Experimental[b]	++	15%	S1	Yes
PDH E1β	14	Apicoplast	Predicted	++	69%	S2	Yes
PDH E2	10	Apicoplast	Experimental[b]	++	81%	S3	Yes
BCKDH E1α	13	Mitochondrion	Predicted	–	99%	M3	Yes
BCKDH E1β	5	Mitochondrion	Predicted	–	99%	M3	Yes
BCKDH E2	3	Mitochondrion	Predicted	–	92%	M4	Yes
KGDH E1	8	Mitochondrion	Predicted	–	91%	M4	Yes
KGDH E2	13	Mitochondrion	Predicted	–	95%	M3	Yes
T-protein	13	Mitochondrion	Predicted	–	82%	M5	Yes
H-protein	11	Mitochondrion	Predicted	–	98%	M5	Yes
aLipDH	8	Apicoplast	Experimental[c]	+	69%	S1	Yes
mLipDH	12	Mitochondrion	Experimental[c]	–	91%	M4	Yes

a. Predictions for the presence of potential mitochondrial targeting sequences or the presence of a potential signal peptide and apicoplast transit peptide were performed using PLASMIT, MITOPROT, TARGETP, SIGNALP and PLASMOAP (Claros and Vincens, 1996; Nielsen *et al*., 1997; Emanuelsson *et al*., 2000; Bender *et al*., 2003; Foth *et al*., 2003). TARGETP prediction scores are abbreviated as M (for mitochondrion) or S (for signal peptide) followed by a number ranging from 1 to 5 that indicates the 'reliability' of the prediction with a smaller number representing a more reliable prediction.
b. N-terminal presequences of the E1α and E2 PDH subunits conferred transport of GFP into the organelle as shown by (Foth *et al*., 2004).
c. N-terminal presequences of aLipDH and mLipDH target GFP fusions into apicoplast and mitochondrion respectively (this study).

amino acids 19 and 20 (using TARGETP) or between amino acids 26 and 27 (using MITOPROT). Analyses of the mitochondrial *lipdh* cDNA showed that the gene contains one intron of 216 bp which separates the two exons (71 bp and 1468 bp) close to the predicted boundary between the mitochondrial targeting peptide and the mature protein.

The apicoplast *lipdh* gene encodes a larger protein of 666 amino acids and a molecular mass of 75.6 kDa. The size difference between mLipDH and aLipDH polypeptides mainly results from the long putative bipartite targeting sequence. This comprises a hydrophobic signal peptide of 34 amino acids (Nielsen *et al*., 1997) followed by a hydrophilic region of approximately 90 amino acids which represents the potential apicoplast transit peptide (Foth *et al*., 2003). The open reading frame (ORF) of the apicoplast LipDH is located on a single exon and the length of the potential bipartite apicoplast targeting peptide can only be estimated by comparison with those known for other proteins. On this basis, it is estimated that the mature protein probably starts before or around amino acid 125.

The deduced amino acid sequences of both *lipdh* genes were aligned to the human and pea mitochondrial LipDH sequences as well as the deduced amino acid sequences of *Plasmodium berghei* obtained from the Sanger genome database (Fig. S1). The mitochondrial LipDH has 35% and 39% identity to the human and pea proteins, respectively, but its identity with the apicoplast LipDH is only 20%. The apicoplast LipDH itself has only 21% identity with the human and pea mitochondrial LipDHs. This is partly attributable to the long presequence of aLipDH – if the N-terminal sequence is omitted from the alignments, the degree of identity increases to 24% with mLipDH and to 25% with both human and pea LipDHs. The mLipDHs of *P. falciparum* and *P. berghei* are 78.1% identical and the aLipDH of the two *Plasmodium* species are 68.3% identical. Both *P. falciparum* and *P. berghei mlipdh* genes appear to possess an intron at the same position. The presence of this intron has been experimentally confirmed for the *P. falciparum* gene, but is only predicted for the *P. berghei* gene. However, the high amino acid sequence similarity between the two *Plasmodium* translated genes suggests that exon 1 also exists in *P. berghei mlipdh.* Phylogenetic analyses of both mLipDH and aLipDH strongly suggest that aLipDH is more closely related to proteins from cyanobacteria and plant plastids whereas the phylogenetic relationships of the mitochondrial protein are uncertain (Foth *et al*., 2004).

Despite the low degree of identity with the human and pea mitochondiral LipDHs, both *Plasmodium* proteins possess the residues required for cofactor binding and catalytic activity. The dinucleotide binding motif GXGXXG is part of the so-called Rossman fold which is characteristic of this class of flavo disulphide oxidoreductases (Schierbeek *et al*., 1989; Mattevi *et al*., 1991). It is present in mLipDH and aLipDH at positions 31–36 and 132–137 respectively. In addition, the catalytic centre containing the redox active cysteine residues (CV/LXGC) that interact with the protein-bound flavin prosthetic group and the substrate (Thorpe and Williams, 1976) are conserved in both *Plasmodium* proteins (positions 63–68 and 165–170 for mLipDH and aLipDH respectively). At positions 219–235 and 328–344 of mLipDH and aLipDH, respectively, the proteins possess a second nucleotide binding motif (GXGXIGXEXXXV/IXXXXG) which is responsible for NAD(H) binding (Bocanegra *et al*., 1993). In the interface domain, which is involved in the formation of the homodimers, both proteins have a conserved HPTXXE motif containing the active site histidine base which acts as a proton acceptor/donor (Williams, 1976). This histidine and the neighbouring glutamic acid are important for the stabilization of the active site thiolate during catalysis (Williams *et al*., 1989; Kim and Patel, 1992).

Expression of KADH in the erythrocytic stages of P. falciparum

Two lines of evidence suggest that the KADHs are expressed in the erythrocytic stages of *P. falciparum*. Reverse transcription polymerase chain reaction (RT-PCR) on total RNA isolated from the parasites resulted in the amplification of bands corresponding to the subunits of the PDH, KGDH and BCKDH complexes after their predicted introns were spliced from the genes, as verified by sequencing the PCR products (Fig. 1). Furthermore, it was shown by Western blotting that mLipDH and branched chain α-ketoacid dehydrogenase dihydrolipoamide transacylase subunit (bcE2) are expressed on the protein level (Fig. 2). Bands corresponding to the predicted mature sizes of mLipDH (56 kDa) and bcE2 (51 kDa) were detectable using polyclonal antibodies raised against each recombinant protein in rabbits. No bands corresponding to the non-processed form of either protein were detectable on the blots, suggesting that the mitochondrial targeting sequences are removed from these proteins faster than has been reported for apicoplast targeting peptides (Waller *et al*., 2000; van Dooren *et al*., 2002).

Characteristics of mitochondrial LipDH

An N-terminally truncated version of mLipDH (amino acids 25–513) lacking the putative mitochondrial targeting sequence was recombinantly expressed. Expression of the construct resulted in the generation of soluble protein that was purified to 98% homogeneity by Ni^{2+}-chelating agarose as shown by SDS-PAGE (Fig. S2). The yield of active protein was about 100 μg per litre of bacterial cul-

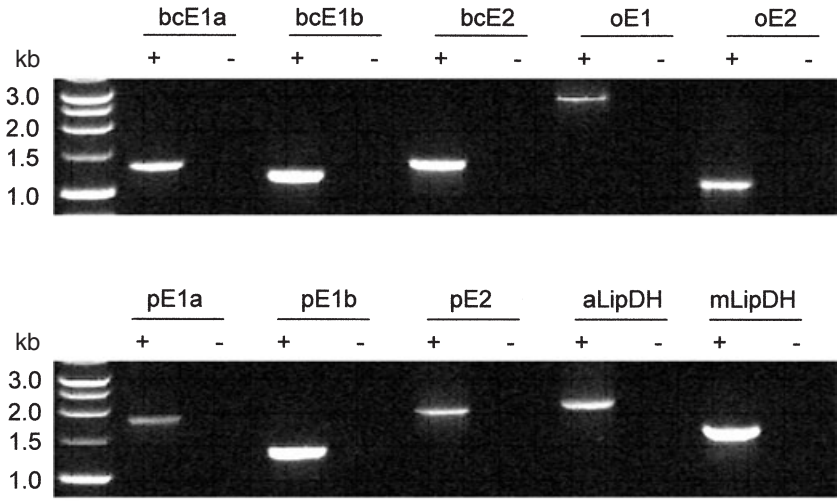

Fig. 1. Reverse transcription polymerase chain reaction (RT-PCR) to identify transcription of α-ketoacid dehydrogenase complexes in *P. falciparum*. Using total RNA isolated from *P. falciparum* erythrocytic stages, components of all three KADH were amplified (+) as specified in *Experimental procedures*. Negative control RT-PCRs (–) were performed on RNA samples not reverse transcribed to test for the presence of DNA in the RNA preparation. bcE1a, branched chain α-ketoacid dehydrogenase decarboxylase subunit E1α; bcE1b, branched chain α-ketoacid dehydrogenase decarboxylase subunit E1 β; bcE2, branched chain α-ketoacid dehydrogenase dihydrolipoamide transacylase subunit E2; oE1, α-ketoglutatrate dehydrogenase decarboxylase subunit E1; oE2, α-ketoglutatrate dehydrogenase subunit dihydrolipoamide transsuccinylase subunit E2; pE1a, pyruvate dehydrogenase dehydrogenase decarboxylase subunit E1α; pE1b, pyruvate dehydrogenase decarboxylase subunit E1β; pE2, pyruvate dehydrogenase dihydrolipoamide transacetylase subunit E2; aLipDH, apicoplast dihydrolipoamide dehydrogenase; mLipDH, mitochondrial dihydrolipoamide dehydrogenase.

ture, as determined by the absorption spectra of the purified protein (Fig. 3). The spectrum of the oxidized protein has a peak at 460 nm with two shoulders at 430 and 480 nm, consistent with the presence of flavin adenine dinucleotide (FAD) as the prosthetic group. When reduced by a 20-fold molar excess of NADH, the absorption spectrum changed – the peak at 460 nm was bleached and shifted towards 440 nm and a new peak at 530 nm appears. This spectral change is indicative of the formation of a charge transfer complex between the protein-bound flavin and one of the active site cysteine residues and is consistent with observations made for flavo disulphide oxidoreductases from other sources (Ghisla *et al*., 1974; Thorpe and Williams, 1976). Addition of FAD during the purification procedure increased the yield of active protein from 57% to 87%.

The optimal pH values for the mLipDH forward and reverse reactions were determined to be pH 9.0 and pH 7.0 respectively (Table 2). The forward reaction was followed by stopped flow fast kinetics as the reaction is inhibited by NADH (Sahlman and Williams, 1989). Using this method, the K_{mapp} for dihydrolipoamide and NAD^+ were found to be 146 ± 15 μM and 0.45 ± 0.03 mM, respectively, which is comparable to those of other LipDH proteins (Fig. S3) (Kim *et al*., 1991; Else *et al*., 1994; Schöneck *et al*., 1997; Argyrou and Blanchard, 2001). The apparent K_m values for NADH and lipoamide for the reverse reaction of mLipDH were determined to be 20.8 ± 5.6 μM and 0.87 ± 0.27 mM respectively (Fig. S4). The *P. falciparum* mLipDH displays ping-pong kinetics, similar to other members of the disulphide oxidoreductase family (Reed, 1973; Vanoni *et al*., 1990), where the protein is reduced by either NADH or dihydrolipoamide before the

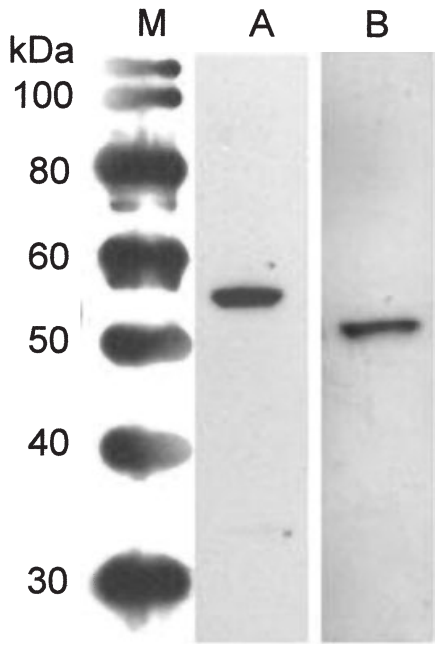

Fig. 2. Western blot analyses of mitochondrial LipDH and mitochondrial branched chain ketoacid dihydrolipoamide transacylase. In order to investigate whether mLipDH (mE3) and another component of a KADH (branched chain α-ketoacid dihydrolipoamide transacylase subunit E2, bcE2) complex are expressed in the erythrocytic stages of *P. falciparum*, Western blot analyses were performed and probed with polyclonal antibodies raised against recombinant mLipDH (1:250) and recombinant bcE2 (1:250) (P.J. McMillan and S. Müller, unpubl.). The bands obtained for mLipDH and bcE2 correspond well with the sizes of the predicted mature proteins (without mitochondrial targeting sequences) of 56 kDa and 51 kDa respectively. Only the mature forms of both proteins were detected, suggesting that the processing of the mitochondrial targeting sequence is an instantaneous process unlike the situation reported for apicoplast proteins (Waller *et al*., 2000; van Dooren *et al*., 2002).

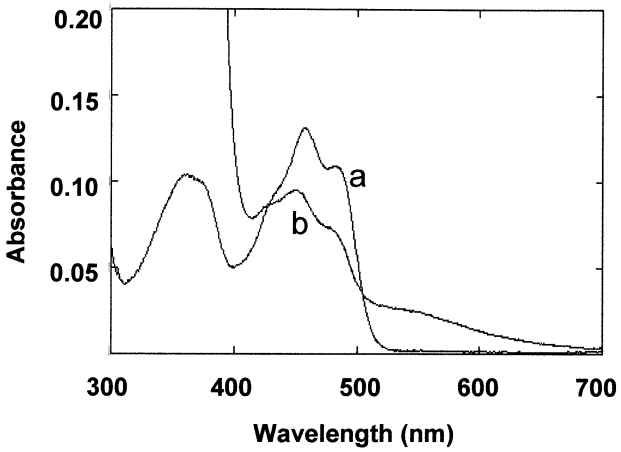

Fig. 3. Oxidized and reduced spectra of mitochondrial LipDH. Purified mLipDH (6.3 μM) was spectrally analysed under oxidizing conditions (line a) and with addition of an excess of NADH (line b). The oxidized spectrum clearly shows the typical peaks at 380 nm and 460 nm, with shoulders at 440 nm and 480 nm. The 460 nm band was lower in the reduced spectrum and a new band at 530 nm appeared which is diagnostic of a flavin-thiolate charge transfer complex.

reducing equivalents are transferred to the second substrate lipoamide or NAD^+ to leave the protein in the oxidized state (Fig. S5). As expected, the true K_m values for NADH and lipoamide established by this method are in good agreement with the apparent K_m values (Table 2).

The recombinant expression of aLipDH resulted in very low yields of <4 μg of protein per litre of bacterial culture. This low amount of protein was insufficient for activity analyses as carried out with the recombinat mLipDH.

Localization of mitochondrial and apicoplast LipDH

Bioinformatic analyses of both *lipdh* genes revealed a potential bipartite apicoplast presequence in one gene and a putative mitochondrial transit peptide in the other (Table 1 and Fig. S1). The subcellular localization of both proteins was analysed by immunofluorescent studies on *P. falciparum* parasites overexpressing fusion proteins of the predicted LipDH presequences including the first few amino acids of the mature proteins (134 amino acids for aLipDH and 33 amino acids for mLipDH) followed by green fluorescent protein (GFP). The presequence of aLipDH resulted in targeting of the GFP marker protein to apicoplast as evidenced by colocalization with the apicoplast-resident acyl-carrier protein (ACP) (Fig. 4A). In addition, microscopy on live parasites transfected with aLipDH–GFP and co-stained with MitoTracker Red clearly showed GFP fluorescence in an organelle closely apposed to but clearly distinct from the mitochondrion (Fig. 4B), as has previously been reported for the apicoplast (Waller *et al.*, 2000). The distinct morphological changes of the apicoplast that occur during intraerythrocytic parasite development from rings to schizont (Waller *et al.*, 2000) were also observed (data not shown) and further supported the correct identification of this organelle.

In contrast, colocalization experiments with the apicoplast marker ACP showed the mLipDH–GFP fusion protein to be present in an organelle different from the apicoplast (Fig. 4C; mitochondrion in green, apicoplast in red). Further colocalization studies employing MitoTracker Red and an antibody directed against the mitochondrial protein HSP60 unambiguously identified the organelle containing the mLipDH–GFP fusion as the mitochondrion (Fig. 4D and E).

Discussion

Dihydrolipoamide dehydrogenases are essential components of KADHs, which are multienzyme complexes usually present in the mitochondria of eukaryotes. Plastid-containing organisms such as plants and algae possess two distinct LipDHs, one in the mitochondria and the other in the plastids (Mooney *et al.*, 2002). In analogy, two different *lipdh* genes were identified in the genome of the malaria parasite *P. falciparum.* Analyses of the deduced amino acid sequences of the two LipDHs revealed that one of the proteins is potentially targeted to the parasite's apicoplast, as it possesses a typical bipartite apicoplast targeting sequence (Foth *et al.*, 2003), whereas the second LipDH potentially is mitochondrial. These predictions

Table 2. Parameters of mitochondrial LipDH.

	mLipDH	*T. cruzi*[a]	*Homo sapiens*[b]
pH optimum (forward)	pH 9.0	pH 7.5	ND
pH optimum (reverse)	pH 7.0	pH 7.0	ND
Subunit size	57.2 kDa	50 kDa	54.2 kDa
Oligomeric state	Dimer[c]	Dimer	Dimer
K_{mapp} NAD^+	0.45 ± 0.03 mM	0.6 mM	ND
K_m NAD^+	ND	ND	0.29 mM
K_{mapp} NADH	20.8 ± 5.6 μM	25 μM	ND
K_m NADH	28 ± 4 μM	ND	51 μM
K_{mapp} lipoamide	0.87 ± 0.27 mM	0.8 mM	ND
K_m lipoamide	1.72 ± 0.23 mM	ND	1.01 mM
K_{mapp} dihydrolipoamide	146 ± 15 μM	130 μM	ND
K_m dihydrolipoamide	ND	ND	570 μM
k_{cat} forward	135.5–337.0 s^{-1}[d]	244 s^{-1}	382 s^{-1}
k_{cat} reverse	448 s^{-1}	248 s^{-1}	167 s^{-1}

a. Data from Schöneck *et al.* (1997).
b. Data from Kim *et al.* (1991).
c. The oligomeric state of mLipDH was estimated by gel filtration on a Superdex S-200 column previously calibrated with appropriate molecular mass standards from Bio-Rad.
d. The k_{cat} determined for the forward reaction was dependent on the enzyme preparation and the assay system used (see Fig. S3).
The steady state kinetic data of *P. falciparum* mLipDH are means (± standard errors) from three independent experiments performed in duplicate.
ND, not determined.

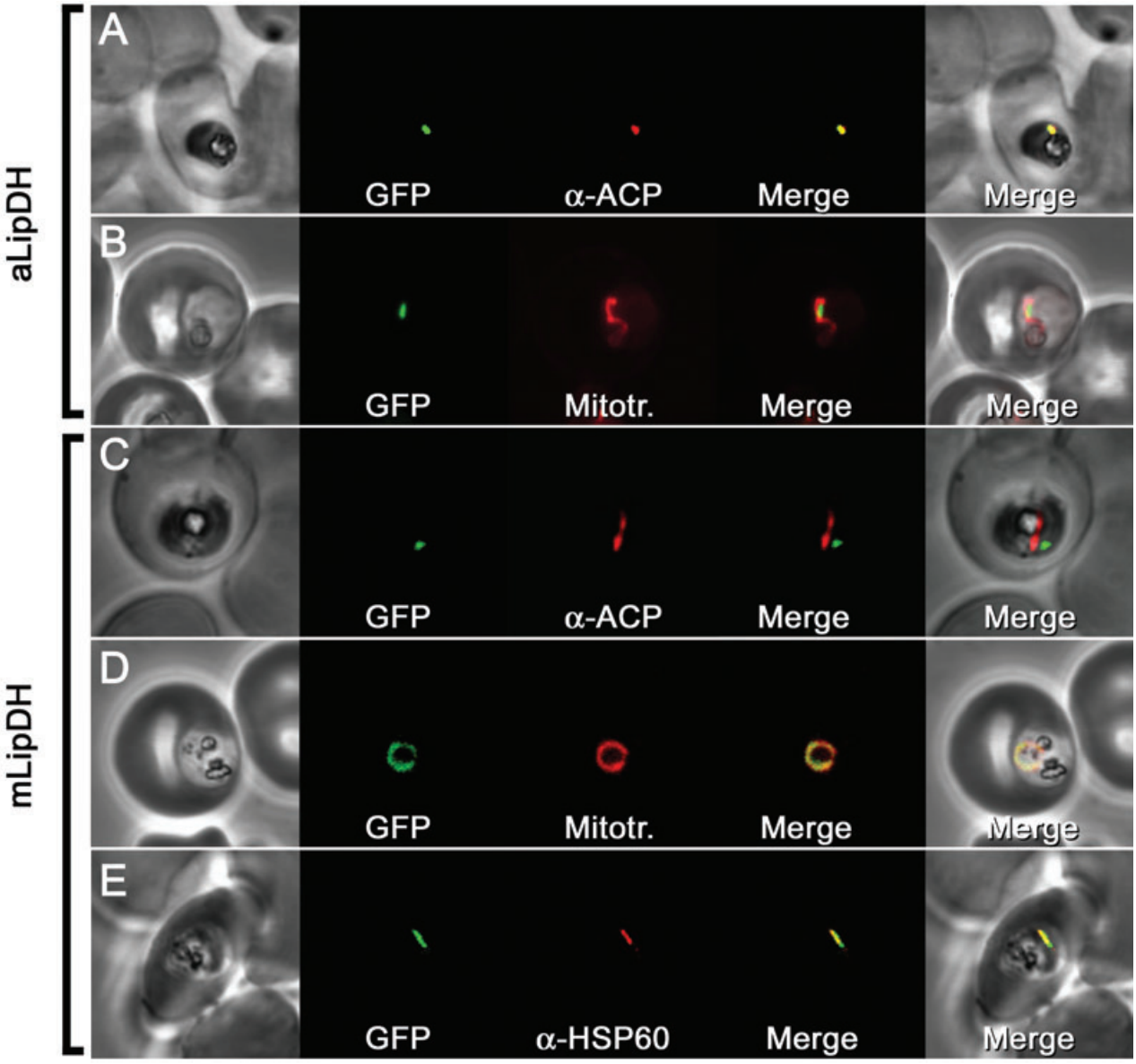

Fig. 4. Localization of apicoplast and mitochondrial LipDH. The localization of aLipDH and mLipDH was investigated by expressing the putative N-terminal presequences fused to GFP in *P. falciparum* intraerythrocytic parasites.
A and B. Colocalization with antibodies directed against the apicoplast-resident acyl-carrier protein (ACP; A) and with MitoTracker Red (B) demonstrates that the putative apicoplast targeting presequence of aLipDH targets the marker protein GFP exclusively to the apicoplast.
C–E. Colocalization experiments with the apicoplast protein ACP (C) and with the two mitochondrial markers MitoTracker Red and HSP60 (D and E) indicate that mLipDH is localized exclusively in the mitochondrion.

were verified by expressing GFP fusion proteins in *P. falciparum* parasites, showing that the N-terminal presequences of aLipDH and mLipDH are sufficient to exclusively target the reporter protein GFP either to the apicoplast or to the mitochondrion respectively. The unambiguous identification of the two organelles was facilitated by colocalization of the GFP fusion proteins with MitoTracker Red as well as with antibodies directed against the organelle-specific proteins ACP (apicoplast) and HSP60 (mitochondrion). These data, together with those of Foth *et al*. (2004), strongly suggest that *P. falciparum* possesses a single PDH targeted to the apicoplast, and that the apicoplast-located LipDH forms an integral part of this plastidic multienzyme complex, whereas the mitochondrial LipDH is a component of the mitochondrial KADHs and glycine decarboxylase complex.

Candidate genes encoding all components of potential BCKDH and KGDH multienzyme complexes are present in the *P. falciparum* genome and appear to possess mitochondrial targeting sequences (see Table 1). Intriguingly

for the glycine decarboxylase complex, only genes potentially encoding the T-protein and H-protein were identified – no gene for the P-protein was found. Possibly the *Plasmodium* P-protein has so little similarity to those of other organisms that it was not recognized in our search or the glycine decarboxylase complex uses the decarboxylase component of one of the other mitochondrial complexes. In addition, the genes for a single PDH complex are present and all have potential apicoplast targeting sequences. The genes encoding subunits of all three KADH complexes are transcribed in the erythrocytic stages of *P. falciparum*. Moreover, mLipDH and bcE2 proteins were also detected by Western blotting in parasite erythrocytic extracts. These results are in agreement with investigations of the *P. falciparum* transcriptome and proteome, which show expression of KADHs in the erythrocytic stages of the parasites (Florens *et al*., 2002; Bozdech *et al*., 2003; Le Roch *et al*., 2003). According to these investigations, the expression levels of KADHs increase in gametocytes compared with asexual erythrocytic stages, implying an alteration in the metabolic requirements upon transformation (Florens *et al*., 2002; Bozdech *et al*., 2003; Le Roch *et al*., 2003).

The occurrence of PDH just in the apicoplast raises the question how the mitochondrial TCA cycle and respiratory chain are fuelled if not by acetyl-CoA and NADH generated by PDH. Possibly the parasites have developed metabolic alternatives that guarantee the supply of essential reducing equivalents to the mitochondrion. One potential way to compensate for the absence of PDH in the mitochondrion is the conversion of phosphoenolpyruvate to oxaloacetate by phosphoenolpyruvate carboxylase in the cytosol and the subsequent generation of malate by cytosolic malate dehydrogenase (Tripathi *et al*., 2004). Malate could then be transported into the mitochondrion where it could provide reducing equivalents to malate:ubiquinone oxidoreductase (Uyemura *et al*., 2004). The genes encoding these proteins were identified in the parasite genome (Gardner *et al*., 2002) and it has been suggested that the supply of malate to the mitochondrion might be one of the roles of the parasite's cytosolic malate dehydrogenase (Tripathi *et al*., 2004). Another interesting finding is that BCKDH is actively expressed in the erythrocytic stages of *P. falciparum*. This enzyme complex is generally involved in the catabolism of the amino acids valine, leucine and isoleucine resulting in the generation of acyl-CoAs including acetyl-CoA. The parasite is thought to acquire these amino acids during its erythrocytic life by degrading haemoglobin and it is possible that the amino acids are not only used for protein *de novo* synthesis but also for the generation of acyl-CoA intermediates that can be fed into the TCA cycle and thus compensate for the absence of a mitochondrial PDH (Ward *et al*., 1999; Holecek, 2002). This hypothesis is consistent with the report that only 20% of the amino acids derived from haemoglobin digestion are used for protein synthesis by *P. falciparum* (Krugliak *et al*., 2002). The presence of these alternative pathways also may be a means to compensate for the potential loss of TCA cycle intermediates such as succinyl-CoA, the product of KGDH, which is required for metabolic processes such as porphyrin biosynthesis (Vaidya, 1996).

The biochemical characterization of recombinant *P. falciparum* mLipDH revealed that the protein forms homodimers and has one FAD bound per subunit, similar to all other LipDH proteins (Williams *et al*., 1984). The steady state kinetic parameters of the protein are in good agreement with those of LipDHs of other organisms (Kim *et al*., 1991; Else *et al*., 1994; Schöneck *et al*., 1997; Argyrou and Blanchard, 2001). The forward reaction is inhibited by NADH, as has been reported for the *Escherichia coli* enzyme (Sahlman and Williams, 1989). Therefore, stopped flow fast kinetics was employed to determine the initial reaction rates of this reaction. They were found to conform to those expected for LipDHs. The spectral analysis of mLipDH revealed the typical spectra of a flavo disulphide oxidoreductase under oxidized and reduced conditions (Ghisla *et al*., 1974). Thus the mLipDH of *P. falciparum* has kinetic and physicochemical characteristics typical of these flavo disulphide oxidoreductases.

This discovery that *P. falciparum* has organelle-specific LipDHs has paved the way for studies aimed at elucidating the precise metabolic functions of the plasmodial KADHs in the two organelles and whether or not they have potential as targets for the design of parasite-specific inhibitors that could be useful anti-malarial drugs.

Experimental procedures

Materials

The ECL$^+$ Western blot detection system was purchased from Amersham Biosciences, UK. Albumax II and RPMI 1640 were from Invitrogen Corporation. WR 99210 was a kind gift of Dr Jacobus, Jacobus Pharmaceuticals, USA. The plasmid cDNA library was a kind gift of Dr D. Kaslow, National Institute of Health, Bethesda, USA.

Database searches and bioinformatics

The genes apparently encoding two distinct *lipdh* genes were identified in the *Plasmodium* genome database PlasmoDB (http://www.PlasmoDB.org) on chromosome 8 and 12, respectively (The *Plasmodium* genome database collaborative, 2001) by performing TBLASTN searches using the human LipDH protein sequence (Accession No. NP 000099). In addition, database searches were performed using the human protein sequences of KADHs and the glycine decarboxylase complex. The translated protein sequences identified were queried back against the SWISSPROT protein database at NCBI (http://www.ncbi.nlm.nih.gov/blast) using BLASTP. Pre-

Table 3. Oligonucleotides used in this study.

Gene	Orientation	sequence of the primer (5′→3′)
PDH E1α	Sense	GCGC**GGATCC**CGATGTTTAATTACGTTTTATGTGTAGG
	Anti-sense	CGCG**GGGCCC**TTAATCTATTATTAAGG
PDH E1β	Sense	GCGC**GGATCC**CGATGGGGAGAAAAAGAAAC
	Anti-sense	CGCG**GGGCCC**TCAAGATGATAGCGAATGAAG
PDH E2	Sense	GTATTTCTAAGAACAATAATTACGG
	Anti-sense	CTATAAAAATATTTTCATAATATCC
BCKDH E1α	Sense	GCGC**CATATG**AGAAATATTGTTCAGAAATACTTACAAAGG
	Anti-sense	CGCG**CTCGAG**TCATCGCTCAAATTTTGATGTATC
BCKDH E1β	Sense	GCGC**CATATG**ATGAGACTATTAAGAAATAACG
	Anti-sense	CGCG**CTCGAG**TTACTTCATCATTTTTTTGACTTCG
BCKDH E2	Sense	GCGC**CATATG**TTTGTGAAGAATGTACTAAACG
	Anti-sense	GCGC**CTCGAG**TTATTCCAATAGTGGTCCTAGGGATGC
KGDH E1	Sense	**GCTAGC**ATGCTTGAAAAAGGGAAAACAG
	Anti-sense	**AAGCTT**TTATAACGCATCGGTATATTTTTG
KGDH E2	Sense	GCGC**AAGCTT**ATGTCCATAGAAACCATTAAAGTACC
	Anti-sense	CGCG**GGATCC**TTAACAATCAATTAACATTAGATTAGG
A1	Sense	GCGC**AAGCTT**ATGGTCATAAGGCAAAATATTAAAC
A2	Anti-sense	GCGC**GGATCC**TAGTGAGTTCTTATTTTTGATATAGATTTAAAAGC
A_exp	Sense	GCGC**AAGCTT**ATGAACATTAATGAAAAAGAATATGATCTTGCTATAATCGG
M1	Sense	GCGC**CATATG**TCAACTAAGAAAGACTATGATGTTATAGTCATTGG
M2	Anti-sense	**GGATCC**TTACATGTGTATAGGTTTATC
M_exp	Sense	**CATATG**TATGATGTTATAGTCATTGG
A_target	Sense	GGGGACAAGTTTGTACAAAAAAGCAGGCTTA**AGATCT**ATGGTCATAAGGCAAAATATTAAACATATCGTTAAAC
	Anti-sense	GGGGACCACTTTGTACAAGAAAGCTGGGTT**CCTAGG**ACCACAACCGATTATAGCAAGATCATATTCTTTTTC
M_target	Sense	GGGGACAAGTTTGTACAAAAAAGCAGGCTTA**AGATCT**ATGAACAGCGTTATTTTTAGAGCACATTG
	Anti-sense	GGGGACCACTTTGTACAAGAAAGCTGGGTT**CCTAGG**CCCTCCTCCAATGACTATAACATCATAG

Restriction sites are in bold letters.

diction of N-terminal targeting sequences was performed using SIGNALP (http://www.cbs.dtu.dk/services/SignalP) (Nielsen *et al*., 1997), TARGETP (http://www.cbs.dtu.dk/services/TargetP) (Emanuelsson *et al*., 2000), MITOPROT (http://ihg.gsf.de/ihg/mitoprot.html) (Claros and Vincens, 1996), PLASMIT (http://gecco.org.chemie.uni-frankfurt.de/plasmit) (Bender *et al*., 2003) and PLASMOAP (http://www.PlasmoDB.org/restricted/PlasmoAPcgi.shtml) (Foth *et al*., 2003). Nucleotide and protein sequence analyses were performed using VECTOR NTI (Informax) and GENERUNNER. Alignments of *P. falciparum* translated *lipdh* genes with those of other *Plasmodium* species were created using CLUSTALW and were manually adjusted.

Parasite culture

Plasmodium falciparum 3D7 (the Netherlands) were cultivated according to Trager and Jensen (1976) with modifications in human A^+ erythrocytes, RPMI 1640 containing 11 mM glucose, with the addition of 0.5% Albumax II. In order to isolate soluble protein from the parasites, infected erythrocytes were saponin-lysed (Umlas and Fallon, 1971) and the resulting pellets were resuspended in phosphate-buffered saline (PBS) containing EDTA-free protease inhibitor cocktail (Roche) and parasites were lysed by freeze/thawing. Isolation of total RNA was performed using saponin-lysed parasites that were immediately transferred into Trizol (Invitrogen) and RNA was isolated according to the manufacturer's instructions.

Reverse transcriptase polymerase chain reaction

In order to confirm that the decarboxylase, dihydrolipoamide transacylase and LipDH subunits of the PDH, KGDH and BCKDH are transcribed in *P. falciparum* erythrocytic stages, an RT-PCR was performed on total RNA from *P. falciparum* isolated as described above. RNA was reverse transcribed for 45 min at 50°C using Superscript II (Invitrogen) and random hexamers (Invitrogen) according to standard protocols (Sambrook *et al*., 1989). Reverse transcribed RNA was used as a template for a subsequent PCR using sense and anti-sense oligonucleotides specific for the subunits of the three KADHs (see Table 3). Negative controls were performed using RNA samples that were not reverse-transcribed before PCR in order to confirm that no DNA contamination was present in the samples. The PCR fragments were cloned into TOPO Blunt (Invitrogen) and their sequence confirmed.

Cloning of putative apicoplast and mitochondrial lipdh *coding regions*

The full-length coding region of the apicoplast *lipdh* gene was amplified by RT-PCR using the sequence specific oligonucleotides A1 and A2 containing *Hin*dIII and *Bam*HI restriction sites and a plasmid cDNA library as a template. Mitochondrial *lipdh* was amplified using the sequence-specific oligonucleotide M1 and anti-sense oligonucleotide M2 from total RNA or the *P. falciparum* cDNA library. The PCR fragments were cloned into TOPO Blunt (Invitrogen) and their nucleotide sequences determined by automated sequencing on an ABI 377 (Bio-Rad).

As the LipDH proteins were predicted to contain N-terminal transit peptides and, in the case of aLipDH, a signal peptide, N-terminally truncated constructs were generated that lacked the potential targeting sequences in order to ensure soluble

recombinant expression of both proteins in *E. coli*. The shorter versions of both genes (aLipDH starts at amino acid 118 and mLipDH starts at amino acid 25) were amplified by PCR as described above using the full-length constructs or the cDNA library as template and performing the PCR with the sequence specific oligonucleotides A_exp and A2 which contained *Hin*dIII and *Bam*HI restriction sites and M_exp sense and M2 anti-sense with *Nde*I and *Bam*HI restriction sites (Table 3). The PCR fragments were cloned into pJC40 (Clos and Brandau, 1994) and their sequences were verified. Subsequently the full-length and N-terminally truncated constructs were transformed into the bacterial expression strain *E. coli* BL21-RIL (DE3) (Stratagene).

Recombinant expression and purification of recombinant proteins

Expression cultures were grown in Luria–Bertani medium containing 50 μg ml^{-1} ampicillin, at 37°C, until they reached an OD_{600} of 0.5. Recombinant expression was induced with 1 mM isopropyl-β-thio-D-galactoside, and expressed for an additional 18 h at 37°C. Bacterial pellets were resuspended in 50 mM sodium phosphate buffer pH 8.0 containing 300 mM NaCl and 10 mM imidazole (lysis buffer) with addition of 1 mM phenylmethanesulphonyl fluoride and 100 μM FAD. Bacteria were lysed by addition of 50 μg ml^{-1} lysozyme, freeze-thawing and by using a French Press (1000 lb in^{-2}; American Instrument Company). The extract was centrifuged for 1.5 h at 50 000 *g* (Beckamn, Avanti J 25) and the supernatant was batch bound to Ni^{2+}-agarose (Qiagen). The resin was washed with 50 mM sodium phosphate buffer pH 8.0 containing 300 mM NaCl and 20 mM imidazole and the recombinant proteins eluted with the same buffer containing 500 mM imidazole. To improve the purity, the proteins were dialysed overnight against two changes of lysis buffer and subsequently applied to a 1 ml Ni^{2+} agarose column (Amersham) with a flow rate of 1 ml min^{-1} using an AKTA FPLC system (Amersham). The resin was washed with lysis buffer before recombinant proteins were eluted with 10 column volumes of a linear gradient of 10 mM to 500 mM imidazole in lysis buffer. The purity of the proteins was assessed by SDS-PAGE. Protein concentrations were determined using the Bradford assay (Bradford, 1976) and by determining the amount of protein-bound FAD at 460 nm using an absorption coefficient for FAD of 11 300 M^{-1} cm^{-1} and the respective molecular mass of the recombinant protein. The absorption spectra of the mLipDH were determined under oxidized conditions and with addition of 20-fold molar excess of NADH.

Enzyme assays

In the physiological forward reaction, LipDH catalyses the oxidation of dihydrolipoamide and the reduction of NAD^+ (Dihydrolipoamide + NAD^+ → Lipoamide + NADH). During the reverse reaction, the enzyme reduces lipoamide at the expense of NADH (Lipoamide + NADH → Dihydrolipoamide + NAD^+). The pH-optima for both reactions can be quite different as has been shown for *Trypanosoma cruzi* LipDH (Schöneck *et al*., 1997) and they were determined for mLipDH using 50 mM Bicine/Bis Tris Propane/MES buffers in the range between pH 5.5 and 10.0. The forward reaction was assayed at 25°C and the reaction mixture contained 2 mM NAD^+, 1 mM dihydrolipoamide (prepared according to Reed *et al*., 1958), 1 mM EDTA and 1 μg of recombinant mLipDH. The increase in absorbance at 340 nm was followed spectrophotometrically (UV-2401 PC, Shimadzu). The reverse reaction was determined at 25°C and contained 200 μM NADH, 2 mM lipoamide (Sigma), 1 mM EDTA and 1 μg of mLipDH and the oxidation of NADH was followed at 340 nm.

After the pH-optima for both reactions were established, apparent K_m values for both substrates of the forward reaction were determined by varying the concentration of dihydrolipoamide (31.25 μM to 2 mM) or NAD^+ (62.5 μM to 2 mM) while keeping the concentration of the second substrate at saturating conditions. As the forward reaction is inhibited by the product NADH (Sahlman and Williams, 1989), it was necessary to use a stopped flow fast kinetic apparatus (SFA-20, Rapid Kinetics Accessory, Hi-Tech Scientific) connected to the spectrophotometer as described by Akerman and Müller (2003) to determine the initial reaction rates of the enzyme. The assays were performed at pH 8.0 to prevent the spontaneous oxidation of dihydrolipoamide which occurs readily at a pH higher than 8.0 (Reed *et al*., 1958). The change in absorbance was followed at 340 nm (reduction of NAD^+) with time points taken every 10 ms to ensure that initial reaction rates were detected.

The apparent kinetic parameters for the reverse reaction were determined in a continuous assay system by varying NADH (10 μM to 200 μM) or lipoamide (50 μM to 2 mM) while using saturating concentrations of the second substrate and following the rate of the reaction spectrophotometrically at 340 nm (oxidation of NADH). Apparent steady-state kinetic parameters were calculated using GraFit 5 (Erithacus Software). In order to investigate the catalytic mechanism of the enzyme, the reverse reaction was studied in more detail by varying both substrate concentrations simultaneously (lipoamide 0.5 mM to 2 mM at three different NADH concentrations: 10 μM, 20 μM and 40 μM) and fitting the results using GraFit 5.

Western blot analysis

To analyse whether mitochondrial LipDH and the potential mitochondrial branched chain ketoacid dehydrolipoamide transacylase subunit (BCKDH E2) are expressed in the erythrocytic stages of *P. falciparum*, soluble protein was isolated from mixed stage parasites and 10 μg per lane were separated on a 4–12% SDS-PAGE (Novagen). After blotting the proteins onto nitrocellulose (Schleicher and Schüll) using standard protocols (Sambrook *et al*., 1989), the blots were probed with the primary anti-mLipDH rabbit anti-serum (generated by Eurogentec) at 1:250 or a primary anti-bcE2 rabbit anti-serum (P.J. McMillan and S. Müller, unpubl.) at 1:250 (Eurogentec) for 1 h followed by extensive washes with PBS containing 0.05% Tween 20. Subsequently the blots were labelled with the secondary anti-rabbit horseradish peroxidase coupled antibody (Scottish Antibody Production Unit) at 1:5000 for 1 h, washed in the same buffer as before and the mLipDH bands were visualized

using the ECL$^+$ system according to the manufacturer's instructions (Amersham).

GFP fusion constructs and P. falciparum *transfection*

The putative N-terminal presequences responsible for protein targeting of the two *lipdh* genes were amplified by PCR from genomic DNA of *P. falciparum* (strain D10) using the primers A_target and M_target listed in Table 3. These oligonucleotides contain *Bgl*II and *Avr*II restriction sites [as well as Gateway™ (Invitrogen) *att* sites]. The resulting PCR products were digested with *Bgl*II and *Avr*II (which removed the *att* sites) and ligated into a GFP expression cassette within a *P. falciparum* vector modified to utilize Gateway™ (Invitrogen) cloning technology. The complete expression cassette flanked by *att* sites was recombinantly inserted into the final transfection vector pCHD-1/2 (kindly provided by C. Tonkin) carrying the human *DHFR* gene for selection with WR99210. Transfection of GFP plasmid constructs into *P. falciparum* D10 parasites was carried out by electroporation as previously described (Wu *et al*., 1995). Transfectant lines were maintained under selection by supplementing media with 0.25 μM WR99210. As the cloned LipDH presequences were amplified from genomic DNA, the mLipDH presequence encompassed exon 1, intron 1 and 29 nucleotides of exon 2 of the *mlipdh* gene. This construct allowed the parasites to splice the intervening sequence between exon 1 and exon 2 upon transcription yielding the correct translation product.

Immunofluorescent studies

Infected erythrocytes were fixed, labelled and prepared for immunofluorescence microscopy as described previously (Tonkin *et al*., 2004). In short, cells were washed (all washes were carried out in PBS) followed by fixation in 4% paraformaldehyde and 0.0075% glutaraldehyde (electron microscopy-grade, ProSciTech) in PBS for 60 min. After washing, cells were permeabilized in 0.1% Triton X-100 in PBS for 10 min and washed again. Cells were then treated with sodium borohydride ($NaBH_4$) in PBS (≈0.1 mg ml^{-1}) for 10 min followed by another wash. Blocking was performed in 3% BSA/PBS for 1 h at room temperature, followed by labelling with anti-ACP antibody (Waller *et al*., 2000) (diluted 1:500) or anti-*E. coli* HSP60 antibody (GroEL; diluted 1:5000; kindly provided by S. Rospert) for a minimum of 1 h in blocking buffer. Cells were washed three times for 10 min each and incubated with AlexaFluor goat anti-rabbit 594 secondary antibody IgG (H+L) (Molecular Probes; diluted 1:1000 in blocking buffer) for 1 h while settling onto a flamed coverslip coated with 1% polyethylenimine (PEI; Sigma). After three washes, cells were mounted in 50% glycerol with 0.1 mg ml^{-1} 1,4-diazabicyclo[2,2,2]octane (DABCO, Sigma). Finally, coverslips were inverted onto a glass microscope slide, mounted and sealed.

Microscopy

Green fluorescence of GFP-expressing transfectant parasites was observed using live parasites and captured using a Leica TCS 4D confocal microscope. Mitochondrial staining of live parasites was carried out using MitoTracker Red CM-H_2XRos (Molecular Probes). Briefly, transfected *P. falciparum* lines were incubated for 15 min at 37°C with MitoTracker diluted to 40 nM in culture medium (RPMI-Hepes supplemented with 0.5% Albumax II), followed by one wash in culture medium before visualization.

Acknowledgements

The authors like to thank the Wellcome Trust for funding this project. B.J.F. was supported by two Melbourne International Research Scholarships (MIRS and MIFRS), and G.I.M. gratefully acknowledges a HHMI International Scholar Grant, a Program Grant from the Australian NHMRC and an ARC Professorial Fellowship. S.M. is a Wellcome Senior Fellow in Basic Biomedical Science. The authors thank C. Tonkin for invaluable help with *P. falciparum* transfection and for providing the transfection vector pCHD-1/2.

Supplementary material

The following material is available from
http://www.blackwellpublishing.com/products/journals/suppmat/mmi/mmi4398/mmi4398sm.htm

Fig. S1. Alignment of mitochondrial and apicoplast LipDH.
Fig. S2. SDS-PAGE of recombinantly expressed mitochondrial LipDH.
Fig. S3. Steady-state kinetic analyses of mLipDH forward reaction.
Fig. S4. Steady-state kinetic analyses of mLipDH reverse reaction.
Fig. S5. mLipDH displays ping-pong kinetics.

References

Akerman, S.E., and Müller, S. (2003) 2-Cys peroxiredoxin PfTrx-Px1 is involved in the antioxidant defence of *Plasmodium falciparum*. *Mol Biochem Parasitol* **130:** 75–81.

Argyrou, A., and Blanchard, J.S. (2001) *Mycobacterium tuberculosis* lipoamide dehydrogenase is encoded by Rv0462 and not by the *lpdA* or *lpdB* genes. *Biochemistry* **40:** 11353–11363.

Bender, A., van Dooren, G.G., Ralph, S.A., McFadden, G.I., and Schneider, G. (2003) Properties and prediction of mitochondrial transit peptides from *Plasmodium falciparum*. *Mol Biochem Parasitol* **132:** 59–66.

Bocanegra, J.A., Scrutton, N.S., and Perham, R.N. (1993) Creation of an NADP-dependent pyruvate dehydrogenase multienzyme complex by protein engineering. *Biochemistry* **32:** 2737–2740.

Bozdech, Z., Llinas, M., Pulliam, B.L., Wong, E.D., Zhu, J., and DeRisi, J.L. (2003) The transcriptome of the intraerythrocytic developmental cycle of *Plasmodium falciparum*. *PLoS Biol* **1:** E5.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254.

Carothers, D.J., Pons, G., and Patel, M.S. (1989) Dihydroli-

poamide dehydrogenase: functional similarities and divergent evolution of the pyridine nucleotide-disulfide oxidoreductases. *Arch Biochem Biophys* **268:** 409–425.

Claros, M.G., and Vincens, P. (1996) Computational method to predict mitochondrially imported proteins and their targeting sequences. *Eur J Biochem* **241:** 779–786.

Clos, J., and Brandau, S. (1994) pJC20 and pJC40 – two high-copy-number vectors for T7 RNA polymerase-dependent expression of recombinant genes in *Escherichia coli*. *Protein Expr Purif* **5:** 133–137.

van Dooren, G.G., Su, V., D'Ombrain, M.C., and McFadden, G.I. (2002) Processing of an apicoplast leader sequence in *Plasmodium falciparum* and the identification of a putative leader cleavage enzyme. *J Biol Chem* **277:** 23612–23619.

Douce, R., Bourguignon, J., Neuburger, M., and Rebeille, F. (2001) The glycine decarboxylase system: a fascinating complex. *Trends Plant Sci* **6:** 167–176.

Else, A.J., Clarke, J.F., Willis, A., Jackman, S.A., Hough, D.W., and Danson, M.J. (1994) Dihydrolipoamide dehydrogenase in the trypanosoma subgenus, trypanozoon. *Mol Biochem Parasitol* **64:** 233–239.

Emanuelsson, O., Nielsen, H., Brunak, S., and von Heijne, G. (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *J Mol Biol* **300:** 1005–1016.

Fichera, M.E., and Roos, D.S. (1997) A plastid organelle as a drug target in apicomplexan parasites. *Nature* **390:** 407–409.

Florens, L., Washburn, M.P., Raine, J.D., Anthony, R.M., Grainger, M., Haynes, J.D., *et al.* (2002) A proteomic view of the *Plasmodium falciparum* life cycle. *Nature* **419:** 520–526.

Foth, B.J., Ralph, S.A., Tonkin, C.J., Struck, N.S., Fraunholz, M., Roos, D.S., *et al.* (2003) Dissecting apicoplast targeting in the malaria parasite *Plasmodium falciparum*. *Science* **299:** 705–708.

Foth, B.J., Stimmler, L.M., Handman, E., Hodder, A.N., Crabb, B.S., and McFadden, G.I. (2004) The malaria parasite *Plasmodium falciparum* has only one pyruvate dehydrogenase complex, which is located in the apicoplast. *Mol Microbiol* (in press).

Gardner, M.J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R.W., *et al.* (2002) Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* **419:** 498–511.

Ghisla, S., Massey, V., Lhoste, J.M., and Mayhew, S.G. (1974) Fluorescence and optical characteristics of reduced flavines and flavoproteins. *Biochemistry* **13:** 589–597.

Holecek, M. (2002) Relation between glutamine, branched-chain amino acids, and protein metabolism. *Nutrition* **18:** 130–133.

Jomaa, H., Wiesner, J., Sanderbrand, S., Altincicek, B., Weidemeyer, C., Hintz, M., *et al.* (1999) Inhibitors of the nonmevalonate pathway of isoprenoid biosynthesis as antimalarial drugs. *Science* **285:** 1573–1576.

Kim, H., and Patel, M.S. (1992) Characterization of two site-specifically mutated human dihydrolipoamide dehydrogenases (His-452–Gln and Glu-457–Gln). *J Biol Chem* **267:** 5128–5132.

Kim, H., Liu, T.C., and Patel, M.S. (1991) Expression of cDNA sequences encoding mature and precursor forms of human dihydrolipoamide dehydrogenase in *Escherichia coli*. Differences in kinetic mechanisms. *J Biol Chem* **266:** 9367–9373.

Koike, M., Reed, L.J., and Carroll, W.R. (1963) alpha-Keto acid dehydrogenation complexes. IV. Resolution and reconstitution of the *Escherichia coli* pyruvate dehydrogenation complex. *J Biol Chem* **238:** 30–39.

Krugliak, M., Zhang, J., and Ginsburg, H. (2002) Intraerythrocytic *Plasmodium falciparum* utilizes only a fraction of the amino acids derived from the digestion of host cell cytosol for the biosynthesis of its proteins. *Mol Biochem Parasitol* **119:** 249–256.

Le Roch, K.G., Zhou, Y., Blair, P.L., Grainger, M., Moch, J.K., Haynes, J.D., *et al.* (2003) Discovery of gene function by expression profiling of the malaria parasite life cycle. *Science* **301:** 1503–1508.

Lutziger, I., and Oliver, D.J. (2001) Characterization of two cDNAs encoding mitochondrial lipoamide dehydrogenase from *Arabidopsis*. *Plant Physiol* **127:** 615–623.

McFadden, G.I., Reith, M.E., Munholland, J., and Lang-Unnasch, N. (1996) Plastid in human parasites. *Nature* **381:** 482.

Mattevi, A., Schierbeek, A.J., and Hol, W.G. (1991) Refined crystal structure of lipoamide dehydrogenase from *Azotobacter vinelandii* at 2.2 A resolution. A comparison with the structure of glutathione reductase. *J Mol Biol* **220:** 975–994.

Mooney, B.P., Miernyk, J.A., and Randall, D.D. (2002) The complex fate of alpha-ketoacids. *Annu Rev Plant Biol* **53:** 357–375.

Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. (1997) Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng* **10:** 1–6.

Perham, R.N., Leistler, B., Solomon, R.G., and Guptasarma, P. (1996) Protein engineering of domains in flavoprotein disulphide oxidoreductases: contributions to folding and assembly. *Biochem Soc Trans* **24:** 61–66.

Reed, J.K. (1973) Studies on the kinetic mechanism of lipoamide dehydrogenase from rat liver mitochondria. *J Biol Chem* **248:** 4834–4839.

Reed, L.J., Koike, M., Levitch, M.E., and Leach, F.R. (1958) Studies on the nature and reactions of protein-bound lipoic acid. *J Biol Chem* **232:** 143–158.

Sahlman, L., and Williams, C.H., Jr (1989) Lipoamide dehydrogenase from *Escherichia coli*. Steady-state kinetics of the physiological reaction. *J Biol Chem* **264:** 8039–8045.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schierbeek, A.J., Swarte, M.B., Dijkstra, B.W., Vriend, G., Read, R.J., Hol, W.G., *et al.* (1989) X-ray structure of lipoamide dehydrogenase from *Azotobacter vinelandii* determined by a combination of molecular and isomorphous replacement techniques. *J Mol Biol* **206:** 365–379.

Schöneck, R., Billaut-Mulot, O., Numrich, P., Ouaissi, M.A., and Krauth-Siegel, R.L. (1997) Cloning, sequencing and functional expression of dihydrolipoamide dehydrogenase from the human pathogen *Trypanosoma cruzi*. *Eur J Biochem* **243:** 739–747.

Surolia, N., and Surolia, A. (2001) Triclosan offers protection against blood stages of malaria by inhibiting enoyl-ACP reductase of *Plasmodium falciparum*. *Nat Med* **7:** 167–173.

The Plasmodium genome database collaborative (2001) PlasmoDB: an integrative database of the *Plasmodium falciparum* genome. Tools for accessing and analyzing finished and unfinished sequence data. The *Plasmodium* Genome Database Collaborative. *Nucleic Acids Res* **29:** 66–69.

Thomsen-Zieger, N., Schachtner, J., and Seeber, F. (2003) Apicomplexan parasites contain a single lipoic acid synthase located in the plastid. *FEBS Lett* **547:** 80–86.

Thorpe, C., and Williams, C.H.Jr (1976) Differential reactivity of the two active site cysteine residues generated on reduction of pig heart lipoamide dehydrogenase. *J Biol Chem* **251:** 3553–3557.

Tonkin, C.J., van Dooren, G.G., Spurck, T.P., Struck, N.S., Good, R.T., Handman, E., *et al.* (2004) Localization of organellar proteins in *Plasmodium falciparum* using a novel set of transfection vectors and a new immunofluorescence fixation method. *Mol Biochem Parasitol* **137:** 13–21.

Trager, W., and Jensen, J.B. (1976) Human malaria parasites in continuous culture. *Science* **193:** 673–675.

Tripathi, A.K., Desai, P.V., Pradhan, A., Khan, S.I., Avery, M.A., Walker, L.A., and Tekwani, B.L. (2004) An alpha-proteobacterial type malate dehydrogenase may complement LDH function in *Plasmodium falciparum*. *Eur J Biochem* **271:** 3488–3502.

Umlas, J., and Fallon, J.N. (1971) New thick-film technique for malaria diagnosis. Use of saponin stromatolytic solution for lysis. *Am J Trop Med Hyg* **20:** 527–529.

Uyemura, S.A., Luo, S., Vieira, M., Moreno, S.N., and Docampo, R. (2004) Oxidative phosphorylation and rotenone-insensitive malate- and NADH-quinone oxidoreductases in *Plasmodium yoelii yoelii* mitochondria *in situ*. *J Biol Chem* **279:** 385–393.

Vaidya, A.B. (1996) Mitochondrial physiology as a target for atovaquone and other antimalarials. In *Malaria: Parasite Biology, Pathogenesis, and Protection.* Sherman, I.W. (ed.). Washington, DC: American Society for Microbiology Press, pp. 355–368.

Vanoni, M.A., Wong, K.K., Ballou, D.P., and Blanchard, J.S. (1990) Glutathione reductase: comparison of steady-state and rapid reaction primary kinetic isotope effects exhibited by the yeast, spinach, and *Escherichia coli* enzymes. *Biochemistry* **29:** 5790–5796.

Waller, R.F., Reed, M.B., Cowman, A.F., and McFadden, G.I. (2000) Protein trafficking to the plastid of *Plasmodium falciparum* is via the secretory pathway. *EMBO J* **19:** 1794–1802.

Waller, R.F., Ralph, S.A., Reed, M.B., Su, V., Douglas, J.D., Minnikin, D.E., *et al.* (2003) A type II pathway for fatty acid biosynthesis presents drug targets in *Plasmodium falciparum*. *Antimicrob Agents Chemother* **47:** 297–301.

Ward, D.E., Ross, R.P., van der Weijden, C.C., Snoep, J.L., and Claiborne, A. (1999) Catabolism of branched-chain alpha-keto acids in *Enterococcus faecalis*: the *bkd* gene cluster, enzymes, and metabolic route. *J Bacteriol* **181:** 5433–5442.

Williams, Ch, Jr (1976) Flavin containing dehydrogenases. In *The Enzymes*, Vol. 13. Boyer, P.D. (ed.). New York: Academic Press, pp. 89–173.

Williams, C.H., Jr, Arscott, D.A., and Swenson, R.P. (1984) Active site chemical modification and sequencing of flavoproteins. In *Flavins and Flavoproteins.* Bray, R.C., Engel, P.C., and Mayhew, S.G. (eds). Berlin: Walter de Gruyter & Co., pp. 95–109.

Williams, C.H., Jr, Allison, N., Russell, G.C., Prongay, A.J., Arscott, L.D., Datta, S., *et al.* (1989) Properties of lipoamide dehydrogenase and thioredoxin reductase from *Escherichia coli* altered by site-directed mutagenesis. *Ann NY Acad Sci* **573:** 55–65.

Wrenger, C., and Müller, S. (2004) The human malaria parasite *Plasmodium falciparum* has distinct organelle-specific lipoylation pathways. *Mol Microbiol* **53:** 103–113.

Wu, Y., Sifri, C.D., Lei, H.H., Su, X.Z., and Wellems, T.E. (1995) Transfection of *Plasmodium falciparum* within human red blood cells. *Proc Natl Acad Sci USA* **92:** 973–977.