A novel octameric AMP-forming acetyl-CoA synthetase from the hyperthermophilic crenarchaeon *Pyrobaculum aerophilum*

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Abstract AMP-forming acetyl-CoA synthetases (ACSs) are ubiquitous in all three domains of life. Here, we report the first characterization of an ACS from a hyperthermophilic organism, from the archaeon *Pyrobaculum aerophilum*. The recombinant ACS, the gene product of ORF PAE2867, showed extremely high thermostability and thermoactivity at temperatures around 100 °C. In contrast to known monomeric or homodimeric mesophilic ACSs, the *P. aerophilum* ACS was a 610 kDa homo-octameric protein, with a significant lower content of thermostable (Cys, Asn, and Gln) and higher content of charged (Glu, Lys, and Arg) amino acids. Kinetic analyses revealed an unusual broad substrate spectrum for organic acids and an extremely high affinity for acetate (K\textsubscript{m} 3 μM).

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1. Introduction

AMP-forming acetyl-CoA synthetases (ACS, E.C. 6.2.1.1) (acetate + ATP + CoA → acetyl-CoA + AMP + PP\textsubscript{i}) – ubiquitous enzymes in all three domains of life – catalyze the activation of acetate to acetyl-CoA, an important intermediate of central metabolic pathways: the activation proceeds in a two step reaction with acetyl-AMP as intermediate [7]. The enzyme belongs to the acyl-adenylate/thioester forming enzyme superfamily, which includes various acyl- and aryl-CoA synthetases, luciferases and the acyl-adenylate/thioester forming enzyme superfamily, which includes various acyl- and aryl-CoA synthetases, luciferases and the adenylation domains of the non-ribosomal peptide synthases, all of which catalyze reactions via adenylylated intermediates [1]. ACSs have been characterized from many eukaryal and bacterial species. These mesophilic enzymes, which are composed of 75 kDa subunits, constitute either monomeric or homodimeric proteins, with exception of the homotrimeric ACSs in the domain of archaea, in particular from hyperthermophiles, which are considered to represent the phylogenetic most ancestral organisms, have not been studied. In this paper, we report the characterization of a novel type of ACS from the hyperthermophilic crenarchaeon *Pyrobaculum aerophilum*, which exhibits unique molecular and catalytic properties.

2. Materials and methods

2.1. Cloning of ORF PAE2867 encoding ACS of *P. aerophilum*, expression in E. coli and purification of the recombinant enzyme

The ORF PAE2867, annotated as *acs* gene, was amplified by PCR. The PCR product was cloned into pET17b via two restriction sites (NheI and BamHI) created with the primers 5′-TCTTTAAT-TTGAGCTAGCCCAATGCTATAG-3′ and 5′-CTAAAACGTA-CTTGAATCTCATGTTCGC-3′. The vector pET17b-acs *Pae* was transformed into *E. coli* BL21 (codon plus(DE3))-RIL cells. For expression, cells 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. After 3 h of further growth, cells were harvested by centrifugation followed by resuspension in 50 mM Tris–HCl pH 8.5. Cells were disrupted by passing through a french pressure cell. After centrifugation, the supernatant was heat-precipitated at 75 °C for 30 min and centrifuged. 1 ml of the supernatant was applied to a Superdex 200 HiLoad 16/60 equilibrated with 50 mM Tris–HCl, pH 7.5. Protein was eluted at a flow rate of 1 ml/min. The fractions with highest ACS activity were pooled and incubated with DNAse I (RNAse free, Roche Diagnostics, Mannheim/Germany) for 2 h at 37 °C followed by an additional heat-precipitation at 86 °C for 30 min and centrifugation. After concentrating by ultrafiltration, the supernatant was applied to a Superdex 200 HiLoad 16/60 equilibrated with 50 mM Tris–HCl, pH 7.5. Protein was eluted at a flow rate of 1 ml/min and the eluted enzyme was apparently homogeneous as judged by SDS–PAGE using 12% gels [13]. Protein concentrations were determined by the method of Bradford with bovine serum albumin as standard [2].

2.2. Characterization of recombinant ACS from *P. aerophilum*

2.2.1. Molecular mass determination. The apparent molecular mass of recombinant ACS from *P. aerophilum* was determined by gel-filtration on a Superdex 200 HiLoad 16/60 column (see 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 ≈2.8 mm column height) and centrifuged at 8000 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(∞)) with the equation:

\[
A(x) = A(\infty) + (A(0) - A(\infty)) \times e^{-x/M}
\]

where A(x) is the absorbance at the position x, A(0) the absorbance at x = 0 and A(∞) the absorbance in the middle of the cell.

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\[ A(x) = A(x_0) \cdot e^{\frac{4\pi \rho_0 x^2}{\rho r^2} \cdot \frac{1}{2} \omega^2} \]

where the partial specific volume \( \bar{\nu} \) was calculated from the amino acid composition of the protein to be \( 7.44 \times 10^{-3} \text{ kg/m}^3 \), is the density of the solution, and \( \omega \) is the angular velocity of the rotor.

2.2.2 Enzyme assays. ACS was measured using three different assay systems (A, B, and C) under aerobic conditions at 85 °C. In two discontinuous assay systems (A and B), the standard assay mixture (500 μl, 100 mM MES, pH 6.5, 10 mM MgCl2, 2 mM ATP, 1 mM CoA and 5 mM sodium acetate or 5 mM of other organic acids) was preincubated and the reaction was started with addition of ACS. After incubation for 0.5–6 min, the reaction was stopped by rapid addition of 500 μl ice-cold buffer containing 200 mM Tris–HCl, pH 7.6, 0.6 mM NAD+ and 5 mM D,L-malate (A) and 200 mM Tris–HCl, pH 7.6, 0.6 mM NADH and 2.5 mM PEP (B), respectively. The amount of acetyl-CoA (A) and AMP (B) formed from acetate, ATP and HSCoA was quantified as described in [5] and [17], respectively. These assay systems were used to determine the apparent \( K_m \) values for acetate, ATP and HSCoA and the temperature optimum (A) as well as the substrate specificity (B) of ACS. To determine (i) ACS activity during purification procedure, (ii) the \( K_m \) values for acetyl-CoA and PPi, and (iii) the pH optimum and the thermostability of ACS, the PPi and AMP dependent HSCoA release from acetyl-CoA was monitored according to Srere et al. [19] with Ellman’s thiol reagent, 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) (ε245 = 13.6 mM⁻¹ cm⁻¹) in a continuous assay system. The assay mixture contained 100 mM MES, pH 6.5, 0.1 mM DTNB, 0.1 mM acetyl-CoA, 0.5 mM AMP and 1 mM PPi.

2.2.3. pH dependence, temperature dependence and thermal stability. The pH dependence of the ACS was measured at 85 °C between pH 5.0 and pH 7.5 using either piperazine (pH 5.0–6.0), MES (pH 5.5–7.0) or triethanolamine (pH 7.0–7.5) (each 100 mM). The temperature dependence was determined between 27 and 97 °C. The long term thermostability (6 μg protein in 60 μl potassium phosphate buffer, pH 7.0) was tested in sealed vials, which were incubated at the temperatures indicated up to 120 min. The vials were cooled for 10 min and the remaining activity was tested.

3. Results

3.1. Cloning, functional overexpression of PAE2867 coding for ACS from P. aerophilum

The ORF PAE2867, previously annotated as putative \( a c s \) gene, consists of 2013 bp coding for a polypeptide of 670 amino acids with a calculated molecular mass of 76.2 kDa. The coding sequence of the protein to have a molar mass of 610 ± 40 kDa, suggested by extrapolation. The pH optimum of ACS was at pH 6.5 with about 60% remaining activity at pH 5.5 and 50% at pH 8.5.

3.2. Properties of recombinant ACS from P. aerophilum

3.2.1. Molecular properties. SDS–PAGE of the recombinant enzyme revealed one subunit of 75 kDa. The native molecular mass was estimated by gel filtration and analytical ultracentrifugation. Gel filtration experiments gave ambiguous results between 340 and 625 kDa. However, the sedimentation equilibrium in the analytical ultracentrifuge showed the protein to have a molar mass of 610 ± 40 kDa, suggesting a homooctameric structure of the native enzyme (Fig. 1A and B).

3.2.2. Catalytic properties. Kinetic parameters of recombinant ACS were determined at 85 °C. In both directions of the reaction, the rate dependence followed Michaelis–Menten kinetics. The apparent \( V_{\text{max}} \) and \( K_m \) values, as calculated from linear Lineweaver–Burk plots, were in the direction of acetyl-CoA formation 37 U/mg, 3 μM (acetate), 70 μM (HSCoA) and 330 μM (ATP), respectively, and in the direction of acetate formation 12 U/mg, 6.6 μM (acetyl-CoA), 7.5 μM (AMP) and 58 μM (PPi), respectively. The apparent \( K_m \) value for acetate was found to be significantly lower than 10–25 μM; the value of 3 μM was obtained by extrapolation. The pH optimum of ACS was at pH 6.5 with about 60% remaining activity at pH 5.5 and 50% at pH 8.5.

3.2.3. Substrate specificities. Various organic acids were tested as substrates for ACS. In addition to acetate (100%), propionate (118%), formate (27%), butyrate (25%) and isobutyrate (28%) were accepted as substrates.

3.2.4. Temperature optimum and thermostability. ACS activity increased exponentially up to 97 °C, the highest temperature tested, indicating an optimum higher than 97 °C (Fig. 2A). From the linear part of the Arrhenius plot between 27 and 97 °C, an activation energy of 118 kJ/mol was calculated (Fig. 2B). The ACS showed high stability against thermal inactivation. No significant loss of activity was observed upon incubation for 120 min at 90 °C. The half life of ACS at 98 °C was 72 min and at 100 °C 8 min. Addition of 1 M (NH4)2SO4 stabilized the enzyme at 100 °C to a half life of 24 min (Fig. 3).

Fig. 1. Sedimentation equilibrium of \( P. \ aerophilum \) acetyl-CoA synthetase (A) (loading concentrations 5.7 μM (\( \bullet \)), 2.3 μM (\( \square \)), and 1.1 μM (\( \bigcirc \) monomer) in 50 mM Tris–HCl, pH 7.4, measured at 8000 rpm. The solid line represents the best fit calculated with a molar mass of 610 ± 40 kDa/molecule (see Section 2). (B) Loading concentration of 2.3 μM Monomer (\( \bigcirc \)) in 50 mM Tris–HCl, pH 7.4, measured at 8000 rpm. The solid line represents the best fit calculated with a molar mass of 604 ± 30 kDa/molecule (see Section 2) and (\( \bullet \)) representing the residues of the fit.

Fig. 2. Arrhenius plots of ACS from \( P. \ aerophilum \). (A) Arrhenius plot of ACS at pH 7.4 tested as substrates for ACS. In addition to acetate (100%), propionate (118%), formate (27%), butyrate (25%) and isobutyrate (28%) were accepted as substrates. (B) Arrhenius plot of ACS at pH 7.4 tested as substrates for ACS. In addition to acetate (100%), propionate (118%), formate (27%), butyrate (25%) and isobutyrate (28%) were accepted as substrates.
4. Discussion

In this paper, we report the first characterization of a hyperthermophilic ACS, from the aerobic crenarchaeon *Pyrobaculum aerophilum*. By functional overexpression in *E. coli*, ORF PAE2867 in the genome sequence of *P. aerophilum* was shown to represent the *acs* gene. As shown in the sequence alignment in Fig. 4, ACS from *P. aerophilum* showed a high degree of sequence identity to other ACSs from all three domains of life (including those of Table 1) and share certain conserved sequence motifs (A1–A10) of the acyl-adenylate/thioester forming enzyme superfamily [7,21]. Most of the residues shown to be involved in substrate binding, as concluded from the crystal structure of the *Salmonella enterica* enzyme, and especially the lysine residue (K609) are well conserved in the *P. aerophilum* ACS [7]. This lysine residue was reported to be crucial for catalysis and to be the site for the proposed acetylation/deacetylation in sirtuin dependent posttranslational regulation of *Salmonella* and *Saccharomyces* ACS [7,20]. Since putative sirtuin homologs could be identified in the genome of *P. aerophilum*, it is likely that this mechanism of posttranslational regulation is also operative in ACS of this hyperthermophilic archaeon.

The biochemical characterization of the recombinant *P. aerophilum* ACS revealed several unique molecular and catalytic properties: (i) The enzyme has temperature optimum above 97 °C and the high thermostability up to 100 °C, which is in accordance with its function at the optimal growth temperature of *P. aerophilum* of 100 °C [24]. (ii) The hyperthermophilic ACS is a 610 kDa homooctamer composed of 75 kDa subunits (calculated 76.2 kDa). All other characterized ACSs, which are mesophilic proteins, are either monomers or homodimers, composed of 75 kDa subunits, with the exception of the homotrimeric *S. cerevisiae* enzyme (Table 1). Thus, the homooctameric structure of the hyperthermophilic *P. aerophilum* ACS supports the idea that a higher oligomerization state could be a significant stabilizing mechanism for hyperthermophilic proteins (for the literature, see [22,23,25]). Furthermore, the *Pyrobaculum* ACS contained significant lower amounts of the thermolabile asparagine, glutamine and cysteine residues and higher amounts of glutamate, lysine, and arginine as compared to mesophilic ACS (Table 2), which was described to be a typical property of several hyperthermophilic proteins [4,25]. (iii) The *P. aerophilum* ACS showed highest affinity for acetate. The apparent $K_m$ value was much lower than 10–25 μM and was calculated to be 3 μM by extrapolation from the Lineweaver–Burk plot. Thus, the $K_m$ value was 20–300 fold below those reported for most of the other characterized ACSs (Table 1). (iv) The *P. aerophilum* ACS exhibits an unusual broad substrate spectrum, converting – in addition to acetate – propionate, formate, butyrate and isobutyrate at significant rates. Most of the characterized ACSs are specific for acetate, additionally utilizing only propionate, however at reduced efficiency. A broad substrate spectrum was reported only for the ACS
from *Penicillium chrysogenum*, where it was implicated in providing activated side chains required for the synthesis of penicillins [15]. It is interesting to note that unusually broad substrate spectra have also been reported for several other crenarchaeal enzymes, including ADP-forming acetyl-CoA synthetase (ACD) from *P. aerophilum* and several glycolytic enzymes, e.g., from *Sulfolobus solfataricus* (for references, see [3,14]).
Table 1
Comparison of molecular and catalytic properties of characterized ACSs

<table>
<thead>
<tr>
<th>Organism</th>
<th>Apparent molecular mass (kDa)</th>
<th>Oligomeric structure</th>
<th>K_m value for acetate (mM)</th>
</tr>
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<tr>
<td>Pyrobaculum aerophilum</td>
<td>610</td>
<td>2</td>
<td>0.003</td>
</tr>
<tr>
<td>Haloarcula marismortui</td>
<td>72</td>
<td>2</td>
<td>0.23</td>
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<tr>
<td>Methanosaeta consilii</td>
<td>148</td>
<td>2</td>
<td>0.30</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>72</td>
<td>2</td>
<td>0.02</td>
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<td>Azotobacter acetii</td>
<td>60</td>
<td>2</td>
<td>0.25</td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td>130</td>
<td>2</td>
<td>0.033</td>
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<tr>
<td>Bradyrhizobium japonicum</td>
<td>150</td>
<td>2</td>
<td>0.146</td>
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<tr>
<td>Saccharomyces cerevisiae</td>
<td>151</td>
<td>2</td>
<td>0.28</td>
</tr>
<tr>
<td>Methanosaeta consilii</td>
<td>139</td>
<td>2</td>
<td>6.6</td>
</tr>
<tr>
<td>Spinacea oleracea</td>
<td>73</td>
<td>2</td>
<td>0.057</td>
</tr>
<tr>
<td>Bos taurus</td>
<td>57</td>
<td>2</td>
<td>0.16</td>
</tr>
</tbody>
</table>

*This work.

1Braes, C. and Schrohe, P., submitted for publication.
2Ref. [9].
3Ref. [10].
5Ref. [12].
6Ref. [13].
7Ref. [14].
8Ref. [15].
9Ref. [16].
10Ref. [17].
11Ref. [18].
12Ref. [20].
13Ref. [21].
14Ref. [8,11].

Table 2
Comparison of amino acid composition of the Pyrobaculum aerophilum ACS with the mesophilic ACSs from the archaeon Methanosaeta consilii, the bacterium Salmonella enterica, and the eukarya Saccharomyces cerevisiae and Homo sapiens

<table>
<thead>
<tr>
<th>Organism</th>
<th>Cys (mol%)</th>
<th>Asn (mol%)</th>
<th>Gln (mol%)</th>
<th>Total (mol%)</th>
<th>Glu (mol%)</th>
<th>Lys (mol%)</th>
<th>Arg (mol%)</th>
<th>Total (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrobaculum aerophilum</td>
<td>0.15</td>
<td>2.39</td>
<td>1.19</td>
<td>3.73</td>
<td>7.91</td>
<td>6.42</td>
<td>5.97</td>
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<tr>
<td>Methanosaeta consilii</td>
<td>0.89</td>
<td>2.08</td>
<td>2.08</td>
<td>5.04</td>
<td>6.25</td>
<td>8.18</td>
<td>4.32</td>
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<tr>
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<td>7.47</td>
<td>5.86</td>
<td>5.86</td>
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<tr>
<td>Homo sapiens</td>
<td>2.71</td>
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<td>3.24</td>
<td>8.41</td>
<td>7.42</td>
<td>5.14</td>
<td>5.14</td>
<td>17.7</td>
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References


