Acetyl Coenzyme A Synthetase (ADP Forming) from the Hyperthermophilic Archaeon *Pyrococcus furiosus*: Identification, Cloning, Separate Expression of the Encoding Genes, *acdAI* and *acdBI*, in *Escherichia coli*, and In Vitro Reconstitution of the Active Heterotetrameric Enzyme from Its Recombinant Subunits

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Received 7 April 1999/Accepted 7 July 1999

Acetyl-coenzyme A (acetyl-CoA) synthetase (ADP forming) represents a novel enzyme in archaea of acetate formation and energy conservation (acetyl-CoA + ADP + P → acetate + ATP + CoA). Two isoforms of the enzyme have been purified from the hyperthermophile *Pyrococcus furiosus*. Isoform I is a heterotetramer (α2β2) with an apparent molecular mass of 145 kDa, composed of two subunits, α and β, with apparent molecular masses of 47 and 25 kDa, respectively. By using N-terminal amino acid sequences of both subunits, the encoding genes, designated *acdAI* and *acdBI*, were identified in the genome of *P. furiosus*. The genes were separately overexpressed in *Escherichia coli*, and the recombinant subunits were reconstituted in vitro to the active heterotetrameric enzyme. The purified recombinant enzyme showed molecular and catalytic properties very similar to those shown by acetyl-CoA synthetase (ADP forming) purified from *P. furiosus*. 

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+ Dedicated to Rolf Thauer on the occasion of his 60th birthday.
Homologous hypothetical proteins of the hyperthermophilic archaea *Methanococcus jannaschii* (704 aa) and *Archaeoglobus fulgidus* (685 aa) and the eubacterium *Escherichia coli* (886 aa) each had a size of about the sum of the amino acids of the α and β subunits of acetyl-CoA synthetase (ADP forming) isoform I from *P. furiosus* (229 aa), *A. fulgidus* (229 aa), and *E. coli* (430 aa).

GAGGT were present, as reported for other *Pyrococcus* genes (e.g., see references 9 and 24). Archaeal promoter regions, TATA boxes, and initiator elements (21) were not found. Downstream from the *acdBI* gene, rather than from the *acdAI* gene, a pyrimidine-rich region within 16 to 19 nucleotides with the consensus sequence TTTTTTYY, indicating a transcription termination site, was identified (22). The G+C contents of the *acdAI* and *acdBI* genes are 43.5 and 40.3 mol%, respectively, and thus are slightly higher than the value of 38.5 mol% reported for the total genome of *P. furiosus* (6).

Comparison of AcdAI and AcdBI sequences with those of other proteins. In a BLASTP search (1), the deduced amino acid sequences of the α (AcdAI) and β (AcdBI) subunits from *P. furiosus* were compared to those of proteins in the database derived from genome sequences (2, 5, 10, 11). Several proteins showing significant amino acid sequence identity were identified (Fig. 1). The highest degrees of identity (93 and 83%) were found with two proteins from *P. horikoshii* (212 aa), *M. jannaschii* (704 aa) and *A. fulgidus* (685 aa), and *E. coli* (886 aa) deduced from genome sequences (2, 5, 10, 11). EMBL accession numbers of the proteins are given in brackets. Amino acid sequence identities given in white boxes are for the homologous α subunits of the *Pyrococcus* proteins and the N-terminal parts (456 aa each) of proteins of *M. jannaschii*, *A. fulgidus*, and *E. coli*. Sequence identities given in shaded boxes are for the homologous β subunits of *Pyrococcus* proteins and the C-terminal parts of proteins of *M. jannaschii* (248 aa), *A. fulgidus* (229 aa), and *E. coli* (430 aa).

Expression of *acdAI* and *acdBI* genes in *E. coli*. The identity of putative *acdAI* and *acdBI* genes as coding genes for the α and β subunits of acetyl-CoA synthetase (ADP forming) isoform I was proved by functional overexpression in *E. coli*. *P*ET-14b and *P*ET-17b protein expression vectors as well as *E. coli* JM109 and BL21(DE3) were purchased from Novagen. *P. furiosus* (DSM 3638) (6) was grown at 90°C with starch as a carbon and energy source, as described previously (8). The *acdAI* and *acdBI* genes were amplified by PCR with genomic DNA of *P. furiosus* as the template. The PCR product of *acdAI* was cloned in *P*ET-17b by using the forward oligonucleotide primer 5′AATTTGACATATGAGTTTGAGGCTCTTTTT TTCTTTGTGTGTTTGGCCTTTC3′ extending by an NdeI restriction site and the reverse complement oligonucleotide primer 5′CCGCTCGAGTACTTTCTTTGTGGTTTGGGCTTTTC3′ containing an *XhoI* site (restriction sites underlined). The PCR product of *acdBI* was cloned in *P*ET-14b by using the restriction sites NcoI and *XhoI* and the primers 5′GATGCGCATGACAGGGTTTGCTAAAG 3′ and 5′GGCGACTGACGTAAGATCATCCTGAC3′. The recombiant plasmids were named p*ET*-17b(*acdAI*) and p*ET*-14b(*acdBI*). The inserted gene sequence was confirmed on each strand by the Sanger method. The two expression vectors p*ET*-17b(*acdAI*) and p*ET*-14b(*acdBI*) were transformed separately into *E. coli* BL21(DE3). Cells were grown in 400 ml of Luria-Bertani medium at 37°C to an optical density at 600 nm of 1.0, and expression was initiated by the addition of 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside). After 3 h of further growth, the cells were harvested by centrifugation at 4°C.
The pellet was frozen at -20°C. Cell extracts were prepared by passing cell suspensions in buffer (150 mM NaCl, 20 mM Tris HCl [pH 8.0]) through a French pressure cell at 150 MPa. After centrifugation at 40,000 x g for 1 h, the resulting supernatant (cell extract, 3 to 4 mg of protein/ml) was analyzed for overexpressed α and β subunits and used for reconstitution experiments.

After induction of the cells with IPTG, proteins with molecular masses of 47 kDa (α subunit [AcdAl]) and 25 kDa (β subunit [AcdBI]) were overexpressed as revealed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of cell extracts. Heat treatment (15 min at 80°C) of the extracts resulted in a significant enrichment (>80%) of the recombinant α or β subunit as judged by SDS-PAGE (data not shown). The oligomeric state of the recombinant subunits obtained after heat treatment was analyzed by gel filtration chromatography on a Superdex 200 HiLoad 26/60 column, equilibrated with 20 mM Tris HCl buffer, pH 8.0, containing 0.15 M NaCl. For the respective subunits more than 80% of the protein applied (1.2 mg each) was eluted at an apparent molecular mass of about 90 kDa (α subunit) or about 55 kDa (β subunit), indicating that the recombinant subunits were present predominantly as dimers.

Reconstitution and purification of recombinant acetyl-CoA synthetase (ADP forming). Equal amounts of E. coli extract (3 mg/ml) containing recombinant α and β subunits (AcdAl and AcdBI) were mixed and incubated on ice (1 h). The combined extracts exhibited acetyl-CoA synthetase (ADP forming) activity of about 1 U/mg at 55°C (for direction of acetate formation, see reference 7), indicating that the subunits had been reconstituted to an active enzyme. The enzyme was purified about 10-fold by heat treatment (15 min at 80°C) and subsequent anion-exchange chromatography, as follows. Heat-precipitated host cell proteins were removed by centrifugation. The resulting supernatant was applied to a 6-ml Resource Q column equilibrated with 20 mM Tris HCl buffer, pH 8.0, containing 10 mM MgCl₂. Protein was eluted with a linear gradient from 0 to 0.4 M NaCl in buffer (150 ml). Highest activity of acetyl-CoA synthetase (ADP forming) was eluted at 0.14 M NaCl. Protein purity was assessed by SDS-PAGE analysis. This two-step purification yielded a homogeneous preparation of recombinant holoenzyme, as indicated by two bands in SDS-PAGE (Fig. 2), which showed the same apparent molecular masses as the enzyme purified from P. furiosus (7).

Biochemical characterization of recombinant acetyl-CoA synthetase (ADP forming). The purified recombinant acetyl-CoA synthetase (ADP forming) was biochemically analyzed with respect to molecular and catalytical properties and compared with the native enzyme purified from P. furiosus (7). Enzyme activity (acetyl-CoA + ADP + P = acetyl + ATP + CoA) was measured in both directions as described previously (7). Optimum temperature and thermostability (between 80 and 110°C) of the enzyme were determined as described previously (7). The apparent molecular masses of the enzyme (determined by gel filtration) and of its subunits (determined by SDS-PAGE) and the apparent Vₘₐₓ values of all substrates were almost identical, as reported for the enzyme isolated from P. furiosus; however, the apparent Vₘₐₓ values were about 40 to 50% lower (Table 1). The recombinant enzyme showed almost identical thermostability and pattern of heat inactivation, i.e., it did not lose activity upon incubation for 3 h at 90°C, but it lost about 60% of its activity after 2 h at 100°C (see Fig. 3 in reference 7).

The data indicate that in vitro reconstitution of separately expressed α and β subunits yielded a recombinant heterotetrameric acetyl-CoA synthetase (ADP forming) with properties very similar to those of the native enzyme isoform I isolated from P. furiosus (7). So far, only three heterooligomeric enzymes from hyperthermophiles have been functionally overexpressed in E. coli by processes involving reconstitution of sep-

![Image](53x550)
arately expressed subunits: heterodimeric reverse gyrase from Methanoparys kandleri (12) and heterotetrameric DNA topoisomerase VI from Sulfolobus shibatae (4) and indolepyruvate ferredoxin oxidoreductase from Pyrococcus kadosakarenas (20). In conclusion, the successful heterologous expression of acetyl-CoA synthetase (ADP-forming) isoform I will allow detailed biochemical analyses of this unusual enzyme in the future, e.g., studies of structure-function relationships following crystallization and mechanistic studies involving site-directed mutagenesis.

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper have been submitted to the EMBL nucleotide database with the accession no. AJ240061 (acdAI) and AJ240062 (acdBII).

We thank K. Lutter-Mohr for skilful technical assistance. This work was supported by a grant from the European Union ("Extremophiles as cell factories") and the Fonds der Chemischen Industrie.

REFERENCES