# The malaria parasite *Plasmodium falciparum* has only one pyruvate dehydrogenase complex, which is located in the apicoplast

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#### Summary

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The relict plastid (apicoplast) of apicomplexan parasites synthesizes fatty acids and is a promising drug target. In plant plastids, a pyruvate dehydrogenase complex (PDH) converts pyruvate into acetyl-CoA, the major fatty acid precursor, whereas a second, distinct PDH fuels the tricarboxylic acid cycle in the mitochondria. In contrast, the presence of genes encoding PDH and related enzyme complexes in the genomes of five Plasmodium species and of Toxoplasma gondii indicate that these parasites contain only one single PDH. PDH complexes are comprised of four subunits (E1a, E1 $\beta$ , E2, E3), and we confirmed four genes encoding a complete PDH in *Plasmodium falciparum* through sequencing of cDNA clones. In apicomplexan parasites, many nuclear-encoded proteins are targeted to the apicoplast courtesy of two-part N-terminal leader sequences, and the presence of such N-terminal sequences on all four PDH subunits as well as phylogenetic analyses strongly suggest that the P. falciparum PDH is located in the apicoplast. Fusion of the two-part leader sequences from the E1α and E2 genes to green fluorescent protein experimentally confirmed apicoplast targeting. Western blot analysis provided evidence for the expression of the E1 $\alpha$  and E1 $\beta$ PDH subunits in blood-stage malaria parasites. The recombinantly expressed catalytic domain of the PDH subunit E2 showed high enzymatic activity in vitro indicating that pyruvate is converted to acetyl-CoA

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in the apicoplast, possibly for use in fatty acid biosynthesis.

#### Introduction

The pyruvate dehydrogenase complex (PDH) is one member of a family of  $\alpha$ -ketoacid dehydrogenase complexes (KADHs). In addition to the PDH, this family of multienzyme complexes also includes the  $\alpha$ -ketoglutarate dehydrogenase complex (KGDH) and the branched-chain  $\alpha$ -ketoacid dehydrogenase complex (BCKDH). All of these multienzyme complexes share similar functionality and structure, i.e. all catalyse the oxidative decarboxylation of particular  $\alpha$ -ketoacids (= 2-oxo acids) and consist of multiple copies of several enzyme subunits. The enzymatic activity of KADHs converts organic acids containing a keto group adjacent to the carboxyl group (i.e. in  $\alpha$ - or 2-position) into acyl-CoA and carbon dioxide (Patel and Roche, 1990; Mooney *et al.*, 2002).

In most eukaryotes, the PDH occupies a central role in mitochondrial metabolism by converting pyruvate into acetyl-CoA (see Fig. 1), thus linking cytoplasmic glycolysis to the tricarboxylic acid (TCA) cycle in the mitochondrial matrix. In addition to the mitochondrial PDH, a second, distinct PDH is present in the plastids of algae and plants. Plastids are the major site of de novo fatty acid synthesis (FAS) in these organisms, and, after much debate, it has now been established that acetyl-CoA needed for FAS is generated from pyruvate by the plastidic PDH (Bao et al., 2000; Ke et al., 2000; Ohlrogge et al., 2000; Rawsthorne, 2002). Moreover, in addition to generating acetyl-CoA, PDH activity also produces part of the reducing power in form of NADH+H<sup>+</sup> (see Fig. 1) that is required for FAS. Thus, PDHs also have a central metabolic role in plastids.

PDHs are found in eubacteria, select members of the Archaea, and in the mitochondria and plastids of most eukaryotes (Jolley *et al.*, 2000; Kleiger *et al.*, 2001). Mitochondrial PDHs are most closely related to the respective enzymes from  $\alpha$ -proteobacteria, whereas plastidic PDH subunits are more similar to those from cyanobacteria, which reflects their origins from endosymbiotic bacteria from these lineages respectively (Mooney *et al.*, 2002; Schnarrenberger and Martin, 2002).

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**Fig. 1.** A simplified reaction scheme of the pyruvate dehydrogenase complex (PDH). The PDH subunits E1, E2 and E3 catalyse successive parts of the overall reaction which converts pyruvate into carbon dioxide and acetyl-CoA. The E3 subunit reoxidizes the reduced lipoamide moiety of the E2 subunit yielding NADH+H<sup>+</sup>. Apart from the subunits and cofactors shown, thiamin pyrophosphate and FAD are also involved in these reactions.

All KADHs consist of multiple copies of at least three different subunits that are referred to as E1, E2 and E3. The PDH E1 subunit represents the 'pyruvate dehydrogenase (lipoamide)' proper (EC 1.2.4.1) and decarboxylates pyruvate (see Fig. 1). It usually occurs as a heterotetramer  $(\alpha_2\beta_2)$  of distinct E1 $\alpha$  and E1 $\beta$  subunits. The PDH E2 subunit - the dihydrolipoamide S-acetyltransferase (EC 2.3.1.12) - contains three distinct protein domains that are separated by variable linker regions. The E2 catalytic domain catalyses the transfer of an acetyl group from an E2-acetyl-lipoamide moiety to free CoA, yielding acetyl-CoA and the reduced dihydrolipoamide form of lipoic acid (see Fig. 1) (Patel and Roche, 1990). The E3 subunit is known as dihydrolipoamide dehydrogenase or LipDH (EC 1.8.1.4) and catalyses the oxidation of the reduced E2-dihydrolipoamide moiety back to lipoamide, concomitantly producing NADH+H<sup>+</sup> (see Fig. 1). While each KADH contains its own specific E1 and E2 subunits, the same E3 subunit is commonly shared between the three KADHs (PDH, KGDH and BCKDH) and the glycine decarboxylase complex within the mitochondria (Johnson et al., 1997; Lutziger and Oliver, 2001). In plastids, an E3 subunit is only found as part of the PDH (Lutziger and Oliver, 2000).

The apicomplexan parasite *Plasmodium falciparum* is the causative agent of the most deadly form of human malaria. Like other apicomplexan parasites, this organism possesses both mitochondria and plastids (the so-called apicoplast), and one might have expected – in analogy to the situation in plants and algae – to find two distinct PDHs, one in the mitochondrion and one in the apicoplast. However, despite the presence of genes encoding two different E3 subunits, the complete *P. falciparum* genome has uncovered only one full set of PDH genes (Gardner *et al.*, 2002). The two distinct E3 subunits (LipDHs) of *P. falciparum* have been localized to the mitochondrion and apicoplast, respectively, and the expression and steadystate enzyme kinetics of the mitochondrial LipDH have been described in detail (McMillan *et al.*, 2004).

In this study, we examine which KADHs are present in apicomplexan parasites, with particular emphasis on Plasmodium. Genes for constituent subunits of three enzyme complexes (one PDH, one KGDH and one BCKDH) were identified in the genomes of several Plasmodium species and of Toxoplasma gondii. cDNA clones of the P. falci*parum* genes for PDH subunits E1 $\alpha$ , E1 $\beta$ , E2 and E3 were sequenced and analysed. Sequence comparisons and phylogenetic analyses provide ample evidence that these genes derive from the endosymbiont that gave rise to the apicoplast. The presence of N-terminal targeting sequences and targeting experiments employing green fluorescent protein (GFP) demonstrate that the PDH enzymes are located in the apicoplast of P. falciparum (this study and McMillan et al., 2004), and anti-sera raised against recombinant PDH subunits  $E1\alpha$  and  $E1\beta$  identified cognate proteins in the erythrocytic stages of malaria parasites. Finally, dihydrolipoamide S-acetyltransferase activity of the recombinantly expressed catalytic domain of the PDH E2 subunit was demonstrated by an in vitro enzyme assay.

#### Results

#### Four genes encode a plastid-like PDH in Plasmodium

The complete nuclear genome sequence of P. falciparum encodes a range of putative KADH subunits (Gardner et al., 2002) (Table 1). We used genomic sequence data from P. falciparum encoding putative PDH subunits to design oligonucleotide primers for subsequent amplification of the corresponding genes via reverse transcription polymerase chain reaction (RT-PCR). Using this strategy, we recovered and sequenced four genes from P. falciparum cDNA whose identities were clearly supported by their similarity to homologues in the protein database: one PDH E1 $\alpha$  subunit, one PDH E1 $\beta$  subunit, one PDH E2 subunit, and one E3 subunit (Fig. 2 and Figs S1-S4 in Supplementary material). These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under Accession No. AY484441-AY484444. According to the annotation of the P. falciparum genome, these nuclear genes are

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encoded on chromosomes 11, 14, 10 and 8 respectively. An additional E3 subunit (Accession No. AJ630268) is also present in *P. falciparum*: this enzyme has been localized to the mitochondrion and is discussed in detail elsewhere (McMillan *et al.*, 2004).

We then surveyed the genomes of several *Plasmodium* species as well as of *T. gondii* to determine whether the presence of only one complete PDH is a general feature of these organisms. Putative KADH genes were initially classified as plastidic or mitochondrial on the basis of the characteristics of their N-terminal presequences (cf. Fig. 2), bioinformatic predictions (PLASMOAP, MITOPROT, TARGETP; see McMillan *et al.*, 2004) and their similarity to

Fig. 2. Schematic summary of the predicted protein sequences of plastidic PDH subunits of Plasmodium falciparum. The schematics indicate signal peptides (grey; as predicted by SIG-NALP), apicoplast transit peptides (black), mature proteins (white; determined by comparison to homologous protein sequences), intron positions (triangles and circled numbers; determined by cDNA sequencing) and estimated transit peptide cleavage sites (scissors symbol; estimated from Western blot analysis). The Cterminal ends of transit peptides are depicted as broad transition regions because of the uncertainty regarding the precise boundary with the mature proteins. The striped regions in the E2 sequence denote distinct protein domains: two lipoyl-binding domains (Lip1 and Lip2), one subunit-binding domain (SB) and one catalytic/self association domain (Cat/SA). The numbers above the schematics indicate amino acid positions.

cyanobacterial or  $\alpha$ -proteobacterial homologues in the database (Schnarrenberger and Martin, 2002). The virtually complete nuclear genome sequences of *P. falciparum*, *Plasmodium yoelii* and *T. gondii* revealed the same pattern of KADH genes: four genes encoding a complete plastid-like PDH complex as well as five genes specific for mitochondrial BCKDH and KGDH complexes (Table 1). The mitochondrial E3 subunit is commonly shared between mitochondrial KADHs and does not indicate the presence of a mitochondrial PDH. In contrast, the lack of specific mitochondrial PDH genes strongly suggests that a PDH is absent from the mitochondria in these organisms (one match for a PDH E1 $\alpha$  subunit in the *T. gondii* data-

Table 1. Occurrence of genes encoding KADH subunits in Plasmodium and Toxoplasma.

Species					Mitochondrial								
	Plastid-like PDH				PDH				BCKDH			KGDH	
	E1α	E1β	E2	E3	E1α	E1β	E2	E3 <sup>a</sup>	E1α	E1β	E2	E1	E2
P. falciparum <sup>b</sup>	✓°	1	✓°	✓d	_	_	_	✓d	1	1	1	1	1
P. vivax	_	1	1	1	_	_	_	_	_	_	1	_	_
P. knowlesi	1	1	1	1	_	_	_	1	_	1	1	1	1
P. voelii	1	1	1	1	_	_	_	1	1	1	1	1	1
P. berghei	1	1	1	1	_	_	_	1	1	1	1	1	1
T. gondii	1	$\checkmark$	$\checkmark$	$\checkmark$	(✔) <sup>e</sup>	-	-	$\checkmark$	1	1	$\checkmark$	1	~

a. The mitochondrial E3 subunit is commonly shared between the PDH, the BCKDH and the KGDH.

**b.** Ticks ( $\checkmark$ ) indicate that a convincing match for the corresponding gene was found in a database containing genomic and/or EST sequences. These matches included full-length sequences (as determined by comparison to homologues), especially for *P. falciparum* and *P. yoelii*, as well as partial sequences (other species).

c. The subcellular localization for this protein has been experimentally verified (this study).

d. The subcellular localization for this protein has been experimentally verified (McMillan et al., 2004).

e. The sole match for the mitochondrial PDH E1α subunit in the *T. gondii* database has greatest similarity to sequences from yeast-like fungi and almost certainly represents a gene from a contaminant and not from *Toxoplasma*.

KADH, α-ketoacid dehydrogenase complex; PDH, pyruvate dehydrogenase complex; BCKDH, branched-chain ketoacid dehydrogenase complex; KGDH, α-ketoglutarate dehydrogenase complex.

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base is clearly yeast-like and almost certainly stems from a contaminant). The pattern of KADH genes present in the partial nuclear genome sequences of *Plasmodium knowlesi*, *Plasmodium vivax* and *Plasmodium berghei* corroborate these findings (Table 1). Apart from the PDH, no other KADH complexes would be expected to be present in the apicoplast, and all other KADH subunits encoded in the *Plasmodium* and *Toxoplasma* genomes appear to be targeted to the mitochondria as judged by manual inspection of N-terminal sequence extensions and targeting predictions using the bioinformatics tools PLAS-MOAP (Foth *et al.*, 2003), MITOPROT (Claros and Vincens, 1996) and TARGETP (Emanuelsson *et al.*, 2000) (see McMillan *et al.*, 2004).

To address the question whether the Plasmodium PDH subunits were originally derived from the genome of a plastid or a mitochondrion, we constructed multiple protein sequence alignments and performed phylogenetic analyses. The phylogenetic trees (Fig. 3) show that the Plasmodium PDH sequences contain a strong phylogenetic signal that consistently groups them with plastidic and cyanobacterial PDH sequences. Very high bootstrap values (≥ 98%) both for Neighbor-Joining (NJ) and Maximum Parsimony (MP) bootstrap analysis support the monophyly of the plastidic/cyanobacterial clade for each of the four PDH subunits. As expected, the trees also recover monophyletic clades consisting of mitochondrial and  $\alpha$ -proteobacterial sequences, and of eubacterial sequences (other than those from cyanobacteria and  $\alpha$ proteobacteria), respectively, which are also supported by high bootstrap values. The tree for the E3 subunit is the only case where these bootstrap values are not entirely convincing (Fig. 3). The low MP bootstrap value (61%) supporting the mitochondrial/a-proteobacterial clade partly results from the fact that two sequences - the mitochondrial E3 subunits of P. falciparum and P. yoelii (referred to as 'P. falciparum 2' and 'P. voelii 2' in Fig. 3) do not clearly cluster within any one of the three major clades. Removal of these sequences from the analysis resulted in an increase of this bootstrap value to 80% (Fig. 3). Furthermore, the mitochondrial Plasmodium E3 genes show greatest sequence similarity to α-proteobacterial and mitochondrial E3 sequences, e.g. the P. falciparum sequence exhibits 39% identity and 59% similarity to the homologous protein from Brucella melitensis versus identity/similarity values of 35%/53%, 34%/53% and 29%/48% in comparison to homologues from Azotobacter vinelandii, Bacillus subtilis and Synechocystis sp. respectively.

To examine whether the *Plasmodium* PDH subunits conformed with the general structure of these enzymes from other organisms, the predicted protein sequences of the *Plasmodium* PDH genes were examined in regard to highly conserved features of corresponding KADH subunits from various sources (Russell and Guest, 1991; Mattevi et al., 1992a; 1993; Toyoda et al., 1998; Aevarsson et al., 1999; 2000; Lutziger and Oliver, 2000). The *P. falciparum* sequences were found to be in very good agreement with virtually all known PDH characteristics regarding amino acids involved in catalytic enzyme activity and substrate binding as well as the presence of distinct protein domains (Fig. 2 and data not shown) (McMillan et al., 2004). In particular, E2 subunits usually comprise one C-terminal catalytic/self-association domain, one small subunit-binding domain, and between one and three N-terminal lipoyl-binding domains to which lipoic acid, a cyclic disulphide (6,8-dithiooctanoic acid), is covalently attached via an amide linkage (Russell and Guest, 1991; Zhou et al., 2001). These protein domains are clearly identifiable in the P. falciparum E2 subunit (Fig. 2) which contains two lipoyl-binding domains, of which the first is more highly conserved compared with homologous sequences than the second (data not shown).

# The gene encoding PDH subunit E2 contains 10 introns in P. falciparum

Comparisons of cDNA and genomic sequences identified 10 spliceosomal introns in the P. falciparum E2 gene (Table 2) of which three (introns 2, 6 and 7) differ from the introns predicted as part of the complete genome annotation (Gardner et al., 2002). Interestingly, the sequencing of multiple cDNA clones also revealed five splice variants (Table 2) that were concluded to represent aberrantly spliced transcripts for the following reasons. Each of the five variants was encountered in only one or two cDNA clones whereas all correctly spliced introns were sequenced from 5 to 10 independent clones. Furthermore, three of the splice variants caused frameshifts that destroy the open reading frame (Table 2). Of the two variants that left the open reading frame intact, variant 1 occurred in a sequence region that is highly conserved between the three Plasmodium species P. falciparum, P. knowlesi and P. yoelii (data not shown), yet the flanking GT and AG dinucleotides responsible for the variant 1 splicing event in P. falciparum are not present in the sequences of P. yoelii and P. knowlesi (data not shown). Similarly, the alternative 3' AG dinucleotide of intron 9 that shortened the start of exon 10 by two amino acids (splice variant 5) did not occur in the sequences of P. yoelii and P. knowlesi, whereas the orthodox 3' boundary of this intron is conserved in all three Plasmodium species (data not shown). These findings suggest that intron excision in P. falciparum is surprisingly prone to error, which may be a consequence of the high AT content of its genome reducing the fidelity of splice site recognition.



**Fig. 3.** Phylogenetic relationships of the *P. falciparum* PDH subunits to related organellar and bacterial proteins. The phylogenetic trees represent weighted Maximum Parsimony trees (see *Experimental procedures*). In all analyses bootstrapping was performed with 1000 bootstrap replicates, and bootstrap values based on Maximum Parsimony and Neighbor-Joining analysis are shown above and below the branches respectively. Only bootstrap values greater than 50% are shown. Bootstrap values in parentheses in the tree for subunit E3 refer to Maximum Parsimony bootstrap analysis in which the mitochondrial sequences '*Plasmodium falciparum* 2' and '*Plasmodium yoelii* 2' were excluded (values shown only where they differed by more than 3% from the analysis including these two sequences). In all trees the two clades consisting of cyanobacterial/plastidic and  $\alpha$ -proteobacterial/mitochondrial PDH sequences are well resolved and supported by high bootstrap values. Only the mitochondrial E3 sequences of *P. falciparum* and *P. yoelii* do not cluster in the respective clade. A. *tumefaciens*, *Agrobacterium tumefaciens*; B. stearothermophilus, *Bacillus stearothermophilus*; C. crescentus, Caulobacter crescentus; C. elegans, Caenorhabditis elegans; L. esculentum, Lycopersicon esculentum; R. prowazekii, Rickettsia prowazekii; S. pombe, Schizosaccharomyces pombe; S. cerevisiae, Saccharomyces cerevisiae.

#### Table 2. Intron splicing in the gene encoding *P. falciparum* PDH subunit E2.

	Intron										
	1	2	3	4	5	6	7	8	9	10	ORF?
Correct intron positions <sup>a</sup>	244– 452	520– 623	1014– 1128	1209– 1289	1339– 1440	1535– 1656	1738– 1841	1885– 1986	2067– 2130	2615– 2884	Yes
Variant 1 <sup>b</sup> Variant 2 <sup>b</sup> Variant 3 <sup>b</sup> Variant 4 <sup>b</sup> Variant 5 <sup>b</sup>	84 bases spliced out of exon 3 (nucleotide positions 888–971) Exon 8 completely missing (introns 7/8 spliced from positions 1738–1986) Intron 8 spliced from positions 1853–1986 (the last 32 bases are missing from exon 8) Intron 9 not spliced Intron 9 spliced from positions 2067–2136 (the first six bases are missing from exon 10)										

a. The correct splice positions for all introns were determined by sequencing of 5-10 independent clones. The intron positions refer to the nucleotide sequence starting from the codon that encodes the translational start-Met.

b. The splice variants were encountered in only one (variants 1, 2, 4, 5) or two (variant 3) independent clones each.

ORF, open reading frame.

#### The PDH of P. falciparum is localized in the apicoplast

The phylogenetic analyses clearly indicate that the four subunits comprising a full PDH in P. falciparum are most closely related to plastidic and cyanobacterial genes, i.e. this set of nuclear PDH genes ultimately derives from the apicoplast genome (or from the genome of the plastid that eventually gave rise to the apicoplast). But are the corresponding gene products targeted back into this organelle in extant apicomplexans? In accordance with the mechanism responsible for protein targeting to the apicoplast, one would expect apicoplast-localized proteins to contain bipartite N-terminal leader sequences (Waller et al., 2000; van Dooren et al., 2001; Foth et al., 2003). Indeed, our alignments clearly show that all plastid-like Plasmodium PDH subunits contain N-terminal presequences that are predicted by SIGNALP (Nielsen et al., 1997) to start with a typical signal peptide consisting of a hydrophobic domain followed by a von Heijne-type cleavage site (Fig. 2 and Figs S1-S4 in Supplementary material). Furthermore, the regions downstream of the predicted signal peptides in the P. falciparum presequences exhibit features characteristic of apicoplast-targeting transit peptides (Zuegge et al., 2001; Foth et al., 2003; Ralph et al., 2004a). They are deficient in acidic residues and rich in basic amino acids and - in the case of the two species with AT-rich genomes, P. falciparum and P. yoelii - contain very high proportions of Asn and Lys (data not shown).

To experimentally verify the predicted apicoplast localization of PDH subunits, we constructed and expressed in erythrocytic stages of *P. falciparum* GFP fusion proteins consisting of the N-terminal 191 and 61 amino acids of PDH subunits E1 $\alpha$  and E2, respectively, followed by GFP. Colocalization with antibodies recognizing the apicoplastresident acyl carrier protein (ACP) or with MitoTracker Red unambiguously revealed that the N-terminal presequences of the plastid-like PDH subunits E1 $\alpha$  and E2 are sufficient to target the reporter protein exclusively to the apicoplast (Fig. 4). Our images show the apicoplast in close proximity to the mitochondrion, as has been reported previously (Waller et al., 2000). Furthermore, the GFP-labelled organelle exhibited the apicoplastcharacteristic morphological changes associated with parasite development throughout the intraerythrocytic life stages (Waller et al., 2000) (Fig. 4 and data not shown). Using the same approach, the plastid-like E3 subunit has also been shown to be localized to the apicoplast (McMillan et al., 2004), whereas experiments employing the N-terminal presequence of the PDH E1ß subunit did not yield reproducible results. Together, these data provide strong evidence for apicoplast localization of the single P. falciparum PDH complex. In contrast, the second, mitochondrial-like E3 subunit was shown to be localized to the mitochondrion (McMillan et al., 2004). Attempts to label the endogenous PDH proteins  $E1\alpha$ ,  $E1\beta$  and E2in P. falciparum blood-stage parasites using our own polyclonal antibodies in immunofluorescence assays remained unsuccessful.

# Plastidic PDH subunits are expressed in blood-stage parasites

Two lines of evidence confirm that the plastidic PDH is expressed in blood-stage parasites of *P. falciparum*. First, amplification of fully spliced cDNA (Fig. 2) encoding the E2 subunit from intraerythrocytic parasites demonstrates that this gene is transcribed in these organisms. Microarray data also indicate transcription of all PDH subunits during the intraerythrocytic cycle (Bozdech *et al.*, 2003; Le Roch *et al.*, 2003), although at relatively low levels, with the transcription profiles of, e.g. PDH subunits E2 and E3 being very similar. Second, Western blot analysis using affinity-purified antibodies against PDH subunits E1 $\alpha$  and E1 $\beta$  revealed bands of appropriate sizes indicating that these subunits are expressed in blood-stage parasites of *P. falciparum* (strain D10) (see Fig. 5).



**Fig. 4.** N-terminal targeting sequences of PDH subunits  $E1\alpha$  and E2 of *P. falciparum* direct GFP to the apicoplast in intraerythrocytic parasites. The panels show images of *P. falciparum* parasites from different blood stages expressing episomally encoded GFP fusion proteins. Colocalization with the apicoplast-resident protein ACP using  $\alpha$ -ACP antibodies unambiguously shows that the presequences of PDH subunits  $E1\alpha$  and E2 are sufficient to target proteins exclusively to the apicoplast (A and C). Red MitoTracker labelling confirms that the GFP staining is – as expected for the apicoplast (cf. Waller *et al.*, 2000) – distinct from but in close proximity to the mitochondrion (B and D). Note also the characteristic shape of the apicoplast in the different parasite stages (cf. Waller *et al.*, 2000).

# The PDH subunit E2 of P. falciparum is enzymatically active

PDH subunits from various sources have been studied for decades, and simple *in vitro* assays have been developed to monitor the enzymatic activity of individual subunits. We employed a spectrophotometric assay (Schwartz and Reed, 1969; Yang *et al.*, 1997) to examine the enzymatic activity of the recombinantly expressed catalytic domain of the PDH E2 subunit (E2CD) from *P. falciparum*. Although E2CD expression in *Escherichia coli* yielded insoluble protein, we were able to obtain protein exhibiting high enzymatic activity by gentle resolubilization (3 M guanidine hydrochloride followed by 4 M urea; see *Experimental procedures*) of purified inclusion bodies (Fig. 6A). The specific activity of recombinant E2CD was determined to be  $\approx 153.8 \pm 16.2 \Delta A232$  per minute per mg

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protein (mean of four independent experiments with standard deviation). This specific activity is given simply as absorbance per minute because the extinction coefficient of the 8-acetyl-dihydrolipoamide product of the enzymatic reaction (about 4 A/mM\*cm) cannot be determined precisely because of the shuffling of acetyl groups within this molecule (Yang et al., 1997). Nevertheless, this specific activity is estimated to be - after compensating for the different molecular masses of the recombinant enzymes involved - about four times higher than that reported for recombinant protein of the human mitochondrial E2 enzyme (Yang et al., 1997) (measurements carried out using the same substrate concentrations). The  $K_m$  value for the substrate acetyl-CoA was estimated to be  $3.08 \ \mu M \pm 0.23$  (averaged data of four independent experiments with standard error; Fig. 6B). Solubilization of E2CD inclusion bodies employing the commonly used



Fig. 5. Western blot analysis confirms expression of PDH subunits  $E1\alpha$  and  $E1\beta$  in asexual parasites. Affinity-purified antibodies raised against recombinantly expressed P. falciparum PDH subunits E1a and E1ß were used to detect the corresponding proteins in Western blot analysis in a mixture of ≈20-60 ng of each of three recombinant proteins (lanes denoted 'rec' containing P. falciparum PDH subunits  $E1\alpha$ ,  $E1\beta$  and full-length E2) and in protein preparations of asynchronous P. falciparum cultures, strain D10 (lanes denoted 'par'). The molecular masses of the parasite proteins are expected to be 68.6 kDa (E1 $\alpha$ ) and 43.2 kDa (E1 $\beta$ ) for the pre-processed proteins (without signal peptide but including transit peptide), and between 48.9 kDa and 68.6 kDa (E1 $\alpha$ ) and between 36.8 kDa and 43.2 kDa (E1ß) for the fully processed proteins (lacking both signal and transit peptide). The molecular masses of the major bands detected (approximately 57 kDa for E1 $\alpha$  and 36 kDa for E1 $\beta$ ) are therefore consistent with these being the respective fully processed PDH subunits of P. falciparum. The identity of a cross-reacting band at about 30 kDa (E1B) is unknown. The apparent molecular masses of the protein size markers are given in kDa.

higher concentrations of 6 M guanidine hydrochloride and 8 M urea yielded E2CD protein with a specific activity of less than 2% of that observed with the gentler solubilization conditions (3 M guanidine hydrochloride and 4 M urea). Similarly, enzyme activity of recombinant E2CD protein was lost almost entirely after freezing at -20°C when no cryoprotectant was present. In contrast, storage at -20°C in the presence of 50% glycerol and 10-20 mM  $\beta$ -mercaptoethanol was found to effectively preserve E2CD enzyme activity over months.

#### Discussion

The present study provides strong evidence for the presence of a single functional PDH in *P. falciparum* and related parasites that is located in the apicoplast. Four genes encoding the subunits of one complete PDH were amplified from cDNA and phylogenetic analyses indicate that these nuclear-encoded genes derive from the endosymbiont that gave rise to the apicoplast. Apicoplast localization of three PDH subunits (subunits E1 $\alpha$ , E2 and E3) courtesy of bipartite N-terminal presequences was demonstrated by expression of GFP fusion proteins in *P. falciparum* (this study and McMillan *et al.*, 2004). Amplification of cDNA corresponding to fully spliced transcripts of subunit E2 as well as Western blot analysis (for subunits



**Fig. 6.** The catalytic domain of the *P. falciparum* PDH subunit E2 (dihydrolipoamide acetyltransferase) is enzymatically active. A. Coomassie-stained SDS-PAGE gel of purified protein preparations from *E. coli* strain BL21DE3. Both Control 1 (Ctrl1) and E2CD refer to the same bacterial strain carrying an expression vector encoding the catalytic domain of PDH subunit E2 of *P. falciparum* (E2CD), but for Ctrl1 without the addition of IPTG for induction of recombinant protein expression. Control 2 (Ctrl2) represents the same bacterial strain but lacking the E2CD expression vector (after addition of IPTG). The first three lanes (denoted by '1x') show a 2 µl aliquot of the initial protein eluate after Ni-agarose purification, whereas the two lanes denoted by '5x' represent 10 µl each of the two negative controls. The major protein band of about 30 kDa size in the E2CD lane represents  $\approx 1 \mu g$  of E2 catalytic domain.

B. The Michaelis constant  $K_m$  for the substrate acetyl-CoA was determined by measuring E2CD enzymatic activity for varying concentrations of Coenzyme A, which is readily and quantitatively converted into the substrate acetyl-CoA in the assay mixture (main plot; shown are means from four independent experiments with standard error). The double-reciprocal diagram (Lineweaver-Burk plot) of enzymatic activity versus substrate concentration specifies a  $K_m$  of

 $3.08~\mu M\pm0.23$  (standard error) for acetyl-CoA.  $\Delta Abs~min^{-1}$  refers to changes of absorbance per minute in the spectrophotometric assay.

E1 $\alpha$  and E1 $\beta$ ) indicate that this PDH is expressed in intraerythrocytic malaria parasites, and enzymatic activity of the recombinantly expressed catalytic domain of the E2 subunit confirms the identity and functionality of this enzyme. The pattern of KADH genes found in the genomes of several *Plasmodium* species and *T. gondii* suggests that a PDH enzyme complex is present only in the plastid in these parasites.

The phylogenetic analyses yielded particularly robust clusters for PDH subunits from the cyanobacterial/plastid and the  $\alpha$ -proteobacterial/mitochondrial lineages. The only exceptions are the mitochondrial E3 subunits of P. falciparum and P. yoelii that defied a clear assignment in an otherwise well-resolved phylogenetic tree (Fig. 3). Nevertheless, these E3 genes still show greatest sequence identity/similarity to  $\alpha$ -proteobacterial and mitochondrial E3 sequences, a finding consistent with a mitochondrial origin of these genes. The ambiguous position of these genes in the phylogenetic tree might at least partly be explained by the amino acid bias caused by the extreme A+T richness of the nuclear genomes of P. falciparum and P. voelii (Carlton et al. 2002; Gardner et al., 2002). This general skew in amino acid composition is expected to unduly bias the phylogenetic analysis such that the plasmodial sequences cluster together more closely. Despite these minor inconsistencies in the phylogenetic analysis, the mitochondrial E3 subunit of P. falciparum has unambiguously been shown to be targeted to the mitochondria (McMillan et al., 2004), where it probably functions as a subunit of both the BCKDH and the KADH, enzyme complexes that are usually involved in the degradation of branched-chain amino acids and in the TCA cycle (see below) respectively.

Apicoplast-targeted proteins contain a bipartite presequence that is processed in two separate steps: the signal peptide is co-translationally removed in the lumen of the ER, whereas the adjacent transit peptide is thought to be cleaved by a processing peptidase within the apicoplast (Waller et al., 2000; van Dooren et al., 2001; 2002). As very little is known about apicoplast transit peptide cleavage sites, the molecular mass of the fully processed proteins can only be estimated approximately by sequence comparisons to homologous proteins. Thus, the E1 $\alpha$  protein of the P. falciparum PDH appears to contain an Nterminal extension of about 183 amino acids length (see Fig. 2) corresponding to a molecular mass of 21.9 kDa. As this extension includes both a signal peptide (predicted by SIGNALP to be 17 amino acids long, or 2.2 kDa) and a transit peptide (see Fig. 2), the transit peptide is expected to comprise up to 166 amino acids (19.7 kDa). The fully processed E1 $\alpha$  protein is therefore estimated to have a molecular mass of somewhere between 48.9 kDa (the mass of the mature protein part that shows sequence similarity to homologous proteins) and less than 68.6 kDa

(mature protein plus transit peptide), depending on where exactly the transit peptide is cleaved. In agreement with this prediction, our Western blot analysis indicates a molecular mass of about 57 kDa for this protein. The discrepancy between this value (57 kDa) and the smallest molecular mass expected for the mature protein part (48.9 kDa) indicates that the transit peptide is cleaved upstream of the point where sequence homology to other  $E1\alpha$  subunits starts (amino acid position 183; see Fig. 2), and suggests that the actual transit peptide cleavage site may be located in the region of amino acid positions 108-125 (VQSLCAIKHGNVLIKNIN) which would yield a molecular mass of 56-58 kDa. In analogy, the fully processed form of the P. falciparum PDH E1ß subunit is estimated to have a molecular mass between 36.8 kDa and 43.2 kDa, and the major band of about 36 kDa detected in our Western blot analysis corresponds well with this estimate. The Western blot analysis for neither protein revealed bands corresponding to the pre-processed species of these proteins (i.e. lacking the signal peptide but including the transit peptide) as it has been described for some apicoplast-targeted proteins (Waller et al., 1998; 2000; Tonkin et al., 2004). Recent microarray data are not unequivocal in regard to PDH expression but do suggest low level transcription of the PDH subunits during the intraerythrocytic cycle. The microarray data also indicate a peak of PDH transcription in gametocytes (Bozdech et al., 2003; Le Roch et al., 2003).

It has been established over the past few years that apicomplexan parasites like Plasmodium and Toxoplasma possess an apicoplast-localized, functional type II FAS machinery allowing for de novo FAS. Importantly, this essential anabolic pathway provides a whole array of new drug targets (Ralph et al., 2001; Foth and McFadden, 2003; Gornicki, 2003; Sharma et al., 2003; Waller et al., 2003). By analogy with plants and algae, we expect the function of the single PDH in apicomplexans to include the provision of acetyl-CoA for FAS within the apicoplast (cf. Ralph et al., 2004b). The metabolic picture for the apicoplast has recently been further complemented by the localization of functional lipoate synthases to the apicoplasts of T. gondii and P. falciparum (Thomsen-Zieger et al., 2003; Wrenger and Muller, 2004). These enzymes, in conjunction with an appropriate lipoyl transferase which has also been shown to be present in the plastid of P. falciparum (Wrenger and Muller, 2004), can thus apparently provide the essential PDH cofactor lipoic acid right within the organelle.

The unique PDH distribution in *Plasmodium* and *Toxoplasma* (PDH present in the plastid but absent from the mitochondrion) raises a couple of issues. First, how do apicomplexans link glycolysis in the cytosol to oxidative phosphorylation in the mitochondrion? Genes necessary for a functional TCA cycle have been found in the

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genome of P. falciparum (Gardner et al., 2002) which begs the question of how this pathway is fuelled. One scenario would be transfer of acetyl-CoA from the apicoplast into the mitochondrion, perhaps using acetyl-CoA transporters. Potential homologues of a putative human acetyl-CoA transporter (Accession No. NP\_004724) (Kanamori et al., 1997) are indeed present in the genomes of several apicomplexans [P. falciparum (NP\_700833), P. yoelii (EAA19825), T. gondii, Cryptosporidium parvum], but their location and function in parasites are unknown. In human cells this transporter is thought to be located in the ER/Golgi membrane, whereas apicoplast-to-mitochondrion transfer of acetyl-CoA would presumably require multiple transporters to cross the multiple membranes involved. Another possibility is that P. falciparum simply does not constitutively use pyruvate as fuel for oxidative phosphorylation, and it has been suggested that the TCA cycle in intraerythrocytic parasites may serve mainly to provide succinyl-CoA for haem biosynthesis (Gardner et al., 2002). The absence of mitochondrial acetyl-CoA could possibly be compensated for by supplying the TCA cycle with  $\alpha$ -ketoglutarate derived from glutamate. One way of generating  $\alpha$ -ketoglutarate from glutamate could be the direct conversion by glutamate dehydrogenase, an enzyme encoded by three different putative genes in P. falciparum (PlasmoDB identifiers PF08-0132, PF14-0164, PF14-0286). Alternatively, aspartate aminotransferase (PFB0200c) (Berger et al., 2001) could produce  $\alpha$ -ketoglutarate from glutamate by simultaneously turning oxaloacetate into aspartate. Glutamate would be readily available from protein digestion or amino acid uptake, whereas oxaloacetate could be provided from phosphoenolpyruvate by the action of phosphoenolpyruvate carboxylase (Gardner et al., 2002). Finally,  $\alpha$ -ketoglutarate would probably have to be imported into the mitochondrion by a dicarboxylate transporter. This scenario of an  $\alpha$ -ketoglutarate-driven TCA cycle would fit well together with findings indicating (i) that the putative aconitase of *P. falciparum* – usually an early TCA cycle enzyme that catalyses the conversion of citrate to isocitrate - does not exhibit aconitase activity (Loyevsky et al., 2001) and (ii) that the isocitrate dehydrogenase of P. falciparum - the enzyme following aconitase in the TCA cycle which converts isocitrate into  $\alpha$ -ketoglutarate – does not utilize NAD<sup>+</sup> but NADP<sup>+</sup> and may thus not be an essential part of the parasite's TCA cycle (Wrenger and Muller, 2003).

Another issue is whether the apicoplast PDH could be a drug target. Disruption of FAS has proven lethal to apicomplexan parasites (Waller *et al.*, 1998; 2003; Surolia and Surolia, 2001; Surolia *et al.*, 2004), and no alternative source of acetyl-CoA in the apicoplast is evident from the predicted proteome (Gardner *et al.*, 2002). As disruption of PDH function is expected to close down FAS, PDH subunits warrant further investigation as potential chemotherapeutic targets (despite the fact that we are not aware of any herbicides that may have already been developed to target this enzyme complex in plants). The presence of a mitochondrial PDH in mammalian cells requires that inhibitors be specific for the plastidic PDH of the pathogen, but this may pose less of a problem than one might initially suspect: amino acid identity/similarity between PDH subunits of humans and P. falciparum are all below 36%/60%, and antibodies raised against mitochondrial PDH subunits (even of the same species) have failed to show significant cross-reactivity with their plastidic counterparts (Taylor et al., 1992; Conner et al., 1996). Furthermore, the three-dimensional structures of several human and bacterial PDH subunits have been solved (e.g. Mattevi et al., 1992b; 1993; Ciszak et al., 2003), and efforts in our laboratories are currently underway to determine the crystal structure of the catalytic domain of the P. falciparum dihydrolipoamide acetyltransferase (E2). This detailed knowledge of structure and catalytic action of PDH subunits in combination with available in vitro enzyme assays will greatly facilitate rational drug design and the search for novel drugs directed against apicomplexan parasites.

#### **Experimental procedures**

#### Database searches

KADH subunit protein sequences were searched for in genomic and EST databases for various Plasmodium species at the NCBI Malaria Genetics and Genomics (http://www.ncbi.nih.gov/projects/Malaria/ web-site plasmodiumblcus.html), at PlasmoDB (http://PlasmoDB.org/) and at the Sanger Institute website (http:// www.sanger.ac.uk/), and for T. gondii at ToxoDB (http:// toxodb.org/). The complete P. falciparum genome and its annotation (Gardner et al., 2002) were also gueried. Database searches were carried out using the programs TBLASTN and BLASTP (Altschul et al., 1997). To confirm whether a resulting match in a database was significant and represented a homologue of the gene in question, such matches were always queried back (using BLASTP) against the non-redundant protein database at NCBI (http:// www.ncbi.nlm.nih.gov/BLAST/). Sequence identities and similarities between pairs of sequences were determined using BLASTP with the low-complexity filter-option disabled (at http://www.ncbi.nlm.nih.gov/blast/bl2seg/bl2.html).

#### Bioinformatic prediction of subcellular localization

To predict the presence of N-terminal targeting sequences and the subcellular localization of proteins, the following bioinformatic tools were employed: SIGNALP (Nielsen *et al.*, 1997) (http://www.cbs.dtu.dk/services/SignalP-2.0/), TARGETP (Emanuelsson *et al.*, 2000) (http://www.cbs.dtu.dk/ services/TargetP/), MITOPROT (Claros and Vincens,

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1996) (http://ihg.gsf.de/ihg/mitoprot.html), PLASMOAP (Foth *et al.*, 2003) (http://www.plasmodb.org/restricted/ PlasmoAPcgi.shtml) and PATS (Zuegge *et al.*, 2001) (http:// gecco.org.chemie.uni-frankfurt.de/pats/pats-index.php).

#### Plasmodium falciparum cell culture and cDNA cloning

Plasmodium falciparum strain D10 was grown according to Trager and Jensen (1976). Before RNA/DNA purification or protein extraction, infected red blood cells were lysed on ice for 10 min with 0.15% saponin in HT-PBS. RT-PCR was used to obtain cDNA clones containing the protein coding regions of the four subunits of the plastidic PDH. The oligonucleotide primers used in these experiments are listed in Table S1 (Supplementary material). These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under Accession No. AY484441 (E1α), AY484442 (E1β), AY484443 (E2) and AY484444 (E3). The PlasmoDB identifiers for all P. falciparum KADH subunits discussed in this study are as follows: PF11-0256 (plastid PDH E1a), PF14-0441 (plastid PDH E1B), PF10-0407 (plastid PDH E2), PF08-0066 (plastid E3), PFL1550w (mitochondrial E3), PF13-0070 (BCKDH E1 $\alpha$ ), PFE0225w (BCKDH E1 $\beta$ ), PFC0170c (BCKDH E2), PF08-0045 (KGDH E1), PF13-0121 (KGDH E2).

#### Sequence alignments and phylogenetic analyses

Multiple protein sequence alignments were generated with CLUSTALX 1.81 and were edited manually. The P. falciparum sequences represent cDNA sequences determined in this study and by McMillan et al. (2004). Sequences of P. yoelii and P. knowlesi were taken from genomic sequence data and have not been confirmed by cDNA sequencing. In silico splicing of the P. yoelii and P. knowlesi sequences for subunit E2 was based on detailed comparison to the P. falciparum E2 cDNA sequence. All other sequences were taken from public databases. Full alignments and accession numbers for all sequences included in the alignments are available in Figs S1-S4. Phylogenetic trees were constructed using PAUP\*4.0b10 (Swofford, 2003). NJ analysis was performed using the Minimum Evolution method and with the mean character difference as distance measure. MP trees were generated using the heuristic search method and tree bisection-reconnection (TBR) as branch-swapping algorithm. The individual weighted MP trees shown in Fig. 3 were searched by starting from random trees with 100 repetitions, whereas for MP bootstrap analysis taxa were added to trees according to the 'closest' criterion. For weighted MP analysis, character weights were assigned according to the rescaled consistency index with a base weight of 1. All MP analyses were based on 329 (E1a), 327 (E1b), 338 (E2) or 442 (E3) characters, of which 273 (E1a), 275 (E1b), 310 (E2) or 382 (E3) were parsimony-informative. All bootstrap calculations were performed by running 1000 replicates.

#### GFP fusion constructs and P. falciparum transfection

The predicted bipartite leader sequences of PDH subunits E1 $\alpha$  and E2 were amplified by PCR from genomic DNA of *P. falciparum* (D10 strain) using primers containing *Bg*/II and

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Avrll restriction sites as well as Gateway<sup>™</sup> (Invitrogen) att sites. The oligonucleotide primers used in these experiments are listed in Table S1 (Supplementary material). The amplified leader sequences comprised the N-terminal 191 and 61 amino acids of PDH subunits  $E1\alpha$  and E2 respectively. They therefore also included the first few amino acids of the putative mature protein regions (cf. Fig. 2). The resulting PCR products were digested with Bg/II and AvrII (which removed the att sites) and ligated into a GFP expression cassette within a *P. falciparum* vector modified to utilize Gateway<sup>™</sup> (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.

#### Immunofluorescence analysis

Infected erythrocytes were fixed, labelled and prepared for immunofluorescence microscopy as described (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 1 h. 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 ( $\approx 0.1 \text{ mg ml}^{-1}$ ) 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) for at least 60 min 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<sup>-1</sup> 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 and captured in live cells using a Leica TCS 4D confocal microscope. Live mitochondria staining was carried out using MitoTracker Red CM-H<sub>2</sub>XRos (Molecular Probes). Briefly, transfected *P. falciparum* lines were incubated with MitoTracker diluted to 40 nM in culture medium (RPMI-Hepes supplemented with 0.5% AlbumaxII) for 15 min at 37°C, and washed once in culture medium.

#### Recombinant protein expression

The *P. falciparum* plastidic PDH subunits E1 $\alpha$ , E1 $\beta$ , and E2 as well as the catalytic domain of E2 (E2CD) were recombinantly expressed following standard protocols (Sambrook

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et al., 1989). Proteins were expressed in E. coli strain BL21DE3 (Stratagene) using the expression system pProEX<sup>™</sup> HT (Life Technologies) with an N-terminal hexahistidine tag. The expression constructs excluded the predicted signal peptides (Nielsen et al., 1997) and most of the predicted transit peptide regions of the genes and encoded the following amino acid positions of the predicted protein sequences (predicted molecular masses of the expressed proteins in parentheses): 156-601 for E1a (58.0 kDa), 86-415 for E1β (40.6 kDa), 32-640 for E2 (74.0 kDa), and 401-640 for E2CD (30.4 kDa). The inserts for the expression plasmids were generated as follows: the  $E1\alpha$  insert was excised from the appropriate cDNA clone in pGEM-T Easy (Promega) using the restriction endonucleases Spel and Xmnl, which created a blunt end after nucleotide position 463 (from the start-Met) because of a naturally occurring restriction site in the E1 $\alpha$  gene. This insert was cloned into pProEXHTc which had been digested with Stul (blunt-end cutter) and Spel. For the E1ß insert, an EcoRI restriction site was introduced by reamplifying the appropriate cDNA clone with the primers listed in Table S1 (Supplementary material). The insert was cloned from pGEM-T Easy into pProEXHTa using EcoRI and Spel. The 'full-length' E2 insert was generated by first cutting the appropriate cDNA clone with Kpnl and blunt-ending with Klenow Fragment followed by a Pstl digest. The resulting piece of DNA was cloned into pProEXHTa, which had been cut with Stul and Pstl. The insert for expressing only the catalytic domain of PDH subunit E2 ('E2CD') was created by PCR amplification from cDNA template (for primers, see Table S1). Cloning of the insert into pProEXHTa was then carried out using Ncol and Kpnl.

The expression plasmids were co-transfected with the RIG plasmid (Baca and Hol, 2000) and - in the case of E2CD with the pREP4-groESL plasmid (Caspers et al., 1994). For antibody production, recombinant protein was obtained from washed and solubilized (6 M guanidine hydrochloride followed by 8 M urea) inclusion bodies and isolated using Nickel-NTA agarose (Qiagen) and SDS-PAGE. The Coomassie Blue-stained protein bands corresponding to the recombinant proteins were excised from several gels and electroeluted over 6 h with a Bio-Rad electroeluter (model 422) according to manufacturer's instructions in a buffer containing 50 mM NH<sub>4</sub>CO<sub>3</sub> and 0.1% SDS (Stearne et al., 1985) and using 15 kDa cutoff membrane cups. The electroeluted protein was precipitated at -20°C for 18 h after addition of 10 volumes of ice-cold acetone, collected by centrifugation in a HB-4 Sorvall rotor at 10 000 r.p.m. for 30 min at -10°C, and resuspended in PBS. To generate enzymatically active protein, recombinant E2CD was obtained from washed and solubilized (3 M guanidine hydrochloride followed by 4 M urea) inclusion bodies and purified using Nickel-NTA agarose (Qiagen). In either purification regimen, guanidine hydrochloride-based buffers were replaced by urea-based buffers after initial binding of protein to Nickel-agarose.

#### Polyclonal antibodies and Western blot analysis

Polyclonal antibodies directed against the recombinantly expressed plastidic PDH subunits E1 $\alpha$  and E1 $\beta$  of *P. falciparum* were affinity-purified from anti-sera raised in rabbits

using recombinant protein bound to nitrocellulose membranes (Schleicher and Schuell). For Western blot analysis, parasite proteins from asynchronous *P. falciparum* cultures (strain D10) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Native PDH proteins were detected using the purified polyclonal antibodies at 1:200– 1:1000 dilutions in blocking buffer (TBS containing 3–5% skim milk powder). Secondary anti-rabbit IgG antibodies conjugated to horseradish peroxidase were employed at 1:20 000 dilution and visualized using SuperSignal<sup>®</sup> (Pierce) chemiluminescent substrates and X-ray film (Fuji).

#### In vitro enzyme assay for PDH subunit E2

The enzyme activity of the catalytic domain (E2CD) of the P. falciparum PDH subunit E2 (dihydrolipoamide acetyltransferase) was measured in vitro using a spectrophotometric assay based on previously published methods (Schwartz and Reed, 1969; Yang et al., 1997). In short, E2 activity was measured in a 1 ml volume of a solution containing 30 mM Tris-HCI (pH 7.4), 1 mM acetyl-phosphate (Sigma), 1 mM dihydrolipoamide, 5 µM CoA (lithium salt, from yeast; Sigma), 1–2 U of phosphotransacetylase (Sigma) and 0.26  $\mu$ g of recombinant E2CD protein. Absorbance at 232 nm was measured at room temperature in quartz cuvettes over a period of 1 min. Dihydrolipoamide was generated from lipoamide (= 6,8-thioctic acid amide; Sigma) (Schoneck et al., 1997). Enzyme kinetic data were analysed, plotted (Fig. 6B) and subjected to non-linear fitting for Michaelis constant determination using GraFit 5 (Erithacus Software).

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### Supplementary material

The following material is available from http://www.blackwellpublishing.com/products/journals/ suppmat/mmi/4407/mmi4407sm.htm **Fig. S1.** Multiple sequence alignment of PDH E1 $\alpha$  protein sequences used for phylogenetic analyses. **Fig. S2.** Multiple sequence alignment of PDH E1 $\beta$  protein sequences used for phylogenetic analyses. **Fig. S3.** Multiple sequence alignment of PDH E2 protein sequences used for phylogenetic analyses. **Fig. S4.** Multiple sequence alignment of PDH E3 protein sequences used for phylogenetic analyses.

 Table S1. Oligonucleotide primers used in this study.

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