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A Novel Metal-Activated L-Serine *O*-Acetyltransferase from *Thermus thermophilus* HB8

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L-Cysteine is an important amino acid in terms of its industrial applications. The biosynthesis of L-cysteine in enteric bacteria is regulated through the feedback inhibition by L-cysteine of L-serine *O*-acetyltransferase (SAT), a key enzyme in L-cysteine biosynthesis. We recently found that L-cysteine is overproduced in *Escherichia coli* strains expressing a gene encoding feedback inhibition-insensitive SAT. Further improvements in L-cysteine production are expected by the use of SAT with high stability. We report here the *sat1* gene encoding SAT of an extreme thermophile, *Thermus thermophilus* HB8. The *sat1* gene was cloned and overexpressed in *E. coli* cells based on the genome sequence in *T. thermophilus* HB8. The predicted amino acid sequence consists of 295 amino acids and is homologous to other *O*-acetyltransferase members. In particular, the carboxyl-terminal region shares approximately 30% identities with SATs found in bacteria and plants, despite showing only about 15% identity in the overall sequence. Enzymatic analysis and an atomic absorption study of the purified recombinant proteins revealed that the enzyme is highly activated by Co^{2+} or Ni^{2+}, and contains Zn^{2+} and Fe^{2+}. These results indicate that the *T. thermophilus* SAT is a novel type of enzyme different from other members of this protein family.

Key words: L-cysteine biosynthesis, metal-activated enzyme, *sat1* gene, L-serine *O*-acetyltransferase, *Thermus thermophilus* HB8.

Abbreviations: OAS, *O*-acetyl-L-serine; SAT, L-serine *O*-acetyltransferase; OASS, *O*-acetyl-L-serine sulfhydrylase; CTS, cystathionine β-synthase; CTL, cystathionine γ-lyase; LB, Luria-Bertani; PCR, polymerase chain reaction; SDS-PAGE, SDS–polyacrylamide gel electrophoresis; IPTG, isopropyl-β-D-thiogalactopyranoside.

L-Cysteine plays crucial roles in the structure, stability, and catalytic function of many proteins. There are two distinct L-cysteine biosynthetic pathways in microorganisms (Fig. 1). In enteric bacteria such as *Escherichia coli* and *Salmonella typhimurium* (*1*, *2*), L-cysteine is synthesized via *O*-acetyl-L-serine (OAS) through the pathway involving L-serine *O*-acetyltransferase (SAT) [EC 2.3.1.30] and *O*-acetyl-L-serine sulfhydrylase (OASS) [EC 4.2.99.8]. Fungi such as *Aspergillus nidulans* (*3*) and *Neurospora crassa* (*4*), are known to produce L-cysteine by both the OAS-pathway and another pathway, the L-cystathionine pathway, which comprises cystathionine β-synthase (CTS) [EC 4.2.1.22] and cystathionine γ-lyase (CTL) [EC 4.4.1.1]. L-Cysteine is also an important amino acid in terms of its industrial applications in the pharmaceutical, food, and cosmetics industries. Due to the feedback inhibition of SAT by L-cysteine (*1*, *2*), high-level production of L-cysteine from glucose has not been successfully achieved in microorganisms. We recently found that L-cysteine is overproduced in *E. coli* strains that express a gene encoding a feedback inhibition-insensitive SAT (*5*–*7*). Further improvements in L-cysteine production are therefore expected to use feedback-insensitive SAT with high stability.

For enzyme stabilization, it is useful to analyze the target enzyme from a thermophilic bacterium. However, little is known about L-cysteine biosynthesis in extremophiles. Some archae have been reported to synthesize L-cysteine from L-methionine through reverse trans-sulfuration from L-homocysteine to L-cysteine (*8*). On the other hand, several archaea are considered to have genes encoding SAT and OASS (*9*). In an extremely thermophilic bacterium, *Thermus thermophilus*, L-cysteine is synthesized using OAS and sulfide, a reduced product of sulfate, by the catalysis of OASS (*10*). This organism catalyzes the trans-sulfuration from L-cysteine to L-homocysteine when ammonium sulfate is used as the sulfur source, but is unable to catalyze the reverse trans-sulfuration because of the absence of CTS (*10*). Recently, OASS has been purified from *T. thermophilus* HB8 and characterized in detail, the results suggest that the enzyme is responsible for the synthesis of L-cysteine when this organism is cultured with either sulfate or L-methionine as the sole sulfur source (*11*). In contrast, no enzymatic information is available regarding the protein and DNA sequences of SAT in *T. thermophilus*. SAT catalyzes the rate-limiting step of L-cysteine biosynthesis in bacteria and plants, and functions in association with

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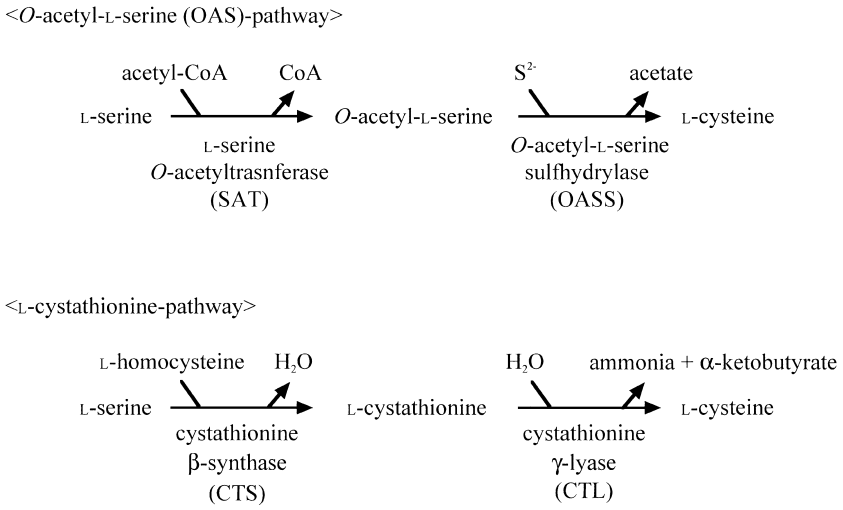

Fig. 1. **Two possible pathways of L-cysteine biosynthesis in microorganisms.**

OASS (*12*, *13*). For a better understanding of L-cysteine biosynthesis in *T. thermophilus*, a detailed analysis of SAT is necessary.

A systematic study of the structures and functions of all proteins from one cell is currently in progress using *T. thermophilus* HB8 (the Whole Cell Project) (*14*, *15*). During the genome sequencing that was performed as part of this project, we found that *T. thermophilus* HB8 has a hypothetical 33.0 kDa protein homologous to *O*-acetyltransferase members. Remarkably, the C-terminal region shows high similarity to the C-termini of SATs from various species. We report here for the first time the gene encoding SAT from *T. thermophilus* HB8 (the *sat1* gene). We also describe the characteristics of a novel SAT purified from *E. coli* transformed cells.

MATERIALS AND METHODS

Strains and Plasmids—*T. thermophilus* strain HB8 was used for the preparation of chromosomal DNA and as an enzyme source. *E. coli* strain DH5α [*F*$^-$λ$^-$Φ80*lacZ*Δ*M15* Δ(*lacZYA argF*)*U169 deoR recA1 endA1 hsdR17*($r_k^-m_k^+$) *supE44 thi-1 gyrA96*] was used as the host strain for plasmid construction. *E. coli* strain BL21 (DE3) and the plasmid vector pET-11a (Novagen, Madison, WI, USA) were used for the overexpression of the *T. thermophilus sat1* gene.

Culture Media—TM complete medium and MM minimal medium (*16*) were used for the cultivation of *T. thermophilus* HB8, and Luria-Bertani (LB) medium (*17*) containing ampicillin (50 μg/ml) was used for the *E. coli* transformants. If necessary, 2% agar was added to solidify the medium.

Construction of the Expression Plasmid for the sat1 Gene—The enzymes used for DNA manipulations were obtained from Takara Shuzo (Kyoto, Japan). Conventional techniques were used for DNA manipulation and transformation as described previously (*17*).

The DNA fragment of the *sat1* gene was prepared by polymerase chain reaction (PCR) with genomic DNA prepared from *T. thermophilus* HB8 and oligonucleotide primers 5′-ATA TCA TAT GCC ATG GCT TCT TCC CCG CGA GAT CGC GC-3′ and 5′-ATA TAG ATC TTT ATT AAG AGC CCG CCT CCC TTG TTC CGA AG-3′ (the underlined sequences are the *Nde*I and *Bgl*II sites, respectively), based on the available nucleotide sequences. Twenty-five PCR cycles (98°C for 1 min, 65°C for 1 min, 72°C for 1 min) were carried out using *LA Taq* polymerase with a GeneAmp PCR system 2400 (PE Biosystems, Foster City, CA, USA). The unique amplified band of 908-bp was digested with *Nde*I and *Bgl*II, and ligated to the large fragment of pET-11a digested with *Nde*I and *Bam*HI to construct pET-SAT1. The nucleotide sequences of the *sat1* gene were confirmed by DNA sequencing with a model 310 DNA sequencer (PE Biosystems) using the dideoxy chain termination method.

Overexpression and Purification of the Recombinant SAT—The plasmid pET-SAT1 was used to transform *E. coli* strain BL21 (DE3). The recombinant strain was cultured at 37°C in LB medium containing ampicillin for 18 h, after which the cells were harvested by centrifugation, resuspended in 20 mM Tris-HCl (pH 8.0) containing 5 mM 2-mercaptoethanol, and disrupted by sonic oscillation. The cell homogeneate obtained was heat-treated at 70°C for 15 min and centrifuged at 10,000 × *g* for 30 min to remove cell debris and denatured proteins. Ammonium sulfate was added to the supernatant to a concentration of 1.2 M and the precipitate was applied to a RESOURCE ISO column (Pharmacia Biotech, Uppsala, Sweden) equilibrated with 50 mM sodium phosphate (pH 7.0) containing 1.2 M ammonium sulfate and connected to an HPLC system (Pharmacia Biotech). The column was eluted with a linear gradient from 1.2 to 0 M ammonium sulfate in 60 ml of 50 mM sodium phosphate (pH 7.0) at a flow rate of 5 ml/min. The pooled fractions containing the protein peak were then loaded onto a RESOURCE Q column (Pharmacia Biotech) equilibrated with 20 mM Tris-HCl (pH 8.0), and eluted by HPLC with a linear gradient from 0 to 1.0 M NaCl in 60 ml of 20 mM Tris-HCl (pH 8.0) at a flow rate of 1 ml/min. As a final step, the pooled fractions containing the protein peak were subjected to gel filtration on a Superdex 200 column (Amersham Pharmacia Biotech) equilibrated with 20 mM Tris-HCl (pH 8.0) and 150 mM NaCl, and eluted by HPLC at a flow rate of 0.5 ml/min. Fractions containing the band corresponding to the relative molecular mass as

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T.thermophilus      1   -MPWLLPREIAP----------------------LHQKALDRYLGSLTERLSDPNVDRNAL  38
D.radiodurans       1   ------------------------------------------------------------  1
Halobacterium sp.   1   ----MTKRHVSLP------------ADAKDVRRAFVSGVDERLSAAETPDELATAVTGTL  44
E.coli              1   ------------------------------------MSCEELEIVWNNIKAEARTLAD  22
A.thaliana          1   MPPAGELRHQSPSKEKLSSVTQSDEAEAASAAISAAAADAEAAGLFTWTQIKAEARRDAE  60

T.thermophilus     39   VREELARLLYG--RPYEELLEANPLAAMGLDPEGITFEAEYYAATDLEKFRRVKPLLWFW  96
D.radiodurans       1   ---MLAQVMYG--RDYAEVLAQMPLAALNLDAHNVTFEAEYYMATDQEKFARVKPLLWLW  55
Halobacterium sp.  45   ADLRGERDAYEAWRDGDDVRPAAAARLATLDPRNASLESEYYAEKDEAAFARSKPLQWLW 104
E.coli             23   CEPMLASFYHATLLKHENLGSALSYMLANKLSSPIMPAIAIREVVEEA--YAADEEMIAS  80
A.thaliana         61   AEPALASYLYSTILSHSSLERSISFHLGNKLCSSTLLSTLLYDLFLFTNTFSSDFSLRNA 120

T.thermophilus     97   KVLDLTPLG-QSV--HSGVAIRRALAPEIFKRVGKN-PKFF-QNVE---FSVGYNLELGD 148
D.radiodurans      56   KNLDLTPFG-QNP--VTGIPLRRVLAGHIFKRVGRN-FKCW-QNVE---FSVGYNMEVGD 107
Halobacterium sp. 105   RQFDTTPLA--DN-VDVALPFRRMLAGHLFAGVGED-VRLF-KGIT---MTYGHNIELGD 156
E.coli             81   AACDIQAVRTRDPAVDKYSTPLLYLKGFHALQAYRIGHWLWNQGRR--ALAIFLQNQVSV 138
A.thaliana        121   TVADLRAARVRDPACISFSHCLLNYKGFLAIQAHRVSHKLWTQSRKPFTLALALHSRISD 180

T.thermophilus     149   --DVVVH------RYVFLDDIGGIKIGDRTSLSDYVNVYSHTHHVLAS--PDVTLKETIT 198
D.radiodurans      108   --DVVVH------RHVLLDDIGGIELHDGASVSDYVNIYSHTHSVLDG--PDVTLRKTVI 157
Halobacterium sp.  157   --NAVVH------DGVHLDDRGELVVGARASVSNGAHLYTHDHDVVDQ--TDVTNYRTEI 206
E.coli             139   TFQVDIHPAAKIGRGIMLDHATGIVVGETAVIENDVSILQSVTLGGTG--KSGGDRHPKI 196
A.thaliana         181   VFAVDIHPAAKIGKGILLDHATGVVVGETAVIGNNVSILHHVTLGGTGFTKACGDRHPKI 240

T.thermophilus     199   GSGVRITYHATVLAGVRIGDDAMVGTGAVVTKDIPPHAIALGIPARPVRYKVRHDCPYCR 258
D.radiodurans      158   GRGARITYHSTVLAGSVVSDDALLATHALLRSDIPPHGIAMGIPAKVTRFKQRPPQ---- 213
Halobacterium sp.  207   GADARVTQGALVRAGVRVGENALVGSRSVVQGDVPAHHIAVGSPAESVRVKPGWEAVAAP 266
E.coli             197   REGVMIGAGAKILGNIEVGRGAKIGAGSVVLQPVPPHTTAAGVPARIVGKP--------- 247
A.thaliana         241   GDGCLIGAGATILGNVKIGAGAKVGAGSVVLIDVPCRGTAVGNPARLVGFT--------- 291

T.thermophilus     259   KGEPHPSDLVPKLPDRKGNPDYPDFLPPGFGTREAGS-----  295
D.radiodurans      214   DGYGVDAALWKRTPDRKANPEFPDPTPNQTRKPDELLVAGEG  255
Halobacterium sp.  267   LDAGGERRQAERRLDDAVPADAAVYDEFQRDRSPPGER----  304
E.coli             248   -DSDKPSMDMDQHFN------GINHT---FEYGDGI------  273
A.thaliana         292   -GKEKPTIHDEECPG-----ESMDHTSFISEWSDYII-----  322
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Fig. 2. **Amino acid sequence of *T. thermophilus* HB8 SAT and its alignment with the sequences of putative acetyltransferases from *D. radiodurans* R1 and *Halobacterium* sp. NRC-1, and with those of SATs from *E. coli* and *A. thaliana*.** Identical and similar amino acids in three or more proteins are shown as dark and light shaded boxes, respectively. Dashes indicate the absence of corresponding amino acid residues at those positions.

determined by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) were collected and used as the purified enzyme for characterization.

Assay of SAT Activity—The purified proteins from the transformed *E. coli* cells were used to determine enzymatic properties. Also, *T. thermophilus* HB8 cells were grown in 500 ml of TM or MM medium at 65°C for 35 h with shaking, washed with 20 mM Tris-HCl (pH 8.0), and resuspended in the same buffer containing 5 mM 2-mercaptoethanol. The supernatants obtained after disruption of the cells by sonic oscillation and centrifugation at 20,000 × *g* for 1 h were used as enzyme sources. The enzyme activity of SAT was assayed at 30°C or 37°C by monitoring the cleavage of the thioester bond of acetyl-CoA as described previously with some modifications (*18*). The initial rate of the decrease in absorbance at 232 nm of the reaction mixture (final volume, 1 ml) containing 50 mM Tris-HCl buffer (pH 7.8), 1 mM L-serine, 0.1 mM acetyl-CoA, 0.1 mM $CoCl_2$ (or other metal ions), and enzyme solution was measured, and that obtained for a solution containing all the materials except L-serine (blank) was subtracted. The reaction rate was calculated using the differential extinction coefficient between acetyl-CoA and CoA of 3.2 mM^{-1} cm^{-1}. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol CoA/min. Protein concentrations were determined using a Bio-Rad protein assay kit (Hercules, CA, USA) with bovine serum albumin as the standard protein.

Metal Atomic Absorbance Measurement—To prevent contamination of the protein samples by adventitious metal ions, purified recombinant SAT was dialyzed against 20 mM Tris-HCl (pH 8.0) and 1 mM EDTA. The zinc and iron contents were determined with a model Z-5010 atomic absorption flame emission spectrophotometer (Hitachi, Tokyo, Japan), and the dialyzed buffer was used as a blank.

Nucleotide Sequence Accession Number—The nucleotide sequence of the *sat1* gene from *T. thermophilus* HB8 has been submitted to the DDBJ/EMBL/GenBank database under accession no. AB159102. The accession numbers for the *Deinococcus radiodurans* R1 putative acatyltransferase gene, the *Halobacterium* sp. NRC-1 putative acetyltransferase gene, *Rhodopseudomonas palustris* putative maltose *O*-acetyltransferase gene, the *E. coli* galactoside *O*-acetyltransferase gene, the *E. coli cysE* gene, and the *Arabidopsis thaliana* cDNA encoding a cytosolic SAT isoform (SAT-c), are AE001916, AE005096, BX572607, U73857, M15745, and U30298, respectively.

RESULTS AND DISCUSSION

Sequence Analysis of the T. thermophilus SAT—We found by a BLAST search (*19*) of protein databases that *T. thermophilus* HB8 contains one open reading frame consisting of 295 amino acids that is partly homologous to sequences of the *O*-acetyltransferase superfamily, including putative acetyltransferases and sugar *O*-

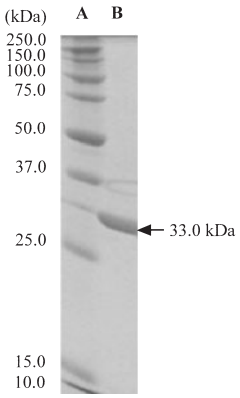

Fig. 3. **SDS-PAGE patterns of SAT from *T. thermophilus* HB8.** SDS-PAGE was performed in a 15% polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue R-250. Lane A, molecular mass markers in kilodaltons (Bio-Rad); lane B, purified enzyme (3 μg). The arrow shows the position of the SAT protein.

acetyltransferases from various species. In particular, the predicted amino acid sequence shares high identities to those of two extremophiles, the *D. radiodurans* R1 and *Halobacterium* sp. NRC-1 putative acetyltransferases (49% and 30%, respectively) (Fig. 2). With regards to sequence homology with SAT, within the C-terminal region (Arg154–Val246), 29% and 32% of the amino acids are identical to the *E. coli* SAT (Arg152–Val244) and the plant *A. thaliana* SAT-c (Lys194–Val288), respectively, despite showing only 14–18% identities for the overall sequence. The well-conserved C-terminal region of this protein family has a hexapeptide-repeat domain that probably corresponds to β-sheets, and is considered to be responsible for feedback inhibition by L-cysteine and the hetero-oligomerization with OASS (*20*, *21*). We, therefore, assumed that the putative acetyltransferase gene (the *sat1* gene) could encode a SAT enzyme from *T. thermophilus* HB8.

Overexpression of the sat1 Gene in E. coli and Purification of the Gene Product—In order to examine the function of the *sat1* gene, we amplified the whole gene by PCR directly from the *T. thermophilus* HB8 genomic DNA using two primers containing an intiation codon (ATG) and a termination codon (TAA), and then constructed an expression plasmid, pET-SAT1, as described in "MATERIALS AND METHODS." After the transformed *E. coli* cells were grown in LB medium without the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to the stationary phase, a large amount of gene product was observed that accounted for approximately 30% of the total cellular proteins (data not shown). It should additionally be noted that the *sat1* gene was fully expressed even in the absence of IPTG, probably due to a derepression of the T7 RNA polymerase under the control of the *lacUV5* promoter in the pET system, as introduced by the supplier, Novagen.

The *sat1* gene product was purified to homogeneity from the recombinant *E. coli* cells by three-column chromatography, and the purified enzyme gave a single band corresponding to a relative molecular mass of approximately 33,000 on SDS-PAGE (Fig. 3). By putting the enzyme together with standard proteins through gel

Table 1. **Effect of divalent cations on enzyme activity.**

Reagent	Concentration (mM)	Specific activity (U/mg)	Ratio
None	–	0.045 ± 0.01	1.0
$CoCl_2$	0.1	1.4 ± 0.02	31.7
$NiCl_2$	0.1	0.53 ± 0.05	12.0
$FeSO_4$	0.1	0.04 ± 0.01	0.9
$MnCl_2$	0.1	0.04 ± 0.02	0.9
$ZnSO_4$	0.1	0.058 ± 0.01	1.3
$MgSO_4$	0.1	0.046 ± 0.01	1.0
$CuCl_2$	0.1	0.036 ± 0.02	0.8
$CaCl_2$	0.1	0.051 ± 0.01	1.1
$NaMoO_4$	0.1	0.036 ± 0.01	0.8
EDTA	1.0	ND	0.0

ND, not detected. Assays were performed in 50 mM Tris-HCl (pH 7.8) at 37°C using 1 μg of purified enzyme. The specific activity values are averages ± standard deviations from the three independent experiments.

Table 2. **Apparent kinetic constants of *T. thermophilus* SAT.**

Substrate	K_m (μM)	V_{max} (nM s^{-1})	k_{cat} (s^{-1})
L-Serine	13 ± 0.5	55 ± 0.5	0.48 ± 0.04
Acetyl-CoA	11 ± 0.5	79 ± 1.0	0.54 ± 0.07

Assays were performed in 50 mM Tris-HCl (pH 7.8) and 0.1 mM $CoCl_2$ at 30°C. The specific activity values are averages ± standard deviations from three independent experiments.

chromatography on a Superdex 200 column, a prominent peak (molecular mass; approximately 460,000) was observed in addition to a homo-hexamer and homo-dimer with apparent molecular masses of 200,000 and 68,000, respectively (data not shown). These results suggest that *T. thermophilus* SAT is a homo-oligomeric protein, although the subunit stoichiometry of the enzyme cannot be determined. According to the reports on bacterial and plant enzymes, SAT consists of two protein-interaction domains, a central-SAT-SAT domain for homomerization, and a C-terminal SAT-OASS domain for heteromerization (*22–24*).

Metal Activation of SAT Activity—The purified enzyme eluted from a Superdex 200 column had a specific activity of 0.045 U/mg. Interestingly, it was found that the specific activities greatly increased to 1.4 U/mg and 0.53 U/mg when the Co^{2+} and Ni^{2+} ions were added to the reaction mixture, respectively. The effect of various divalent metal cations is shown in Table 1. The Co^{2+} ion stimulated the enzyme to give the highest acetyltransferase activity among all cations tested (approximately a 30-fold increase). Therefore, its enzymatic properties were characterized in the presence of 0.1 mM Co^{2+} ion. The apparent K_m, V_{max}, and k_{cat} values in the presence of Co^{2+} ion were determined at 30°C from the initial rate measurements of L-serine and acetyl-CoA (Table 2). The k_{cat} and K_m values of the enzyme for both L-serine and acetyl-CoA were significantly lower than those of bacterial and plant SAT proteins (*25*, *26*). In particular, the enzyme showed a high affinity for substrates (a small K_m value), suggesting that a limited amount of L-serine or acetyl-CoA present in the cell can be preferably used by SAT. The enzyme was found to be active over a wide range of temperatures, with maximum activity at around 75°C (Fig. 4). The optimum pH for the activity was approximately pH 7.8 (data not shown).

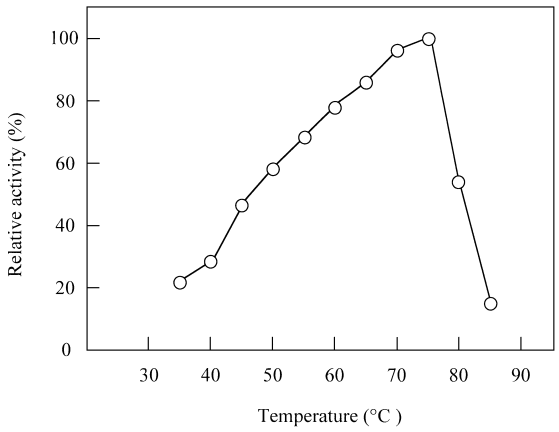

Fig. 4. **Effect of temperature on enzyme activity.** Assays were performed in 50 mM Tris-HCl (pH 7.8) containing 0.1 mM $CoCl_2$ at various temperatures using 1 μg of purified enzyme.

As described above, the deduced amino acid sequence of the *sat1* gene product was also shown to be homologous to those of maltose *O*-acetyltransferases from *R. palustris* and galactoside *O*-acetyltransferase from *E. coli*. However, the chromogenic sugar substrates, including *p*-nitrophenyl β-D-galactopyranoside, were not acetylated by the recombinant SAT. We also examined the acetyltransferase activities for L-serine at 37°C in total cell extracts of *T. thermophilus* HB8 grown in TM liquid medium. When 0.1 mM Co^{2+} ion was added to the reaction mixture, considerable SAT activity was expressed (0.14 mU/mg). These results indicate that the *sat1* gene encodes a SAT enzyme of *T. thermophilus* HB8 that is highly activated by metal ions such as Co^{2+} and Ni^{2+}.

As for the cation-activated enzyme, Wada *et al*. found a novel levodione reductase from *Corynebacterium aquaticum* M-13 that is highly activated by monovalent cations, such as K^+, Na^+, and NH_4^+ (*27*). Recent studies have shown that eight cobalt-containing enzymes have been isolated and characterized (*28*). A cobalt transporter is also involved in the metallocenter biosynthesis of the host cobalt-containing enzyme, nitrile hydratase of *Rhodococcus rhodochrous* J1, which catalyzes the hydration of nitriles to amides (*29*, *30*). To our knowledge, this is the first report of a novel metal cation–activated SAT, although the physiological function of cobalt is unknown. Also, it would be interesting to analyze the membrane protein(s) required for cobalt transport into the *T. thermophilus* cells, because the steady state concentration of intracellular cobalt is probably insufficient to activate the enzyme.

Regulation of SAT Activity—L-Cysteine biosynthesis in wild-type strains of *E. coli* and *S. typhimurium* is strictly regulated through feedback inhibition of SAT by L-cysteine (*1*, *12*). We analyzed the feedback inhibition of the *T. thermophilus* SAT activity by L-cysteine using the purified enzymes (data not shown). A significant decrease in activity occurred in the presence of 100 μM L-cysteine, although the activity was virtually invariant up to 10 μM L-cysteine. These results suggest that *T. thermophilus* SAT is sensitive to feedback inhibition by L-cysteine, but at a weaker order of magnitude than *E. coli* SAT (concentration for 50% inhibition: IC_{50} = 6.0 μM) (*5*) and the *Arabidopsis thaliana* SAT-c (IC_{50} = 1.8 μM) (*26*). We successfully constructed mutant *cysE* genes in *E. coli* that were genetically desensitized to feedback inhibition by L-cysteine, by replacing Met256 with 19 other amino acid residues using site-directed mutagenesis (*5*, *6*). Mino *et al*. also showed that a truncated SAT with a 20 amino acid deletion from residues 254–273, was much less sensitive to feedback inhibition than the wild-type enzyme, and that it did not form a complex with OASS (*31*). Therefore, the C-terminal region downstream from Met254 in the *E. coli* SAT is considered to be involved in feedback inhibition by L-cysteine, and in complex formation with OASS. It should be additionally noted that the corresponding region in *T. thermophilus* SAT showed no significant similarity to that of *E. coli* SAT, which may be the cause of the different feedback-sensitivities to L-cysteine.

In addition, *T. thermophilus* HB8 was cultivated in TM or MM liquid medium, and the SAT activities in the soluble cell extracts were then examined. In the presence of 0.1 mM Co^{2+} ion, the activity was low in cells grown in TM medium (0.14 mU/mg) and high in cells grown in MM medium (0.68 mU/mg). In contrast, the SAT activity was no longer detectable in the absence of $CoCl_2$ (data not shown). In *E. coli* and *S. typhimurium*, L-cysteine down-regulates the sulfate reduction pathway by inhibiting the synthesis of OAS, which isomerizes to *N*-acetylserine. *N*-Acetylserine, together with the transcriptional activator CysB, acts as an inducer of the genes for enzymes in the sulfate reduction pathway (*1*, *12*). This result raises the possibility that the expression of the *sat1* gene is regulated by an unknown mechanism. From these observations, our speculation is that *T. thermophilus* SAT might be regulated at both the transcriptional and post-translational levels.

Metal Binding to the SAT Protein—During the process of enzymatic analysis, it was also found that the addition of EDTA to the reaction mixture resulted in a complete loss of SAT activity, suggesting that some divalent metal ions are attached to the enzyme. Following dialysis and gel filtration to remove free metal ions, the amount of bound metal ions in the enzyme was determined by atomic absorption spectrometry. The stoichiometry of Zn^{2+} and Fe^{2+} ion binding per mol of subunit was estimated to be approximately 1 mol and 0.25 mol, respectively, suggesting that the recombinant enzyme could contain four zinc atoms and one iron atom per four subunits. The binding of the Zn^{2+} ion was also substantiated by the preliminary crystallographic analysis of this enzyme (data not shown). However, no bound Co^{2+} or Ni^{2+} ions were detectable in the recombinant SAT in this study.

We isolated the gene encoding a novel SAT from *T. thermophilus* HB8. The question arose as to why *T. thermophilus* SAT is activated by Co^{2+} and Ni^{2+}, and binds Zn^{2+} and Fe^{2+} in the native form. Although much more research is needed to answer this question, it is probable that Zn^{2+} and/or Fe^{2+} ions may stabilize the structure of the SAT protein, even at high temperatures. Another possibility is that the Co^{2+} ion may bind to the enzyme, or that the Fe^{2+} ion may be replaced by a Co^{2+} ion for its catalytic activity. We are currently attempting to determine

the tertiary structure of the enzyme in order to elucidate the mechanism. Also, the purification of the enzyme from *T. thermophilus* and gene disruption are essential to clarify the physiological role of SAT.

Although a thermostable SAT from *T. thermophilus* HB8 should be mutated to desensitize it to feedback inhibition based on our previous reports (*5*–*7*), an engineered SAT with high stability could be a useful enzyme for further improvement in L-cysteine production.

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