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Characterization of Two Members among the Five ADP-Forming Acyl Coenzyme A (Acyl-CoA) Synthetases Reveals the Presence of a 2-(Imidazol-4-yl)Acetyl-CoA Synthetase in *Thermococcus kodakarensis*

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The genome of *Thermococcus kodakarensis*, along with those of most *Thermococcus* and *Pyrococcus* species, harbors five paralogous genes encoding putative α subunits of nucleoside diphosphate (NDP)-forming acyl coenzyme A (acyl-CoA) synthetases. The substrate specificities of the protein products for three of these paralogs have been clarified through studies on the individual enzymes from *Pyrococcus furiosus* and *T. kodakarensis*. Here we have examined the biochemical properties of the remaining two acyl-CoA synthetase proteins from *T. kodakarensis*. The TK0944 and TK2127 genes encoding the two α subunits were each coexpressed with the β subunit-encoding TK0943 gene. In both cases, soluble proteins with an $\alpha_2\beta_2$ structure were obtained and their activities toward various acids in the ADP-forming reaction were examined. The purified TK0944/TK0943 protein (ACS III_{Tk}) accommodated a broad range of acids that corresponded to those generated in the oxidative metabolism of Ala, Val, Leu, Ile, Met, Phe, and Cys. In contrast, the TK2127/TK0943 protein exhibited relevant levels of activity only toward 2-(imidazol-4-yl)acetate, a metabolite of His degradation, and was thus designated 2-(imidazol-4-yl)acetyl-CoA synthetase (ICS_{Tk}), a novel enzyme. Kinetic analyses were performed on both proteins with their respective substrates. In *T. kodakarensis*, we found that the addition of histidine to the medium led to increases in intracellular ADP-forming 2-(imidazol-4-yl)acetyl-CoA synthetase activity, and 2-(imidazol-4-yl)acetate was detected in the culture medium, suggesting that ICS_{Tk} participates in histidine catabolism. The results presented here, together with those of previous studies, have clarified the substrate specificities of all five known NDP-forming acyl-CoA synthetase proteins in the *Thermococcales*.

Hyperthermophilic archaea that belong to the genera *Thermococcus* and *Pyrococcus* are capable of utilizing peptides/amino acids for cell growth (1). The breakdown of amino acids in these organisms is considered to be composed of three steps: (i) a deamination reaction forming 2-oxo acids, (ii) coenzyme A (CoA)-dependent oxidative decarboxylation leading to CoA-thioester compounds, and (iii) hydrolysis of the thioester bond releasing a carboxylic acid and CoA accompanied by substrate-level phosphorylation. Step i is catalyzed by either glutamate dehydrogenase (GDH) in the case of Glu or multiple aminotransferases coupled with GDH for other amino acids (2). Step ii is catalyzed by various ferredoxin-dependent oxidoreductases. A number of enzymes with distinct substrate specificities, including pyruvate:ferredoxin oxidoreductase (POR) (3, 4), 2-oxoisovalerate:ferredoxin oxidoreductase (VOR) (5), indolepyruvate:ferredoxin oxidoreductase (IOR) (6), and 2-oxoglutarate:ferredoxin oxidoreductase (KGOR) (7), have been identified in *Pyrococcus furiosus*, and closely related genes are also present in other members of the *Thermococcales*, including *T. kodakarensis* (8). The final step (step iii) is made possible by various nucleoside diphosphate (NDP)-forming acyl-CoA synthetases. These enzymes catalyze the following reversible reaction: acyl-CoA + NDP + Pi ⇄ acid + CoA + NTP.

The archaeal NDP-forming acyl-CoA synthetases were first identified in *P. furiosus* and were designated ADP-forming acetyl-CoA synthetase I (ACS I_{Pf}) and ACS II_{Pf} (9–15). The enzymes exhibit activity not only toward acetyl-CoA but also toward branched-chain acyl-CoAs (ACS I_{Pf}/ACS II_{Pf}) and aryl-CoAs (ACS II_{Pf}) (12). ADP-forming ACSs have also been identified in *Archaeoglobus fulgidus* (16, 17), *Methanocaldococcus jannaschii* (17), *Pyrobaculum aerophilum* (18), *Thermococcus kodakarensis* (ACS II_{Tk}) (19), and the halophilic *Haloarcula marismortui* (18). The enzymes display a common structure and are comprised of two α and two β subunits ($\alpha_2\beta_2$) or are homodimers of α-β fusion proteins. They have been proposed to be members of the nucleoside diphosphate (NDP)-forming acyl-CoA synthetase superfamily (20).

Almost all genomes of *Thermococcales* members harbor five α subunit and two β subunit paralogs. It has been suggested that the two β subunits can assemble in *T. kodakarensis* with multiple α subunits and that the α subunits are responsible for the substrate specificity of the enzymes in terms of acid/acyl-CoA compounds (19). As described below, the α subunits in *Thermococcales* can be phylogenetically divided into five groups (Fig. 1). One group is represented by ACS I_{Pf} (α subunit; PF1540), and another group includes ACS II_{Pf} (α subunit; PF0532) and ACS II_{Tk} (α subunit; TK0139), while a third group includes the α subunit of an ADP-forming acyl-CoA synthetase specific to succinate/succinyl-CoA

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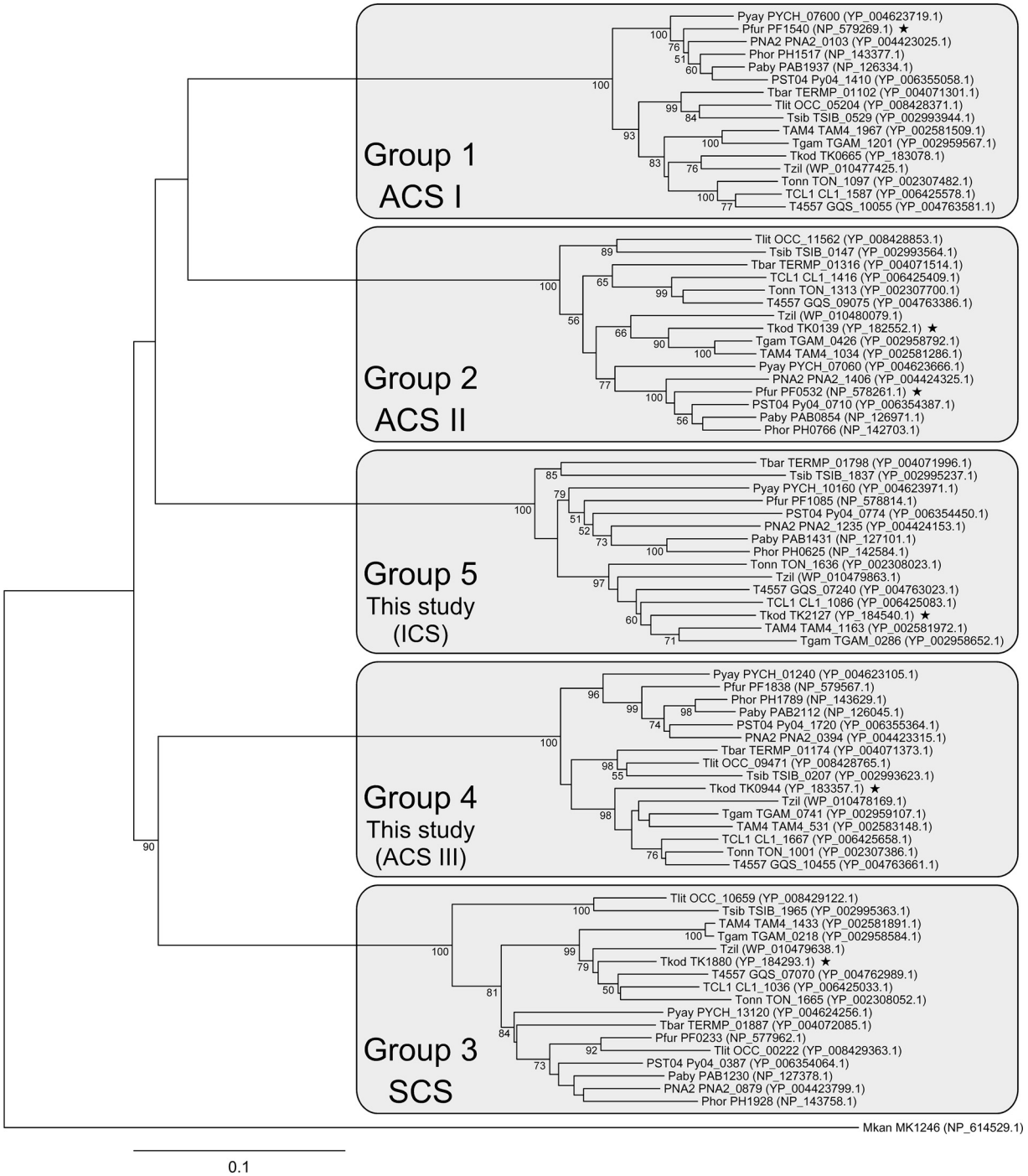

FIG 1 Phylogenetic analysis of NDP-forming acyl-CoA synthetase α subunits of the *Thermococcales*. The five NDP-forming acyl-CoA synthetase α subunit homologs present on the genomes of various members of the *Thermococcales* were aligned, and a phylogenetic tree was constructed with the neighbor-joining method. Species are *Thermococcus kodakarensis* (Tkod), *Thermococcus barophilus* (Tbar), *Thermococcus gammatolerans* (Tgam), *Thermococcus litoralis* (Tlit), "*Thermococcus onnurineus*" (Tonn), *Thermococcus sibiricus* (Tsib), *Thermococcus zilligii* (Tzil), *Thermococcus* sp. AM4 (TAM4), *Thermococcus* sp. CL1 (TCL1), *Thermococcus* sp. 4557 (T4557), *Pyrococcus furiosus* (Pfur), "*Pyrococcus abyssi*" (Paby), *Pyrococcus horikoshii* (Phor), *Pyrococcus yayanosii* (Pyay), *Pyrococcus* sp. NA2 (PNA2), and *Pyrococcus* sp. ST04 (PST04). Both locus tags and accession numbers are shown except for the proteins from *T. zilligii*, which are identified only with accession numbers. An NDP-forming acyl-CoA synthetase α subunit homolog from *Methanopyrus kandlerii* (Mkan, MK1246) was used as the outgroup. Bootstrap values above 50 are shown. Stars indicate proteins that have been examined experimentally.

(succinyl-CoA synthetase [SCS_{Tk}]) (α subunit; TK1880) from *T. kodakarensis* (19). The enzyme has recently been shown to be involved in Glu catabolism and succinate generation (2).

In this study, we examined the biochemical properties of the NDP-forming acyl-CoA synthetases from *T. kodakarensis* whose α subunits are composed of TK0944 and TK2127, with TK0943 as the β subunit. These proteins are included in the two remaining groups of ACS α subunits, whose members have not yet been biochemically characterized. While TK0944/TK0943 recognized a relatively broad range of substrates, we found that TK2127/TK0943 displayed strict substrate specificity, leading us to conclude that the enzyme is an ADP-forming 2-(imidazol-4-yl) acetyl-CoA synthetase.

MATERIALS AND METHODS

Phylogenetic tree analysis. Sequences of the ACS α subunits from all members of the *Thermococcales* available from NCBI were collected and aligned using the ClustalW program provided by the DNA Databank of Japan (DDBJ). Multiple sequence alignment was performed with the following default parameters: Protein Weight Matrix, Gonnet; GAP OPEN, 10; GAP EXTENSION, 0.20; GAP DISTANCES, 5; NO END GAPS, no; ITERATION, none; NUMITER, 1; and CLUSTERING, NJ. The phylogenetic tree was constructed by the neighbor-joining method (21). Bootstrap resampling was performed 1,000 times.

Microorganisms, plasmids, and media. *Thermococcus kodakarensis* KOD1 (22, 23) cells were grown under anaerobic conditions at 85°C in a nutrient-rich growth medium (ASW-YT-S^0 medium). ASW-YT-S^0 medium contains 5.0 g $liter^{-1}$ yeast extract, 5.0 g $liter^{-1}$ tryptone, 16 g $liter^{-1}$ NaCl, 2.4 g $liter^{-1}$ $MgCl_2 \cdot 6H_2O$, 4.8 g $liter^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.8 g $liter^{-1}$ $(NH_4)_2SO_4$, 0.16 g $liter^{-1}$ $NaHCO_3$, 0.24 g $liter^{-1}$ $CaCl_2 \cdot 2H_2O$, 0.4 g $liter^{-1}$ KCl, 0.336 g $liter^{-1}$ KH_2PO_4, 40 mg $liter^{-1}$ NaBr, 16 mg $liter^{-1}$ $SrCl_2 \cdot 6H_2O$, 8 mg $liter^{-1}$ ferric ammonium citrate, with 2.0 g $liter^{-1}$ elemental sulfur (S^0). The medium was supplemented with 0.1 g $liter^{-1}$ Na_2S and 0.25 mg $liter^{-1}$ resazurin. *Escherichia coli* DH5α and plasmid pUC118 were used for general DNA manipulation and sequencing. *E. coli* BL21-CodonPlus(DE3)-RIL (Stratagene, La Jolla, CA) and pET21a(+) (Novagen, Madison, WI) were used for gene expression. *E. coli* strains were cultivated in Luria-Bertani (LB) medium (10 g $liter^{-1}$ tryptone, 5 g $liter^{-1}$ yeast extract, 10 g $liter^{-1}$ NaCl, pH 7.0) at 37°C. Ampicillin was added to the medium at a concentration of 100 μg ml^{-1}. Unless mentioned otherwise, all chemicals were purchased from Wako Pure Chemicals (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan).

DNA manipulation and sequencing. KOD Plus polymerase (Toyobo, Osaka, Japan) was used for PCR. Restriction and modification enzymes were purchased from Toyobo. Plasmid DNA was isolated with a Qiagen Plasmid Mini Kit (Qiagen, Hilden, Germany) or a Quantum Prep Mini Kit (Bio-Rad, Hercules, CA). DNA fragments were recovered from agarose gels with a Wizard SV gel and PCR clean-up system (Promega, Madison, WI). DNA sequencing was performed with a BigDye Terminator cycle sequencing kit (v.3.1) and a model 3100 capillary DNA sequencer (Applied Biosystems, Foster City, CA).

Cloning and expression of genes TK0944 and TK2127 with gene TK0943 in *E. coli*. The TK0943, TK0944, and TK2127 genes were amplified from *T. kodakarensis* genomic DNA with the following primers: for TK0943, F0943 (5′-CCCTCTAGAATTCAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGAGCGCCAAAGAAGAGGCCC-3′) and R0943 (5′-CTTGTCGACTCACTCTTTCTTTTCTGGAGCTTTC-3′) (underlined sequences indicate EcoRI and SalI sites); for TK0944, F0944 (5′-CCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGTCAGAGAAAATCGTCGAAGAACTC-3′) and R0944 (5′-TTAGAATTCGGAACCTCATTCCCCCTTAACCTTGCGG-3′) (underlined sequences indicate XbaI and EcoRI sites); and for TK2127, F2127 (5′-CCTTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGAGCCTAGACTTCTTTTTCTACCCG-3′) and R2127 (5′-TTCGAATTCGGAACCCTATCTCATCGAGAGCCACTTC-3′) (underlined sequences indicate XbaI and EcoRI sites). The amplified fragments were individually inserted into pUC118 and sequenced. After confirming the absence of unintended mutations, the EcoRI-SalI-digested fragment of TK0943 was inserted into pET21a(+), resulting in plasmid pET-TK0943. The XbaI-EcoRI-digested fragments of TK0944 and TK2127 were inserted into pET-TK0943 upstream of TK0943, resulting in plasmids pET-TK0944/0943 and pET-TK2127/0943, respectively. In these plasmids, besides the ribosomal binding site upstream of TK0944 and TK2127, another ribosomal binding site is inserted upstream of TK0943. After sequence confirmation, the plasmids were introduced into *E. coli* BL21-CodonPlus(DE3)-RIL and grown in LB medium at 37°C to an optical density (at 660 nm) of 0.5. Gene expression was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside at the mid-exponential-growth phase followed by further incubation for 4 h. Cells were harvested by centrifugation (6,000 × *g*, 4°C, 15 min), washed with 50 mM Tris-HCl buffer (pH 7.5), and centrifuged again (4,500 × *g*, 4°C, 20 min).

Purification of recombinant TK0944/TK0943 and TK2127/TK0943 proteins. The collected cells were resuspended in 50 mM Tris-HCl buffer (pH 7.5) and sonicated for 10 min (output:pause = 0.5:0.5 s; output power = ~60 W) with a UD-201 Ultrasonic Disruptor (Tomy, Tokyo, Japan). The lysates were centrifuged (15,000 × *g*, 4°C, 15 min), and the respective supernatants were subjected to heat treatment (15 min at 85°C). After removing heat-labile proteins by centrifugation (15,000 × *g*, 4°C, 30 min), the supernatants were applied to a Resource Q anion exchange column (GE Healthcare, Little Chalfont, United Kingdom) (6 ml) equilibrated with buffer A (50 mM Tris-HCl buffer, pH 7.5). Recombinant proteins were eluted with a linear gradient of 0 to 1 M NaCl in buffer A. Fractions containing recombinant TK0944/TK0943 or TK2127/TK0943 were concentrated and applied to a Superdex 200 HR 10/30 gel filtration column (GE Healthcare) with a mobile phase of 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl at a flow rate of 0.5 ml min^{-1}. Protein concentrations were determined with a protein assay kit (Bio-Rad) with bovine serum albumin as a standard. The approximate sizes and molecular weights of the proteins were examined by gel filtration with a Superdex 200 HR 10/30 column using the standard Blue Dextran 2000, a gel filtration HMW calibration kit, and a gel filtration LMW calibration kit (GE Healthcare).

Activity measurements of recombinant TK0944/TK0943 and TK2127/TK0943 proteins. For kinetic analyses and the examination of activity toward various acid compounds, ADP formation was coupled with pyruvate kinase and lactate dehydrogenase (PK/LDH), and the oxidation of NADH was monitored spectrophotometrically at 340 nm. Reactions were performed at 55°C. The reaction mixture contained the acid substrate (10 mM), ATP (1 mM), CoA (1.5 mM), $MgCl_2$ (5 mM), phosphoenolpyruvate (1.5 mM), NADH (0.15 mM), recombinant protein (2 μg ml^{-1}), and pyruvate kinase/lactate dehydrogenase from rabbit muscle (Sigma, St. Louis, MO) (PK/LDH, 20 U/14 U) in 50 mM MES (2-morpholineethanesulfonic acid)-NaOH buffer (pH 6.5). The equation $v = V_{max}\{[S]/K_{s1} + [S]^2/(K_{s1}K_{s2})\}/\{1 + 2[S]/K_{s1} + [S]^2/(K_{s1}K_{s2})\}$ was used to calculate the kinetic parameters with respect to acid substrates, where v is the reaction velocity, V_{max} is the maximum velocity, [S] is the substrate concentration, and K_{s1} and K_{s2} are the dissociation constants of the first and second acid substrates, respectively. When measuring activity directed at CoA thioester cleavage, the formation of free CoASH was determined using 5,5′-dithiobis(2-nitrobenzoic acid) with methods described in a previous study (19). The reaction mixture contained 0.5 mM acetyl-CoA (Sigma), isovaleryl-CoA (Sigma), or isobutyryl-CoA (Sigma) or 1 mM succinyl-CoA (Sigma), 0.4 mM ADP (Oriental Yeast, Tokyo, Japan), 20 mM sodium phosphate, 0.1 mM 5,5′-dithiobis(2-nitrobenzoic acid), 10 mM $MgCl_2$, and recombinant protein (2 μg ml^{-1}) in 50 mM MES-NaOH (pH 6.5). Reactions were performed at 55°C, and the increase in absorbance at 412 nm was monitored continuously.

Effects of exogenous histidine on 2-(imidazol-4-yl)acetyl-CoA synthetase activity in *T. kodakarensis* cells. 2-(Imidazol-4-yl)acetyl-CoA synthetase activity was measured in the extracts of cells grown in the

absence or presence (0.95, 2.39, or 4.77 mM) of exogenous histidine in ASW-YT-S^0 medium. Activity was measured according to the methods described above using 34.5 μg of cell extract instead of the recombinant protein. Background levels observed in the absence of 2-(imidazol-4-yl)acetate were subtracted to calculate activity levels.

Detection of 2-(imidazol-4-yl)acetate in the growth medium. *T. kodakarensis* KOD1 cells were cultivated in ASW-YT-Pyr medium (2) supplemented with 0.2 g $liter^{-1}$ of His at 85°C. After 10 h, aliquots of the culture were taken, cooled on ice, and centrifuged (20,000 × *g*, 4°C, 5 min). The supernatant was filtered with USY-1 disposable ultrafilter units (Advantec, Tokyo, Japan). A 10-μl volume of aliquot was applied to a VP-ODS Shim-pack (Shimadzu, Kyoto, Japan) (150 mm by 4.6 mm) at 40°C. The mobile phase was a gradient of acetonitrile in 50 mM sodium phosphate buffer (pH 2.3) containing 5 mM sodium hexanesulfonate at a flow rate of 0.8 ml min^{-1}. 2-(Imidazol-4-yl)acetate was detected with a SPD-M20A photodiode array detector (Shimadzu) with a wavelength of 230 nm.

RESULTS

Phylogenetic examination of the α subunits of NDP-forming acyl-CoA synthetase in the *Thermococcales*. As mentioned above, almost all *Thermococcales* members harbor five ACS α subunit paralogs on their genomes, along with two β subunit paralogs. A phylogenetic examination was performed on the ACS α subunits from all members of the *Thermococcales* whose genome sequences were available (Fig. 1). The analysis revealed that the proteins clustered into five groups, which we designated groups 1 to 5. The structural similarities among members of a single group are high. In group 1, the protein most distantly related to TK0665 is the protein from *Pyrococcus* sp. NA2, which is 84.7% identical to the TK0665 protein. On the other hand, the protein most closely related to TK0665 from the other groups is the PAB1230 protein from "*Pyrococcus abyssi*," which is only 45.2% identical. Likewise, the proteins most distantly related to TK0139 (group 2), TK1880 (group 3), TK0944 (group 4), and TK2127 (group 5) but belonging to the same group displayed identities of 77.9%, 76.1%, 80.2%, and 77.5%, respectively. The proteins from other groups that were most closely related to these proteins displayed 47.4%, 50.7%, 50.8%, and 47.0% identity, respectively. This suggests that each α subunit from one organism corresponds to a single α subunit in most of the other organisms. This raises the possibility that members of a particular group share similar substrate specificities with one another. Supporting this, it has been shown that ACS II_{Pf} (α: PF0532) and ACS II_{Tk} (α: TK0139), both with α subunit members of group 2, display similar substrate preferences (12, 19).

Expression of the TK0944/TK0943 and TK2127/TK0943 genes in *E. coli*. Among the five groups of NDP-forming acyl-CoA synthetase α subunits of the *Thermococcales* (Fig. 1), at least one member of groups 1 to 3 has been biochemically examined with enzymes from either *P. furiosus* or *T. kodakarensis* (2, 9–15, 19). In this study, we set out to determine the biochemical properties of enzymes that are composed of the group 4 and group 5 α subunits from *T. kodakarensis*. It has previously been shown that β subunits can assemble with multiple α subunits *in vivo*. Furthermore, the recombinant TK0943 protein (β subunit) assembles with the TK1880 protein (α subunit) to form an active and thermostable succinyl-CoA synthetase, as well as with the TK0139 protein (α subunit), resulting in an active acetyl-CoA synthetase II *in vitro* (19). The results indicated that the substrate specificities of these enzymes are dependent on the α subunit, most likely through substrate recognition and binding by domain 5 of the α subunit (19). Here we expressed the TK0944 or TK2127 gene with the TK0943 gene in *E. coli*. Recombinant proteins with the expected molecular masses (for TK0944, 51.9 kDa; for TK2127, 47.6 kDa; and for TK0943, 27.1 kDa) were recovered in a soluble form, and the cell extracts were subjected to heat treatment, anion exchange, and gel filtration chromatography. SDS-PAGE analysis showed that the recombinant proteins were purified to apparent homogeneity (Fig. 2). The two proteins, although to different extents, displayed acyl-CoA synthetase activity with acetate as the acid substrate.

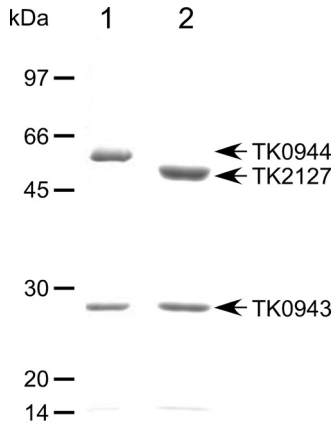

FIG 2 SDS-PAGE analysis of the purified, recombinant TK0944/TK0943 and TK2127/TK0943 proteins. A 3-μg volume of TK0944/TK0943 (lane 1) and TK2127/TK0943 (lane 2) was applied, and the gels were stained with Coomassie brilliant blue.

Assembly of the TK0944/TK0943 and TK2127/TK0943 proteins. Gel-filtration chromatography and SDS-PAGE revealed that the TK0944 or TK2127 protein coeluted with the TK0943 protein, indicating that each α subunit assembled with the TK0943 β subunit. Estimated molecular weights were consistent with an $\alpha_2\beta_2$ subunit assembly and were the same as those observed for previously characterized NDP-forming acyl-CoA synthetases of the *Thermococcales*. In order to examine the stability of these complexes, we measured the thermostability of TK0944/TK0943 and TK2127/TK0943. The half-lives at 80 and 90°C were 380 and 170 min for TK0944/TK0943 and 140 and 120 min for TK2127/TK0943, respectively. The results of gel-filtration chromatography and the high thermostability of the two proteins indicated that the recombinant TK0944/TK0943 and TK2127/TK0943 proteins were properly assembled and thus were subjected to further enzymatic analyses.

Activity toward various acid substrates. ADP-forming acyl-CoA synthetase activities of the TK0944/TK0943 and TK2127/TK0943 proteins were examined in the acid-utilizing direction with 18 different acid compounds, corresponding to metabolites that are expected to be formed via oxidative degradation of amino acids in the *Thermococcales*. Each acid substrate was present at a concentration of 10 mM, and ATP was used as the nucleoside 5′-triphosphate. As shown in Fig. 3, the TK0944/TK0943 protein displayed significant levels of acetyl-CoA synthetase activity that were higher than those seen for ACS II_{Tk}. Activity was also observed toward a broad range of other substrates, with a preference for those derived from the oxidation of hydrophobic amino acids. As these properties are similar to those observed for ACS II_{Tk} (19) and ACS II_{Pf} (11, 12), we gave the TK0944/TK0943 enzyme the

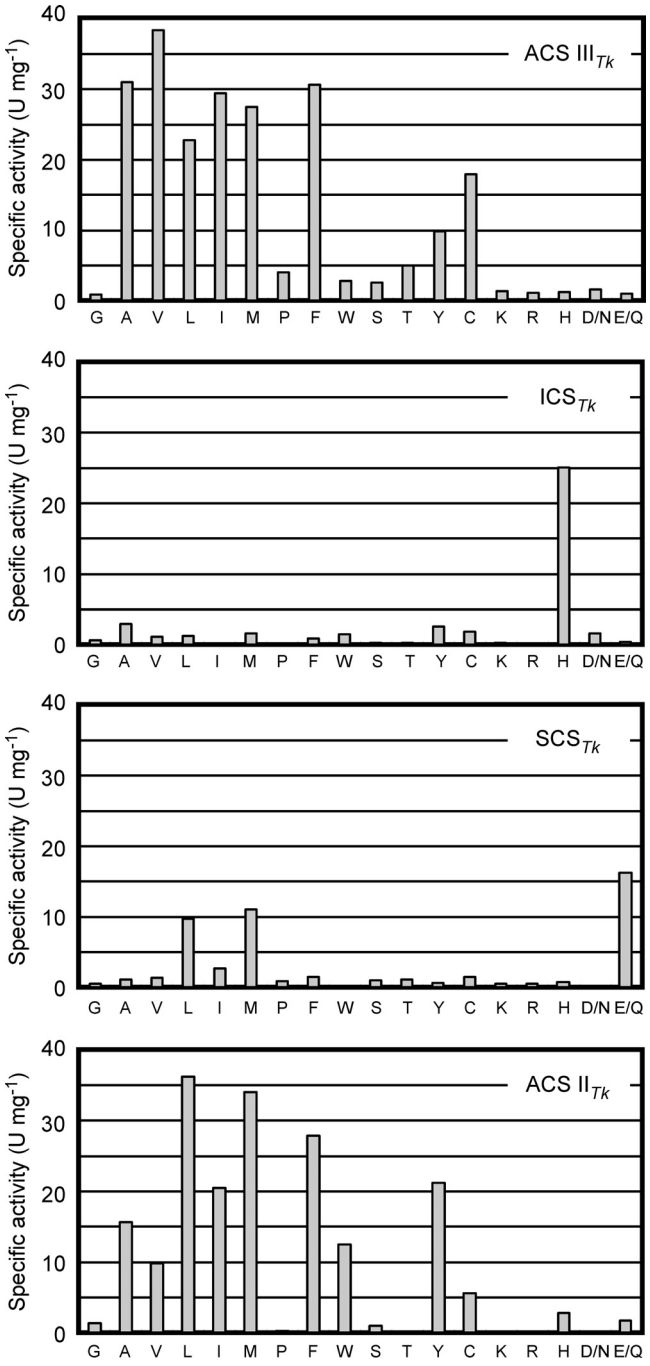

FIG 3 Activity levels of TK0944/TK0943 and TK2127/TK0943 proteins for various acid compounds. Amino acids, represented by single-letter codes, and the corresponding acid compounds are as follows: G, formate; A, acetate; V, isobutyrate; L, isovalerate; I, 2-methylbutyrate; M, 3-methylthiopropionate; P, 4-aminobutyrate; F, phenylacetate; W, 2-(indol-3-yl)acetate; S, glycolate; T, lactate; Y, 2-(4-hydroxyphenyl)acetate; C, thioglycolate; K, 5-aminovalerate; R, 4-guanidinobutyrate; H, 2-(imidazol-4-yl)acetate; D/N, malonate; and E/Q, succinate. Activities of TK0944/TK0943 (top [first] panel) and TK2127/TK0943 (second panel) proteins are shown, along with previously reported results for SCS$_{Tk}$ (third panel) and ACS II$_{Tk}$ (bottom panel) (19).

designation ACS III$_{Tk}$. ACS III$_{Tk}$ displayed activities with isobutyrate and thioglycolate, products of Val and Cys degradation, respectively, that were higher than those seen with ACS II$_{Tk}$. In contrast to the broad specificity of ACS III$_{Tk}$, the TK2127/TK0943 protein exhibited a strict preference for 2-(imidazol-4-yl)acetate, the metabolite expected in the degradation of histidine (Fig. 3). The enzyme was therefore designated 2-(imidazol-4-yl)acetyl-CoA synthetase (ICS$_{Tk}$). To our knowledge, this is the first report identifying an enzyme with this activity.

Enzyme activities were also examined in the ATP-forming (acid-generating) direction with several commercially available substrates at a concentration of 0.5 mM. In the case of ACS III$_{Tk}$, ATP-forming activity was observed with acetyl-CoA, isobutyryl-CoA, and isovaleryl-CoA but not with succinyl-CoA (see Table S1 in the supplemental material). In the case of ICS$_{Tk}$, clear levels of activity could not be detected, but low levels of activity were observed with acetyl-CoA at a concentration of 1 mM. The substrate specificities are in good agreement with the results obtained for these enzymes in the ADP-forming direction.

Kinetic analyses of ACS III$_{Tk}$ and ICS$_{Tk}$. As ACS III$_{Tk}$ displayed significant levels of activity toward several acid substrates, we performed kinetic analyses of the reactions with isobutyrate, acetate, thioglycolate, and phenylacetate at substrate concentrations of 0.2 to 20 mM. Kinetic parameters for CoA and ATP were also determined (Table 1). When various CoA (12 μM to 1,600 μM) and ATP (20 μM to 1,000 μM) concentrations were employed in the presence of 10 mM isobutyrate, the reactions followed Michaelis-Menten kinetics. The K_m values of CoA and ATP were 5.6 ± 0.4 and 56 ± 3 μM, respectively. As in the case of SCS$_{Tk}$ (19), the ACS III$_{Tk}$ reaction did not follow Michaelis-Menten kinetics when the concentration of acid substrates was adjusted. The data fit well with the assumption that the K_s value of the second acid substrate to the dimeric $(\alpha\beta)_2$ catalytic unit (K_{s2}) differs from that of the first (K_{s1}) by a factor of a ($= K_{s2}/K_{s1}$). The parameters are shown in Table 1. Among the four acid substrates, acetate displayed a relatively high K_{s1} value and low k_{cat}/K_{s1}, indicating that acetate is not the preferred substrate of ACS III$_{Tk}$. However, the K_{s1} value (1.5 mM) is not high compared to those of ACS I$_{Pf}$ (1.1 mM) and ACS II$_{Pf}$ (10.7 mM) toward acetate (11, 12) and thus does not necessarily rule out the involvement of ACS III$_{Tk}$ in the conversion of acetyl-CoA to acetate. Kinetic analyses were also performed on ICS$_{Tk}$ with CoA, ATP, and the only acid substrate with relevant levels of activity, 2-(imidazol-4-yl)acetate (Table 1). The K_m value toward CoA (62.5 μM) was over 10-fold higher than that observed for ACS III$_{Tk}$ but was approximately equivalent to those observed for SCS$_{Tk}$ (60 μM) and ACS II$_{Pf}$ (74 μM). The k_{cat}/K_{s1} value with 2-(imidazol-4-yl)acetate (69.7 s^{-1} mM^{-1}) was higher than that observed for SCS$_{Tk}$ with succinate (10 s^{-1} mM^{-1}) (19), indicating that its catalytic efficiency is comparable to those of previously reported ACS enzymes.

Detection of ICS activity in *T. kodakarensis* cells. It is generally accepted that the NDP-forming acyl-CoA synthetases of the *Thermococcales* play a catabolic role in amino acid metabolism. As ACS II$_{Tk}$, ACS III$_{Tk}$, and SCS$_{Tk}$ do not exhibit relevant levels of acyl-CoA synthetase activity toward 2-(imidazol-4-yl)acetate, we anticipated that it would be possible to examine the intracellular levels of ICS$_{Tk}$ by measuring ICS activity. This would not be possible with the individual ACS enzymes, as ACS activity would be a sum of the activities of ACS I to III$_{Tk}$. Although we have not examined the substrate preference of ACS I$_{Tk}$, we assumed it unlikely that the enzyme displays ICS activity, considering the properties of ACS I$_{Pf}$ (11, 12). *T. kodakarensis* cells were grown in ASW-YT-S^0 medium in the absence or presence (0.95, 2.39, or 4.77 mM) of exogenous histidine. While activity levels were 0.020 μmol min^{-1}

TABLE 1 Kinetic parameters of ACS III_{Tk} and ICS_{Tk}[a]

Substrate	Kinetic model	V_{max} (U mg^{-1}) ± SD	k_{cat} (s^{-1} active $site^{-1}$) ± SD	K_{s1} or K_m (mM) ± SD	K_{s2} (mM) ± SD	k_{cat}/K_{s1} or k_{cat}/K_m (s^{-1} active $site^{-1}$ mM^{-1})
ACS III_{Tk} (TK0944/TK0943)						
Isobutyrate	Adair-Pauling	38.7 ± 0.2	50.9 ± 0.3	0.132 ± 0.054	0.0860 ± 0.0156	385
Acetate	Adair-Pauling	37.4 ± 0.6	49.3 ± 0.7	1.50 ± 0.07	2.06 ± 0.21	32.8
Thioglycolate	Adair-Pauling	19.8 ± 0.3	26.1 ± 0.4	0.322 ± 0.038	1.12 ± 0.14	81.0
Phenylacetate	Adair-Pauling	30.8 ± 0.2	40.6 ± 0.2	0.329 ± 0.094	0.0706 ± 0.0140	123
CoA	Michaelis-Menten	40.4 ± 0.2	53.1 ± 0.3	0.00560 ± 0.00040		9,490
ATP	Michaelis-Menten	40.8 ± 0.4	53.7 ± 0.5	0.0556 ± 0.0026		965
ICS_{Tk} (TK2127/TK0943)						
2-(Imidazol-4-yl)-acetate	Adair-Pauling	30.3 ± 0.5	37.8 ± 0.7	0.543 ± 0.036	2.12 ± 0.26	69.7
CoA	Michaelis-Menten	31.7 ± 0.2	39.6 ± 0.3	0.0625 ± 0.0025		633
ATP	Michaelis-Menten	30.4 ± 1.0	37.9 ± 1.2	0.0300 ± 0.0011		1,260

[a] Kinetic analyses on acid substrates were performed in the presence of 1 mM ATP and 1.5 mM CoA. Kinetic analyses on CoA were performed in the presence of 10 mM isobutyrate (ACS III_{Tk}) or 2-(imidazol-4-yl)acetate (ICS_{Tk}) and 1 mM ATP. Kinetic analyses on ATP were performed in the presence of 10 mM isobutyrate (ACS III_{Tk}) or 2-(imidazol-4-yl)acetate (ICS_{Tk}) and 1.5 mM CoA.

(U) mg $extract^{-1}$ in the absence of histidine, levels of 0.045 to 0.053 μmol min^{-1} mg $extract^{-1}$ were observed with the addition of exogenous histidine. The levels of acetyl-CoA synthetase activity did not change under these conditions, with 0.169 μmol min^{-1} mg $extract^{-1}$ in the absence of histidine and 0.150 to 0.171 μmol min^{-1} mg $extract^{-1}$ in the presence of histidine, supporting the assumption that the ICS activity was due to the function of ICS_{Tk} and not to the function of ACS I_{Tk}. Furthermore, by examining the culture supernatant after 10 h of growth in ASW-YT-Pyr medium containing 0.95 mM His, we were able to detect the production of 5.3 mg $liter^{-1}$ of 2-(imidazol-4-yl)acetate, the expected end product of His degradation. The results suggest that ICS_{Tk} plays a role in histidine catabolism in *T. kodakarensis*.

DISCUSSION

Taken together with the results from previous studies, the data presented here provide the first overall indications as to what types of NDP-forming acyl-CoA synthetases are present in the *Thermococcales* (summarized in Table 2). We cannot rule out the possi-

TABLE 2 The substrate specificities of the five ADP-forming acyl-CoA synthetases in *Thermococcales*

Enzyme	Abbreviation	Activity detected with:	Involved in the metabolism of:	References or source
Acetyl-CoA synthetase I	ACS I	Acetate	Ala	10, 12, 13
		Isobutyrate	Val	
		Isovalerate[a]	Leu[a]	
Acetyl-CoA synthetase II	ACS II	Acetate	Ala	12, 19
		Isobutyrate	Val	
		Isovalerate	Leu	
		2-Methylbutyrate	Ile	
		3-Methylthiopropionate	Met	
		Phenylacetate	Phe	
		2-(4-Hydroxyphenyl)acetate	Tyr	
		2-(Indol-3-yl)acetate	Trp	
Acetyl-CoA synthetase III	ACS III	Acetate	Ala	This study
		Isobutyrate	Val	
		Isovalerate	Leu	
		2-Methylbutyrate	Ile	
		3-Methylthiopropionate	Met	
		Phenylacetate	Phe	
		Thioglycolate	Cys	
		2-(4-Hydroxyphenyl)acetate[a]	Tyr[a]	
Succinyl-CoA synthetase	SCS	Succinate	Glu	2, 19
		Isovalerate	Leu	
		3-Methylthiopropionate	Met	
2-(Imidazol-4-yl)acetyl-CoA synthetase	ICS	2-(Imidazol-4-yl)acetate	His	This study

[a] Relatively low activity levels.

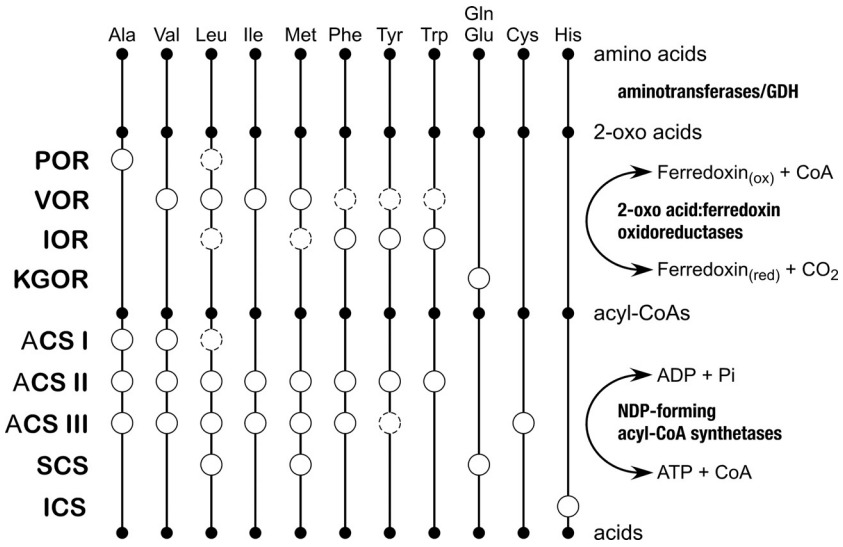

FIG 4 A diagram illustrating the possible involvement of different 2-oxo acid:ferredoxin oxidoreductases and NDP-forming acyl-CoA synthetases of the *Thermococcales* in the catabolism of individual amino acids. Open circles with solid lines indicate that the enzyme has been shown to exhibit relatively high levels of activity toward the corresponding substrate. Open circles with dotted lines indicate the presence of lower degrees of activity.

bility that members of a single group from different organisms exhibit entirely different substrate specificities. However, the striking relatedness among the members within a single group (>76% identical to *T. kodakarensis* homologs), compared to those between members of different groups (<51% identical to *T. kodakarensis* homologs), suggests otherwise (Fig. 1).

Group 1 enzymes, represented by ACS I_{Pf}, exhibit relatively high levels of activity toward acetate, propionate, butyrate, isobutyrate, and, to a lesser extent, isovalerate in the acid-utilizing direction. 2-(Indol-3-yl)acetate, phenylacetate, and succinate could not be utilized. Acid-forming activity has also been observed for acetyl-CoA, propionyl-CoA, butyryl-CoA, and isobutyryl-CoA but not for phenylacetyl-CoA (10, 12). The results suggest that group 1 enzymes are involved in the metabolism of Ala, Val, and possibly Leu. Members of group 2, represented by ACS II_{Pf} and ACS II_{Tk}, accommodate a broader range of substrates, and besides the substrates recognized by ACS I_{Pf}, the enzymes also display activity toward aromatic acids such as phenylacetate, 2-(indol-3-yl)acetate, and 2-(4-hydroxyphenyl)acetate, as well as 2-methylbutyrate and 3-methylthiopropionate (12, 19). This implies the involvement of group 2 enzymes in the metabolism of Ala, Val, Leu, Ile, Met, Phe, Trp, and Tyr. It should be noted that ACS I_{Pf} and ACS II_{Pf} display comparable K_m values for acetyl-CoA and isobutyryl-CoA, making it difficult to estimate which enzyme plays a larger role in the metabolism of Ala and Val. Group 3, represented by SCS_{Tk}, recognizes only a few acid compounds, namely, succinate, 3-methylthiopropionate, and isovalerate, corresponding to acid compounds implicated in Glu/Gln, Met, and Leu metabolism, respectively (19). SCS_{Tk} seems to be the only enzyme involved in succinate formation in *T. kodakarensis*, as formation of succinate could no longer be detected in the disruption strain of the α subunit gene (19). Group 4, represented by ACS III_{Tk}, displays a broad substrate preference similar to that seen with group 2 enzymes. Relatively high levels of activity were also observed for thioglycolate and isobutyrate, suggesting the involvement of this group in the degradation of a wide range of amino acids which may include Cys and Val. ICS_{Tk} of group 5 displays strict substrate specificity, and relevant levels of activity were observed only for 2-(imidazol-4-yl)acetate, strongly suggesting an exclusive role in the metabolism of His. Judging from these results (Table 2), NDP-forming acyl-CoA synthetases are not present for the breakdown of Gly, Asp, Asn, Arg, and Lys, and this may also be the case for Thr, Ser, and Pro.

Four enzymes from the *Thermococcales* that are responsible for the oxidation of 2-oxo acids, POR, IOR, VOR, and KGOR, have been examined. POR from *P. furiosus* has been found to be relatively specific for pyruvate, indicating a role of the enzyme in Ala metabolism (3, 7). Analysis of the IOR from *P. furiosus* indicates that the enzyme is involved in the oxidation of aromatic amino acids Trp, Phe, and Tyr and possibly Met and Leu (6, 7). VOR from various *Pyrococcus* and *Thermococcus* strains displays activity toward 2-oxo acid derivatives of branch-chained Val, Leu, Ile, and Met (5, 7). KGOR from *Thermococcus litoralis* has been shown to be specific to 2-oxoglutarate, indicating its role in the metabolism of Glu/Gln (7). KGOR from *T. kodakarensis* has been genetically shown to be the major 2-oxo acid:ferredoxin oxidoreductase in Glu/Gln metabolism, as succinate generation was no longer observed in its disruption strain (2). As a whole, the amino acids for which a 2-oxo acid:ferredoxin oxidoreductase is present correspond well to those suggested to be metabolized by the five groups of NDP-forming acyl-CoA synthetases. Enzymes catalyzing both steps are clearly present for Ala, Val, Leu, Ile, Met, Phe, Tyr, Trp, and Glu/Gln. In contrast, it is most likely that Gly, Arg, Lys, Pro, Ser, Thr, and Asp/Asn are not degraded through these pathways, suggesting either that other catabolic mechanisms for these amino acids are present or that these amino acids are utilized for anabolic purposes. As we have identified an acyl-CoA synthetase apparently specific for His degradation and, moreover, detected 2-(imidazol-4-yl)acetate in the culture medium, it is most likely that a 2-oxo acid:ferredoxin oxidoreductase(s) responsible for the degradation of His is present. The enzyme may well be one of the four 2-oxo acid:ferredoxin oxi-

doreductases described above, as activity of these enzymes toward the corresponding substrate 3-(imidazol-4-yl)pyruvate has not been examined. It remains to be determined whether Cys is also degraded by the 2-oxo acid:ferredoxin oxidoreductases, followed by ACS III. Based on our present knowledge, we can now summarize which enzymes among the four 2-oxo acid:ferredoxin oxidoreductases and the five NDP-forming acyl-CoA synthetases are responsible for the degradation of individual amino acids in the *Thermococcales* (Fig. 4).

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