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Purification and Characterization of Two Reversible and ADP-Dependent Acetyl Coenzyme A Synthetases from the Hyperthermophilic Archaeon *Pyrococcus furiosus*

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Pyrococcus furiosus **is a strictly anaerobic archaeon (archaebacterium) that grows at temperatures up to 105°C by fermenting carbohydrates and peptides. Cell extracts have been previously shown to contain an unusual acetyl coenzyme A (acetyl-CoA) synthetase (ACS) which catalyzes the formation of acetate and ATP from acetyl-CoA by using ADP and phosphate rather than AMP and PP_i. We show here that *P. furiosus* contains two distinct isoenzymes of ACS, and both have been purified. One, termed ACS I, uses acetyl-CoA and isobutyryl-CoA but not indoleacetyl-CoA or phenylacetyl-CoA as substrates, while the other, ACS II, utilizes all four CoA derivatives. Succinyl-CoA did not serve as a substrate for either enzyme. ACS I and ACS II have similar molecular masses (approximately 140 kDa), and both appear to be heterotetramers ($\alpha_2\beta_2$) of two different subunits of 45 (α) and 23 (β) kDa. They lack metal ions such as Fe^{2+}, Cu^{2+}, Zn^{2+}, and Mg^{2+} and are stable to oxygen. At 25°C, both enzymes were virtually inactive and exhibited optimal activities above 90°C (at pH 8.0) and at pH 9.0 (at 80°C). The times required to lose 50% of their activity at 80°C were about 18 h for ACS I and 8 h for ACS II. With both enzymes in the acid formation reactions, ADP and phosphate could be replaced by GDP and phosphate but not by CDP and phosphate or by AMP and PP_i. The apparent K_m values for ADP, GDP, and phosphate were approximately 150, 132, and 396 μM, respectively, for ACS I (using acetyl-CoA) and 61, 236, and 580 μM, respectively, for ACS II (using indoleacetyl-CoA). With ADP and phosphate as substrates, the apparent K_m values for acetyl-CoA and isobutyryl-CoA were 25 and 29 μM, respectively, for ACS I and 26 and 12 μM, respectively, for ACS II. With ACS II, the apparent K_m value for phenylacetyl-CoA was 4 μM. Both enzymes also catalyzed the reverse reaction, the ATP-dependent formation of the CoA derivatives of acetate (I and II), isobutyrate (I and II), phenylacetate (II only), and indoleacetate (II only). The N-terminal amino acid sequences of the two subunits of ACS I were similar to those of ACS II and to that of a hypothetical 67-kDa protein from *Escherichia coli* but showed no similarity to mesophilic ACS-type enzymes. To our knowledge, ACS I and II are the first ATP-utilizing enzymes to be purified from a hyperthermophile, and ACS II is the first enzyme of the ACS type to utilize aromatic CoA derivatives.**

Acetyl coenzyme A (acetyl-CoA) is a central metabolite at the junction of various anabolic and catabolic pathways, and its interconversion with acetate occurs by two distinct mechanisms (4). One involves acetyl-CoA synthetase (ACS), which catalyzes the following reaction: acetyl-CoA + AMP + PP_i ↔ acetate + CoA + ATP. This reaction proceeds through an acetyladenylate intermediate and is used by microorganisms as a catabolite-repressible, acetate-inducible, high-affinity acetate uptake system to scavenge low concentrations of extracellular acetate. The second main mechanism for acetate/acetyl-CoA interconversion involves two enzymes: phosphotransacetylase (PTA), which catalyzes the reaction acetyl-CoA + phosphate ↔ acetyl phosphate + CoA, and acetate kinase (ACK), which catalyzes the reaction acetyl phosphate + ADP ↔ acetate + ATP. In contrast to ACS, these two enzymes function primarily in the catabolic direction, whereby acetate is excreted and ATP is synthesized. Hence, in most anaerobic bacteria, the formation of acetate from acetyl-CoA is accomplished by the ACK-PTA pathway, while in mammals, plants, fungi, and some acetoclastic methanogens, the activation of acetate to acetyl-CoA is catalyzed by ACS. Only the unrelated species *Escherichia coli* and *Bacillus subtilis* are known to utilize both the ACS and the ACK-PTA pathways (11, 22).

ACS has been purified from several organisms able to utilize acetate, including bacteria (32), methanogenic archaea (18), and eukaryotes (6, 17, 28). All are homodimeric enzymes with a subunit M_r of about 70,000. The genes that encode several of these enzymes have been cloned and sequenced (6–8, 10, 22, 33), as have the genes for two ACSs that have yet to be purified (10, 33). Their overall sequence identity is over 45% at the amino acid level. All ACSs purified so far generate AMP and PP_i during acetate activation, and in the reverse reaction, acetate synthesis, these cannot be replaced by ADP and phosphate (28). However, two eukaryotic protists, *Entamoeba histolytica* (34) and *Giardia lamblia* (35), and the subject of this study, the anaerobic hyperthermophilic archaeon *Pyrococcus furiosus* (37, 38), have been reported to contain an ADP-dependent ACS. All three organisms produce acetate as an end product of metabolism, but they lack the ACK-PTA pathway. Instead, they appear to use the ADP-dependent ACS reaction, acetyl-CoA + ADP + phosphate ↔ acetate + ATP + CoA, as a means of conserving energy.

P. furiosus grows at temperatures up to 105°C by fermenting carbohydrates and peptides (9). Both the nature and the role of the proposed ADP-dependent ACS in this organism were of considerable interest because it contains several enzymes that generate various CoA derivatives. In addition to pyruvate

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ferredoxin oxidoreductase (POR), which generates acetyl-CoA from pyruvate (3), *P. furiosus* contains three other types of ferredoxin-dependent, 2-keto acid oxidoreductase. These utilize as primary substrates either 2-ketoglutarate (KGOR [27]), aromatic 2-keto acids such as indolepyruvate (IOR [26]), or branched-chain 2-keto acids such as 2-ketoisovalerate (VOR [13]). Moreover, the acid derivatives of the products of the VOR reaction, such as isovalerate and isobutyrate, have been found in the culture media of several species of *Pyrococcus* (14, 15, 24). In this study, we sought to purify the ADP-dependent ACS from *P. furiosus* to determine (i) if the ADP-dependent production of acetate from acetyl-CoA was catalyzed by a single enzyme, (ii) how its structural and catalytic properties compared with those of the AMP-dependent ACS from mesophiles, and (iii) if it utilized the products of the IOR, VOR, and KGOR reactions, in addition to that of POR. Herein, we describe the purification and properties of two distinct ACSs from *P. furiosus* which differ in their substrate specificities.

MATERIALS AND METHODS

Growth of organism. *P. furiosus* (DSM 3638) was grown in a 600-liter fermentor with maltose as a carbon source as described previously (5).

Enzyme assays. (i) Acid formation from the CoA derivative. ACS I activity in the direction of acetate formation was routinely measured by coupling the reaction to *P. furiosus* POR (38). In this system, POR generates acetyl-CoA from pyruvate, using methyl viologen (MV) as the electron acceptor in the presence of limiting amounts of CoA. Further reduction of MV is only possible if CoA is regenerated by ACS I when it converts acetyl-CoA to acetate. Thus, ACS activity can be measured spectrophotometrically by monitoring the reduction of MV. Assays were carried out in serum-stoppered cuvettes under Ar at 80°C (5). The standard reaction mixture (2.0 ml) for ACS I contained pyruvate (10 mM), $MgCl_2$ (5 mM), thiamine PP_i (0.4 mM), CoA (0.025 mM), MV (5 mM), ADP (1 mM), K_2HPO_4 (10 mM), and *P. furiosus* POR (40 μg) in 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid (EPPS) buffer (pH 8.4). Changes in A_{600} were measured with a DMS 200 spectrophotometer (Varian Associates) equipped with a thermostatted cuvette holder and a thermoinsulated cell compartment. ACS II activity was routinely measured by the same method used for ACS I except that the assay mixture contained *P. furiosus* IOR (40 μg [26]) instead of POR and indolepyruvate (10 mM) instead of pyruvate. Hence, indoleacetyl-CoA served as the routine substrate for determining ACS II activity. In addition to the IOR and POR reactions, the substrate specificities of ACS I and II for CoA derivatives were studied by using succinyl-CoA, generated by *Thermococcus litoralis* KGOR (40 μg [27]) from 2-ketoglutarate (10 mM), and using 2-isobutyryl-CoA, produced by *T. litoralis* VOR (40 μg [13]) from 2-ketoisovalerate. For substrate specificity assays, GDP and phosphate, CDP and phosphate, or AMP and PP_i were used in place of ADP and phosphate. All results were calculated as units per milligram of protein, where 1 U equals the reduction of 2 μmol of MV per min, which is equivalent to 1 μmol of the corresponding acid produced per min.

For kinetic analyses using CoA derivatives as substrates, the activities of ACS I and II were measured by CoASH formation as described previously (39). The reaction mixture (2.0 ml) contained the CoA derivative (acetyl-CoA or isobutyryl-CoA for ACS I and acetyl-CoA, isobutyryl-CoA, or phenylacetyl CoA for ACS II) (0.1 mM), ADP (2 mM), K_2HPO_4 (10 mM), $MgCl_2$ (2 mM), and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB; 0.1 mM) in 50 mM EPPS buffer (pH 8.4). The activity was measured spectrophotometrically at 80°C under anaerobic conditions as described above except that absorbance changes were monitored at 412 nm. An extinction coefficient of 13,600 M^{-1} cm^{-1} was used for the DTNB derivative. All results were calculated as micromoles of CoASH produced per minute per milligram of protein.

(ii) Formation of the CoA derivative from the acid. For kinetic analyses, the activities of ACS I and II were measured in the reverse direction by determining the amount of phosphate formed. The assay mixture (0.5 ml) for ACS I contained CoA (0.5 mM), $MgCl_2$ (10 mM), and sodium acetate (10 mM) in 50 mM EPPS buffer (pH 8.4). ATP (2 mM) and the enzyme were added after the reaction mixture had been incubated at 80°C for 3 min. After a further 3 min, the reaction was terminated by the addition of 100 μl of 6 N H_2SO_4, and phosphate was determined (12). The data were corrected in control experiments conducted without enzyme, which showed that about 10% of the added ATP (2 mM) hydrolyzed nonenzymatically under the experimental conditions. ACS II was assayed by the same method except that indoleacetate (10 mM) replaced acetate. Where indicated, GTP was used in place of ATP and various acids were used in place of acetate and indoleacetate. All results were calculated as micromoles of phosphate produced per minute per milligram of protein.

Purification of ACS I. The enzyme was routinely purified from 500 g (wet weight) of frozen *P. furiosus* cells. Cell extracts of were prepared as described previously (26). All procedures were carried out at 23°C under strictly anaerobic conditions (26) since other oxygen-sensitive enzymes were also purified from the same batch of cells. All solutions were repeatedly degassed and flushed with Ar, prepurified with heated BASF catalyst (Kontes, Vineland, N.J.), and were maintained under a positive pressure of Ar. In addition, all solutions used to prepare the cell extract and for the first chromatography step contained sodium dithionite (2 mM), glycerol (10% [vol/vol]), and dithiothreitol (2 mM). The buffer used throughout the purification was 50 mM Tris-HCl (pH 8.0). All chromatography columns were operated with a fast protein liquid chromatography system (Pharmacia LKB, Piscataway, N.J.). The procedure for ACS I purification was the same as for the purification of IOR from *P. furiosus* (26) up to and including the first chromatography step, which used a column (7.5 by 21 cm) of DEAE-Sepharose Fast Flow (Pharmacia LKB). ACS I activity eluted at 180 to 200 mM NaCl in a gradient (9 liters) from 0 to 500 mM NaCl in buffer. Fractions (100 ml) from this column with ACS I activity above 4.0 U/mg were combined (600 ml) and loaded directly onto a column (5 by 10 cm) of hydroxyapatite (high resolution; Behring Diagnostics) equilibrated with buffer. The absorbed protein was eluted with a gradient (1.2 liters) from 0 to 0.2 M potassium phosphate in buffer at a flow rate at 3 ml/min. Fractions of 42 ml were collected, and ACS I activity eluted as 70 to 110 mM phosphate was applied. Fractions with ACS I activity above 4.8 U/mg were combined (336 ml), diluted with an equal volume of buffer containing ammonium sulfate (2.0 M), and applied to a column (3.5 by 10 cm) of phenyl-Sepharose (Pharmacia LKB) previously equilibrated with buffer containing ammonium sulfate (1.0 M) at 6 ml/min. The absorbed proteins were eluted with a decreasing gradient (600 ml) of ammonium sulfate (1.0 M to 0). ACS I activity was detected in the eluent as 520 mM ammonium sulfate was applied. Fractions (45 ml) with ACS I activity above 29.6 U/mg were combined (180 ml) and concentrated to approximately 20 ml by ultrafiltration (Amicon PM-30 filter). The concentrated sample of ACS I from the previous step was applied to a column (6 by 60 cm) of Superdex 200 (Pharmacia LKB) equilibrated at 3 ml/min with buffer containing 200 mM NaCl. Fractions (10 ml) with ACS I activity above 62.5 U/mg were separately analyzed by nondenaturing and sodium dodecyl sulfate (SDS)-gel electrophoresis. Those judged pure were combined, concentrated by ultrafiltration, and stored at −80°C.

Purification of ACS II. The purification procedure for ACS II was the same as that used for ACS I up to and including the first chromatography step, except that ACS II activity eluted at 67 to 138 mM NaCl in a gradient (9 liters) from 0 to 500 mM NaCl in buffer. Fractions (100 ml) from this column with ACS II activity above 1.2 U/mg were combined (400 ml) and loaded directly onto a column (5 by 10 cm) of hydroxyapatite (high resolution; Behring Diagnostics) equilibrated with buffer. The absorbed protein was eluted with a gradient (1.2 liters) from 0 to 0.2 M potassium phosphate in the same buffer at a flow rate at 3 ml/min. Fractions of 45 ml were collected, and ACS II activity eluted as 65 to 70 mM phosphate was applied. Fractions with ACS II activity above 2.7 U/mg were combined (180 ml), diluted with an equal volume of buffer containing ammonium sulfate (2.0 M), and applied to a column (3.5 by 10 cm) of phenyl-Sepharose (Pharmacia LKB) previously equilibrated with buffer containing ammonium sulfate (1.0 M) at 6 ml/min. The absorbed proteins were eluted with a decreasing gradient (600 ml) of ammonium sulfate (1.0 M to 0). ACS II activity was detected in the eluent as 468 mM ammonium sulfate was applied. Fractions (20 ml) with ACS II activity above 14.0 U/mg were combined (40 ml) and concentrated to approximately 5 ml by ultrafiltration (Amicon PM-30 filter). The concentrated sample of ACS II from the previous step was applied to a column (3.5 by 60 cm) of Superdex 200 (Pharmacia LKB) equilibrated at 1 ml/min with buffer containing 200 mM NaCl. Fractions (10 ml) with ACS II activity above 30 U/mg were separately analyzed by nondenaturing and SDS-gel electrophoresis. Those judged pure were combined, concentrated by ultrafiltration, and stored at −80°C.

Amino-terminal amino acid sequencing. The amino-terminal sequences of the subunits of both ACS I and ACS II were determined with an Applied Biosystems model 477 sequencer. The subunits of the enzyme were separated by SDS-gel electrophoresis and electroblotted onto polyvinylidene difluoride protein-sequencing membranes, using a Bio-Rad electroblotting system. Electroblotting was carried out in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer (pH 11.0) containing methanol (10% [vol/vol]) for 1 h at 50 V.

Molecular weight determinations. Electrophoresis in the presence and absence of SDS, using 6, 15, or 20% (wt/vol) acrylamide, was performed as described previously (40). Samples were prepared by heating at 100°C for 10 min in the presence of SDS (2.5% [wt/vol]) and mercaptoethanol (1% [vol/vol]). Subunit molecular weights were estimated by comparison with the following standard proteins (their molecular masses in kilodaltons are given in parentheses): bovine serum albumin (BSA; 66), ovalbumin (45), glyceraldehyde-3-phosphate dehydrogenase (36), carbonic anhydrase (29), trypsinogen (24), soybean trypsin inhibitor (20.1), and 2-lactalbumin (14). The molecular weight of the holoenzyme was estimated by gel filtration using a column (1.6 by 60 cm) of Superdex 200. The proteins used to calibrate the column (and their molecular masses in kilodaltons) were ferritin (450), catalase (240), amylase (200), alcohol dehydrogenase (150), BSA (66), and carbonic anhydrase (29). The column was run at 0.5 ml/min, using 50 mM Tris-HCl buffer (pH 8.0) containing NaCl (200 mM) as the eluent.

Other methods. POR (3) and IOR (26) from *P. furiosus* and KGOR (27) and VOR (13) from *T. litoralis* were purified as described in the references. Protein concentrations were routinely estimated by the Lowry method (23), using BSA as

TABLE 1. Purification of *P. furiosus* ACS I[a]

Step	Activity (U)	Protein (mg)	Sp act (U/mg)	Recovery (%)	Purification (fold)
Extract	42,100	15,200	2.77	100	1.0
DEAE-Sepharose	14,700	2,220	6.64	35	2.4
Hydroxyapatite	12,300	1,550	7.97	29	2.9
Phenyl-Sepharose	9,030	202	44.7	21	16.1
Superdex 200	5,230	81	64.6	12	23.3

[a] From 300 g (wet weight) of cells.

the standard. A complete metal analysis (40 elements) was carried out by plasma emission spectroscopy, using a Jarrel Ash Plasma Comp 750 instrument at the Chemical Analysis Laboratory of the University of Georgia. The search in the GenBank for sequence similarities was carried out with the programs RETRIVE and BLAST (1). Multiple alignments were carried out with the programs PILEUP and LINEUP, and comparisons between any two sequences were carried out with the BESTFIT program.

RESULTS

Purification of ACS I and ACS II. Cell extracts of *P. furiosus* contained a significant amount of ACS activity (approximately 3 U/mg) when coupled to the POR reaction. Activity albeit much lower (approximately 0.1 U/mg), was also detected when the reaction was coupled to IOR. In both cases, no activity was observed if ADP or phosphate was omitted from the reaction mixture, and neither activity could be detected in the particulate fraction of cell extracts. That the cytoplasm of this organism might contain two types of enzyme able to use CoA derivatives was supported by the separation of the POR- and IOR-dependent activities by anion-exchange chromatography. When the cell extract was applied to a DEAE-Sepharose column (pH 8.0), the POR-dependent activity eluted between 180 to 200 mM NaCl whereas IOR-dependent ACS activity eluted between 70 to 140 mM NaCl. Hence, the enzymes responsible for these two activities were designated ACS I, which uses acetyl-CoA as a substrate, and ACS II, which uses indoleacetyl-CoA. The recovery of each activity from the first DEAE-Sepharose column was 50% or less, which might be expected since there are several factors in the cell extract that could interfere with the coupled assay system for ACS. For example, *P. furiosus* contains three CoA-independent, aldehyde-oxidizing enzymes that reduce MV (31). ACS I and ACS II were further purified by multistep chromatography, and in both cases there were no dramatic losses of activity at each step, in support of the presence of interfering substances in the cell extract. The results of typical purification procedures for the two enzymes are given in Tables 1 and 2. The specific activities of the two pure enzymes are within the same range, 65 U/mg for ACS I and 30 U/mg for ACS II, but the purification factors indicate that the cellular concentration of ACS I is about an order of magnitude greater than that of ACS II.

Molecular compositions of ACS I and ACS II. The two enzymes each gave rise to a single protein band with comparable R_f values after nondenaturing electrophoresis (using 6% [wt/vol] acrylamide [data not shown]), and each yielded two protein bands after SDS-electrophoresis (using 15% [wt/vol] acrylamide), which corresponded to M_rs of 45,000 (α) and 23,000 (β) (Fig. 1). In addition, each protein eluted from a Superdex 200 gel filtration column as a single peak with an apparent M_r of 140,000 ± 10,000. These data suggested that both ACS I and ACS II are heterotetramers ($\alpha_2\beta_2$). Similarly, neither protein exhibited absorption in the visible region of the spectrum, and neither contained iron or other metals, such as copper, zinc, or magnesium. That ACS I and ACS II were, in fact, distinct enzymes was shown by the amino-terminal sequences for their two subunits, which, as shown in Fig. 2, were similar but not identical. Their α subunits have 58% identity over 40 amino acids, while the β subunits are 40% identical in the first 26 amino acids. Database searches using these amino-terminal sequences revealed only one sequence with significant similarity, and that was the sequence of a hypothetical 67-kDa protein fragment of unknown function in the uracil DNA glycosylase 3′ region from *E. coli* (GenBank accession number D64044). Its amino-terminal sequence has 58% identity with the α subunit of ACS I and 42% identity with the α subunit of ACS II (Fig. 2). Although ACS I and ACS II were purified anaerobically, neither showed any loss of activity after exposure to O_2 (air) for 24 h, and both enzymes could presumably be purified aerobically without deleterious effects. The times required for a 50% loss of activity upon incubation of ACS I (0.4 mg/ml) and ACS II (0.5 mg/ml) at 80°C in EPPS buffer (pH 8.0) were 18 and 8 h, respectively.

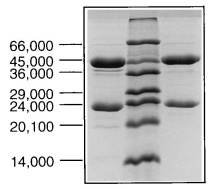

FIG. 1. SDS-polyacrylamide electrophoresis of *P. furiosus* ACS I and ACS II. The right lane contained purified ACS I (15 μg), the center lane contained marker proteins with the molecular weights indicated at the left, and the left lane contained purified ACS II (15 μg).

Catalytic properties of ACS I. With acetyl-CoA as the substrate generated by POR, ACS I was virtually inactive at ambient temperature and showed a dramatic increase in activity above 70°C, with an optimum above 90°C (at pH 8.0) (Fig. 3). The optimum pH for activity (at 80°C) was about 9.0 (Fig. 3). ACS I activity was absolutely dependent on ADP and phosphate. Under standard assay conditions, a linear double-reciprocal plot was obtained when the concentration of ADP was varied from 0.03 to 2.0 mM (using 10 mM phosphate), and the apparent K_m and k_{cat} values were 150 μM and 203 s^{-1}, respectively (Table 3). From a similar analysis with various phosphate concentrations (from 0.05 to 10 mM, using 1 mM ADP), the

TABLE 2. Purification of *P. furiosus* ACS II[a]

Step	Activity (U)	Protein (mg)	Sp act (U/mg)	Recovery (%)	Purification (fold)
Extract	3,430	27,000	0.13	100	1
DEAE-Sepharose	1,800	838	2.19	54	17
Hydroxyapatite	1,160	372	3.12	34	24
Phenyl-Sepharose	908	32	28.4	26	218
Superdex 200	600	20	30.0	17	231

[a] From 500 g (wet weight) of cells.

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Pf ACS I α       GLEALFNPKS VAVIGASAKP GKIGYAIMKN LIEYGYQGKI YPVNXK
Pf ACS II α      MLDYFFNPRG IAVIGASNDP KKLGYEVFKN LKEYQ-GGKV
Ec 67kDa     MSQRGLEALLRPKS IAVIGASMKP NRAGYLMMRN LLAGGFNGPV LPVTPA

Pf ACS I β       MDRVAKARE IIEKAKAENR PLVQP-AAK
Pf ACS II β      MKGE--ALK IIEEVLAQGR TAMVEYEAKQ VLK
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FIG. 2. Amino-terminal sequences of the subunits (α and β) of ACS I and ACS II from *P. furiosus* (Pf) and of the hypothetical 67-kDa protein fragment in the uracil DNA glycosylase 3′ region of *E. coli* (Ec 67kDa) Gaps (−) have been inserted to maximize identity, and identical residues are shown in boldface.

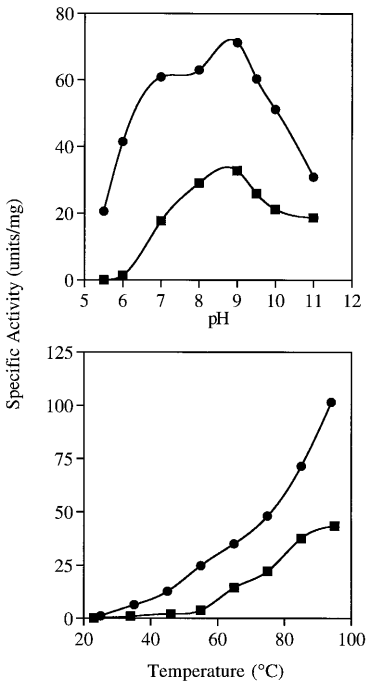

FIG. 3. Effects of pH (top) and temperature (bottom) on the catalytic activities of *P. furiosus* ACS I and ACS II. The activities of ACS I (circles) and ACS II (squares) were determined under standard assay conditions at 80°C and pH 8.0, except that the pH or the temperature was varied as indicated. The buffers used for the pH dependence assays were 4-morpholinethanesulfonic acid (pH 5.5 and 6.0), 4-morpholinepropanesulfonic acid (pH 7.0), EPPS (pH 8.0), glycine (9.0, 9.5), and CAPS (pH 10.0 and 11.0).

calculated apparent K_m and k_{cat} values were 396 μM and 182 s^{-1}, respectively (Table 3). No activity was detected under standard conditions when ADP and phosphate were replaced by CDP and phosphate or by AMP and pyrophosphate. However, GDP did substitute for ADP, and on the basis of apparent K_m and k_{cat} values (determined by using GDP concentrations of 0.02 to 2.0 mM), the enzyme exhibited similar affinities for the two nucleotides but was twice as active with GDP (Table 3). When POR was replaced in the standard assay with either IOR or KGOR, ACS I was inactive, showing that it cannot use indoleacetyl-CoA or succinyl-CoA as a substrate. However, the enzyme exhibited a specific activity of 52 U/mg when POR was replaced by VOR. Thus, isobutyryl-CoA does serve as a substrate for ACS I, and the activity was comparable to that obtained with acetyl-CoA (65 U/mg). To determine kinetic constants for acid formation catalyzed by ACS I, acetyl-CoA and isobutyryl-CoA were used as substrates and the reaction was measured by reaction of the CoASH that was generated with DTNB. Under standard assay conditions, linear double-reciprocal plots were obtained when the concentrations of acetyl-CoA and isobutyryl-CoA were varied from 0.01 to 0.2 mM (using 2 mM ADP, 10 mM phosphate, 2 mM $MgCl_2$, and 0.1 mM DTNB). The apparent K_m and k_{cat} values are summarized in Table 3. Notably, the apparent K_m values were all less than 29 μM. ACS I did not generate CoASH when phenylacetyl-CoA was used as a substrate, consistent with the results described above for the IOR-linked assay.

ACS I also catalyzed the reverse reaction, the formation of acetyl-CoA, ADP, and phosphate by using acetate, ATP, and CoA as the substrates, as measured by phosphate formation. Under standard assay conditions, the specific activity was 27 μmol of phosphate produced per min per mg, which is about 41% of the rate of the forward reaction. As summarized in Table 3, kinetic constants were calculated for acetate (concentration range, 0.1 to 10 mM), ATP (0.2 to 3.0 mM), and CoA (0.005 to 0.5 mM). Notably, the enzyme had a very high affinity for CoA (apparent K_m, 18 μM). As with the acetate formation reaction, for acetyl-CoA synthesis the guanine nucleotide (GTP) would replace the adenine one (ATP). From the kinetic data shown in Table 3 (obtained by using 0.04 to 2.0 mM GTP), ACS I had similar affinities for GTP and ATP, although as with acetate formation, the activity with GTP was higher than with ATP (in this case, by about 50%). However, with both adenine and guanine nucleotides, the enzyme was about an order of

TABLE 3. Kinetics parameters of *P. furiosus* ACS I and ACS II

Reaction and variable substrate	ACS I			ACS II		
	App.[a] K_m (μM)	App. k_{cat} (s^{-1})	App. k_{cat}/app. K_m ($\mu M^{-1}\ s^{-1}$)	App. K_m (μM)	App. k_{cat} (s^{-1})	App. k_{cat}/app. K_m ($\mu M^{-1}\ s^{-1}$)
Acid formation						
ADP[b]	150	203	1.35	61	115	1.89
GDP[b]	132	411	3.11	236	21	0.09
Phosphate[b]	396	182	0.46	580	117	0.20
Acetyl-CoA[c]	25	157	6.28	26	42	1.62
Isobutyryl-CoA[c]	29	121	4.17	12	8	0.67
Phenylacetyl-CoA[c]		0		4	138	34.5
Acid utilization[d]						
ATP[e]	477	82	0.17	326	68	0.21
GTP[e]	430	121	0.28	770	27	0.04
CoA[e]	18	73	4.06	74	70	0.95
Acetate	1,100	65	0.06	10,700	67	0.006
Isobutyrate	457	55	0.12	5,800	22	0.004
Indoleacetate		0		2,000	66	0.03
Phenylacetate		0		768	89	0.12
Succinate		0			0	

[a] App., apparent.
[b] The activities of ACS I and ACS II were determined by coupling the reactions to POR and IOR, respectively.
[c] The activities were determined by CoASH formation.
[d] The activities were determined by phosphate formation.
[e] The activities of ACS I and ACS II were determined by using acetate and indoleacetate, respectively, as the substrates.

magnitude more efficient in acetate formation than in acid utilization, as judged from the apparent k_{cat}/apparent K_m values (Table 3). As might be expected from the specificity of ACS I for CoA derivatives, neither indoleacetate nor succinate served as a substrate for the enzyme, but isobutyrate was converted to isobutyryl-CoA. From kinetic analyses (using 0.1 to 10 mM isobutyrate), ACS I exhibited comparable activities with acetate and isobutyrate as substrates and had a twofold-higher affinity for the latter acid (Table 3).

Catalytic properties of ACS II. With indoleacetyl-CoA as the substrate (generated by IOR), the temperature and pH dependence of the ACS II reaction were similar to those of ACS I, with an optimum above 90°C (pH 8.0) and pH 9 (at 80°C) (Fig. 3). The activity of ACS II was dependent on ADP and phosphate, and kinetic analyses showed that the catalytic efficiencies of ACS II with these substrates were similar to those determined for ACS I (Table 3). Like ACS I, ACS II did not use CDP plus phosphate or AMP plus PP_i in place of ADP and phosphate. On the other hand, while GDP was a better substrate than ADP for ACS I, ACS II exhibited a 21-fold-higher apparent k_{cat}/K_m value for ADP (Table 3). When indoleacetyl-CoA (generated by IOR) was replaced in the standard assay with acetyl-CoA (generated by POR) or with isobutyryl-CoA (generated by VOR), the specific activity of ACS II was 50 or 12% of that measured with using the IOR-coupled reaction. No activity was detected with succinyl-CoA as the substrate (generated by KGOR). Kinetic constants were determined with acetyl-CoA, isobutyryl-CoA, or phenylacetyl-CoA as the substrate (we were unable to find a commercial source of indoleacetyl-CoA) by measuring CoASH production with DTNB. Under standard assay conditions, linear double-reciprocal plots were obtained when the concentrations of the three CoA derivatives were varied from 0.001 to 0.2 mM (using 2 mM ADP, 10 mM phosphate, 2 mM $MgCl_2$, and 0.1 mM DTNB). Apparent K_m and k_{cat} values are given in Table 3. Interestingly, the apparent K_m values for all three CoA derivatives were below 26 μM, and the enzyme had the highest affinity for phenylacetyl-CoA (apparent K_m, 4 μM).

ACS II also catalyzed the reverse reaction, the synthesis of indoleacetyl-CoA with indoleacetate, ATP, and CoA as substrates, and the catalytic efficiency with respect to ATP was similar to that found for ACS I in acetyl-CoA synthesis (apparent k_{cat}/K_m, 0.2 $\mu M^{-1}\ s^{-1}$; see Table 3). GTP would replace ATP in the ACS II reaction, but the apparent k_{cat}/K_m value was fivefold lower (Table 3). ACS II exhibited a high affinity for CoA (apparent K_m, 74 μM), although less than that observed with ACS I (apparent K_m, 18 μM). ACS II also utilized acetate, isobutyrate, and phenylacetate as substrates, with specific activities comparable to those measured with ACS I; however, the apparent K_m values were an order of magnitude greater with ACS II (Table 3). Like ACS I, succinate was not a substrate for ACS II.

DISCUSSION

ACSs previously purified from prokaryotic sources, which include the bacteria *E. coli* and *Bradyrhizobium japonicum* (32) and the methanogen *Methanothrix soehngenii* (18), are involved in acetate utilization and generate AMP and PP_i. The enzyme has also been purified from mammals (17) and from several species of fungus (6, 28, 32), and these have the same substrate specificity as the prokaryotic enzymes. Hence, the discovery of an ADP-dependent ACS in cell extracts of the hyperthermophilic, acetate-producing archaeon *P. furiosus* by Schäfer and Schönheit (37) was of some significance. These researchers also detected this activity in other hyperthermophilic archaea and in the mesophilic archaeon *Halobacterium saccharovorum* but not in the hyperthermophilic bacterium *Thermotoga maritima* (38). It was therefore proposed that the ADP-dependent ACS is a property of acetate-producing archaea rather than a characteristic of hyperthermophiles, whereas acetate-forming bacteria utilize the ACK-PTA pathway (37). We show herein that the hyperthermophilic archaea, or at least those represented by *P. furiosus*, contain two distinct ADP-dependent ACS enzymes which differ in their substrate specificities. These include the first known ACS to utilize aromatic derivatives of CoA (ACS II) and one (ACS I) which exhibits twice the catalytic efficiency with guanine nucleotides (Table 3).

ADP-dependent, acetate-forming activities have also been reported in two eukaryotes, both of which are human parasites, although the enzymes involved have not been completely characterized. That of *E. histolytica* was identified in cell extracts (34), while the enzyme from *G. lamblia* was partially purified (35). Unfortunately, the latter enzyme was extremely unstable, and although acetate formation was shown to be dependent on ADP, the reverse reaction could not be demonstrated. In contrast to the *P. furiosus* enzymes, *G. lamblia* ACS used succinyl-CoA as a substrate for ATP synthesis but would not utilize GDP in replace of ADP. Another enzyme that could be included in the ADP-dependent ACS category is the so-called acyl-CoA esterase of the anaerobic bacterium *Selenomonas ruminantium* (29). This enzyme was partially purified and catalyzed the ADP-dependent conversion of acetyl-CoA, propionyl-CoA, and succinyl-CoA to the corresponding acid, with concomitant ATP synthesis. However, the esterase appears to have a much higher molecular weight (~230,000 from an activity stained, nondenaturing electrophoresis gel) than the ACSs of *P. furiosus* or the AMP-dependent enzymes from mesophiles (see below), and its relationship to them is unclear at present.

All of the ACSs purified so far from mesophilic organisms, including both prokaryotes and eukaryotes, are homodimers with M_rs of about 140,000 (18, 28, 33), and the corresponding genes encode proteins with M_rs of about 70,000 (6–8, 10, 22, 33). These enzymes show between 45 and 68% sequence identity at the amino acid level. Some regions, such as the putative nucleotide-binding site, are highly conserved, although the enzymes show no similarity at their amino-terminal sequences (10). There was also no correspondence between the amino-terminal sequences of ACS I and ACS II from *P. furiosus*, which are highly similar to each other (Fig. 2), and the amino-terminal sequences of the mesophilic ACSs. Interestingly, the two *P. furiosus* enzymes have the same molecular mass (140,000) as the mesophilic enzymes, and the aggregate size of their two subunits (45,000 and 23,000) approximates the size of the single subunit of other ACSs. We therefore wondered if the single genes for the latter enzymes evolved by gene fusion of two ancestral genes now represented by those encoding the two subunits of the hyperthermophilic ACSs. For example, the gene encoding the single large subunit of mesophilic 2-keto acid ferredoxin oxidoreductases appears to have evolved from the fusion of four ancestral genes of the type now present in the hyperthermophilic archaea (20). However, no similarity was evident between the amino-terminal sequences of ACS I and ACS II of *P. furiosus* and the complete sequences of the mesophilic ACSs, and so the evolutionary relationship between them remains to be established. Database searches using the amino-terminal sequences of ACS I and ACS II of *P. furiosus* revealed only one sequence with significant similarity, that of a hypothetical protein (M_r 67,000) in the uracil DNA glycosylase 3′ region from *E. coli* (GenBank accession number D64044) (Fig. 2). Unfortunately, no information is available on the

likely function of the *E. coli* protein, which shows no sequence similarity to the ACS from *E. coli* (22).

The presence of two distinct ACSs in a single organism, as in *P. furiosus*, has not been previously reported for prokaryotes, although *Saccharomyces cerevisiae* appears to contain two AMP-dependent ACS isoenzymes which differ in their immunological and regulatory properties (21, 36). The ACSs of *P. furiosus* also differ from their mesophilic counterparts in their substrate specificity. For example, the majority of the AMP-dependent ACSs only use acetate as the organic acid substrate. The exceptions are the ACSs from *Penicillium chrysogenum* (28), which utilizes acetate, propionate, butyrate, and valerate, and the enzymes from *Alcaligenes eutrophus* (33) and *M. soehngenii* (18), which also utilize propionate, but in all cases, the activities are less than 50% of those observed with acetate. In contrast, compared with acetate as the substrate, ACS I and ACS II from *P. furiosus* exhibited two- and fivefold-higher efficiencies with isobutyrate and indoleacetate, respectively (Table 3). A similar trend is seen when nucleotide specificities are compared. For example, some of mesophilic ACSs can replace ATP with other nucleotides for acetate activation, but these support much lower catalytic activities. Thus, the activity of *Penicillium chrysogenum* ACS with GTP was 3% of that observed with ATP (28). In contrast, *P. furiosus* ACS I exhibited more than twice the catalytic efficiency with guanine nucleotides compared with adenine nucleotides in both the synthesis and the utilization of acetate, and ACS II was about 50% as active with GTP as it was with ATP in indoleacetate utilization (Table 3). The significance of guanine nucleotide utilization is not clear at present, but these data suggest that GTP might be used as a means of energy conservation in *P. furiosus*. Unfortunately, ACS is the only ATP-utilizing enzyme to be purified from hyperthermophilic archaea such as *P. furiosus* at present, and so it remains to be seen if other enzymes exhibit similar specificities for ATP and GTP.

We now turn to the physiological role of ACS I and ACS II in *P. furiosus* and why two enzymes of this type are present. This organism ferments certain carbohydrates (5) via an unusual Embden-Meyerhof pathway (19, 30), and the pyruvate produced is converted to acetyl-CoA via POR (3). An ADP-dependent ACS was proposed to catalyze the generation of acetate, the main fermentation product, from acetyl-CoA (37, 38). This function can now be assigned to both ACS I and ACS II (Fig. 4), which exhibit high affinities for acetyl-CoA (apparent K_m values, ~25 μM [Table 3]). *P. furiosus* also grows by fermenting peptides (5), and other organic acids, such as isovalerate, isobutyrate, and phenylacetate, have been detected in the growth media of all proteolytic hyperthermophiles so far investigated (9, 14–16, 25). In this case, peptide-derived amino acids are transaminated to the corresponding 2-keto acids (2), each of which in turn is converted to the CoA derivative by the three other 2-keto acid oxidoreductases found in *P. furiosus*, IOR (26), KGOR (27), and VOR (13). VOR is most active with the 2-keto acids originating from the amino acids valine, leucine, isoleucine, and methionine (13), IOR is specific for aromatic 2-keto acids corresponding to the aromatic amino acids (26), and the only known substrate for KGOR is 2-ketoglutarate (27). Thus, the fermentation end product, phenylacetate, must be produced from phenylacetyl-CoA, which in turn is derived from phenylalanine via transamination and the IOR reaction. Accordingly, ACS II, which was purified by its ability to use indoleacetyl-CoA as a substrate, has a very high affinity (apparent K_m, 4 μM) for phenylacetyl-CoA, while ACS I showed no activity with this compound (Table 3). Therefore, ACS II is proposed to catalyze phenylacetate production (Fig. 4).

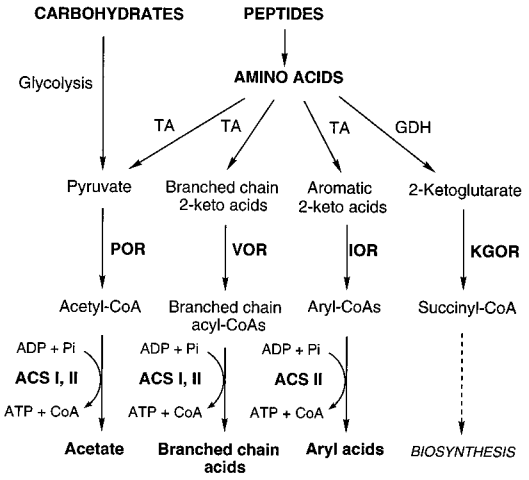

FIG. 4. Proposed physiological roles of ACS I and ACS II during fermentation by *P. furiosus*. TA, transaminase; GDH, glutamate dehydrogenase.

Both ACS I and ACS II were also able to utilize isobutyryl-CoA, a metabolite derived from valine and the product of the VOR reaction. With both enzymes, the activities and apparent K_m values (<30 μM) were comparable to the values obtained with acetyl-CoA (Table 3). Thus, both ACS I and ACS II must participate in producing branched-chain organic acids from amino acids (Fig. 4). In contrast, neither enzyme utilized 2-ketoglutarate, the product of the KGOR reaction, and we assume that the succinyl-CoA produced is used for biosynthetic rather than energy-conserving purposes (Fig. 4). Thus, two ACS enzymes are required in *P. furiosus* to accommodate the various CoA derivatives that are produced during peptide fermentation, represented by acetyl-CoA, isobutyryl-CoA, and phenylacetyl-CoA. In addition, although this organism cannot grow with organic acids as sole carbon sources (5), the affinities (as measured by the apparent K_m values) of ACS I for acetate and isobutyrate, and of ACS II for phenylacetate (Table 3), suggest that these enzymes might, under nutrient limitation, function in reverse to provide carbon skeletons for amino acid biosynthesis (13). In any event, the catalytic properties of the two *P. furiosus* enzymes are very different from those of the AMP-dependent ACSs that are involved in acetate utilization in mesophilic archaea and bacteria. An understanding of the phylogenetic relationship between these two enzyme types will have to await the complete sequences of the hyperthermophilic enzymes, and such studies are under way.

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