Mechanism of Substrate Recognition and Insight into Feedback Inhibition of Homocitrate Synthase from Thermus thermophilus

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Homocitrate synthase (HCS) catalyzes aldol-type condensation of acetyl coenzyme A (acetyl-CoA) and α-ketoglutarate (α-KG) to synthesize homocitrate (HC), which is the first and committed step in the lysine biosynthetic pathway through α-amino adipate. As known in most enzymes catalyzing the first reactions in amino acid biosynthetic pathways, HCS is regulated via feedback inhibition by the end product, lysine. Here, we determined the crystal structures of HCS from Thermus thermophilus complexed with α-KG, HC, or lysine. In the HC complex, the C1-carboxyl group of HC, which is derived from acetyl-CoA, is hydrogen-bonded with His292* from another subunit, indicating direct involvement of this residue in the catalytic mechanism of HCS. The crystal structure of HCS complexed with lysine showed that lysine is bound to the active site with rearrangement of amino acid residues in the substrate-binding site, which accounts for the competitive inhibition by lysine with α-KG. Comparison between the structures suggests that His72, which is conserved in lysine-sensitive HCSs and binds the C5-carboxyl group of α-KG, serves as a switch for the conformational change. Replacement of His72 by leucine made HCS resistant to lysine inhibition, demonstrating the regulatory role of this conserved residue.

Lysine is known to be biosynthesized by two completely different pathways. Fungi and yeast synthesize lysine through the α-amino acid pathway (1, 2), while most bacteria and plants synthesize amino acid through the diaminopimelate (DAP) pathway (3, 4). Since higher animals cannot biosynthesize lysine, the enzymes of these pathways are attractive targets for designing antibiotics against pathogenic microorganisms (5, 6). We found that the extremely thermophilic bacterium, Thermus thermophilus, biosynthesizes lysine through the AAA pathway (7). In biosynthesis, the enzymes involved in the former part, conversion of α-ketoglutarate (α-KG, 2-oxopentanedioate) to AAA, have amino acid sequence similarity to those of the tricarboxylic acid cycle and leucine biosynthesis as fungi and yeast, whereas the enzymes involved in the latter part, AAA to lysine biosynthesis, have amino acid sequence similarity to those involved in the conversion of glutamate to ornithine in arginine biosynthesis (7-14), although lysine biosynthesis differs from arginine biosynthesis in that the former involves proteinaceous amino group modification of biosynthetic intermediates instead of the acetylation used in the latter (15).

Amino acid biosynthesis is often controlled at levels of gene expression and enzyme activity, depending on nutrient availability in the environment. Homocitrate synthase (HCS, EC2.3.3.14), responsible for the first reaction in lysine biosynthesis, transfers the acetyl group from acetyl-CoA to α-KG to yield homocitrate (HC, 2-hydroxybutane-1,2,4-tricarboxylate) and CoA (Scheme 1). As commonly observed in enzymes responsible for the first reaction of amino acid biosynthesis, HCS from T. thermophilus (TtHCS) is feedback-inhibited by the end-product, lysine (11). We have previously revealed that the promoter of the major lysine biosynthetic gene cluster, starting from the hcs gene of T. thermophilus, is controlled by lysine through leader peptide-mediated transcriptional attenuation (16).

α-Isopropylmalate synthase (IPMS, EC2.3.3.13) is the enzyme responsible for the
first reaction of leucine biosynthesis. The enzyme catalyzes transfer of the acetyl group from acetyl-CoA to \( \alpha \)-ketoisovalerate (\( \alpha \)-KIV) to yield isopropylmalate in a manner similar to that of HCS (17). IPMS is inhibited by the end-product, leucine (18). The crystal structures of IPMS from *Mycobacterium tuberculosis* (MtIPMS) were determined in \( \alpha \)-KIV-bound and leucine-bound forms (19). The structure of \( \alpha \)-KIV-bound MtIPMS reveals the active site organization of IPMS and the recognition mechanism of the hydrophobic isopropyl moiety of \( \alpha \)-KIV. In the leucine-bound form, leucine is bound at the dimer interface formed between C-terminal regulatory domains, both of which are from different chains of the IPMS dimer. The leucine-binding site is located close to the site of mutation giving resistance to a leucine analog (20), which was previously found in IPMS from *Saccharomyces cerevisiae*. These observations indicate that end-product inhibition occurs through leucine binding to the C-terminal domain; however, the detailed allosteric regulatory mechanism remains to be elucidated, because no apparent conformational change around the active site is observed between leucine-bound and -unbound forms of MtIPMS. Recently, the crystal structure of the catalytic domain of citramalate synthase (CMS, EC2.3.1.182) from *Leptospira interrogans* (LiCMS) complexed with acetyl-CoA and pyruvate was reported (21). CMS is another HCS paralogue, which catalyzes the first reaction of the isoleucine biosynthetic pathway, converting pyruvate and acetyl-CoA to citramalate and CoA. Interestingly, HCS is shorter than IPMS and CMS by about 150 amino acid residues, lacking the C-terminal portion corresponding to the regulatory domain for sensing allosteric inhibitors in these paralogues. This observation suggests that the regulatory mechanism of HCS is different from those of IPMS and CMS. Here, we report the crystal structures of TtHCS complexed with \( \alpha \)-KG, HC, or lysine, which reveal the mechanism of substrate recognition and feedback inhibition.

**EXPERIMENTAL PROCEDURES**

*Protein preparation -* We designed three expression systems for the hcs gene: i) TtHCS without tag (TtHCSwt) used for crystallization of \( \alpha \)-KG and HC, and ii) HCS with N-terminal (his)\(_8\)-tag (TtHCS-Nhis) for enzymatic assays of wild-type and mutant enzymes. To prepare the DNA fragment for TtHCSwt, the hcs gene from *T. thermophilus* was amplified by polymerase chain reaction (PCR) using the oligonucleotides 5'-GGCGAATTCCATATGCGGGAGTGGAAGATT-3' and 5'-GGCAAGCTTTTAGTGGTGGTGGTGGTGGCGCCGTGATCCACTCCCG-3'. The amplified DNA fragment, designed to direct the production of full-length TtHCS (Met1-Ala376), was cloned into the *EcoRI/HindIII* site of pBluescript II SK (+). To prepare the DNA fragment for TtHCS-Chis, similar PCR was performed using the oligonucleotides 5'-GGCGAATTCCATATGCGGGAGTGGAAGATT-3' and 5'-GGCAAGCTTTTAGTGGTGGTGGTGGTGGCGCCGTGATCCACTCCCG-3'. The amplified DNA fragment, designed to direct the production of full-length TtHCS (Met1-Ala376) with a (his)\(_6\)-tag extension at the C-terminal end, was cloned into the *EcoRI/HindIII* site of pBluescript II SK (+). To prepare the DNA fragment of TtHCS-Nhis, the hcs gene was amplified by PCR using oligonucleotides 5'-CCCGAATTCATGCGGGAGTGGAAGATT-3' and 5'-CCCCTCAGCTTTACCAGCGCGTGACCTCC-3'. The amplified DNA fragment, which was designed to direct the production of full-length HCS with 24 additional residues containing a (his)\(_8\)-tag extension and thrombin site at the N-terminal end, was cloned into the *EcoRI/SalI* site of pBluescript II SK (+). DNA fragments with the correct sequence were cloned into the *NdeI/HindIII* site of pET-26b(+) for TtHCSwt and TtHCS-Chis, and the *EcoRI/SalI* site of pHIS8 (22) for TtHCS-Nhis, and introduced into *Escherichia coli* BL21-CodonPlus (DE3)-RIL cells. *E. coli* cells harboring pET26b(+)-TtHCSwt or pET26b(+)-TtHCS-Chis were grown in 2 × YT medium (1.6% tryptone, 1% yeast extract, and 0.5% sodium chloride) supplemented with kanamycin (50 \( \mu \)g ml\(^{-1}\)) and chloramphenicol (30 \( \mu \)g ml\(^{-1}\)) at 310 K. Cells harboring pHIS8-TtHCS-Nhis were grown in 2 × YT medium containing kanamycin (50 \( \mu \)g ml\(^{-1}\)) and chloramphenicol (30 \( \mu \)g ml\(^{-1}\)) at 310 K. In each case, when optical density at 600 nm of the
culture reached about 0.6, gene expression was
induced by adding 0.5 mM isopropyl
\( \beta \)-D-thiogalactopyranoside and the culture was
continued for an additional 12 h at 298 K.

TtHCS-Chis was purified as follows. The
cells were harvested, washed, and suspended in
buffer \( A \) (20 mM Tris-HCl, pH 8.0, 150 mM
NaCl) containing 10 mM \( \alpha \)-KG and 10 % (v/v)
glycerol. Suspended cells were disrupted by
sonication and centrifuged at 40,000 \( \times \) g for 15
min. The supernatant was heated at 343 K for 30
min to denature proteins from \( E. \ coli \). The
heated solution was centrifuged at 40,000 \( \times \) g for
15 min and the supernatant was applied to a
\( \text{Ni}^{2+}\)-NTA column (Novagen, Darmstadt,
Germany). The concentrated sample was applied
to a HiLoad 26/60 Superdex 75 gel-filtration FPLC
column (GE Healthcare UK, Buckinghamshire, UK)
equilibrated with buffer \( A \) (20 mM Tris-HCl, pH 8.0, 150 mM
\( \alpha \)-KG, 5 mM \( \text{MgSO}_4 \), and 10 % glycerol) containing
25 mM imidazole. After washing with buffer \( B \) containing 25 mM
imidazole, the proteins bound to the resin were
eluted with buffer \( B \) containing 500 mM imidazole.
Fractions containing TtHCS-Chis were pooled and concentrated to about 10 mg
\( \text{ml}^{-1} \) using Vivaspin-20 centrifugal filtration with
10 kDa cutoff (Vivasience, Goettingen, Germany). The concentrated sample was applied to a HiLoad 26/60 Superdex 75 gel-filtration FPLC column equilibrated with buffer \( A \) containing 10 mM \( \alpha \)-KG, 5 mM
\( \text{MgSO}_4 \), and 10 % glycerol at a flow rate of 1.25
\( \text{ml} \text{ min}^{-1} \). The protein fractions were pooled and
concentrated to 10 mg \( \text{ml}^{-1} \).

TtHCSwt was purified as follows. The cells
were harvested, washed, and suspended in buffer
\( C \) (10 mM \( N \)-2-hydroxyethylpiperazine-\( N' \)-\( N \)-2'-
ethanesulfonic acid (HEPES), pH 7.5, 1 mM
EDTA). Suspended cells were disrupted by
sonication and centrifuged at 40,000 \( \times \) g for 15
min. The supernatant was heated at 343 K for 30
min to denature proteins from \( E. \ coli \). The
heated solution was centrifuged at 40,000 \( \times \) g for
15 min. Ammonium sulfate was added at a final
50% to the supernatant. After centrifugation, the
precipitant was suspended in buffer \( C \) containing
1.5 M ammonium sulfate and applied onto a
HiLoad 26/60 Phenyl Sepharose column (GE
Healthcare UK). Protein was eluted with a linear
gradient of 0 to 1 M ammonium sulfate in 250
\( \text{ml} \) buffer \( C \) at a flow rate of 5 \( \text{ml} \text{ min}^{-1} \) and
fractions containing TtHCSwt were pooled and
concentrated to about 4 mg \( \text{ml}^{-1} \). The concentrated sample was applied to a Resource Q anion exchange column (GE Healthcare UK)
equilibrated with buffer \( C \) and eluted with a
linear gradient of 0 to 1 M ammonium sulfate in
buffer \( C \) at a flow rate of 3 \( \text{ml} \text{ min}^{-1} \). Fractions
containing TtHCSwt were pooled and concentrated to about 10 mg \( \text{ml}^{-1} \), and applied to a HiLoad 26/60 Superdex 75 gel-filtration FPLC
column equilibrated with buffer \( D \) (10 mM
HEPES, pH 7.5, 150 mM \( \text{NaCl} \), 5 mM \( \text{MgSO}_4 \)) and eluted at 2.5 \( \text{ml} \text{ min}^{-1} \). TtHCSwt had over
95% purity on SDS-PAGE. Protein was
concentrated to 10 mg \( \text{ml}^{-1} \) in buffer \( D \) and used for
crystallization.

**Crystallization** - Crystallization conditions
of the \( \alpha \)-KG complex were screened by the
hanging-drop vapor-diffusion method using
Crystal Screen kits (Hampton Research, CA).
Drops of 2 \( \mu \)l consisting of 1 \( \mu \)l reservoir
solution and 1 \( \mu \)l TtHCS-Chis solution of 10 mg

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\( \text{MgSO}_4 \cdot 7\text{H}_2\text{O} \), and 16 mg \( \text{FeSO}_4 \cdot 7\text{H}_2\text{O} \), 16 ml vitamin
supplement (Sigma-Aldrich-Japan, Tokyo), 50 \( \mu \)g \( \text{ml}^{-1} \) kanamycin, and 40 mg SeMet (Wako Pure
Chemical). The protein was purified using the
protocol shown above. Native and SeMet-substituted proteins were concentrated to
10 mg \( \text{ml}^{-1} \) in buffer \( A \) supplemented with 10
mM \( \alpha \)-KG, 5 mM \( \text{MgSO}_4 \), and 10 % glycerol, and used for crystallization. The method of
purifying TtHCS-Nhis was essentially the same
as that of TtHCS-Chis except that 10 mM \( \alpha \)-KG,
5 mM \( \text{MgSO}_4 \), and 10 % glycerol were not
added in the purification steps. Protein samples
to determine the crystal structure of the
HCS/Mg\(^{2+}\)/\( \alpha \)-KG complex were prepared as
follows. After purification by \( \text{Ni}^{2+}\)-NTA column,
4 mM ethylenediaminetetraacetic acid (EDTA)
was added to the sample and incubated at 277 K
for 1 h. The sample was then dialyzed against
buffer \( A \) for 2 h twice. The concentrated sample
was applied to a HiLoad 26/60 Superdex 75
gel-filtration FPLC column equilibrated with
buffer \( A \) containing 10 mM \( \alpha \)-KG, 5 mM
\( \text{MgSO}_4 \), and 10 % glycerol at a flow rate of 1.25
\( \text{ml} \text{ min}^{-1} \). The protein fractions were pooled and
concentrated to 10 mg \( \text{ml}^{-1} \).

TtHCSwt was purified as follows. The cells
were harvested, washed, and suspended in buffer
\( C \) (10 mM \( N \)-2-hydroxyethylpiperazine-\( N' \)-\( N \)-2'-
ethanesulfonic acid (HEPES), pH 7.5, 1 mM
EDTA). Suspended cells were disrupted by
sonication and centrifuged at 40,000 \( \times \) g for 15
min. The supernatant was heated at 343 K for 30
min to denature proteins from \( E. \ coli \). The
heated solution was centrifuged at 40,000 \( \times \) g for
15 min. Ammonium sulfate was added at a final
50% to the supernatant. After centrifugation, the
precipitant was suspended in buffer \( C \) containing
1.5 M ammonium sulfate and applied onto a
HiLoad 26/60 Phenyl Sepharose column (GE
Healthcare UK). Protein was eluted with a linear
gradient of 1.5 to 0 M ammonium sulfate in 250
\( \text{ml} \) buffer \( C \) at a flow rate of 5 \( \text{ml} \text{ min}^{-1} \) and
fractions containing TtHCSwt were pooled and
concentrated to about 4 mg \( \text{ml}^{-1} \). The concentrated sample was applied to a Resource Q anion exchange column (GE Healthcare UK)
equilibrated with buffer \( C \) and eluted with a
linear gradient of 0 to 1 M ammonium sulfate in
buffer \( C \) at a flow rate of 3 \( \text{ml} \text{ min}^{-1} \). Fractions
containing TtHCSwt were pooled and concentrated to about 10 mg \( \text{ml}^{-1} \), and applied to a HiLoad 26/60 Superdex 75 gel-filtration FPLC
column equilibrated with buffer \( D \) (10 mM
HEPES, pH 7.5, 150 mM \( \text{NaCl} \), 5 mM \( \text{MgSO}_4 \)) and eluted at 2.5 \( \text{ml} \text{ min}^{-1} \). TtHCSwt had over
95% purity on SDS-PAGE. Protein was
concentrated to 10 mg \( \text{ml}^{-1} \) in buffer \( D \) and used for
crystallization.

**Crystallization** - Crystallization conditions
of the \( \alpha \)-KG complex were screened by the
hanging-drop vapor-diffusion method using
Crystal Screen kits (Hampton Research, CA).
Drops of 2 \( \mu \)l consisting of 1 \( \mu \)l reservoir
solution and 1 \( \mu \)l TtHCS-Chis solution of 10 mg
ml$^{-1}$ supplemented with 5 mM MgSO$_4$ and 10 mM $\alpha$-KG were equilibrated against 500 $\mu$L reservoir solution at 293 K. In addition, a similar TtHCS-Chis solution supplemented with 5 mM MgSO$_4$, 10 mM $\alpha$-KG, and 0.2 mM acetyl-CoA was used for crystallization of the HC complex. A few crystals were obtained from both droplets using solution No. 32 (2.0 M ammonium sulfate) of Crystal Screen I over a few days. Crystals of 0.1 x 0.2 x 0.3 mm, formed in 2.0 M ammonium sulfate, were used for X-ray diffraction. We also obtained several crystals of similar shape and size to SeMet-containing TtHCS-Chis under the same conditions. Crystals of the Mg$^{2+}$/-$\alpha$-KG complex were obtained from a droplet with a reservoir solution of 0.1 M 2-morpholinoethanesulfonic acid (MES)-NaOH, pH 6.0, and 1.6 M ammonium sulfate. Although we also attempted to crystallize TtHCS-Nhis, no crystals suitable for crystallographic analysis were obtained.

Crystallization conditions of TtHCSwt complexed with lysine were screened by the hanging-drop vapor-diffusion method using Crystal Screen Kits. Drops of 2 $\mu$L consisting of 1 $\mu$L reservoir solution and 1 $\mu$L TtHCSwt solution of 10 mg ml$^{-1}$ added to 10 mM lysine and 5 mM MgSO$_4$ were equilibrated against 500 $\mu$L reservoir solution at 293 K. A crystal obtained from a droplet using solution No. 25 (0.1 M MES-NaOH, pH 6.5, 0.01 M CoCl$_2$6H$_2$O, and 1.8 M ammonium sulfate) of Crystal Screen 2 was used for crystallographic analysis.

**Data collection, structure determination, and refinement** - Prior to data collection, crystals were briefly soaked in reservoir solution supplemented with 20% (v/v) glycerol as a cryoprotectant, flash-cooled in a nitrogen gas stream at 95 K, and stored in liquid nitrogen. Diffraction data of the $\alpha$-KG and HC complexes were collected at the BL-5A station of the Photon Factory, High Energy Accelerator Research Organization (KEK) (Tsukuba, Japan). Diffraction data of the lysine complex were collected at the NW12 station of the Photon Factory. Diffraction images were indexed, integrated, and scaled using the HKL-2000 program suite (24). SeMet multiple wavelength anomalous diffraction (Se-MAD) data were also collected at the BL-5A station of the Photon Factory. Details of data collection statistics are summarized in Table 1. Crystals of SeMet-labeled TtHCS-Chis contain one monomer per asymmetric unit. Se-MAD data were analyzed using SOLVE (25). All six Se atoms were identified from the six possible Se atoms in the primary sequence. Automated model building was performed by cycling with RESOLVE (26). The data of native TtHCS-Chis crystals collected at a wavelength of 1.0 Å were used for subsequent molecular replacement and crystallographic refinement. Molecular replacement was performed with MOLREP (27) in the CCP4 program suite (28) using the program Refmac 5.2 (29) and model correction in the electron density map was performed with Coot (30). Data collection, refinement statistics, and the results of Ramachandran plots produced by the program PROCHECK (31) are summarized in Table 1. Although Ile279 falls into the disallowed region in each complex, excellent map fitting ensures unambiguous model building. The figures were prepared using PyMol (http://pymol.sourceforge.net/). The atomic coordinates of the crystal structures determined in this study have been deposited in the RCSB PDB with accession number 2ZTJ, 2ZTK, 2ZYF, and 3A9I, respectively.

**Preparation of mutant enzymes** - The His72Leu mutation was introduced into the HCS-Nhis gene on pBluescript II SK(+) vector by a QuikChange kit (Stratagene-Japan, Tokyo) using oligonucleotide primers 5'-GCGGACTGGTAGGATGAGGGTCACCACCTT-3' and 5'-GCAGCTGGTACCCGCATCTCTGAGGTCACCACCTTG-3'. The mutated gene with the desired mutation was introduced into pHIS8 as described for constructing pHIS8-TtHCS-Nhis. Protein expression and purification of the His72Leu mutant enzyme were performed by the method described above.

**Enzyme assay** - TtHCS activity was measured at 323 K using 2,6-dichlorophenolindophenol (DCPIP) by monitoring the decrease in absorbance at 600 nm (32). To determine the $K_m$ of wild-type TtHCS-Nhis for acetyl-CoA, the reaction mixture contained 2-400 $\mu$M acetyl-CoA, 200 $\mu$M $\alpha$-KG, 5 mM MgSO$_4$ in 1 ml of 100 mM HEPES, pH 7.5. To determine the $K_m$ of wild-type TtHCS-Nhis for $\alpha$-KG, the reaction mixture contained 50 $\mu$M acetyl-CoA, 2-50 $\mu$M $\alpha$-KG, 5 mM MgSO$_4$ in 1 ml of 100 mM HEPES, pH 7.5. The reaction was initiated by the
addition of 30 μg enzyme to the reaction mixture. To determine the \( K_m \) of α-KG and acetyl-CoA of the His72Leu mutant, the concentrations of α-KG and acetyl-CoA varied in the range of 10–500 and 5–100 μM, respectively. Inhibition by lysine was analyzed in a reaction mixture containing 50 μM acetyl-CoA, 2-100 μM α-KG, 0-100 μM lysine, 5 mM MgSO\(_4\) in 1 ml of 100 mM HEPES, pH7.5, for wild-type TtHCS, and in a reaction mixture containing 200 μM acetyl-CoA, 100–2000 μM α-KG and 0–2000 μM lysine for TtHCS-His72Leu-Nhis.

To examine the effects of metal ions on the enzymatic activity of TtHCS, we first removed the bound metal ions co-purified with the enzyme by the following procedure. The purified protein was first dialyzed six times for 30 min against 20 mM Tris-HCl, pH8.0, containing 10 mM EDTA to remove the divalent metal ions, and then dialyzed for 30 min against 20 mM Tris-HCl, pH8.0, eight times. The sample was used for the activity assay in the presence of various metal ions, as described above.

### RESULTS AND DISCUSSION

**Overall structure of HCS** - The crystal structure of TtHCS complexed with α-KG was determined by the MAD method and subsequent molecular replacement, and refined to 1.80 Å (Fig. 1). This complex contains the electron density corresponding to α-KG in the catalytic pocket. In addition, in this complex there was strong electron density near α-KG. TtHCS prepared without EDTA treatment binds divalent metal ions co-purified with TtHCS from the *E. coli* expression system, as described below. Although Mg\(^{2+}\) ion was added to the crystallization buffer and a substantial portion of initially bound metal ion was expected to be replaced by Mg\(^{2+}\) ion, which is biologically relevant for TtHCS, the electron density of the metal ion was too strong to be identified as Mg\(^{2+}\) ion. We also prepared TtHCS crystals in buffer containing α-KG, acetyl-CoA, and Mg\(^{2+}\) ions, and determined the crystal structure at 1.96 Å resolution. The complex has electron density similar to that of α-KG. The strong electron density observed in both complexes probably corresponds to that of mixtures of several metal ions, Mg\(^{2+}\), Cu\(^{2+}\), Zn\(^{2+}\), and Fe\(^{2+}\), contained in the crystallization buffer. Since Cu\(^{2+}\) contains more electrons than Mg\(^{2+}\), although Cu\(^{2+}\) was not the most abundant metal ion in the crystallization buffer, the electron density of the metal ion was well-assigned as Cu\(^{2+}\) with a B-factor of 21.5 Å\(^2\), which is comparable to the average B-factor of protein in structural refinement (Table 1). Therefore, we tentatively named the complexes TtHCS/Cu\(^{2+}/\alpha\)-KG and TtHCS/Cu\(^{2+}/HC\) for substrate-bound and product-bound complexes, respectively, for convenience. We also determined the crystal structure of another TtHCS complex containing α-KG and Mg\(^{2+}\) at 2.15 Å resolution, which was prepared using TtHCS by EDTA treatment, as described in the Experimental Procedure. This complex was named TtHCS/Mg\(^{2+}/\alpha\)-KG and its electron density for metal ion matched Mg\(^{2+}\) very well. For each crystal structure, an asymmetric unit contained a monomer of TtHCS, sharing a very similar structure with the root mean square deviation (RMSD) below 0.2 Å. We here describe the overall structure of the TtHCS/Cu\(^{2+}/\alpha\)-KG complex with the highest crystallographic resolution. TtHCS is composed of a (β/α)\(_4\) TIM barrel domain (from Met1 to Gln245), which is named after triosephosphate isomerase, and C-terminal small domain I (from Pro246 to Ala320) forming an α9-α10-β9-α11-β10 fold (Fig. 1A). In the TIM barrel domain, the electron density of the loop region (Ser98 to His105) between β4 and α4 was not observed, indicating that this region is disordered in the crystal. Although some electron density was observed for the C-terminal small domain II (from Ser321 to Ala376), which is expected to form a 3-helix bundle by homology with small domain II in the linker domain of MtIPMS and by secondary structure prediction with PSIPRED server (33), we could not build a model of this region due to very weak density. When crystals of TtHCS/Cu\(^{2+}/\alpha\)-KG and TtHCS/Cu\(^{2+}/HC\) were dissolved and subjected to SDS-PAGE, protein bands corresponding to the original size of full-length TtHCS were observed (Fig. 1B); therefore, the weak electron density suggests that this small domain has high mobility in the crystal.

In these crystal structures of TtHCS, two TtHCS molecules related by two-fold
crystallographic symmetry form a dimer (Fig. 1C). The surface area (3,508 Å²) buried by dimerization is large (21.6%) compared with the total surface area of the monomer (16,240 Å²), indicating that the structural unit of TtHCS is a dimer. Three helices, α6, α7, and α8, mainly contribute to the stabilization of dimer formation. The α8 helix interacts with β7-α7 and β6-α6 loops of the TIM barrel domain from another subunit, and the α7 helix interacts with the α7 helix in the TIM barrel domain from another subunit. Arg173 on the α6 helix from one subunit interacts with Tyr176* on the α6 helix from another subunit by π-cation interaction (asterisks denote residues from another subunit hereafter). The C-terminal small domain I of one monomer extends to cover the active site of another monomer. Glu282 in the α10-β9 loop and Lys316 in the α11-β10 loop from C-terminal small domain I form ion pairs with Lys19* and Glu18* in the β1-α1 loop of the TIM barrel domain from another subunit, respectively (Fig. 1C). These ion bonds between chains might be a factor elevating thermostolerance over 70 °C of TtHCS.

DALI search (33) was performed to find proteins with a similar structure. HCS from Schizosaccharomyces pombe (SpHCS), whose crystal structure was determined most recently (34), showed the highest similarity (Z-score = 42.9, RMSD = 1.9 Å, PDB ID 3IVS). The TIM barrel domain structure of TtHCS is also similar to those of LiCMS and MtIPMS with RMSD of 42.9, RMSD = 1.9 Å, PDB ID 3IVS). The TIM barrel domain (34), showed the highest similarity (Z-score = 42.9, RMSD = 1.9 Å, PDB ID 3IVS). The TIM barrel domain. To identify the bound metal ion, we performed ICP analysis of the purified enzymes. The results showed that purified protein contains Cu²⁺, Fe²⁺, and Zn²⁺ at 0.25, 0.12, and 0.04 mol mol⁻¹, respectively. These metal ions could be removed to the background level by treatment of the purified enzyme with EDTA. We analyzed metal ion dependency for the reaction of TtHCS after EDTA treatment. In the absence of metal ions, TtHCS exhibited only negligible activity. On the other hand, the addition of Mg²⁺, Mn²⁺, and Co²⁺ to the reaction buffer significantly restored TtHCS activity (Table 2). Thus, we assume that Mg²⁺ or Mn²⁺ ion is a biologically relevant divalent cation. Both metal ions (Cu²⁺ and Mg²⁺) are bound to TtHCS with six ligands in similar octahedral geometry (Fig. 2AB), coordinated by Oe1 of Glu13 (2.2 and 2.4 Å bond length for HCS/Cu²⁺/α-KG and HCS/Mg²⁺/α-KG complexes, respectively), Ne2 of His197 (2.1 and 2.4 Å), C1-carboxyl group (2.3 and 2.4 Å), and C2-oxo group (2.0 and 1.9 Å) of α-KG in the equatorial position, and Nε2 of His195 (2.2 and 2.3 Å) and a water molecule (2.3 and 2.1 Å) in the axial position. A metal atom is also bound in octahedral geometry in the HCS/Cu²⁺/HC complex, which is mostly similar to that of the HCS/Cu²⁺/α-KG complex (Fig. 2AC).

In the TtHCS/Co²⁺/Lys complex, Co²⁺ was bound with a coordination similar to those of Cu²⁺ and Mg²⁺ in other complexes. Co²⁺ is coordinated by the α-carboxyl group (2.2 Å) and α-amino group (2.1 Å) of bound lysine in place of the C1-carboxyl group and the C2-keto group.

**Bound metal ion** - In TtHCS/Cu²⁺/α-KG and TtHCS/Cu²⁺/HC complexes, the electron density of the metal ion was at the center of the TIM barrel domain. To identify the bound metal ion, we performed ICP analysis of the purified enzymes. The results showed that purified protein contains Cu²⁺, Fe²⁺, and Zn²⁺ at 0.25, 0.12, and 0.04 mol mol⁻¹, respectively. These metal ions could be removed to the background level by treatment of the purified enzyme with EDTA. We analyzed metal ion dependency for the reaction of TtHCS after EDTA treatment. In the absence of metal ions, TtHCS exhibited only negligible activity. On the other hand, the addition of Mg²⁺, Mn²⁺, and Co²⁺ to the reaction buffer significantly restored TtHCS activity (Table 2). Thus, we assume that Mg²⁺ or Mn²⁺ ion is a biologically relevant divalent cation. Both metal ions (Cu²⁺ and Mg²⁺) are bound to TtHCS with six ligands in similar octahedral geometry (Fig. 2AB), coordinated by Oe1 of Glu13 (2.2 and 2.4 Å bond length for HCS/Cu²⁺/α-KG and HCS/Mg²⁺/α-KG complexes, respectively), Ne2 of His197 (2.1 and 2.4 Å), C1-carboxyl group (2.3 and 2.4 Å), and C2-oxo group (2.0 and 1.9 Å) of α-KG in the equatorial position, and Nε2 of His195 (2.2 and 2.3 Å) and a water molecule (2.3 and 2.1 Å) in the axial position. A metal atom is also bound in octahedral geometry in the HCS/Cu²⁺/HC complex, which is mostly similar to that of the HCS/Cu²⁺/α-KG complex (Fig. 2AC).

In the TtHCS/Co²⁺/Lys complex, Co²⁺ was bound with a coordination similar to those of Cu²⁺ and Mg²⁺ in other complexes. Co²⁺ is coordinated by the α-carboxyl group (2.2 Å) and α-amino group (2.1 Å) of bound lysine in place of the C1-carboxyl group and the C2-keto group.
of α-KG in other complexes, respectively.

**Determinants of the substrate specificity of TtHCS by comparison of structures between TtHCS and homologous enzymes** - Although TtHCS has low sequence similarity to MtIPMS (22%) and LiCMS (25%), the residues forming the active site are present at similar positions in the 3-D structures of these enzymes, sharing similar conformations (Fig. 3). This observation indicates that these enzymes catalyze the aldol-type condensation reaction by a common mechanism. While α-KG, the substrate for HCS, contains the carboxyl group at C5 position, α-KIV and pyruvate, the substrates for IPMS and CMS, respectively, have hydrophobic moieties. According to the characteristics of the substrates, the mechanism to recognize the distal portion of the substrate differs among TtHCS, MtIPMS, and LiCMS. MtIPMS recognizes the isopropyl chain of α-KIV by the hydrophobic wall formed by Leu143, His167, Tyr169, and Pro252 (Fig. 3A). LiCMS recognizes the C3 methyl group of pyruvate by the more projected hydrophobic wall formed by Leu81, Leu104, Tyr144, and Pro177 (Fig. 3B). In the TtHCS/Cu²⁺/α-KG complex, α-KG is bound to the enzyme by specific interactions with several residues (Figs. 2A and 3AB). The C1-carboxyl group forms a hydrogen bond with Thr166. The C2-keto group is recognized by the guanidium group of Arg12. The hydrophobic portion, C3-C4 atoms, of the substrate makes van der Waals contacts with the side chains of Ala164 and Leu94. The C5-carboxyl group is stabilized by hydrogen bonds with Ne2 (2.7 Å) of His72 and with Nε1 (2.8 Å) and Nε2 (3.3 Å) of Arg133. Furthermore, the C5-carboxyl group makes a water molecule-mediated hydrogen bond with Oγ of Ser135 (distances between the C5-carboxyl group and water and between water and Oγ of Ser135 are 2.6 and 3.3 Å, respectively). Thus, the well-designed recognition of α-KG assures that TtHCS recognizes α-KG with high affinity (11).

**Catalytic mechanism of TtHCS** - In the HC complex, HC, which is probably synthesized from α-KG and acetyl-CoA added to crystallization droplets by the HCS reaction, is bound to the active site of TtHCS in a manner similar to that of α-KG in the TtHCS/Cu²⁺/α-KG complex (Fig. 2C). The C1-carboxyl group, which is transferred from acetyl-CoA, forms hydrogen bonds with Ne of Gln16 and Ne2 of His292* from small domain I of another subunit. Interestingly, when the active sites are superimposed between TtHCS/Cu²⁺/HC and LiCMS/Zn²⁺/pyruvate/acetyl-CoA complexes, the C1-carboxyl group of HC is located close to the distal methyl group of acetyl-CoA (Fig. 4). The catalytic mechanism of HCS has been analyzed for HCS from *S. cerevisiae* (35). After α-KG and acetyl-CoA are bound, the catalytic reaction proceeds as follows. A general base abstracts a proton from the reactive methyl group of acetyl-CoA for enolization of the acetyl group, and a general acid stabilizes the enolated form of acetyl-CoA, which nucleophilically attacks the C2 atom of α-KG to form homocitreryl-CoA. In the final step, a metal-bound water hydrolyzes homocitreryl-CoA to yield homocitrate and CoA, aided by the general acid acting as a base. Through site-directed mutagenesis of HCS from *S. cerevisiae*, Qian et al proposed that the catalytic dyad of Glu155 and His309* corresponding to Glu137 and His292* in TtHCS, respectively, abstracts a proton from the methyl group of acetyl-CoA, where His309* is responsible for deprotonation of the methyl group of acetyl-CoA, and Glu155 stabilizes the protonated form of His309* by electrostatic interaction (35). The conserved Arg12 acts as a general acid in the catalytic mechanism. The present TtHCS/Cu²⁺/HC structure indicates that His292*, which interacts with the C1-carboxyl group of HC, is the most probable candidate to aid the hydrolysis of homocitreryl-CoA by acting as a base. The putative location of the C1-carboxyl group of HC close to the reactive methyl group of acetyl-CoA may support the hypothesis that His292* also acts as a general base for proton abstraction from the reactive methyl group of acetyl-CoA. The crystal structure of TtHCS complexed with HC for the first time revealed direct structural evidence for the involvement of His292* in the catalytic mechanism in this protein family. In the structures determined in this study, Glu137 resides somewhat apart from His292* (4.6 Å from Oε2 of Glu137 to Ne2 of His292*). Glu137 forms a hydrogen bond with Tyr303*. Since Tyr303* stabilizes His292* orientation by π-π stacking, we assume that Glu137 plays a role in the catalytic mechanism indirectly by placing the active site residue His292* in the correct orientation in TtHCS.
Most recently, the crystal structure of HCS from *S. pombe* (SpHCS) in apo-form and complexes with α-KG were determined (34). The structures of SpHCS are very similar to those determined in this study. However, His321*, corresponding to His292* in ThHCS is located 13Å apart from Glu167 (Glu137 in ThHCS). On this point, authors argued the involvement of this residue in the acid-base mechanism by constructing a mutant of this residue and speculated the possible conformational change by acetyl-CoA binding. In this study, the ThHCS structure for the first time provided evidence of an interaction between His292* and the acetyl moiety. Therefore, it is likely that the structures determined in this study are the highly catalytically relevant form of HCS.

**Acetyl-CoA recognition** - The crystal structure of LiCMS was determined as a complex with acetyl-CoA and pyruvate (38). Acetyl-CoA is bound to LiCMS in a U-shaped conformation. The acetyl group of acetyl-CoA is positioned near pyruvate with the carbonyl oxygen of the acetyl group hydrogen-bonded with the side chain Nζ2 of Arg16 and the methyl group sandwiched between the side chain of Or2 of Glu146 and the C2 atom of pyruvate. Arg16 and Glu146 are conserved as Arg12 and Glu137 in ThHCS. Thus, it can be expected that ThHCS recognizes acetyl-CoA in a similar manner. In LiCMS, the cysteamine moiety of acetyl-CoA interacts with Gln20 and Glu239 and the carbonyl group of β-alanine moiety of acetyl-CoA is recognized by Ser51. These residues are conserved as Gln16, Glu227, and Thr46 in ThHCS (Supplementary Fig. 1A). The dimethyl group of panthetheinyl moiety is stabilized by hydrophobic interaction with Val54 and Ala299* in LiCMS, which are conserved as Ala49 and Ala289* in ThHCS. Gly300* at the N-terminal helix dipole of α11 makes contact with the pyrophosphate group of acetyl-CoA. The residue is also conserved in ThHCS as Gly290*. In contrast to the strong conservation of amino acid residues for recognition of the diphosphopanthetheinyl moiety of acetyl-CoA, residues recognizing the adenine ring and 3’-phosphoribose are not conserved in ThHCS. In LiCMS, the adenine moiety of acetyl-CoA is stabilized through π-π stacking interaction with Phe83 of β3. The corresponding residue is replaced by Leu77 in ThHCS. Arg53, which electrostatically recognizes the 3’-phosphate group of the adenine ribose in LiCMS, is replaced by Val48 in ThHCS. In addition, comparison of the protein surface around the acetyl-CoA binding site shows that the cavity recognizing the adenosine moiety has a different shape in ThHCS (Supplementary Fig. 1B). These observations may suggest that adenine and 3’-phosphoribose moieties are recognized in a different way in ThHCS.

**Mechanism of feedback inhibition by lysine** – Despite the difference in the structure between lysine and α-KG, in the ThHCS/Co2+/lysine complex a lysine molecule occupies the active site in a manner similar to that of α-KG in the ThHCS/Cu2+/α-KG complex. The α-carboxyl and α-amino groups of bound lysine form hydrogen bonds with Thr166 and Tyr297*, respectively. The aliphatic side chain of lysine is stabilized by van der Waals contact with Ala164 and Leu94. The ε-amino group of bound lysine is stabilized by electrostatic interaction with Asp92 and Glu193 and by a hydrogen bond with Ser135. The ε-amino group of bound lysine is further stabilized by Glu43 and Asp219 via bridging water molecules. Lysine binding to the active center is accompanied by displacement of a set of amino acid residues surrounding the C5-carboxyl group of α-KG (Figs. 2AD and 5). The most significant change is found in the side chain rotation of Arg133, Asp92, and His72 (Fig. 5). Arg133, which forms an ion pair with α-KG in the ThHCS/Cu2+/α-KG complex, moves away from the active site with maintaining the electrostatic interaction with Glu193 and forms a new ion pair with Glu43. Asp92, which recognizes Arg160 in the ThHCS/Cu2+/α-KG complex, moves into the active site and interacts with bound lysine. Upon lysine binding, His72, which forms a hydrogen bond with α-KG, changes its side chain orientation to stack with Arg12. In the inhibitory complex, Arg12, which also interacts with Glu43, makes a van der Waals contact with the side chain of bound lysine. Conformational changes are also found in the opposite entrance side of the TIM barrel structure. Glu131, which recognizes Lys68 in the ThHCS/Cu2+/α-KG complex, rotates and forms a hydrogen bond with Tyr41 and electrostatic interaction with Arg160. These residues in the TIM barrel are all well conserved among HCSs (Supplementary Fig. 2), suggesting that concerted conformational change upon
binding the substrate/inhibitor is common mechanism of inhibition in HCS.

To investigate the conformational change upon the transition between active and inhibitory forms, we compared overall structures between TtHCS/Cu\(^{2+}\)/\(\alpha\)-KG, TtHCS/Co\(^{2+}\)/Lys, and SpHCS/Zn\(^{2+}\) complexes as an active form, an inhibitory form, and an apo form, respectively. Comparison suggests that the TIM barrel domain does not show gross conformational change (Fig. 1AEF). In addition, the structure of the C-terminal small domain I of the TtHCS/Cu\(^{2+}\)/\(\alpha\)-KG complex is similar to that of the corresponding domain of SpHCS. In the TtHCS/Co\(^{2+}\)/lysine complex, two \(\alpha\) of three helices in small domain II are seen. When this structure is compared with that of SpHCS in the closed form, \(\alpha\)11 and \(\alpha\)12 of small domain II, which correspond to \(\alpha\)12 and \(\alpha\)13 in SpHCS, are displaced in the lysine complex. In the SpHCS/Zn\(^{2+}\) complex, Asp394 on \(\alpha\)13 electrostatically interacts with Arg397 on \(\alpha\)13 helix from another subunit. In the TtHCS/Co\(^{2+}\)/lysine complex, the interactions are lost by gross displacement; \(\alpha\)11 helix is displaced by 44 Å with 308\(^\circ\) rotation. On the other hand, His292* and Tyr303*, which stacks with His292* to keep it in correct orientation, move 13 Å away from the active site, and instead Tyr297* occupies the vacant space to form a hydrogen bond with bound lysine in the TtHCS/Co\(^{2+}\)/Lys complex (Fig. 2D). His321*, which corresponds to His292* in TtHCS, is also found to be present 13 Å apart from the catalytic site in SpHCS. Therefore, the large displacement of His292* found in the lysine-bound complex may not be restricted to feedback inhibition.

As described above, substantial differences in structure are found in the C-terminal domains. Feedback-resistant HCSs (Lys20p and Lys21p) from Saccharomyces cerevisiae (ScHCS), which share the same domain structure with TtHCS, were obtained by random mutagenesis (36). Arg270 and Ser386 are replaced with Lys and Phe, respectively, in ScHCS\(^{Lys20p}\), while Gln366 is replaced with Arg in ScHCS\(^{Lys21p}\). Arg270 and Ser386 in ScHCS\(^{Lys20p}\) and Gln366 in ScHCS\(^{Lys21p}\) correspond to Pro259, Arg368, and Glu335, respectively, in TtHCS. When these residues are mapped on the structure of the TtHCS/Co\(^{2+}\)/Lys complex (Fig. 6), Arg368 is on the loop between \(\alpha\)11 and \(\alpha\)12 helices of the C-terminal small domain II forming an ionic pair with Glu256 on \(\alpha\)9 from the small domain I, on which Pro259 is located. Glu335 could not be mapped on the determined structure because this residue is expected to be located on the first \(\alpha\)-helix of the 3-helix bundle, which was not modeled in this study. This residue is near Gly337 at the N-terminal end of \(\alpha\)11 of small domain II. Information on mutation mapping and the substantial displacement of the C-terminal domains upon lysine binding suggest that inhibitory complex formation is accompanied by rearrangement of the interaction between the TIM barrel domain and small domain II.

In contrast to TtHCS, the feedback inhibitor, leucine, is bound at the regulatory domain in MtIPMS, where the regulatory domain is fused via the linker domain to C-terminus of the catalytic domain. The same mechanism is expected for CMS because the enzyme also possesses the C-terminal extension that could serve as a regulatory domain. Most recently, it was suggested that interdomain communication between the regulatory domain and catalytic domain is important for allosteric regulation in MtIPMS (37) and LiCMS (38), although it is unclear how the effector causes structural change at the catalytic site in both cases. Interdomain communication between the regulatory domain and catalytic domain might be important for allosteric regulation in this protein family.
of ScHCS by one–two orders of magnitude. We can therefore assume different inhibitory mechanisms between TtHCS and ScHCS although both HCSs exhibit apparent competitive inhibition by lysine with α-KG and, as described above, all amino acid residues responsible for lysine recognition are conserved in lysine-sensitive HCSs, including ScHCS.

Identification of His72 as a residue involved in feedback inhibition by lysine - Feedback inhibition by lysine in a competitive manner with α-KG (11, 39-41) and the present crystal structure of TtHCS/Co^{2+}/Lys complex indicate that lysine competes with α-KG for the overlapping sites in TtHCS. On the other hand, NifV, which is also the homocitrate synthase required to synthesize HC as the organic constituent of the Fe-Mo cofactor of nitrogenase in N2-fixing bacteria, is not regulated by lysine (42), although lysine-sensitive and -insensitive HCSs share amino acid sequence identity of about 25%. Comparison of the amino acid sequence between TtHCS and homologous enzymes indicates that His72 is conserved only among lysine-sensitive HCSs, suggesting that this residue is involved in feedback inhibition by lysine in TtHCS: the residue is replaced with Trp in lysine-insensitive HCS (NifV) and hydrophobic residues in IPMS and CMS (Supplementary Fig. 2). The crystal structures determined in this study reveal that His72 is located in the substrate-binding pocket and interacts with the C5-carboxyl group of α-KG in the TtHCS/Cu^{2+}/α-KG complex, while the same residue changes its side chain orientation to stabilize Arg12 to stack bound lysine in the TtHCS/Co^{2+}/Lys complex. These observations suggest that His72 serves as a structural switch determining which form, active or inactive, should be taken, depending on the availability of α-KG or lysine. We therefore constructed mutant His72Leu and examined the kinetic properties, including sensitivity to lysine. The kinetic parameters of the His72Leu mutant are comparable to those of the wild-type enzyme, except that the $K_m$ value of α-KG is 32-fold larger than that of the wild-type enzyme (Table 3). The increased $K_m$ value of α-KG is expected because His72 is also involved in binding the C5-carboxyl group of α-KG (Figs. 2 and 3). The wild-type enzyme is inhibited by lysine with $K_i$ of 70.7 μM in a competitive manner with α-KG. In contrast, the His72Leu mutant exhibited a significantly increased $K_i$ value, larger than 2,000 μM for lysine. These results indicate that His72 functions not only as a residue recognizing the C5-carboxyl group of α-KG but also as a switch controlling the activity. His72Leu mutation may sterically hinder Arg12 to stack the bound lysine.

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FOOTNOTES

Abbreviations: HCS, homocitrate synthase; HC, homocitrate; α-KG, α-ketoglutarate; acetyl-CoA, acetyl coenzyme A; IPMS, α-isopropylmalate synthase; CMS, citramalate synthase; ThHCS, Thermus thermophilus HCS; MtIPMS, Mycobacterium tuberculosis IPMS; LiCMS, Leptospira interrogans CMS; SpHCS, Schizosaccharomyces pombe HCS; ScHCS, Saccharomyces cerevisiae HCS; MES, 2-morpholinoethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2'-ethanesulfonic acid; RMSD, root mean square deviation; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; SeMet, Selenomethionine; Se-MAD, SeMet multiple wavelength anomalous diffraction.

FIGURE LEGENDS

FIGURE 1. Structures of ThHCS. A, Monomer structure of the ThHCS/Cu²⁺/α-KG complex. The (β/α)₈ TIM barrel domain and C-terminal small domain I are in green and yellow, respectively. Metal Cu²⁺ is shown as orange spheres and α-KG is shown as orange sticks. The disordered region (Ser98-His105) is shown as a dotted line. B, SDS-PAGE of ThHCS proteins dissolved from crystals. A single crystal was dissolved in 20 mM Tris-HCl, pH 8.0, and applied to 15 % SDS-PAGE. Lane 1, freshly purified ThHCS-Chis; lane 2, ThHCS/Cu²⁺/α-KG dissolved from a crystal; lane 3, ThHCS/Cu²⁺/HC dissolved from a crystal. C, Dimer structure of ThHCS/Cu²⁺/α-KG complex. Bound Cu²⁺ ion is shown as orange spheres and α-KG as sticks. One monomer is in green and the adjacent subunit is in gray. Intersubunit hydrogen bonds are shown in enlarged insets. D, Monomer structure of the ThHCS/Co²⁺/Lys complex. The (β/α)₈ TIM barrel domain, C-terminal small domain I, and C-terminal small domain II are in green, yellow, and purple, respectively. Metal Co²⁺ is shown as pink spheres and bound lysine is shown as magenta sticks. The disordered region (Leu317-Leu336) is shown as a dotted line. E, Monomer structure of the SpHCS/Zn²⁺ complex. Domain colors are the same as D. Metal Zn²⁺ is shown as pink spheres. F, Superposition of the structure of the ThHCS/Cu²⁺/α-KG complex (green) with those of the ThHCS/Co²⁺/Lys complex (cyan) and the SpHCS/Zn²⁺ complex (magenta). α-KG and Lys are shown as green and cyan sticks, respectively.

FIGURE 2. α-KG/HC/lysine-binding residues of ThHCS. A, ThHCS/Cu²⁺/α-KG complex; B, ThHCS/Mg²⁺/α-KG complex; C, ThHCS/Cu²⁺/HC complex; and D, ThHCS/Co²⁺/Lys complex. Residues interacting with ligands are shown as sticks. Bound Cu²⁺, Mg²⁺, and Co²⁺ ions are shown as orange, purple, and pink spheres, respectively. α-KG, HC, and Lys are shown as yellow, orange, and purple sticks, respectively. Water molecules are shown as red spheres. Hydrogen bonds are shown as dashed lines. F₀ - Fₑ omit electron density maps for α-KG, HC, and Lys are shown as yellow, orange, and purple mesh, respectively, and contoured at 3.0 σ, 3.0 σ, 3.6 σ, and 3.5 σ, for A-D, respectively. Residues from the adjacent subunit are indicated by asterisks.

FIGURE 3. Superposition of substrate-binding sites of ThHCS and related enzymes. A, ThHCS/Cu²⁺/α-KG complex (green) and MtIPMS/Zn²⁺/α-KIV complex (cyan); B, ThHCS/Cu²⁺/α-KG complex (green) and LiCMS/Zn²⁺/Pyr complex (pink). Bound Cu²⁺ and Zn²⁺ ions are shown as orange and gray spheres, respectively. α-KG, α-KIV, and Pyr are shown as yellow, dark blue, and magenta sticks, respectively. Hydrogen bonds between ligands and proteins are shown as dashed lines.

FIGURE 4. Superposition of catalytic site between ThHCS/Cu²⁺/HC complex and LiCMS/Zn²⁺/Pyr/acetyl-CoA complex. The ThHCS/Cu²⁺/HC complex and the LiCMS/Zn²⁺/Pyr/acetyl-CoA complex are shown as green and cyan sticks, respectively. Hydrogen bonds between ligand and proteins are shown as dashed lines. His302* of LiCMS, corresponding to His292* of ThHCS, is not seen due to disorder of the regions containing this residue in the LiCMS.
FIGURE 5. Comparison of recognition by TtHCS between α-KG and lysine. A, Recognition of C5-carboxyl group of α-KG; B, Recognition of ε-amino group of Lys; C and D, Schematic drawing of recognition of α-KG and Lys, respectively.

FIGURE 6. Mapping of mutations conferring feedback-resistance to ScHCSs on structure of TtHCS/Co^{2+}/Lys complex. A dimer structure of the TtHCS/Co^{2+}/Lys complex is shown. In a monomer, (β/α)_8 TIM barrel domain, C-terminal small domain I, and C-terminal small domain II are shown in green, yellow, and purple, respectively. The adjacent subunit is in gray. Metal Co^{2+} is shown as pink spheres and bound lysine is shown as magenta sticks.
Table 1. X-ray data collection and refinement statistics

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<td><strong>Phasing</strong></td>
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<td>FOMc</td>
<td>0.39 (0.67)c</td>
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<td><strong>Refinement</strong></td>
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<tr>
<td>Resolution (Å)</td>
<td>30.66-1.80</td>
<td>44.36-1.96</td>
<td>31.5-2.15</td>
<td>35.6-1.80</td>
<td>17.1-21.3</td>
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<tr>
<td>R-factor/Rfree (%)</td>
<td>19.9/22.4</td>
<td>19.4/22.2</td>
<td>20.5/23.2</td>
<td>17.1/21.3</td>
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<td>No. of protein atoms</td>
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<td>2,502</td>
<td>2,745</td>
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<td>No. of α-KG</td>
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<td>1</td>
<td>-</td>
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<tr>
<td>No. of HC</td>
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<td>-</td>
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<tr>
<td>No. of Lys</td>
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<tr>
<td>No. of water molecules</td>
<td>423</td>
<td>370</td>
<td>234</td>
<td>185</td>
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<td>Average B-factor (Å²)</td>
<td>19.3</td>
<td>22.5</td>
<td>27.3</td>
<td>16.5</td>
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<td>α-KG</td>
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<td>56.0</td>
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<td>HC</td>
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<td>20.4</td>
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<tr>
<td>Metal ion</td>
<td>21.5</td>
<td>26.5</td>
<td>37.3</td>
<td>16.4</td>
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<td>Water</td>
<td>55.5</td>
<td>42.0</td>
<td>48.9</td>
<td>26.3</td>
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<td>R.m.s.d from ideal values</td>
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<tr>
<td>Bond length (Å)</td>
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<td>0.013</td>
<td>0.014</td>
<td>0.012</td>
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<tr>
<td>Bond angles (deg.)</td>
<td>1.4</td>
<td>1.6</td>
<td>1.4</td>
<td>1.3</td>
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<tr>
<td>Ramachandran plot (%)</td>
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<tr>
<td>Favored</td>
<td>90.8</td>
<td>89.4</td>
<td>90.9</td>
<td>91.4</td>
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<td>Additional allowed</td>
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<td>9.2</td>
<td>7.7</td>
<td>7.6</td>
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<tr>
<td>Generously allowed</td>
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<td>1.1</td>
<td>1.1</td>
<td>0.7</td>
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<tr>
<td>Disallowed</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.3</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

aValues in parentheses are for the highest resolution shell.

b\(R\text{merge} = \Sigma|I_i - <I>|/\Sigma|I|\).

cFOMc was calculated with the program SOLVE.
Table 2. Effect of metal ion on enzymatic activity

<table>
<thead>
<tr>
<th>Additive</th>
<th>Specific activity (mU/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>41.5 ± 1.4</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>39.2 ± 3.8</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>33.3 ± 1.9</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>7.5 ± 1.6</td>
</tr>
<tr>
<td>NiCl₂</td>
<td>3.6 ± 0.6</td>
</tr>
<tr>
<td>ZnSO₄</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>CuCl₂</td>
<td>0.3 ± 0.0</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>0.8 ± 0.2</td>
</tr>
</tbody>
</table>
Table 3. Kinetic parameters of HCS and His72Leu mutant

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$K_i$ for lysine (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α-KG</td>
<td>acetyl-CoA</td>
<td></td>
</tr>
<tr>
<td>HCS$^a$</td>
<td>5.4 ± 0.7</td>
<td>10.9 ± 0.4$^b$</td>
<td>70.7 ± 6.1</td>
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<tr>
<td></td>
<td>33 ± 5.3</td>
<td>41 ± 4</td>
<td></td>
</tr>
<tr>
<td>His72Leu$^a$</td>
<td>174 ± 13</td>
<td>14.9 ± 0.5$^b$</td>
<td>&gt;2000$^c$</td>
</tr>
<tr>
<td></td>
<td>37 ± 4.2</td>
<td>31 ± 1</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Recombinant enzymes with N-terminal (his)$_8$-tags were used.

$^b$k$_{cat}$ for α-KG is lower than that for acetyl-CoA because HCS and the mutants are significantly subject to substrate inhibition by acetyl-CoA.

$^c$The His72Leu mutant was significantly desensitized to feedback inhibition and 50% inhibition was not achieved even in the presence of 2000 μM lysine.
Okada, T., et al., Scheme 1

\[
\text{\(\alpha\)-Ketoglutarate} + \text{Acetyl-CoA} \rightarrow \text{Homocitrate} + \text{CoA-SH}
\]
Okada, T., et al., Fig. 1
Okada, T., et al., Fig. 2
Okada, T., et al., Fig. 6
Mechanism of substrate recognition and insight into feedback inhibition of homocitrate synthase from thermus thermophilus
Takuya Okada, Takeo Tomita, Asri Peni Wulandari, Tomohisa Kuzuyama and Makoto Nishiyama

J. Biol. Chem. published online December 7, 2009

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