Characterization of bacterial homocitrate synthase involved in lysine biosynthesis

Asri Peni Wulandari[a], Junichi Miyazaki[a], Nobuyuki Kobashi[a], Makoto Nishiyama[a,*], Takayuki Hoshino[b], Hisakazu Yamane[a]

[a] *Biotechnology Research Center, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan*
[b] *Institute of Applied Biochemistry, Tsukuba University, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan*

Received 7 March 2002; revised 16 May 2002; accepted 21 May 2002

First published online 3 June 2002

Edited by Stuart Ferguson

Abstract In *Thermus thermophilus* homocitrate synthase (HCS) catalyzes the initial reaction of lysine biosynthesis through α-aminoadipic acid, synthesis of homocitrate from 2-oxoglutarate and acetyl-CoA. HCS is strongly inhibited by lysine, indicating that the biosynthesis is regulated by the endproduct at the initial reaction in the pathway. HCS also catalyzes the reaction using oxaloacetate in place of 2-oxoglutarate as a substrate, similar to citrate synthase in the tricarboxylic acid cycle. Several other properties of *Thermus* HCS and an evolutionary relationship of the biosynthetic pathway in the bacterium to other metabolic pathways are also described.

Key words: α-Aminoadipic acid pathway; Feedback inhibition; Homocitrate synthase; Lysine biosynthesis; *Thermus thermophilus*

1. Introduction

Thermus thermophilus HB27 is an extreme thermophile showing optimal growth around 70°C. In most bacteria lysine has been believed to be synthesized from aspartic acid through the diaminopimelic pathway. However, recently it was found that in *T. thermophilus* lysine is synthesized via α-aminoadipic acid [1,2], which is a precursor for the alternative lysine biosynthetic pathway in fungi and yeast [3–5]. Our previous studies on cloning of the genes encoding enzymes involved in the lysine biosynthesis revealed that the *Thermus* lysine biosynthetic pathway is similar to those of fungi and yeast in that the pathway starts with homocitrate synthesis and includes α-aminoadipic acid as a biosynthetic intermediate [6] (Fig. 1). The reactions from homocitrate to α-aminoadipic acid are also similar to the reactions for leucine biosynthesis and a portion of the tricarboxylic acid (TCA) cycle, and distinct similarity in amino acid sequence is observed between the corresponding enzymes [6,7]. On the other hand, our recent studies showed that the second half of the pathway, conversion of α-aminoadipic acid to lysine, differs from that of the lower eukaryotes [8,9]. In the lower eukaryotes α-aminoadipic acid is converted to lysine through an adenylated derivative and saccharopine as the biosynthetic intermediates. In *T. thermophilus* the corresponding conversion proceeds in a manner similar to that of a portion of arginine biosynthesis. Based on these findings, we suggested that enzymes involved in *Thermus* lysine biosynthesis are evolutionarily related to the enzymes required for biosynthesis of leucine and arginine along with the related portion of the TCA cycle [6,8,9].

Amino acid biosynthesis is usually regulated at the protein level by an excess amount of the endproduct in the pathway, via so called feedback inhibition, where the enzyme involved in the first step of the pathway is the target for the regulation in most cases. In the lysine biosynthesis of lower eukaryotes, homocitrate synthase (HCS), that also catalyzes the synthesis of homocitrate with 2-oxoglutarate and acetyl-CoA, is regulated by lysine, while in other organisms synthesizing lysine through the diaminopimelic acid pathway, aspartate kinase is the target for feedback regulation by lysine. *Thermus* lysine biosynthesis resembles biosynthesis of arginine, and some of the lysine biosynthetic enzymes in the *Thermus* pathway were recently suggested to play a role in the biosynthesis of arginine as well [8,9]. It is, therefore, particularly interesting to elucidate how the lysine biosynthesis is regulated in this microorganism in order to further clarify the regulation and relationship of these biosynthetic pathways.

In this paper, we describe the catalytic properties of HCS from *T. thermophilus* and the regulation of the lysine biosynthesis in this microorganism.

2. Materials and methods

2.1. Enzymes and chemicals

Restriction endonucleases and *Z-Taq* were purchased from Takara Shuzo (Kyoto, Japan), and oligonucleotides were purchased from Genset (Tokyo, Japan). 2-Oxoglutarate and acetyl-CoA were purchased from Wako Pure Chemicals (Tokyo, Japan). Lysine, arginine, ornithine, leucine, α-aminoadipate, α-ketoadipate, 2-isovaleric acid, 2-aminoethylcysteine, and glutaraldehyde were purchased from Kanto Chemicals (Tokyo, Japan).

2.2. Bacterial strains

Escherichia coli DH5 α [Δ(U169(ϕ80*lacZ*ΔM15)) *recA*1 *endA*1 *hsdR*17($r_k^- m_k^+$) *supE*44 λ^- *thi*-1 *gyrA*96 *relA*1] was used for DNA manipulation, and *E. coli* BL21(DE3) [*ompT* *hsdS*$_B$($r_b^- m_b^-$) *gal* (λ cI^{ts857} *ind*1 *Sam*7 *nin*5 *lac*UV7-T7 gene1) *dcm* (DE3)] was used as the host for gene expression.

2.3. Construction of expression plasmid

Polymerase chain reaction (PCR) was performed using pET151$^+$ containing the *hcs* gene from *T. thermophilus* HB27 [1] as the template

*Corresponding author. Fax: (81)-3-5841 8030.
E-mail address: umanis@mail.ecc.u-tokyo.ac.jp (M. Nishiyama).

0014-5793/02/$22.00
PII: S0014-5793(02)02877-6

and the two following oligonucleotides as the primers, 5′-GGGCATATGCGGGAGTGGAAGATTATTGAC-3′ and 5′-TTAAAAGCTTCGCCGTGATCCACTCCCGGAGGAT-3′. The former was designed to contain a *Nde*I site around the translational initiation codon, ATG, and the latter was designed to contain a *Hin*dIII site and direct the 6×histidine-tag ((His)$_6$-tag) sequence at the COOH-terminus of HCS. PCR conditions were as follows: 95°C for 5 min, 25 cycles of 95°C for 20 s and 72°C for 20 s, and 72°C for 5 min. The amplified DNA fragment was digested with *Nde*I and *Hin*dIII, and ligated with *Nde*I/*Hin*dIII-digested pET26b$^+$ (Novagen, Madison, WI, USA). The resulting plasmid was named pET-*hcs*-(His)$_6$-tag. Another plasmid (called *pET-hcs*) that directed the production of HCS without the (His)$_6$-tag was also constructed using 5′-AAAAAGCTTTTACGCCGTGATCCACTCCCGGAG-3′, in place of the latter primer for PCR in the same way as described above. The nucleotide sequences of the amplified fragments were directly confirmed by sequencing.

2.4. Production and purification of HCS

E. coli BL21(DE3) cells harboring the pET-*hcs*-(His)$_6$-tag were cultured in 10 ml 2×YT medium [10] supplemented with kanamycin (100 μg/ml) and incubated overnight at 25°C. A small portion (2.5 ml) was taken from the preculture and transferred to 250 ml of fresh medium, and the culture was continued at 25°C until the optical density of the culture reached 0.6. Isopropyl-β-D-thiogalactopyranoside was then added to the culture to a final concentration of 1 mM. The culture was further continued for an additional 12 h at 25°C. The cells were disrupted by ultrasonication after being collected by centrifugation and suspended in 10 mM Tris–HCl (pH 7.5) containing 0.1% Triton X-100, 0.5 M NaCl, and 5 mM imidazole. The sonicate was centrifuged at 5000×*g* for 15 min and the supernatant was collected. The supernatant was then applied onto a Ni-nitroacetic acid (NTA) column (ϕ 10 mm, 3 cm) at room temperature. Non-specifically bound proteins were washed out with 40 ml washing buffer, 10 mM Tris–HCl (pH 7.5) containing 10 mM imidazole. HCS was eluted with the same buffer containing 70 mM imidazole. The eluate was dialyzed against 10 mM Tris–HCl (pH 7.5) containing 0.5 M NaCl and 0.1% Triton X-100. HCS thus prepared showed over 95% purity on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [11], and was used for the enzymatic characterization.

2.5. Estimation of subunit organization

Sedimentation equilibrium analysis of the purified HCS (0.225–0.9 mg/ml in 10 mM Tris–HCl buffer, pH 7.5, containing 10 mM NaCl) was carried out with a Beckman Optima XL-A analytical centrifuge fitted with a Beckman An-60Ti analytical rotor. The quaternary structure of the enzyme was analyzed according to the procedure of Van Holde and Baldwin [12]. Molecular size calibration of HCS was also done with size exclusion gel column chromatography using Superose 6HR 10/30 FPLC column (Amersham) with an Amersham gel filtration calibration kit. The column was equilibrated with 10 mM Tris–HCl, pH 7.5, containing 100 mM NaCl, and proteins were eluted at a flow rate of 0.5 ml/min.

2.6. Enzyme assay

The standard reaction mixture for measuring HCS activity contained 50 μM acetyl-CoA, 0.5 mM 2-oxoglutarate, 5 mM $MgSO_4$ in 1 ml of 0.1 M Tris–HCl (pH 7.5). After addition of about 35 μg of HCS, the reaction mixture was incubated at 50°C for 10 min. The reaction was then terminated by addition of an equal amount (v/v) of ethanol. The control reaction was done without 2-oxoglutarate. After that, 5 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was added to the mixture and incubated for another 5 min at room temperature. The amount of 5-thio-2-nitrobenzoic acid generated from DTNB by reduction with CoASH released by the enzyme reaction, was determined by measuring the absorbance at 412 nm (ε = 13 600 M^{-1} cm^{-1}). One unit was defined as the amount of the enzyme that released 1 μmol 5-thio-2-nitrobenzoic acid, which is produced equimolarly to CoASH, per minute. Protein was determined by the method of Bradford [13] using a Bio-Rad protein assay kit (Nippon Bio-Rad, Tokyo, Japan). Kinetic parameters, K_m and k_{cat} values, were determined by varying concentrations of both substrates. Data were analyzed by using initial velocity programs, HYPER, of Cleland [14].

The reaction mixture for measuring citrate synthase activity contained 25–125 μM acetyl-CoA and 0.05–1.25 mM oxaloacetate, in 1 ml of 0.1 M Tris–HCl (pH 7.5) supplemented with 10 mM KCl. The reaction and data analysis were done as above.

2.7. Dependence of enzyme activity on temperature and thermal stability

To examine the temperature dependence of the reaction of HCS, the enzyme reaction was performed at various temperatures ranging from 20 to 90°C. Thermal stability was analyzed by measuring the activity remaining after heating the enzyme solution at various temperatures for an appropriate time.

3. Results

3.1. Purification of HCS and estimation of its structural organization

A large amount of the protein with a molecular weight of about 45 kDa (data not shown) was produced in *E. coli* cells by using either system for directing the production of HCS or (His)$_6$-tagged HCS. The specific activities in crude extracts containing HCS or (His)$_6$-tagged HCS were almost the same (0.50 and 0.41 U/mg, respectively), suggesting that the (His)$_6$-tag attached to the C-terminus of HCS had no great effect on the catalytic activity of HCS. Since we had difficulty in purifying HCS without (His)$_6$-tag (several column chromatographic steps were required), and a single step with a Ni-NTA column was sufficient to give a single band on SDS–PAGE, (His)$_6$-tagged HCS attached to the COOH-terminus was used for characterization of HCS. (His)$_6$-tagged HCS is called HCS throughout the text.

To evaluate the quaternary structure of HCS, we carried out sedimentation equilibrium analysis using a partial specific volume of 0.7423 calculated from the amino acid composition. The sedimentation equilibrium profile could be fitted by postulating an equilibrium between monomer and homodimer with an equilibrium constant of 4.19 M^{-1}. We therefore concluded that HCS was present as a mixture of monomer and homodimer in our experimental concentration range of the enzyme. In activity measurements using an HCS concentration of 35 μg/ml, most of the HCS protein (95%) was present in a monomeric form. On the other hand, size exclusion gel chromatography for 2 mg/ml HCS, where monomer and homodimer are present in the ratio of about 1 to 1 considering the dilution during the chromatography, gave a somewhat broad elution peak with the peak top corresponding to approximately 60 kDa. Since the concentrations of HCS are not high in *Thermus* cells, we conclude that HCS functions as a monomer.

3.2. Properties of Thermus HCS

We next analyzed the effect of temperature on the enzyme properties. We at first determined an activity–temperature

Table 1
Kinetic parameters of HCS from *T. thermophilus*

Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (M^{-1} min^{-1})
2-Oxoglutarate	44 ± 5	(59 ± 1)[a]	(1.3×10^6)[a]
Acetyl-CoA[b]	32 ± 6	92 ± 10	2.9×10^6

[a] k_{cat} value was underestimated because a saturating amount of acetyl-CoA could not be added due to the inhibition by the compound.
[b] Determined in the presence of a saturating amount of 2-oxoglutarate.

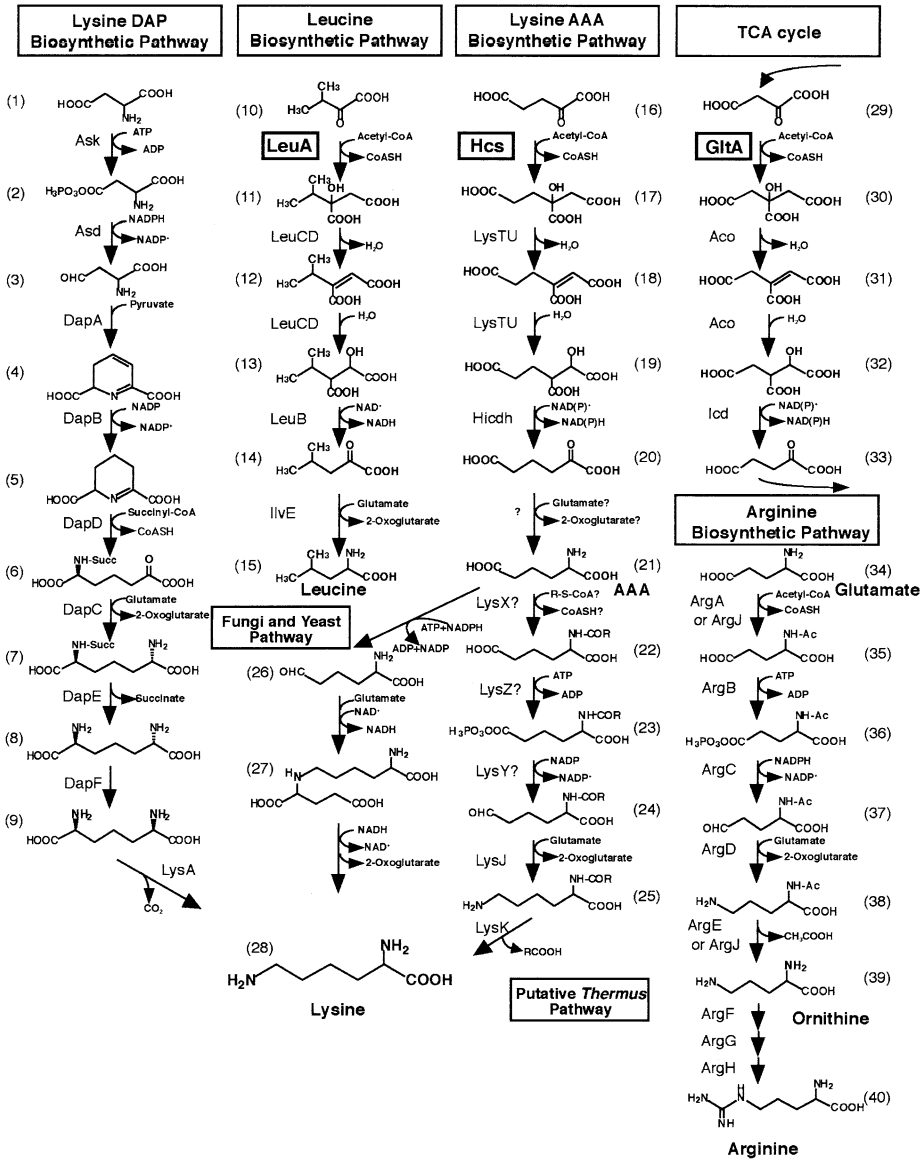

Fig. 1. Lysine AAA biosynthetic pathway and related metabolic pathways. (1) L-aspartate; (2) L-aspartyl-γ-phosphate; (3) L-aspartate semialdehyde; (4) L-dihydrodipicolinate; (5) L-tetrahydrodipicolinate; (6) N^2-succinyl-L-2-amino-6-oxopimelate; (7) N^2-succinyl-L,L-diaminopimelate; (8) L,L-diaminopimelate; (9) D,L-diaminopimelate; (10) 2-oxoisovalerate; (11) 2-isopropy-L-malate; (12) dimethylcitraconate; (13) 3-isopropyl-L-malate; (14) 2-oxoisocaproate; (15) L-leucine; (16) 2-oxoglutarate; (17) homocitrate; (18) homoaconitate; (19) homoisocitrate; (20) 2-oxoadipate (α-ketoadipate); (21) α-aminoadipate; (22) N^2-acetyl-L-aminoadipate; (23) N^2-acetyl-L-aminoadipyl-δ-phosphate; (24) N^2-acetyl-L-aminoadipate semialdehyde; (25) N^2-acetyl-L-lysine; (26) α-aminoadipate semialdehyde; (27) L-saccharopine; (28) L-lysine; (29) 2-oxoacetate; (30) citrate; (31) aconitate; (32) isocitrate; (33) 2-oxoglutarate; (34) L-glutamate; (35) N^2-acetyl-L-glutamate; (36) N^2-acetyl-L-glutamyl-γ-phosphate; (37) N^2-acetyl-L-glutamate semialdehyde; (38) N^2-acetyl-L-ornithine; (39) L-ornithine; (40) L-arginine. Enzymes involved in the reactions are also shown as their gene names. GltA (citrate synthase), LeuA (2-isopropylmalate synthase), and Hcs (homocitrate synthase) are boxed.

profile by measuring the enzyme activity at various temperatures. The profile was bell-shaped with an optimal temperature for the reaction around 60°C (Fig. 2A). Considering that a chemical reaction proceeds with a faster velocity as the temperature for the reaction is raised, it was probable that the decrease in the activity above 70°C was due to denaturation of HCS. Consistent with this, HCS lost its activity rapidly above 70°C (Fig. 2B).

Kinetic analysis of *Thermus* HCS yielded K_m values of 44 μM and 32 μM for 2-oxoglutarate and acetyl-CoA, respectively (Table 1). A low value of 92 min^{-1} for the k_{cat} value of *Thermus* HCS was obtained. HCS was found to be inhibited by excess amounts of acetyl-CoA (data not shown).

3.3. Regulation of HCS by endproduct

Since in most cases amino acid biosynthesis is regulated in the first step of the biosynthetic pathway, we added lysine and its related compounds to the HCS reaction mixture and examined their effects on the activity. This analysis revealed that HCS was highly sensitive to lysine (Fig. 3). The fact that all three lines in the double reciprocal plots (1/*v* vs 1/[2-oxoglutarate]) cross at the same point of the vertical axis (1/*v*) indicates that lysine inhibited HCS competitively with 2-oxoglutarate. The K_i value for lysine in the inhibition was calculated to be 9.4 μM.

We next examined the effect of other compounds on the activity of HCS. Among seven other compounds examined,

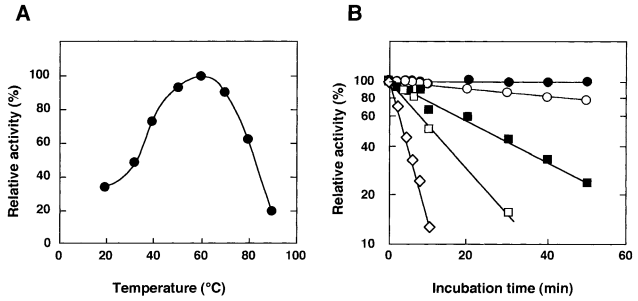

Fig. 2. Effects of temperature on the enzyme activity. A: Temperature–activity profile of HCS. B: Thermal stability of HCS. HCS (1 mg/ml) was incubated at the following temperatures: closed circle, 50°C; open circle, 60°C; closed square, 70°C; open square, 75°C; diamond, 85°C. After incubation for the indicated period, the enzyme was rapidly chilled on ice for 10 min. The remaining activity was then measured by the standard assay method.

two compounds, arginine and 2-aminoethylcysteine, exhibited an inhibitory effect on HCS (Fig. 4), although the effect was smaller than that of lysine. Thus it is obvious that HCS is regulated by lysine in *T. thermophilus*.

3.4. Substrate specificity of HCS

When a homology search was carried out using the amino acid sequence of HCS as the query, LeuA was found to show the highest similarity. LeuA is a member of a group of leucine biosynthetic enzymes and catalyzes the initial reaction, synthesis of 2-isopropylmalate from 2-isovaleric acid and acetyl-CoA (see Fig. 1) [6]. Despite the similarity in amino acid sequence, HCS did not show any detectable activity for 2-oxoisovaleric acid (Table 2). On the other hand, citrate synthase, a member of the TCA cycle enzymes, catalyzes a similar reaction where oxaloacetate is used as a substrate in place of 2-oxoglutarate in the lysine biosynthesis. Interestingly, citrate synthase shows no similarity in amino acid sequence to HCS or LeuA, despite the similarity in the reaction and in substrate structures. When oxaloacetate was added in place of 2-oxoglutarate to the reaction mixture for HCS, no activity was detected in the absence of KCl. However, in the presence of KCl, HCS showed significant activity using oxaloacetate as the substrate. By kinetic analysis, the K_m values for oxaloacetate and acetyl-CoA were determined to be 255 and 28 μM, respectively, and the k_{cat} value was 58 min^{-1}. In the reaction no inhibition by acetyl-CoA was observed.

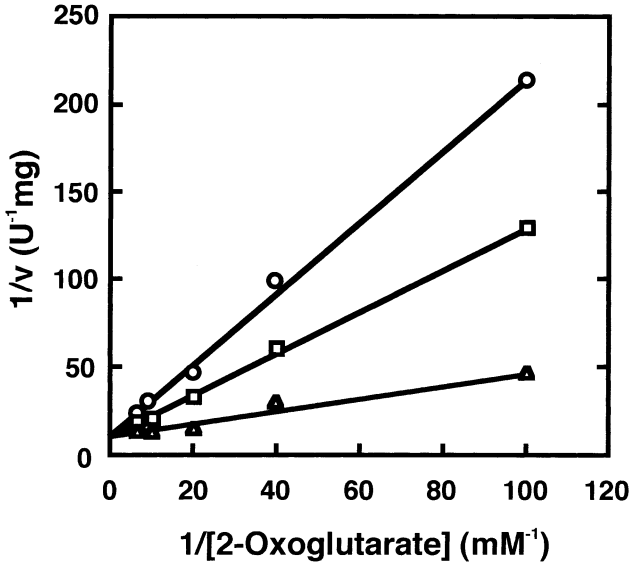

Fig. 3. Feedback inhibition by lysine. Triangle, without lysine; square, 20 μM lysine; circle, 40 μM lysine.

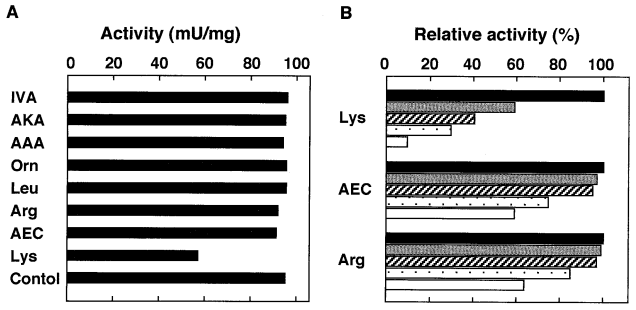

Fig. 4. Effect of addition of lysine and related compounds on the enzyme activity. Catalytic reactions were performed in the presence of lysine and its related compounds. A: HCS activity in the presence of the indicated compounds at 10 μM. B: The relative HCS activity in the presence of inhibitors at the following concentrations: closed bar, without additives; halftone bar, 10 μM; diagonally striped bar, 20 μM; dotted bar, 50 μM; open bar, 100 μM. Abbreviations are: IVA, 2-isovaleric acid; AKA, α-ketoadipic acid; AAA, α-aminoadipic acid; Orn, ornithine; Leu, leucine; Arg, arginine; AEC, 2-aminoethylcysteine; Lys, lysine.

4. Discussion

In the present study, we characterized HCS involved in lysine biosynthesis in *T. thermophilus* HB27. There are multimeric and monomeric types of HCSs. HCS (NifV) from *Azotobacter vinelandii*, which synthesizes homocitrate as the organic constituent of FeMo cofactor of nitrogenase for nitrogen fixation, has been shown to be a dimeric enzyme composed of two identical subunits [15]. In the fungus, *Penicillium chrysogenum*, the molecular weight of the native HCS responsible for its lysine biosynthesis was reported to be 155 ± 10 kDa [16], suggesting that the enzyme consists of multiple identical subunits of 54 kDa. Our analyses suggest that *Thermus* HCS is present mostly as a monomer although the enzyme forms a homodimer at higher concentrations. Thus, it appears that bacterial HCS has a simple subunit organization and fungal enzyme has a complicated one. The difference in the subunit organization may reflect evolutionary differences between bacteria and fungi. On the other hand, like the fungal HCS, many allosteric enzymes that are inhibited in a feedback manner are present in oligomeric forms [17]. *Thermus* HCS is, however, inhibited by lysine. It is therefore of interest to ex-

Table 2
Kinetic analysis of HCS for other related compounds

Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (M^{-1} min^{-1})
Oxaloacetate[a,b]	255 ± 60	58 ± 4	2.3×10^5
Acetyl-CoA[a,b]	28 ± 5	58 ± 3	2.1×10^6
Oxaloacetate[c]	N.D.	N.D.	
2-Oxoisovalerate[b]	N.D.	N.D.	

[a]Determined in the presence of saturating amounts of the other substrates.
[b]In the presence of 10 mM KCl.
[c]In the absence of KCl.
N.D.: not detected.

amine whether or not the presence of lysine affects the subunit organization of *Thermus* HCS.

The K_m values of *Thermus* HCS for 2-oxoglutarate and acetyl-CoA were determined to be 44 and 32 μM, respectively, in our HCS assay. These values are significantly smaller than those of HCSs from most other sources; the K_m value for 2-oxoglutarate ranging from 2.2 to 11.8 mM and that for acetyl-CoA ranging from 0.06 to 0.2 mM [15,18–20]. An exception is HCS from *Candida maltosa*. This HCS from the yeast has K_m values of 25 and 33 μM for 2-oxoglutarate and acetyl-CoA, respectively [21]. At this point, since the gene encoding HCS has not yet been cloned from *C. maltosa*, we cannot compare the amino acid sequence of *Thermus* and *Candida* enzymes. Comparison of the primary sequences and three-dimensional structures between these HCSs will reveal the amino acid residues affecting the affinity to the substrates as well as the mechanism for the substrate recognition.

T. thermophilus grows at elevated temperatures and can grow up to 82°C. Enzymes contained in *Thermus* are known to possess extremely high thermostability, some of them stable even at 90°C [22,23]. Compared to those cases, HCS of *T. thermophilus* is not as stable (Fig. 2B). This may suggest that the enzyme could be a candidate that determines an upper limit of the temperature for the growth of this bacterium.

We showed here that *Thermus* HCS has the ability to utilize oxaloacetate as substrate with similar turnover numbers and K_m values for acetyl-CoA. When the catalytic efficiency was compared using $k_{cat}/K_m^{2\text{-oxoglutarate/oxaloacetate}}$, the *Thermus* enzyme catalyzed the HCS reaction about 13 times higher, compared to using oxaloacetate as the substrate. Therefore, it is likely that the enzyme is designed to have homocitrate synthesis as its primary function. However, the catalytic efficiency also suggests that HCS can potentially substitute for citrate synthase in the synthesis of citrate in vivo. Four reactions starting from citrate synthesis in the TCA cycle proceed in a manner similar to those in the corresponding reactions in leucine biosynthesis and lysine biosynthesis in *T. thermophilus*. In addition to similarity in the reactions, most of the enzymes involved in the corresponding conversions share distinct similarity in amino acid sequence [6,7]. However, there is an exception: the amino acid sequence of citrate synthase in the TCA cycle is similar to neither that of HCS nor 2-isopropylmalate synthase. We previously suggested the possibility that an enzyme of the TCA cycle evolved from an ancestral enzyme involved in lysine (leucine) biosynthesis, based on the assumption that the earliest organisms have lived under hot and anaerobic conditions where the TCA cycle was not required for growth [6]. If this assumption is correct, an ancestral enzyme of HCS could have had a primary function in both the lysine (leucine) synthesis and the TCA cycle during an early stage of evolution during which concentrations of molecular oxygen in the air were low. However, all the HCSs including the *Thermus* enzyme so far investigated have a very low turnover number for the reaction [16,24], while citrate synthase has a much higher turnover number (over 10 000 min^{-1}) [25]. Thus it might be more likely that another type of enzyme, an ancestor of the current citrate synthase with a higher turnover number, could have been recruited as the enzyme specific to the TCA cycle, in order to obtain enough energy to assure the rapid growth of the cells.

As described in our previous reports, lysine biosynthesis is related to arginine biosynthesis, and some of the enzymes involved in lysine synthesis could catalyze the reactions using the corresponding substrates in arginine biosynthesis [8,9]. From these observations, we speculate that the enzymes involved in lysine and arginine biosyntheses share ancestors that could function in both syntheses. HCS of *T. thermophilus* is strongly regulated by lysine with the K_i value of 9.4 μM which is comparable to that (8 μM) reported for the enzyme from *P. chrysogenum* [16]. Our analysis also revealed that arginine also has an inhibitory effect on HCS, although the effect was smaller than that of lysine. We assume that this might be a trace of an ancient time when the enzyme played a primary role in syntheses of arginine as well as lysine. It should be noted that ornithine, which is structurally more similar to lysine than to arginine, has no inhibitory effect on HCS (Fig. 4).

To gain the highest lysine production from microorganism, mutants resistant to 2-aminoethylcysteine have been developed [26]. Such mutants are expected to have altered aspartate kinases desensitized to feedback inhibition by lysine and therefore able to accumulate a large amount of lysine [26]. In *T. thermophilus*, lysine is synthesized via α-aminoadipic acid but not via diaminopimelic acid. Despite the totally different synthetic pathway, *Thermus* HCS was also inhibited by 2-aminoethylcysteine weakly but as significantly as arginine. This suggests that the same strategy could be used to isolate a mutant of *T. thermophilus* that could produce lysine in large amounts. Thus, this study suggests an alternative tool for lysine fermentation.

Acknowledgements: We thank Dr. Elinor T. Adman (University of Washington School of Medicine) for providing useful comments for completing the draft. This work was supported in part by a grant-in aid for scientific research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

[1] Kosuge, T. and Hoshino, T. (1998) FEMS Microbiol. Lett. 169, 361–367.
[2] Kobashi, N., Nishiyama, M. and Tanokura, M. (1999) J. Bacteriol. 181, 1713–1718.
[3] Vogel, H.J. (1964) Am. Nat. 98, 446–455.
[4] Vogel, H.J. (1965) in: Evolving Genes and Proteins (Bryson, V. and Vogel, H.J., Eds.), pp. 25–40, Academic Press, New York.
[5] Broquist, H.P. (1971) Methods Enzymol. 17, 112–129.
[6] Nishida, H., Nishiyama, M., Kobashi, N., Kosuge, T., Hoshino, T. and Yamane, H. (1999) Genome Res. 9, 1175–1183.
[7] Irvin, S.D. and Bhattacharjee, J.K. (1998) J. Mol. Evol. 46, 401–408.
[8] Miyazaki, J., Kobashi, N., Nishiyama, M. and Yamane, H. (2001) J. Bacteriol. 183, 5069–5073.
[9] Miyazaki, J., Kobashi, N., Nishiyama, M. and Yamane, H. (2002) FEBS Lett. 512, 269–274.
[10] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[11] Laemmli, U.K. (1970) Nature 227, 680–685.
[12] Van Holde, K.E. and Baldwin, R.L. (1958) J. Phys. Chem. 62, 734–743.
[13] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[14] Cleland, W.W. (1979) Methods Enzymol. 78, 103–138.
[15] Zheng, L., White, R.H. and Dean, D.R. (1997) J. Bacteriol. 179, 5963–5966.
[16] Jaklitsch, W.M. and Kubicek, C.P. (1990) Biochem. J. 269, 247–253.

[17] Helmstaedt, K., Krappmann, S. and Braus, G.H. (2001) Microbiol. Mol. Biol. Rev. 65, 404–421.
[18] Masurekar, P.S. and Demain, A.L. (1974) Appl. Microbiol. 28, 265–270.
[19] Takenouchi, E., Tanaka, H. and Soda, K. (1981) J. Ferment. Technol. 59, 429–433.
[20] Banuelos, O., Casqueiro, J., Fierro, F., Hijarrubia, M.J., Gutierrez, S. and Martin, J.F. (1999) Gene 226, 51–59.
[21] Schmidt, H., Bode, R. and Lindner, M. (1985) J. Basic Microbiol. 25, 675–681.
[22] Nishiyama, M., Matsubara, N., Yamamoto, K., Iijima, S., Uozumi, T. and Beppu, T. (1986) J. Biol. Chem. 261, 14178–14183.
[23] Kunai, K., Machida, M., Matsuzawa, H. and Ohta, T. (1986) Eur. J. Biochem. 160, 433–440.
[24] Feller, A., Ramos, F., Pierard, A. and Dubois, E. (1999) Eur. J. Biochem. 261, 163–170.
[25] Rault-Leonardon, M., Atkinson, M.A.L., Slaughter, C.A., Moomaw, C.R. and Srere, P.A. (1995) Biochemistry 34, 257–263.
[26] Tosaka, O., Enei, H. and Hirose, Y. (1983) Trends Biotechnol. 1, 70–74.