Enzyme-Catalyzed Acylation of Homoserine: Mechanistic Characterization of the *Escherichia coli metA*-Encoded Homoserine Transsuccinylase†

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ABSTRACT: The first unique step in bacterial and plant methionine biosynthesis involves the activation of the γ-hydroxyl of homoserine. In *Escherichia coli*, this activation is accomplished via a succinylation reaction catalyzed by homoserine transsuccinylase. The activity of this enzyme is closely regulated in vivo and therefore represents a critical control point for cell growth and viability. We have cloned homoserine transsuccinylase from *E. coli* 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 succinyl group of succinyl-CoA is initially transferred to an enzyme nucleophile before subsequent transfer to homoserine to form the final product, *O*-succinylhomoserine. The maximal velocity, $V/K_{\text{succinyl-CoA}}$, and $V/K_{\text{homoserine}}$ all exhibited a bell-shaped pH dependence with apparent p*K*'s of 6.6 and ~7.9. The enzyme was inhibited by iodoacetamide in a pH-dependent manner, with an apparent p*K* of the group being inactivated of 6.4. This suggests the presence of an active site cysteine which forms a succinyl−cysteine intermediate during enzymatic turnover. Solvent kinetic isotope effect studies yielded inverse effects of 0.7 on *V* and 0.61 on *V*/*K* in the reverse reaction only. On the basis of these observations, we propose a detailed chemical mechanism for this important member of the acyltransferase family.

Methionine is one of 10 essential dietary amino acids in mammals. Both microorganisms and plants synthesize methionine from aspartic acid, which also serves as the precursor of lysine, threonine, and isoleucine. In addition to its role as a building block of proteins, adenosylation of methionine produces *S*-adenosylmethionine (SAM),[1] a biological methylating agent which may also serve as a precursor of the "quorum sensing" acyl-homoserine lactone signal molecules (*1*, *2*). Since both methionine and SAM are required for cell viability and growth, investigations into the biochemistry of methionine biosynthesis may yield information that is useful in the development of inhibitors effective against pathogenic bacteria.

Beginning with the last common precursor, homoserine, methionine biosynthesis occurs in four steps (Figure 1). The initial reaction, acylation of the γ-hydroxyl of homoserine, serves as an activation step which allows the succeeding condensation with cysteine for forming the thioether-containing compound cystathionine. Hydrolysis of cystathionine produces homocysteine, which is then methylated in a vitamin B_{12}-dependent or -independent reaction to form methionine. Subsequent adenosylation produces *S*-adenosylmethionine.

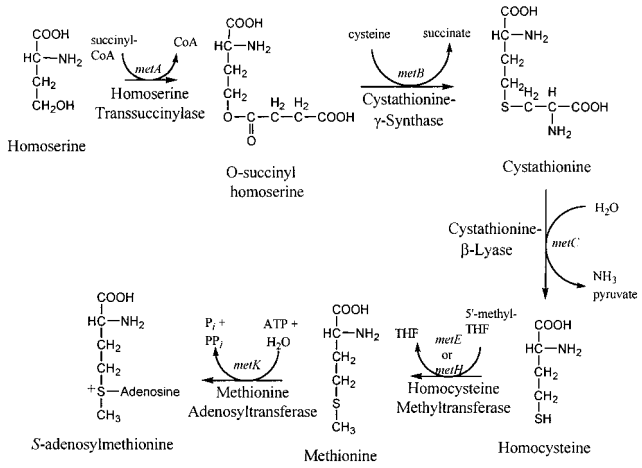

FIGURE 1: Biosynthetic pathway of methionine and *S*-adenosylmethionine in *E. coli*.

In *Escherichia coli*, the initial step of methionine biosynthesis is catalyzed by homoserine transsuccinylase (HTS, EC 2.3.1.46), which transfers a succinyl group from succinyl-CoA to homoserine. This enzyme is allosterically inhibited by methionine and SAM (*3*), and is also inhibited by elevated temperatures (*4*, *5*). In other bacteria, such as *Haemophilus influenzae*, the initial step is catalyzed by homoserine transacetylase (HTA, EC 2.3.1.31), which transfers the acetyl group of acetyl-CoA to homoserine. This enzyme is also allosterically inhibited by methionine and SAM (*6*−*8*). Although these enzymes are responsible for the same biological function, and utilize similar substrates, they exhibit

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[1] Abbreviations: CoA, coenzyme A; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); ESI-MS, electrospray ionization mass spectrometry; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HTA, homoserine transacetylase; HTS, homoserine transsuccinylase; IAA, iodoacetamide; IPTG, isopropyl thio-β-D-galactoside; OSH, *O*-succinylhomoserine; SAM, *S*-adenosylmethionine; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TEA, triethanolamine.

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no sequence similarity. A thorough mechanistic and structural study of both enzymes will be required to determine how these significantly dissimilar enzymes catalyze an exceedingly similar reaction.

Mechanistic studies which have been performed on a number of acyl transferases have demonstrated that members of this enzyme class catalyze acyl transfer by ping-pong, rapid equilibrium random, and rapid equilibrium sequential mechanisms. Although the majority of enzymes of the ping-pong subclass utilize a nucleophilic cysteine residue to form the acyl−enzyme intermediate (*9*−*12*), glutamic acid can also act as the nucleophile (*13*). For other members of this class, the nucleophile has not been determined (*14*−*16*). Despite the extensive literature that is available on this class of enzymes, relatively little is known about the subset in which the acyl group is transferred to an alcohol. Both HTS and HTA are important members of this class.

Many acyl transferases are of significant biological importantance. These include serotonin-*N*-acetyltransferase, required for synthesis of melatonin (*17*); arylamine-*N*-acetyltransferase, required for the acylation and subsequent degradation of a variety of drugs (*18*, *19*); and thiaminase I, which degrades thiamine and is implicated in thiamine deficiency (*10*). Therefore, members of this enzyme class represent potential targets for the design of novel antibacterial compounds. Specifically, HTS and HTA possess a number of characteristics that make them attractive targets. (1) Enzymes in the methionine biosynthetic pathway are not present in mammals. (2) HTS and HTA catalyze the first unique step in the synthesis of methionine, and this initial step is closely regulated in vivo. (3) Both enzymes are subject to feedback control by products, and HTS has been shown to be inactivated in *E. coli* at elevated temperatures, leading to the arrest of cell growth. These observations strongly suggest that inhibition of either of these enzymes will be bactericidal or bacteriostatic.

In this paper, we report the cloning, overexpression, and purification of the *metA*-encoded homoserine transsuccinylase from *E. coli*. We have determined the kinetic parameters for its natural substrates, as well as a number of alternative substrates, which suggest a ping-pong kinetic mechanism. The pH dependence of the enzyme activity, in conjunction with inactivation studies with iodoacetamide, has allowed us to identify the presence of an active site sulfhydryl residue and to determine its p*K* value. Additionally, solvent kinetic isotope effect studies indicate an inverse isotope effect on the reverse reaction, but no effect on the forward reaction. A model of the enzymatic chemical mechanism that incorporates these data is proposed.

MATERIALS AND METHODS

Materials. Oligonucleotide primers used for PCR amplification were synthesized by Life Technologies (Gaithersburg, MD). *Nde*I and *Bam*HI restriction enzymes were purchased from New England BioLabs (Beverly, MA), and pET23a(+) vector DNA and *E. coli* BL21(DE3) cells were from Novagen (Madison, WI). Chromatographic supports for protein purification were obtained from Pharmacia (Piscataway, NJ). D_2O (99.9%) was from Cambridge Isotope Laboratories (Andover, MA). All other materials were from Sigma (St. Louis, MO). DNA sequencing was performed by the Albert Einstein College of Medicine DNA sequencing facility.

Cloning and Overexpression of E. coli Homoserine Transsuccinylase. The sequence of the putative *E. coli metA* gene has been reported (*20*). Two oligonucleotides (5′-AATTCCATATGCCGATTCGTGTGCCGGACG-3′ and 5′-GCGGATCCCTTAATCCAGCGTTGGATTCATG-3′) which were complementary to the amino-terminal coding and carboxyl-terminal noncoding strands were synthesized containing *Nde*I and *Bam*HI restriction sites (underlined), respectively, in noncomplementary overhangs. These primers were used to amplify the *E. coli metA* gene from genomic DNA using AmpliTac DNA polymerase (Perkin-Elmer) under standard PCR conditions. The PCR product was purified by electrophoresis on low-melting point agarose and directly ligated into the pCR2.1 TA cloning vector (Invitrogen). Plasmid DNA was purified from ampicillin resistant colonies and digested with *Nde*I and *Bam*HI, 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 competent *E. coli* BL21(DE3) cells. The cells were induced with IPTG, and analysis by SDS−PAGE indicated the significant overexpression of a 35−40 kDa protein.

Protein Purification. Four liters of LB media containing ampicillin (50 mg/L) was inoculated with a single bacterial colony and grown to an OD_{600} of 0.6 at 37 °C before induction with 1 mM IPTG. After induction for 2 h, approximately 16 g of cells (wet weight) was recovered by centrifugation. The cells were resuspended in 75 mL of 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 the solution was stirred for 30 min, the cells were lysed by sonicating four times for 1 min intervals with a Branson model 450 sonifier 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 concentration) to the supernatant, and the solution was stirred for 1 h 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 2 h. 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 a rate of 2 mL/min with a 1500 mL linear 0 to 1 M NaCl gradient. The active fractions were pooled, concentrated (YM-10 membrane, Amicon), and loaded onto a 2.4 cm × 57 cm Sepharose S-200 gel filtration column. The protein was eluted at a rate of 0.5 mL/min in 50 mM HEPES (pH 7.5). The active fractions, which yielded a single band on SDS−PAGE with Coomassie blue staining, were concentrated to 10 mg/mL and stored at −20 °C.

Measurement of Enzyme Activity. Reaction rates were determined by monitoring the decrease in absorbance at 232 nm due to hydrolysis of the thioester bond of succinyl-CoA ($\epsilon = 4500\ M^{-1}$) in a UVIKON 9310 or 943 spectrophotometer equipped with thermospacers and connected to a

Table 1: Kinetic Parameters for the Reaction Catalyzed by Homoserine Transsuccinylase

substrate	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} M^{-1})
succinyl-CoA	0.17 ± 0.03	24 ± 2	1.4×10^5
coenzyme A	0.64 ± 0.10	5.23 ± 0.57	8.2×10^3
glutaryl-CoA	0.18 ± 0.03	1.6 ± 0.1	8.9×10^3
acetyl-CoA		inactive	
L-homoserine	1.6 ± 0.2	24 ± 2	1.5×10^4
O-succinylhomoserine	3.5 ± 0.6	5.23 ± 0.57	1.5×10^3
D-homoserine	10 ± 2	12 ± 2	1.2×10^3
3-amino-1-propanol	20 ± 13	0.13 ± 0.06	6.7
L-serine	25 ± 10	0.023 ± 0.006	0.92
L-threonine	31 ± 18	0.015 ± 0.007	0.49
γ-hydroxybutyric acid		inactive	
γ-aminobutyric acid		inactive	

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, and initial velocity kinetic data were analyzed by Lineweaver−Burk analysis and fitted to eq 1 using the programs of Cleland (*21*)

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

where V is the maximal velocity, A and B are the concentrations of substrates A and B, respectively, and K_A and K_B are the Michaelis constants for substrates A and B, respectively. Initial velocity patterns were obtained by measuring the extent of substrate depletion as a function of the concentration 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 of 6.0−8.75 using phosphate (6.0−8.0) and Tris (7.8−8.75) 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 and fitted to eq 2 using the program Sigma Plot (version 3.03, Jandel Scientific Software)

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

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

Inactivation by Iodoacetamide. A solution containing 25 μg of enzyme in 100 mM phosphate buffer was inactivated at 25 °C by the addition of 12.5 μM iodoacetamide (100 μL final volume). Aliquots (5 μL) were removed at various time points and added to an assay solution containing 100 mM K_2HPO_4 (pH 7.5), 5 mM homoserine, and 200 μM succinyl-CoA in a final volume of 500 μL. The residual enzyme activity was measured by monitoring the change in absorbance at 232 nm as described above. The rate of inactivation was measured over a pH range of 6.0−8.0 using phosphate buffer, and the data were fitted to eq 3 using Sigma Plot

$$\log V = \log[C/(1 + H/K_a)] \quad (3)$$

were V is the rate of inactivation and C, H, and K_a are as defined in eq 2.

Solvent Kinetic Isotope Effects. Solvent kinetic isotope effects on V and V/K were determined by measuring the initial velocity of thioester cleavage or thioester formation at 232 nm. The mixtures used in experiments for measuring thioester cleavage contained 0.5 μg of enzyme, 50 mM K_2HPO_4 (pH 7.5), 5 mM homoserine, and varying concentrations of succinyl-CoA in H_2O or 94% D_2O. Assays were performed at 25 °C and were initiated by the addition of enzyme. Mixtures used in experiments for measuring the extent of thioester formation contained 2.5 μg of enzyme, 50 mM K_2HPO_4 (pH 7.5), 15 mM *O*-succinylhomoserine (OSH), and varying concentrations of CoA in H_2O or 80% D_2O, or 0.4 mM CoA and varying concentrations of OSH in H_2O or 80% D_2O. Assays were performed at 25 °C and were initiated by the addition of enzyme. Solvent deuterium isotope effects were calculated from eq 4 using the programs of Cleland (*21*)

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

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 described above. Substrate concentrations were as follows: 0.5 mM CoA and 15 mM OSH.

RESULTS

Expression and Purification of Homoserine Transsuccinylase. The *E. coli* homoserine transsuccinylase was overexpressed by cloning the corresponding gene into the pET23a-(+) expression vector. Upon IPTG induction, BL21(DE3) cells containing this vector expressed a soluble protein with the expected molecular mass, with the transsuccinylase representing more than 60% of the total soluble protein as determined by SDS−PAGE. Following cell lysis, HTS was purified by anion exchange over a Fast-Q Sepharose column followed by gel filtration over a Sepharose S-200 column, yielding approximately 0.6 g of HTS from 16 g of cells. Automated Edman amino-terminal sequencing indicated that the N-terminal methionine had been cleaved and confirmed that the 20 subsequent residues were identical to the amino acid sequence derived from the published DNA sequence. Complete nucleotide sequencing of the cloned gene detected a possible substitution at codon 199, resulting in the replacement of Val with Ile at position 67, while the rest of the sequence matched the previously published sequence (*20*). HTS eluted at an apparent molecular mass of 86 kDa when analyzed on a Superdex 75 gel filtration column, while the monomer molecular mass is 35.6 kDa.

Determination of Steady-State Parameters. Enzyme activity was directly measured by following the change in absorbance at 232 nm due to the hydrolysis of the thioester bond of succinyl-CoA. Using this assay, initial velocities were linear and proportional to the amount of added enzyme (data not shown). Initial velocities were determined at varying concentrations of each substrate, and the data were plotted as a double-reciprocal plot (Figure 2). The parallel lines in Figure 2A suggest a ping-pong mechanism, and an

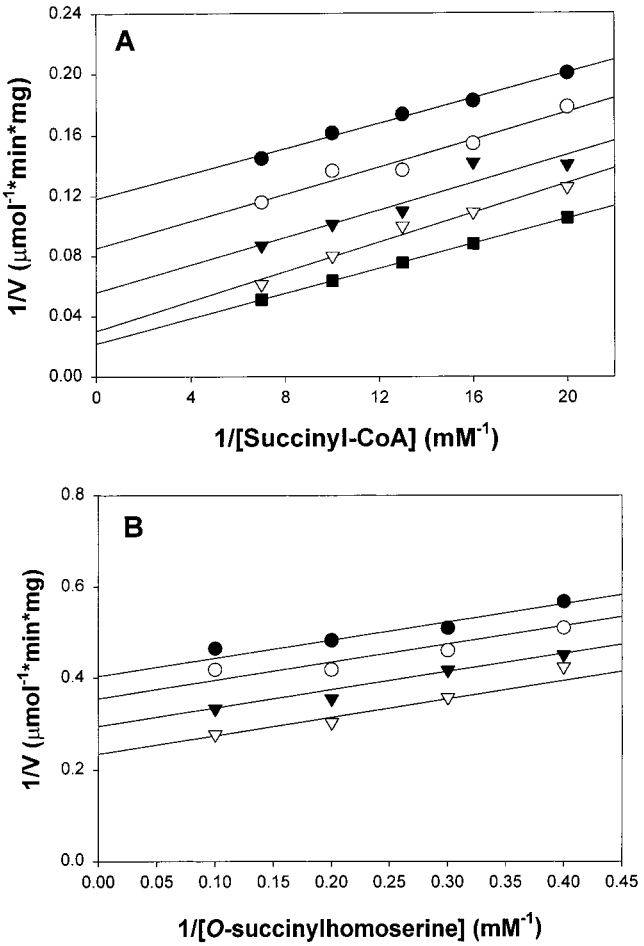

FIGURE 2: Initial velocity patterns of homoserine succinylation catalyzed by homoserine transsuccinylase. The symbols represent the experimentally determined values, while the curves are the best linear fits to the data. (A) Varying concentrations of succinyl-CoA at L-homoserine concentrations of (●) 0.5, (○) 1.0, (▼) 2.0, (▽) 5.0, and (■) 10 mM. (B) Varying concentrations of *O*-succinylhomoserine at CoA concentrations of (●) 250, (○) 300, (▼) 400, and (▽) 600 μM.

identical pattern of lines is obtained when monitoring the formation of succinyl-CoA from *O*-succinylhomoserine (OSH) and coenzyme A (CoA) (Figure 2B). The kinetic parameters for the natural substrates in both directions, as well as a variety of alternative substrates, are summarized in Table 1. The alternative substrates were assayed using constant concentrations of succinyl-CoA (200 μM) or homoserine (5 mM).

pH Dependence of Homoserine Transsuccinylase. The pH dependence of the transsuccinylase reaction was measured over the pH range of 6.0−8.75 using phosphate and Tris as buffers due to their low absorbance at 232 nm. As can be seen in Figure 3A, the plot of the pH dependence of the maximal velocity is a bell-shaped curve, with *V* decreasing with an apparent slope of 1 at lower pH values and a slope of −1 at higher pH values. Similarly, the plot of the pH dependence of $V/K_{\text{succinyl-CoA}}$ is also a bell-shaped curve, decreasing with a slope of 1 at lower pH values and a slope of −1 at higher pH values (Figure 3B). The p*K* of the acidic group is identical in these two data sets, as listed in Table 2. The pH dependence of $V/K_{\text{homoserine}}$ (Figure 3C) is similar to that seen for the maximal velocity and for $V/K_{\text{succinyl-CoA}}$. The bell-shaped curve yields a p*K* for the acidic group that

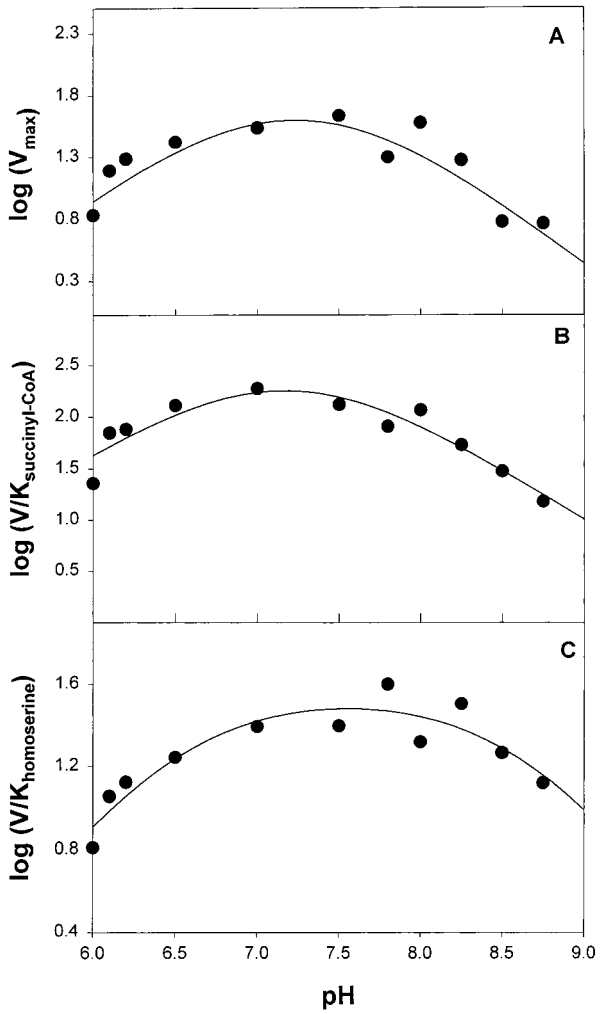

FIGURE 3: pH dependence of the kinetic parameters of homoserine transsuccinylase-catalyzed acylation of homoserine. Experiments were conducted as described in Materials and Methods. The symbols represent the experimentally determined values, while the curves are fits to the data calculated from eq 2. (A) Dependence of maximal velocity on pH. The units of maximal velocity are micromoles per minute per milligram. (B) Dependence of $V/K_{\text{succinyl-CoA}}$ on pH. The values of *V*/*K* are measured in units of maximal velocity per millimolar succinyl-CoA. (C) Dependence of $V/K_{\text{homoserine}}$ on pH. The values of *V*/*K* are measured in units of maximal velocity per millimolar homoserine.

Table 2: Calculated p*K* Values of Residues Whose Ionization State Is Critical for HTS Activity

	pK_a	pK_b
V_{max}	6.6 ± 0.3	7.9 ± 0.3
$V/K_{\text{succinyl-CoA}}$	6.7 ± 0.2	7.5 ± 0.2
$V/K_{\text{homoserine}}$	6.5 ± 0.2	8.6 ± 0.3
$k_{\text{inact,IAA}}$	6.4 ± 0.1	

is indistinguishable from that seen in the other two pH profiles, while the p*K* of the basic group is slightly higher than that seen previously (Table 2).

Inactivation by Iodoacetamide. Iodoacetamide (IAA) was determined to be an inactivator of HTS. Incubation of HTS with IAA resulted in a time-dependent loss of activity which was first-order over a 20 min time period. Moreover, the inactivation proved to be pH-dependent (Figure 4). As the pH was increased from 6.0, the rate of inactivation increased correspondingly, reaching a plateau value above pH 7.5. As shown in Figure 4, the rate of inactivation decreased with

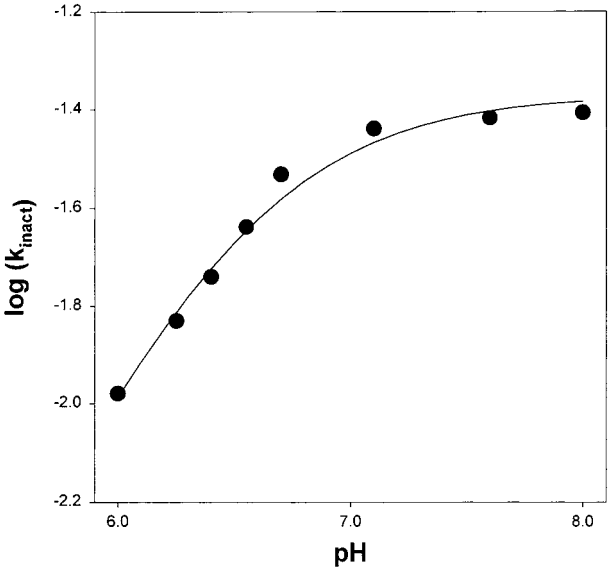

FIGURE 4: pH dependence of inactivation of homoserine transsuccinylase by iodoacetamide. Experiments were conducted as described in Materials and Methods. The symbols represent the experimentally determined values, while the curve is a fit to the data calculated from eq 3. The rate of inactivation is measured in units of remaining initial velocity (micromoles per minute per milligram) per minute.

an apparent slope of 1 below pH 7.5, indicating that inactivation was due to deprotonation of a single ionizable group exhibiting a p*K* value of 6.4. The p*K* value determined for this group agrees closely with those identified in the pH dependence of the kinetic parameters (Table 2).

Solvent Kinetic Isotope Effects. Solvent kinetic isotope effects were investigated by measuring initial velocities in both the forward and reverse direction in H_2O and 94% or 80% D_2O. The measurements were made holding one substrate concentration constant while varying the concentration of the second substrate. Buffers were prepared by dissolving K_2HPO_4 and KH_2PO_4 in either H_2O or D_2O in ratios calculated from the Henderson−Hasselbalch equation to produce solutions with a pH of 7.5, a value where the kinetic parameters are maximal and relatively pH-independent. No solvent kinetic isotope effects were observed on the forward reaction. For the reverse reaction, effects of 0.61 ± 0.06 on *V*/*K* and 0.67 ± 0.09 on *V* are measured when holding OSH at saturating levels (Figure 5A). When the CoA concentration was held at 0.4 mM, the observed effect on *V*/*K* was 0.96 ± 0.08 and on *V* was 0.70 ± 0.04 (Figure 5B). These values of the solvent kinetic isotope effect were calculated using eq 4 assuming a linear dependence of the isotope effect on fractional deuterium abundance. A proton inventory, obtained under saturating conditions, is shown in Figure 5C. The inventory is linear, suggesting the transfer of a single proton in the isotopically sensitive step.

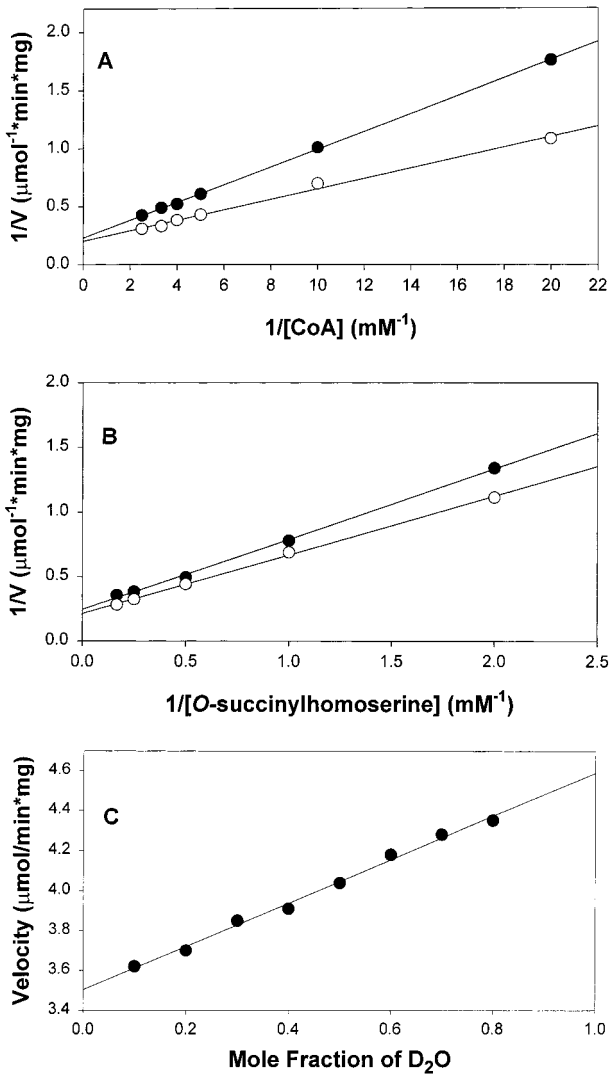

FIGURE 5: Double-reciprocal plot of the formation of succinyl-CoA catalyzed by homoserine desuccinylase in H_2O and D_2O. Experiments were performed as described in Materials and Methods: (○) experiments performed in 80% D_2O and (●) experiments performed in H_2O. (A) The concentration of OSH was held constant at 15 mM, while the concentration of CoA was varied. (B) The concentration of CoA was held constant at 0.4 mM, while the concentration of OSH was varied. (C) Proton inventory. Maximal velocities were measured at increasing mole fractions of D_2O in the presence of 0.5 mM CoA and 15 mM OSH.

DISCUSSION

Methionine, an essential amino acid, is required for the initiation and elongation of proteins, for the biosynthesis of polyamines, and in a number of methylation reactions. Therefore, it is not surprising that its synthesis is tightly controlled, with the major point of regulation being at the first biosynthetic step. This reaction, acylation of the γ-hydroxyl group of homoserine, is catalyzed by either homoserine transsuccinylase (HTS) or homoserine transacetylase (HTA), depending on the bacterial species. *E. coli* utilizes the succinylase pathway, and evidence for the existence of *O*-succinylhomoserine was first postulated by Rowbury (*22*). The gene encoding HTS, *metA*, has been cloned from both *E. coli* and *Salmonella typhimurium* (*23*, *24*), and is reported to be under control of the σ-32 promoter (*25*), which also regulates the heat shock proteins groE and dnaK. In fact, it has been suggested that HTS itself is a heat shock protein since its cellular levels increase at elevated temperatures (*25*). Paradoxically, HTS is highly sensitive to increased temperatures and loses 70% of its activity when the temperature is shifted from 37 to 42 °C (*4*). This drop in activity is seen in extracts from *E. coli*, *Aerobacter aerogenes*, *Klebsiella pneumonia*, *Serratia marcescens*, and *S. typhimurium* (*5*). HTS regains activity once the temperature is restored to 37 °C. If β-mercaptoethanol is added to the

extracts at the elevated temperature, however, HTS fails to regain activity once returned to 37 °C (*4*).

In this paper, we describe the first detailed kinetic investigation of HTS. The gene from *E. coli* was cloned on the basis of the previously published sequence (*20*), and purification of the overexpressed protein produced 0.6 g of homogeneous protein from 4 L of cells. Analysis of HTS by ESI-MS indicated a discrepancy between the predicted and experimental molecular mass. Subsequent sequencing of the cloned gene identified a single apparent mutation, based on the original published sequence (*20*), which resulted in the substitution of Ile for Val at position 67. This apparent substitution probably represents the correct sequence since the more recently published *E. coli* genome also contains Ile at position 67 (*26*). The monomer molecular mass is predicted to be 35 596 Da on the basis of the nucleotide sequence, while the observed molecular mass from ESI-MS analysis is 35 598 Da. Gel filtration analysis yielded an estimated molecular mass of 86 kDa, suggesting that HTS may exist as a dimer in vivo, as was previously reported (*27*).

Initially, we attempted to measure HTS activity continuously by following the release of CoA using DTNB (*28*); however, we were unable to detect any activity with this assay. When a discontinuous DTNB assay was employed, as described by De Angelis et al. (*17*), significant enzyme activity was apparent. This suggested that DTNB is an inhibitor of HTS and allowed us to surmise a catalytic role for a cysteine residue. Development of a direct assay in which the hydrolysis (or formation) of the succinyl-CoA thioester bond was directly measured at 232 nm allowed the steady-state kinetic parameters to be determined.

The parallel initial velocity profiles seen in Figure 2 suggest that HTS utilizes a ping-pong mechanism in which the succinyl group of succinyl-CoA is initially transferred to the enzyme to form a succinyl−enzyme intermediate. This group is then transferred to homoserine to form the final product, *O*-succinylhomoserine. As expected for a bona fide ping-pong kinetic mechanism, an identical series of parallel lines is obtained when the reverse reaction, formation of succinyl-CoA from OSH and CoA, is monitored.

The steady-state kinetic parameters of the natural substrates in both directions were measured. The K_M value for succinyl-CoA is 170 μM, while the K_M value for CoA is 640 μM. The K_M values for homoserine and OSH were nearly 10-fold higher, at 1.6 and 3.5 mM, respectively. A number of substrate analogues of both succinyl-CoA and homoserine were also tested. We were surprised to find that D-homoserine was active as a substrate analogue, with a K_M that was 6-fold higher and a k_{cat} that was only 2-fold lower than those of L-homoserine. Samples of authentic D- and L-homoserine were analyzed by HPLC on a chirobiotic T column (Alltec) in 50% methanol. Baseline separation between the two isomers was obtained, and no contamination of D-homoserine by L-homoserine could be detected, indicating that HTS catalyzes turnover with both homoserine isoforms. 3-Amino-1-propanol, which lacks the carboxyl group of homoserine, was 2500-fold less active than homoserine, while γ-hydroxybutyric acid, which lacks the α-amino group of homoserine, was inactive. γ-Aminobutyric acid was also inactive as an acyl acceptor. Threonine and serine both exhibited approximately 16000-fold less activity than homoserine. Glutaryl-CoA is a substrate for HTS, with a K_M that is equivalent to that of succinyl-CoA and a k_{cat} that is 15-fold lower, but acetyl-CoA, propionyl-CoA, butyryl-CoA, crotonyl-CoA, and malonyl-CoA were all inactive as substrates. These studies indicate that the position and identity of the acyl acceptor are extremely important. Additionally, it appears that active acyl donors require the presence of a terminal carboxyl group, but that the presence of this group is not sufficient for activity. It is particularly interesting to note that acetyl-CoA, the corresponding acyl donor in other organisms, is not a substrate for HTS.

The equilibrium constant for the reaction lies decidedly in the biologically relevant direction, and can be estimated from eq 5 (*29*):

$$K_{eq} = (V_f/V_r)^2(K_P K_Q/K_A K_B) \quad (5)$$

where V_f and V_r are the maximal velocities in the forward and reverse directions, respectively, and K_P, K_Q, K_A, and K_B are the Michaelis constants for the two products (CoA and OSH) and the two substrates (succinyl-CoA and homoserine), respectively. Using this equation, the equilibrium constant is estimated to be ca. 170, compared to a value of 15−40 measured for serine transacetylase (*14*). Since oxygen esters are of lower energy, and thus more stable, than thioesters, it is not surprising that the equilibrium constant should lie in favor of the production of OSH from succinyl-CoA.

The pH dependencies of the maximal velocity and $V/K_{succinyl-CoA}$ are qualitatively identical; the plots are both bell-shaped curves. In each case, the p*K* of the group that must be deprotonated is approximately 6.6 while the p*K* of the residue which must be protonated is approximately 7.7. As discussed in more detail below, the group functioning as a general base is probably a catalytic cysteine residue which forms an acyl−cysteine intermediate during the first half-reaction. The residue that is observed in the pH profiles functioning as a general acid is likely responsible for the proton transfer required in the second step of the first half-reaction, breakdown of a tetrahedral intermediate to form the acyl−enzyme intermediate and free CoA. Breakdown of this intermediate will be facilitated by donation of a proton to the leaving $CoAS^-$. The $V/K_{homoserine}$ pH profile also appears to be bell-shaped. In this case, the group functioning as a general base, which exhibits a p*K* of 6.5, is presumably required to activate homoserine. A group exhibiting a p*K* value of 8.6 is also observed in this pH profile, although there is no mechanistic requirement for general acid assistance. The data for this profile can also be fit to an equation that describes a pH profile that goes from a slope of 1 to 0. While this fit is not as optimal as the bell-shaped fit, it may better describe the data mechanistically.

The initial observation that HTS was inactive in the presence of DTNB suggested the presence of an active site cysteine residue. An analysis of the acyl transferase literature revealed a number of examples of enzymes which utilize an active site cysteine as the enzyme nucleophile responsible for formation of the acyl−enzyme intermediate (*9*−*12*). Incubation of HTS with IAA resulted in enzyme inactivation, further supporting the notion of a catalytic cysteine residue. The pH dependence of the rate of enzyme inactivation by IAA has been used to estimate the p*K* of an active site cysteine in other enzymes (*30*, *31*). When the pH dependence

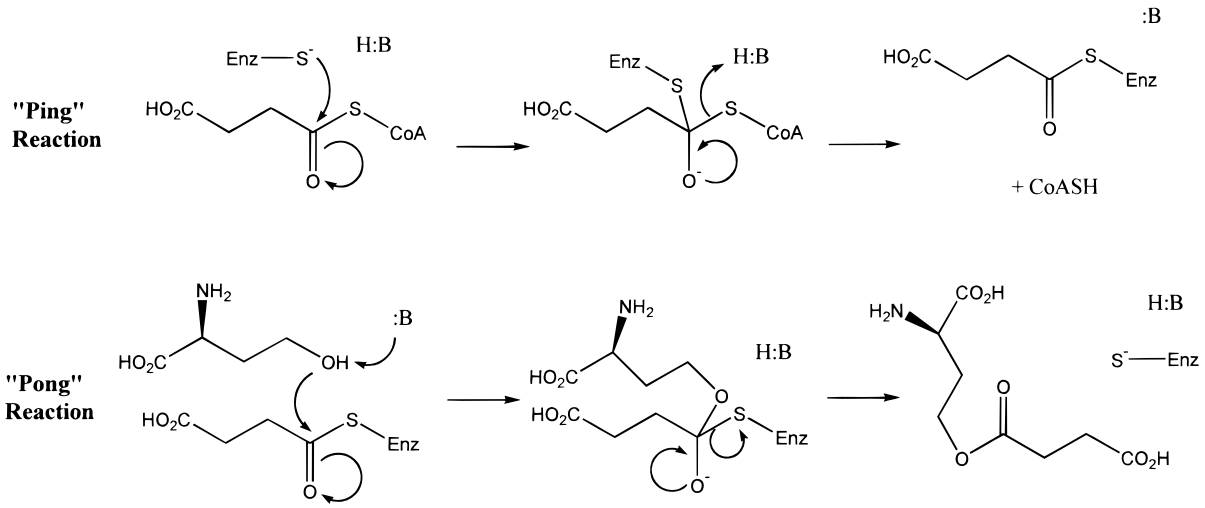

FIGURE 6: Proposed mechanism for transfer of succinate from succinyl-CoA to homoserine catalyzed by homoserine transsuccinylase.

of the rate of IAA-induced inactivation of HTS was measured, the profile suggested that inactivation depended on a group exhibiting a p*K* value of 6.4. This value agrees closely with that determined by the pH studies on *V* and *V*/*K* and strongly suggests that we are assessing the same group in these experiments, namely, the catalytic cysteine residue. The p*K* of 6.4 indicates that this cysteine residue is physiologically present as the thiolate anion, and the enzyme must therefore stabilize this negative charge at the active site. HTS from *E. coli* contains only two cysteine residues, one of which is conserved in the putative *Bacillus subtilis* HTS sequence. This cysteine residue, Cys142, is likely the active site cysteine nucleophile.

Solvent kinetic isotope effect studies demonstrated that there was essentially no effect of D_2O on the reaction rate in the physiological direction, production of OSH. However, an inverse solvent kinetic isotope effect was observed when the reaction was monitored in the reverse direction, formation of succinyl-CoA. The inverse effect can be most easily explained by the occurrence of a proton transfer involving a thiol, which exhibits a uniquely inverse fractionation factor of 0.4−0.6 (*32*). The absence of an effect in the forward direction suggests that any isotopically sensitive steps are fast compared to the overall rate in that direction. In the reverse direction, however, the reaction is slower and solvent isotope effects are observed.

When OSH is held at a saturating level and the CoA concentration is varied, we are able to monitor the solvent isotope effect on the "pong" reaction in the reverse direction, transfer of succinate from the succinyl−enzyme intermediate to CoA to form succinyl-CoA. In this case, we see inverse and nearly equivalent effects on *V* (0.67 ± 0.09) and *V*/*K* (0.61 ± 0.06). The inverse effect can be interpreted to suggest the involvement of a thiol in the reaction that is being monitored. The two potential thiols that may be responsible are the putative active site cysteine and free CoA. We suggest that the inverse fractionation factor of the thiol of CoA is responsible for the inverse solvent kinetic effect observed in this half-reaction since a proton must be abstracted from CoA to produce the nucleophilic thiolate anion which attacks the succinyl−enzyme intermediate and forms succinyl-CoA. The proton inventory observed for this reaction is linear, consistent with the removal of the single proton of CoASH by an enzymic base.

When the CoA concentration is held constant and the OSH concentration is varied, we are able to monitor the solvent isotope effect on the "ping" reaction in the reverse direction, transfer of the succinate group from OSH to the enzyme. The observed effect on *V* (0.70 ± 0.04) is inverse and equal to that seen in the pong reaction, as expected, while the effect on *V*/*K* is essentially unity (0.96 ± 0.08). The active site cysteine, which attacks OSH to form the succinyl−enzyme intermediate, contains the only thiol involved in this reaction. The pH studies described above indicate that this cysteine is primarily present as the thiolate anion, exhibiting a p*K* value of ca. 6.4. These experiments, performed at pH 7.5, would not require base-assisted deprotonation of the enzyme thiol, and thus, we observe no effect of solvent isotopic composition on V/K_{OSH}.[2]

A chemical mechanism for the succinyl transferase reaction catalyzed by HTS is shown in Figure 6. In the first half-reaction, succinyl-CoA binds to the enzyme and is attacked by an active site thiolate to form the first tetrahedral intermediate. This intermediate breaks down to form the succinyl−enzyme intermediate and free CoA, which is presumably facilitated by protonation of the thiolate anion of the product CoA by an active site acid. Homoserine binds in the second half-reaction to the succinyl−enzyme intermediate, and an active site base can remove the γ-hydroxyl proton, allowing for the attack on the succinyl−enzyme intermediate to form the second tetrahedral intermediate. Finally, this intermediate breaks down to form the final product, OSH, and to regenerate the active enzyme. The participation of the acid which protonates CoA as the first tetrahedral intermediate breaks down and the ionization of the thiolate are both reflected in the pH profiles of $V/K_{succinyl-CoA}$. The base required to activate homoserine presumably is the group observed exhibiting a p*K* value of 6.5 in the $V/K_{homoserine}$ pH profile.

[2] It should be noted that nonlinear proton inventories can be obtained by adjusting the OSH concentrations such that they are near K_M values. Thus, if the concentration of OSH is kept at or below K_M, the relative rates of the two half-reactions can be poised such that they are nearly equal and the resulting proton inventory is nonlinear (data not shown).

In the reverse direction, a base is required to abstract a proton from CoA before it can form the second tetrahedral intermediate (in the pong reaction). We observe this proton abstraction in the solvent isotope effect studies when the concentration of CoA is varied.

The data presented in this paper represent an initial kinetic and mechanistic characterization of the enzyme which catalyzes the succinylation of homoserine in *E. coli* as the first unique step in methionine biosynthesis. Additional kinetic and structural information about both HTS and HTA will be required before these enzymes can be fully evaluated as potential drug targets.

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