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ORIGINAL PAPER

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Purification and characterization of *Thermotoga maritima* homoserine transsuccinylase indicates it is a transacetylase

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Abstract The methionine biosynthetic pathway found in bacteria is controlled at the first step, acylation of the γ-hydroxyl of homoserine. This reaction is catalyzed by one of two unique enzymes, homoserine transacetylase or homoserine transsuccinylase, which have no amino acid sequence similarity. We cloned, expressed, and purified homoserine transsuccinylase from the thermophilic bacterium *Thermotoga maritima*. Substrate specificity experiments demonstrated that acetyl-coenzyme A (CoA) is the preferred acyl donor and is used at least 30-fold more efficiently than succinyl-CoA. Steady-state kinetic experiments confirm that the enzyme utilizes a ping-pong kinetic mechanism in which the acetate group of acetyl-CoA is initially transferred to an enzyme nucleophile before subsequent transfer to homoserine. The maximal velocity, $V/K_{\text{acetyl-CoA}}$ and $V/K_{\text{homoserine}}$, all exhibited bell-shaped pH curves with apparent p*K*s of 6.0–6.9 and 8.2–8.8. The enzyme was inactivated by iodoacetamide in a pH-dependent manner, with an apparent p*K* of 6.3, suggesting the presence of an active-site cysteine residue which forms an acetyl-enzyme thioester intermediate during catalytic turnover, similar to observations with other transsuccinylases. In addition, the enzyme is highly stable at elevated temperatures, maintaining full activity at 70°C. Taken together, these data suggest that the *T. maritima* enzyme functions biochemically as a transacetylase, despite having the sequence of a transsuccinylase.

Keywords Homoserine transsuccinylase · Homoserine transacetylase · Acyltransferase · Methionine biosynthesis · *Thermotoga maritima* · Hyperthermophile

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Abbreviations *EcHTS*: *Escherichia coli* homoserine transsuccinylase · *HiHTA*: *Haemophilus influenzae* homoserine transacetylase; · *HTA*: Homoserine transacetylase · *HTS*: Homoserine transsuccinylase · *IAA*: Iodoacetamide · *OAH*: *O*-acetylhomoserine · *OSH*: *O*-succinylhomoserine · *PLP*: Pyridoxal phosphate · *SAM*: *S*-adenosylmethionine · *SDS-PAGE*: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis · *SELDI-TOF MS*: Surface-enhanced laser desorption/ionization-time of flight mass spectrometry · *TEA*: Triethanolamine · *TmHTA*: *Thermotoga maritima* homoserine transacetylase

The methionine biosynthetic pathway is a critical pathway in microorganisms and plants which is required for cellular activities such as protein synthesis, DNA and RNA synthesis, transmethylation reactions involving *S*-adenosylmethionine (SAM), and the synthesis of polyamines (Flavin 1975; Saint-Girons et al. 1988). The complete pathway, which is essential to the growth and survival of microorganisms, is absent in mammals, suggesting that it might be a target for novel antibacterial agents. The first unique step in the methionine biosynthetic pathway is the acylation of the C_4-hydroxyl of homoserine to form either *O*-succinylhomoserine (OSH), as occurs in *Escherichia coli* and is catalyzed by homoserine transsuccinylase (HTS, EC 2.3.1.46), or *O*-acetylhomoserine (OAH), as occurs in *Haemophilus influenzae* and is catalyzed by homoserine transacetylase (HTA, EC 2.3.1.31), as shown in Fig. 1. This step presumably provides the free-energy input necessary to commit homoserine to methionine biosynthesis, while also preparing the C_4 carbon atom of homoserine for subsequent attack by cysteine to form cystathionine, catalyzed by the pyridoxal phosphate (PLP)-containing enzyme cystathionine-γ-synthase. This enzyme appears to be able to accept both OSH and OAH as substrates (Aitken and Kirsch 2005; Hachem et al. 2003). Cystathionine breaks down in another PLP-dependent

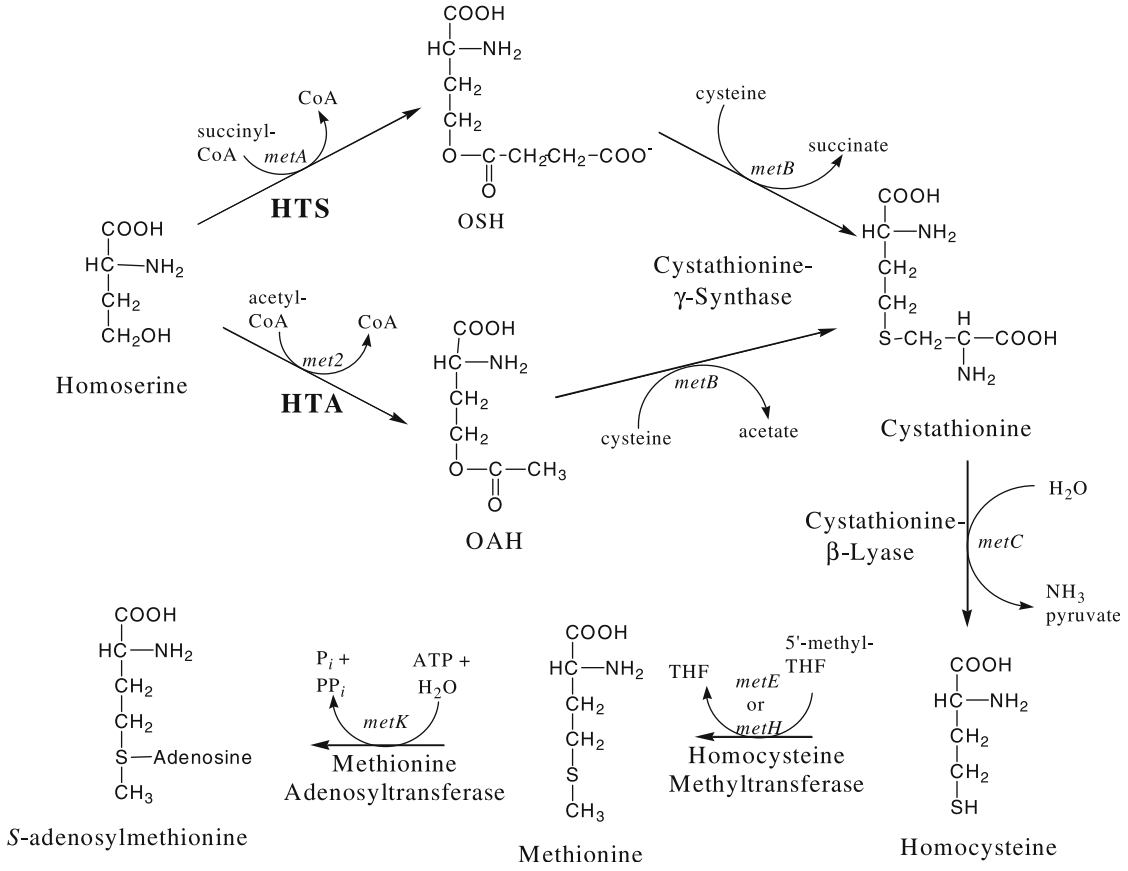

Fig. 1 Biosynthetic pathways of methionine and *S*-adenosylmethionine by microorganisms. Gene and enzyme names are listed *above* and *below* the *reaction arrows*, and the structures of primary intermediates are included. Special emphasis is placed on the two acylated versions of homoserine, both of which are substrates for cystathionine-γ-synthase

dreaction to form homocysteine, NH_3, and pyruvate. Finally, methylation of homocysteine in either a vitamin B_{12}-dependent or -independent reaction produces L-methionine (Saint-Girons et al. 1988).

Microorganisms employ different synthetic and regulatory paths for methionine biosynthesis. The major differences are found in the nature of the acylhomoserine intermediate formed in the first step of the pathway, in the enzyme used to convert homocysteine to methionine, in the method of assimilation of the sulfur atom (direct sulfhydrylation vs transsulfuration), and in the level of feedback inhibition by methionine and SAM (Anderson et al. 1998; Foglino et al. 1995; Kerr and Flavin 1970; Savin and Flavin 1972). In the first step of methionine biosynthesis in *E. coli*, HTS catalyzes the transfer of a succinyl group from succinyl-CoA to homoserine. Studies of *E. coli* HTS (EcHTS) have shown that this enzyme utilizes a ping-pong catalytic mechanism and that it is inactive with acetyl-CoA as an acyl donor (Born and Blanchard 1999). Inactivation by iodoacetamide (IAA) suggested the involvement of a cysteine residue in catalysis, possibly as the nucleophile required for succinyl transfer. Mass spectrometry experiments have recently identified a succinylated lysine residue that might serve as the nucleophile instead of cysteine (Rosen et al. 2004). Additional studies demonstrated that HTS activity decreases drastically at temperatures higher than 37°C and that temperatures greater than 44°C bring the methionine biosynthetic pathway to a halt and limit the growth of bacterial cells in media lacking a source of methionine (Ron and Davis 1971; Ron and Shani 1971; Wyman et al. 1975). HTS has been proposed to play a central role in the regulation of *E. coli* growth rate and cell survival at elevated temperature based on the fact that HTS occupies the control point of methionine biosynthesis, and that its activity is regulated by temperature.

Homoserine transacetylase fills an equivalent biological role to HTS in some microorganisms by catalyzing the transfer of acetate from acetyl-CoA to homoserine in the first step of the methionine biosynthetic pathway. Like the transsuccinylase, it uses a ping-pong kinetic mechanism (Born et al. 2000), although the catalytic nucleophile is thought to be a serine residue based on its membership in the α/β hydrolase family of enzymes. Further support for the use of serine as the nucleophile is provided by work with the yeast enzyme (Nazi and Wright 2005). Members of this family contain a catalytic triad of serine–histidine–aspartic acid, with the serine residue found in a G-X-S/C-X-G signature motif (Born et al. 2000; Hemila et al. 1994; Jaeger et al. 1994; Brumlik and Buckley 1996; Schrag and Cygler

1997). HTA has broader substrate specificity than HTS, preferring acetyl-CoA as its acyl donor and using succinyl-CoA 1000-fold less efficiently. The effect of temperature on enzymatic activity or protein expression has not been studied for HTA.

Thermotoga maritima is a thermophilic bacterium that was isolated from geothermally heated marine sediment and grows optimally at 80°C (Huber et al. 1986; Jaenicke and Bohm 2001; Gluch et al. 1995; Pysz et al. 2004). The homoserine acyltransferase encoded within the *T. maritima* genome (Nelson et al. 2001) is 50% identical to the *E. coli* enzyme and has no amino acid sequence similarity to *Haemophilus influenzae* HTA (HiHTA). Considering that succinyl transfer during the first step of methionine biosynthesis in *E. coli* is highly temperature sensitive, we decided to examine the enzyme from *T. maritima*. In this study we report the cloning, expression, purification, and characterization of *T. maritima* HTS and compare its properties with the *E. coli* and *H. influenzae* enzymes. Although this enzyme has an amino acid sequence consistent with that of a transsuccinylase, it has the substrate specificity of a transacetylase and is thus an HTS-like HTA and should be referred to as a transacetylase [*T. maritima* HTA (TmHTA)]. Other characteristics, including pH profiles and sensitivity to IAA, are consistent with its similarity to other transsuccinylases and raise questions concerning the influence of environmental pressures on the choice of acyl-CoA substrate.

Materials and methods

Materials

The oligonucleotide primers used for PCR amplification were synthesized by Invitrogen Custom Primers (Carlsbad, CA). The pBAD102/D-TOPO expression vector was purchased from Invitrogen (Carlsbad, CA) and *E. coli* BL21 (DE3) cells were purchased from Novagen (Madison, WI). *T. maritima* genomic DNA (source strain DSM 3109) was purchased from ATCC (Manassas, VA), DNaseI was from Epicenter (Madison, WI), and TALON metal affinity resin was purchased from BD Bioscience Clontech (Palo Alto, CA). D_2O (99.9%) and 4-nitrophenyl acetate were from Aldrich Chemical Co. (Milwaukee, WI). Protease inhibitors and substrates listed in Tables 1 and 2 were purchased from Sigma Chemical Co. (St. Louis, MO), except for OAH, which was synthesized as described (Nagai and Flavin 1971). DNA sequencing was performed by Northwoods DNA, Inc. (Bemidji, MN).

Cloning and expression of TmHTA

The sequence of the putative *metA*-encoded HTS gene (TM0881) from *T. maritima* has been reported (Nelson et al. 2001). Two oligonucleotide primers (5'-CAC CTTGCCAATAAACGTTCCAAGCG-3' and 5'-GTG TATGTCTTCCAATCTGTAAG-3'), which were complementary to the amino-terminal coding and carboxyl-terminal noncoding strands were synthesized and used in a standard PCR reaction to amplify the *T. maritima metA* gene. The PCR product was cloned into the pBAD102/D-TOPO expression vector in frame with the *N*-terminal thioredoxin gene and the *C*-terminal histidine tag. *E. coli* BL21(DE3) competent cells were transformed with the resulting product and plasmid DNA was purified from ampicillin-resistant colonies and sequenced to verify the cloning of the correct gene. The transformed *E. coli* cells were grown at 37°C in LB media containing 50 μg/ml ampicillin to an OD_{600} of 0.5, induced with varying levels of arabinose, and then grown for an additional 3 h. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie blue staining indicated the overexpression of a 50–55-kDa protein in cell extracts which were induced with arabinose at a concentration equal to or greater than 0.002% (w/v).

Protein purification

Two liters of LB containing ampicillin (50 mg/l) were inoculated with a single bacterial colony and grown to an OD_{600} of 0.5 at 37°C. The temperature was then lowered to 25°C and the cells were induced with 0.02% arabinose. After 3 h of induction, the cells were harvested by centrifugation and the cell pellet was resuspended in 50 ml of 25 mM triethanolamine (TEA, pH = 7.8). The cells were lysed by incubation with 0.2 mg/ml lysozyme in the presence of protease inhibitors, DNase I, and 10 mM $MgCl_2$ for 30 min with stirring at 4°C. The cells were further lysed by five rounds of sonication at 70% amplitude, 1 min each time, with an Ultrasonic Processor (model GEX 600) and the cell debris was removed by centrifugation at $12{,}000 \times g$. Protein was purified from the supernatant using TALON metal affinity resin charged with Co^{2+}, which

Table 1 Kinetic parameters for the preferred *Thermotoga maritima* homoserine transacetylase (TmHTS) substrates in the forward and reverse reactions

Substrate	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1}\ M^{-1}$)
Acetyl-CoA	130 ± 20	23 ± 2	1.8×10^5
L-Homoserine	240 ± 30	23 ± 2	9.6×10^4
O-acetylhomoserine (OAH)	1,700 ± 300	1.9 ± 0.3	1.1×10^3
Coenzyme A	140 ± 40	1.9 ± 0.3	1.4×10^4

Table 2 Kinetic parameters of alternate substrates for *Thermotoga maritima* homoserine transacetylase (TmHTS)

Substrate	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1}M^{-1}$)
Acetyl-CoA	83 ± 8	9.49 ± 0.42	1.1×10^5
n-Butyryl-CoA	ND[a]	ND[a]	
β-Hydroxybutyryl-CoA	58 ± 13	0.12 ± 0.01	2.1×10^3
Glutaryl-CoA	180 ± 80	0.25 ± 0.07	1.4×10^3
Isobutyryl-CoA	68 ± 12	0.19 ± 0.01	2.8×10^3
Malonyl-CoA	17 ± 3	0.12 ± 0.01	7.1×10^3
Propionyl-CoA	44 ± 5	2.6 ± 0.1	5.9×10^4
Succinyl-CoA	51 ± 27	0.19 ± 0.03	3.7×10^3
4-Nitrophenyl acetate	3,800 ± 600	29 ± 3	7.6×10^3
L-Homoserine	130 ± 10	18 ± 1	1.4×10^5
D-Homoserine	280 ± 30	16 ± 1	5.7×10^4
O-acetylhomoserine (OAH)	1,700 ± 300	1.9 ± 0.3	1.1×10^3
Coenzyme A	140 ± 40	1.9 ± 0.3	1.4×10^4

[a] K_M and k_{cat} values could not be determined for this substrate

binds the 6x-histidine tag at the *C*-terminus of the protein. Three milliliter of resin slurry was washed with 9 ml of 25 mM TEA (pH = 7.8), and then incubated with the supernatant with rocking at 4°C for 30 min. The unbound material was removed by gravity filtration and the resin was washed with 9 ml of 25 mM TEA (pH = 7.8). Subsequent elutions with wash buffer containing imidazole at concentrations of 5 mM (6 ml), 200 mM (9 ml), and 500 mM (9 ml) released the protein from the resin. These elutions were examined for the presence of protein by SDS-PAGE. A protein band at the anticipated size of ~52 kDa indicated that all imidazole elutions contained highly pure protein. The three imidazole elutions were combined, concentrated with an Amicon concentrator over a YM-10 membrane, and brought to 50% glycerol for long-term storage at −20°C.

Measurement of enzyme activity

The change in absorbance at 232 nm due to hydrolysis or formation of the thioester bond of acyl-CoA (ε = 4,500 M^{-1}) is indicative of enzyme activity. Experiments were conducted using a Uvikon 933 double beam UV/VIS spectrophotometer (Research Instruments International) connected to a constant-temperature circulating water bath (T = 25°C unless otherwise noted). Assays were performed with varying concentrations of acetyl-CoA (25, 33, 50, 100, and 200 μM) and homoserine (55.6, 71.4, 100, 166.7, and 500 μM), and the initial velocities were obtained by measuring substrate depletion as a function of one of the substrates at different fixed concentrations of the second substrate. The pH was held constant at 7.5 using 50 mM K_2HPO_4. The observed initial velocities were analyzed using SigmaPlot (version 7.0 SPSS) and fit to Eq. 1:

$$v = VAB/(K_A[B] + K_B[A] + [A][B]), \quad (1)$$

where V is the maximal velocity, K_A and K_B are the Michaelis constants for substrates A and B, and [A] and [B] are the concentrations of substrates A and B. The kinetics of the reverse reaction were studied in the same way using OAH at concentrations of 400, 500, 667, 1,000, and 2,000 μM and CoA at concentrations of 83.3, 100, 125, 167, and 250 μM. The alternative substrates listed in Table 2 were analyzed by varying the concentration of the substrate of interest, while holding the companion substrate constant in a pseudo-first-order reaction.

pH profiles

Enzyme activity was measured over the pH range 5.5–9.6 using phosphate (5.5–8.1) and Tris-Cl (8.0–9.6) as buffers. Assays were performed at 25°C in 50 mM buffer. The kinetic parameters V and V/K were determined using homoserine concentrations of 200, 250, 333, and 500 μM with phosphate buffers and 250, 333, 500, and 1,000 μM with Tris-Cl buffers, and acetyl-CoA concentrations of 50, 100, 150, and 200 μM at each pH value. The pH-dependent data were fit to Eq. 2 using SigmaPlot:

$$\log(V/K) = \log[C/(1 + H/K_a + K_b/H)] \quad (2)$$

where C is the pH-independent plateau value, H is the hydrogen ion concentration, K_a is the ionization constant for the acidic group, and K_b is the ionization constant for the basic group.

Inactivation by IAA

IAA was used to inactivate TmHTA via alkylation of a free thiol. The 75 μl-inactivation mixture contained 41.2 μg of enzyme in 50 mM phosphate buffer (pH = 7.5) to which different amounts of IAA were added for each assay. Aliquots of 10 μl were taken from the inactivation mixture at various time points and added to an assay mixture containing 50 mM phosphate buffer (pH = 7.5), 1 mM homoserine, and 200 μM acetyl-CoA in a final volume of 500 μl. Enzyme activity was then measured as described previously. The data were fit to Eq. 3 using SigmaPlot:

$$\log k_{inact} = \log[C/(1 + H/K_a)] \quad (3)$$

where k_{inact} is the rate of inactivation and C, H, and K_a are as defined in Eq. 2. The pH dependence of IAA inactivation was determined in an identical manner, except that the pH of the inactivation solution was varied and the IAA concentration was held constant at 250 μM.

Solvent kinetic isotope effects

The solvent kinetic isotope effects on V and V/K of the forward and reverse reactions were studied by measuring the change in absorbance at 232 nm due to cleavage or formation of thioester bonds, respectively. Solvent kinetic isotope effects on the forward reaction were measured using 1.0 μg of enzyme in 50 mM phosphate buffer (pH = 7.1), and varying concentrations of acetyl-CoA or homoserine in H_2O and 80% D_2O. The concentration of homoserine was held constant at 1 mM when acetyl-CoA was varied, and the concentration of acetyl-CoA was held constant at 200 μM when homoserine was varied. Assays of the reverse reaction contained 5 μg of enzyme in 50 mM phosphate buffer (pH = 7.1). The concentration of OAH was held constant at 5 mM when CoA was varied, and the concentration of CoA was held constant at 300 μM when OAH was varied. The solvent deuterium isotope effects were fit to Eq. 4 using SigmaPlot:

$$v = VA/[K(1 + F_iE_{V/K}) + A(1 + F_iE_V)] \quad (4)$$

where V, A, and K are as described in Eq. 1, F_i is the fraction of isotope label (either 0 or 0.8), and $E_{V/K}$ and E_V are the isotope effects on V/K and V, respectively.

Temperature dependence of TmHTA

The effect of temperature on the activity of TmHTA was studied by measuring acetyl-CoA hydrolysis while increasing the temperature from 25 to 75°C by 5°C intervals. The temperature was controlled using a circulating water bath directly connected to the spectrophotometer cuvette holders. At any given temperature five assays were conducted, each containing 2 mM homoserine, 50 mM phosphate buffer (pH = 7.0), 1.0 μg of TmHTA, and acetyl-CoA concentrations of 40, 80, 120, 160, or 200 μM. Assay mixtures, without acetyl-CoA, were incubated in a water bath at the appropriate temperature for 5 min before the addition of acetyl-CoA and immediate activity measurement. An identical experiment was performed using 0.4 μg of HiHTA per assay.

In a second experiment, the reversibility of thermal denaturation of HiHTA was examined using the assay described previously, except that the assay mixture was allowed to cool to room temperature for 5 min before the addition of acetyl-CoA and the measurement of enzyme activity.

Results

Expression and purification of TmHTA

TmHTA was overexpressed by cloning the *metA* gene into the pBAD102/D-TOPO expression vector to produce a protein fused to thioredoxin at the *N*-terminus and containing a *C*-terminal 6x-histidine tag. Induction of *E. coli* cells containing this vector with 0.02% arabinose resulted in overproduction of a soluble protein with the expected molecular mass, as determined by SDS-PAGE. Following cell lysis, TmHTA was purified on TALON metal affinity resin charged with Co^{2+} by following the manufacturer's instructions. SDS-PAGE analysis confirmed the presence of a band with a MW matching that expected for the fusion protein. The purified protein yielded a single species of mass 52,500 Da when analyzed by surface-enhanced laser desorption/ionization-time of flight mass spectrometry (SELDI-TOF MS).

Determination of steady-state parameters

Enzyme activity was measured by monitoring the change in absorbance at 232 nm due to hydrolysis of the acyl-CoA thioester bond. Surprisingly, little activity was observed when succinyl-CoA was used as a substrate, but acetyl-CoA was an excellent acyl donor. Initial velocities were determined in triplicate at five different concentrations of homoserine and acetyl-CoA, and the data were fit to a ping-pong kinetic mechanism (Eq. 1) to determine K_M values for each substrate and the k_{cat} value for the enzyme. Fitting of the data to a sequential mechanism yielded significantly poorer results. A similar set of data was obtained for the enzyme in the reverse reaction by determining initial velocities at five different concentrations of OAH and CoA. The resulting kinetic parameters are summarized in Table 1.

The activity of the enzyme was measured with a variety of alternative acyl donors and acceptors, as summarized in Table 2. These experiments were performed by holding one substrate concentration constant and varying the concentration of the other substrate in a pseudo-first-order reaction. Homoserine was held constant at 1 mM when alternate acyl donors were examined, and acetyl-CoA was held constant at 100 μM when alternate acyl acceptors were examined. The data indicate that two of the acyl donors, acetyl-CoA and propionyl-CoA, are preferred. The data also indicate that TmHTA does not distinguish well between D-homoserine and L-homoserine. The values listed in Table 2 for acetyl-CoA and homoserine differ slightly from those in Table 1, because they were determined at a single concentration of the second substrate and not at an array of concentrations. All the values listed in Table 2 were collected under identical conditions, however, permitting direct comparison of these data.

pH dependence of HTS

The pH dependence of the reaction catalyzed by TmHTA was monitored over the pH range 5.5–9.6 using phosphate and Tris-Cl as buffers. The results indicate that the maximal velocity, $V/K_{homoserine}$ and $V/K_{acetyl\text{-}CoA}$ all show bell-shaped pH curves (Fig. 2), suggesting that a general acid and a general base are required in each half reaction. The data for each curve was fit to Eq. 2 and estimates of the ionization constants for each group were extracted (Table 3). The pK values of the acidic groups in the plots of V_{max} and $V/K_{acetyl\text{-}CoA}$ are similar, suggesting that they monitor the same ionization.

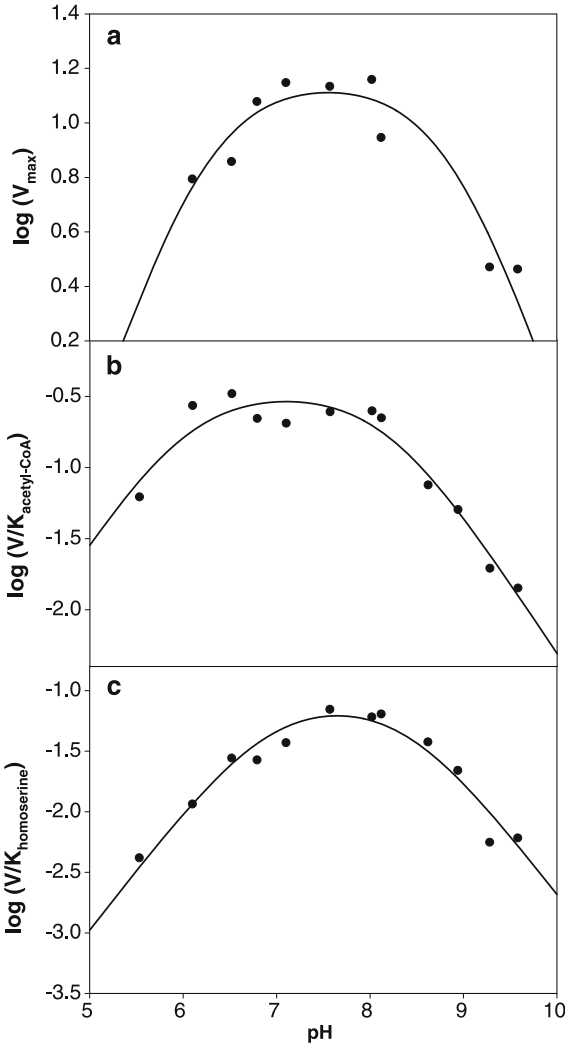

Fig. 2 pH dependence of the kinetic parameters of the *Thermotoga maritima* homoserine transacetylase (*TmHTA*)-catalyzed acylation of homoserine. Experiments were conducted as described in 'Materials and methods'. *Symbols* represent the experimental data and the *curves* are fits of V_{max}, $V/K_{acetyl\text{-}CoA}$, and $V/K_{homoserine}$ data to Eq. 2. **a** Dependence of maximal velocity on pH, where V is in units of μmol/min mg, indicating that the maximal velocity occurs at a pH of ~7.5 and decreases at higher and lower pH values. **b** Dependence of $V/K_{acetyl\text{-}CoA}$ on pH, where V is in units of V_{max} and $K_{acetyl\text{-}CoA}$ is in μM units, indicating that the maximal rate of the first half reaction occurs at a pH of ~7.3 and decreases at higher and lower pH values. **c** Dependence of $V/K_{homoserine}$ on pH, where V is in units of V_{max} and $K_{homoserine}$ is in μM units, indicating that the maximal rate of the second half reaction occurs at a pH of ~7.7 and decreases at higher and lower pH values

Inactivation by IAA

A previous study demonstrated that EcHTS could be inactivated by IAA (Born and Blanchard 1999). Although the *T. maritima* enzyme has the activity of a transacetylase, it contains the putative active-site cysteine residue of the HTS family members. Inactivation of TmHTA with IAA resulted in a first-order, time-dependent loss of activity over a 20-min time period. Similar to the *E. coli* enzyme (Born and Blanchard 1999), the inactivation of TmHTA by IAA is pH-dependent. As the pH of the inactivation solution was decreased from 8.0 to 6.0, a decrease in the rate of inactivation was observed. Fitting of the data to Eq. 3 yielded a pK of 6.3 for the modified cysteine residue, suggesting that this residue exists as a thiolate anion under physiological conditions.

Solvent kinetic isotope effects

Solvent kinetic isotope effects on both the forward and reverse reactions were measured by determining initial velocities in H_2O and 80% D_2O. Isotope effects were measured for the forward reaction by holding the homoserine concentration at 1 mM and varying acetyl-CoA, or by holding acetyl-CoA at 200 μM and varying homoserine. Solvent kinetic isotope effects were measured for the reverse reaction in the same way by holding the OAH concentration constant at 5 mM and varying CoA, or by keeping CoA constant at 300 μM and varying the OAH concentration (Fig. 3). In the forward direction, a slightly normal isotope effect is observed on V when varying the concentrations of acetyl-CoA (1.3 ± 0.2) and homoserine (1.13 ± 0.3). Similar effects are seen on V/K for acetyl-CoA and homoserine (1.08 ± 0.09 and 1.16 ± 0.3, respectively). Inverse isotope effects are observed in the reverse direction on V when varying CoA (0.84 ± 0.12) and OAH (0.87 ± 0.14), and on V/K when varying CoA (0.72 ± 0.07) and OAH (0.76 ± 0.05).

Table 3 Calculated pK values of residues whose ionization state is critical for *Thermotoga maritima* homoserine transacetylase (TmHTS) activity

Parameter	pK_a	pK_b
V_{max}	6.3	8.8
$V/K_{acetyl\text{-}CoA}$	6.0	8.2
$V/K_{homoserine}$	6.9	8.4
$k_{inact,IAA}$	6.3	

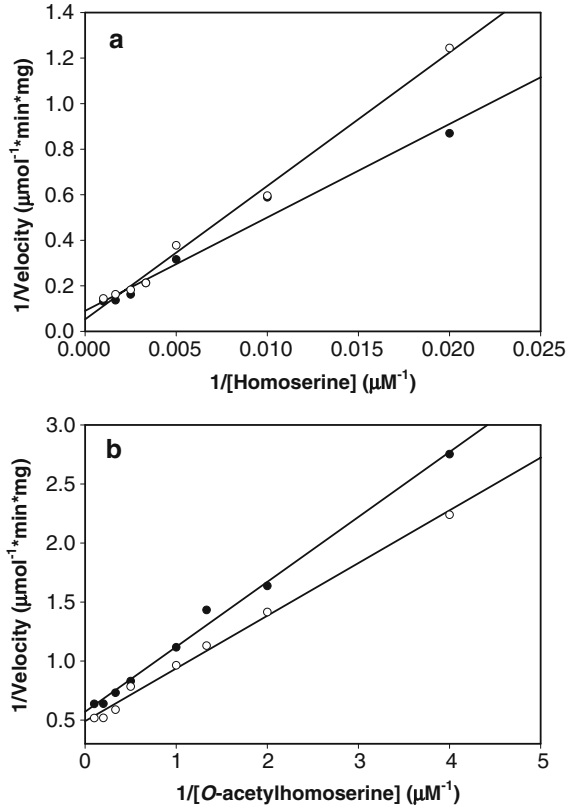

Fig. 3 Solvent kinetic isotope effects for the reaction catalyzed by *Thermotoga maritima* homoserine transacetylase (*TmHTA*). **a** A double-reciprocal plot of the forward reaction in H_2O (*closed circles*) and 80% D_2O (*open circles*). The concentration of acetyl-CoA was held constant at 200 μM while the concentration of homoserine was varied. **b** A double-reciprocal plot of the reverse reaction in H_2O (*closed circles*) and 80% D_2O (*open circles*). The concentration of CoA was held constant at 300 μM while the concentration of *O*-acetylhomoserine (*OAH*) was varied. The *symbols* represent experimental values and the *lines* are linear fits to the data

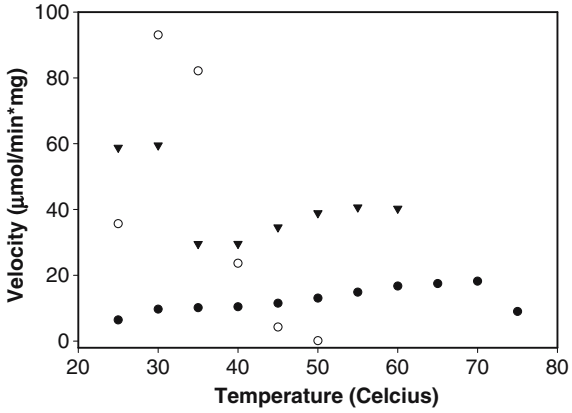

Fig. 4 Comparison of the temperature dependence of *Thermotoga maritima* homoserine transacetylase (*TmHTA*) and *Haemophilus influenzae* homoserine transacetylase (*HiHTA*). Assays were conducted at the indicated temperatures as described in the 'Materials and methods'. *Closed circles* represent the maximal velocity determined at the indicated temperatures for TmHTA by varying the concentration of acetyl-CoA. *Open circles* represent the maximal velocity determined at the indicated temperatures for HiHTA by varying the concentration of acetyl-CoA. *Closed triangles* represent the maximal velocity obtained for HiHTA when the enzyme was returned to 25°C prior to analysis, illustrating the reversibility of HiHTA inactivation by elevated temperatures. Activity is in units of V_{max} (μmoles/min mg)

Temperature dependence of TmHTA

Since *T. maritima* is a thermophilic organism, it was assumed that TmHTS would retain significant activity at elevated temperatures, in contrast to that observed with EcHTS. It was difficult to analyze the activity of EcHTS at elevated temperatures due to the inherent instability of succinyl-CoA, which has a half-life of 1.9 min at 75°C versus an acetyl-CoA half-life of 80 min at the same temperature, and hence the TmHTA activity was compared with that of HiHTA, as both enzymes use acetyl-CoA as their acyl donor. Activities were measured as the temperature was increased from 25 to 75°C in 5°C intervals. As Fig. 4 illustrates, the activity of HiHTA decreases as the temperature is raised above 35°C and is undetectable by 50°C, while the TmHTA activity remains steady up to 70°C. The reversibility of HiHTA temperature inactivation was investigated by incubating the enzyme at elevated temperature for 5 min followed by a return to room temperature, addition of acetyl-CoA and determination of enzyme activity. As can be seen in Fig. 4, temperature inactivation of HiHTA is readily reversible.

Discussion

The methionine biosynthetic pathway is of general interest because its products are essential for cellular activities, such as protein and spermidine synthesis, because it supplies homocysteine and SAM to other metabolic pathways, and because inactivation of the pathway has been shown to limit the growth of microorganisms (Nagai and Flavin 1967). In this paper, the enzyme catalyzing the first step of the pathway in *T. maritima* was studied and its properties were compared with those of HTS from *E. coli* and HTA from *H. influenzae*. The gene coding for TmHTA was cloned into the pBAD102/D-TOPO expression vector. This vector includes a *C*-terminal histidine tag, which aids in purification. More importantly, it also appends an *N*-terminal thioredoxin tag, which can be cleaved with enterokinase and which greatly increases the level of soluble protein. Expression of TmHTA without the thioredoxin tag resulted in the formation of insoluble inclusion bodies. The fusion protein was purified using TALON metal affinity resin and appeared as a single species when examined by SELDI-TOF MS. The purified protein had significant activity with the nonphysiological substrate 4-nitrophenyl acetate, and hence its enzymatic activity was further characterized.

It was assumed that the *T. maritima* acyl transferase would be a succinyl transferase based on a 50% sequence identity and 68% sequence similarity to EcHTS and a complete lack of sequence similarity to HiHTA. Surprisingly, little activity was observed using succinyl-CoA as the acyl donor. Activity was evident, however, when acetyl-CoA and homoserine were used as the substrate pair, suggesting that TmHTA is actually an acetyltransferase. Initial velocities were determined at varying substrate concentrations in both the forward and reverse directions and analyzed via double-reciprocal plots. The parallel lines of these plots suggest that TmHTA utilizes a ping-pong kinetic mechanism, as has been observed for EcHTS and HiHTA (Born and Blanchard 1999; Born et al. 2000). The results obtained with several other acyl-donor substrates are listed in Table 2. Based on the k_{cat}/K_M values, acetyl-CoA is the favored acyl donor and propionyl-CoA is also a good acyl-donor substrate. Succinyl-CoA, isobutyryl-CoA, β-hydroxybutyryl-CoA, butyryl-CoA, glutaryl-CoA, and malonyl-CoA displayed minimal activity with TmHTA. The nearly constant K_M values of the acyl-CoA substrates suggest that the enzyme has a binding site that accommodates the CoA portion of the substrate, but is optimally arranged to react with C_2 and C_3 acyl side chains that are fully reduced. This is in contrast to EcHTS, which has maximal activity with succinyl-CoA and glutaryl-CoA (Born and Blanchard 1999). These two compounds have longer acyl side chains which contain a terminal carboxylate group. The TmHTA substrate profile is more similar to HiHTA, which also prefers fully reduced side chains, although the *H. influenzae* enzyme is able to accommodate four carbon acyl side chains, such as butyryl-CoA (Born et al. 2000). It was observed that L-homoserine was only a twofold better substrate than D-homoserine and that the turnover numbers for the isomers were identical, while similar preferences were 12.5-fold for EcHTS and 40-fold for HiHTA. This result indicates that there is surprising freedom within the active site for binding of the amino acid moiety of homoserine.

Analysis of an enzyme reaction over a range of pH values will often reveal information on the ionization states of residues that are involved in catalysis. When the pH dependence of TmHTA was determined, bell-shaped curves were obtained for plots of V_{max}, $V/K_{acetyl\text{-}CoA}$, and $V/K_{homoserine}$, suggesting that each half reaction requires at least one protonated group and one deprotonated group. In the first half reaction, the group functioning as the catalytic nucleophile is probably a cysteine residue (see next), which must be deprotonated for maximal activity. The observed pK value of 6.0 for $V/K_{acetyl\text{-}CoA}$ is close to that predicted from the pH dependence of IAA inactivation, indicating that the group observed in this profile is probably the catalytic cysteine. The residue with a pK value of 8.2 is similar to that seen in the V_{max} profile and is probably involved in the donation of a proton to $CoAS^-$ to facilitate productive breakdown of a tetrahedral intermediate. The two groups observed in the $V/K_{homoserine}$ pH profile have ionizations constants of 6.9 and 8.4. The group functioning as a general base in this half reaction is probably responsible for the deprotonation of homoserine, and a similar pK of 6.5 is seen with EcHTS (Born and Blanchard 1999). The group that must be deprotonated for maximal activity in the second half reaction is most likely to be involved in the stabilization of a negatively charged intermediate. This ionization is also observed with EcHTS (Born and Blanchard 1999). The ionizations observed in the V/K profiles may alternatively reflect binding groups and not catalytic residues.

Analysis of the inactivation of TmHTA by IAA suggested that the enzyme active site contains an essential cysteine residue, which presumably acts as a nucleophile in the first half reaction to accept acetate from acetyl-CoA and form an acetylated enzyme. The recent identification of a succinylated lysine residue in EcHTS has led to the suggestion that cysteine is not the catalytic nucleophile (Rosen et al. 2004). The evidence presented is not conclusive, however, and a nucleophilic cysteine residue is more likely than a nucleophilic lysine based on precedence with other acyl transferases (Andres et al. 1988; Costello et al. 1996; Weber and Cohen 1967; Miziorko et al. 1975). The rate of IAA inactivation was linear over the initial time period and was dependent on the concentration of IAA, as would be expected for chemical modification of a catalytic residue. Previous studies with EcHTS demonstrated that the inactivation by IAA was pH dependent (Born and Blanchard 1999), and a similar experiment with TmHTA clearly showed that inactivation by IAA was dependent on pH over the pH range 6.0–8.0. A pK value of 6.3 was determined from the data, suggesting that the cysteine residue is normally present as the catalytically active thiolate anion and is possibly part of a catalytic triad or dyad, as seen in other enzyme systems. This pK value agrees closely with the pK value of 6.4 observed for EcHTS.

Solvent kinetic isotope effect studies (Fig. 3) suggest a small and normal effect in the forward reaction on V, $V/K_{acetyl\text{-}CoA}$, and $V/K_{homoserine}$ of 1.3, 1.08, and 1.16, respectively. These values are similar to what has been observed for EcHTS and HiHTA and demonstrate that D_2O has little or no effect on the forward reaction. An inverse effect was observed in the reverse direction, however, with effects on V, V/K_{CoA}, and V/K_{OAH} of 0.87, 0.72, and 0.76, respectively. These values are also similar to what was observed with EcHTS and HiHTA, suggesting that they have a common cause. As inverse isotope effects are known to occur when a proton is being transferred to or from a thiol, and deprotonation of the CoA thiol is required for all three enzymes, this is the simplest explanation for the observed inverse effect. An inverse effect of 0.4 is expected if deprotonation is completely rate limiting, and hence the deprotonation of the CoA thiol is only partially rate limiting during the reverse reaction of TmHTA. A second potential cause is the deprotonation of the catalytic cysteine residue during the transfer of the acyl group from OSH or OAH to the enzyme. This is only

applicable to the EcHTS and TmHTA enzymes, however, since HiHTA appears to utilize a catalytic serine residue, and thus is less likely to explain the inverse isotope effects observed in the reverse direction.

The EcHTS is the best-studied version of this enzyme and it has been reported to be extremely sensitive to elevated temperatures (Ron and Davis 1971; Ron and Shani 1971; Wyman et al. 1975). The current hypothesis is that the catalytic cysteine residue of EcHTS is located close to the enzyme periphery and moves from the active site to the surface as the temperature is raised. Since *T. maritima* is a thermophilic organism, one would predict that the activity of its acetyltransferase is thermostable. This could either be an intrinsic property of the enzyme or it may be the result of other factors found in the organism's cytosol. The TmHTA activity was measured over a range of temperatures, starting at 25°C and continuing up to 75°C. The enzyme not only maintained its activity during this experiment, but also showed an increase between 25 and 70°C. This demonstrates that thermal stability is an intrinsic property of TmHTA and it indicates that the structures of EcHTS and TmHTA have significant differences that relate not only to their choice of acyl donor, but also to their relative stabilities. The maximal velocity of TmHTA tripled from 6.42 μmol/min×mg at 25°C to 18.2 μmol/min×mg at 70°C, much less than that which would be predicted from the general observation that the rate of a reaction doubles for every 10°C rise in temperature. Enzyme-catalyzed reactions tend to have more complex temperature dependencies, however, and rarely follow this general principle. Moreover, the maximal velocity of the TmHTA reaction does not appear to have a distinct temperature maximum, in contrast to what is seen for HiHTA. Such a maximum is more likely to be seen at the typical growth temperature of the *T. maritima* rather than at the lower temperatures examined in this study. A more complete characterization of the temperature dependence of TmHTA will be required to determine the optimal temperature of this enzyme, but it is clear that the maximal TmHTA activity occurs at a higher temperature than for EcHTS or HiHTA.

A similar experiment was not attempted with EcHTS due to the thermal lability of succinyl-CoA, but the activity of HiHTA was tested to determine if thermal stability is a common property of all homoserine transacetylases. In contrast to TmHTA, the activity of HiHTA decreased rapidly as the temperature was raised above 35°C. In fact, no activity was detected above 50°C. Activity was recoverable if HiHTA was allowed to cool to room temperature, indicating that thermal inactivation is readily reversible.

Interestingly, the only HTS analog currently identified in thermophiles/hyperthermophiles is the enzyme in *T. maritima*, while multiple HTA analogs are found in these organisms. The *T. maritima* genome also contains genes for both cystathionine-γ-synthase and *O*-acetyl-L-homoserine sulfhydrylase. These proteins are responsible for the step immediately following OAH synthesis in the transsulfuration and the direct sulfhydrylation pathways, respectively, and indicate that this organism may be capable of synthesizing methionine via either route. Cystathionine-γ-synthase can accept both OAH and OSH as substrates and the reaction rate appears to be controlled by substrate availability rather than substrate specificity (Hacham et al. 2003). As the transsulfuration pathway primarily uses OAH (Hacham et al. 2003), this may explain why *T. maritima* employs a transacetylase rather than a transsuccinylase.

In this report, we demonstrate that the homoserine acyltransferase from *T. maritima* is not a succinyltransferase, as originally assigned, but is actually an acetyltransferase. It is currently unknown whether TmHTA uses acetyl-CoA because of selective pressures applied by its unusual environment or whether other HTS family members also utilize acetyl-CoA. Characterization of putative HTS enzymes from additional organisms will be required to answer this question. The results described here illustrate the limitations of bioinformatic approaches to protein function assignment and reinforce the need for direct biochemical characterization whenever possible. In addition, data such as that described in this report are required for proper modeling of metabolic flux within model organisms. The use of incomplete or incorrect data in these models will almost certainly lead to erroneous conclusions, and although these errors are unlikely to have significant consequences when applied to *E. coli*, the results may be different when they are applied to relevant pathogens.

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