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# Important roles of hydroxylic amino acid residues in the function of *Bacillus subtilis* adenylosuccinate lyase

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## Abstract

Thr<sup>93</sup>, Ser<sup>94</sup>, Thr<sup>140</sup>, and Ser<sup>306</sup> are conserved in all adenylosuccinate lyases (ASL) and are close to other amino acids previously identified by mutagenesis as being in the active site. To test their involvement in the enzyme's function, each of these amino acids was replaced by alanine. All the mutants exhibit circular dichroism spectra which are similar to that of wild-type enzyme, indicating there is no appreciable change in secondary structure. T93A exhibits 0.5% of the  $V_{\max}$  of wild-type ASL with a 10-fold increase in  $K_m$  for adenylosuccinate. S94A has 65% of the  $V_{\max}$  of wild-type ASL with little change in  $K_m$ . T140A exhibits 0.03% of the activity of wild-type enzyme with an 11-fold increase in  $K_m$ . S306A has 0.4% of the  $V_{\max}$  of wild-type ASL with a sevenfold increase in  $K_m$ . Measurements of the pH- $V_{\max}$  profile reveal a  $pK_2$  value for S94A of 7.83 and S306A of 7.65, in contrast to 8.24 for the wild-type enzyme and 8.42 for T93A. Thr<sup>93</sup> may orient adenylosuccinate optimally for catalysis, while Ser<sup>94</sup> stabilizes protonated His<sup>89</sup>, a determinant of  $pK_2$ . Thr<sup>140</sup> may, through hydrogen bonding, interact with Asn<sup>270</sup>, an amino acid essential for catalysis. Ser<sup>306</sup> may be involved in a hydrogen bond network that ultimately stabilizes protonated His<sup>68</sup>, which is probably the general acid in the reaction of enzyme with substrate. The results of this paper demonstrate the importance in the catalytic function of ASL of hydrogen bonds and hydrogen bonding networks involving serine and threonine.

**Keywords:** adenylosuccinate lyase; purine biosynthesis; site-directed mutagenesis; hydrogen bonding

Adenylosuccinate lyase (ASL) catalyzes the conversion of adenylosuccinate (SAMP) to AMP and fumarate, a key step in purine biosynthesis (Ratner 1972). Point mutations at various positions within the human form of this enzyme result in the disorder Adenylosuccinate Lyase Deficiency, which is characterized by psychomotor retardation, autistic features, epilepsy, and muscle wasting (Jaeken and Van den Berghe 1984; Jaeken et al. 1988; Marie et al. 1999; Race et al. 2000; Van den Berghe and Jaeken 2001; Spiegel et al. 2006).

The crystal structure of *Thermatoga maritima* ASL indicates that the enzyme is a highly helical homotetramer,

with each of its four active sites composed of different portions of three subunits (Toth and Yeates 2000). Biochemical evidence in support of the multisubunit active site has been provided by experiments in which two inactive variants of *Bacillus subtilis* ASL, with mutations in regions contributed to an active site by different subunits, functionally complement each other with substantial recovery of enzymatic activity (Lee et al. 1999; Brosius and Colman 2000, 2002; Segall and Colman 2004). Although a high-resolution structure has not been determined for *B. subtilis* ASL, a homology model has been constructed using the structure of *T. maritima* ASL as a template (Brosius and Colman 2002; Segall and Colman 2004). Since the ASLs from these two bacteria share 49% identity plus an additional 23% strong similarity in amino acid sequence, this homology model should provide an excellent indication of the structure of *B. subtilis* ASL.

Affinity labeling and mutagenesis studies of *B. subtilis* ASL have implicated His<sup>141</sup> and His<sup>68</sup> as the general

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Abbreviations: ASL, adenylosuccinate lyase; SAMP, adenylosuccinate; *B. subtilis*, *Bacillus subtilis*; *T. maritima*, *Thermatoga maritima*.

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base/acid in the catalytic reaction (Lee et al. 1997, 1998, 1999). Mutagenesis studies have revealed additional amino acid residues, including His<sup>89</sup>, Lys<sup>268</sup>, and Asn<sup>270</sup>, that assist in the catalytic reaction, most likely by contributing to substrate binding and orientation in the active site (Brosius and Colman 2000, 2002; Segall and Colman 2004). It appears that a network of amino acid residues is required to coordinate the multiple negatively charged groups on SAMP through electrostatic and hydrogen bonding interactions, and that each of these residues is indispensable for high catalytic activity.

The importance of hydrogen bonding networks in enzymes has been well documented in terms of maintaining active site integrity and optimal substrate orientation for catalysis (Radisky et al. 2005; Jao et al. 2006; Zimmerman and Ferry 2006). Thr<sup>93</sup>, Ser<sup>94</sup>, Thr<sup>140</sup>, and Ser<sup>306</sup> are conserved in all ASLs, as illustrated in Figure 1 for representative species from bacteria (e.g., *B. subtilis* and *T. maritima*) to humans. Among these, Thr<sup>93</sup>, Ser<sup>94</sup>, and Thr<sup>140</sup> are located close to other amino acids that have previously been identified as within the active site, including His<sup>68</sup>, His<sup>89</sup>, His<sup>141</sup>, and Asn<sup>270</sup> (Figs. 5, 6, see below). Ser<sup>306</sup> is further from the active site but is positioned to hydrogen bond to Arg<sup>310</sup>, a residue recently shown to influence the catalytic rate, probably through a chain of electrostatic interactions involving His<sup>68</sup> (Sivendran et al. 2005). Thus, these four amino acids were selected as targets for site-directed mutagenesis. Alanine was substituted for each of these four hydroxylic amino acids, one at a time, to determine whether their –OH functionalities were involved in the ASL-catalyzed conversion of SAMP to AMP and fumarate. A preliminary version of some of this work has been presented (Cashman et al. 2005).

## Results

### *Activity and purity of wild-type and variant adenylosuccinate lyases*

The mutant ASLs (T93A, S94A, T140A, and S306A) were all expressed well. Each yielded a single protein band on polyacrylamide gel electrophoresis in the presence of SDS, with a migration consistent with the known subunit molecular mass (~50 kDa). Each ASL sample gave a single N-terminal sequence on Edman degradation

for the first 15 amino acids, confirming the purity of these ASL preparations.

All four mutant enzymes retained sufficient catalytic activity to determine steady-state kinetic parameters at pH 7.0, as shown in Table 1. S94A ASL exhibited values for  $K_m$  and  $V_{max}$  that were very close to those observed for the wild-type enzyme. In contrast, the replacement of Thr<sup>93</sup> by Ala resulted in a 200-fold decrease in  $V_{max}$  and a 10-fold increase in  $K_m$ , while the mutation at S306 caused a 250-fold decrease of  $V_{max}$  and sevenfold increase of  $K_m$ . However, replacement of T140 had the most profound effect on catalysis, causing a 3600-fold decrease of  $V_{max}$  and 11-fold increase of  $K_m$ .

### *pH- $V_{max}$ profiles of wild-type and mutant ASLs*

The pH dependence of  $V_{max}$  for wild-type ASL is bell-shaped (Palenchar et al. 2003), indicating that  $V_{max}$  is influenced by two ionization events. The pH- $V_{max}$  profiles of the T93A, S94A, and S306A mutant enzymes are also bell-shaped. Figure 2 shows a representative graph of  $V_{max}$  versus pH for the S94A mutant. Table 2 summarizes the values of  $pK_1$  and  $pK_2$  for wild-type and the three mutant enzymes. The pH- $V_{max}$  profile for T93A ASL is minimally perturbed, suggesting that this mutation does not influence the local environment of the amino acid residues that determine the effects of pH on enzyme activity. In contrast, the S94A mutant displays a marked decrease in  $pK_2$ , as compared to wild-type ASL (Table 2). A large decrease in  $pK_2$  is also apparent in the pH- $V_{max}$  profile for the S306A mutant. (Because of the very low specific activity of the T140A enzyme, the pH dependence of  $V_{max}$  could not be obtained.)

### *Circular dichroism of wild-type and mutant enzymes*

To determine whether the global secondary structure of the enzyme was affected by the mutations to Ala at positions 93, 94, 140, and 306, far-UV circular dichroism (CD) spectra were obtained for wild-type ASL and each of these mutants. As shown in Figure 3, the spectra from the wild-type and mutant enzymes exhibited pronounced minima at both 208 and 222 nm, indicating a predominance of  $\alpha$ -helical structure, as earlier studies of ASL have shown (Palenchar et al. 2003; Segall and

<i>B. subtilis</i>	<sup>89</sup> HYGL <b>TS</b> TDVVD <b>TAL</b> <sup>102</sup>	<sup>138</sup> GRTHGVHAEPTTFGLK <sup>153</sup>	<sup>300</sup> WHERDISHSSAER <sup>310</sup>
<i>T. maritima</i>	HYGL <b>TS</b> SDVLD <b>TAN</b>	GRTHGVHAEPT <b>S</b> FGLK	WHERDISH <b>S</b> SVER
<i>H. sapiens</i>	HYGAT <b>S</b> CYVGD <b>NTD</b>	GF <b>TH</b> FQPAQLTT <b>VG</b> KR	WFERTLDD <b>S</b> ANRR
<i>M. musculus</i>	HYGAT <b>S</b> CYVGD <b>NTD</b>	GF <b>TH</b> FQPAQLTT <b>VG</b> KR	WFERTLDD <b>S</b> ANRR
<i>G. gallus</i>	HYGAT <b>S</b> CYVGD <b>NTD</b>	GF <b>TH</b> YQPAQLTT <b>VG</b> KR	WFERTLDD <b>S</b> ANRR

**Figure 1.** Amino acid sequence alignment of ASLs from two bacterial species, as well as from human, mouse, and chicken in the region of Thr<sup>93</sup>, Ser<sup>94</sup>, Thr<sup>140</sup>, and Ser<sup>306</sup> (shown in bold). The amino acid numbers are those of the *B. subtilis* ASL.

**Table 1.** Kinetic parameters for wild-type and mutant enzymes

Enzyme	$V_{\max} \pm \text{S.E.}$ ( $\mu\text{mol min}^{-1} \text{mg}^{-1}$ )	$K_m \pm \text{S.E.}$ ( $\mu\text{M}$ )	$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	$k_{\text{cat}}/K_m$ ( $\text{M}^{-1} \text{s}^{-1}$ )
Wild type	$2.08 \pm 0.06$	$1.77 \pm 0.26$	1.74	$9.83 \times 10^5$
T93A	$0.012 \pm 0.0008$	$17.7 \pm 4.3$	0.010	$0.00565 \times 10^5$
S94A	$1.36 \pm 0.03$	$2.24 \pm 0.35$	1.14	$5.09 \times 10^5$
T140A	$0.00057 \pm 0.00005$	$19.2 \pm 5.5$	0.00048	$0.00025 \times 10^5$
S306A	$0.00822 \pm 0.00031$	$12.8 \pm 2.2$	0.00689	$0.00538 \times 10^5$

The conversion of adenylosuccinate to AMP and fumarate was measured at 25°C in HEPES buffer, pH 7.0. The  $K_m$  and  $V_{\max}$ , along with the standard errors, were obtained by fitting to the Michaelis-Menten equation using Sigma Plot software.

Colman 2004). The CD spectra of T140A and S306A are similar to that of wild-type enzyme (Fig. 3). CD spectra of T93A and S94A are superimposable on that of wild-type enzyme (data not shown). We conclude that the mutations at positions 93, 94, 140, and 306 did not have an appreciable effect on the secondary structure of the enzyme.

#### Molecular masses of wild-type and mutant ASLs

ASL is a tetramer of identical subunits (Palenchar and Colman 2003). Although wild-type ASL has been shown to exist as an equilibrium mixture of dimeric ( $\sim 100,000$  Da) and tetrameric ( $\sim 200,000$  Da) forms, only the tetrameric form is catalytically functional (Palenchar and Colman 2003). In the present study, native polyacrylamide gel electrophoresis and analytical ultracentrifugation were used to evaluate whether the mutations affected the oligomeric state of the enzyme at pH 7.0. The results are shown in Table 3. All the enzymes exhibit molecular weights of about 200 kDa, showing that the wild-type and mutant enzymes exist predominantly in a tetrameric state. Thus, no major changes in the oligomeric state of ASL resulted from the four amino acid replacements made in this study.

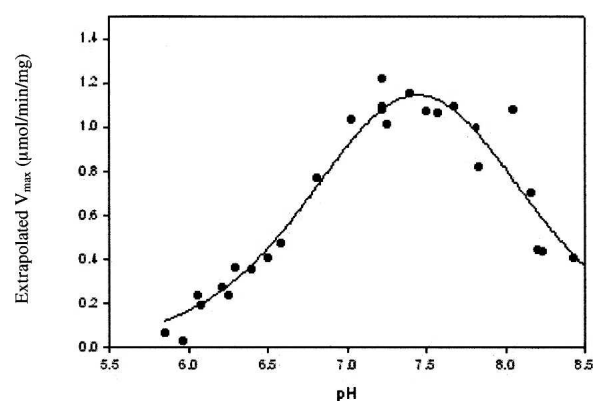
#### Intersubunit complementation

Although ASL contains both four identical subunits and four active sites, each of these active sites is formed from contributions of different regions from three subunits (Brosius and Colman 2002). As a result of this structural arrangement, we have previously shown that two different ASL mutant enzymes, in which each harbors a mutation in one of these separate regions, can complement each other forming some functionally intact active sites in a process referred to as intersubunit complementation (Lee et al. 1999; Brosius and Colman 2000; Segall and Colman 2004). We have also demonstrated that mixtures of mutants with changes at amino acids provided to the active site by the same subunit do not show an increase in

activity (Segall and Colman 2004). For a tetramer with random dissociation and random reassociation of subunits, recovery of activity up to 25% that of wild-type enzyme is expected (Lee et al. 1999). Representative results of the complementation experiments are illustrated in Figure 4, which shows the time-dependent reactivation of the inactive T140A and S306A mutant enzymes when paired with variant enzymes whose point mutations come from different subunits.

Table 4 summarizes the maximum regain of activity of the inactive T93A, T140A, and S306A when mixed with each of three other mutant enzymes. (Because of the high specific activity of the S94A mutant enzyme under standard assay conditions, intersubunit complementation studies of this type could not be conducted for this mutant.) When the T93A mutant was combined with K268A ASL (a completely inactive mutant enzyme described in an earlier study; Brosius and Colman 2002), the observed ASL activity reached 10% of that of the wild-type enzyme, while T93A plus completely inactive H141Q mutant (characterized previously; Lee et al. 1999) yielded 3% of wild-type activity. In contrast, no activity was gained when T93A was paired with H68Q, indicating that Thr<sup>93</sup> and His<sup>68</sup> are contributed by the same subunit to the active site. (H68Q alone has a specific activity of only  $0.016 \mu\text{mol min}^{-1} \text{mg}^{-1}$ ; Lee et al. 1998.)

T140A achieved maximum activity that is 15.4% that of the wild-type enzyme when paired with K268Q, and 11.5% when incubated with H68Q, but gained no activity when paired with H141Q. S306A when combined with K268Q reached 15.4% of the activity of wild-type enzyme, and 4.6% of the wild-type enzyme activity when paired with H141Q. In contrast, no reactivation was observed when S306A and H68Q were mixed. These results demonstrate that Thr<sup>140</sup> and His<sup>141</sup> are contributed to the active site by the same subunit, and that Ser<sup>306</sup> and His<sup>68</sup> come from the same subunit.

**Figure 2.** pH dependence of  $V_{\max}$  for the S94A mutant ASL.

**Table 2.** *pK* values determined from *pH-V<sub>max</sub>* profiles of wild-type and mutant ASLs

Enzyme	<i>pK</i> <sub>1</sub> ± S.E.	<i>pK</i> <sub>2</sub> ± S.E.
Wild type	6.67 ± 0.07	8.24 ± 0.09
T93A	7.02 ± 0.16	8.42 ± 0.14
S94A	7.05 ± 0.11	7.85 ± 0.11
S306A	7.18 ± 0.14	7.65 ± 0.15

## Discussion

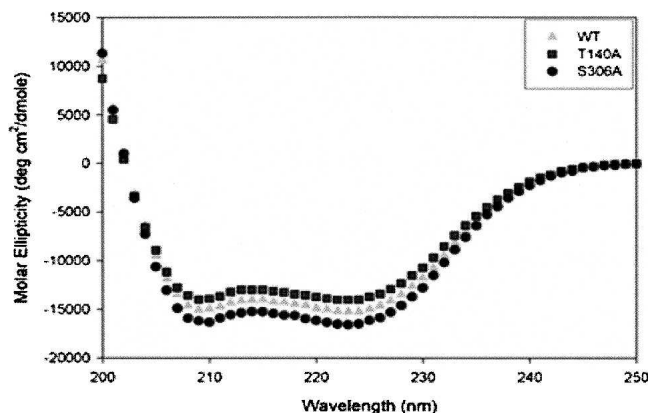
The intersubunit region of ASL previously identified as the active site (Lee et al. 1999; Toth and Yeates 2000; Brosius and Colman 2002; Segall and Colman 2004) also contains serine and threonine residues that are conserved in ASLs of many species. The present study sought to examine, by site-directed mutagenesis, the roles of the hydroxyl groups of these conserved serines and threonines. Alanine was substituted for each of the amino acids presently studied, since this replacement results in the elimination of a side-chain that can participate in hydrogen bonding without appreciably changing residue volume. In fact, each of these four mutations has minimal effect on the secondary or quaternary structure of ASL as indicated by their circular dichroism spectra and molecular weights.

As shown in Figure 5, the hydroxyl group of Thr<sup>93</sup> is positioned within hydrogen bonding distance (2.6 Å) of one of the carboxylate oxygens on the succinyl portion of adenylosuccinate. The large decrease in *V<sub>max</sub>* and increase in *K<sub>m</sub>* for the T93A variant, along with the absence of a significant effect on the *pH*-rate profile for this mutant, are consistent with a role for this threonine in orienting adenylosuccinate within the active site. Since the T93A mutant enzyme regains activity by complementation, the global structure is largely unchanged and the loss of activity is strictly due to the point mutation at position 93. Other amino acid residues have also been shown to contribute to the positioning of SAMP within the active site by hydrogen bonding or electrostatic interactions: Asn<sup>270</sup>, Gln<sup>212</sup>, and Arg<sup>301</sup> (Segall and Colman 2004). The succinyl moiety of SAMP is the location of the chemical transformation. Therefore, precise orientation is necessary for the enzymic general acid and base to function properly.

Ser<sup>94</sup> does not have a major influence on the catalytic function of the enzyme at *pH* 7.0, but it helps to establish the *pH* range over which the enzyme is active, since replacement of this residue with alanine (in the S94A enzyme) lowers the *pK*<sub>2</sub> for the enzyme. Evidence has previously been presented that the alkaline side of the *pH-V<sub>max</sub>* curve is due to the deprotonation of histidine, based on the temperature dependence of *pK* (Brosius and Colman 2000). The *pK*<sub>2</sub> value appears to reflect the deprotonation

of both His<sup>68</sup> and His<sup>89</sup> (Lee et al. 1999; Brosius and Colman 2000). Figure 5 shows that the hydroxyl group on the side-chain of Ser<sup>94</sup> is nearly close enough to the side-chain δN of His<sup>89</sup> to hydrogen bond to this residue. The X-ray crystal structure of the enzyme from *T. maritima* includes a water molecule that is well positioned to mediate interaction between the corresponding Ser and His in this enzyme, and presumably also in the *B. subtilis* ASL. Typically the *pK* of the imidazole of a protein histidine is 6–6.5 (Tipton and Dixon 1979); however, a water-mediated interaction between Ser<sup>94</sup> and His<sup>89</sup> could raise the *pK* of the histidine by favoring the protonated form of the imidazole. It has been proposed that the protonated His<sup>89</sup> contributes to the catalytic function of ASL by coordination of a ribose hydroxyl of SAMP (Brosius and Colman 2000, 2002). In the H94A mutant, the absence of a hydroxyl group at position 94 would disrupt the hydrogen bonding network. This structural model (Fig. 5) can thus explain the decrease in *pK*<sub>2</sub> observed for the S94A variant.

The importance of Thr<sup>140</sup> for the catalytic function of ASL is indicated by the 3600-fold decrease in *V<sub>max</sub>* along with an 11-fold increase in *K<sub>m</sub>* for adenylosuccinate in the T140A mutant. Thr<sup>140</sup> is located immediately adjacent in sequence to His<sup>141</sup> (the histidine that has been postulated to be the general base in ASL catalysis) and it is conserved in at least 46 species. The structural model shown in Figure 6 suggests that Thr<sup>140</sup> functions indirectly in catalysis by maintaining the correct orientation of amino acid residues that interact directly with the substrate SAMP. The hydroxyl group of Thr<sup>140</sup> is only 3.12 Å from the side-chain carbonyl oxygen of N270, which is close to hydrogen bonding distance of one of the carboxyls of the substrate, SAMP. The backbone carbonyl oxygen of Thr<sup>140</sup> is only 2.88 Å from the side-chain carbonyl oxygen of Asn<sup>270</sup>, also sufficiently close for hydrogen bonding. A recent report suggested that Asn<sup>270</sup> functions in a critical role in ASL catalysis through

**Figure 3.** CD spectra of wild-type, T140A, and S306A enzymes.

**Table 3.** Molecular weight of wild-type and mutant ASLs at pH 7.0

Enzyme	Molecular weight (kDa) $\pm$ S.D.
Wild type	196 $\pm$ 13 <sup>a</sup> 199.7 $\pm$ 0.3 <sup>b</sup>
T93A	194 $\pm$ 1 <sup>a</sup>
S94A	193 $\pm$ 20 <sup>a</sup>
T140A	194.8 $\pm$ 0.3 <sup>b</sup>
S306A	216.4 $\pm$ 0.4 <sup>b</sup>

<sup>a</sup>Determined by native polyacrylamide gel electrophoresis.

<sup>b</sup>Determined by analytical ultracentrifugation at pH 7.0 and 25°, using 0.4 mg/mL enzyme.

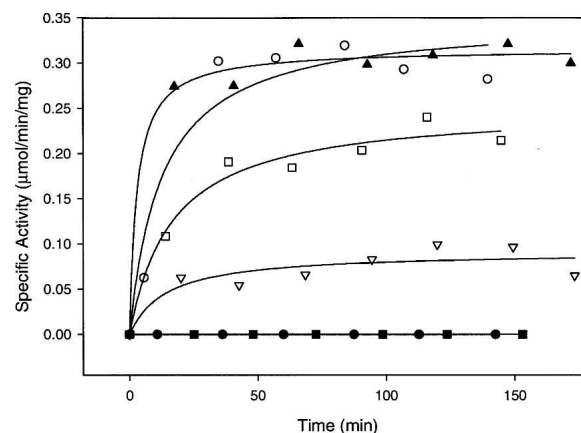
binding to one of the carboxylate oxygens on the succinyl moiety of SAMP (Segall and Colman 2004). Therefore, the importance of maintaining Asn<sup>270</sup> in a catalytically productive orientation provides a reasonable explanation for the severe decrease in  $V_{\max}$  and modest increase in  $K_m$  at pH 7.0 for the T140A mutant. (Additional amino acids in the region of Thr<sup>93</sup>, Ser<sup>94</sup>, and Thr<sup>140</sup> are indicated by the amino acid sequences shown in Fig. 1.)

Ser<sup>306</sup> also makes a contribution to the function of ASL since the S306A mutant enzyme has a  $V_{\max}$  that is decreased 250-fold and a  $K_m$  that is increased sevenfold, as compared to wild-type enzyme. Furthermore, the S306A enzyme exhibits a markedly decreased  $pK_2$ . While Thr<sup>140</sup> likely mediates ASL catalysis through one intervening amino acid residue, Ser<sup>306</sup>, which is completely conserved in at least 46 species, probably affects the catalytic reaction through a chain of three other amino acids: Arg<sup>310</sup>, Asp<sup>69</sup>, and His<sup>68</sup>. There is evidence that Asp<sup>69</sup> interacts electrostatically with His<sup>68</sup>, stabilizing the functionally important protonated state of this histidine (Sivendran et al. 2005). The positively charged guanidinium group of Arg<sup>310</sup>, in turn, is sufficiently close to the negatively charged carboxylate of Asp<sup>69</sup> to promote the optimal orientation of this residue by electrostatic interaction. These interactions are illustrated in the ASL structure shown in Figure 7. The hydroxyl group of Ser<sup>306</sup> is only 3.1 Å from a N of the guanidinium group of Arg<sup>310</sup>, allowing for hydrogen bonding between these two amino acids, and there is a comparable distance between the backbone oxygen of Ser<sup>306</sup> and the backbone nitrogen of Arg<sup>310</sup>. Thus, Ser<sup>306</sup> may constitute an additional amino acid in this functional chain of amino acid residues extending to His<sup>68</sup>. The characteristics of the S306A mutant enzymes suggest a role for this serine in the optimal positioning of Arg<sup>310</sup> and imply that perturbation of this orientation has a similar effect on catalysis as loss of the positive charge at position 310, as in R310Q, the drastic consequences of which have recently been reported (Sivendran et al. 2005).

Each of the mutant enzymes also exhibits a small increase in the  $pK_1$  value of its pH- $V_{\max}$  profile as compared to wild-type ASL. Although the identity of the functional group responsible for this ionization remains uncertain, a previous study postulated that  $pK_1$  reflects the deprotonation of carboxylic acid groups on the succinyl portion of enzyme-bound SAMP. We thus interpret the increases observed in this value in terms of changes in the active site orientation of SAMP for each of the mutants; small differences in the environment of these carboxylic acid groups may cause a shift in their  $pK$  values.

#### Intersubunit complementation

Since each of the four active sites of the ASL homotrimer is composed of different regions of three identical subunits, pairs of ASL variants with amino acid substitutions at sites contributed to an active site by different subunits can functionally complement one another when mixed at equal concentrations (Lee et al. 1999; Brosius and Colman 2002; Segall and Colman 2004). As no activation occurs when T93A is paired with H68Q, His<sup>68</sup> and Thr<sup>93</sup> are contributed to the active site by the same subunit. The results of the present study also demonstrate that Ser<sup>306</sup> is contributed to an intersubunit active site of ASL by the same subunit that provides His<sup>68</sup>. Thr<sup>140</sup> and His<sup>141</sup> originate from a second, separate subunit and Lys<sup>268</sup> is contributed by a third. In addition, the abilities of the variant enzymes in the present study to complement ASL mutants bearing substitutions at different sites indicate that the mutations do not substantially affect the capabilities of the enzymes to form functional intersubunit contacts, and confirm the evidence that shows no major structural perturbations as a result of the amino acid substitutions.



**Figure 4.** Complementation studies by reactivation of inactive mutant enzymes. The following mutant enzymes were mixed: T140A + K268Q (open circles); S306A + K268Q (closed upright triangles); T140A + H68Q (open squares); S306A + H141Q (upside-down open triangles); T140A + H141Q (closed circles); and S306A + H68Q (closed squares).

**Table 4.** Complementation of inactive mutant ASLs

Enzyme pairs	Maximum specific activity ( $\mu\text{mol min}^{-1} \text{mg}^{-1}$ )	% wild-type activity reached <sup>a</sup>
T93A + K268Q	0.21	10.1
T93A + H141Q	0.068	3.0
T93A + H68Q	0	0
T140A + K268Q	0.32	15.4
T140A + H141Q	0	0
T140A + H68Q	0.24	11.5
S306A + K268Q	0.32	15.4
S306A + H141Q	0.096	4.6
S306A + H68Q	0	0

<sup>a</sup>Specific activity for wild-type ASL is  $2.08 \mu\text{mol min}^{-1} \text{mg}^{-1}$ .

The maximum extent of reactivation varies considerably among the different pairs of mutant enzymes (from 3.0% to 15.5% of wild-type activity, as shown in Table 4). For a tetramer with random dissociation of subunits, and random reassociation of monomers, the maximum extent of reactivation is expected to be 25% (Lee et al. 1999). The lower-than-theoretical degree of reactivation indicates that these processes are not random. There are three types of subunit interface in the tetramer, which differ in the contact area between the subunits (Segall and Colman 2004). The variation in the extent of reactivation has been attributed to the differences in the particular subunit interface involved for each pair of mutants tested for complementation, the exact location of the mutated amino acid within the subunit interface, and whether subunit dissociation or reassociation is rate-limiting in the overall complementation process (Lee et al. 1999; Brosius and Colman 2000, 2002; Segall and Colman 2004).

In conclusion, Thr<sup>93</sup>, Ser<sup>94</sup>, Thr<sup>140</sup>, and Ser<sup>306</sup> perform very important roles in the function of ASL through hydrogen bonding interactions directly with adenylosuccinate, as well as indirectly through hydrogen bonds to active site amino acid residues. This is not surprising in light of earlier work that has demonstrated the remarkable sensitivity of ASL catalysis to the integrity of the network of amino acid residues in proximity to an active site (Lee et al. 1999; Brosius and Colman 2002; Segall and Colman 2004; Sivendran et al. 2005; Spiegel et al. 2006). The results of this study provide a clear example of the importance and variety of roles that the hydroxylic amino acid residues serine and threonine can perform in an enzyme and also emphasize the importance to catalysis by ASL of amino acid residues that interact with bound substrate through other residues, rather than directly.

## Materials and methods

### Materials

BioSynthesis Inc. provided the oligonucleotide primers used for site-directed mutagenesis and sequencing. Adenylosuccinate,

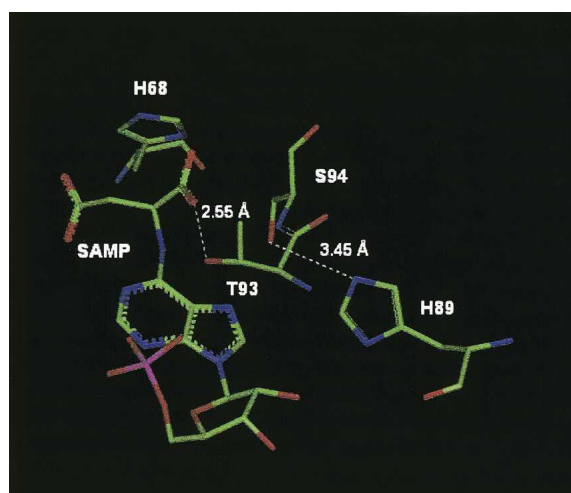
as well as imidazole, and HEPES were supplied by Sigma Chemical Co. Concentrated protein assay reagent was purchased from BioRad. All other chemicals were of reagent grade.

### Site-directed mutagenesis

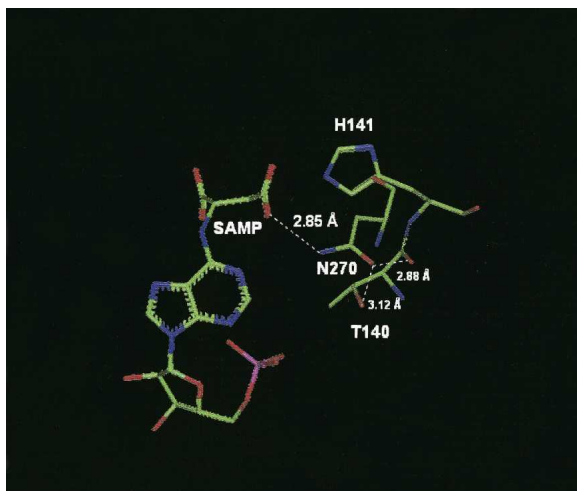
The pBHis plasmid containing *B. subtilis* ASL was provided to us as a generous gift from Dr. Jack E. Dixon (UCSD Sch. Med., La Jolla, CA). The Stratagene QuikChange Mutagenesis kit, along with the plasmid, was used to construct mutations to this gene. The oligonucleotide primers used to produce the mutants were the following (and their respective complementary sequences): CATTACGGCT TAGCGTCAACTGACGTTG (T93A), CGGCTTAACGGC GACTGACGTTGTTG (S94A), GATGGG GCGCGCA CACGGCGTAC (T140A), and GCGATATTTCTC ATGCTT CAGCAGAACG (S306A). DNA sequencing to ensure the presence of the engineered mutations was conducted by the University of Delaware Center for Agricultural Biotechnology with an ABI Prism model 377 DNA sequencer (PE systems). The *B. subtilis* ASLs were expressed in *Escherichia coli* strain BL21(DE3) as His-tag fusion proteins, as described previously (Lee et al. 1997).

Wild-type and mutant enzymes were purified in a single step procedure using nickel affinity chromatography, as reported in an earlier study (Segall and Colman 2004; Lee et al. 1997); and protein purity was assessed electrophoretically using a 12% polyacrylamide gel in the presence of 0.1% sodium dodecyl sulfate. N-terminal amino acid sequencing in the gas phase, utilizing an Applied Biosystems Procise sequence analyzer, was also used to confirm protein purity. Except where indicated differently, absorbance at 280 nm ( $E^{1\%} = 10.6$ ) was used to determine protein concentration (Lee et al. 1997). Purified protein was stored at  $-80^\circ\text{C}$  in separate aliquots.

It should be noted that the mutant as well as wild-type *B. subtilis* ASLs studied here all have six additional histidines at the N terminus to facilitate the purification. Thus, each mutant differs from the wild type by only one amino acid. While the properties of the His-tagged and non-His-tagged



**Figure 5.** Structural model of *B. subtilis* ASL in the vicinity of Thr<sup>93</sup> and Ser<sup>94</sup>. The distance between the  $-\text{OH}$  of Thr<sup>93</sup> and a carboxyl oxygen of adenylosuccinate (SAMP) as well as that between the  $-\text{OH}$  of Ser<sup>94</sup> and the  $\delta\text{N}$  of His<sup>89</sup> are shown.



**Figure 6.** Structural model of *B. subtilis* ASL in the region of Thr<sup>140</sup>. The model shows distances between Thr<sup>140</sup>, Asn<sup>270</sup>, and the substrate adenylosuccinate (SAMP).

*B. subtilis* ASLs have not been compared explicitly, the corresponding forms of human ASL have been studied. The His-tagged and non-His-tagged human ASLs were shown to exhibit very similar values of  $V_{\max}$  and  $K_m$  for adenylosuccinate (Lee and Colman 2007).

#### Kinetics of wild-type and mutant adenylosuccinate lyase

Catalytic activity toward SAMP was measured by following the decrease in absorbance at 282 nm that accompanies the conversion of SAMP to AMP plus fumarate. (The difference in extinction coefficient between SAMP and AMP = 10,000  $M^{-1}cm^{-1}$ ; Tornheim and Lowenstein 1972.) For assays in the presence of SAMP concentrations >120  $\mu M$ , the decrease in absorbance at 290 nm ( $\Delta\epsilon = 4050 M^{-1}cm^{-1}$ ) was monitored instead. Assays under standard conditions were performed at 25°C and pH 7.0, using about 8  $\mu g/mL$  wild-type ASL (and higher concentrations of mutant enzymes) in the presence of 60  $\mu M$  SAMP and 50 mM HEPES buffer. Specific activity was defined as micromoles of SAMP reacted per minute per milligram of enzyme under these conditions.  $K_m$  and  $V_{\max}$  values toward SAMP were obtained for wild-type and mutant enzymes under standard assay conditions, except for the variation in the concentration of SAMP (between 2 and 300  $\mu M$ ), and the data were calculated via SigmaPlot using the Michaelis-Menten equation. All enzymes were preincubated at 25°C for at least 30 min prior to the assay.

#### pH- $V_{\max}$ profiles for wild-type and mutant ASL enzymes

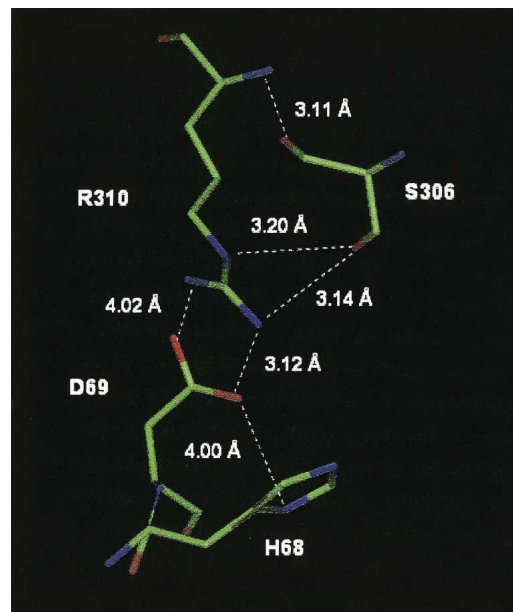
$V_{\max}$  determinations were performed for wild-type, T93A, S94A, and S306A enzymes as a function of pH using MES (pH 6.0–6.9), HEPES (pH 6.8–8.0), and TAPS (pH 7.9–9.0) buffers. (These experiments were not done for the T140A ASL because of its extremely low specific activity.) In all of these assays, the SAMP concentration was held constant at 300  $\mu M$ , which is high relative to the wild-type enzyme's  $K_m$  for adenylosuccinate (Brosius and Colman 2000), and the reaction

rates were monitored by following the change in absorbance at 290 nm. For the mutant enzymes,  $K_m$  values were determined at several of the pH conditions tested and used to extrapolate to  $V_{\max}$  using the Michaelis-Menten equation. The pH profile data were fit to the equation:  $V_{\max} = V_0 / (1 + 10^{(pK_1 - pH)} + 10^{(pH - pK_2)})$  where  $V_{\max}$  is the maximum velocity at a given pH,  $V_0$  is the intrinsic, pH-independent value of  $V_{\max}$ , and  $pK_1$  and  $pK_2$  are the pK values obtained for the left and right sides of the curve (Dixon and Webb 1964).

#### Circular dichroism of adenylosuccinate lyase mutants

Far-UV CD spectra were obtained for wild-type ASL and each of these mutants. For the T93A and S94A mutants, spectra were acquired (at 0.2-nm intervals) on a Jasco J710 spectropolarimeter and averaged over five scans. For the T140A and S306A enzymes, an Aviv Quick Start 215 Circular Dichroism Spectrometer was used and spectra (with points at 1-nm intervals) were averaged over sets of three acquisitions. Wild-type ASL was measured for comparison, using both instruments. Final spectra were expressed in terms of molar ellipticity values ( $[\theta]$ , deg  $cm^2/dmol$ ) as a function of wavelength, using the equation  $[\theta] = \theta/10nCl$ . In this expression,  $\theta$  is the experimentally determined ellipticity,  $n$  is the amount of residues in each enzyme subunit (437 including the His<sub>6</sub>-tag),  $C$  is the molar concentration of enzyme subunits, and  $l$  is the cuvette path-length (0.1 cm).

Protein samples at concentrations of 0.2–0.5 mg/mL were prepared in 20 mM potassium phosphate buffer, pH 7.0, containing 20 mM potassium chloride. Enzymes were preincubated for at least 30 min at 25°C before obtaining measurements. Scans of buffer alone were performed over the same wavelength range and used as a background correction. Protein concentrations were measured using an assay based on that of



**Figure 7.** Structural model of *B. subtilis* ASL in the region of Ser<sup>306</sup>. The chain of hydrogen bonding and electrostatic interactions between Ser<sup>306</sup>, Arg<sup>310</sup>, Asp<sup>69</sup>, and His<sup>68</sup> are shown.

Bradford, in which wild-type ASL at a known concentration served as the standard (Bradford 1976).

### Molecular mass determination of mutant enzymes

Molecular masses were obtained for wild-type and mutant ASLs using analytical ultracentrifugation or native polyacrylamide gel electrophoresis. Analytical ultracentrifugation was performed using a Beckman Optima XL-A analytical ultracentrifuge. The instrument was run at 8000 rpm, 10,000 rpm, and 14,000 rpm, using an An-60 Ti rotor for sedimentation equilibrium studies and UV absorbance was monitored at 280 nm. All measurements were obtained at 25°C with enzyme samples adjusted to 0.4 mg/mL in 20 mM potassium phosphate buffer with 20 mM potassium chloride, pH 7.0. Three determinations of molecular weight were made for each type of enzyme used.

For native polyacrylamide gel electrophoresis, a series of gels was run at pH 7.0, 25°C ranging from 5% to 10% (acrylamide); the ratio of acrylamide:bisacrylamide was maintained constant at 37.5:1, as previously described (Palenchar et al. 2003). Molecular weight standards consisted of ferritin (450 kDa) alcohol dehydrogenase (140 kDa), bovine serum albumin (67 kDa), and ovalbumin (45 kDa). Each protein sample (8  $\mu$ L at a concentration of 2 mg/mL) was applied to each type of gel. Prior to measurement, enzyme samples were preincubated at 25°C for 30 min.

### Intersubunit complementation of mutant enzymes

Equal-volume (100  $\mu$ L) mixtures of mutant enzymes in 0.1 M sodium phosphate buffer, pH 7.0, to give a final concentration of 0.7 mg/mL for each enzyme, were slowly frozen at  $-20^{\circ}$ C. Upon slowly thawing at room temperature, each sample was gently mixed and incubated at 25°C. Assays under standard conditions were then performed at various timepoints with the enzyme remaining at this temperature, until a maximum level of activity was reached. Previous studies have suggested that slow freezing of multisubunit proteins in sodium phosphate buffer results in a decrease in pH that promotes subunit dissociation (Chilson et al. 1965), and this approach has been used successfully with ASL (Segall and Colman 2004). Each individual enzyme used in the experiment was also separately assayed (as a control) under the same conditions and at the same total concentration of enzyme following at least 30 min of preincubation at 25°C.

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