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Unusual ADP-forming acetyl-coenzyme A synthetases from the mesophilic halophilic euryarchaeon *Haloarcula marismortui* and from the hyperthermophilic crenarchaeon *Pyrobaculum aerophilum*

Abstract ADP-forming acetyl-CoA synthetase (ACD), the novel enzyme of acetate formation and energy conservation in archaea (Acetyl - $CoA + ADP + P_i \rightleftharpoons$ acetate + ATP + CoA), has been studied only in few hyperthermophilic euryarchaea. Here, we report the characterization of two ACDs with unique molecular and catalytic features, from the mesophilic euryarchaeon *Haloarcula marismortui* and from the hyperthermophilic crenarchaeon *Pyrobaculum aerophilum*. ACD from *H. marismortui* was purified and characterized as a salt-dependent, mesophilic ACD of homodimeric structure (166 kDa). The encoding gene was identified in the partially sequenced genome of *H. marismortui* and functionally expressed in *Escherichia coli*. The recombinant enzyme was reactivated from inclusion bodies following solubilization and refolding in the presence of salts. The ACD catalyzed the reversible ADP- and P_i-dependent conversion of acetyl-CoA to acetate. In addition to acetate, propionate, butyrate, and branched-chain acids (isobutyrate, isovalerate) were accepted as substrates, rather than the aromatic acids, phenylacetate and indol-3-acetate. In the genome of *P. aerophilum*, the ORFs PAE3250 and PAE 3249, which code for α and β subunits of an ACD, overlap each other by 1 bp, indicating a novel gene organization among identified ACDs. The two ORFs were separately expressed in *E. coli* and the recombinant subunits α (50 kDa) and β (28 kDa) were in-vitro reconstituted to an active heterooligomeric protein of high thermostability. The first crenarchaeal ACD showed the broadest substrate spectrum of all known ACDs, catalyzing the conversion of acetyl-CoA, isobutyryl-CoA, and phenylacetyl-CoA at high rates. In contrast, the conversion of phenylacetyl-CoA in euryarchaeota is catalyzed by specific ACD isoenzymes.

Keywords ADP-forming acetyl-coenzyme A synthetase · Halophilic and hyperthermophilic archaea · *Haloarcula marismortui* · *Pyrobaculum aerophilum* · Gene organization

Dedicated to Prof. Dr. Dr. h.c. mult. Hans Günter Schlegel on the occasion of his 80th birthday.

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Introduction

ADP-forming acetyl-CoA synthetase (ACD) (Acetyl - $CoA + ADP + P_i \rightleftharpoons$ acetate + ATP + CoA) is a novel enzyme that catalyzes the conversion of acetyl-CoA and other acyl-CoA esters to the corresponding acids and couples this reaction with the synthesis of ATP (Schönheit and Schäfer 1995). This unusual synthetase was first detected in the domain of eukarya, in the protists *Entamoeba histolytica* and *Giardia lamblia*, where it is involved in acetate formation and ATP production during fermentative metabolism (Reeves et al. 1977; Sanchez and Müller 1996). In prokaryotes, ACD activities have been found in all acetate-forming archaea tested, including anaerobic hyperthermophilic euryarchaeota and crenarchaeota, and mesophilic aerobic halophiles (see Schäfer et al. 1993; Schönheit and Schäfer 1995). The first detailed description of ACD activity was carried out in the hyperthermophilic euryarchaeon *Pyrococcus furiosus*, where the enzyme catalyzes the major energy-conserving reaction during pyruvate and sugar fermentation to acetate (Schäfer and Schönheit 1991; Schönheit and Schäfer 1995). The conversion of acetyl-CoA to acetate by one enzyme is unusual in prokaryotes and appears to be restricted to archaea, since all acetate-forming bacteria studied so far, including the hyperthermophile *Thermotoga maritima*, utilize two enzymes, phosphate acetyltransferase and

acetate kinase, for acetate formation and ATP synthesis (Bock et al. 1999; Schönheit and Schäfer 1995).

So far, ACDs in archaea have been characterized only from the anaerobic, hyperthermophilic euryarchaeota *P. furiosus*, *Archaeoglobus fulgidus* and *Methanococcus jannaschii* (Glasemacher et al. 1997; Mai and Adams 1996; Musfeldt et al. 1999; Musfeldt and Schönheit 2002). In *P. furiosus*, two isoenzymes, ACD I and ACD II, were characterized. They constitute heterotetramers ($\alpha_2\beta_2$) of 145 kDa composed of α (47 kDa) and β (25 kDa) subunits. The isoforms differed in their specificities and kinetic constants towards acetyl-CoA/ acetate and various acyl-CoA and aryl-CoA esters and their corresponding acids. ACD I preferentially uses acetyl-CoA as substrate and apparently constitutes the physiologically relevant enzyme of acetate formation and energy conservation in the course of sugar and pyruvate fermentation (Glasemacher et al. 1997; Mai and Adams 1996). ACD II utilizes the aryl-CoA esters phenylacetyl-CoA and indolacetyl-CoA as substrates and has been implicated primarily in the fermentation of aromatic amino acids (Adams et al. 2001; Mai and Adams 1996; Schut et al. 2003). The genes encoding α and β subunits of *P. furiosus* ACD isoforms are located on different sites in the *Pyrococcus* chromosome, i.e., they are not arranged in an operon.

By sequence comparison of the *acd* genes of *P. furiosus*, homologous open reading frames (ORFs) were identified in genomes of various hyperthermophilic archaea, including the sulfate reducer *A. fulgidus* (5 ORFs) and the methanogen *M. jannaschii* (1 ORF) (Musfeldt and Schönheit 2002; Sanchez et al. 2000). All these ORFs represent fusions of the homologous α- and β-subunit-encoding genes from *P. furiosus* (Musfeldt and Schönheit 2002). Two ORFs from *A. fulgidus*, AF1211 and AF1938, and ORF MJ0590 from *M. jannaschii* were expressed in *Escherichia coli* and the recombinant proteins were characterized as distinctive ACD isoenzymes with different substrate specificities. *A. fulgidus* ACDs AF1211 and AF1938 were similar to ACD I and ACD II of *P. furiosus* (Musfeldt and Schönheit 2002). In accordance with the fused gene structure, the ACDs of *A. fulgidus* and *M. jannaschii* are homodimers of about 140 kDa composed of 70-kDa subunits, representing fusions of the *P. furiosus* α and β subunits (Musfeldt and Schönheit 2002).

So far, ACDs have been characterized neither from the mesophilic Haloarchaea nor from any crenarchaeon; however, recently, ACD activities were found in various halophilic archaea, all of which formed acetate during exponential growth on glucose (Bräsen and Schönheit 2001).

In this report, we describe the purification and characterization of ACD and the identifcation of the encoding gene from the halophilic archaeon *Haloarcula marismortui*. The enzyme is a homodimeric protein made up of 74-kDa subunits, showed a temperature optimum in the mesophilic range, and required high salt concentrations for stability. Substrate specificities were similar to those of ACD I isoforms from *P. furiosus* (Glasemacher et al. 1997; Mai and Adams 1996; Musfeldt et al. 1999) and *A. fulgidus* (Musfeldt and Schönheit 2002). Furthermore, the first crenarchaeal ACD, from the hyperthermophile *Pyrobaculum aerophilum*, was characterized as a heterooligomer of 28-kDa and 50-kDa subunits. The enzyme differs from all known ACDs by a novel organization of the encoding genes and an unusually broad substrate spectrum.

Materials and methods

Purification of ACD from *H. marismortui*

H. marismortui (DSM 3752) (Oren et al. 1990) was grown aerobically in a 8-l fermentor (FairmenTec, Germany) on a medium containing glucose as carbon and energy source (for detailed description of the media, see Bräsen and Schönheit 2001). Cell extracts were prepared from 25 g (wet weight) of frozen cells that had been harvested in the late-exponential growth phase and then suspended in 50 mM Tris–HCl (pH 7.0) with 20 mM MgCl$_2$ (buffer A) containing 20% glycerol (v/v) and 2 M (NH$_4$)$_2$SO$_4$. Cells were disrupted by passage through a French pressure cell at 1.3×10^8 Pa. Cell debris was removed by centrifugation for 90 min at 100,000g. The supernatant was incubated at 70°C for 20 min followed by an additional centrifugation step at 100,000g for 90 min. All chromatographic steps were done at 4°C under oxic conditions. The 100,000 g supernatant was dialyzed with buffer A containing 20% (v/v) glycerol. The protein solution was applied to a Q-Sepharose HiLoad 26/10 column equilibrated with the same buffer. Protein was desorbed at a flow rate of 1 ml/ min with buffer A containing 20% glycerol (v/v) and 0.25 M NaCl (200 ml) followed by a linear gradient from 0.25 M to 0.5 M NaCl. The fractions with the highest ACD activity (0.4–0.5 M NaCl) were pooled, concentrated by ultrafiltration and applied to a Superdex 200 HiLoad 16/60 column equilibrated with buffer A containing 10% glycerol (v/v) and 2 M NaCl. The protein was eluted at a flow rate of 1 ml/min. The fractions containing the highest ACD activity were pooled, adjusted to a final concentration of 2 M (NH$_4$)$_2$SO$_4$ and applied to a Phenyl-Sepharose 6ff (low sub) [(1 ml)] column. Protein was desorbed with buffer containing 20% glycerol (v/v) at a flow rate of 1 ml/min with two decreasing gradients, from 2 M to 1.7 M (NH$_4$)$_2$SO$_4$ and from 1.7 M to 0.5 M (NH$_4$)$_2$SO$_4$. The fractions containing the highest ACD activity were eluted between 0.6 M and 0.8 M (NH$_4$)$_2$SO$_4$. At this stage ACS was essentially pure.

Cloning and expression of *H. marismortui* ACD in *E. coli*

In contig 158 of the partially sequenced genome of *H. marismortui* (Zhang et al. 2003), the gene putatively encoding ACD was identified by BLAST search using

the N-terminal amino acid sequence of the purified enzyme. The ORF, designated *acd*, was amplified from genomic DNA of *H. marismortui* by PCR. The PCR product was cloned into pET17b (Novagene) via two restriction sites (*Nde*I and *Xho*I) created with the primers 5′-CCACCGAGTAGACATATGGGACGATTATCG-3′ and 5′-GTGACGACTCGAGTGTTCGTGTCGG-3′. For expression, *E. coli* BL21 codon plus(DE3)-RIL cells (Stratagene) transformed with vector pET17b-*acd*-Har were grown in Luria–Bertani medium at 37°C. The expression was started by inducing the promotor with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). After 3 h of further growth, cells were harvested by centrifugation.

Solubilization, refolding, and purification of recombinant *H. marismortui* ACD

Refolding of the insoluble recombinant ACD was carried out using a modified method according to Connaris et al. (1999). *E. coli* BL21 codon plus(DE3)-RIL cell pellets, transformed with pET17b-*acd*Har, were resuspended in 20 mM Tris–HCl buffer (pH 7.5) containing 3 M KCl, 30 mM MgCl$_2$, and 10% (v/v) glycerol (buffer B), treated with 100 μg lysozyme/ml and 0.1 volume of 1% (v/v) Triton-X100, and incubated at 30°C for 1 h before transferring the solution onto ice for 15 min. The suspension was then sonicated and centrifuged at 40,000g for 30 min at 4°C. The pellet was washed twice in buffer B and centrifuged as before. The insoluble fraction was dissolved in solubilization buffer [20 mM Tris–HCl (pH 7.5) containing 8 M urea, 2 mM EDTA, 50 mM dithioerythritol]. Refolding was initiated by slowly diluting the solubilized pellet into reactivation buffer to a final protein concentration of 0.02 mg/ml followed by incubation for 6 days at 4°C. The renaturation buffer consisted of buffer B supplemented with 2 mM ATP, 5 mM sodium acetate, 10 μM CoA, 3 mM reduced glutathione (GSH) and 0.3 mM oxidized glutathione (GSSG).

The reactivated protein was concentrated by ultrafiltration (cutoff 30 kDa) and adjusted to 2 M (NH$_4$)$_2$SO$_4$. The protein solution was than centrifuged at 100,000g for 45 min at 4°C. The supernatant was concentrated by ultrafiltration and applied to a Superdex 200 HiLoad 16/60 column equilibrated with 50 mM Tris–HCl (pH 7.5) (buffer C), containing 2 M KCl, 20 mM MgCl$_2$ and 10% glycerol (v/v). Protein was eluted at a flow rate of 1 ml/min. The fractions containing the highest ACD activity were pooled, adjusted to a final concentration of 2 M (NH$_4$)$_2$SO$_4$ and applied to a Phenyl-Sepharose 6ff (low sub) [(1 ml)] column equilibrated with buffer C, containing 20 mM MgCl$_2$ and 2 M (NH$_4$)$_2$SO$_4$. Protein was desorbed with buffer C containing 20% glycerol (v/v) at a flow rate of 0.5 ml/min with a decreasing gradient from 1.6 M to 0 M (NH$_4$)$_2$SO$_4$. The fractions containing the highest ACD activity were eluted between 0.9 M and 0.5 M (NH$_4$)$_2$SO$_4$. At this stage ACD was essentially pure.

Characterization of ACD from *H. marismortui*

Enzyme assays

Acetyl-CoA synthetase (ADP-forming) from H. marismortui. (Acetyl - CoA + ADP + P$_i$ ⇌ acetate + ATP + HSCoA) was measured in both directions under oxic conditions at 37°C using two different assay systems (A, B): (A) P$_i$- and ADP-dependent HSCoA release from acetyl-CoA was monitored according to the method of Srere et al. (1963) with Ellman's thiolreagent, 5′5-dithiobis (2-nitrobenzoic acid) [DTNB] by measuring the formation of thiophenolate anion at 412 nm ($\epsilon_{412} = 13.6$ mM^{-1} cm^{-1}). The assay mixture contained 100 mM Tris–HCl (pH 7.5), 3 M KCl, 30 mM MgCl$_2$, 0,1 mM DTNB, 1.5 mM acetyl-CoA, 2 mM ADP and 5 mM KH$_2$PO$_4$. This assay was used to determine (1) the K_m values for acetyl-CoA, ADP and P$_i$, (2) the specificity for CoA-esters of organic acids and for nucleosid diphosphates, and (3) the salt dependence and salt stability of ACD. (B) HSCoA- and acetate-dependent ADP formation from ATP was measured by coupling the reaction with the oxidation of NADH ($\epsilon_{365} = 3.4$ mM^{-1} cm^{-1}) at 365 nm via pyruvate kinase and lactate dehydrogenase (Schäfer and Schönheit 1991). The assay mixture contained 100 mM Tris–HCl (pH 7.5), 3 M KCl, 30 mM MgCl$_2$, 10 mM sodium acetate, 2 mM ATP, 1 mM HSCoA, 2.5 mM phosphoenolpyruvate, 0.3 mM NADH, 6 U lactate dehydrogenase and 4 U pyruvate kinase. This assay was used (1) to routinely assay ACD activity during the purification procedure, (2) to determine the apparent K_m values for acetate, ATP and HSCoA, (3) to test the specificity of the enzyme for organic acids and phosphoryldonors, and (4) to determine the temperature and the pH optima as well as the salt dependence of the ACD. The auxiliary enzymes were tested to ensure that they were not rate limiting.

pH dependence, temperature dependence, and substrate specificity

The pH dependence of the ACD was measured between pH 5.0 and pH 10.0 in the direction of acetyl-CoA formation in the coupled assay system (see above) using either MES (pH 5.0–7.0), Tris–HCl (pH 7.0–9.0) and glycin (pH 9.0–10.0) at 100 mM each. The temperature dependence of ACD activity was determined between 16°C and 50°C in the direction of acetyl-CoA formation. The substrate specificities of ACD were examined in the direction of both acetate formation and acetyl-CoA formation, replacing acetate with 2.5–10 mM propionate, butyrate, fumarate, succinate, isobutyrate, isovalerate, phenylacetate or indol-3-acetate or exchanging

acetyl-CoA with phenylacetyl-CoA. ATP was replaced with GTP and ADP with GDP.

Salt dependence and salt stability

The salt dependence of the ACD activity was measured between 0 M and 3 M KCl and NaCl, and between 0 mM and 150 mM $MgCl_2$ in the assay systems described above. In order to investigate the relationship between stability and salt concentration, ACD (8 µg in 1 ml) was incubated at 4°C in the following buffers: (1) 100 mM Tris–HCl (pH 7.5) containing 30 mM $MgCl_2$, (2) 100 mM Tris–HCl (pH 7.5) containing 30 mM $MgCl_2$ and 20% glycerol, and (3) 100 mM Tris–HCl (pH 7.5) containing 30 mM $MgCl_2$ and 3 M KCl. The enzyme was incubated up to 48 h. At the times indicated, the remaining activity was tested in the direction of acetate formation (see above).

Cloning of ORF PAE3250 and PAE 3249 encoding ACD in P. aerophilum

In the sequenced genome of *P. aerophilum*, the genes putatively encoding the α and β subunits of ACD were identified by BLAST search using the amino acid sequences of the α and β subunits of the ACD isoenzyme I from *P. furiosus*. The ORFs PAE3250 and PAE3249, designated *acd*A and *acd*B, respectively, were amplified by PCR. The PCR product of *acd*A was cloned into pET17b (Novagene) via two restriction sites (*Nde*I and *Eco*RI) created with the primers 5′-CTTCAATACAATTCA-TATGCTCTCTAAG-3′ and 5′-CAGAATTCGGTGG-GTCATAG-3′. The PCR product of *acd*B was also cloned into pET17b using the restriction sites *Nhe*I and *Xho*I and the primers 5′-TAAATACGGGGAGCTAGCGAG-GAGGCTATG-3′ and 5′-TAATCCTATGGATTT-CTCGAGTTTAGGCCATCC-3′. For expression, *E. coli* BL21 codon plus(DE3)-RIL cells (Stratagene) transformed with vector pET17b-*acd*APae and pET17b-*acd*BPae, respectively, were grown in Luria–Bertani medium at 37°C. Expression was started by inducing the promotor with 0.4 mM IPTG. After 4 h of further growth, cells were harvested by centrifugation.

Reconstitution and purification of recombinant P. aerophilum ACD

E. coli BL21 codon plus(DE3)-RIL cell pellets, transformed with pET17b-*acd*APae and pET17b-*acd*BPae, respectively, were resuspended in 50 mM Tris–HCl, pH 8.5. Cell extracts were prepared by passing each resuspended pellet through a French pressure cell at 1.3×10^8 Pa. After centrifugation at 100,000g for 45 min at 4°C, equal amounts of the α and β subunits contained in the two extracts were mixed and incubated on ice for 1 h followed by heat precipitation for 30 min at 75°C. The solution was centrifuged at 100,000g for 45 min at 4°C.

The resulting supernatant exhibited ACD activity, indicating that the subunits had been reconstituted to an active enzyme. The solution containing the reconstituted ACD was adjusted to pH 9.0 and applied to a Q-Sepharose HiLoad column equilibrated with 50 mM Tris–HCl (pH 8.8). Protein was eluted with a pH gradient from pH 8.8 to pH 7.5 in 50 mM Tris–HCl followed by a gradient from 0 M to 0.75 M NaCl in 50 mM Tris–HCl (pH 7.5). Fractions with the highest ACD activity were adjusted to 10 mM $MgCl_2$ and treated with DNase I (RNase-free, Roche Diagnostics, Germany) for 2 h at 37°C followed by heat precipitation for 30 min at 80°C. After an additional centrifugation step at 100,000g for 30 min at 4°C, the supernatant was concentrated by ultrafiltration and applied to a Superdex 200 HiLoad 16/60 column equilibrated with 50 mM Tris–HCl (pH 7.5). Protein was eluted at a flow rate of 1 ml/min with the same buffer. At this stage ACD was apparently homogeneous.

Characterization of ACD from P. aerophilum

Determination of molecular mass of P. aerophilum ACD

The apparent molecular mass of the recombinant ACD from *P. aerophilum* was determined by gelfiltration on a Superdex 200 HiLoad 16/60 column as described above and by analytical ultracentrifugation. Sedimentation equilibrium experiments were done in a Beckman–Coulter XLA analytical ultracentrifuge equipped with UV absorption optics. Samples were filled into 6-channel charcoal-filled epon centerpieces (125-µl volume and approx. 2.8-mm column height) and centrifuged at 8,000 rpm. It was assumed that equilibrium was reached when the observed concentration gradient (measured by 280 nm absorption) of the protein did not change measurably for at least 12 h. All scans taken during that time were averaged to reduce random noise. Finally, the protein was sedimented for 8 h at 44,000 rpm to determine the blank absorption of the buffer. Data were evaluated by non-linear least-square fitting of the molar mass (M) and the absorption in the middle of the cell [$A(x_0)$] with the equation

$$A(x) = A(x_0)\exp\left\{\frac{M(1-\bar{v}\rho)}{2RT}\left(x^2 - x_0^2\right)\omega^2\right\}$$

where the partial specific volume $\bar{v}$ was calculated from the amino acid composition of the protein to be 7.44×10^{-4} kg/m³, ρ is the density of the solution, and ω is the angular velocity of the rotor.

Enzyme assays

Acetyl-CoA synthetase from *P. aerophilum* was measured under oxic conditions at 85°C. The P_i- and ADP-dependent HSCoA release from acetyl-CoA was determined according to the method of Srere et al. (1963) with DTNB by measuring the formation of

thiophenolate anion at 412 nm ($\epsilon_{412} = 13.6$ mM^{-1} cm^{-1}). The assay mixture contained 100 mM MES (pH 6.5), 5 mM MgCl$_2$, 0.1 mM DTNB, 1.5 mM acetyl-CoA (or other CoA esters), 2 mM ADP and 5 mM KH$_2$PO$_4$. This assay was used to determine (1) the K_m values for acetyl-CoA, isobutyryl-CoA, phenylacetyl-CoA, ADP and P$_i$, (2) the pH optimum, the temperature optimum and thermal stability of ACD.

pH dependence, temperature dependence, and thermal stability

The pH dependence of the ACD activity was measured at 85°C between pH 5.0 and pH 7.5 in the direction of acetate formation with Ellman's thiolreagent (see above) using either piperazin (pH 5.0–5.5), MES (pH 5.5–7.0) or triethanolamine (pH 7.0–7.5) at 100 mM each. The temperature dependence of the enzyme activity was determined between 27°C and 93°C in the direction of acetate formation. The long term thermostability of the enzyme (1 µg in 60 µl potassium phosphate buffer, pH 7.0) was tested in sealed vials that were incubated at temperatures between 80 and 100°C up to 120 min. The vials were cooled for 10 min, and the remaining activity was tested at 85°C in the direction of acetate formation.

Analytical assays

The purity of the preparations was documented by SDS-PAGE on a 12% polyacrylamide gel stained with Coomassie brilliant blue R250 (Laemmli 1970). Protein concentrations were determined by the method of Bradford with bovine serum albumin as standard (Bradford 1976).

Results

ACD from the halophilic euryarchaeon H. marismortui

H. marismortui was reported to form significant amounts of acetate during exponential growth on glucose as substrate. It was shown that the formation of acetate was catalyzed by an ACD (Bräsen and Schönheit 2001). Thus, ACD was purified from glucose-grown cells of H. marismortui harvested in the exponential growth phase.

Purification of ACD

ACD was purified 234-fold from cell extracts of H. marismortui by anion-exchange chromatography on Q-Sepharose, gel-filtration chromatography on Superdex 200 and hydrophobic interaction chromatography on Phenyl-Sepharose to a specific activity of 33 U/mg (at 37°C) with a yield of 6% (Table 1). The purified protein was apparently homogenous as judged by denaturing SDS-PAGE. ACD thus represents about 0.4% of the cellular protein of H. marismortui.

Molecular and catalytic properties

The apparent molecular mass of native ACD, determined by gel filtration on Superdex 200, was approximately 166 kDa. SDS-PAGE revealed only one subunit with an apparent molecular mass of 87 kDa, suggesting a homodimeric (α_2) structure of the native enzyme.

Kinetic parameters of purified ACD were determined at 37°C. In both directions of the reaction (Acetyl - CoA + ADP + P$_i$ $\rightleftharpoons$ acetate + ATP + CoA) the rate dependence on the substrate concentrations followed Michaelis–Menten kinetics. The apparent V_{max} and K_m values in the direction of acetate formation were 48 U/mg and 0.45 mM (acetyl-CoA), 0.046 mM (ADP) and 2.0 mM (P$_i$), respectively, and in the direction of acetyl-CoA formation 40 U/mg and 1.1 mM (acetate), 0.08 mM (HSCoA) and 0.3 mM (ATP), respectively. The pH optimum of ACD was at pH 7.5 with about 60% remaining activity at pH 6.5 and pH 8.5. The temperature optimum was at 43°C, thus describing the first mesophilic archaeal ACD (Fig. 2). From the linear part of the Arrhenius plot between 24°C and 37°C, an activation energy of 57.3 kJ/mol was calculated (Table 2).

Substrate specificities Various organic acids, CoA esters and nucleotides were tested as substrates for ACD. In addition to acetate (100%), the enzyme effectively utilizes propionate (108%) and butyrate (98%) as well as the branched-chain fatty acids isobutyrate

Table 1 Purification of ADP-forming acetyl-CoA synthetase from *Haloarcula marismortui*. Enzyme activity was measured at 37°C in the direction of acetyl-CoA formation in the coupled assay system using pyruvate kinase and lactate dehydrogenase as described in "Materials and methods"

Purification step	Protein (mg)	Activity (U)	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Cell-free extract	1,496	209	0.14	100	1.0
Heat precipitation	817	160	0.2	77	1.5
Q-Sepharose	8.96	75	8.9	33	56
Superdex 200	1.2	30	26	14	185
Phenyl-Sepharose	0.35	12	33	6	234

Table 2 Molecular and catalytic properties of native and recombinant ACD from *H. marismortui*. Kinetic constants were determined in the direction of both acetate formation and acetyl-CoA formation as described in "Materials and methods." The molecular mass of native enzyme was determined by gel filtration, of subunit by SDS-PAGE. *ND* Not determined

Parameter	Substrate	Native enzyme	Recombinant enzyme
Apparent molecular mass of enzyme (kDa)			
Native		166	162
Subunit		87	85
Calculated		–	74
Oligomeric structure		α_2	α_2
Apparent V_{max} value (U/mg)	Acetyl-CoA	48	34
(direction of acid formation)	Phenylacetyl-CoA	0.2	ND
Apparent K_m value (mM)	Acetyl-CoA	0.45	0.41
	ADP	0.05	0.02
	P_i	2.0	1.30
Apparent V_{max} value (U/mg)	Acetate	40	36
(direction of CoA ester formation)	Phenylacetate	1	1.5
Apparent K_m value (mM)	Acetate	2.6	1.7
	ATP	0.33	0.38
	CoA-SH	0.08	0.06
pH optimum		7.5	ND
Temperature optimum (°C)		43	ND
Arrhenius activation energy (24–37°C) (kJ/mol)		57	ND

(38%) and isovalerate (25%) as substrates. With isobutyrate, the ACD showed a substrate inhibition, above concentrations of 2.5 mM (maximal activity); complete inhibition was observed at 10 mM isobutyrate. The aryl acids phenylacetate (3%) and indol-3-acetate (2%), and succinate and fumarate were not accepted as substrates. ATP (100%) could only be replaced by GTP, but with significant reduced activities (11%). In the opposite direction, the enzyme showed highest activity with acetyl-CoA (100%) and ADP (100%), which could not be substituted by phenylacetyl-CoA (1%) and by GDP (5%), CDP (1%) and UDP (8%), respectively.

Salt dependence and stability   The effect of KCl and NaCl on enzyme activity was tested in the presence of 30 mM MgCl₂. Enzyme activity (short time kinetics, measured up to 10 min reaction time) was not affected by the KCl and NaCl concentrations up to 3 M and 2 M, respectively. Increased NaCl concentration from 2 M to 3 M resulted in a decrease of activity by 40%. The optimal MgCl₂ concentration for ACD activity was 30 mM in the absence of KCl and 20 mM in the presence of 3 M KCl. ACD activity required high salt concentrations for long-term stability, tested in the presence of 30 mM MgCl₂. The enzyme did not lose activity after incubation at 3 M KCl for 48 h. In the absence of KCl, at 30 mM MgCl₂, the activity decreased by 50% after 8 h; after 48 h, activity was lost completely. KCl could be effectively replaced by 20% glycerol.

Identification, functional overexpression of the gene encoding ACD in H. marismortui

Using the N-terminal amino acid sequence determined for the subunit of the purified enzyme (GRLSTLFA-PERVAVVGATDS), an ORF with a nucleotide se-quence that exactly matched the 20 amino acids was identified in contig 158 of partially sequenced genome of *H. marismortui* (Zhang et al. 2003). The ORF consists of 2,106 bp coding for a polypeptide of 701 amino acids with a calculated molecular mass of 74.3 kDa. The protein contains high amounts of negatively charged amino acids, 9% Asp and 9% Glu, which is typical for halophilic enzymes (Cendrin et al. 1993; Pire et al. 2001) and had a predicted pI of 3.8. The G+C content of *acd* is 63 mol%. The coding sequence starts with ATG and stops with TGA. The ORF was identified as *acd* encoding ACD by its functional overexpression in *E. coli*. The gene was amplified by PCR, cloned into vector pET17b, and the recombinant plasmid was used to transform *E. coli* BL21 codon plus (DE3)-RIL. After induction with IPTG, a polypeptide of about 85 kDa was overproduced, almost completely in the form of inclusion bodies. The protein was purified from inclusion bodies as catalytically active, extremely halophilic ACD as follows.

Reactivation, purification and characterization of recombinant ACD

Recombinant ACD was purified from inclusion bodies by dissolving in 8 M urea in the presence of dithioerythritol, followed by refolding in buffer containing a high salt concentration (3 M KCl), substrates and glutathione (see "Materials and methods"). Maximal ACD activity was achieved after 20 h of incubation at 4°C. The reactivated recombinant ACD was purified by ammonium sulfate precipitation, gel filtration and chromatography on phenyl sepharose. The recombinant ACD purified from transformed *E. coli* and native ACD purified from *H. marismortui* showed almost identical properties, including molecular masses of enzyme and subunits, apparent V_{max} and K_m values and substrate specificities (Fig. 1a, Table 2).
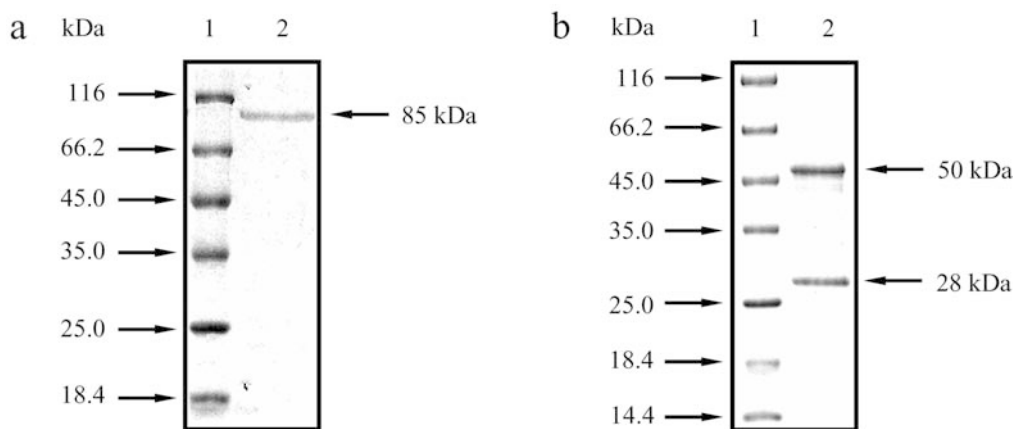
Fig. 1 **a** Recombinant ADP-forming acetyl-CoA synthetase (ACD) from *Haloarcula marismortui* isolated from transformed *Escherichia coli* as analyzed by SDS-PAGE. *Lane 1* Molecular mass standards, *lane 2* recombinant enzyme purified from *E. coli*. **b** Purified recombinant *Pyrobaculum aerophilum* ACD from transformed *E. coli* as analyzed by SDS-PAGE. *Lane 1* Molecular mass standards, *lane 2* recombinant *P. aerophilum* ACD purified from *E. coli*

ACD from the hyperthermophilic crenarchaeon *P. aerophilum*

Cell extracts of *P. aerophilum* grown with yeast extract and nitrate as carbon and energy source (Völkl et al. 1993) catalyzed the ADP- and P_i-dependent conversion of acetyl-CoA to acetate with activities of 0.013 U/mg (85°C), indicating the presence of a functional ACD in this organism.

Identification and functional overexpression of the genes encoding ACD in P. aerophilum and purification of the recombinant protein

By sequence comparison using the α- and β-subunit-encoding genes of ACD I from *P. furiosus*, two homologous ORFs were identified in the sequenced genome of *P. aerophilum*. The ORFs PAE3250 and PAE 3249, designated *acd*A and *acd*B, respectively, which code for α and β subunits of a functional ACD (see below), had a novel genome organization compared to those of all ACD genes identified so far. In the *P. aerophilum* genome, both ORFs overlap each other by 1 bp with PAE3250 preceeding PAE3249 (Fig. 3). Thus, neither gene is in-frame; hence they encode two separate subunits α and β. In contrast, the α and β subunits of all other ACD genes identified so far are located either at different sites of the chromosome, as in *P. furiosus*, or are fused to one single ORF, as described for *A. fulgidus* and *M. jannaschii*. The *P. aerophilum acd*A consists of 1,386 bp coding for a polypeptide of 461 amino acids with a calculated molecular mass of 50.1 kDa. *acd*B comprises 699 bp coding for a polypeptide of 232 amino acids with a calculated molecular mass of 25.7 kDa. Both genes were separately cloned and overexpressed. The native enzyme was reconstituted by mixing nearly

equal amounts of recombinant α (50 kDa) and β (28 kDa) subunits contained in the transformed *E. coli* extracts, followed by incubation on ice. The reconstituted protein showed ACD activity and was purified to homogeneity by three steps, including heat precipitation, anion-exchange chromatography on Q sepharose and gel filtration on Superdex 200 (Fig. 1b). The purified enzyme showed a specific activity of 12 U/mg.

Properties of recombinant ACD from P. aerophilum

Molecular properties SDS-PAGE of the recombinant enzyme revealed two subunits of 50 kDa and 28 kDa,
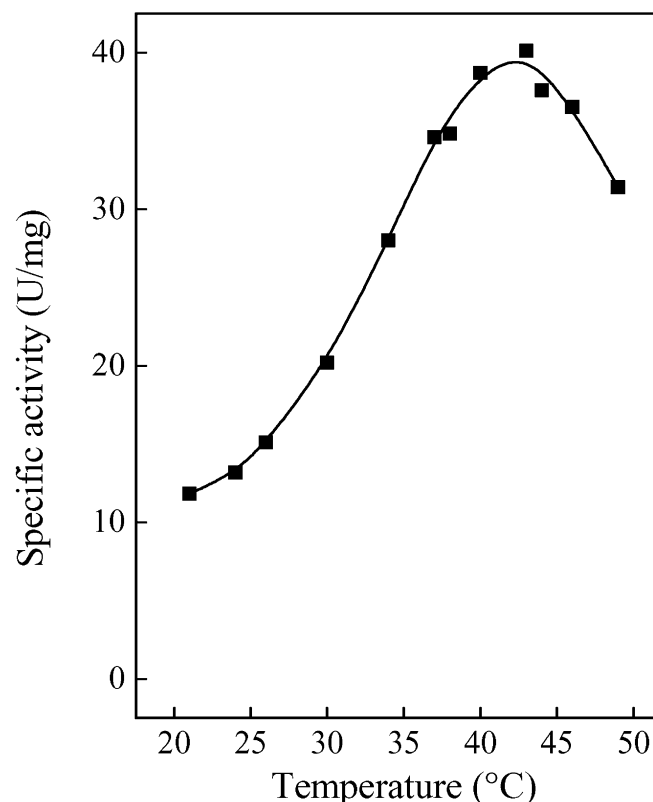

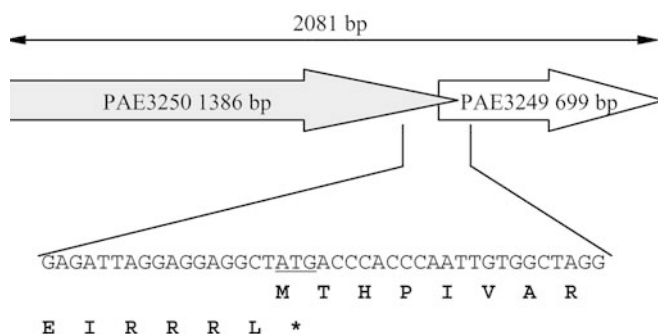Fig. 2 Effect of temperature on the specific activity of purified ACD from *H. marismortui*

Fig. 3 Organization of the ORFs PAE3250 and PAE3249, designated as *acd*A and *acd*B , respectively, in the genome of *Pyrobaculum aerophilum*. *Arrows* represent the ORFs PAE3250 and PAE3249 and their orientation. The *enlargement* shows the overlapping regions of *acd*A and *acd*B in *P. aerophilum*, and the respective protein sequence is shown in *bold letters*. The *acd*A stop codon is marked by an *asterisk*, and the ATG start codon of *acd*B is *underlined*

respectively (Fig. 1b). The native molecular mass was estimated by gel filtration and analytical ultracentrifugation. Gel filtration experiments revealed a molecular mass of the *P. aerophilum* ACD between 450 kDa and 920 kDa. The sedimentation equilibrium analysis showed that the main part of the protein formed higher aggregates, with molecular masses of 2,000–3,000 kDa and to a small extent revealed a second species with a molecular mass between 300 and 800 kDa. Thus, with both methods, the molecular mass and the oligomeric structure of the native enzyme could not be determined unambiguously, most likely due to unspecific aggregation. However, the results obtained with both methods suggest that the protein had a molecular mass higher than 150 kDa and thus a higher oligomerization state, as described for previously characterized ACDs.

Catalytic properties Recombinant ACD from *P. aerophilum* catalyzed the ADP- and P_i-dependent conversion of acetyl-CoA to acetate with an apparent V_{max} of 12 U/mg (85°C) and apparent K_m values for P_i, ADP and acetyl-CoA of 1.3, 0.14, and 0.12 mM, respectively. In addition to acetyl-CoA, ACD also accepted isobutyryl-CoA and phenylacetyl-CoA as substrates at high rates. The apparent V_{max} and K_m values (at 85°C) with isobutyryl-CoA were 11 U/mg and 0.04 mM, and with phenylacetyl-CoA 7 U/mg and 0.11 mM. The V_{max}/K_m values indicate that isobutyryl-CoA is converted more efficiently than acetyl-CoA and phenylacetyl-CoA (Table 3). Significant rates of the *P. aerophilum* ACD measured in the reverse direction, i.e., the ATP- and CoA-dependent conversion of acetate to acetyl-CoA, could not be demonstrated. The pH optimum of *P. aerophilum* ACD was 6.5, with about 40% of activity at pH 5.5 and 20% of activity at pH 7.5.

Temperature optimum and thermostability The temperature dependence of ACD activity was measured up to 93°C. The activity increased exponentially up to 93°C, the highest temperature measured, indicating an optimum higher than 93°C (Fig. 4a). From the linear part of the Arrhenius plot between 27 and 93°C, an activation energy of 114 kJ/mol was calculated. The ACD showed high stability against thermal inactivation. No significant loss of activity could be detected upon incubation for 120 min at 80°C. The half-life of the enzyme at 90°C was 105 min and at 100°C 13 min. Addition of 1 M $(NH_4)_2SO_4$ stabilized ACD effectively against heat inactivation at 100°C, retaining almost 100% of its activity after incubation for 120 min (Fig. 4b).

Discussion

In this study, we characterized two unsual ACDs, from the mesophilic extremely halphilic euryarchaeon *H. marismortui* ,and from the first crenarchaeon, the

Table 3 Molecular and catalytic properties of recombinant ACD from *Pyrobaculum aerophilum*. Kinetic constants were determined at 85°C in the direction of free acid formation as described in "Materials and methods." The molecular mass of native enzyme was determined by gel filtration and analytical ultracentrifugation, of subunit by SDS-PAGE. The K_m value for ADP and P_i was measured with acetyl-CoA as substrate

Parameter	Substrate	ACD
Apparent molecular mass of enzyme (kDa)		
Native		> 150
Subunits		α 50, β 28
Calculated		α 50.1, β 25.7
Apparent V_{max} value (U/mg)	Acetyl-CoA	12
	Isobutyryl-CoA	11
	Phenylacetyl-CoA	7
Apparent K_m value (mM)	Acetyl-CoA	0.12
	Isobutyryl-CoA	0.04
	Phenylacetyl-CoA	0.11
	ADP	0.14
	P_i	1.30
V_{max}/K_m value	Acetyl-CoA	100
	Isobutyryl-CoA	300
	Phenylacetyl-CoA	64
pH optimum		6.5
Temperature optimum		> 93
Arrhenius activation energy (27–93°C) (kJ/mol)		114

Fig. 4 Effect of temperature on the specific activity and on the stability of recombinant *P. aerophilum* ACD. **a** Temperature dependence of the specific activity; **b** thermostability at 80°C (*filled triangles*), 90°C (*filled circles*), 100°C (*filled squares*) and at 100°C in the presence of 1 M $(NH_4)_2SO_4$ (*filled diamonds*). A 100% activity corresponded to a specific ACD activity of 8 U/mg
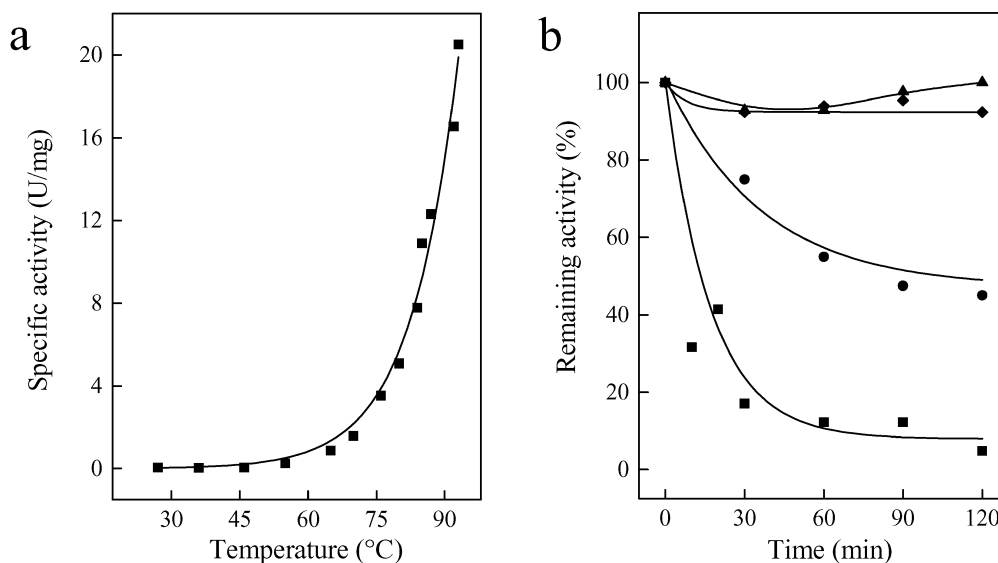
hyperthermophile *P. aerophilum*. The two ACDs differ in various aspects from characterized ACDs in archaea and from that of the eukaryotic protist *G. lamblia*.

The *H. marismortui* ACD is the first mesophilic ACD from the archaeal domain, with a temperature optimum of 43°C. All other archaeal ACDs were characterized from hyperthermophiles and thus showed extremely thermophilic properties. For the partially characterized ACD from *G. lamblia*, no temperature optimum was reported (Sanchez and Müller 1996). The salt-dependent *H. marismortui* ACD was a homodimer of 166 kDa; the molecular mass of subunits on SDS-PAGE of 87 kDa were overestimated, a feature reported for several halophilic proteins, probably due to high amounts of negatively charged amino acids (see in Bonete et al. 1996). The calculated molecular mass is 74.3 kDa.
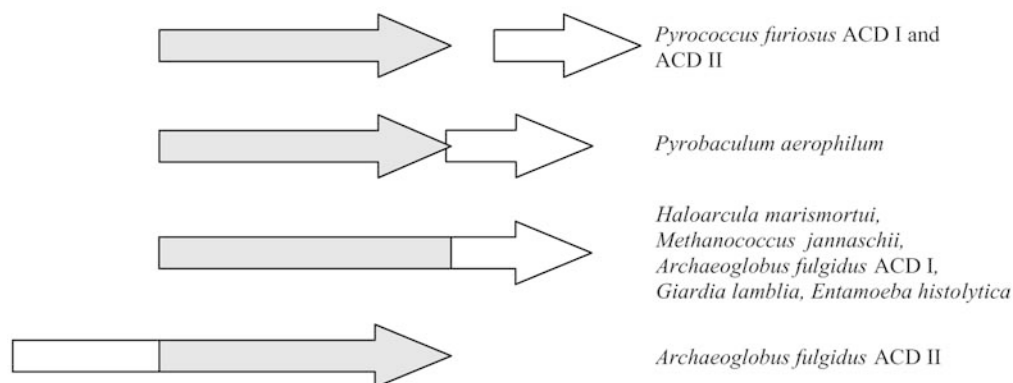
Using the N-terminal amino acid sequence of the subunit, the gene encoding ACD was identified in the partial sequenced genome of *H. marismortui* (Zhang et al. 2003) by functional overexpression in *E. coli*. The recombinant halophilic enzyme, which had to be reactivated from inclusion bodies, showed almost identical molecular and kinetic properties as the native enzyme, a prerequisite for future structural and mutagenesis studies of the recombinant enzyme.

The halophilic ACD catalyzed the reversible conversion of acetyl-CoA and acetate and, in addition to acetate, accepted branched-chain acids rather than aryl acids as substrates. Thus, the substrate specificity of the *H. marismortui* ACD is similar to that of ACD I from *P. furiosus* and *A. fulgidus*, where it has been implicated in sugar metabolism (Glasemacher et al. 1997; Mai and Adams 1996; Musfeldt and Schönheit 2002). These findings are in accordance with the proposed function of the *H. marismortui* ACD in acetate formation during growth on glucose (Bräsen and Schönheit 2001). A second ACD gene could not be identified in the genome sequence, suggesting that *H. marismortui*—in contrast to *P. furiosus* and *A. fulgidus*—does not contain a homolog to ACD II, utilizing phenylacetyl-CoA/phenylacetate.

The *P. aerophilum* ACD is the first crenarchaeal ACD to be characterized. By sequence comparison with *P. furiosus* ACD, two ORFs were identified coding for α and β subunits of a putative ACD. The ORFs were

Fig. 5 Organization and orientation of genes encoding characterized ACDs. *Arrows* represent separate ORFs; α-subunit-homologous regions are *shaded*; β-subunit-homologous regions are *white*. The amino acid sequence identities of α subunits to each other were between 31 and 47%, and of the β subunits between 31 and 56%

Pyrococcus furiosus ACD I and ACD II

Pyrobaculum aerophilum

Haloarcula marismortui,
Methanococcus jannaschii,
Archaeoglobus fulgidus ACD I,
Giardia lamblia, Entamoeba histolytica

Archaeoglobus fulgidus ACD II

separately expressed in *E. coli* and the recombinant α (50 kDa) and β (28 kDa) subunits were reconstituted to an active heterooligomeric enzyme. In accordance with the optimal growth temperature of the organism (100°C), the enzyme showed extremely thermophilic properties, with a temperature optimum higher than 93°C and high thermostability up to 100°C. Similar thermophilic properties were described for ACDs from the hyperthermophile *P. furiosus* (Glasemacher et al. 1997; Mai and Adams 1996; Musfeldt et al. 1999); less pronounced thermophilic features were reported for the ACD I from *A. fulgidus*, due to the lower temperature optimum (83°C) of the organism (Musfeldt and Schönheit 2002).

The *P. aerophilum* ACD exhibited two novel features among all characterized ACDs: first, the *P. aerophilum* ACD showed the broadest substrate specificity of all ACDs towards CoA esters. Besides acetyl-CoA, it converts isobutyryl-CoA and phenylacetyl-CoA at high rates. The effective conversion of these three types of CoA esters by one enzyme is an unusual property and has not been reported for any ACD. In the euryarchaeota *P. furiosus* and *A. fulgidus*, the effective conversion of these three CoA esters requires the activity of two isoenzymes (Mai and Adams 1996; Musfeldt and Schönheit 2002). ACD I in both organisms catalyzes predominantly the conversion of acetyl-CoA and various branched-chain acyl-CoA esters and does not accept aryl-CoA esters (phenylacetyl-CoA) as substrates. The conversion of aryl-CoA esters in both organisms is catalyzed by a specific isoenzyme, i.e., ACD II. This ACD has been primarily implicated in the fermentation of aromatic amino acids (Mai and Adams 1996). For *P. furiosus* induction of ACD II during growth on peptides has been indicated (Schut et al. 2003). Since in *P. aerophilum* a second ACD homolog was not found, the single, "multifunctional" ACD might play a role in both sugar and peptide metabolism. It is interesting to note that unusually broad substrate spectra have also been reported for several enzymes in other crenarchaeota. These include glucose dehydrogenase and KDG aldolase from *Sulfolobus solfataricus* (Lamble et al. 2003), and, from *Aeropyrum pernix*, a glucokinase with an unusual "hexokinase like" broad substrate spectrum (Hansen et al. 2002), an ATP-dependent 6-phosphofructokinase (Hansen and Schönheit 2001) and a bifunctional glucose-6-phosphate isomerase/mannose-6-phosphate isomerase (Hansen et al. 2004).

The *P. aerophilum* ACD only showed activity in the direction of CoA ester conversion to the corresponding acids, i.e., the physiological direction, rather than in the reverse direction. The reasons for the apparent irreversibility of this ACD are not known. A similar property, using only CoA ester as substrates, has also been reported for ACDs from *M. jannaschii* and from *G. lamblia* (Musfeldt and Schönheit 2002; Sanchez and Müller 1996).

The second novel feature exhibited by the *P. aerophilum* ACD is the organization of the coding genes. The *P. aerophilum* ACD is composed of separate α and β subunits, with the encoding genes overlapping each other by 1 bp. In contrast, the organization of the coding genes of characterized ACDs from archaea (*P. furiosus*, *A. fulgidus*, *M. jannaschii* and *H. marismortui*) and the eukaryote *G. lamblia* (Fig. 5), revealed three types different from that in *P. aerophilum*: (1) The ACD isoenzymes I and II from *P. furiosus* are α₂β₂ heterotetramers composed of two different subunits, α and β, and the encoding genes are separated and located on different sites of the chromosome, not forming an operon (Musfeldt et al. 1999). The most frequent genome organization is that genes coding both α and β subunits and the respective polypeptides are fused. (2) The fusions are either in αβ direction, as found in *A. fulgidus* (ACD I, AF1211), *M. jannaschii*, the protist *G. lamblia* and—as shown in this work—from *H. marismortui*, or (3) the fusions are in the βα direction, as in ACD II (AF1938) from *A. fulgidus* (Musfeldt and Schönheit 2002; Sanchez et al. 1999, 2000). Thus, the organization of ACD-encoding genes in *P. aerophilum* is unique and has not been reported for any other characterized or putative ACD homolog. The rationale behind these different genome organizations of the ACD genes and the fused polypeptides is not known. The fused ACDs might be originated from an ancestral ACD with separate genes, whereby the *P. aerophilum* ACD, showing overlapping genes, might represent a sort of an intermediate situation. Alternatively, the separate organization of genes and polypeptides could be the result of splitting event from ACDs with fused genes.

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