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Assessing the roles of essential functional groups in the mechanism of homoserine succinyltransferase

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Abstract

Homoserine acyltransferases catalyze the commitment step to methionine and other important biological precursors which make this class of enzymes essential for the survival of bacteria, plants and fungi. This class of enzymes is not found in humans, making them an attractive new target for antimicrobial design. Homoserine *O*-succinyltransferase (HST) is a representative from this class, with little known about the key amino acids involved in substrate specificity and catalysis. HST from *Escherichia coli* has been cloned, purified and kinetically characterized. Through site-directed mutagenesis and steady-state kinetic studies the residues that comprise a catalytic triad for HST, the catalytic cysteine nucleophile, an active site acid–base histidine, and the base orienting glutamate, have been identified and characterized. Several residues which confer substrate specificity for both homoserine and succinyl-CoA have also been identified and kinetically evaluated. Mutations of an active site glutamate to either aspartate or alanine drastically increase the K_m for homoserine, assigning this glutamate to a binding role for the α-amino group of homoserine. An active site arginine orients the carboxyl moiety of homoserine, while the carboxyl moiety of succinyl-CoA is positioned for catalysis by a lysine residue. Removing functionality at either of these positions alters the enzyme's ability to effectively utilize homoserine or succinyl-CoA, respectively, reflected in an increased K_m and decreased catalytic efficiency. The data presented here provides new details of the catalytic mechanism of succinyltransferases, resolves a controversy between alternative mechanistic hypotheses, and provides a starting point for the development of selective inhibitors of HST.
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Keywords: Enzyme mechanism; Homoserine succinyltransferase; Site directed mutagenesis; Catalytic mechanism

The increased resistance of infectious microorganisms to the available arsenal of antibiotics has prompted searches for new antibiotics against new microbial targets. Bacterial survival requires the proper functioning of a number of fundamental metabolic pathways that are not found in mammals, thus providing a variety of candidates for possible intervention. The aspartate biosynthetic pathway is rich in potential targets, producing a range of essential metabolites in bacteria, fungi, and plants, including the end product amino acids lysine, methionine, threonine and isoleucine. In addition, critical intermediates in this pathway provide the precursors for the cross-linking of the peptidoglycan layer during gram-negative bacterial cell wall biosynthesis [1], sporulation in gram-positive bacteria [2], and quorum sensing [3].

The central reactions of the aspartate pathway that convert aspartic acid to homoserine are quite similar across the microbial and plant species [4], with only minor differences in isoenzyme composition [5,6] and slight variations in end product regulation [7,8]. The enzymes that catalyze the reactions in the lysine and the threonine/isoleucine branches of this pathway are also closely related from species to species. However, there are significant species differences in the methionine branch of this pathway. Homoserine must be activated prior to the incorporation of a sulfur atom to produce methionine. In plants this activation is accomplished primarily through phosphorylation [9]. The majority of microorganisms carry out an acetylation reaction for this activation step [10], while most enteric bacteria use succinylation [11]. There are also substantial differences in the routes by which sulfur is added to this activated metabolite [12].

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The acylation of homoserine in bacteria is catalyzed by two functionally related enzymes, homoserine *O*-succinyltransferase (HST, E.C. 2.3.1.46) and homoserine *O*-acetyltransferase (HAT, E.C. 2.3.1.31). Kinetic characterization of HST and HAT revealed a ping-pong mechanism by which a succinyl or an acetyl group is first transferred from the respective acyl-coenzyme A donor to a functional group on the enzyme and then to homoserine [13,14]. A cysteine residue is proposed to be the catalytic nucleophile in HST that accepts the succinyl group [13], while in HAT a serine has been assigned as the acetyl group acceptor [14,15].

Recent mass spectrometry studies have identified a succinate covalently bonded to a lysine residue at either position 45 or 46 in HST and, since only lysine 46 is completely conserved, makes it the more likely candidate to carry out an essential catalytic function [16]. In this alternative mechanism the lysine is proposed to be the site of formation of the acyl-enzyme intermediate instead of the previously identified cysteine, with this latter residue relegated to involvement in dimer association or conformational integrity [16]. No other catalytic or substrate binding residues have been definitively identified, and the controversy regarding the identity of the active site nucleophile has not been resolved. The structures of HST from *Thermotoga maritima* and *Bacillus cereus* were recently determined and deposited (PDB codes 2H2W and 2GHR, respectively).

Because of its critical role in the commitment to methionine synthesis in enteric bacteria, HST represents an important potential target for the design of novel antimicrobials. A detailed understanding of the residues involved in the catalytic mechanism is necessary to guide inhibitor design and synthesis. A number of mutants of HST have been constructed and characterized, with the goals of defining the catalytic functional groups and resolving the discrepancies between alternate mechanistic proposals. Our studies provide a detailed elucidation of the roles of these essential functional groups and implicate several residues which confer substrate specificity in the reaction catalyzed by homoserine *O*-succinyltransferase.

Materials and methods

Cloning, expression, and purification of homoserine succinyltransferase

The *metA* gene was PCR-amplified from *Escherichia coli* genomic DNA using standard PCR methods. The purified PCR product was ligated into a pET41a(+) expression vector (Novagen) with the restriction enzymes, *Nde*I and *Eco*RI. The cloned gene was sequenced (MWG, Inc) and showed no deviation from the published gene sequence [17]. This metA/pET41a(+) construct was transformed into *E. coli* BL21-(DE3) cells, protein expression was induced after initial cell growth by the addition of 1 mM isopropylthio-β-D-galactopyranoside (IPTG),[1] and the cells were allowed to grow for an additional 4 h at 33 °C. The cells were harvested by centrifugation, resuspended in extraction buffer (50 mM Hepes, pH 7.5), and disrupted by sonication. The soluble protein extract was fractionated on a 50 mL high capacity Q Sepharose XL column (Amersham Bioscience) and the HST enzyme was eluted with a linear salt gradient (0–750 mM NaCl) over 6 column volumes, followed by dialysis into a low salt buffer for a subsequent polishing anion exchange column step (Source Q15) using an ÄKTA Explorer Chromatography system (Amersham Bioscience). HST was purified to greater than 98% purity in these successive anion exchange chromatographic steps to yield 800 mg of pure enzyme from 12 g of cell paste. The purified HST enzyme was visualized by SDS–PAGE with Coomasie blue staining, indicating a molecular weight of ~36 kDa compared to the calculated value of 35,727 Da. Active HST enzyme was dialyzed into a final storage buffer of 50 mM Hepes, pH 7.5, and concentrated to 5–20 mg/mL, with protein concentrations determined by the Bradford method [18].

Site-directed mutagenesis

Mutants of HST were prepared by using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutagenic primers were designed to introduce the desired mutation using the metA/pET41a(+) recombinant DNA which contained the wild-type gene as a template. Each HST mutant was cloned, overexpressed, and purified under similar conditions and with similar yields as the native enzyme. The mutated gene for each construct was verified by nucleotide sequencing (MWG, Inc) to contain the desired mutation. Each purified mutant was isolated from fractions based on specific activity. For mutants that did not show measurable activity, SDS–PAGE was used to verify the presence of single protein band with the appropriate molecular weight.

Kinetic measurements

Enzyme activity was directly measured by using a modification of a previously described protocol [13]. All enzymatic assays were carried out in a SpectraMax 190 microplate reader at 25 °C using a 96-well UV transparent flat bottom plate in a final volume of 100 μL. Enzyme reactions were initiated by the addition of HST and activities of the native and mutant enzymes were measured in the biologically relevant direction by following the hydrolysis of the succinyl-CoA thioester bond at 232 nm. Typical enzyme concentrations were between 0.2 μg and 1.5 μg for suitable linear activity detection. Assays to determine kinetic parameters were conducted by varying one substrate and holding the other substrate at several fixed concentrations. L-Homoserine concentrations were varied between 0.01 mM and 10 mM for mutant *Ec*HSTs, except for mutations at position 246 where the concentrations were varied between 1 mM and 300 mM. The concentration of succinyl-CoA was varied from 0.05 mM to 0.8 mM for all constructs expect those involving residues at position 45, 46, and 156, where a concentration range of 0.5 mM to 3.0 mM succinyl-CoA was used. All reactions were carried out in a 100 mM potassium phosphate assay buffer, pH 7.5. The enzymatic rate measured for the low activity mutants was higher than the non-enzymatic background hydrolysis of succinyl-CoA. There is no observable rate measured above this background in the absence of the over-expressed HST in *E. coli* cells. Initial velocities were fitted to the equation for a Ping-Pong kinetic mechanism (Eq. 1) using an enzyme-kinetics package adapted from earlier kinetic programs [19]:

$$v = \frac{VAB}{(K_A B + K_B A + AB)} \tag{1}$$

where V is the maximal velocity, A and B are the concentrations of succinyl-CoA and homoserine, and K_A and K_B are the Michaelis constants for the respective substrates.

[1] *Abbreviations used*: HST, homoserine succinyltransferase; HAT, homoserine acetyltransferase; *Ec*HST, *Escherichia coli* homoserine succinyltransferase; ESI-MS, electrospray ionization-mass spectrometry; Suc-CoA, succinyl-coenzyme A; DLS, dynamic light scattering; DSC, differential scanning calorimetry.

Electrospray ionization-mass spectrometry

ESI-MS, conducted at The Ohio State University Mass Spectrometry and Proteomics Facility, was used to verify the correct mass of the purified HST. Enzyme samples were concentrated to 35 μM and prepared for mass spectrometry by dialysis into 100 mM ammonium acetate, pH 7.6. ESI-MS, conducted at The University of Toledo, was used to verify the formation of reaction products catalyzed by the HST. Enzyme samples were concentrated and added to a reaction mixture containing 10 mM homoserine and 5 mM succinyl-CoA, at a final concentration of 10 μM for the lower activity *Ec*HST mutants and 4 μM for the native enzyme and the more active mutants. Aliquots were removed and measured immediately upon addition to the reaction solution and then again at 30 min. Measurements were made using Esquire-LC Ion Trap LC/MS System (Bruker Daltonics, Billerica, MA) with pneumatically assisted electrospray ionization source in the positive ion mode. Mass fragments were detected with a multipole ion trap and mass spectra were analyzed with Esquire NT 4.5 (Bruker Daltonics).

Differential scanning calorimetry

The *E. coli* HST wild-type and mutant enzymes were dialyzed against 50 mM Hepes, pH 7.5, and concentrated to 28 μM. The solutions were degassed for 10 min before being loaded into their respective sample and reference cells in a VP-Differential Scanning Calorimeter (MicroCal Inc., Amherst, MA, USA). A stable reference baseline was established prior to sample analysis and each enzyme sample shows a stable baseline before the thermal transition occurs and returns to baseline upon unfolding. The protein samples were scanned relative to the buffer reference solution over a temperature range of 25–85 °C at a scan rate of 90 °C h^{-1}, with data points taken every 16 s. Each data set was shown to be completely reproducible by conducting replicate analyses. Data analysis was performed by subtracting reference data from the sample data and normalized by concentration using the *Origin* software package (OriginLab Corp., Northampton, MA, USA). The deconvolution of unfolding endotherms was prepared with the *Origin* peak-fitting module using a non-two-state fitting parameter. The deconvoluted data allowed for the determination of the calorimetric transition midpoint, T_m.

Dynamic light scattering

Dynamic light scattering was used to determine homogeneity of the protein solution and to verify the predicted molecular weight of ~70 kDa, corresponding to the dimeric form of HST. Typical solutions containing from 0.5 mg/mL to 1.0 mg/mL of HST were examined by using a Dyna Pro Titan DLS (Wyatt Technologies) at 25 °C. The hydrodynamic radius was determined in 100 mM potassium phosphate buffer, pH 7.5, and converted to a calculated molecular mass.

Results and discussion

Catalytic properties of acyltransferases

Because of conflicting results between several previous studies [13,14,16] no definitive roles have been determined for functional groups in any homoserine *O*-succinyltransferase. The *Ec*HST enzyme catalyzes the *O*-succinylation of homoserine using succinyl-coenzyme A via a ping-pong mechanism [13] in which succinate is transferred from succinyl-CoA to an active site functional group to form an acyl-enzyme intermediate. Subsequent transfer of succinate from the enzyme to homoserine completes the reaction cycle. There are several acyltransferase families, of which the homoserine *O*-succinyltransferases represents a class with little known about the details of regulation and catalytic function. The acyltransferases that have been studied in detail utilize one of three possible residues as the nucleophile. Glutamic acid is the catalytic nucleophile employed by tetrahydrodipicolinate *N*-succinyltransferase [20–22], while serine is utilized by homoserine *O*-acetyltransferase [14,15], and a cysteine is used by lecithin retinol acyltransferases [23]. The mechanisms of the enzymes in the carboxylate and serine classes have been extensively characterized, while members of the cysteine nucleophile group are less well studied. The first question to be resolved is the identity of the catalytic nucleophile that will assign HST to one of these mechanistic classes.

The identity of the catalytic nucleophile

The pH dependence of *Ec*HST and the iodoacetamide-induced inactivation have previously suggested the involvement of a cysteine as the active site nucleophile [13]. A *Prosite* scan revealed a conserved L–x–x–**C**W–x–x–QA sequence that is found in all HSTs and contains the only conserved cysteine residue in the HST family. In order to identify whether this conserved cysteine is involved in nucleophilic attack, several mutants were prepared and kinetically evaluated. C142 was mutated to a serine (C142S), a mutation that substitutes a less nucleophilic hydroxyl group and should impair the enzyme's catalytic ability if this cysteine is the nucleophile. In fact, the k_{cat} for this C142S mutant is <0.1% of the native HST (Table 1), consistent with the expected loss for the removal of a catalytic functional group. However, an accurate determination of the low rate of catalysis of C142S is complicated by a measurable non-enzymatic hydrolysis of the succinyl-CoA thioester bond. This background rate is negligible when compared to the catalyzed rate for the native enzyme; however, for very low activity mutants there is a question of how much of the slow decrease in absorbance at 232 nm is a consequence of the non-enzymatic hydrolysis of succinyl-CoA. ESI-MS was used to directly monitor the production of the catalytic product, and showed the appearance of succinyl-homoserine ($m/z = 220.1$) along with a proportional decrease in the homoserine peak observed at m/z of 120.1 (Fig. 1). A second, more drastic, cysteine to alanine (C142A) mutant was also prepared and in this case no detectable catalytic activity was measured above the background hydrolysis reaction and no succinyl-homoserine was observed by ESI-MS. These results implicate the thiolate of C142 as an essential catalytic functional group and support its assignment as the active site nucleophile.

To further probe the mechanism of *Ec*HST a sequence alignment between the HSTs from *Thermotoga maritima* and *Bacillus cereus* and that of *Ec*HST was used to identify additional residues which could potentially serve a catalytic role (Fig. 2). Adjacent to the catalytic cysteine is a conserved histidine residue, which was examined as a potential candidate for an acid–base residue to abstract a proton

Table 1
Kinetic parameters for native and mutant HST enzymes

Enzyme	k_{cat} (s^{-1})	Homoserine		Succinyl-CoA	
		K_m (mM)	k_{cat}/K_M (M^{-1} s^{-1})	K_m (mM)	k_{cat}/K_M (M^{-1} s^{-1})
Native	25.7 ± 0.5	0.38 ± 0.03	6.8×10^4	0.28 ± 0.04	9.2×10^4
Catalytic residues[a]					
E237D	6.7 ± 0.4	0.36 ± 0.01	1.8×10^4	0.24 ± 0.05	2.8×10^4
E237A	0.80 ± 0.08	1.1 ± 0.3	7.3×10^2	0.15 ± 0.04	5.3×10^3
Succinyl carboxyl binding residues[b]					
K46L	1.26 ± 0.01	0.044 ± 0.005	2.8×10^4	2.9 ± 0.3	4.3×10^2
K45L	40.1 ± 0.6	2.26 ± 0.15	1.5×10^4	1.78 ± 0.05	2.3×10^4
Homoserine α -amino binding residues					
Y238F	13.0 ± 0.3	2.0 ± 0.1	6.5×10^3	0.35 ± 0.11	3.7×10^4
E246D	15.0 ± 0.4	9.1 ± 0.7	1.6×10^3	0.043 ± 0.008	3.5×10^5
E246A	6.9 ± 0.4	59.1 ± 5.8	1.2×10^2	0.094 ± 0.004	7.3×10^4
Y238F/E246A	8.9 ± 0.9	95.5 ± 17.8	9.3×10^1	0.050 ± 0.007	1.8×10^5
Homoserine carboxyl binding residues					
R193K	3.4 ± 0.2	0.31 ± 0.07	1.1×10^4	0.40 ± 0.04	8.5×10^3
R193A	3.3 ± 0.2	1.1 ± 0.1	3.0×10^3	0.43 ± 0.01	7.7×10^3
R249K	23.7 ± 0.8	1.51 ± 0.05	1.6×10^4	0.31 ± 0.03	7.6×10^4
R249A	10.0 ± 0.3	1.15 ± 0.10	8.7×10^3	0.23 ± 0.01	4.3×10^4
R193A/E246A	1.20 ± 0.04	93.8 ± 19.3	1.3×10^1	0.20 ± 0.01	6.0×10^3

[a] The catalytic activities of the C142S and H235N mutants are <0.01 s^{-1}, and the C142A mutant has an activity <0.001 s^{-1}.
[b] The mutants K45A/K46A and K156L have activities below the detectable background rates, <0.001 s^{-1}.

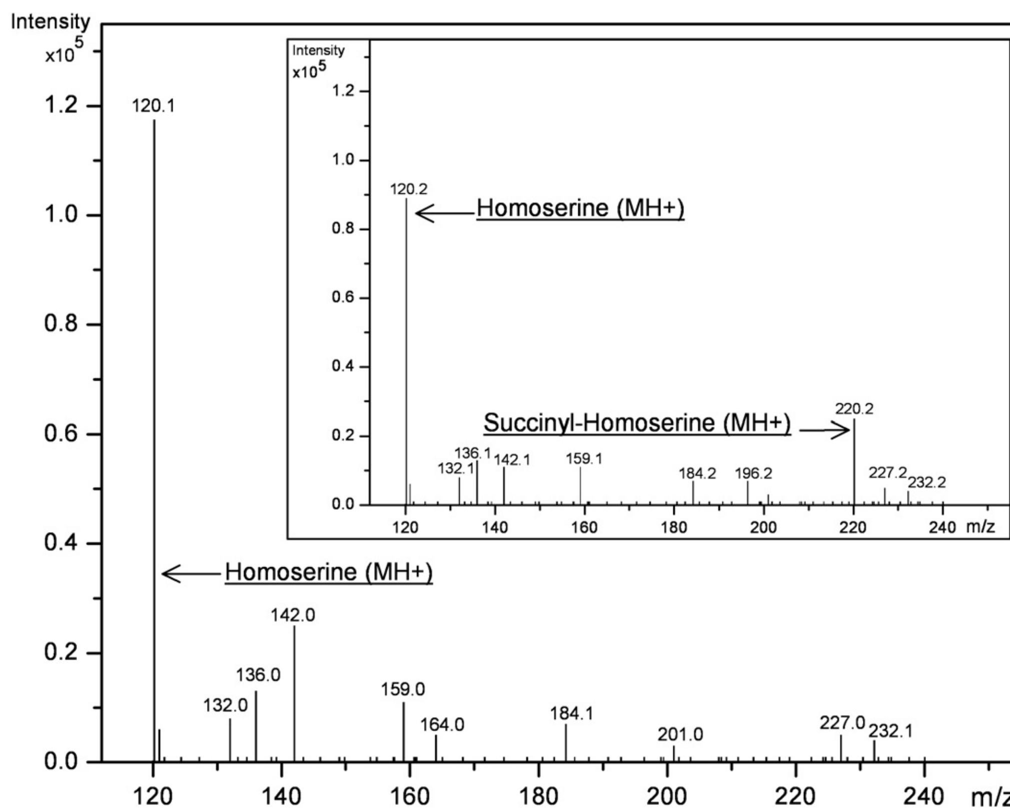

Fig. 1. Electrospray ionization mass spectrum assay. ESI chromatogram plotted with relative intensity vs. m/z with the homoserine peak annotated ($m/z = 120.1$) at time = 0 min. Inset, expansion of ESI-MS chromatogram of the reaction mixture with K46L *Ec*HST, showing both the homoserine substrate and the succinyl-homoserine product peak ($m/z = 220.2$) at time = 30 min.

from the catalytic nucleophile. H235 was mutated to asparagine (H235N), a mutation which should impair the enzyme's ability to perform this role. The perturbation caused by this mutation does in fact drastically affect enzyme activity, decreasing k_{cat} to <0.1% of the native enzyme (Table 1), with the low enzymatic activity of this

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                                                              K45 K46
EcHST     MPIRVPDELPAVNFLREENVFVMTTSRASGQEIRPLKVLILNLMPKKIETENQFLRLLSN 60
TmHST     MPINVPSGLPAVKVLAKEGIFVMTEKRAIHQDIRPLEILILNLMPDKIKTEIQLLRLLGN 60
BcHST     MPIIIDKDLPARKVLQEENIFTMTKERAEKQDIRALKIAILNLMPKKQETEAQLLRLLGN 60
          *** : . *** :.* :*.:*.** .**  *:**.*:: ******.* :** *:****.*

                               C142           K156
EcHST     WPQIKQVLEWSKDHVTSTLFVCWAVQAALNILYGIPKQTRTEKLSGVYEHHILHPHALLT 180
TmHST     WEELTEIMEWSRHNVYSTMFICWAAQAGLYYFYGIPKYELPQKLSGVYKHRVAKDSV-LF 179
BcHST     WEELKKIMDYSKTNVTSTLHICWGAQAGLYYHYGIPKYPLAEKMFGVFEHEVLEQHVKLL 180
          * ::.:::::*: :* **:.:**..**.*   *****   .:*: **::*.: .  . *

                      R193                                H235 E237 Y238
EcHST     RGFDDSFLAPHSRYADFPAALIRDYTDLEILAETEEGDAYLFASKDKRIAFVTGHPEYDA 240
TmHST     RGHDDFFWAPHSRYTEVKKEDIDKVPELEILAESDEAGVYYVVANKSERQIFVTGHPEYDR 239
BcHST     QGFDELFFAPHSRHTEVHAADIEKVEDLKLLAISEEAGVYLVIGQNGKHIFVLGHSEYSC 240
          :*.*: * *****:::.    * .  :*::** ::*...*:. .:. :  ** **.**.

               E246 R249
EcHST     QTLAQEFFRDVEAGLDPDVPYNYFPHNDPQNTPRASWRSHGNLLFTNWLNYYVYQITPYD 300
TmHST     YTLRDEYYRDIGRNLKVPIPANYFPNDDPTKTPILTWWSHAHLFFSNWLNYCIYQKTPYR 299
BcHST     DTLKREYERDIQRGLNIAVPKNYFKYNNPDEKPLVRWRSHGNLLFSNWLNYYVYQETPYI 300
           **  *: **:  .*.  :* ***  ::* :.*   * ** .:*:*:***** :** ***
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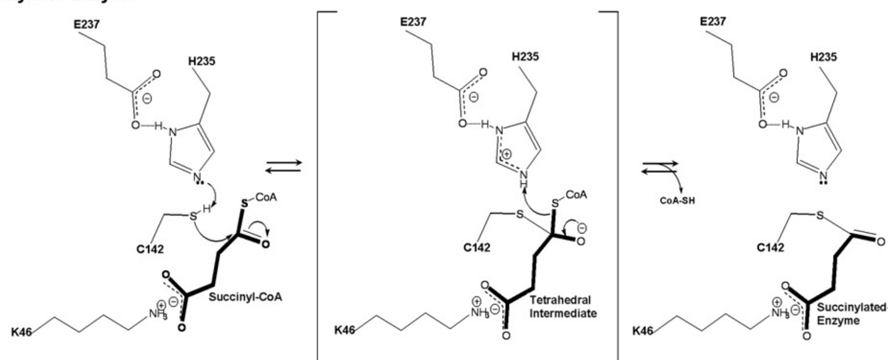
Fig. 2. Sequence alignment of *Escherichia coli* HST, *Ec*HST; *Thermotoga maritima* HST, *Tm*HST; and *Bacillus cereus* HST, *Bc*HST. The conserved residues studied by mutagenesis are indicated, with the residues that are absolutely conserved throughout the HST family colored red. The residues from 61 to 120, and beyond 301 are not shown in this alignment that was created with clustal W [25].

mutant again precluding an accurate determination of its kinetic parameters. This dramatic effect on k_{cat} is consistent with H235 playing an essential acid–base catalytic function, and also supports the role of C142 as the active site nucleophile, since this cysteine would not be fully deprotonated without base assistance. This observation is consistent with previous kinetic and structural studies showing a histidine in this position serving as an active site base to deprotonate the cysteine nucleophile in aspartate semialdehyde dehydrogenase, an upstream enzyme in the aspartate pathway [24].

Homoserine succinyltransferase catalyzes a nearly identical reaction as that of homoserine acetyltransferase, which was previously shown kinetically to employ a catalytic triad (Ser-Asp-His) to support transfer of the acetyl group [15]. Based on this similarity, a search for a carboxylate candidate using the *Tm*HST structure as a model identified a conserved glutamate that could potentially serve this role in the succinyltransferases. The glutamate at position 237 was mutated to an aspartic acid (E237D) to investigate its proposed role in hydrogen bonding with H235 to help orient this base for proton abstraction. This E237D mutation causes a 3-fold reduction in k_{cat} while the K_m values for homoserine and succinyl-CoA remain unchanged (Table 1). Thus, removing one methylene carbon in the E237D mutation reduces catalysis, presumably by decreasing the population of H235 in the correct conformation to effectively deprotonate C142. If E237 is responsible for maintaining H235 in the proper orientation, then removing functionality at this position with a glutamate to alanine (E237A) mutation should have a more dramatic effect on catalysis. In fact, the E237A mutation reduces the enzyme activity to ~3% of the native k_{cat} without affecting the K_m parameters of either substrate (Table 1).

The kinetic evidence collected for this set of mutants convincingly establishes C142 as the catalytic nucleophile, activated through deprotonation by H235 to form a thiolate ion. H235 is properly oriented by E237, allowing effective deprotonation of C142 for nucleophilic attack on the thioester carbonyl center of succinyl-CoA. Based on the information obtained from the mutations at these positions, a proposed mechanism incorporating this catalytic triad for the formation of the acyl-enzyme intermediate is outlined in Fig. 3a.

To test whether the loss of activity with any of these mutants is caused by the selective removal of an essential functional group, or is a more global consequence of an improperly folded enzyme caused by the mutations, differential scanning calorimetry (DSC) was used to measure the stability of the mutated enzymes. The thermal stability of the native and mutant enzymes was determined by DSC over a temperature range of 25–85 °C. The thermal transition midpoint (T_m) for the native enzyme is 51.7 °C, and the T_ms for each mutant are found to be within 2.0 °C of the native enzyme (Table 2). These results indicate that the overall stability of the mutant enzymes does not deviate substantially and that they are therefore likely to be properly folded. The loss of activity observed with each mutation must therefore be a direct catalytic effect, confirming our functional assignments.



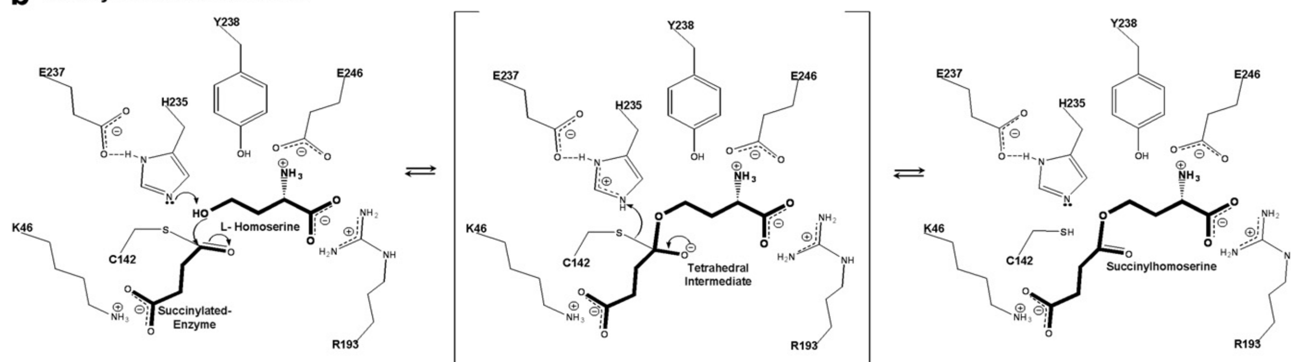


Fig. 3. Proposed catalytic mechanism of *Ec*HST. (a) Illustrates the mechanism of acyl-enzyme formation involving the catalytic triad (C142, H235, E237). C142 is activated for nucleophilic attack on the carbonyl center of the thioester of succinyl-CoA through deprotonation by H235, which is oriented by E237. Lysine 46 is shown interacting with the carboxyl moiety of succinyl-CoA. (b) Illustrates the deacylation mechanism involving the residues which confer substrate specificity. Y238 and E246 interact with the α-amino group of homoserine, while R193 is shown interacting with the carboxyl moiety of homoserine in a bidentate fashion. The γ-hydroxyl group of homoserine is deprotonated by H235 allowing attack on the succinyl-enzyme intermediate to form the product, succinylhomoserine. The geometric arrangement of the active site functional groups is adapted from the deposited structure of *Thermotoga maritima* apo-HST (PDB code 2H2W).

Table 2
Differential scanning calorimetric parameters for the HST mutants

Sample	T_m(°C)[a]	ΔH[a]	ΔH_v[a]	$\Delta H/\Delta H_v$
WT	51.7	7.4×10^4	1.2×10^5	0.62
C142A	49.8	7.9×10^4	1.8×10^5	0.44
K46L	50.6	6.8×10^4	1.5×10^5	0.45
C142S	51.0	6.8×10^4	1.9×10^5	0.36
K45A/K46A	51.0	7.2×10^4	1.3×10^5	0.55
K156L	51.6	7.6×10^4	9.5×10^4	0.80

[a] Errors associated with each datum are <1% for T_ms, <1.4% for ΔH, <2% for ΔH_v.

The binding groups that confer substrate specificity

Succinate carboxyl binding residues

The identification of residues involved in substrate binding and orientation is critical to an understanding of how the HST and HAT enzyme families can distinguish between structurally related substrates. Since both enzymes utilize homoserine as the acyl-group acceptor, positively charged residues such as arginine or lysine are likely candidates to bind this substrate. However, because of the low overall sequence similarity between HST and HAT (<10%), no conserved arginine residues could be reliably aligned between these enzyme families. The major structural difference between the substrates for these related enzymes is the presence of a terminal carboxyl on the succinyl group that is absent in the acetyl group. A sequence comparison among just the HST family of enzymes identified eight conserved positively charged residues (5 arginines and 3 lysines) that are possible candidates for electrostatic interactions with the carboxylate anion of the succinyl group. We expect that a positively charged residue would be required to help orient the succinyl group in HST, and that this group would be absent in the HAT enzymes. Based on the *differences* between the respective transferase families, two conserved lysine residues in HSTs were identified to serve this possible role, lysines 46 and 156, with MS evidence supporting lysine 46 as the putative acyl-group acceptor at the enzyme active site [16].

The conserved K156 was mutated to a leucine to remove the positive-charged functionality at this position; however, this mutation (K156L) renders the HST enzyme inactive, with no detectable activity above background. This complete loss of activity was unexpected, and may be due to a more global change within the HST structure. DSC will determine the calorimetric heat ΔH, referring to the heat change per mole of enzyme, as well as the van't Hoff heat

change ΔH_v, which can reveal the heat change per unfolding unit. The ratio, $\Delta H/\Delta H_v$, is a measure of the number of cooperative units that undergo a thermal transition per mole of enzyme. All of the enzyme mutants analyzed by DSC gave a $\Delta H/\Delta H_v$ ratio of 0.5, corresponding to a dimerized protein sample where the dimer undergoes a coupled transition. However, the K156L mutant has a $\Delta H/\Delta H_v$ ratio closer to one, indicative of a protein sample composed of a single domain unfolding as one unit. This suggests that K156 may be involved in dimer association or a domain interaction. DLS studies do not show a substantial difference in the hydrodynamic radius between the native *Ec*HST and the K156L mutant, indicating that there are no gross conformational changes or subunit dissociation in the K156L mutant enzyme.

Previous MS studies showed that succinate was bound to a lysine residue at either position 45 or 46 [16]. These findings were interpreted as evidence that these lysines are in the active site and essential for catalysis, and subsequently concluded that the completely conserved K46 is the site for succinate binding. If lysine 46 is involved as the acyl-group acceptor, then removal of the lysine functionality would eliminate the enzyme's catalytic capability. However, when K46 is mutated (K46L) the k_{cat} is still 5% of the native activity (Table 1). Surprisingly, the K_m for homoserine is reduced 8-fold in this mutant, the K_m for succinyl-CoA increased 10-fold, and the catalytic efficiency (k_{cat}/K_m) is decreased by 2-orders of magnitude. These data suggest that K46 plays a role in orienting the succinate moiety of succinyl-CoA rather than functioning as the acyl group acceptor. The adjacent lysine, K45 is not absolutely conserved, but is present in a large fraction of the HST enzymes. To determine if K45 is functioning as the active site nucleophile, the single mutant, K45L, was prepared, however this mutant has comparable kinetic parameters to the native enzyme, with K_m values within a factor of three and k_{cat} increased by 1.5-fold. However, when the lysines at both of these positions are replaced by alanines (K45A/K46A) the enzyme becomes completely inactive. Removing two positively charged amino acids from the same region in HST will likely cause local structural perturbations that can alter the enzyme's ability to perform catalysis; however, the unchanged T_m for this mutant suggests no gross conformational changes. The results of the mutational studies serve to eliminate K45 and K46 from consideration as active site nucleophiles, and support the original assignments of C142 to fulfill this function.

The structure of homoserine succinyltransferase from *Thermotoga maritima* was recently solved as an apo structure (PDB code 2H2W). Based on this structure the ε-nitrogen of K46 is 14.5 Å from the thiolate of C142, too distant to serve as a substrate binding group. However, it is not uncommon for a key amino acid to be engaged with a surrogate binding partner in the apo-enzyme. Once the substrate enters the active site this side chain will then reorient itself towards the substrate. The K46 residue can be rotated into a conformation that places the ε-nitrogen within 8.5 Å of the thiolate of C142, which is a reasonable distance to engage in an electrostatic interaction with the carboxyl of the succinyl group covalently attached to C142. The enzyme also loses activity when lysine 156 is mutated to a leucine, but this lysine is located more than 11 Å from any active site residues and does not appear to be in a position to play a direct role in catalysis. Rather, the complete loss of activity upon removal of K156 may be related to a more global change in enzyme structure. In the newly deposited apo-enzyme structure K156 is located at the junction of two domains and interacts with the carbonyl backbone of proline 114 (a glutamate occupies this position in *Ec*HST) in the adjacent domain. The putative active site, the site of residues C142, H235, and E237, is located in the middle of this junction. Disruption of the K156 interaction with the adjacent loop could lead to a conformational change that alters the orientation of the critical binding and catalytic residues at the active site of *Ec*HST.

Homoserine α-amino binding residues

γ-Hydroxybutyric acid is not a substrate for HST [13], nor is it an inhibitor when examined at concentrations up to 100 mM. The absence of an α-amino group is the only difference between γ-hydroxybutyric acid and homoserine, suggesting an essential role for this group in conferring substrate specificity. Several polar or negatively charged residues that could possibly interact with the α-amino group of homoserine were identified by sequence alignment using the apo-HST structure as a model (PDB code 2H2W). A tyrosine was replaced by phenylalanine (Y238F) to test if the *para*-hydroxyl group of this residue is participating in a hydrogen bond with homoserine. The K_m for homoserine only increases a modest 5-fold in this mutant while the k_{cat} is slightly reduced (Table 1). Another possible candidate is glutamate 246, which was assessed with a conservative aspartate substitution (E246D). The catalytic activity of this enzyme form is only slightly reduced from the native enzyme, while the K_m for homoserine has increased ~24-fold (Table 1). A more drastic glutamate to alanine (E246A) mutation further decreases the activity of the enzyme, and causes a more dramatic 150-fold increase in the K_m for homoserine (Table 1). While each of these mutations at position 246 affect the K_m of homoserine the K_m for succinyl-CoA remains essentially unchanged, implicating E246 as a binding group that interacts with the positively charged α-amino group of homoserine. However, removal of this functional group still does not allow γ-hydroxybutyric acid to bind in a productive orientation and serve as a substrate for HST. The E246A mutant still retains significant catalytic capability (>25% of native HST) suggesting that elimination of this electrostatic interaction with homoserine is not sufficient to significantly alter substrate binding. The tyrosine at position 238 may also interact directly with homoserine to support substrate binding in this mutant. To test this hypothesis the double mutant Y238F/E246A was designed

and kinetically evaluated. The k_{cat} of this mutant is comparable to that of the E246A mutant, however the K_m for homoserine is increased an additional 1.5-fold from 59 mM to 95 mM, with the catalytic efficiency (k_{cat}/K_m) now decreased nearly 3-orders of magnitude below that of the native enzyme. The tyrosine hydroxyl and glutamate carboxyl groups may work in tandem to orient the α-amino group of homoserine, with E246 directly participating in this interaction.

Homoserine carboxyl binding residues

Several conserved, positively charged arginine residues that could potentially function to bind and orient the α-carboxyl group of homoserine were also examined. The arginine at position 249 was mutated to lysine (R249K) to eliminate a possible bidentate interaction with the substrate carboxyl group. The R249K mutation caused a 4-fold increase in K_m for homoserine while the k_{cat} and K_m for succinyl-CoA are comparable to the native enzyme (Table 1). To further investigate this position an alanine was substituted for arginine (R249A) to remove any possible interaction with homoserine, however no dramatic effect was observed on either the k_{cat} or the K_m for homoserine, thus eliminating this residue from further consideration as a substrate binding group.

A second conserved arginine at position 193 was mutated to a lysine (R193K) to test its possible role in substrate binding. The k_{cat} of this mutant enzyme is reduced by 8-fold, while the K_m for homoserine and succinyl-CoA remain unchanged (Table 1). An R193A mutation also causes a similar 8-fold reduction in k_{cat} and a 3-fold increase in the K_m of homoserine. Thus, eliminating the arginine at position 193 has only a modest overall effect on the catalytic efficiency. To examine if elimination of both the α-amino and the α-carboxyl binding groups will have a synergistic effect on homoserine binding the double mutant R193A/E246A was prepared and kinetically evaluated. This double mutant causes a >20-fold reduction in the catalytic activity of the enzyme and an increase in the K_m of homoserine by nearly 250-fold (Table 1). This corresponds to a decrease in k_{cat}/K_m of over 3 orders of magnitude from the native enzyme, however the increase in K_m of homoserine from E246A to the double mutant R193A/E246A is only 1.5-fold, supporting the conclusion of a relatively minor role for Arg193 in substrate binding.

A proposed mechanism which incorporates the roles of these substrate orienting residues in the deacylation reaction is outline in Fig. 3b. In this half-reaction residues Y238 and E246 are in position to orient the α-amine of homoserine while R193 is shown interacting electrostatically in a bidentate fashion with the carboxyl moiety of homoserine. These interactions help orient homoserine properly to be deprotonated by the active site acid–base, H235, which activates homoserine for nucleophilic attack on the succinyl-thioester enzyme intermediate. The negatively charged carbonyl intermediate formed during this process is most likely stabilized by a yet unidentified residue. The collapse of the homoserine-succinylated-enzyme intermediate is facilitated by the protonation of C142, releasing succinylhomoserine to complete a full catalytic cycle.

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