Enzyme-Catalyzed Acylation of Homoserine: Mechanistic Characterization of the *Haemophilus influenzae met2*-Encoded Homoserine Transacetylase†

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ABSTRACT: The first unique step in bacterial and plant methionine biosynthesis involves the acylation of the γ-hydroxyl of homoserine. In *Haemophilus influenzae*, acylation is accomplished via an acetyl-CoA-dependent acetylation catalyzed by homoserine transacetylase. The activity of this enzyme regulates flux of homoserine into multiple biosynthetic pathways and, therefore, represents a critical control point for cell growth and viability. We have cloned homoserine transacetylase from *H. influenzae* and present the first detailed enzymatic study of this enzyme. Steady-state kinetic experiments demonstrate that the enzyme utilizes a ping-pong kinetic mechanism in which the acetyl group of acetyl-CoA is initially transferred to an enzyme nucleophile before subsequent transfer to homoserine to form the final product, *O*-acetylhomoserine. The maximal velocity and $V/K_{homoserine}$ were independent of pH over the range of values tested, while $V/K_{acetyl-CoA}$ was dependent upon the ionization state of a single group exhibiting a pK value of 8.6, which was required to be protonated. Solvent kinetic isotope effect studies yielded inverse effects of 0.75 on V and 0.74 on V/K_{CoA} on the reverse reaction and effects of 1.2 on V and 1.7 on $V/K_{homoserine}$ on the forward reaction. Direct evidence for the formation of an acetyl-enzyme intermediate was obtained using rapid-quench labeling studies. On the basis of these observations, we propose a chemical mechanism for this important member of the acyltransferase family and contrast its mechanism with that of homoserine transsuccinylase.

The biosynthesis of methionine is essential to the survival of microorganisms and plants. This pathway provides organisms with not only methionine, required for protein synthesis, but also *S*-adenosylmethione (SAM).[1] SAM is the most important biological methylating agent and additionally serves as a precursor for the polyamines spermine and spermidine (*1, 2*). It has recently been suggested that SAM is a substrate for the *luxI* family of enzymes that synthesize a series of acylhomoserine lactone signaling molecules identified in Gram-negative bacteria (*3, 4*).

L-Methionine is derived from aspartic acid, as are the essential amino acids L-lysine, L-threonine, and L-isoleucine. L-Homoserine, generated by the reduction of L-aspartate semialdehyde, is the last common precursor for methionine, threonine, and isoleucine. The first unique step in methionine biosynthesis is the acylation of homoserine to form either *O*-succinylhomoserine or *O*-acetylhomoserine (OAH) (Figure 1). This step serves to activate the C4 carbon atom of homoserine for the subsequent nucleophilic attack by cysteine, generating cystathionine and acetate, catalyzed by the PLP-containing enzyme cystathionine synthase. Cystathionine is then broken down in a second PLP-dependent reaction to form homocysteine, NH_3, and pyruvate. Methylation of homocysteine in either a vitamin B_{12}-dependent or -independent reaction produces L-methionine. Subsequent adenosylation results in the formation of SAM. Because mammals obtain methionine exclusively in their diets, enzymes involved in methionine biosynthesis represent potential targets for the design of novel antibacterial compounds.

The acylation of homoserine is catalyzed by one of two acyl transferases: in *Escherichia coli*, homoserine transsuccinylase (HTS, EC 2.3.1.46) catalyzes the transfer of succinate from succinyl-CoA to homoserine (*5*), while in *Haemophilus influenzae*, homoserine transacetylase (HTA, EC 2.3.1.31) catalyzes the transfer of acetate from acetyl-CoA to homoserine. Both of these enzymes can be feedback-inhibited by L-methionine and SAM (*6*−*10*), although HTAs from all sources are not inhibited by these compounds (*11, 12*). HTA is reversibly inhibited by zinc (*8*) while HTS is inhibited at elevated temperatures (*13, 14*). Remarkably, although HTA and HTS catalyze the same biological reaction and have nearly identical substrates, they exhibit no primary sequence similarity.

Previously we investigated the mechanism of HTS (*15*) and demonstrated that it utilizes a ping-pong kinetic mechanism in which the succinyl group of succinyl-CoA is transferred to an active-site cysteine residue before subsequent transfer to the γ-hydroxyl group of homoserine. Studies of the pH dependence of the kinetic parameters allowed us to identify the nucleophilic cysteine residue, a general acid

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[1] BSA, bovine serum albumin; CoA, coenzyme A; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); ESI-MS, electrospray ionization-mass spectrometry; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); HTA, homoserine transacetylase; HTS, homoserine transsuccinylase; IPTG, isopropylthio-β-D-galactoside; OAH, *O*-acetylhomoserine; SAM, *S*-adenosylmethionine; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TEA, triethanolamine.

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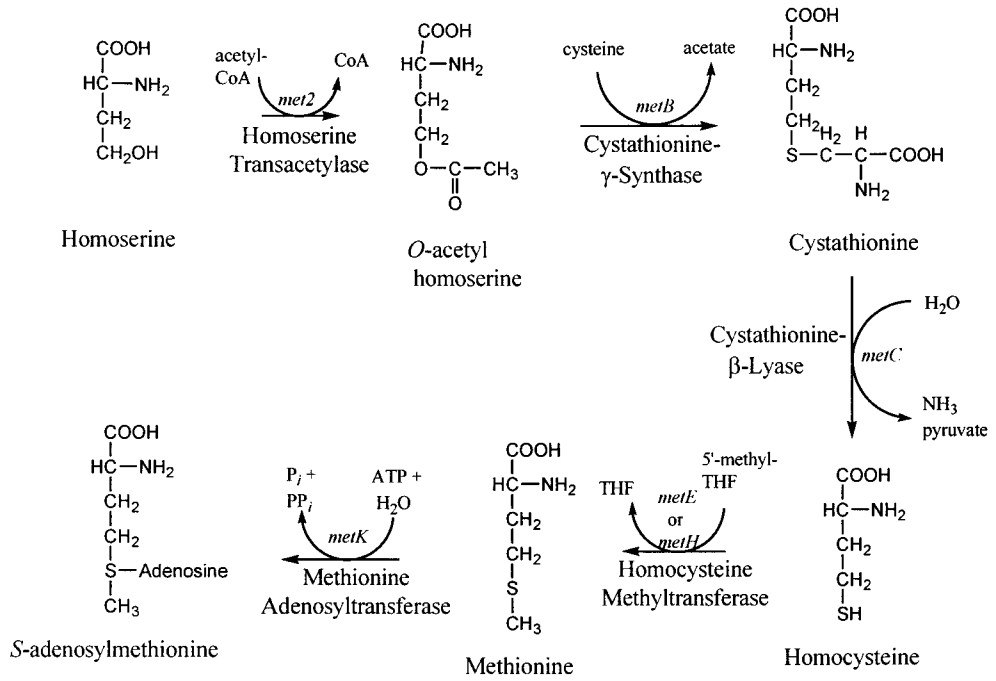

FIGURE 1: Biosynthetic pathway of methionine and *S*-adenosylmethionine in *H. influenzae*.

required for protonation of $CoAS^-$, a general base that activates the γ-hydroxyl group of homoserine, and a residue believed to be involved in substrate binding. Solvent kinetic isotope effect studies revealed inverse isotope effects of 0.7 on V and 0.61 on V/K on the reverse reaction only.

Because HTA and HTS catalyze the same biological reaction, yet share no sequence similarity, we were curious about the two enzymes' possibly having convergently evolved a common catalytic mechanism. In this paper we report the characterization of the *H. influenzae* transacetylase and compare its mechanism to that of the *E. coli* transsuccinylase. HTA was cloned, overexpressed, purified, and shown to utilize a ping-pong kinetic mechanism. The kinetic parameters were determined for the natural substrates as well as a number of alternative substrates, highlighting differences between HTA and HTS. The rate of the HTA reaction has been shown to be independent of pH over the range of values studied, and no evidence has been found for an active-site cysteine nucleophile. Solvent kinetic isotope effect studies revealed a small, normal effect on the forward reaction and an inverse effect on the reverse reaction. Finally, direct evidence has been obtained, using rapid-quench labeling studies, for an acetyl-enzyme intermediate, allowing us to propose a chemical mechanism which incorporates these data.

EXPERIMENTAL PROCEDURES

Materials. Oligonucleotide primers used for PCR amplification were synthesized by Life Technologies (Gaithersburg, MD). *Nde*I and *Hin*dIII restriction enzymes were purchased from New England BioLabs (Beverly, MA). pET23a(+)-vector DNA and *E. coli* BL21(DE3) cells were purchased from Novagen (Madison, WI). *H. influenzae* genomic DNA was purchased from ATCC (Rockville, MD). Chromatographic supports for protein purification were obtained from Pharmacia (Piscataway, NJ). D_2O (99.9%) was purchased from Cambridge Isotope Laboratories (Andover, MA). [^{3}H]-Acetyl-CoA was purchased from ICN (Costa Mesa, CA). All other materials were from Sigma (St. Louis, MO). OAH was synthesized as described (*16*), and the identity of the final product was verified by ^{1}H and ^{13}C NMR and mass spectrometry.

Cloning and Expression of H. influenzae Homoserine Transacetylase. The sequence of the putative *met2*-encoded homoserine transacetylase gene from *H. influenzae* has been reported (*17*). Two oligonucleotide primers (5′-AATTC-CATATGTCTGTGCAAAATGTAGTGC-3′ and 5′-GCG-GATCCGTTAATTACCTGCCAAACCATC-3′) which were complementary to the amino-terminal coding and carboxyl-terminal noncoding strands were synthesized. These primers were used to amplify the *H. influenzae met2* gene from genomic *H. influenzae* RD using standard PCR conditions (Perkin-Elmer). The PCR product was purified by electrophoresis on low-melting agarose and was directly ligated into the pCR 2.1 TA cloning vector (Invitrogen). Plasmid DNA was purified from kanamycin-resistant colonies and digested with *Nde*I and *Hin*dIII, and the insert was purified by electrophoresis on low-melting point agarose. The insert was ligated into a pET23a(+) expression vector which had previously been digested with the same restriction enzymes. The recombinant plasmid was transformed into *E. coli* BL21-(DE3) cells. The transformed cells were grown at 37 °C in LB media containing 50 μg/mL ampicillin until OD_{600} = 0.5, were induced by addition of 1 mM isopropylthio-β-D-galactoside (IPTG), and were grown for an additional 3 h. Analysis by SDS−PAGE with Coomassie blue staining indicated the overproduction of a 35−40 kDa protein in the cell extracts.

Protein Purification. Six liters of LB media containing ampicillin (50 μg/mL) was inoculated with a single bacterial colony and grown to an OD_{600} of 1.0 at 37 °C before induction with 1 mM IPTG. After 2 h of induction, approximately 23.4 g of cells (wet weight) was recovered by centrifugation. The cells were resuspended in 75 mL of

Table 1: Kinetic Parameters for the Reaction Catalyzed by HTA.

substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}M^{-1}$)
acetyl-CoA	140 ± 10	92 ± 5	6.5×10^5
propionyl-CoA	90 ± 10	30 ± 1	3.3×10^5
butyryl-CoA	210 ± 20	27 ± 2	1.3×10^5
crotonyl-CoA	130 ± 30	15 ± 2	1.1×10^5
succinyl-CoA	360 ± 80	.21 ± 0.03	5.7×10^2
glutaryl-CoA	280 ± 200	.033 ± 0.014	1.2×10^2
4-nitrophenyl acetate	1400 ± 200	7.8 ± 0.5	5.6×10^3
L-homoserine	130 ± 10	92 ± 5	7.1×10^5
D-homoserine	4700 ± 700	78 ± 5	1.7×10^4
γ-hydroxybutyric acid	19 000 ± 7000	50 ± 13	1.8×10^3
3-amino-1-propanol	71 000 ± 144 000	5 ± 13	4.6×10^1
OAH	850 ± 140	30 ± 4	3.6×10^4
coenzyme A	620 ± 100	30 ± 4	5.0×10^4

25 mM triethanolamine (TEA, pH 7.8) containing lysozyme (0.2 mg/mL) and protease inhibitors (Complete protease inhibitor cocktail tablets, Boehringer Mannheim). All subsequent steps were performed at 4 °C. After being stirred for 30 min, the cells were lysed by sonicating five times for 1-min intervals with a Branson Sonifier 450, and the cell debris was removed by centrifugation at 12000*g* for 30 min. Nucleic acids were precipitated by the addition of streptomycin sulfate (1%, w/v, final) to the supernatant, and the solution was stirred for 30 min before pelleting the nucleic acids at 17000*g* for 30 min. The nucleic acid-free supernatant was dialyzed against 14 L of 20 mM TEA (pH 7.8) for 3.5 h, and the precipitate which formed during dialysis was removed by centrifugation at 17000*g* for 30 min before loading the clear supernatant onto a 400-mL fast-flow Q-Sepharose anion-exchange column which had been equilibrated with 20 mM TEA (pH 7.8). The protein was eluted at 2 mL/min with a 1600-mL linear 0−1 M NaCl gradient. The active fractions were pooled, concentrated (YM-10 membrane, Amicon), and loaded onto a 2.4 × 57 cm Sepharose S-200 gel filtration column. The protein was eluted at 0.5 mL/min in 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2ethanesulfonic acid) (HEPES) (pH 7.8). The active fractions were concentrated and loaded onto an 8-mL Mono-Q column (Pharmacia). The protein was eluted at 1 mL/min with a 500 mL linear 0−0.6 M NaCl gradient in 20 mM TEA. The active fractions, which yielded a single band on SDS−PAGE with Coomassie blue staining, were combined and stored at −20 °C.

Measurement of Enzyme Activity. Reaction rates were determined by monitoring the change in absorbance at 232 nm due to hydrolysis or formation of the thioester bond of acetyl-CoA ($\epsilon = 4500\ M^{-1}$), in a UVIKON 9310 or 943 spectrophotometer equipped with thermospacers and connected to a constant-temperature circulating-water bath. Assays were performed in 100 mM K_2HPO_4 (pH 7.5) at a temperature of 25 °C. Reactions were initiated by the addition of enzyme. Initial velocity kinetic data were analyzed by Lineweaver−Burk analysis and fit to eq 1 using the programs of Cleland (*18*)

$$v = VAB/(K_AB + K_BA + AB) \quad (1)$$

where V is the maximal velocity, A and B are the concentrations of substrates A and B, and K_A and K_B are the Michaelis constants for substrates A and B. Initial velocity patterns were obtained by measuring substrate depletion as a function of one of the substrates at different fixed concentrations of the second substrate. The alternative substrates listed in Table 1 were assayed in the same manner, substituting the alternative compound for the corresponding natural substrate.

pH Profiles. Enzyme activity was measured over the pH range 5.5−9.0 using phosphate (5.5−8.0) and Tris (7.8−9.0) as buffers to avoid spectrophotometric interference. Assays were performed at 25 °C in 100 mM buffer, and reactions were initiated by addition of enzyme. The kinetic parameters V and V/K were determined using four concentrations of each substrate at each pH value. The pH-dependent data were fit to eq 2 using the program Sigma Plot (version 3.03, Jandel Scientific Software)

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

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

Solvent Kinetic Isotope Effects. Solvent kinetic isotope effects on V and V/K were determined by measuring the initial velocity of either thioester cleavage or thioester formation at 232 nm. Assays measuring thioester cleavage contained 0.375 μg of enzyme, 50 mM K_2HPO_4 (pH 7.5), 400 μM acetyl-CoA, and varying concentrations of homoserine in H_2O or 80% D_2O. Assays measuring thioester formation contained 2.5 μg of enzyme, 50 mM K_2HPO_4 (pH 7.5), 400 μM CoA, and varying concentrations of OAH in H_2O or 80% D_2O. All assays were performed at 25 °C and were initiated by the addition of enzyme. Solvent deuterium isotope effects were calculated from eq 3 using the programs of Cleland (*18*)

$$v = VA/[K(1 + F_iE_{V/K}) + A(1 + F_iE_V)] \quad (3)$$

where V, A, and K are equivalent to those same parameters in eq 1, F_i is the fraction of isotopic label, and $E_{V/K}$ and E_V are the isotope effects on V/K and V, respectively.

Proton inventories were determined by varying the atom fraction of D_2O from 0 to 0.8 in increments of 0.1 in the same buffer as above. Substrate concentrations were 300 μM acetyl-CoA and 100 μM homoserine.

Rapid-Quench Experiments. The detection of an acetyl-enzyme intermediate was performed using a rapid-quench apparatus (KinTek Instruments, model RQF-3). The reactions were initiated by rapidly mixing 20 μL of HTA (final concentration, 275 μM) with [^{3}H]-acetyl-CoA (final concentrations, 400 μM and 20.3 μCi/μmol) in 50 mM HEPES (pH 7.7), and the reaction was quenched after various times with 25% trichloroacetic acid (TCA). The quenched reactions were collected in a microcentrifuge tube containing 25% TCA and placed on ice for a minimum of 5 min. The precipitates were centrifuged at maximum speed for 5 min, washed once with ice-cold 25% TCA, centrifuged again, and resuspended in 0.1 N NaOH. The amount of tritium associated with the precipitated protein was determined by scintillation counting in an LKB 1219 Rackbeta liquid scintillation counter (ScintiSafe Econo 2). Control reactions were performed with similar concentrations of bovine serum albumin (BSA) and HTS.

RESULTS

Expression and Purification of HTA. The *H. influenzae* HTA was overexpressed by cloning the *met2* gene into the pET23a(+) expression vector. Induction of BL21(DE3) cells containing this vector with IPTG resulted in overproduction of a soluble protein of the expected molecular mass. Following cell lysis, HTA was purified by anion exchange over a Fast-Q Sepharose column, by gel filtration over a Sepharose S-200 column, and by anion exchange over a Mono-Q column. This purification procedure yielded 370 mg of pure protein from 23.4 g of cells. Automated Edman amino-terminal sequencing revealed that the eleven amino-terminal residues were identical to the published sequence (*17*) and indicated that the N-terminal methionine had been cleaved. The molecular mass of the purified protein was determined by electrospray ionization-mass spectrometry (ESI-MS) to be 39 859 Da, matching the molecular mass predicted from the published sequence after subtracting the mass of the N-terminal methionine (39 858 Da). HTA eluted at an apparent molecular mass of 73 kDa on a Superdex 75 gel filtration column, which suggests it exists as a dimer in solution.

Determination of Steady-State Parameters. Enzyme activity was measured by determining the change in absorbance at 232 nm due to the hydrolysis of the thioester bond of acetyl-CoA. Initial velocities were determined at five different concentrations of each substrate, and the data were plotted as a double reciprocal plot (Figure 2). The parallel lines in the double reciprocal plot suggest a ping-pong mechanism. Fitting the data to eq 1 yields K_m values for acetyl-CoA and homoserine of 140 and 130 μM, respectively, and a k_{cat} of 92 s^{-1}. Initial velocities were also measured for the reverse reaction, the formation of acetyl-CoA. These data were fit to eq 1, yielding K_m values for OAH and CoA of 850 and 620 μM, respectively, and a k_{cat} of 31 s^{-1}. The steady-state kinetic parameters for the natural substrates, as well as a variety of alternative substrates, are summarized in Table 1. Alternative substrates were assayed using constant concentrations of either acetyl-CoA (200 μM) or homoserine (500 μM).

pH Dependence of Homoserine Transacetylase. The pH dependence of the transacetylase reaction was monitored over the pH range 5.5−9.0, using phosphate and Tris as buffers. The results are shown in Figure 3. Both the maximal velocity and $V/K_{homoserine}$ are pH independent over the range tested. The parameter $V/K_{acetyl-CoA}$, however, is dependent on the ionization state of a single group exhibiting a pK value of 8.6, which must be protonated.

Solvent Kinetic Isotope Effects. Solvent kinetic isotope effects were measured for the forward reaction by determining initial velocities in both H_2O and 80% D_2O. Measurements were made while holding the concentration of one substrate constant and varying the concentration of the second substrate. When acetyl-CoA was held at 400 μM, solvent kinetic isotope effects of 1.2 ± 0.1 on V and 1.7 ± 0.1 on $V/K_{homoserine}$ were observed (Figure 4A). When homoserine was held constant at 500 μM, effects of 1.2 ± 0.1 on V and 0.95 ± 0.03 on $V/K_{acetyl-CoA}$ were observed (data not shown). These values assume a linear dependence of the isotope effect on fractional deuterium abundance. A proton inventory was performed by varying the atom fraction of D_2O while holding the concentrations of acetyl-CoA and homoserine constant at 300 and 100 μM, respectively. Each data point was determined in triplicate. The points yielded a linear relation between the rate and mole fraction of deuterium (data not shown).

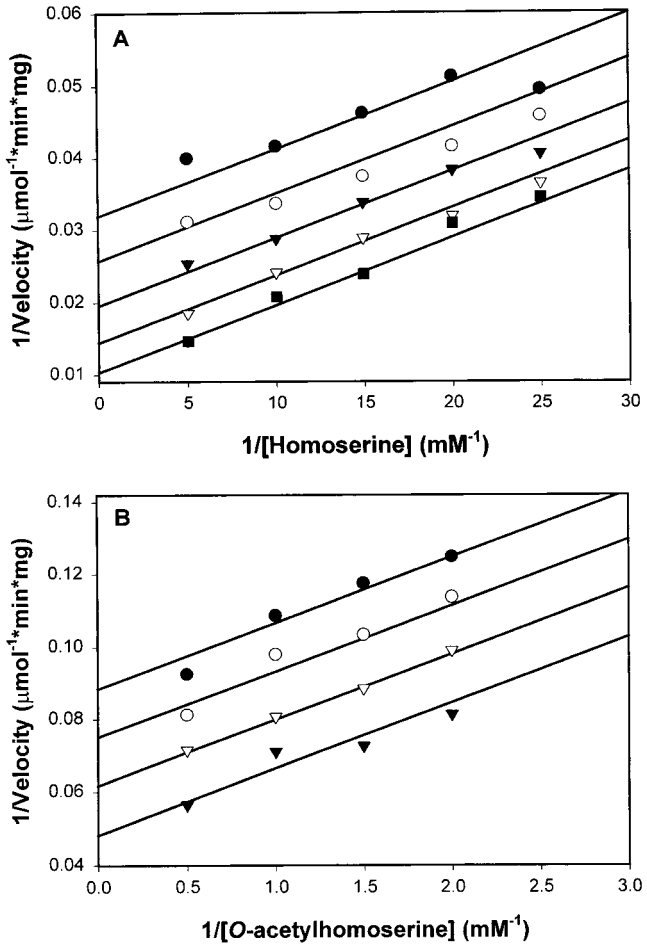

FIGURE 2: Initial velocity patterns of homoserine acetylation catalyzed by the *H. influenzae* HTA. The symbols represent the experimentally determined values, while the straight lines are the best fits to the data, assuming a ping-pong kinetic mechanism, calculated using eq 1. (A) Varying concentrations of homoserine at acetyl-CoA concentrations of (●) 41.7, (○) 55.6, (▼) 83.3, (Δ) 143, and (■) 333 μM. (B) Varying concentrations of OAH at CoA concentrations of (●) 200, (○) 250, (Δ) 333, and (▼) 500 μM.

Solvent kinetic isotope effects were measured for the reverse reaction in the same manner. In these experiments, the concentration of OAH was held constant at 5 mM and the concentration of CoA was varied. Solvent kinetic isotope effects of 0.75 ± 0.07 and 0.74 ± 0.06 were observed on V and V/K_{CoA}, respectively (Figure 4B).

Identification of an Acetyl-Enzyme Intermediate. A ping-pong kinetic mechanism predicts that an acetyl-enzyme intermediate is formed in the reaction of enzyme and acetyl-CoA and that acetate is subsequently transferred to homoserine in the second half-reaction. HTA catalyzes the slow hydrolysis of acetyl-CoA in the absence of homoserine, with a measured value of 4.5 s^{-1} at saturating concentrations of acetyl-CoA, compared to a k_{cat} of 92 s^{-1} for acetyl transfer in the presence of saturating homoserine (Table 1). To detect the formation of the acetyl-enzyme intermediate, HTA was incubated with [^{3}H]-acetyl-CoA for various times, and the reaction was quenched with 25% TCA. A rapid-quench

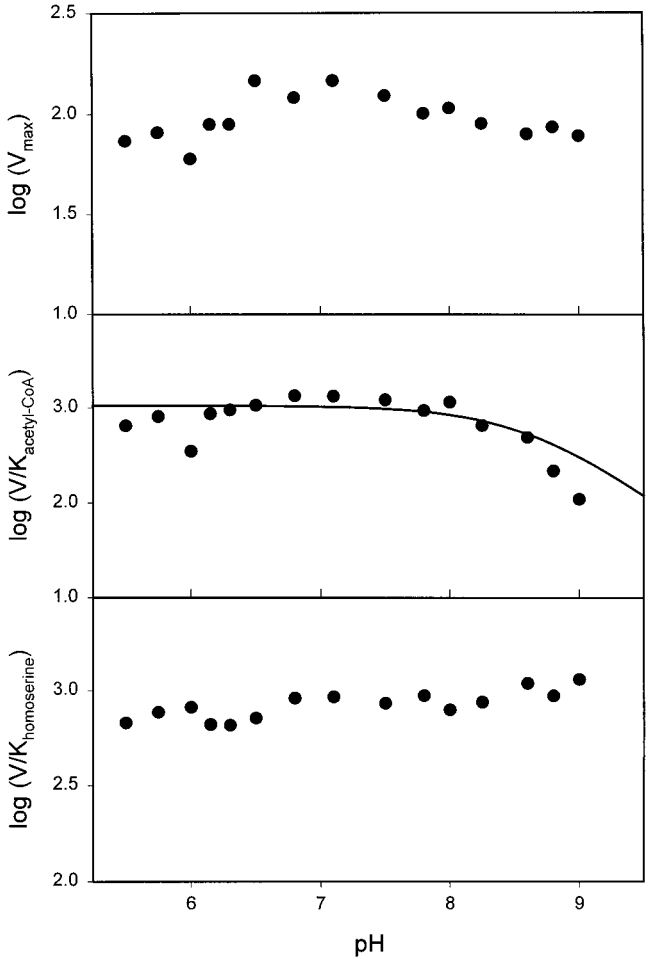

FIGURE 3: pH dependence of the kinetic parameters of the HTA-catalyzed acylation of homoserine. Experiments were conducted as described in Experimental Procedures. The symbols represent the experimentally determined values, while the curve is a fit of the $V/K_{acetyl-CoA}$ data to eq 2. (A) Dependence of maximal velocity on pH. The units of maximal velocity are micromoles per minute per milligram of enzyme. (B) Dependence of $V/K_{acetyl-CoA}$ on pH. The values of V/K are measured in units of maximal velocity per millimolar acetyl-CoA. (C) Dependence of $V/K_{homoserine}$ on pH. The values of V/K are measured in units of maximal velocity per millimolar homoserine.

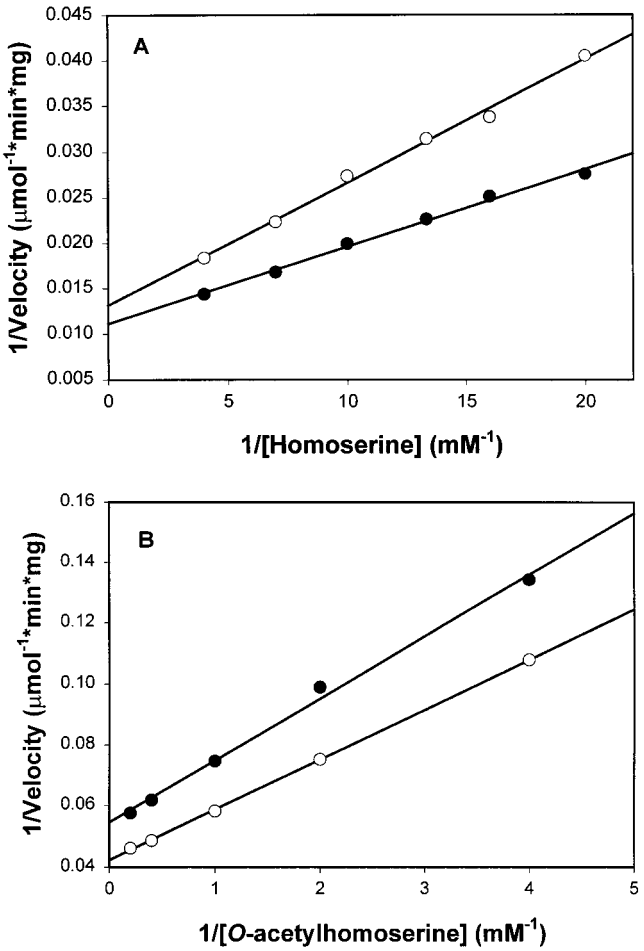

FIGURE 4: Solvent kinetic isotope effects for the HTA-catalyzed reaction. Experiments were performed as described in Experimental Procedures: experimental values in H_2O (●) and in 80% D_2O (○). Symbols represent the experimentally determined values, while the lines are the linear fits to the data. (A) The concentration of acetyl-CoA was held constant at 400 μM, while the concentration of homoserine was varied. (B) The concentration of CoA was held constant at 400 μM, while the concentration of OAH was varied.

apparatus was employed to allow accurate quenching of the reactions on a millisecond time scale. As shown in Figure 5, acylation of the enzyme occurs rapidly and reaches a maximum of ca. 50% active-site labeling in 60 ms. After 100 ms the acetyl-enzyme intermediate begins to hydrolyze. When the same experiment was performed using either BSA or HTS in place of HTA, no radioactivity was associated with the precipitated proteins (<1% labeling).

DISCUSSION

Homoserine is the last common precursor in the bacterial biosynthetic pathways of three essential amino acids: threonine, isoleucine, and methionine. Control of homoserine flux into these pathways is therefore important in regulating the biosynthesis of these three amino acids as well as *S*-adenosylmethionine. Control of methionine and SAM biosynthesis occurs at the first unique enzymatic step in the pathway, the acylation of homoserine to form either OAH or *O*-succinylhomoserine (Figure 1). The enzymes catalyzing this reaction are feedback-inhibited by both the final product of the pathway, methionine, and SAM (*6*−*10*). These enzymes can also be inhibited by elevated temperatures (*13*, *14*) or the presence of metal ions such as zinc (*8*). Genes encoding the *metA*-encoded HTS have been identified in *E. coli* (*19*, *20*), *Bacillus subtilus* (*21*), *Thermotoga maritima* (*22*), and *Salmonella typhimurium* (*23*), and the enzymatic activity has been reported in cell extracts from *Aerobacter aerogenes*, *Klebsiella pneumonia*, and *Serratia marcescens* (*13*). The *met2*-encoded HTA appears to have a wider phylogenetic distribution, with genes having been identified in *H. influenzae* (*17*), *Mycobacterium tuberculosis* (*24*), *Saccharomyces cerevisiae* (*25*), and *Leptospira meyeri* (*12*), among many others. No organism containing genes for both acyltransferases has been identified to date. In addition to their variant organismal distribution, the two enzymes display absolutely no sequence similarity. Because these two enzymes are involved in the same biosynthetic pathway, are responsible for catalyzing the same reaction, use nearly identical substrates, and have similar control mechanisms, we were intrigued by the lack of primary sequence conservation and were interested in investigating whether they had evolved similar or different chemical mechanisms.

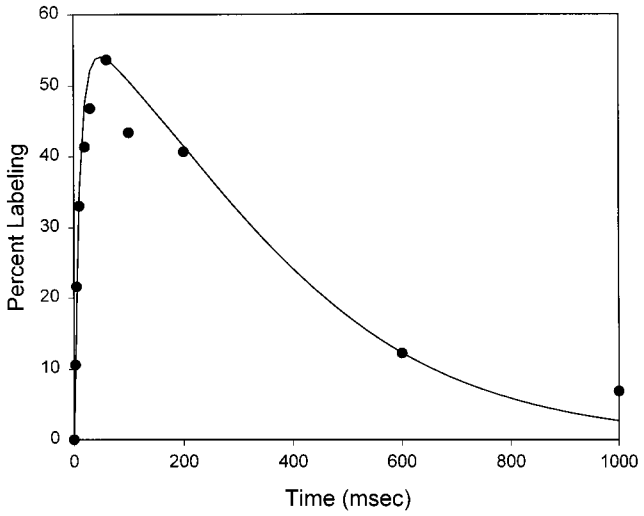

FIGURE 5: Isolation of an acetyl-enzyme intermediate by rapid quench. HTA and [^{3}H]-acetyl-CoA were combined, and the reaction was quenched as described in Experimental Procedures. The percent of enzyme labeled is shown as a function of the time of incubation, in milliseconds, before quench by TCA. The simulated time-course was performed using the Scientist program (MicroMath Scientific Software, Inc., Salt Lake City) using the kinetic scheme shown in Scheme 1.

The transacetylase from *H. influenzae* was cloned and overexpressed to high levels, greatly simplifying purification and making it possible to obtain hundreds of milligrams of protein in a single preparation. Although the enzyme from *Bacillus polymyxa* has been reported to exist as a monomer (*8*), the *H. influenzae* enzyme elutes from a gel filtration column at an apparent molecular mass of 73 kDa, suggesting it exists as a dimer under these conditions. HTS has also been reported to exist as a dimer (*15, 26*).

The kinetic data reported in this paper were obtained spectrophotometrically by continuously following the change in absorbance at 232 nm due to the hydrolysis or the formation of the thioester bond of acetyl-CoA. Using this assay, initial velocities could be determined by varying substrate concentrations in both the forward and the reverse directions. As shown in Figure 2, the resulting double-reciprocal plots are parallel, suggesting that HTA utilizes a ping-pong kinetic mechanism. This predicts that during catalysis the acetyl group of acetyl-CoA is transferred to an active-site nucleophile during the first half-reaction, followed by transfer to homoserine during the second half-reaction. The steady-state kinetic parameters were measured for a number of acyl donors and acceptors (Table 1). Acetyl-CoA is the favored acyl donor, as measured by k_{cat}/K_m, but propionyl-CoA, butyryl-CoA, and crotonyl-CoA are also good substrates for HTA. Additionally, succinyl-CoA and glutaryl-CoA can be used as acyl donors, but are much poorer substrates. This contrasts with HTS, which is able to use succinyl-CoA and glutaryl-CoA as substrates, but is unable to use other CoA-thioesters. Due to the favorable spectrophotometric properties of the resulting nitrophenol product, 4-nitrophenylacetate was explored as an acyl donor, but it exhibited a K_m value that was too high to be kinetically useful. When acyl acceptors were analyzed, it was found that L- and D-homoserine were the best substrates, while both γ-hydroxybutyric acid and 3-amino-1-propanol had <1% the activity of L-homoserine. We were surprised to find that D-homoserine was a good substrate, although D-homoserine has been demonstrated to be a substrate for HTS (*15*). While HTA and HTS have similar structural requirements for their acyl acceptors, it should be noted that HTA has a 16-fold lower K_m value for homoserine.

The equilibrium constant for the reaction can be estimated from the Haldane equation (*27, 28*)

$$K_{eq} = (V_f/V_r)^2(K_PK_Q/K_AK_B) \quad (4)$$

where V_f and V_r are the maximal velocities in the forward and reverse directions, respectively, and K_A, K_B, K_P, and K_Q are the Michaelis constants for the two substrates, acetyl-CoA and homoserine, and the two products, CoA and OAH, respectively. Using this equation, the equilibrium constant for HTA can be calculated to be ca.. 250, similar to the estimated equilibrium constant of 170 for HTS (*15*) and a value of 15−40 measured for serine transacetylase (*29*).

From the data presented in Figure 3A, there is no dependence of the maximal velocity on pH. However, the pH was varied over a fairly narrow range, 5.5−9, and there may be catalytic groups whose ionizations occur at pH values outside this range. The continuous spectrophotometric assay used, monitoring the change in absorbance at 232 nm, limits the use of buffers to those with low absorbance at this wavelength, and only phosphate and Tris were found to be acceptable for HTA. As will be discussed below in the proposed mechanism for HTA, one would expect the maximal velocity to depend on the ionization of at least two groups, one of which would be required to be protonated and function as a general acid, and another to be deprotonated and function as a general base. When the dependence of $V/K_{acetyl-CoA}$ on pH is examined (Figure 3B), we observe a single group exhibiting a pK value of ~8.6 that must be protonated. The pH dependence of $V/K_{acetyl-CoA}$ reports on the ionization behavior of groups on the free enzyme or free acetyl-CoA involved in binding but may include ionization behavior of groups through the first irreversible step. For acetyl-CoA, this includes acetyl transfer to the enzyme and CoA release (see below). The only ionizable group on acetyl-CoA is the 3′-phosphate, and this cannot be the group observed in the $V/K_{acetyl-CoA}$ pH profile. This leaves enzyme groups and could include a cationic residue that interacts electrostatically with the 3′-phosphate of acetyl-CoA or a group that functions to protonate the thiolate anion of the product CoA. These possibilities will be discussed further below.

As is observed for the maximal velocity, $V/K_{homoserine}$ appears to be independent of pH over the range tested. The pH dependence of $V/K_{homoserine}$ should report on the ionization behavior of groups on substrate or enzyme responsible for homoserine binding and catalysis of the second half-reaction through the first irreversible step of this half-reaction. We would have expected to observe a group functioning as a general base to generate the nucleophilic alkoxide of homoserine; however, we see no evidence for such a group that must be deprotonated for activity. The pH-dependencies observed for HTA are very different from those observed for HTS, where the three analogous curves were bell-shaped. Even though the mechanism that we propose is similar for both acyltransferases, these data suggest that the residues involved in catalysis will be different.

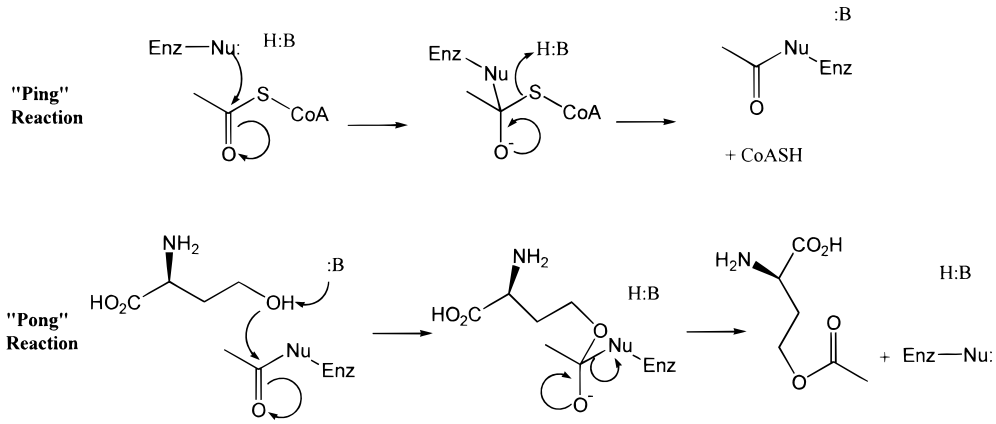

FIGURE 6: Proposed mechanism for transfer of acetate from acetyl-CoA to homoserine catalyzed by HTA.

Scheme 1

$$\text{E} + [^3\text{H}]\text{-acetyl-CoA} \underset{k_3}{\overset{k_1}{\rightleftharpoons}} \text{E-}[^3\text{H}]\text{-acetyl} + \text{CoA} \xrightarrow{k_2} \text{E} + [^3\text{H}]\text{-acetate}$$

Solvent kinetic isotope effect studies demonstrated that there was an inverse effect when the reaction was monitored in the reverse direction, formation of acetyl-CoA. The ^{D_2O}V was 0.75 while the $^{D_2O}V/K_{CoA}$ was 0.74, values that are similar to those observed for HTS. The inverse solvent kinetic isotope effects suggest that there is a proton transfer from a sulfhydryl group exhibiting an inverse fractionation factor, and we suggest that this is reporting on the deprotonation of CoASH occurring during the slower reverse reaction. A small, normal solvent kinetic isotope effect is observed in the forward reaction on both V and $V/K_{acetyl-CoA}$, and this might reflect the effect of solvent isotopic composition on homoserine alkoxide formation or thiol protonation.

The demonstration of a ping-pong kinetic mechanism by HTA requires the formation of an acetyl-enzyme intermediate. Attempts were made to identify this intermediate by incubating HTA with [^{3}H]-acetyl-CoA in the absence of homoserine. Initial experiments using gel filtration were unsuccessful in identifying a labeled acetyl-enzyme, arguing that the rate of hydrolysis of the intermediate was fast. A rapid-quench apparatus was employed to allow accurate quenching of the reaction on a millisecond time scale. These experiments provided compelling evidence for the rapid formation of an acetyl-enzyme intermediate, with a maximum labeling of ~50% of enzyme active sites within 60 ms. To ensure that labeling was not due to acetyl-CoA nonspecifically associating with the enzyme, we repeated these experiments using both BSA and HTS. No radioactivity was found to be associated with either of the negative controls, suggesting that HTA does, indeed, form a covalent intermediate.

The relative rates of acetylation and hydrolysis were estimated by fitting the data in Figure 5 to Scheme 1. In this scheme, HTA combines with acetyl-CoA to form the acetyl-enzyme intermediate plus CoA. This intermediate either can be hydrolyzed by water to form acetate or can recombine with CoA to form acetyl-CoA. When the rapid-quench data were fitted and analyzed using the program Scientist (MicroMath, Inc, version 2.01), estimates of the relative rate constants k_1, k_2, and k_3 were 0.4, 0.6, and 2.0, respectively. If both steps in Scheme 1 were assumed to be irreversible (i.e. $k_3 = 0$), it was not possible to accurately fit the data using combinations of k_1 and k_2. Simulations where $k_3 = 0$ either resulted in >50% labeling of the active sites by [^{3}H]-acetyl-CoA or the time required to reach maximum labeling was much longer than 60 ms. From these simulations, recombination of the acetyl-enzyme with CoA to form acetyl-CoA is favored over hydrolysis by H_2O. General acid-catalyzed protonation of the CoA thiolate would disfavor back reaction, and the group observed in the $V/K_{acetyl-CoA}$ pH profile is thus more likely to be a group involved in binding, rather than a group that could fulfill the function of a general acid. These data suggest that acetylation of HTA is the slowest step in the first half-reaction.

Taken together, these data allow us to propose a chemical mechanism for HTA, shown in Figure 6. In the "ping" half-reaction, an enzyme nucleophile attacks the thioester bond of acetyl-CoA to form an initial tetrahedral intermediate. The nucleophile has been proposed to be cysteine in HTS, while the HTA nucleophile is not known at this time, although it seems unlikely that it is a cysteine residue for at least three reasons. First, thiol modifying agents, such as iodoacetamide, have no effect on the activity of HTA, but inactivate HTS. Second, 5,5′-dithio-bis(2-nitrobenzoic acid), (DTNB), an inhibitor of HTS, does not inhibit HTA and can be used in a coupled assay for HTA. Third, we failed to identify any residues with a pK greater than 5.5 which are required to be unprotonated for maximal activity. If the active-site nucleophile were a cysteine residue, its ionization would be expected to be within the range of values tested, as observed in HTS (*15*).

Once the initial tetrahedral intermediate is formed between HTA and acetyl-CoA, it must break down to form the acetylated enzyme and CoASH. Breakdown of this intermediate would be facilitated by protonation of the thiolate anion by an active-site general acid, but simulation of our rapid-quench data argues against efficient general acid-catalyzed thiolate anion protonation. Homoserine binds to the acetyl-enzyme intermediate in the pong reaction, and an active-site base is required to remove the proton from the γ-hydroxyl group before it can attack the acetyl-enzyme to form a tetrahedral intermediate. We do not observe this base

[2] While it is most plausible to assign the group that results in an inverse solvent kinetic isotope effect to the free sulfhydryl of CoA, this is not the only possible explanation. ^{1}H NMR studies have identified proton-exchangeable sites on catalytic residues with fractionation factors less than unity (low-barrier hydrogen bonds). However, in the context of the reaction catalyzed by HTA, we feel that assignment of the observed inverse effect to the fractionation factor of CoA is justifiable.

in the $V/K_{homoserine}$ pH profile, suggesting that the p*K* of this base is higher than 9. This contrasts with the situation for HTS, where evidence for a general base exhibiting a p*K* value of 8.6 was obtained. Finally, this tetrahedral intermediate breaks down to form the final product, OAH, and regenerate the active enzyme.

Our solvent kinetic isotope effects suggest the transfer of a single proton from a thiol during the reverse reaction. In the mechanism proposed, a base is required to abstract a proton from CoASH in the reverse reaction before it can attack the acetyl-enzyme intermediate and ultimately form acetyl-CoA. It is the abstraction of this proton that we observe in our solvent kinetic isotope effects.[2]

At this time the identity of the HTA catalytic nucleophile is unknown. Precedence from other acyltransferases, including HTS, suggests cysteine as a potential nucleophile. However, as outlined above, there is no physicochemical evidence with HTA for the involvement of a cysteine residue, and there are no conserved cysteine residues among the known, aligned, HTA sequences. There are, however, three conserved serine residues, two conserved histidine residues, and six conserved aspartic acid residues. Each of these residues is precedented as functioning as a nucleophile in various enzyme systems (*30*−*34*).

One of the three conserved serine residues in HTA, S143, is found in a G-G-S-X-G-G sequence that is absolutely conserved among HTAs. This motif, G-X-S/C-X-G has been identified as a signature sequence for the lipase superfamily (*35*, *36*). These lipases also have acyl transferase activity, with the conserved serine or cysteine functioning as the nucleophile in the formation of a covalent intermediate. Members of the lipase superfamily contain a catalytic "triad" of serine, aspartic acid, and histidine (*37*−*39*), with the residues always found in this order in the primary sequence. Histidine has been assigned the role of a general base to activate serine for nucleophilic attack. Essential histidines have been identified in monoglyceride lipase (*39*), rat carnitine palmitoyltransferase (*40*), polyhydroxybutyrate synthase (*41*) and glycerophospholipid-cholesterol acyltransferase (*37*), among others. The HTAs contain only one conserved histidine C-terminal to S143, H337, which may function as the general base. The role of the third residue of the "triad", aspartic acid, is somewhat ambiguous, and in some cases the acid has been shown not to be essential. Four conserved aspartates are found in the intervening sequence between the conserved serine (S143) and histidine (H337) residues, Asp-158, Asp-190, Asp-262, and Asp-304. Site-directed mutagenesis studies will be used to begin to define the roles of each of these residues in the catalytic mechanism of HTA.

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