Identification of two [4Fe–4S]-cluster-containing hydro-lyases from *Pyrococcus furiosus*

Barbara M. A. van Vugt-Lussenburg, Laura van der Weel, Wilfred R. Hagen and Peter-Leon Hagedoorn

Department of Biotechnology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands

The hyperthermophilic archaeon *Pyrococcus furiosus* is a strict anaerobe. It is therefore not expected to use the oxidative tricarboxylic acid (TCA) cycle for energy transduction. Nonetheless, its genome encodes more putative TCA cycle enzymes than the closely related *Pyrococcus horikoshii* and *Pyrococcus abyssi*, including an aconitase (PF0201). Furthermore, a two-subunit fumarase (PF1755 and PF1754) is encoded on the *Pr. furiosus* genome. In the present study, three of these genes were heterologously overexpressed in *Escherichia coli* to enable characterization of the enzymes. PF1755 and PF1754 were shown to form a [4Fe–4S]-cluster-containing heterodimeric enzyme, able to catalyse the reversible hydration of fumarate. The aconitase PF0201 also contained an Fe–S cluster, and catalysed the conversion from citrate to isocitrate. The fumarase belongs to the class of two-subunit, [4Fe–4S]-cluster-containing fumarate hydratases exemplified by MmcBC from *Pelotomaculum thermopropionicum*; the aconitase belongs to the aconitase A family. Aconitase probably plays a role in amino acid synthesis when the organism grows on carbohydrates. However, the function of the seemingly metabolically isolated fumarase in *Py. furiosus* has yet to be established.

**INTRODUCTION**

The genes PF1755 and PF1754 from the hyperthermophilic archaeon *Pyrococcus furiosus* are annotated as the α- and β-subunits of a two-subunit fumarate hydratase (fumarase, EC 4.2.1.2) based on sequence homology to class I fumarases. The PF1755 and PF1754 proteins are highly homologous to MmcB and MmcC of the thermophilic bacterium *Pelotomaculum thermopropionicum*, which was the first two-subunit fumarase described in the literature (Kosaka et al., 2006; Shimoyama et al., 2007).

Fumarase plays a key role in the tricarboxylic acid (TCA) cycle, where it catalyses the reversible hydration of fumarate to L-malate. No alternative substrates have been reported for the *Pel. thermopropionicum* fumarase MmcBC, but several other fumarases have been described to be able to catalyse the dehydration of the stereoisomer Δ-tartrate, but not L-tartrate or meso-tartrate, to oxaloacetate (Flint, 1994; Kim et al., 2007; Nakamura & Ogata, 1967, 1968a, b).

Although the strict anaerobe *Py. furiosus* is not likely to use the oxidative TCA cycle for energy transduction, structural genes for several of the other TCA cycle enzymes have been identified in the genome of *Py. furiosus* as well. Indeed, *Py. furiosus* contains three TCA cycle enzymes that appear to be absent in the closely related *Py. abyssi* and *Py. horikoshii* (Table 1) and in many other archaea (Huynen et al., 1999). For instance, *Py. furiosus* contains a gene coding for aconitase. Aconitase is responsible for the second step of the TCA cycle, the conversion of citrate to isocitrate. This *Py. furiosus* aconitase gene, PF0201, has been annotated based on sequence homology, but the enzyme has not been investigated experimentally. The aim of this study was to characterize the putative fumarase (PF1755-1754) and aconitase (PF0201) and to confirm their identity as TCA cycle enzymes. Our approach to accomplish this was to overexpress both enzymes in *Escherichia coli*, and study their enzymic properties.

**METHODS**

**Materials.** Pig-heart malate dehydrogenase was purchased from Roche; isocitrate dehydrogenase was purchased from Sigma. The restriction enzymes and T4 DNA ligase were obtained from New England Biolabs. PCR primers were purchased from Thermo Scientific. Pfx polymerase was purchased from Invitrogen. For subcloning pCR2.1-TOPO (Invitrogen) was used and for heterologous expression pET15b and pETDuet-1 vectors (Novagen) were used. All other chemicals were of the highest grade and obtained from standard suppliers.

**Organisms and growth conditions.** *E. coli* strain DH5α (Invitrogen) was used as a host for the construction of pET15b and pETDuet-1 derivatives. *E. coli* strain BL21(DE3) Codon+ RP (Stratagene) was used as an expression host. Both strains were
Table 1. Gene numbers of annotated TCA cycle enzymes in Pyr. furiosus, Pyr. abyssi and Pyr. horikoshii

<table>
<thead>
<tr>
<th>TCA cycle enzyme</th>
<th>Pyr. furiosus</th>
<th>Pyr. abyssi</th>
<th>Pyr. horikoshii</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Citrate synthase</td>
<td>PF0203</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2. Aconitase</td>
<td>PF0201</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3. Isocitrate dehydrogenase</td>
<td>PF0202</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4. 2-Ketoglutarate:ferredoxin oxidoreductase</td>
<td>PF1768/69/70; PF1771/72/73</td>
<td>PAB2359; PAB0344/45; PF0346/47/48</td>
<td>PH1662/61/60; PH1666/65/63</td>
</tr>
<tr>
<td>5. Succinyl-CoA synthetase</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>6. Succinate dehydrogenase</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>7. Fumarase</td>
<td>PF1754/55</td>
<td>PAB2030/31</td>
<td>PH1683/84</td>
</tr>
<tr>
<td>8. Malate dehydrogenase</td>
<td>–</td>
<td>PAB1791</td>
<td>PH1277</td>
</tr>
</tbody>
</table>

grown under standard conditions following instructions from the manufacturer.

Cloning of PF1754, PF1755 and PF0201. The genes PF1754, PF1755 and PF0201 were amplified from P. furiosus chromosomal DNA using the following primers: BamHI-1755-F (CTGGATCCGAGGACATAGTGGA); PF1755-SalI-R (CTGCGATTACCTGCGATATCAGG); NdeI-1754-F (CCATATGCGGTCGTAAGGCTAAAAACT); 1754-Xhol-R (GCTCGAGTTAATTTTTTCTGTATAGGGAT); Xhol-Aco-F2 (GCTGCGATTATTTAGAAGAGAGGATGGA); Aco-BamHI-R (CGGATCCCTAGCCCAACAGTTC).

PCR products of PF1754 and PF1755 and PF0201 were treated with Taq polymerase (Amersham Biosciences) for 10 min at 72°C to obtain single 3'-adenine overhangs for subcloning into the pCR2.1-TOPO vector. TOPO constructs were transformed into chemically competent DH5α TOPO vector. TOPO polymerase (Amersham Biosciences) for 10 min at 72°C and the PCR products of PF1754 and PF1755 and PF0201 were treated with Taq polymerase (Amersham Biosciences) for 10 min at 72°C to obtain single 3'-adenine overhangs for subcloning into the pCR2.1-TOPO vector. TOPO constructs were transformed into chemically competent DH5α TOPO vector. TOPO polymerase (Amersham Biosciences) for 10 min at 72°C and the PCR products of PF1754 and PF1755 and PF0201 were treated with Taq polymerase (Amersham Biosciences) for 10 min at 72°C to obtain single 3'-adenine overhangs for subcloning into the pCR2.1-TOPO vector. TOPO constructs were transformed into chemically competent DH5α TOPO vector. 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NADP-dependent enzyme isocitrate dehydrogenase, resulting in NADPH formation. The reaction was carried out by aconitase (cytosolic extract after heat treatment), regenerated with Fe$^{2+}$ and L-cysteine as described above for fumarase, at 75 °C in assay buffer containing 1 mM citrate. The reaction was terminated after 10 min by lowering the temperature to 0 °C. Subsequently, 1 mM NADP$^+$, 1.5 mM MnSO$_4$ and 0.5 mg isocitrate dehydrogenase ml$^{-1}$ were added, and NADPH formation was monitored using the $A_{340}$ at 25 °C.

**RESULTS**

**Sequence comparison**

The amino acid sequences of PF1755 and PF1754 were compared to *E. coli* fumarase FumA and *Pel. thermopropionicum* fumarases MmcB and MmcC. PF1755 shows high homology to MmcB (41% identity, 57% similarity), while PF1754 is highly homologous to MmcC (39% identity, 58% similarity). In addition, PF1755 has significant homology to the N-terminal part of FumA (24% identity, 40% similarity), while PF1754 is homologous to the C-terminal part (31% identity, 49% similarity) (Fig. 1). The three cysteine residues that are expected to participate in the binding of iron in class I fumarases (Reaney et al., 1993) are also present in PF1755, which indicates that PF1755-1754 contains a [4Fe–4S] cluster, similar to FumA, FumB and MmcBC (Flint et al., 1992; Reaney et al., 1993; Shimoyama et al., 2007) (B. M. A. van Vugt-Lussenburg and others, unpublished). Sequence comparison showed that PF0201 is highly homologous to *E. coli* aconitase (47% identity, 65% similarity). Also for this protein, the cysteine residues that are involved in binding of the [4Fe–4S] cluster are conserved (Hentze & Argos, 1991) (data not shown).

**Enzyme expression and purification**

The genes encoding PF1754 and PF1755 were over-expressed in *E. coli* using the pETDuet vector, which is designed for the co-expression of two genes, and which introduces an N-terminal 6-His tag into one of the genes. SDS-PAGE analysis of the *E. coli* cells after overnight induction showed good expression of PF1754 (18 kDa) and significantly lower expression of PF1755 (30 kDa). After elution from the nickel Sepharose, equal quantities of PF1755 and PF1754 were detected by SDS-PAGE, which

![Fig. 1. Alignment of *Pyrococcus furiosus* PF1754 and PF1755, *E. coli* FumA and *Pel. thermopropionicum* MmcB and MmcC. PF1755 and MmcB align with the N-terminal part of FumA, and PF1754 and MmcC align with the C-terminal part of FumA. The cysteine residues that are expected to be involved in iron binding are indicated with arrows. (†), identical residue; (‡), conservative substitution; (§), semi-conservative substitution.](http://mic.sgmjournals.org)
indicates that PF1754 is associated with the His-tagged PF1755 during the nickel affinity purification to form a heterodimer. The molecular mass as determined by size-exclusion chromatography was 48 kDa, which corresponds to one PF1755 and one PF1754 subunit. This establishes the heterodimeric state of the native enzyme, as was observed previously for Pel. thermopropionicum MmcBC (Shimoyama et al., 2007). Iron determination showed that only approximately 2% of the enzyme contained an Fe–S cluster, the remainder being apoprotein (data not shown). SDS-PAGE analysis of cultures expressing aconitase (PF0201) showed efficient overexpression of the protein (93 kDa). For aconitase, no size-exclusion chromatography or iron determination was performed.

EPR spectroscopy

The EPR spectrum of fumarase (PF1754-1755) as isolated is characteristic of an S=1/2 species (gmax = 2.019, gCrossover = 2.011) from a [3Fe-4S]+ cluster (Duderstadt et al., 1999; Telser et al., 2000) (Fig. 2a). The spectral amplitude was very low due to low cluster content of the sample. The spectrum is similar to the previously reported spectra of the [3Fe-4S]+ cluster of m-aconitase (Kennedy et al., 1992). After regeneration of the enzyme by the addition of ferrous iron and l-cysteine followed by reduction with sodium dithionite the previous signal disappeared. Only after incubation of the reduced regenerated enzyme with fumarate did an S=1/2 signal (g = 2.021, g = 1.902, g = 1.820) from the [4Fe–4S]+ cluster arise, apparently with substrate and/or product bound (Fig. 2b). The spectra are very similar to those of E. coli FumA and FumB, and Pel. thermopropionicum MmcBC (Flint et al., 1992; Shimoyama et al., 2007) (B. M. A. van Vugt-Lussenburg and others, unpublished).

Enzyme activity

Enzyme activity of fumarase could only be measured when the enzyme was regenerated with Fe2+ in the presence of l-cysteine prior to measurement. The enzyme was able to perform fumarate hydration as well as l-malate dehydration, with similar kinetic parameters (Table 2). After correction for apoenzyme, the catalytic efficiency kcat/Km of the reactions was in the same range as the catalytic efficiency of E. coli FumA and FumB, 1.4 × 106 s–1 M–1 for malate and 4.5 × 106 s–1 M–1 for fumarate (B. M. A. van Vugt-Lussenburg and others, unpublished), but one to two orders of magnitude greater than the catalytic efficiency of Pel. thermopropionicum MmcBC. For the D-tartrate dehydratase reaction, the catalytic efficiency of fumarase was two orders of magnitude greater than that of FumA, 3 × 105 s–1 M–1, and FumB, 1.2 × 104 s–1 M–1, while all Kms values were similar (B. M. A. van Vugt-Lussenburg and others, unpublished). Similar to FumA and FumB, fumarase was not able to dehydrate D-malate, l-tartrate or meso-tartrate (data not shown) (B. M. A. van Vugt-Lussenburg and others, unpublished).

Aconitase was found to be able to catalyse the isomerization of citrate to isocitrate. Activity could only be measured after regeneration with Fe2+, suggesting that aconitase contains an Fe–S cluster like all other members of the aconitase family (Hentze & Argos, 1991). Because aconitase was not purified to homogeneity, and because the cluster content was not determined, it was not possible to calculate the specific activity for this enzyme.

Discussion

Similar to what has been reported for other hyperthermophilic archaea, Pyr. furiosus contains two genes that have been annotated as fumarases based on sequence similarity (Siebers et al., 2004). In the present study we show that PF1755 and PF1754 indeed form a two-subunit fumarase
Table 2. Kinetic parameters of fumarase and tartrate dehydratase activity of Pyr. furiosus fumarase and Pel. thermopropionicum MmcBC

<table>
<thead>
<tr>
<th>Substrate/enzyme</th>
<th>l-Malate</th>
<th>Fumarate</th>
<th>d-Tartrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$</td>
<td>$V_{max}$</td>
<td>$k_{cat}/K_m$</td>
</tr>
<tr>
<td>Pf fumarase</td>
<td>0.41</td>
<td>1892</td>
<td>$3.7 \times 10^6$</td>
</tr>
<tr>
<td>MmcBC*</td>
<td>0.59</td>
<td>23.7</td>
<td>$4.3 \times 10^4$</td>
</tr>
</tbody>
</table>

*Data from Shimoyama et al. (2007).

Table 2. Kinetic parameters of fumarase and tartrate dehydratase activity of Pyr. furiosus fumarase and Pel. thermopropionicum MmcBC

Values are corrected for Fe–S cluster content. Units are as follows: $K_m$, mM; $V_{max}$, μmol product min$^{-1}$ (mg enzyme)$^{-1}$; $k_{cat}/K_m$, s$^{-1}$ M$^{-1}$. ND, Not determined. Substrate concentrations ranged from 0.07 mM to 5.5 mM (malate) and 0.1 mM to 2.7 mM (fumarate).

containing a [4Fe–4S] cluster, similar to the Pel. thermopropionicum fumarase MmcBC (Shimoyama et al., 2007).

It is interesting to note that in addition to the fumarase reaction, Pyr. furiosus is able to perform the first three steps of the TCA cycle, catalysed by citrate synthase (annotated), aconitase (this study) and isocitrate dehydrogenase (annotated), while these three enzymes are not present in the closely related Pyr. abyssi and Pyr. horikoshii. Schut et al. (2003) have reported that citrate synthase, aconitase and isocitrate dehydrogenase in Pyr. furiosus are dramatically upregulated when the organism is grown on maltose instead of peptides, presumably for the generation of x-ketoglutarate required for glutamate biosynthesis (Schut et al., 2003). For fumarase, on the other hand, no effect on transcript levels was observed. Pyr. horikoshii and Pyr. abyssi are unable to grow on carbohydrates (Erauso et al., 1993; Gonzalez et al., 1998), which justifies the absence of citrate synthase, aconitase and isocitrate dehydrogenase in these organisms. An alternative function of Pyr. furiosus aconitase may be regulatory, as certain types of eukaryotic and bacterial aconitases, in their apo-form, are known to bind mRNA. These aconitases are known to be able to block translation of certain mRNAs (e.g. encoding ferritin), by binding to an iron-responsive element in the 5′ untranslated region of the mRNA, or enhance translation of other mRNAs (e.g. encoding the E. coli aconitases AcnA and AcnB) by increasing stability by binding to an iron-responsive element in the 3′ untranslated region of the transcript (Tang & Guest, 1999 and references quoted therein).

While this explains the function of aconitase in Pyr. furiosus, the physiological role of fumarase remains unclear. Microarray data indicate that Pyr. furiosus fumarase is approximately twofold upregulated in sulfur-grown cells, which could mean that it plays a role in the bioenergetics of the cell (Schut et al., 2001). Fumarase could also have a function in the entry of carbon into the central metabolism of the cell. Pyr. furiosus contains a malic enzyme (PF1026) that is highly homologous to the malic enzyme of Thermococcus kodakarenensis (TK1963). For T. kodakarenensis, it has been shown that this enzyme catalyses the reversible decarboxylation of malate to pyruvate and CO$_2$ (Fukuda et al., 2005). The malate required for this reaction could be supplied by fumarase, while the fumarate originates from the biosynthesis of arginine from ornithine via carbamoyl phosphate synthetase and argininosuccinate lyase (http://www genome.jp/kegg/pathway/ pfu/pfu00330.html). One can imagine that fumarase supplements the pyruvate pool for gluconeogenesis when Pyr. furiosus is grown on amino acid carbon sources.

Alternatively, fumarate has been known to act as a switch factor for the flagellar motor in several prokaryotes. In E. coli, Halobacterium salinarum and Salmonella typhimurium, fumarate is essential to change the flagellar rotation from counter-clockwise to clockwise (Barak et al., 1996; Marwan et al., 1990). The physiological function of fumarase in Pyr. furiosus could therefore be fumarate production to regulate flagellar rotation. Malic enzyme could supply the malate required for the reaction; the highly homologous malic enzyme of T. kodakarenensis has been shown to perform the interconversion of malate and pyruvate in both directions with similar catalytic efficiency (Fukuda et al., 2005).

The mode of action of fumarate as a switch factor occurs through interaction with fumarate reductase in E. coli (Cohen-Ben-Lulu et al., 2008). Pyr. furiosus does not possess fumarate reductase, but neither does the archaeon H. salinarum, which is known to depend on fumarate for flagellar switching (Marwan et al., 1990). Therefore, it is possible that flagellar switching by fumarate occurs via a different mechanism in archaea, one that does not require the presence of fumarate reductase.

In conclusion, we have isolated and characterized two hydro-lyases from Pyr. furiosus that are part of the TCA cycle. The presence of citrate synthase, aconitase, isocitrate dehydrogenase and x-ketoglutarate:ferredoxin oxidoreductase suggests that Pyr. furiosus uses the first four steps of the oxidative TCA cycle for glutamate and tetrapyrusre synthesis. However, fumarate and malate seem to be metabolically isolated in Pyr. furiosus, since the links to succinate and oxaloacetate are missing (Table 1). Possibly, fumarase fulfils a function that is not related to the TCA cycle, such as flagellar switching or sulfur-related bioenergetics.
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