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Enzymatic synthesis of diastereospecific carbacephem intermediates using serine hydroxymethyltransferase

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The serine hydroxymethyltransferase (SHMT) gene *glyA* was over-expressed in *Escherichia coli* and the enzyme was purified to near homogeneity. Reaction conditions for *E. coli* and rabbit liver SHMTs were optimized using succinic semialdehyde methyl ester (SSAME) and glycine. The catalytic efficiency (k_{cat}/K_m) of *E. coli* SHMT for SSAME was 2.8-fold higher than that of rabbit liver enzyme. *E. coli* SHMT displayed a pH-dependent product distribution different from that of rabbit liver enzyme. For the pyridoxal-5′-phosphate (PLP)-dependent reaction, *E. coli* and rabbit liver SHMTs showed a high product diastereospecificity. The stoichiometric ratio of PLP to the dimeric *E. coli* SHMT was 0.5–0.7, indicating a requirement for external PLP for maximal activity. Using SSAME or its analog at a high temperature, *E. coli* SHMT mediated efficient condensation via a lactone pathway. In contrast, at a low temperature, the enzyme catalyzed efficient conversion of 4-penten-1-al via a non-lactone mechanism. Efficient conversion of either aldehyde type to a desirable diastereospecific product was observed at a pilot scale. *E. coli* SHMT exhibited a broad specificity toward aldehyde substrates; thus it can be broadly useful in chemo-enzymatic synthesis of a chiral intermediate in the manufacture of an important carbacephem antibiotic.

Keywords: chemo-enzymatic synthesis; serine hydroxymethyltransferase; PLP; diastereospecificity; carbacephem

Introduction

Serine hydroxymethyltransferase (SHMT) from rabbit liver catalyzes several chemical reactions, including C-C cleavage/condensation, transamination and racemization. Each of the three reaction types is reversible. For C-C condensation of an aldehyde and glycine, the SHMT exhibits a broad specificity toward the aldehyde co-substrate [11–13]. As described previously [8], the enzyme catalyzes condensation of glycine and succinic semialdehyde methyl ester (SSAME) to form diastereospecific l-allo-α-amino-β-hydroxyadipyl (l-allo-aAbHA) methyl ester (Figure 1), a potential intermediate for synthesis of a carbacephem [6].

The *gly*A gene encoding SHMT from *E. coli* was over-expressed intracellularly in *E. coli* and purified to near homogeneity. Purified *E. coli* SHMT was shown to catalyze condensation of SSAME and glycine to l-allo-α-amino-β-hydroxyadipic acid (l-allo-aAbHAA or l-erythro-aAbHAA) as a detectable reaction product; but not to l-allo-aAbHA methyl ester as described for the rabbit enzyme [8]. We report here: (a) intrinsic kinetic properties of *E. coli* and rabbit liver SHMTs; and (b) with *E. coli* SHMT, the kinetic mechanism with two types of aldehyde substrates, cofactor requirement, conversion efficiency and substrate specificity. Our chemo-enzymatic study, using *E. coli* SHMT and one aldehyde co-substrate, has been effectively applied in the generation of a chiral intermediate at liter-pilot scale for carbacephem synthesis.

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Materials and methods

Chemicals

Glycine and analogs were obtained from Sigma (St Louis, MO, USA) and 4-penten-1-al (pentenal) was purchased from Lancaster Research Chemicals (Morcambe, Lancs, UK). All other aldehydes were synthesized at Lilly Research Laboratories (Lilly); the synthesis of four aldehydes is described briefly below. 4-Pentenoic acid was purchased from Aldrich (Milwaukee, WI, USA) and converted to methyl or ethyl ester by refluxing in acidic methanol or ethanol, respectively. The methyl or ethyl ester was then converted to SSAME or SSAEE by ozonolysis followed by quenching with triphenylphosphine in dichloromethane/methanol. 4-Pentenoic acid was converted to a *t*-butyl ester by reaction with isobutylene in ether. The *t*-butyl ester was then converted to SSATBE as described for SSAME. Furyl acrolien from Aldrich Chemical Co was converted to FPA by hydrogenation in the presence of triphenylphosphine rhodium chloride in ethanol. After the synthesis, each aldehyde was isolated by low vacuum distillation.

Enzyme sources

Purified (>95%) rabbit liver SHMT was provided by LaVerne Schirch of Virginia Commonwealth University (Richmond, VA, USA). The bacterial enzyme was purified to ~95% from recombinant *E. coli*, as described below.

Activity assay

For the study on gene cloning and over-expression and for enzyme purification, the activity of SHMT was determined using phenylserine as substrate and by monitoring product formation as modified from that described previously [12]. Benzaldehyde, one cleavage product, was quantified by

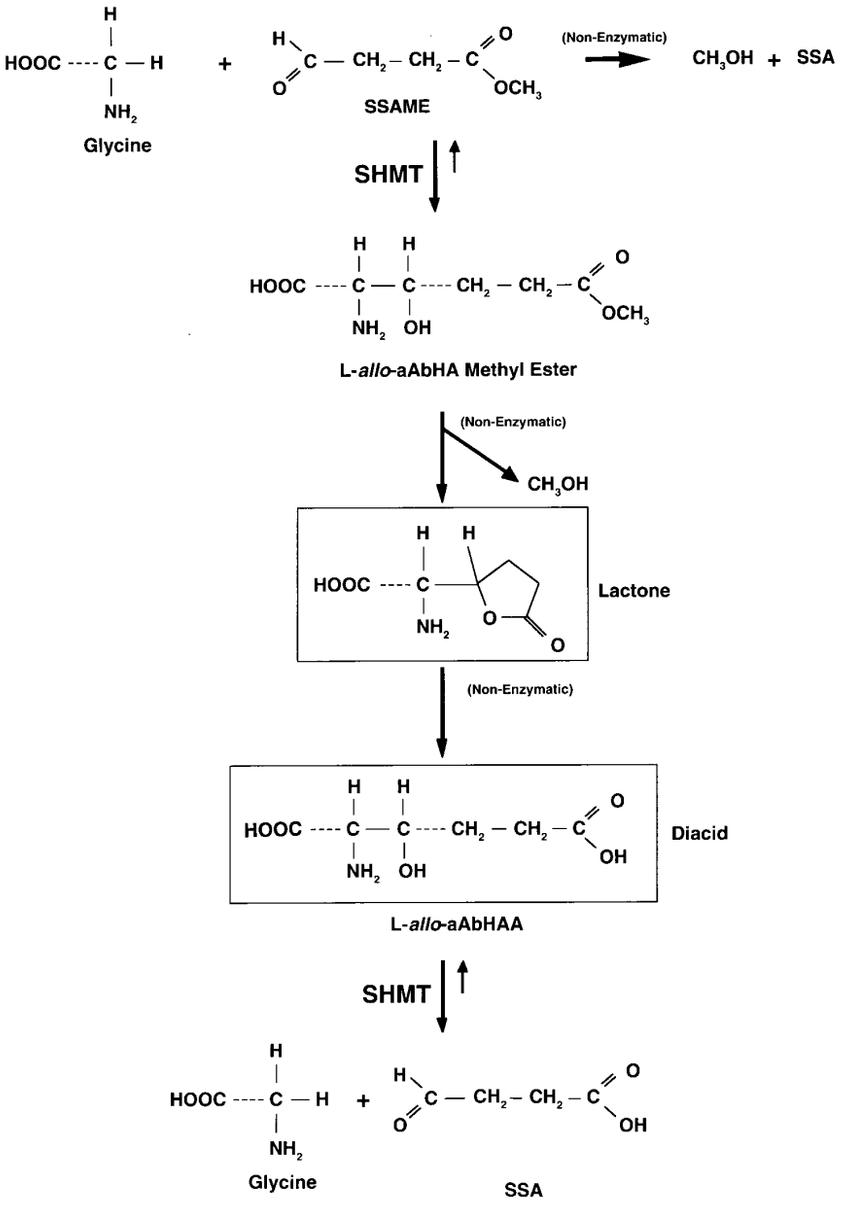

Figure 1 Proposed reaction pathway of *E. coli* SHMT with SSAME as aldehyde co-substrate. A bold/long arrow indicates a high conversion and a light/short arrow a low conversion.

HPLC at 290 nm. For all biochemical studies, the SHMT activity or its conversion efficiency was determined using either the OPA-NAC ([1]; C Higginbotham, Lilly, personal communication) or the DABSYL method [3]. A typical HPLC assay by the OPA-NAC method is shown in Figure 2. For each activity assay, one unit of SHMT is defined as the amount of the enzyme that causes formation of one μmole of the product in one minute under the reaction conditions.

Cloning and over-expression of E. coli glyA (SHMT) gene

Plasmid pGS29 containing the glyA gene encoding SHMT [9] and a glyA$^-$ *E. coli* host GS245 were provided by George Stauffer, Department of Microbiology, University of Iowa (Iowa City, IA, USA). Plasmid pHKY390, a temperature-inducible heterologous expression vector, was created at Lilly (Figure 3a; JL Larson, Lilly, personal communication). All reagents and *E. coli* strains were obtained from commercial sources or at Lilly. All genetic manipulations performed (including transformation, plasmid isolation, DNA fragment isolation and ligation) were carried out as described by Maniatis *et al* [7] and Ausubel [2]. Strains Dgly and Rgly were induced to produce SHMT by growing cultures at 30°C until mid-log phase and then switched to 40°C for 1–8 h.

Three genetic manipulations were conducted to improve SHMT gene expression: (1) a superior host was selected; (2) unnecessary DNA and a copy control element were removed from plasmid pGS29; and (3) the *gly*A open read-

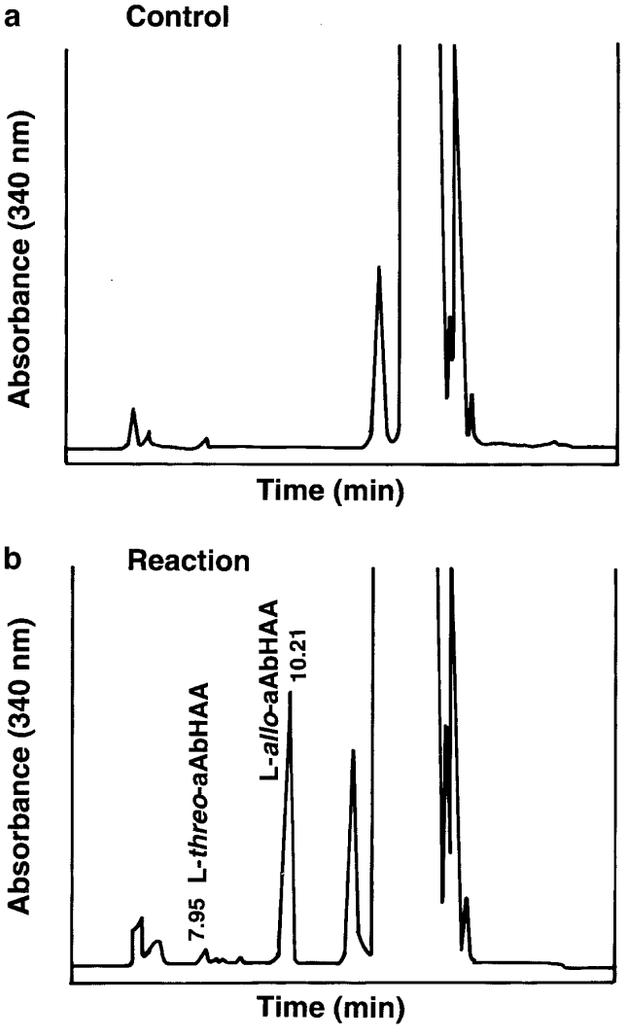

Figure 2 HPLC assay for SHMT-catalyzed condensation reaction: glycine + SSAME→l-*allo*-aAbHAA + methanol. The reaction mixture was derivatized using an OPA-NAC method [1]. The derivatives were analyzed on a YMC Basic C-8 column (4.6 mm × 25 cm) from YMC Inc, Morris Plains, NJ, USA, at a flow rate of 1 ml min^{-1} with detection at 340 nm. The derivatives were eluted from the column by a mobile phase gradient consisting of (a) 0.05 M NaH_2PO_4, pH 4.7–5.5 and (b) 80% methanol in (a). (a) Control: SSAME and glycine were in the mixture containing no enzyme; (b) reaction; the enzymatic reaction was carried out under the optimized conditions as described in Table 2.

ing frame (ORF) was inserted into a temperature-inducible expression vector. Stauffer's original expression system, G.1 (pGS29 in *E. coli* strain GS245), produced SHMT with a specific activity of 0.4 U mg^{-1}. This represented a >100-fold increase compared to that from *E. coli* strain GS245 (Table 1). (1) Transformation of *E. coli* strains DH5α and RV308 with pGS29 resulted in a several-fold increase in both total and specific activity (Table 1). *E. coli* DH5α was chosen as a host for its superior plasmid stability and RV308 for its superior growth in stirred fermentors. (2) Plasmid pGS29N was constructed to eliminate the non-essential portions of pGS29 including a competing open reading frame and the copy control gene (rop) of the vector pBR322. Substantial increases in SHMT levels were seen when pGS29N was placed into DH5α and RV308, creating

Table 1 Activity analysis of SHMT from *E. coli* strains

Strain	Host/plasmid	Activity (U ml^{-1})	Protein (mg ml^{-1})	Sp. Act. (U mg^{-1})
GS245	No plasmid	0.09	29	0.003
G.1	GS245/pGS29	14.4	36	0.4
DH5α	No plasmid	1.48	27	0.05
D.1	DH5α/pGS29	50.84	36	1.41
DN.1	DH5α/pGS29N	53.28	33	1.61
Dgly	DH5α/pZPI-glyA	92.16	31	2.97
RV308	No plasmid	0.97	31	0.03
R.1	RV308/pGS29	24.6	30	0.82
RN.1	RV308/pGS29N	26.54	31	0.86
Rgly	RV308/pZPI-glyA	44.01	33	1.33

Table 2 Intrinsic reaction properties of *E. coli* and rabbit liver SHMTs

Property	*E. coli* SHMT	Rabbit liver SHMT
Optimal temperature	61°C	50°C
Optimal pH	8.0	7.7–8.5
Optimal buffer	0.05 M KPi[a]	0.01 M MOPS[a]
Linear rate time	≤30 min	≤20 min
Limiting enzyme	≤0.16 mg	≤0.14 mg
Saturated cofactor	≥40 μM	≥80 μM
Saturated co-substrates		
SSAME	≥65 mM	≥25 mM
Glycine	100 mM	150 mM
Kinetic constants		
K_m (SSAME)	12.5 mM	12.5 mM
K_m (Glycine)	20 mM	7.5 mM
V_{max}	1.35 μmol min^{-1} mg^{-1}	0.7 μmol min^{-1} mg^{-1}
Catalytic efficiency[b]		
k_{cat}/K_m (SSAME)	6.7	2.4
k_{cat}/K_m (Glycine)	4.2	4

[a]KPi: potassium phosphate; MOPS: 4-morpholinepropanesulfonic acid.
[b]Defined as μmoles of aAbHAA formed per minute per μmole of enzyme subunit over per mmolarity of either co-substrate.

strains DN.1 and RN.1, respectively (Table 1). SHMT increases in these two strains were due mainly to the gene dosage effect reflecting an increased plasmid copy number per cell. (3) Finally, plasmid pZPI-glyA (Figure 3b) was constructed by placing the glyA ORF and downstream termination sequences into pHKY390, a vector containing a temperature-inducible and modified pL promoter to drive expression of heterologous genes. The use of an inducible promoter system results in increased cell density due to nonstressed growth conditions prior to induction as well as a stronger promoter driving higher expression per cell. Placing this plasmid into strains DH5a and RV308 created strains Dgly and Rgly which produced greatly increased levels of SHMT (Table 1). The expression levels of these constructs were impressive, with Dgly producing SHMT as 40–50% of the total soluble protein as estimated by SDS-PAGE. A specific activity of 2.97 U mg^{-1} for this strain represents a 7.5-fold improvement over strain G.1 and 1000-fold improvement over *E. coli* strain GS245 (Table 1). Strain Rgly also increased the specific activity of SHMT to 1.33 U mg^{-1}, a 3-fold improvement over strain G.1. In addition, no granules were observed in any of the

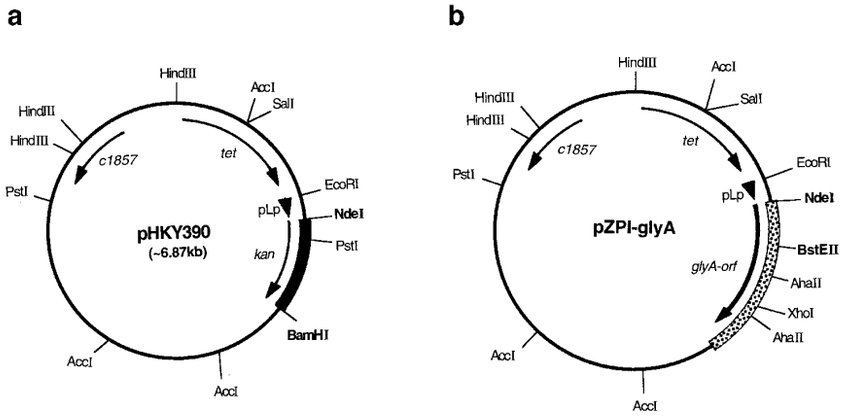

Figure 3 Restriction enzyme site and function maps of (a) plasmid pHKY390 and (b) plasmid pZPI-glyA.

induced strains, even after extended induction periods (eg 24 h). This indicates that all of the SHMT produced is in a soluble form.

Large-scale fermentation of these strains raised SHMT levels even further to more than 9 g of SHMT per liter of culture. The high enzyme yield led to its single step purification, as described below, and contributed to the cost effectiveness in its biocatalytic application as described in Results and Discussion.

Purification of E. coli SHMT

Recombinant *E. coli* cells were resuspended in 50 mM Tris-HCl, pH 7.8, containing 1 mM EDTA and 0.1 mM PLP (TEP buffer), sonicated at 4°C and centrifuged at $30\,000 \times g$ for 10 min. The supernatant fraction was filtered through glass wool and loaded on to a DEAE-Sepharose column previously equilibrated with TEP buffer. The column was washed with TEP buffer and the enzyme was eluted with a 0–300 mM KCl gradient in TEP buffer. *E. coli* SHMT was purified ~3-fold to near homogeneity (~95% pure) by single anion-exchange chromatography.

Cofactor determination

The stoichiometric ratio of pyridoxal-5′-phosphate (PLP) from holo-SHMT was determined by activity analysis, pyridoxamine-phosphate formation [14], electrospray mass spectroscopy [10] and capillary electrophoresis [15]. The cofactor requirement for maximal activity was determined from apo-SHMT by addition of exogenous PLP.

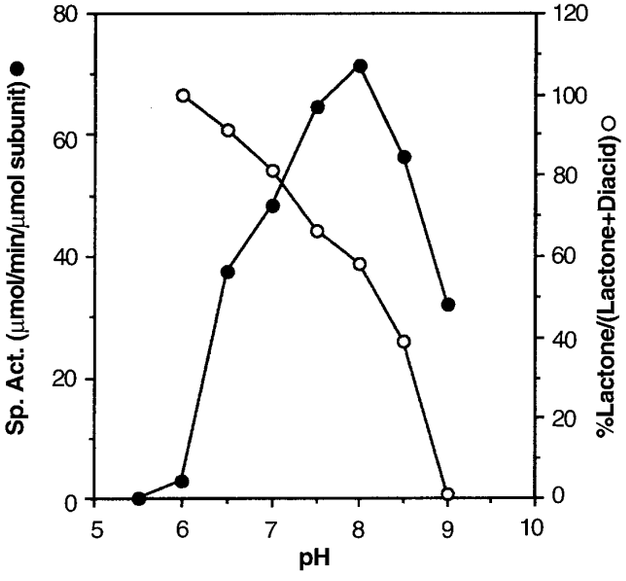

Figure 4 *E. coli* SHMT-catalyzed reaction: pH effect and product distribution. An enzymatic reaction was carried out at various pHs under the conditions: 80 mM glycine, 21.5 mM SSAME, 4.25 μM SHMT subunit, 61°C, 40 μM PLP and 50 mM KPi. The reaction time was 20 min.

Table 3 Diastereospecificity and rate enhancement of *E. coli* and rabbit liver SHMT-catalyzed reactions

Catalyst	Product distribution (% of total aAbHAA)				k_{cat}(fold) (μmol aAbHAA min^{-1} μmol^{-1} catalyst)
	allo- l	allo- d	threo- l	threo- d	
PLP[a]	41.5	41.5	8.5	8.5	0.000031 (1)
E. coli SHMT[b]	>99	0	<1	0	83.08 (2.68×10^6)
Rabbit liver SHMT[b]	98	0	2	0	30.06 (0.97×10^6)

[a]200 μM PLP only.
[b]Based on enzyme subunit in the presence of 40 μm PLP, at which no product was formed from the cofactor alone.

Results and discussion

Intrinsic kinetic properties

Using SSAME and glycine as co-substrates, reaction conditions were optimized for *E. coli* and rabbit liver SHMTs, respectively. Under these optimal conditions (Table 2), K_m and V_{max} values for each enzyme were determined. The catalytic efficiency of *E. coli* SHMT for SSAME was 2.8-fold higher than that of rabbit liver enzyme and, with glycine as substrate, it was about the same for both enzymes (Table 2). *E. coli* SHMT catalyzed a rapid and highly diastereospecific condensation of SSAME and glycine to l-allo-aAbHAA as compared to rabbit liver enzyme or PLP (Table 3). Thus, *E. coli* SHMT was catalytically superior to rabbit liver enzyme for generation of chiral l-allo-aAb-

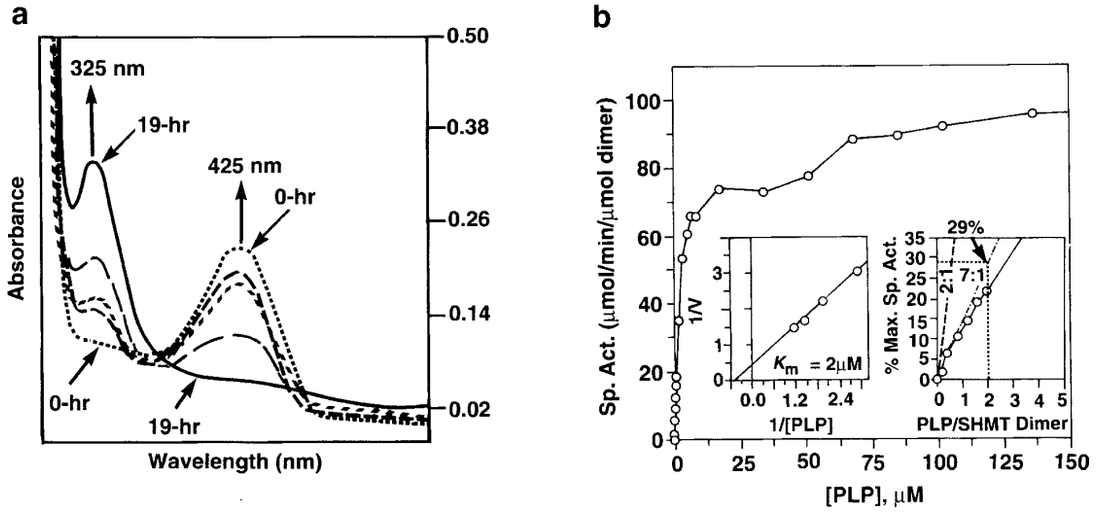

Figure 5 PLP stoichiometry of *E. coli* SHMT. (a) By pyridoxamine-phosphate analysis. The transamination reaction between d-alanine and SHMT-PLP led to the generation of apo-enzyme and pyridoxamine phosphate which was monitored spectrophotometrically [14]. (b) By activity reconstitution. The holo-enzyme could be reconstituted from the apo-enzyme, generated from the d-alanine transamination reaction, by addition of external PLP. The titration of apo-enzyme with PLP showed a $K_m = 2\ \mu$M (left insert) and a requirement of 7 : 1 molar ratio for PLP : apo-SHMT to reconstitute full activity.

Table 4 Cofactor stoichiometry of *E. coli* SHMT

Analytical procedure	PLP/SHMT dimer[a] (mol mol^{-1})
Activity determination of Holo-SHMT	0.54
Electrospray analysis of Holo-SHMT	0.5–0.6
Pyridoxamine-phosphate quantitation from Holo-SHMT	0.6
Capillary electrophoresis quantitation from Holo-SHMT	0.6–0.7
Activity reconstitution from Apo-SHMT	7

[a]Theoretical molar ratio of PLP/SHMT dimer: 2.0.

HAA. Our enzymatic optimization of *E. coli* SHMT (57-fold higher reaction rate), in combination with the ultra-high gene expression (7.5-fold higher enzyme activity than that from G.1; [9]), led to a greater than 400-fold rate enhancement for l-allo-aAbHAA formation.

Kinetic mechanism with SSAME

For *E. coli* SHMT, the distribution of l-allo-aAbhAA as lactone and diacid (Figure 1) was pH-dependent (Figure 4). In the absence of *E. coli* enzyme, no chemical conversion of the diacid to the lactone was detected at pH 6 under the reaction conditions. The observation of product formation as only the lactone in the presence of *E. coli* SHMT indicates that the lactone preceded the diacid in the reaction pathway (Figure 1). In the absence of *E. coli* enzyme, between pH 6.5 and 9.0, conversion of the lactone to the diacid increased with increasing pH, suggesting that the lactone→diacid conversion is non-enzymatic (Figure 1). The lactone→diacid conversion also increased with increasing temperature with or without the enzyme, substantiating it as a non-enzymatic reaction. l-Allo-aAbHA methyl ester, the direct condensation product from SSAME and glycine as previously suggested [8], was not detected. However, during *E. coli* SHMT-catalyzed condensation of succinic semialdehyde ethyl ester (SSAEE) and glycine, we detected l-allo-aAbHA ethyl ester as a minor (~4%) and transient reaction product. In the absence of *E. coli* SHMT, complete lactonization of d/l-allo-aAbHA ethyl ester to the corresponding racemic lactone occurred at pH 8, 50°C in 10 min, suggesting a non-enzymatic lactonization in the presence of the enzyme. The non-enzymatic lactonization might minimize the reverse hydrolysis of l-allo-aAbHA methyl ester to SSAME and glycine (Figure 1). The enzyme catalyzed hydrolysis of the diacid (but not the lactone) to succinic semialdehyde (SSA) and glycine. It also catalyzed condensation of SSA (albeit as a poor co-substrate) and glycine back to the diacid. The kinetic mechanism of *E. coli* SHMT with SSAEE involving a lactonization step is most likely the same as that with SSAME (Figure 1).

Effectors and cofactor stoichiometry

External PLP was greatly stimulatory to *E. coli* SHMT (up to 3-fold activity increase), suggesting that the cofactor was loosely bound to the enzyme. None of the metal ions tested was stimulatory to the enzyme and several metal ions (particularly Cu^{2+}, Co^{2+}, Zn^{2+} and Mn^{2+}) were inhibitory. In the presence of Cu^{2+} with or without PLP, more minor products of d-allo-aAbHAA and d/l-threo-aAbHAA were generated (data not shown). A slight stimulation was observed with dithiothreitol, presumably as a result of improved enzyme stabilization. The observation of Zn^{2+} inhibition and dithiothreitol stimulation is consistent with a catalytically essential sulfhydryl group of the enzyme as speculated previously [13].

The stoichiometric molar ratio of PLP to dimeric holo-SHMT was determined by four independent analytical methods as 0.5–0.7 (Figure 5a and Table 4; [15]). A stoichiometry of two molecules of PLP for maximal activity of the dimeric enzyme [4] suggests loose binding of the cofactor to the enzyme. This is substantiated by a requirement

of a minimum of 7 PLP per dimer for maximal reconstitution of the enzyme activity from apo-SHMT (Figure 5b and Table 4). Thus, PLP contributes not only to the activity of SHMT but also to product diastereospecificity. In addition, the cofactor improves the stability of the enzyme in the conversion of either type of an aldehyde substrate (data not shown), as described below.

Conversion efficiency with SSAEE and SSAME

Using SSAME, our initial studies with *E. coli* SHMT identified three critical conversion variables: enzyme inhibition by SSAME, non-enzymatic SSAME hydrolysis and association of conversion with the ratio of glycine to SSAME. We observed little enzyme inhibition by SSA and methanol, two side-reaction products. Aldehyde inhibition of SHMT was observed; however, it could be minimized by maintaining a low aldehyde concentration. Controlled multiple additions of acetaldehyde to glycine led to SHMT-catalyzed serine formation at 90% conversion efficiency on a large scale [5]. Within 100 min of SHMT-catalyzed condensation of SSAEE and glycine (100 mM) to l-allo-aAbHAA, at pH 8 and 60°C, we observed that multiple additions of SSAEE to a total concentration of 2 mM resulted in an almost complete (ie 95–100%) conversion (Figure 6a). The maximal conversion efficiency for SSAME was similarly determined as 75% (Figure 6b). Only a low chemical hydrolysis of SSAEE to SSA (3%) occurred by multiple additions of the aldehyde co-substrate. In comparison to SSAME (with 10% hydrolysis to SSA), the low hydrolysis of SSAEE might partially account for its higher conversion efficiency. The stoichiometry of l-allo-aAbHAA formation to SSAEE disappearance (0.95–1.0) suggests the absence of any significant competing and non-productive side reaction. The enzyme process with SSAEE or SSAME has been effectively scaled-up 100-fold or more at a high temperature (37–50°C). Up to 500 mM (including 50 or 100 mM) SSAEE or SSAME, we observed a conversion efficiency of at least 80% for SSAEE and at least 60% for SSAME (data not shown).

Conversion efficiency with 4-penten-1-al (pentenal) and kinetic mechanism

As described above, *E. coli* SHMT exhibited a high temperature optimum toward an aldehyde co-substrate, SSAEE or SSAME, which yielded a diastereospecific product via a lactone pathway. In contrast, using purified *E. coli* SHMT or recombinant *E. coli* cells, SHMT-catalyzed condensation of pentenal (0.05 M) and glycine (0.2 M) to l-allo-α-amino-β-hydroxyhexanoic acid (l-allo-AHHA) exhibited a low temperature optimum at 15 and 20°C (Figure 7). Formation of l-threo-AHHA, an undesirable product, increased with increasing temperature. The enzymatic reaction, using either whole cells or purified enzyme in excess for several cycles, was carried out by controlled multiple additions of pentenal up to 0.25–1 M glycine at 15°C and pH 6.5 (below the pH optimum of 8.0 for the enzyme). We observed for each cycle about 80% conversion to AHHA, with >90% as l-allo-AHHA. A reaction pathway for condensation of glycine and pentenal, which did not involve a lactonization step, is proposed (Figure 8). We also observed a minor non-enzymatic pentenal auto-aggregation. Thus, under the optimized conditions described above, we could minimize non-enzymatic aggregation and enzymatic l-threo-AHHA accumulation and also maximize l-allo-AHHA formation (Figure 8).

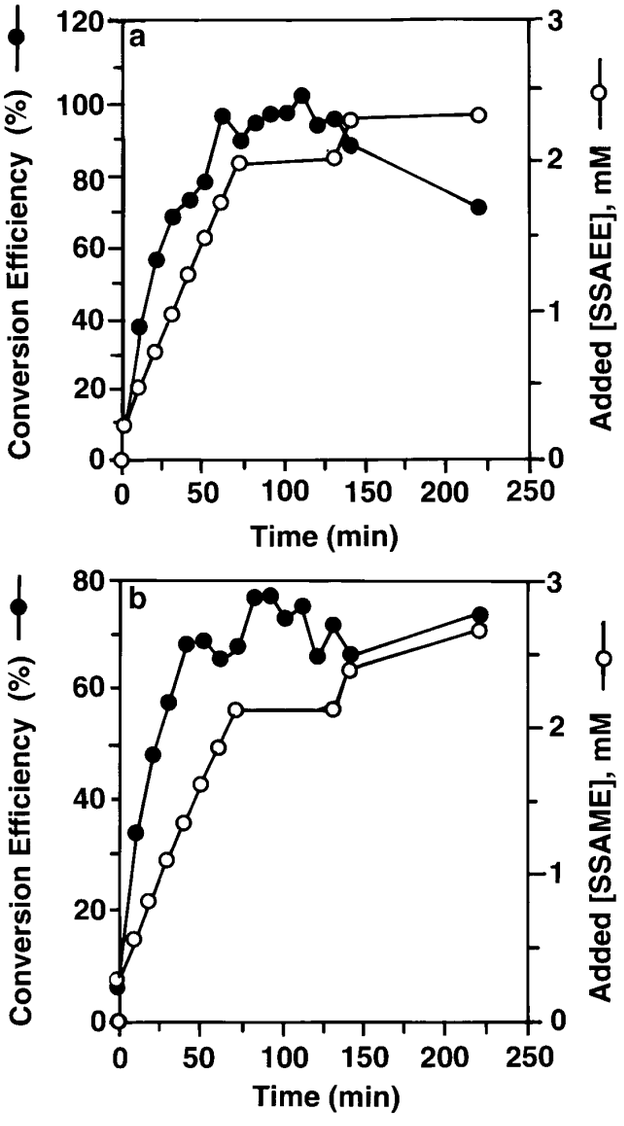

Figure 6 Conversion efficiency of *E. coli* SHMT with SSAEE or SSAME as aldehyde co-substrate. The total reaction mixture (10 ml) contained 100 mM glycine, the aldehyde added in step-wise fashion as indicated in the figure, in 50 mM potassium phosphate, pH 8.0, 24.1 μM of the enzyme and 40 μM PLP. The enzymatic reaction was carried out at 60°C for 220 min.

Substrate specificity

At 61°C, *E. coli* SHMT exhibited a broad specificity toward a large variety of aldehyde co-substrates but strongly preferred glycine as the amine co-substrate (Table 5). Based on k_{cat} and K_m values as well as product diastereospecificity, SSAEE, SSATBE and SSAME were good aldehyde co-substrates for the enzyme. The enzymatic condensation of each aldehyde with glycine involved a lactonization step and, under the reaction conditions, the chemical hydrolysis of SSAEE (or likely SSATBE) was slower than that of

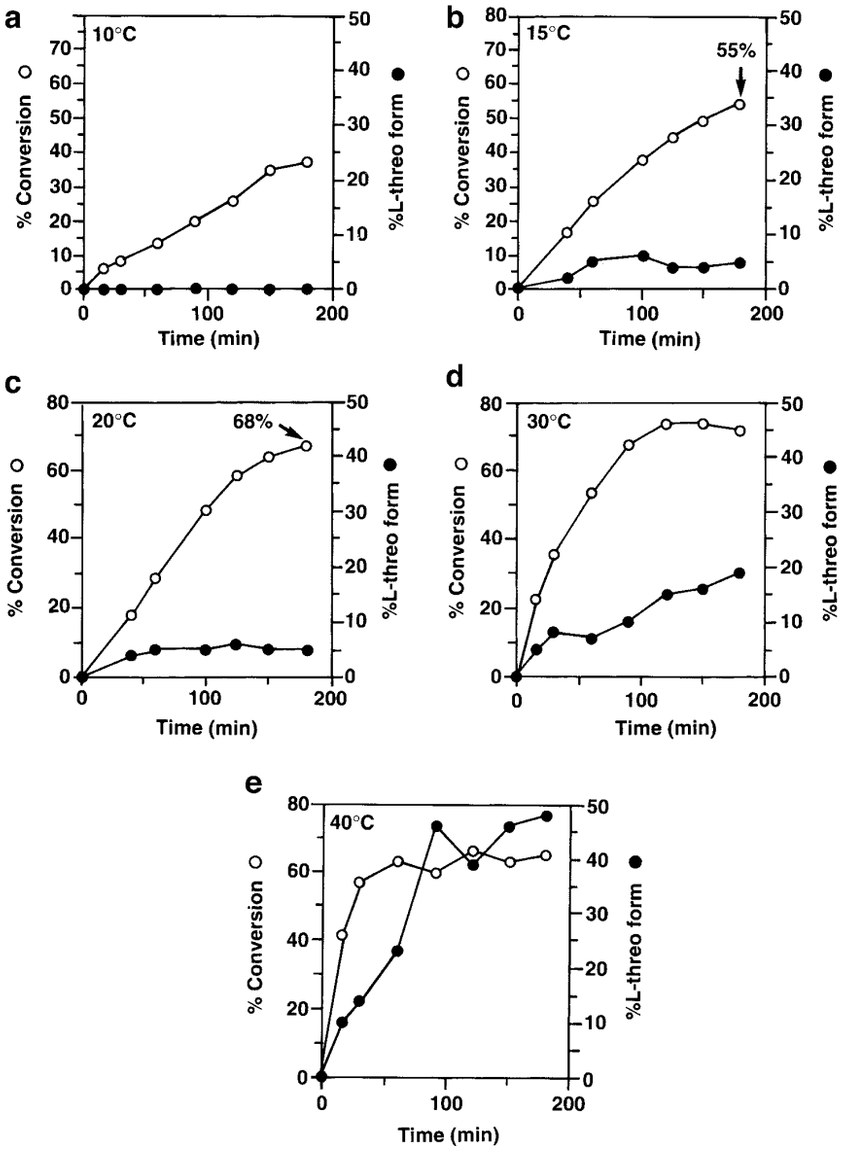

Figure 7 Whole-cell conversion with pentenal: temperature effect. The reaction mixture (10 ml) contained 200 mM glycine, 50 mM pentenal, in 50 mM potassium phosphate, pH 7.0, 0.5 g (wet weight) of *E. coli* cells and 200 μM PLP. The whole-cell conversion was carried out for 180 min at the temperatures indicated in the figure. The % conversion is the sum of l-allo and l-threo forms of AHHA as detected by DABSYL derivatization. l-Threo is the undesirable form. A high % conversion and a low % l-threo form were observed at 15 and 20°C. Since, under the reaction conditions, l-allo and l-threo forms were the two primary amine products, DABSYL (rather than an OPA-NAC) method [1] was used in quantifying the two products.

SSAME. SSAPE and SSA were poor co-substrates. SSA was a poor inhibitor for SHMT-catalyzed condensation of SSAME with glycine (not shown). As 'non-lactonization' aldehyde co-substrates, pentenal and FPA were also good aldehyde co-substrates for the enzyme. As described above, the SHMT-catalyzed condensation of pentenal and glycine to l-allo-AHHA at low temperature (15°C) was effectively scaled up.

Conclusion

Starting from a crude extract of recombinant *E. coli* providing an ultra-high expression of SHMT, we can purify the enzyme to ~95% by a single anion-exchange chromatography. *E. coli* SHMT, with SSAME as aldehyde co-substrate, is catalytically superior to the rabbit liver enzyme. The low stoichiometry of PLP to the dimeric enzyme suggests loose cofactor binding and the requirement for exogenous cofactor for maximal activity was substantiated. *E. coli* SHMT-catalyzed condensation of SSAEE and glycine to l-allo-aAbHAA, involving a lactonization step, exhibits a very high conversion efficiency and product diastereospecificity at a high temperature. In contrast, the enzymatic condensation of pentenal, as a non-lactonization aldehyde co-substrate, with glycine to l-allo-AHHA shows a very high conversion efficiency and product diastereospecificity only at a low temperature. Using either SSAEE or pentenal, an effective biocatalytic process with *E. coli* SHMT or whole cells has been developed at a large scale. With a broad substrate specificity, particularly toward aldehyde co-sub-

Table 5 Substrate specificity of *E. coli* SHMT[a]

Co-substrate[b]	k_{cat}[c]	K_m[d]	Diastereospecificity[e]
SSAME	57.1	8.3	98.2
SSAEE	88.7	7	98
SSATBE	65.5	7.6	96.9
SSAPE	1.6	–[f]	88
SSA	3.3	–	94.5
PA	111.5	9.5	98–100
FPA	116.5	4.5	95.7
FBA	51	18	73.3–82.6
Glycine	59.5	20	98
Glycine methyl ester	2	–	86.9
Glycine amide	0	–	–

[a]100 mM glycine or 30 mM SSAME, 4.25 μM SHMT subunit, 61°C, 40 μM PLP, and 50 mM KPi, pH 8.0.
[b]SSa, succinic semialdehyde; SSAEE, succinic semialdehyde ethyl ester; SSATB, succinic semialdehyde τ-butyl ester; SSAPE, succinic semialdehyde phenyl ester; PA, pentene aldehyde (pentenal); FPA, 3,2-furanyl-1 propene aldehyde; and FBA, 4-formyl butaldehyde.
[c]μmol l-*allo*-aAbHAA min^{-1} μmol^{-1} SHMT subunit.
[d]mM.
[e]% μmol l-*allo*-aAbHAA μmol^{-1} total aAbHAA.
[f]Not applicable.

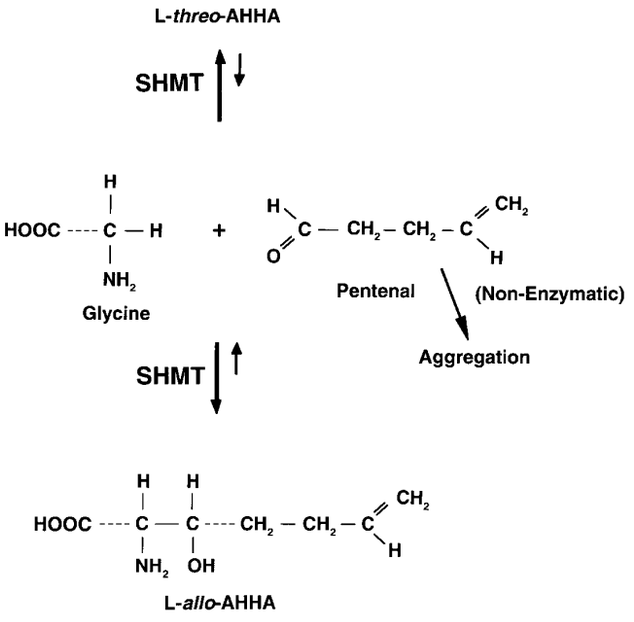

Figure 8 Proposed reaction pathway of *E. coli* SHMT with pentenal as aldehyde co-substrate. A bold/long arrow indicates a high conversion and a light/short arrow a low conversion.

strates, *E. coli* SHMT has shown utility in its synthesis of a diastereospecific intermediate in the manufacture of an important carbacephem antibiotic.

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References

1 Aswad DW. 1984. Determination of d- and l-aspartate in amino acid mixtures by high-performance liquid chromatography after derivatization with a chiral adduct of *o*-phthaldehyde. Anal Biochem 137: 405–409.
2 Ausubel FM (ed). 1989. Current Protocols in Molecular Biology, pp 40–360, Wiley-Interscience, New York, NY.
3 Beckman Instruments. 1988. Beckman Dabsylation Kit Instruction Manual, pp 1–22, Fullerton, CA.
4 Cai K, D Schirch and V Schirch. 1995. The affinity of pyridoxal 5′-

phosphate for folding intermediates of *Escherichia coli* serine hydroxymethyltransferase. J Biol Chem 270: 19294–19299.
5 Hsiao HY and T Wei. 1986. Enzymatic production of l-serine with a feedback control system for formaldehyde addition. Biotech Bioeng 28: 1510–1518.
6 Jackson BG, CW Doecke, E Farkas, JW Fisher, JP Gardner, RJ Gazak, EP Kroeff, JA Misner, SW Pederson, MA Stazak, KL Trinkle and JT Vicenzi. 1994. Proceedings of Chiral '94 USA Symposium, p 61, Reston, VA.
7 Maniatis T, EF Fritch and J Sambrook (eds). 1982. Molecular Cloning: A Laboratory Manual, pp 50–250, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
8 Miller M. 1991. Process for beta-hydroxy-alpha-amino acids. European Patent Application No. 91304977.1.
9 Plamann M and G Stauffer. 1983. Characterization of the *Escherichia coli* gene for serine hydroxymethyltransferase. Gene 22: 9–18.
10 Sall DE and RE Kaiser JR. 1993. Characterization of the interaction between human α-thrombin and methyl 3- (2-methyl-oxopropoxy)-[1]benzothieno[3,2-b]furan-2-carboxylate (LY806303) using electrospray mass spectrometry and tandem mass spectrometry. J Med Chem 36: 2350–2355.
11 Schirch L. 1982. Serine hydroxymethyltransferase. In: Advances in Enzymology (Meister A, ed), vol 53, pp 83–112, Wiley-Interscience, New York, NY.
12 Schirch L. 1984. Folates in serine and glycine metabolism. In: Folates and Pterins: Chemistry and Biochemistry of Folates (Blakley RL and SJ Benkovic, eds), pp 399–431, Wiley-Interscience, New York, NY.
13 Schirch V, S Hopkins, E Villar and S Angelaccio. 1985. Serine hydroxymethyltransferase from *Escherichia coli*: purification and properties. J Bacteriol 163: 1–7.
14 Schirch L and WT Jenkins. 1964. Serine transhydroxymethylase: transamination of d-alanine. J Biol Chem 239: 3797–3800.
15 Strege M, D Schmidt, AJ Kreuzman, JE Dotzlaf, WK Yeh and A Lagu. 1994. Capillary electrophoretic analysis of serine hydroxymethyltransferase in *E. coli* fermentation broth. Anal Biochem 223: 198–204.