A Novel ADP-forming Succinyl-CoA Synthetase in *Thermococcus kodakaraensis* Structurally Related to the Archaeal Nucleoside Diphosphate-forming Acetyl-CoA Synthetases*

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We have identified and characterized a structurally novel succinyl-CoA synthetase (SCS) from the hyperthermophilic archaea *Thermococcus kodakaraensis*. The presence of an SCS completes the metabolic pathway from glutamate to succinate in Thermococcales, which had not been clarified because of the absence of classical SCS homologs on their genomes. The SCS from *T. kodakaraensis* (SCS$_{Tk}$) is a heteromeric enzyme ($\alpha_3\beta_2$) encoded by TK1880 ($\alpha$-subunit) and TK0943 ($\beta$-subunit). Although both SCS$_{Tk}$ and classical SCSs harbor the five domains present in enzymes of the acyl-CoA synthetase (nucleoside diphosphate-forming) superfamily, the domain order and distribution among subunits in SCS$_{Tk}$ ($\alpha$-subunit, domains 1-2-5; $\beta$-subunit, domains 3-4) are distinct from those of classical SCSs ($\alpha$-subunit, domains 1-2; $\beta$-subunit, domains 3-4-5) and instead resemble the acetyl-CoA synthetases from *Pyrococcus furiosus* (SCSs I$_{Pf}$ and II$_{Pf}$). Comparison of the four Thermococcales genomes revealed that each strain harbors five $\alpha$- and two $\beta$-subunit homologs. Sequence similarity suggests that the $\beta$-subunit of SCS$_{Tk}$ is also a component of the presumed ACS II from *T. kodakaraensis* (ACS II$_{Tk}$). We coexpressed the $\alpha$/$\beta$-genes of SCS$_{Tk}$ (TK1880/TK0943) and of ACS II$_{Tk}$ (TK0139/TK0943). ACS II$_{Tk}$ recognizes a broad range of hydrophobic/aromatic acid compounds, as is the case with ACS II$_{Pf}$ whereas SCS$_{Tk}$ displays a distinct and relatively strict substrate specificity for several acids, including succinate. This indicates that the $\alpha$-subunits are responsible for the distinct substrate specificities of SCS$_{Tk}$ and ACS II$_{Tk}$.

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Thermococcus and Pyrococcus species are hyperthermophilic archaea that preferentially utilize peptides/amino acids for cell growth (1). It is presumed that amino acids are first deaminated to 2-oxo acids by aminotransferases, with 2-oxoglutarate as a key amino acceptor (2). The generated glutamate is converted back to 2-oxoglutarate by oxidative deamination catalyzed by glutamate dehydrogenase (3). The 2-oxo acids are then converted to CoA thioester compounds by oxidative decarboxylation. Ferredoxin-dependent oxidoreductases catalyze these reactions, and a number of enzymes with distinct substrate specificities have been identified in *Pyrococcus furiosus* (4–7). Closely related genes are also found on the genomes of *Thermococcus kodakaraensis* (8), *Pyrococcus abyssi* (9), and *Pyrococcus horikoshii* (10). The final step is the hydrolysis of the thioester bond, releasing a carboxylic acid and CoA accompanied by substrate level phosphorylation, an important energy conservation reaction in these hyperthermophiles. The ADP-forming acetyl-CoA synthetases I and II from *P. furiosus* (ACSs I$_{Pf}$ and II$_{Pf}$) have been identified to be involved in this step (11–14) and exhibit activity not only for acetyl-CoA but also for branched-chain acyl-CoAs (ACSs I$_{Pf}$ and II$_{Pf}$) and aryl-CoAs (ACS II$_{Pf}$) (13). The substrate specificities of the two enzymes are consistent with the preferential consumption of Leu, Ile, and Phe by *P. furiosus* during growth on amino acids (15). The same tendencies are also observed in *T. kodakaraensis* (16). However, although to a smaller extent compared with hydrophobic/aromatic amino acids, consumption of other amino acids is consistently observed during growth of *T. kodakaraensis*. The enzyme(s) involved in the metabolism of these amino acids have not been identified.

In contrast to the wide distribution of AMP-forming ACSs, the presence of ADP-forming ACSs seems to be limited to protists such as *Entamoeba histolytica* (17) and *Giardia lamblia* (18) and the archaea. Archaeal ADP-forming ACSs have been characterized from the hyperthermophilic *P. furiosus* (11–13), *Archaeoglobus fulgidus* (19, 20), *Methanocaldococcus jannaschii* (20), and *Pyrococcus furiosus* (4–7).

The abbreviations used are: ACS$_{I_{Pf}}$, acetyl-CoA synthetase I from *P. furiosus*; NDP, nucleoside diphosphate; SCS, succinyl-CoA synthetase; SCS$_{Tk}$, succinyl-CoA synthetase from *T. kodakaraensis*; MES, 4-morpholineethanesulfonic acid; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; SCS$_{I_{Pf}}$, succinyl-CoA synthetase from *E. coli*; ACS II$_{I_{Pf}}$, acetyl-CoA synthetase II from *T. kodakaraensis*.
Novel ADP-forming Succinyl-CoA Synthetase

Gene expression was induced at the mid-exponential growth phase by the addition of 0.1 mM isopropyl β-D-thiogalactopyranoside, followed by additional incubation for 4 h at 37 °C. Protein concentration was determined with a Bio-Rad protein assay kit using bovine serum albumin as a standard. The N-terminal amino acid sequences of protein bands separated by SDS-PAGE were determined by using a Model 491 cLC protein sequencer (Applied Biosystems, Foster City, CA) after electroblotting the proteins onto a polyvinylidene difluoride membrane (Millipore Corp.).

Overexpression of SCS_{T_k} and ACS II_{T_k} Genes in E. coli—The expression plasmids for the SCS_{T_k} and ACS II_{T_k} genes were constructed as follows. First, the TK1880 and TK0139 genes encoding α-subunits and the TK0943 gene encoding the common β-subunit were independently amplified from T. kodakaraensis genomic DNA with the following primer sets: TK1880, 5’-CCCTCTAGAAATAATTTTGTATTTAACTTTAGAAGGATATACATATGGAGACCCGAACTGGACCTTCTG-3’ (sense) and 5’-TTAGGATCTCAAGCTCCCGTCCTCC-CTC-3’ (antisense); TK0139, 5’-ATACATAATGACGTTGACTACCTTCTTCTCAAGGC-3’ (sense) and 5’-TTAGGATCTCCTACTCCTCCCTCAGAAGCACCAG-3’ (antisense); and TK0943, 5’-CCCTCTAGAATTTCAATAATTGTTTTAC-TTTAAAGAGAATATCATATCAATGAGCGCCAAAGAGAGGGCC-3’ (sense) and 5’-CTTGGACTCCTCTTTTCCTTTCTGGAGCTTTTCC-3’ (antisense). The underlined sequences indicate restriction sites for XbaI, BamHI, NdeI, BamHI, EcoRI, and Sall, respectively, and the italic sequences indicate ribosome-binding sequences aimed to promote efficient translation in E. coli. The amplified fragments were subcloned into pUC118 and then digested with the appropriate restriction enzymes. The fragments for the TK1880 and TK0139 genes were individually inserted into pET21a(+) at the corresponding sites, and the fragment for TK0943 was further inserted into each of the resulting plasmids so that the TK0943 gene was properly oriented downstream of the α-subunit gene. The plasmids obtained (pET-scs and pET-acs2) were sequenced to confirm the absence of unintended mutations and used to transform E. coli BL21-CodonPlus(DE3)-RIL cells. Gene expression was induced at the mid-exponential growth phase by the addition of 0.1 mM isopropyl β-D-thiogalactopyranoside, followed by additional incubation for 4 h at 37 °C. After expression, cells were harvested, suspended in buffer A, and disrupted by sonication. The soluble protein fraction after centrifugation (15,000 × g, 15 min) was heat-treated at 80 °C for 10 min and centrifuged to remove heat-labile proteins from the host. The supernatant was subjected to sequential chromatography using Resource Q, Resource ISO, and Superdex 200 HR 10/30 columns according to the methods described above with a slight modification in the linear gradient for the hydrophobic column (1.8 to 0 mM ammonium sulfate in buffer A). The final protein sample was dialyzed against buffer A and subjected to enzyme assay.

Enzyme Assays—Succinyl-CoA-forming activity in T. kodakaraensis extracts and in fractions during the purification procedure was measured by the hydroxamate method (26). The reaction was performed in a mixture (500 μl) composed of 1 mM CoASH, 5 mM ATP or GTP, 50 mM hydroxylamine, and 2.5 mM MgCl₂ in 25 mM Tris succinate buffer at 70 °C for the appropriate...
ate periods of time. After the reaction, 350 μl of 20% trichloroacetic acid and 150 μl of 1 m FeCl₃ were added to the mixture, and formation of the iron-succinoylhydroxamate complex derived from succinyl-CoA was determined spectrophotometrically at 520 nm. Authentic succinyl-CoA (Sigma) was used as a standard for calibration.

A continuous spectrophotometric assay monitoring CoA thioester formation from acids was applied for examination of the substrate specificities and kinetic properties of recombinant SCS₉₂ and ACS II₉₂ (21). The reaction mixture (1000 μl) was composed of 10 mM carboxylic acid, 1 mM ATP, 1.5 mM CoA, 5 mM MgCl₂, 1.5 mM phosphoenolpyruvate, and 1.5 mM NADH in 50 mM MES-NaOH buffer (pH 6.5) together with a mixture of pyruvate kinase/lactate dehydrogenase from rabbit muscle (Sigma) as coupling enzymes. The ADP formation accompanied by CoA thioester formation at 55 °C was continuously monitored as a decrease in the absorbance of NADH at 340 nm. We confirmed that the stability and activity levels of the coupled enzymes were sufficient during the assay at 55 °C.

When measuring activity in the direction of CoA thioester cleavage, the formation of free CoASH was determined using 5,5'-dithiobis(2-nitrobenzoic acid) (13). The reaction was carried out in a mixture (1000 μl) containing 0.5 mM CoA thioester (isovaleryl-CoA or succinyl-CoA), 0.1 mM 5,5'-dithiobis(2-nitrobenzoic acid), 0.4 mM ADP, and 10 mM MgCl₂ in 50 mM MES-NaOH buffer (pH 6.5) at 55 °C, and the increase in absorbance at 412 nm was continuously recorded.

**Immunoprecipitation**—Protein A-Sepharose Fast Flow (GE Healthcare) was used as the affinity resin for immunoprecipitation experiments. Five-hundred microliters of rabbit antiserum (anti-TK0943 or anti-TK0465 serum) containing 1% bovine serum albumin was mixed with 100 μl of the resin suspension at 4 °C for 12 h. Antibody-bound resin was centrifuged, and the supernatant was removed. The pellet was washed five times with 1.5 ml of phosphate-buffered saline (136.9 mM NaCl, 8.1 mM Na₂HPO₄, 12H₂O, 2.68 mM KCl, and 1.47 mM KH₂PO₄), followed by the addition of 100 μl of cell extracts (2 mg/ml protein) of T. kodakaraensis grown in MA-YT-Pyr medium.

After incubation at 4 °C for 1.5 h, the resin was washed three times with phosphate-buffered saline and then resuspended in 30 μl of the buffer. The suspension was heat-treated at 90 °C for 30 min and centrifuged to remove heavy and light chains of the antibodies, followed by SDS-PAGE analysis. The protein bands with a molecular mass of ~50 kDa (corresponding to the α-subunits) were sliced out and subjected to MALDI-TOF tandem mass spectrometry analysis at Hitachi High-Technologies Corp. (Hitachinaka, Japan).

**RESULTS**

**Identification of an SCS in Cell-free Extracts of T. kodakaraensis**—We first examined the presence of SCS activity in T. kodakaraensis. The extract was prepared from cells grown in MA-YT-Pyr medium containing amino acids peptides and pyruvate without elemental sulfur, and ATP-dependent succinyl-CoA formation from succinate and CoA was measured by the hydroxamate procedure. As a result, we observed 0.18 μmol of succinyl-CoA-forming activity/min/mg of total protein in the extract at 70 °C.

![FIGURE 1. Purification of SCS from cell extracts of T. kodakaraensis. Arrowheads indicate the major proteins along with their respective N-terminal amino acid sequences and corresponding gene numbers on the T. kodakaraensis genome.](image)

The protein responsible for the SCS activity was purified from the cell extracts. The crude extract was subjected to sequential column chromatography, and the enzyme was purified 221-fold based on the ATP-dependent activity (39.4 units/mg) (Table 1). SDS-PAGE analysis of the active fractions after the final purification step showed two major bands with molecular masses of 50 and 27 kDa, respectively (Fig. 1). The elution of a single peak with a molecular mass of ~160 kDa was observed by gel filtration chromatography, which supported a heterotetrameric conformation (α₂β₂) of the enzyme composed of 50-kDa (α) and 27-kDa (β) subunits. It should be noted that a lower GTP-dependent activity was always associated with the ATP-dependent activity through all purification steps, and neither ATP- nor GTP-dependent SCS activity could be detected in other fractions. These facts strongly suggested that

| Step             | Protein | Total activity | Specific activity | Purification |
|------------------|---------|----------------|-------------------|--------------|
| Extract          | 659     | 117            | 0.18              | 1.0          |
| Resource Q       | 30.8    | 109            | 2.82              | 15.7         |
| Mono Q           | 5.35    | 35.3           | 2.75              | 15.5         |
| HiLoad 26/60 Superdex 200 pg | 3.43    | 33.1           | 11.6              | 63.1         |
| Resource ISO     | 0.581   | 16.4           | 30.0              | 168          |
| Superdex 200 HR 10/30 | 0.298   | 12.0           | 39.4              | 221          |

**TABLE 1**

Purification profile of SCS₉₂

![Large subunit (N-XETPNL) TK1880](image)

![Small subunit (N-SAXEEAL) TK0943](image)
Novel ADP-forming Succinyl-CoA Synthetase

SCS<sub>Tk</sub> Belongs to the Acyl-CoA Synthetase (NDP-forming) Superfamily—The deduced amino acid sequence of TK1880 displays high similarity (47% identical) to the α-subunits of the NDP-forming ACSs (I and II) from the closely related Archaea *P. furiosus* (PF1540 and PF0532, respectively). In addition, TK0943 displays 55% identity to the β-subunit of ACS I<sub>Pr</sub> (PF1787) and much higher identity (89%) to that of ACS II<sub>Pr</sub> (PF1837). The TK1880 and TK0943 proteins also displayed similarity to other members of the NDP-forming acyl-CoA synthetase superfamily, although to a lesser extent.

Bacterial and eukaryotic SCSs are the most studied enzymes in this superfamily. The detailed three-dimensional structure of SCS from *E. coli* (SCS<sub>E</sub>), which consists of two small α-subunits (SucD) and two large β-subunits (SucC), indicates the presence of five (sub)domains in the protein, domains 1-2 provided by the α-subunit and domains 3-4-5 composed of the β-subunit, as shown in Fig. 2 (23, 27). Based on the detailed structure of SCS<sub>E</sub> and information provided in the Molecular Modeling Database structure summary (Protein Data Bank code 2SCU), the five domains of SCS<sub>E</sub> are designated here as domain 1 (α-subunit residues 1-122), domain 2 (α-subunit residues 123-288), domain 3 (β-subunit residues 19-102), domain 4 (β-subunit residues 1-18 and 103-229), and domain 5 (β-subunit residues 230-388). The α-subunit is responsible for binding with the CoA substrate, and the active-site histidine (His<sup>246</sup>) resides in domain 2. Domains 3-4 of the β-subunit harbor the ATP-grasp fold, and domain 5 harbors one of the three nucleotide-binding motifs found in the αβ-dimer. It has been pointed out that other members in this superfamily also harbor these five domains, although the order and distribution of the domains between the two subunits display variation (18). Although all previously characterized classical SCSs share the same subunit and domain structure (domain order: α-subunit, 1-2; and β-subunit, 3-4-5) (23, 24, 27, 28), the SCS identified here from *T. kodakaraensis* exhibits a distinct structure: domain 5 is fused to the α-subunit (α-subunit, domains 1-2-5; and β-subunit, domains 3-4) (23, 24, 27, 28), the SCS<sub>Tk</sub> exhibits a distinct structure: domain 5 is fused to the α-subunit (α-subunit, domains 1-2-5; and β-subunit, domains 3-4) (Fig. 2). The structure is therefore closer to those of ACSs of Thermococcales (11-14) and the single polypeptide ACSs in *G. lamblia* (18). It should also be noted that the individual domains of SCS<sub>Tk</sub> exhibit higher similarity to the domains of the Thermococcales and *G. lamblia* ACSs (>38% identical) than to those of the classical SCSs (<28% identical). These facts explain why functional prediction of TK1880 and TK0943 as SCSs was not possible based on the primary structure in *T. kodakaraensis*.

**Overexpression and Purification of Recombinant SCS<sub>Tk</sub> and ACS II<sub>Tk</sub>**—The *T. kodakaraensis* genome harbors four genes (TK0139, TK0665, TK0944, and TK2127) paralogous to the α-subunit of SCS<sub>Tk</sub> (TK1880) and one gene (TK0465) paralogous to the β-subunit (TK0943). Members of the five- and two-paralog groups are similar in length (440-474 and 212-243 amino acids) and display overall similarity (41-50 and 55% identical) to the other members of the respective groups, indicating that their domain arrangements are common. Equivalent paralogous gene sets are also present on the genomes of *P. abyssi* (9), *P. furiosus* (29), and *P. horikoshii* (10), and each member exhibits particularly high identity (>80% at the amino acid levels) to only one member in the other strains (Fig. 3). The larger number of α-subunit genes implies that each β-subunit may accommodate more than one type of α-subunit. Interestingly, the β-subunit of SCS<sub>Tk</sub> identified here (TK0943) corresponds to the β-subunit of ACS II<sub>Pr</sub> (PF1837), suggesting that
SCS and ACS II share the same β-subunit in these hyperthermophiles.

To examine whether TK0943 can act as the β-subunit for both SCS and an ACS, we expressed the gene together with the TK1880 gene (encoding the SCS α-subunit) or the TK0139 gene (the \( T. kodakaraensis \) counterpart of the α-subunit gene of ACS II\(_{pp} \)) in \( E. coli \). Recombinant proteins of TK1880/TK0943 (SCS\(_{Tk} \)) and TK0139/TK0943 (ACS II\(_{Tk} \)) were recovered as soluble proteins after heat treatment at 80 °C for 10 min and subsequently purified. SCS\(_{Tk} \) and ACS II\(_{Tk} \) were determined to be \( α_3β_2 \)-heterotetramers by gel filtration chromatography, the same quaternary structures as those reported for native SCS\(_{Tk} \) and ACS II\(_{pp} \). The formation of \( α_3β_2 \)-heterotetramers and their high thermostability (see below) strongly suggest that TK0943 can assemble with either TK1880 or TK0139 as the α-subunit.

**Biochemical Characterization of Recombinant SCS\(_{Tk} \) and ACS II\(_{Tk} \)—** Purified recombinant SCS\(_{Tk} \) and ACS II\(_{Tk} \) exhibited ATP-dependent succinyl-CoA- and acetyl-CoA-forming activities at high temperature, respectively. Both enzymes showed the highest activity at pH 6.5, and the optimum temperatures were 75–80 °C for SCS\(_{Tk} \) and 75 °C for ACS II\(_{Tk} \). The enzymes were highly thermostable, with half-lives of 90 min (SCS\(_{Tk} \)) and 120 min (ACS II\(_{Tk} \)) at 95 °C. At 98 °C, both enzymes showed half-lives of 50 min. The high thermostability of the proteins suggests that the decrease in activity observed in both enzymes at temperatures above 80 °C is most likely due to the instability of CoA thioesters in our assay system at high temperatures.

We coupled the ATP-dependent CoA thioester-forming activity with pyruvate kinase and lactate dehydrogenase at 55 °C to quantify ADP formation. As expected, high rates of NADH oxidation were observed with succinate (SCS\(_{Tk} \)) and acetate (ACS II\(_{Tk} \)), confirming that SCS\(_{Tk} \) and ACS II\(_{Tk} \) are ADP-forming enzymes. Using this assay, we examined the substrate specificity of SCS\(_{Tk} \) and ACS II\(_{Tk} \) with various organic acids likely to be generated through amino acid metabolism. As shown in Fig. 4, SCS\(_{Tk} \) showed high activity for succinate (16.2 units/mg), but malonate was not recognized as a substrate. Isovalerate and 3-methyl thiopropionate were converted by SCS\(_{Tk} \) with activity levels of approximately two-thirds compared with the succinate-convert- ing activity. Although not direct intermediates of amino acid catabolism, glutarate (121%), adipate (59%), and butyrate (48%) also served as good substrates for SCS\(_{Tk} \), whereas propionate (10%) and oxalate (9%) did not (data not shown). Considering the structures of these compounds, SCS\(_{Tk} \) seems to prefer mono- or dicarboxylates with a backbone of four or more carbons. In contrast, ACS II\(_{Tk} \) exhibited higher levels of activity for isovalerate (36.1 units/mg) than for acetate (15.6 units/mg) and could accept various acids with diverse hydrophobic structures such as 3-methyl thio propionate (94% to isovalerate), 2-methyl butyrate (57%), isobutyrate (27%), and aromatic acids (phenyl acetate (77%), 4-hydroxyphenyl acetate (59%), and 3-indole acetate (34%). The broad substrate specificity of ACS II\(_{Tk} \) for hydrophobic and aromatic acids corresponds well with that of the previously reported ACS II\(_{pp} \). Our results clearly demonstrate that, despite the common β-subunit, swapping of α-subunits in SCS\(_{Tk} \) and ACS II\(_{Tk} \) results in entirely different substrate specificities.

We further examined the kinetic properties of the SCS\(_{Tk} \)-catalyzed reactions in both directions (Table 2). The CoA thioester-forming activity of SCS\(_{Tk} \) was determined by monitoring ADP formation at 55 °C as described above. Interestingly, SCS\(_{Tk} \) displayed strong negative cooperation in succinate binding, and nonlinear regression analysis of the velocity data gave a dissociation constant of 2.8 mM for the first succinate molecule (\( K_{d1} \)), whereas the dissociation constant for the second substrate molecule (\( K_{d2} \)) was much larger (29 mM). Such negative cooperation was also observed in the reactions with isovalerate and 3-methyl thio propionate as substrates (Table 2). The kinetic parameters indicated that succinate was the preferred substrate in the acyl-CoA-forming reaction. Acetate, the major substrate for ACS, was not efficiently converted to acetyl-CoA by SCS\(_{Tk} \). Increases in activity were still observed at concentrations as high as 600 mM, and reliable curve fitting could therefore not be performed. The velocity of the reverse reaction was determined by monitoring the release of free CoA at 55 °C. Kinetic analysis with succinyl-CoA could not be performed because of the labile nature of succinyl-CoA at high reaction temperatures. We found that isovaleryl-CoA was much more stable than succinyl-CoA at these temperatures, and kinetic examinations were carried out with this substrate. SCS\(_{Tk} \) showed typical Michaelis-Menten kinetics with \( K_m \) and \( k_{cat} \) values of 9.1 \( \mu \)M and 54 s\(^{-1} \) active site\(^{-1} \), respectively. The \( k_{cat}/K_m \) value for isovaleryl-CoA was >10-fold higher than that for acetyl-CoA, again suggesting that SCS\(_{Tk} \) is not an enzyme involved mainly in acetate/acyetyl-CoA conversion. Furthermore, the much higher affinity and \( k_{cat}/K_m \) values of the enzyme for isovaleryl-CoA compared with isovalerate (Table 2) support an \textit{in vivo} function of SCS\(_{Tk} \) in catalyzing CoA thioester

![FIGURE 4. Levels of acyl-CoA-forming activity (ADP-forming) of recombinant SCS\(_{Tk} \) and ACS II\(_{Tk} \) for various organic acid compounds](image)
**Novel ADP-forming Succinyl-CoA Synthetase**

**TABLE 2**

| Substrate                           | $K_m$ or $K_{ij}$ | $K_{cat}$ | $k_{cat}$/| $k_{cat}/K_m$ |
|-------------------------------------|------------------|-----------|----------|--------------|
| Acyl-CoA-forming reaction*          |                  |           |          |              |
| ATP                                 | 0.0050 ± 0.0008  | 23 ± 1    | 4600     |              |
| CoA                                 | 0.060 ± 0.008    | 25 ± 1    | 420      |              |
| Succinate                           | 2.8 ± 0.2        | 29 ± 6    | 10       |              |
| 3-Methyl thioipropionate            | 5.3 ± 1.1        | 130 ± 48  | 5.3      |              |
| Isovalerate                         | 14 ± 8           | 560 ± 270| 2.8      |              |
| Acetate                             | >50              |           |          |              |
| CoA-releasing reaction†             |                  |           |          |              |
| ADP                                 | 0.0077 ± 0.0008  | 22 ± 1    | 2900     |              |
| GDP                                 | 0.84 ± 0.12      | 22 ± 1    | 26       |              |
| Phosphate                           | 1.4 ± 0.1        | 19 ± 6    | 14       |              |
| Acetyl-CoA                          | 0.041 ± 0.004    | 23 ± 1    | 560      |              |
| Isovaleryl-CoA                      | 0.0091 ± 0.0006  | 54 ± 1    | 5900     |              |

* Activity was measured by quantifying ADP generation rates using pyruvate kinase and lactate dehydrogenase. ATP and CoA measurements were carried out in the presence of 10 mM succinate, 1 mM ATP (for CoA kinetics), and 1.5 mM CoA (for ATP kinetics). Kinetic measurements for the acids were performed in the presence of 1 mM ATP and 1.5 mM CoA.
† Increases in reaction velocity were still observed at 600 mM acetate, preventing reliable curve fitting and calculation of kinetic parameters.

breakdown resulting in ATP production rather than acyl-CoA synthesis.

**Immunoprecipitation of SCS$_{Tk}$ and ACS II$_{Tk}$—** As our in vitro data clarified that the single $\beta$-subunit TK0943 can form an active enzyme with two distinct $\alpha$-subunits, we next examined whether this is the case in vivo. We performed an immunoprecipitation analysis using specific antisera prepared against the individual purified $\beta$-subunits TK0943 and TK0465. The precipitates were heated to denature and remove heavy and light chains of the antibodies, followed by SDS-PAGE analysis. Multiple proteins with a molecular mass of $\sim$50 kDa coprecipitated with the individual $\beta$-subunits, and MALDI-TOF tandem mass spectrometry analyses identified TK0944, TK0665, TK1880, and TK0139 associated with TK0943 and also TK0944, TK0665, and TK0139 with associated TK0465 (data not shown). Although cross-reaction of the antibodies with the other $\beta$-subunit was not observed in Western blot analysis, there is a possibility that cross-reaction occurred in the immunoprecipitation experiments. However, the presence of more than two types of $\alpha$-subunits in each precipitate indicates that the $\beta$-subunits assemble with multiple $\alpha$-subunits in vivo.

**DISCUSSION**

Paralog sets of five $\alpha$-subunits and two $\beta$-subunits of acyl-CoA synthetase (NDP-forming) genes are completely conserved in the Thermococcales genomes. Among the 10 combinations possible, only two had been identified until now, ACSs I$_{pf}$ and II$_{pf}$ (11–14). In this study, we found that one of the $\alpha$-subunits (TK1880) in *T. kodakaraensis* is a component of a novel ADP-forming SCS with a distinct domain order and distribution among subunits compared with the classical SCS enzymes.

Despite the different order and distribution of the five domains among the $\alpha$- and $\beta$-subunits, it is presumed that members of the acyl-CoA synthetase (NDP-forming) superfamily share a common catalytic mechanism (18). In the case of CoA thioester formation by SCS$_{Ec}$, the $\gamma$-phosphate of ATP is first transferred to the catalytic His$^{\text{261}}$ residue in the $\alpha$-subunit, and the phosphorylated imidazole ring reacts with acid and CoA to form the corresponding acyl-CoA molecule. The ATP- and CoA-binding sites are located within the ATP-grasp fold domain in the $\beta$-subunit (domains 3-4) and the CoA-binding domain in the $\alpha$-subunit (domain 1), respectively (23, 27, 30, 31). The catalytic His residue (corresponding to His$^{\text{261}}$ in the TK1880 $\alpha$-subunit) and several important residues that function in binding with the ATP-Mg$^{2+}$ complex and CoA are well conserved among this superfamily (18). In contrast, the substrate-binding site has yet to be clarified even in the well studied SCS$_{Ec}$. The alteration of substrate specificity between SCS$_{Tk}$ and ACS II$_{Tk}$ in this study indicates the presence of the substrate-binding site in the $\alpha$-subunits. In particular, the CoA ligase domains (domains 2 and 5) attached to the N-terminal CoA-binding domain in the $\alpha$-subunit can be considered to be candidates for the substrate-binding domain. In the classical ATP- and GTP-dependent SCs from pigeon, distinct $\beta$-subunits (domain order 3-4-5) are responsible for large differences in the apparent $K_m$ values for succinate, in addition to a change in nucleotide specificity probably caused by replacement of the ATP-grasp fold (domains 3-4) (32). Taking these facts into consideration, domain 5 in the $\alpha$-subunit of SCS$_{Tk}$ and ACS$_{Tk}$ seems likely to play a role in substrate recognition and binding.

Although not published in the literature, the crystal structures of PH0766 and PH1788 are available in the Protein Data Bank and provide valuable information in understanding how the archaeal SCS and ACS and the classical SCs exhibit similar activity with different domain orientations (Fig. 5). *P. horikoshii* PH0766 and PH1788 are the $\alpha$- and $\beta$-subunits that correspond to the subunits of ACS II and ACS II/SCS in *T. kodakaraensis*, respectively. As in the case of the $\alpha$-subunit of SCS$_{Tk}$ (TK1880), PH0766 consists of domains 1-2-5. The crystal structure reveals a dimeric structure of the protein, in which domains 1-2 of one monomer ($\alpha_1$-subunit) make contact with domain 5 of the opposite monomer ($\alpha_2$-subunit). The arrangement of the three domains ($\alpha_1$-subunit, domains 1-2; and $\alpha_2$-subunit, domain 5) is strikingly similar to that observed in SCS$_{Ec}$ ($\alpha$-subunit,
domains 1-2; and β-subunit, domain 5). Furthermore, the surface of the α1-subunit (domains 1-2)/α2-subunit (domain 5) structure of PH0766 corresponding to the surface that interacts with domains 3 and 4 in SCS₄ₑ, seems to be exposed to the solvent, indicating that a domain 3-4 protein can make contact in an orientation similar to that observed in SCS₄ₑ. As anticipated from its primary structure, the fold of PH1788 consisting of domains 3-4 (β-subunit) is an ATP-grasp fold. As TK1880 and TK0943 can be expected to display a structure similar to those of the Pyrococcus counterparts, this model explains how SCS₄ₑ and SCS₄ₑ, with differences in their domain order and subunit distribution, can assemble into functional units of basically the same topology.

The physiological functions of ACSs I and II have been supposed to be in the breakdown of the acyl-CoA molecules coupled to ATP generation (11–14). Although ACS IIₜₜ (13, 14) and ACS IIₜₜ (this study) exhibit broad substrate specificities for a variety of acids with hydrophobic side chains, SCS₄ₑ is relatively specific, displaying relevant levels of activity for only a few acids. The activity levels and kinetic parameters of SCS₄ₑ for

![Model indicating the proposed topology of SCS₄ₑ that would allow the enzyme to exhibit a domain orientation similar to those of the classical SCS proteins.](image)
Novel ADP-forming Succinyl-CoA Synthetase

![Diagram illustrating the catabolism of glutamate in T. kodakaraensis](image)

**FIGURE 6.** Diagram illustrating the catabolism of glutamate in *T. kodakaraensis*. 5: elemental sulfur; AT: amino acid-2-oxoacid aminotransferase; GDH: glutamate dehydrogenase; AlaAT: alanine aminotransferase; POR: pyruvate:ferredoxin oxidoreductase; VOR: 2-oxoisovalerate:ferredoxin oxidoreductase; IOR: indolepyruvate:ferredoxin oxidoreductase; KGOR: 2-oxoglutarate:ferredoxin oxidoreductase; Fdox: oxidized ferredoxin; Fdred: reduced ferredoxin.

succinate, isovalerate, and 3-methyl thipropionate suggest the involvement of this enzyme in the catabolism of Glu, Leu, and Met. However, considering that ACS II is also displays high levels of activity for the latter two substrates, the major physiological role of ACS II is most likely in the generation of ATP from succinyl-CoA. Indeed, in addition to large amounts of acetate production (±35.6 mM), we observed accumulation of succinate (±1.03 mM) stoichiometric to Glu/Gln consumption (±1.04 mM) in the medium when *T. kodakaraensis* was grown in the presence of amino acids along with pyruvate. As shown in Fig. 6, the identification of ACS II completes a metabolic route for energy generation from glutamate to succinate via 2-oxoglutarate and succinyl-CoA by the function of glutamate dehydrogenase, 2-oxoglutarate:ferredoxin oxidoreductase, and ACS II.

REFERENCES

1. Itoh, T. (2003) *J. Biosci. Bioeng.* 96, 203–212
2. Adams, M. W. W. (1994) *FEMS Microbiol. Rev.* 15, 261–277
3. Robb, F. T., Park, J.-B., and Adams, M. W. W. (1992) *Biochim. Biophys. Acta* 1120, 267–272
4. Ma, K., Hutchins, A., Sung, S.-J., and Adams, M. W. W. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 9608–9613
5. Heider, J., Mai, X., and Adams, M. W. W. (1996) *J. Bacteriol.* 178, 780–787
6. Mai, X., and Adams, M. W. W. (1996) *J. Bacteriol.* 178, 5890–5896

7. Mai, X., and Adams, M. W. W. (1994) *J. Biol. Chem.* 269, 16726–16732
8. Fukui, T., Atomi, H., Kanai, T., Matsumi, R., Fujiwara, S., and Imanaka, T. (2005) *Genome Res.* 15, 352–363
9. Cohen, G. N., Barbe, V., Flament, D., Galperin, M., Heilig, R., Lecompte, O., Poch, O., Prieur, D., Quérelou, J., Ripp, R., Thierry, J.-C., Van der Oost, J., Weissenbach, J., Zivanovic, Y., and Forterre, P. (2003) *Mol. Microbiol.* 47, 1495–1512
10. Kawarabayasi, Y., Sawada, M., Horikawa, H., Haikawa, Y., Hino, Y., Yamamoto, S., Sekine, M., Baba, S., Kosugi, H., Hosoyama, A., Nagai, Y., Sakai, M., Ogura, K., Otsuka, R., Nakazawa, H., Takamiya, M., Ohfuku, Y., Funahashi, T., Tanaka, T., Kudoh, Y., Yamazaki, J., Kushida, N., Oguchi, A., Aoki, K., and Kikuchi, H. (1998) *DNA Res.* 5, 55–76
11. Schäfer, T., and Schönheit, P. (1991) *Arch. Microbiol.* 155, 366–377
12. Schäfer, T., Selig, M., and Schönheit, P. (1993) *Arch. Microbiol.* 159, 72–83
13. Mai, X., and Adams, M. W. W. (1996) *J. Bacteriol.* 178, 5897–5903
14. Musfeldt, M., Selig, M., and Schönheit, P. (1999) *J. Bacteriol.* 181, 5885–5888
15. Krahe, M., Antranikian, G., and Märkl, H. (1996) *FEMS Microbiol. Rev.* 18, 271–285
16. Mai, X., and Adams, M. W. W. (1996) *J. Bacteriol.* 178, 271–282
17. Field, J., Rosenthal, B., and Samuelson, J. (2000) *Mol. Microbiol.* 38, 446–455
18. Sánchez, L. B., Galperin, M. Y., and Müller, M. (2000) *J. Biol. Chem.* 275, 5794–5803
19. Meschede, A., and Schönheit, P. (2001) *Arch. Microbiol.* 176, 329–338
20. Musfeldt, M., and Schönheit, P. (2002) *J. Bacteriol.* 184, 636–644
21. Bräsen, C., and Schönheit, P. (2004) *Arch. Microbiol.* 182, 277–287
22. Bräsen, C., and Schönheit, P. (2004) *FEMS Microbiol. Lett.* 241, 21–26
23. Fraser, M. E., James, M. N. G., Bridger, W. A., and Wolodko, W. T. (1999) *J. Mol. Biol.* 285, 1633–1653
24. Fraser, M. E., Hayakawa, K., Hume, M. S., Ryan, D. G., and Brownie, E. R. (2005) *J. Mol. Biol.* 352–363
25. Atomi, H., Fukui, T., Kanai, T., Matsumi, R., Fujiwara, S., and Imanaka, T. (2004) *Archaea* 1, 263–267
26. Takeda, Y., Suzuki, F., and Inoue, H. (1969) *Methods Enzymol.* 13, 153–160
27. Wolodko, W. T., Fraser, M. E., James, M. N. G., and Bridger, W. A. (1994) *J. Biol. Chem.* 269, 10883–10890
28. Fraser, M. E., James, M. N. G., Bridger, W. A., and Wolodko, W. T. (2000) *J. Mol. Biol.* 299, 1325–1339
29. Robb, F. T., Maeder, D. L., Brown, J. R., DiRuggiero, J., Stump, M. D., Yeh, R. K., Weiss, R. B., and Dunn, D. M. (2001) *Methods Enzymol.* 330, 134–157
30. Joyce, M. A., Fraser, M. E., James, M. N. G., Bridger, W. A., and Wolodko, W. T. (2000) *Biochemistry* 39, 17–25
31. Fraser, M. E., Joyce, M. A., Ryan, D. G., and Wolodko, W. T. (2002) *Biochemistry* 41, 537–546
32. Johnson, J. D., Muñoz, W. W., and Lambeth, D. O. (1998) *J. Biol. Chem.* 273, 27573–27579
33. DeLano, W. L. (2002) *The PyMOL Molecular Graphics System*, DeLano Scientific, Palo Alto, CA