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Substrate Analysis of Homoserine Acyltransferase from *Bacillus cereus*[†]

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Abstract

Substrate specificity within the family of enzymes designated as homoserine transsuccinylases is variable, with some organisms utilizing succinyl-CoA and other organisms utilizing acetyl-CoA. In this study it is shown that the enzyme from *Bacillus cereus* uses acetyl-CoA as its acyl donor, but its catalytic rate is significantly lower than other HTS family members. BcHTS is inactivated by both iodoacetamide and diethyl pyrocarbonate and the enzyme can be partially protected from inactivation by the presence of succinyl-CoA. This leads to the conclusion that BcHTS can bind both acetyl-CoA and succinyl-CoA and suggests that it may represent an intermediate between the succinate-transferring HTS family members and the acetate-transferring HTS family members. The *B. cereus* enzyme was unable to rescue growth of an *E. coli* strain lacking a functional transsuccinylase, however.

Keywords

Homoserine transsuccinylase; homoserine transacetylase; acyltransfer; methionine biosynthesis

Homoserine transsuccinylase (HTS)¹ (EC 2.1.3.46) is an enzyme in the methionine biosynthetic pathway found in a variety of microorganisms which transfers succinate from succinyl-CoA to homoserine to form *O*-succinylhomoserine (Figure 1). Methionine and its derivatives *S*-adenosylmethionine and acylhomoserine lactones are essential compounds that fill numerous biological roles in microorganisms [1;2]. Mutations or deletions of enzymes involved in methionine biochemistry lead to various metabolic defects in humans [3], and inhibition of methionine biosynthesis in microorganisms leads to the arrest of cell growth and may attenuate pathogenicity in some fungi [4;5]. These properties make enzymes in the methionine biosynthetic pathway attractive targets for the design of novel inhibitors.

A second enzyme, homoserine transacetylase (HTA) (EC 2.1.3.31), catalyzes the biologically equivalent reaction in many microorganisms using acetyl-CoA as the acyl donor in place of succinyl-CoA [6;7]. The products of these two enzymes are both substrates for the subsequent enzyme in the pathway, yielding cystathionine in each case (Figure 1). No amino acid sequence homology exists between HTS and HTA and they appear to have evolved independently. It

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has recently been reported that *Thermotoga maritima* HTS (TmHTS) uses acetyl-CoA rather than succinyl-CoA [8], suggesting the possibility that other HTS family members may also be acetyl transferases. Previous studies had suggested that *Bacillus subtilis* and *Bacillus polymyxa* utilize a transacetylase in methionine biosynthesis [9;10;11], while genomic sequencing has shown that *Bacilli* species contain the gene for HTS but not HTA [12;13]. Thus, *Bacillus* species may represent a second example of HTS family members that catalyze acetate transfer preferentially to succinate transfer. We cloned, expressed, and purified homoserine transsuccinylase from *Bacillus cereus* (BcHTS). The enzyme had good catalytic activity with the non-physiological substrate 4-nitrophenylacetate (PNPA), low catalytic activity with acetyl-CoA, and no catalytic activity with succinyl-CoA. BcHTS is able to bind succinyl-CoA, as was demonstrated with chemical modification and substrate protection experiments.

Materials and Methods

Materials

Oligonucleotide primers used for PCR amplification were synthesized by Invitrogen Custom Primers (Carlsbad, CA). The pBAD102/D-TOPO expression system and *Escherichia coli* BL21 (DE3) cells were purchased from Novagen (Madison, WI). *B. cereus* genomic DNA was purchased from ATCC (Manassas, VA) and DNaseI was from Epicenter (Madison, WI). The AB1932 *E. coli* strain (CGSC#1932) was obtained from the *E. coli* Genetic Stock Center (Yale University, New Haven, CT). Arabinose was purchased from Acros Organics (Morris Plains, NJ). TALON Metal Affinity Resin was purchased from BD Bioscience (San Jose, CA) and

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BcHTS	<i>Bacillus cereus</i> homoserine transsuccinylase
CHES	2-(<i>N</i> -cyclohexylamino)ethane sulfonic acid
CoA	coenzyme A
DEPC	diethyl pyrocarbonate
EcHTS	<i>Escherichia coli</i> homoserine transsuccinylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HiHTA	<i>Haemophilus influenzae</i> homoserine transacetylase
HTA	homoserine transacetylase
HTS	homoserine transsuccinylase
IAA	iodoacetamide
PNPA	4-nitrophenyl acetate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TmHTS	<i>Thermotoga maritima</i> homoserine transsuccinylase

S-200 gel filtration resin was from GE Healthcare Life Sciences (Piscataway, NJ). All other chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Cloning and Overexpression of *B. cereus* Homoserine Transsuccinylase and Generation of Mutants

The sequence of the putative *metA*-encoded homoserine transsuccinylase gene from *B. cereus* has been reported [12] and was amplified from genomic DNA (ATCC 14579 strain) under standard PCR conditions. The PCR product was cloned into the pBAD102/D-TOPO expression vector in frame with the N-terminal thioredoxin gene and the C-terminal histidine tag. The recombinant plasmid was transformed into competent *E. coli* BL21 (DE3) cells, which were used to express the protein under arabinose induction.

Likewise, oligonucleotide primers which contained a mutation from cysteine to alanine at amino acid 142 or from histidine to alanine at amino acid 235 were used to create the desired *B. cereus metA* mutants using the QuikChange® Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). Recombinant plasmids containing the mutations were transformed into competent *E. coli* BL21 (DE3) cells and protein expression was induced with arabinose. All recombinant DNAs were sequenced to confirm successful cloning and mutant generation.

Protein Purification

Two liters of LB containing ampicillin (50 mg/L) were inoculated with 500 µl of an overnight culture started from a single bacterial colony. The cultures were grown to an OD₆₀₀ of 0.5 - 0.8 before induction with 0.02% arabinose for 3h, harvested by centrifugation, and resuspended in 50 mL of lysis buffer (25 mM HEPES (pH 7.5), 25 mM NaCl, lysozyme (0.2 mg/mL), 10 mM MgCl₂ and 10 units of DNaseI). Cells were lysed by sonication and the cell debris was removed by centrifugation at 12,000×g for 30 min at 4 °C. Protein was purified from the supernatant using TALON Metal Affinity Resin charged with Co²⁺ by following the manufacturer's instructions. To further purify the protein and remove excess imidazole, the concentrated sample was loaded onto a 58 cm × 2.8 cm Sepharose S-200 gel filtration column. Fractions containing active, pure protein, as determined by SDS-PAGE and enzyme assays, were concentrated and stored at -20 °C in 50% glycerol.

Measurement of Enzyme Activity

Reaction rates were determined by monitoring the decrease in absorbance at 232 nm for acyl-CoA donors or by monitoring the increase in absorbance at 410 nm for PNPA in a UV/VIS spectrophotometer. Assays were performed in 50 mM K₂HPO₄ (pH 7.2) at 25 °C. Reactions were initiated by the addition of enzyme, and initial velocity kinetic data were fit to eq. 1 using SigmaPlot (version 7.0 SPSS):

$$v_o = V_{\max} [S] / (K_m + [S]) \quad (1)$$

where v_o is the observed initial velocity, $V_{\max}$ is the maximal velocity, $[S]$ is the substrate concentration, and K_m is the Michaelis constant.

pH Profiles

Enzyme activity was measured with PNPA over the pH range of 6.0 - 9.6 using phosphate (6.0 - 7.0), Tris (7.55 - 9.3) and CHES (9.6) as buffers. The kinetic parameters $V_{\max}$ and K_m were determined at each pH value using seven concentrations of each substrate, while holding the other substrate at a constant concentration (500 µM for homoserine, 1 mM for PNPA).

Inactivation by Iodoacetamide

A 100 μ l solution containing 23.4 μ g of enzyme in 100 mM phosphate buffer was inactivated at 25 °C by the addition of 0 (control), 50, 100, 250, 500 and 1000 μ M iodoacetamide (IAA). At various time points, aliquots (10 μ l) were removed and added to an assay solution containing 100 mM KH_2PO_4 (pH 7.0), 500 μ M homoserine, and 1 mM PNPA for a final volume of 500 μ l.

The pH-dependence of IAA inactivation was determined by measuring the rate of inactivation by 250 μ M iodoacetamide over a pH range of 6.0 - 8.0 using phosphate buffer, and the data were fit to eq. 2

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

where V is the rate of inactivation, C is the pH-independent plateau value, H is the hydrogen ion concentration, and K_a is the ionization constant for the cysteine residue.

To study the effect of putative substrates on iodoacetamide inactivation, various acyl-CoA donors or PNPA were included in the inactivation mixture described above for the pH dependence, and residual enzyme activity was monitored at 410 nm.

Inactivation by Diethyl Pyrocarbonate

A solution containing 23.4 μ g of enzyme in 100 mM phosphate buffer (pH 6.0) was inactivated at 25 °C by the addition of 0 (control), 50, 100, 250, and 500 μ M diethyl pyrocarbonate (DEPC) dissolved in acetonitrile (100 μ l final volume). At various time points, 10 μ l aliquots were removed and added to an assay solution containing 100 mM KH_2PO_4 (pH 7.0), 500 μ M homoserine, and 1 mM PNPA. To study the effect of substrate on DEPC inactivation, succinyl-CoA was added to an inactivation mixture containing 250 μ M DEPC and residual enzyme activity was monitored at 410 nm.

Rescue of *E. coli* Methionine Auxotrophs

The AB1932 *E. coli* strain was obtained from the *E. coli* Genetic Stock Center at Yale University. This strain is an arginine and methionine auxotroph, with the defect in methionine biosynthesis located in the HTS gene. Cells were transformed with plasmids expressing *E. coli* HTS (EcHTS), TmHTS, BcHTS, and *Haemophilus influenzae* HTA (HiHTA). Transformed cells were grown in LB, protein expression was induced once A_{600} reached 0.9, the cells were grown for an additional 45 minutes, and then 1.5 mL of cells were harvested by centrifugation. The cell pellets were washed twice with 750 μ L of minimal media (25 mM KH_2PO_4 , 10 mM NaCl, 50 mM Na_2HPO_4 , 20 mM NH_4Cl , 2 mM MgSO_4 , 0.2% glucose, 0.1 mM CaCl_2 , 0.0002% thiamine) to remove all traces of nutrient broth and used to inoculate 50 mL cultures of minimal media. The cultures were grown at 37 °C in the presence of ampicillin, 100 μ M L-arginine, and 0.02% arabinose. Negative controls lacked arginine and positive controls included 100 μ M L-methionine.

Results and Discussion

Cloning, Expression and Purification of *B. cereus* HTS

The *metA* gene encoding for homoserine transsuccinylase was amplified from *B. cereus* genomic DNA and cloned into the pBAD102/D-TOPO expression vector. This vector includes an optional thioredoxin tag at the N-terminus and a C-terminal 6x-histidine tag, both of which were included in the recombinant fusion protein. The resulting plasmid was used to transform *E. coli* cells, which were then induced with 0.02% arabinose in order to express the cloned enzyme. After cell lysis, BcHTS was purified in a two column procedure, first using TALON Metal Affinity Resin charged with Co^{2+} followed by purification on a Sepharose S-200 gel

filtration column. SDS-PAGE analysis and enzyme activity assays were used to confirm the presence of a pure and active protein at the correct molecular mass of 52 kDa.

Determination of Steady-State Parameters

Members of the HTS family have been reported to use either succinyl-CoA (EcHTS) or acetyl-CoA (TmHTS) as their acyl-donor substrates [8;14]. Enzyme activity in the forward direction was measured by monitoring the decrease in absorbance at 232 nm due to hydrolysis of the thioester bond of acyl-CoA donors or by monitoring the increase in absorbance at 410 nm for PNPA due to the formation of the nitrophenylate anion. Of the nine acyl-CoA donors tested, activity was only observed with acetyl-CoA and this activity was ~1% of the activity seen with other HTS family members, regardless of their substrate preference. Acyl-CoA donors that did not react included propionyl-CoA, malonyl-CoA, succinyl-CoA, crotonyl-CoA, butyryl-CoA, isobutyryl-CoA, β -hydroxybutyryl-CoA, and glutaryl-CoA (Table 1). Other homoserine acyltransferases that utilize acetyl-CoA as the acyl donor substrate, such as TmHTS and HiHTA, also use propionyl-CoA and it was surprising that this compound was not a substrate for BcHTS. The K_m for acetyl-CoA is three times higher than what is observed for other HTS and HTA enzymes, while the homoserine K_m is almost 20-fold lower than that of EcHTS but similar to that of HiHTA and TmHTS [6;7;8;14].

The compound PNPA, while not a natural substrate, did act as an excellent acyl donor and was a 25-fold better substrate than acetyl-CoA when judged by a comparison of k_{cat}/K_m values. Substrate specificity for L- or D-homoserine was evaluated at six different concentrations and the data indicate that BcHTS prefers L-homoserine to D-homoserine by a factor of 14, similar to what has been observed for EcHTS, TmHTS, and HiHTA [6;7;8;14]. Attempts to detect enzyme activity in the reverse direction using *O*-acetylhomoserine or *O*-succinylhomoserine and CoA were unsuccessful. All kinetic data obtained from these experiments are listed in Table 1.

It appears that recombinant BcHTS is not able to efficiently catalyze acyl transfer. Possible explanations for this observation include purification of a modified form of BcHTS that is unable to utilize acyl-CoA substrates, a deleterious effect of the thioredoxin fusion, or high levels of conformational flexibility for the purified enzyme. TmHTS has been cloned into the identical plasmid in-frame with the thioredoxin tag and this protein has significant activity with acetyl-CoA [8], indicating that the presence of thioredoxin does not necessarily result in loss of activity. In addition, the PNPA activity ($k_{cat}/K_m = 1.5 \times 10^4 \text{ s}^{-1}\text{M}^{-1}$) is similar to that observed with HiHTA ($k_{cat}/K_m = 5.6 \times 10^3 \text{ s}^{-1}\text{M}^{-1}$) and EcHTS ($k_{cat}/K_m = 5.1 \times 10^3 \text{ s}^{-1}\text{M}^{-1}$) and demonstrates that the enzyme retains acyltransfer activity. A recent crystal structure of BcHTS revealed that portions of the enzyme are conformationally flexible [15]. This flexibility may be responsible for the low activity of the purified protein since it has been removed from the cellular environment, where ionic strength, pH, levels of reductant, interactions with other proteins, and other factors likely combine to hold the protein in an active conformation.

It is also possible that unknown cofactors are required for maximal BcHTS activity. These cofactors could be small molecules required for interactions with substrate or stabilization of protein conformation or they could be other proteins required to form a functional complex. To determine if a metal cofactor was required for BcHTS activity, enzyme activity was monitored in a reaction containing 500 μM homoserine and 200 μM succinyl-CoA, 200 μM acetyl-CoA, or 1 mM PNPA in the presence of a 500 μM concentration of various metal ions: NaCl, KCl, LiCl, CsCl, MgCl_2 , MnCl_2 , CaCl_2 , NiSO_4 , $\text{Zn(NO}_3)_2$, and $\text{Cu(NO}_3)_2$. Enzyme activity was measured and the results compared to that of an identical reaction containing no metal. None of the metals significantly increased the reaction rate, suggesting that they are not required for enzyme activity.

pH Dependence of Homoserine Transsuccinylase

The pH dependence of BcHTS activity was monitored over the pH range of 6.0 - 9.6 using phosphate, Tris-Cl and CHES as buffers and PNPA as the acyl donor (Figure 2). The results indicate that the maximal velocity of the enzyme decreases as the pH is lowered, suggesting the presence of an amino acid residue that must be deprotonated for enzyme activity. This trend is most evident when PNPA is varied (Figure 2A), but also occurs when homoserine is varied (Figure 2C). On the other hand, the $\log(V_{\max}/K_M)$ with both substrates varies little with pH, except for a possible downward trend at the highest pH values tested (Figures 2B and 2D).

Similar pH studies have been performed for EcHTS and TmHTS and bell-shaped pH curves are seen for the maximal velocity and for $V_{\max}/K_M$ with each substrate. Ionizations are only observed in pH studies when they result in changes in a rate-limiting step, and the rate of the BcHTS reaction ($k_{\text{cat}} = 0.29 \text{ s}^{-1}$) is nearly two orders of magnitude lower than the rates for EcHTS ($k_{\text{cat}} = 24 \text{ s}^{-1}$) and TmHTS ($k_{\text{cat}} = 23 \text{ s}^{-1}$). Since the chemistry of the three enzymes is the same, the BcHTS reaction may be partially or fully controlled by conformational steps rather than chemical steps, and this may explain the limited influence of pH on enzyme activity.

Inactivation by Iodoacetamide

Previous studies have shown that IAA inactivates homoserine transsuccinylases [8;14]. When BcHTS was incubated with IAA, the enzyme lost activity in a time- and concentration-dependent manner (Figure 3A). In addition, this inactivation is pH-dependent, as is indicated by a decrease in the rate of inactivation as the pH is lowered from 8.0 to 6.0 (Figure 3B). The data were fit to equation 2 and a pK of 6.6 for the modified cysteine residue was determined, suggesting the residue exists as a thiolate anion under physiological conditions.

To determine if the modification of BcHTS occurs at the active site, PNPA was added to the inactivation mixture as a protective agent and the residual activity was monitored as before. When 1 mM PNPA was added to the inactivation solution protection from inactivation was observed. A variety of acyl-CoA donors were then tested for their ability to prevent inactivation by iodoacetamide. When added to the inactivation mixture at a concentration of 250 μM , succinyl-CoA offered the greatest level of protection, followed by acetyl-CoA (Table 2), suggesting that succinyl-CoA is capable of binding to the enzyme active site even though it is not a substrate for the enzyme.

Inactivation by Diethyl Pyrocarbonate

A sequence comparison of EcHTS and BcHTS confirmed the presence of a putative active site histidine residue contained in homoserine transsuccinylase family members [16;17]. Inactivation with DEPC was time- and concentration-dependent (Figure 4). Substrate protection experiments were performed to determine if the DEPC inactivation occurred at the active site. Addition of 250 μM succinyl-CoA resulted in protection of 30% of enzyme activity after an 11 minute inactivation, providing further evidence that succinyl-CoA can bind to the active site of BcHTS.

BcHTS Mutant Activity

Sequence comparisons with HTS-family members from other organisms indicated one completely conserved cysteine residue (C142) and one completely conserved histidine residue (H235). These two residues were individually mutated to alanine, and the resulting mutant proteins (C142A and H235A) were analyzed for residual activity. No activity was detected with the C142A mutant and slight activity was detected with the H235A mutant when 40 μg of enzyme were added to the assay mixture. To further investigate the activity of the H235A mutant, initial velocities were determined at six different concentrations of PNPA ranging from

100 μM to 2 mM while the concentration of homoserine was held constant at 500 μM . The data were fit to eq. 1 and the K_m of PNPA and the k_{cat} of the H235A mutant were determined to be 2.4 mM and 0.058 s^{-1} , respectively. The H235A mutant has a 200-fold lower reaction rate than the wild-type enzyme and a 3-fold greater K_m for PNPA, suggesting that H235 is required both for catalysis and for substrate binding or orientation. No activity was observed for the H235A mutant when using acetyl-CoA as the acyl donor substrate.

Rescue of *E. coli* Methionine Auxotrophs

BcHTS appears to have many of the properties characteristic of EcHTS, but its substrate specificity and pH dependence are more similar to HiHTA. We therefore wondered whether BcHTS could functionally complement EcHTS. To test this, we used the AB1932 strain of *E. coli*, which is an arginine and methionine auxotroph. The defect in methionine biosynthesis is located in the *metA* gene and these cells will grow in the presence of methionine or *O*-succinylhomoserine. Cells were transformed with plasmids expressing EcHTS, TmHTS, HiHTA or BcHTS, induced, and grown in rich media. A 1 mL aliquot of each growth was pelleted, washed extensively with minimal media and then used to seed a 50 mL culture in minimal media supplemented with arginine and inducer. Growth curves were measured for each transformant over a 48-hour period. Transformation with EcHTS resulted in rapid growth of the AB1932 cells while transformation with TmHTS and HiHTA permitted growth on a much slower time scale. Cells transformed with BcHTS failed to grow within the 48-hour time frame of the experiment, however, indicating that this protein cannot functionally substitute for EcHTS.

Conclusion

The homoserine acyltransferase from *Bacillus cereus* has properties associated with transsuccinylases and transacetylases. The recombinant enzyme poorly uses acetyl-CoA as a substrate and does not use succinyl-CoA. Succinyl-CoA is capable of binding to the active site, however, since it protects the enzyme from IAA and DEPC inactivation. This suggests that BcHTS has a conformationally flexible active site that prefers the shorter acetyl group, but can accommodate the larger, negatively-charged succinyl group. Further biochemical characterization can be used in conjunction with the recently reported structure to shed light on these properties.

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Figure 1. Homoserine acylation pathways. Homoserine destined for methionine biosynthesis is activated either by HTS using succinyl-CoA or by HTA using acetyl-CoA.

Figure 2.

pH dependence of BcHTS. (A) Dependence of maximal velocity on pH when varying PNPA. The units of maximal velocity are micromoles per minute per milligram of protein. (B) Dependence of V/K_{PNPA} on pH. The values of V are measured in units of maximal velocity and K_{PNPA} is in μM units. (C) Dependence of maximal velocity on pH when varying homoserine. (D) Dependence of $V/K_{\text{homoserine}}$ on pH.

Figure 3. Inactivation of BcHTS by iodoacetamide and effect of pH on iodoacetamide inactivation. (A) BcHTS was incubated with IAA for varying periods of time and the remaining activity was determined. The log (% activity), in comparison to a control reaction without IAA, was then calculated at each time point and the data plotted. (●) 0 μ M IAA; (○) 50 μ M IAA; (▼) (100 μ M IAA; (△) 250 μ M IAA; (■) 1000 μ M IAA. (B) Dependence of the rate of inactivation of BcHTS by iodoacetamide on pH. The rate of inactivation is measured in units of remaining initial velocity per minute.

Figure 4.

Inactivation of BcHTS by diethylpyrocarbonate. BcHTS was incubated with DEPC for varying periods of time and the remaining activity was determined. The log (% activity), in comparison to a control reaction without DEPC, was then calculated at each time point and the data plotted. (●) 0 μM DEPC; (○) 50 μM DEPC; (▼) 100 μM DEPC; (△) 250 μM DEPC; (■) 500 μM DEPC.

Table 1

Kinetic Parameters for the preferred and alternate substrates for BcHTS in the forward and reverse reactions.

Substrate	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\text{s}^{-1}\text{M}^{-1}$)
Acetyl-CoA	460 ± 130	0.29 ± 0.06	630
4-Nitrophenyl acetate	780 ± 260	12 ± 2	1.5×10^4
L-Homoserine	50 ± 20	4.7 ± 0.5	9.4×10^4
D-Homoserine	270 ± 140	1.8 ± 0.5	6.7×10^3
Succinyl-CoA	IA ^a	IA ^a	
O-acetylhomoserine	IA ^a	IA ^a	
O-succinylhomoserine	IA ^a	IA ^a	
Coenzyme A	IA ^a	IA ^a	

^a No activity could be determined for these compounds under the conditions tested.

Table 2

Protection of BcHTS from IAA inactivation by acyl-CoA compounds.

Acyl-CoA Compound	% Activity ^a
Succinyl-CoA	33.5
Acetyl-CoA	13.2
Propionyl-CoA	7.6
Malonyl-CoA	6.7
Butyryl-CoA	4.8
Glutaryl-CoA	4.0
CoA	3.3

^a BcHTS was inactivated for 11 minutes with 250 μ M IAA in the presence of the acyl-CoA compound and remaining enzyme activity was measured with PNPA and compared to a control reaction that lacked IAA. Acyl-CoA compounds were kept at a concentration of 250 μ M in the inactivation mixture.