Effect of Asp[69] and Arg[310] on the pK of His[68], a key catalytic residue of adenylosuccinate lyase

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Abstract

Adenylosuccinate lyase (ASL) of *Bacillus subtilis* contains three conserved histidines, His[68], His[89], and His[141], identified by affinity labeling and site-directed mutagenesis as critical to the intersubunit catalytic site. The pH-V_{max} profile for wild-type ASL is bell-shaped ($pK_1 = 6.74$ and $pK_2 = 8.28$). Only the alkaline side changes with temperature, characteristic of histidine p*K*s. To identify determinants of pK_2 in the enzyme-substrate complex, we replaced residues at two positions close to His[68] (but not to His[89] or His[141]) in the structure. Compared with the specific activity of 1.75 μmol adenylosuccinate reacting/min/mg of wild-type enzyme at pH 7.0, mutant enzymes D69E, D69N, R310Q, and R310K exhibit specific activities of 0.40, 0.04, 0.00083, and 0.10, respectively. While D69E has a K_m for adenylosuccinate similar to that of wild-type ASL, D69N and R310K exhibit modest increases in K_m, and R310Q has an 11-fold increase in K_m. The mutant enzymes show no significant change in molecular weight or secondary structure. The major change is in the pH-V_{max} profile: pK_2 is 8.48 for the D69E mutant and is decreased to 7.83 in D69N, suggesting a proximal negative charge is needed to maintain the high p*K* of 8.28 observed for wild-type enzyme and attributed to His[68]. Similarly, R310Q exhibits a decrease in its pK_2 (7.33), whereas R310K shows little change in pK_2 (8.24). These results suggest that Asp[69] interacts with His[68], that Arg[310] interacts with and orients the β-carboxylate of Asp[69], and that His[68] must be protonated for ASL to be active.

Keywords: adenylosuccinate lyase; pH-V_{max} profile; site-directed mutagenesis

Supplemental material: see www.proteinscience.org

Adenylosuccinate lyase (ASL) plays a critical role in both cellular replication and metabolism via its involvement in the de novo purine biosynthetic pathway, the end products of which serve as precursors for DNA and RNA synthesis, as intermediates in biosynthetic reactions, as energy storage depots, and as metabolic regulators (Ratner 1972). The importance of ASL is indicated by the existence of ASL deficiency, a human genetic disease resulting in autism, mental retardation, muscle wasting, and/or epilepsy (Jaeken and Van den Berghe 1984; Van den Berghe and Jaeken 2001).

ASL catalyzes the cleavage of adenylosuccinate (SAMP) to AMP and fumarate, the second step in the conversion of inosine monophosphate (IMP) to adenosine monophosphate (AMP) (Ratner 1972). Steady-state kinetics and inhibition studies suggest a uni–bi mechanism with a strong preference for product release in which fumarate leaves the enzyme before AMP (Bridger and Cohen 1968). It has been proposed that the cleavage proceeds by a β-elimination mechanism, involving the attack on the β-H of adenylosuccinate

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Abbreviations: ASL, adenylosuccinate lyase; SAMP, adenylosuccinate; AMP, adenosine monophosphate; AMPS, adenosine 5′-O-thiomonophosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; TAPS, *N*-tris-(hydroxymethyl)methyl-3-aminopropanesulfonic acid; CD, circular dichroism; *B. subtilis, Bacillus subtilis*; *T. maritima*, *Thermatoga maritima*

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by an amino acid of the enzyme functioning as the general base, and the protonation of either the N1 or N6 position of adenylosuccinate by an enzymic amino acid functioning as a general acid (Hanson and Havir 1972).

ASL of *Bacillus subtilis* is a homotetramer, with a molecular weight of ~200,000, composed of subunits with 431 amino acids. Studies on the *B. subtilis* ASL have shown that the enzyme has four active sites, with each active site formed by three subunits each contributing one or more amino acids (Brosius and Colman 2002). Previous affinity labeling and site-directed mutagenesis studies of the *B. subtilis* ASL enzyme have identified three histidines as critical residues of the intersubunit catalytic site: His^{68}, His^{141}, and His^{89} (Lee et al. 1997, 1998, 1999; Brosius and Colman 2000). It has been postulated that one of these histidines acts as the general base and one histidine functions as the general acid to protonate the AMP leaving group; His^{141} and His^{68} have been suggested to act as the general base–general acid (Lee et al. 1999).

Kinetic studies over the pH range 6–9 reveal a bell-shaped pH-V_{max} profile for the wild-type ASL enzyme with pK_1 and pK_2 values of about 6.7 and 8.3, respectively. These p*K*s presumably represent those of ionizable groups of the enzyme–substrate complex. Studies of the temperature dependence of the pH-V_{max} profile of wild-type ASL have shown that the pK_1 value does not change with temperature (excluding histidine as that ionizable group), while the pK_2 value decreases with increasing temperature, with a ΔH_i value of 9.9 *k*cal/mol, characteristic of histidines (Brosius and Colman 2000). We have recently shown that mutation of Ser^{94}, which is close to His^{89} but not to the other two important histidines, results in a decrease in pK_2 (Segall et al. 2007). To test whether the ionization of another amino acid is also reflected in the pK_2 of the wild-type pH-V_{max} profile, we now focus on Asp^{69}, which is 3.63 Å from His^{68}, 9.31 Å from His^{89}, and 10.74 Å from His^{141}, based on the structure of ASL (Toth and Yeates 2000; Brosius and Colman 2002; Segall and Colman 2004). Replacement of Asp^{69} would be expected to perturb the ionization of His^{68} but not of the other two histidines. Thus, if substitution for Asp^{69} results in a change in pK_2, His^{68} would be implicated as an ionizable group reflected in pK_2. This paper reports the results of replacing, by site-directed mutagenesis, amino acids close to or interacting with His^{68}. A preliminary version of this paper has been presented (Sivendran et al. 2005).

Results

Expression and purification of wild-type and mutant enzymes

His^{68} is one of the three conserved histidines that are critical for the intersubunit catalytic site (Lee et al. 1998, 1999). Because the negatively charged Asp^{69} is close to the critical His^{68} (but not to His^{89} or His^{141}) in the crystal structure of ASL, mutations were made at position 69. Since the positively charged Arg^{310} is close to Asp^{69} in the ASL crystal structure, mutations were also made at position 310. Asp^{69} and Arg^{310} are conserved in ASLs from bacteria to humans, as indicated in the representative sequences shown in Figure 1. To study the effect of these conserved charged residues on the p*K* of histidine 68, the negatively charged aspartate at 69 was mutated to the uncharged asparagine and the negatively charged glutamate. The arginine at 310 was replaced by the neutral glutamine and the positively charged lysine. The wild-type and mutant pBHis plasmid encoding *B. subtilis* ASL were expressed in *Escherichia coli*, and the enzymes were purified as described in Materials and Methods. The wild-type and all mutant enzymes were homogeneous as indicated by the single band for each on SDS-PAGE (Supplemental Fig. S1).

Circular dichroism spectroscopy of wild-type and mutant enzymes

Circular dichroism (CD) spectra of wild-type and mutant enzymes were measured to evaluate any changes in the secondary structure of the enzymes. The CD spectra of all mutant and wild-type enzymes are shown in Supplemental Figure S2. The spectra of all mutant enzymes are very similar to that of wild type, indicating that the mutations

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B. subtilis   63EKDTRH68 D69 VVA73   298WHERDISHSSAER310  IILPDA316
T. Maritima     EEKTNH   D   VVA       WHERDISHSSVER      YVFPDA
G. gallus       EKKLRH   D   VMA       WFERTLDDSANRR      VCLAEA
M. musculus     EKRLRH   D   VMA       WFERTLDDSANRR      ICLAEA
H. sapiens      EKRLRH   D   VMA       WFERTLDDSANRR      ICLAEA
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Figure 1. Amino acid sequence alignments of adenylosuccinate lyase from *B. subtilis*, *T. maritima*, *Gallus gallus*, *Mus musculus, and Homo sapiens* according to CLUSTALW. The asterisks indicate that the amino acids at the position are identical.

do not cause any detectable changes in the secondary structure of the enzymes.

Molecular weight determination

The oligomeric state of the wild-type and mutant enzymes was analyzed by light-scattering photometry. ASL has been shown to exist in solution as an equilibrium mixture of dimer and tetramer with their relative proportions dependent on the protein concentration (Palenchar and Colman 2003). Thus, the molecular weights of wild-type and mutant enzymes were measured at the same protein concentration (0.25 mg/mL); the results are shown in Table 1. The M_r values of wild-type and mutant enzymes are similar; these are consistent with an equilibrium mixture of dimer and tetramer, with the tetramer predominating. Light scattering could not be used on the R310Q mutant enzyme because of its tendency to aggregate. Therefore, native polyacrylamide gel electrophoresis was used to compare the sizes of wild-type and R310Q enzymes. Figure 2 shows a representative gel (9%). The wild-type and R310Q mutant enzymes migrate to similar positions, yielding molecular weights of 147 and 148 kDa, respectively; both enzymes were added to the gels at a lower protein concentration, accounting for the lower molecular weight observed by this method as compared with light scattering.

Kinetics of mutant enzymes

The kinetic properties at pH 7.0 for wild-type and mutant enzymes are summarized in Table 2. Compared with the wild-type V_{max} of 1.87 μmol/min/mg, the mutant enzymes D69E, D69N, R310K, and R310Q display markedly decreased V_{max} values. Of the Asp^{69} mutants, and of the Arg^{310} mutants, those in which the original charge was preserved (D69E and R310K) exhibit the smallest changes in V_{max}, and the K_m values for SAMP of these mutant enzymes are not very different from the wild-type value of 2 μM. In contrast, the K_m for SAMP is elevated about 11-fold in R310Q. Table 2 also reports values of k_{cat}/K_m. The mutant enzymes, in which charged amino acids were replaced with neutral residues, as in D69N and R310Q, exhibit the most striking decreases in these values.

Table 1. *Molecular weight determined by light scattering*

Enzyme	M_r
Wild type	191,000 ± 2000
D69E	194,000 ± 3000
D69N	176,000 ± 200
R310K	182,000 ± 1000

The molecular weight was determined at a concentration of 0.25 mg/mL in 20 mM potassium phosphate buffer at pH 7.0 containing 20 mM sodium chloride.

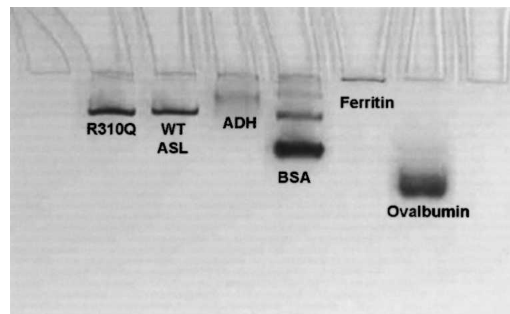

Figure 2. Native polyacrylamide gel electrophoresis. A representative 9% gel is shown: lane *1*, R310Q; lane *2*, wild type (WT ASL); lane *3*, yeast alcohol dehydrogenase (ADH) (141,000); lane *4*, bovine serum albumin (BSA) (67,000); lane *5*, horse spleen ferritin (450,000); lane *6*, chicken egg ovalbumin (43,000).

pH Dependence on V_{max} for wild-type and mutant ASL enzymes

To evaluate the effect of the mutations on the kinetically detectable ionizable groups, the velocity at 300 μM SAMP was measured as a function of pH (from pH 6.0 to pH 9.3) for all the enzymes in the AMP and fumarate formation reaction. The K_m for SAMP increases with pH over the pH range used. For example, the K_m varies from 1.9 to 33 μM for wild type, from 1.3 to 14 μM for the D69E mutant, and from 9 to 43 μM for the D69N mutants. In most cases, SAMP concentration is high relative to the K_m over the pH range used; therefore, the velocity at 300 μM SAMP measures the V_{max} at each pH. For the R310Q mutant, however, the 300 μM SAMP was only ~6 times the K_m in the high pH range; therefore, for this mutant the rates measured at 300 μM SAMP were used to calculate V_{max} from the equation: $V_{max} = v_{obs}(1 + K_m/[SAMP])$. The wild-type and all mutant enzymes show a bell-shaped pH-V_{max} curve. Representative graphs for wild type, D69E, and D69N are shown in Figure 3. The pK values were determined by using the equation $V_{max\ (at\ each\ pH)} = V_{max\ intrinsic}/[1 + 10^{(pK1\ -\ pH)} + 10^{(pH\ -\ pK2)}]$, where p$K_1$ and pK_2 are the pK values for the ionizable groups of the enzyme–substrate complex responsible for the left and right limbs, respectively, of the pH-V_{max} curve; $V_{max\ (at\ each\ pH)}$ is the value at saturating SAMP concentration at each pH; and $V_{max\ intrinsic}$ is the pH-independent maximum velocity for the enzyme. The pK_1, pK_2, and $V_{max\ intrinsic}$ values are summarized in Table 3. The ionization of His^{68} has been postulated to be reflected in pK_2 (Lee et al. 1999; Brosius and Colman 2000), even though this pK_2 is higher than the value of 6.0 given for a

Table 2. *Kinetics of wild-type and mutant* B. subtilis *adenylosuccinate lyases*

	V_{max}, pH 7.0 (μmol/min/mg)	K_m (SAMP), pH 7.0 (μM)	k_{cat}/K_m (/M/s)
Wild type	1.87 ± 0.04	2.1 ± 0.5	7.5×10^5
D69E	0.41 ± 0.01	1.5 ± 0.4	2.3×10^5
D69N	0.05 ± 0.002	9.3 ± 1.9	0.037×10^5
R310K	0.11 ± 0.002	7.1 ± 0.5	0.12×10^5
R310Q	0.0009 ± 0.00009	23.0 ± 8.0	0.00029×10^5

The activities were measured by the decrease in absorbance at 282 nm using SAMP in 50 mM HEPES (pH 7) at 25°C.

typical histidine (Garrett and Grisham 1999). The wild-type enzyme has pK_1 and pK_2 values of 6.74 and 8.28, respectively. In contrast, the D69N and R310Q mutant enzymes have pK_2 values of 7.83 and 7.33, respectively. Thus, removing the charge at position 69 and 310 lowers the pK_2 value, whereas changing the amino acid but maintaining the same charge does not alter pK_2 appreciably (e.g., for D69E enzyme pK_2 is 8.48, while for R310K enzyme, pK_2 is 8.24, similar to that of a wild-type enzyme). Since the amino acid at position 69 is close to His[68] in the homology model, pK_2 must reflect the effect on the ionization of His[68] resulting from the charge at position 69.

Discussion

This study seeks to identify an amino acid whose ionization is reflected in pK_2 of the wild-type pH-V_{max} profile. We constructed, expressed, purified, and characterized enzymes with site-directed mutations of residues Asp[69] and Arg[310] of the *B. subtilis* ASL to test their effect on pK_2. Sequence alignment revealed that amino acid residues Asp[69] and Arg[310] are conserved in all species and are close to or interact with His[68], according to the homology model.

The *Thermatoga maritima* enzyme was the first ASL crystal structure to be published (Toth and Yeates 2000). The *B. subtilis* and *T. maritima* enzymes share 50% identity plus 23% similarity at the amino acid sequence level. Based on the strong resemblance of the amino acid sequences of the two enzymes, the homology model of *B. subtilis* ASL (which is based on the crystal structure of

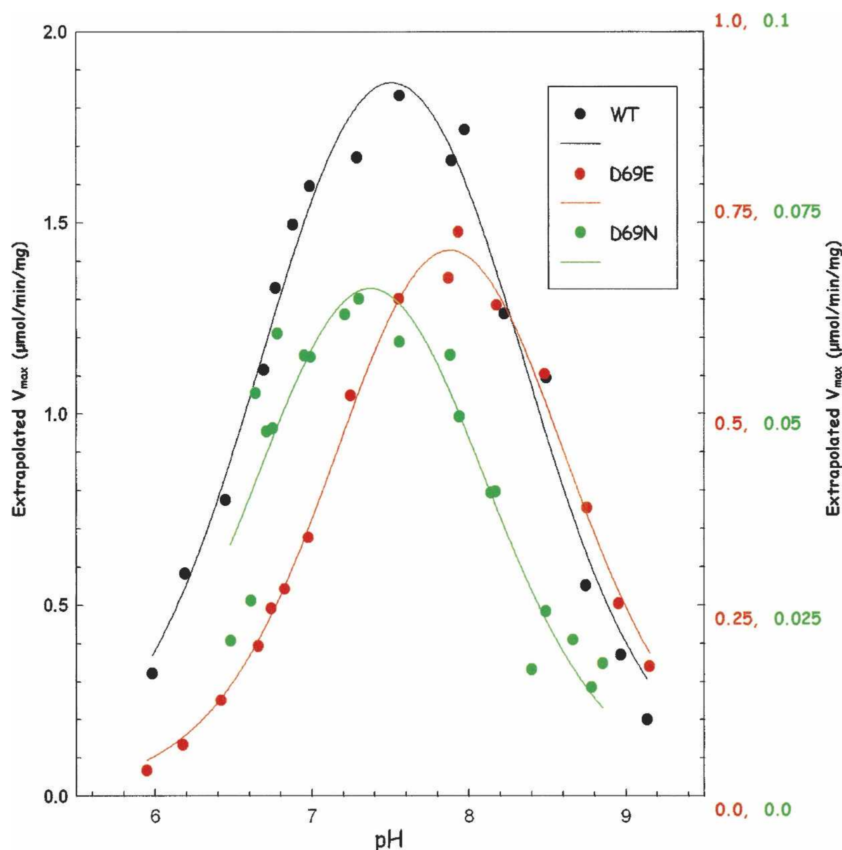

Figure 3. pH-V_{max} profiles of wild-type (WT) and mutant adenylosuccinate lyase enzymes. The pH dependence of V_{max} of WT (black), D69E (red), and D69N (green). Note the different scales for V_{max} for the three enzyme samples.

Table 3. *pK values determined from pH-V_{max} profiles of wild-type and mutant adenylosuccinate lyase enzymes*

Enzyme	pK_1	pK_2	$V_{max,\ intrinsic}$ (μmol/min/mg)
Wild type	6.74 ± 0.06	8.28 ± 0.06	2.50 ± 0.14
D69E	7.29 ± 0.04	8.48 ± 0.04	1.07 ± 0.04
D69N	6.89 ± 0.26	7.83 ± 0.24	0.10 ± 0.03
R310K	6.82 ± 0.06	8.24 ± 0.07	0.23 ± 0.01
R310Q	7.23 ± 0.03	7.33 ± 0.02	0.005 ± 0.0002

The pK values were determined as described in Materials and Methods and Results, using the type of data represented by Figure 3.

T. maritima enzyme) is likely to be a reasonable representation of the actual structure (Segall and Colman 2004). We have previously postulated that the protonated form of His[68] acts as a general acid in the catalytic reaction and/or is involved in an electrostatic interaction with a carboxylate of SAMP to facilitate the binding of substrate (Segall and Colman 2004). Recently, Tsai et al. have determined the structure of an inactive mutant of *E. coli* ASL with bound SAMP and they found that the histidine equivalent to His[68] in the *B. subtilis* enzyme is actually closer to the carboxylate of SAMP (Tsai et al., in press).

In the homology model of the *B. subtilis* ASL in Figure 4, His[68] is 3.6 Å from the carboxylate of Asp[69], and a proximal negative charge would raise the pK of the protonated form of histidine. A similar strategy was used for acetoacetate decarboxylase; in that case, the pK of the active site Lys[115] was markedly changed by mutating the amino acid at position 116 (Highbarger et al. 1996). Also shown in Figure 4 for ASL, Asp[69] is 3.6 Å from the guanidino group of Arg[310]. An electrostatic interaction between the negatively charged carboxylate of Asp and the positively charged Arg could properly orient Asp[69] to interact with His[68].

The kinetic data show that all mutant enzymes display greatly decreased specific activities, especially in the cases where charged amino acids were replaced with neutral residues, suggesting that Asp[69] and Arg[310] are important for catalytic function. The biophysical characteristics of these mutant enzymes are not appreciably different from those of wild-type enzyme in their secondary or quaternary structure. The Asp[69]-to-Asn mutant enzyme has a 50-fold lower activity and Arg[310]-to-Gln has a 2500-fold lower activity. Removing the charge from Asp[69] by replacing it with Asn also causes a fourfold increase in K_m; and replacing positively charged Arg[310] by neutral Gln causes an 11-fold increase in the K_m, indicating that there is weakened affinity between the enzyme and substrate.

The pH-V_{max} profiles of the Asp[69] mutant enzymes show that when the same negative charge is maintained (as in D69E), pK_2 is similar to that of wild-type enzyme, with a pK_2 value of 8.48, and His[68] is maintained in a positively charged form. The small increase in pK_2 in D69E may be due to the negatively charged COO^- of Glu[69] being closer than that of Asp[69] to His[68]. When the negative charge is removed (D69N), the positively charged form of His[68] is not stabilized and the pK_2 decreases, as seen by the pK_2 value of 7.83. In the pH-V_{max} profile of the Arg[310] mutant enzyme, in which the positive charge is maintained (R310K), the electrostatic interaction with Asp[69] remains, and the pK_2 (8.24) is high, as in wild-type enzyme. In contrast, when the positive charge is replaced by a neutral amino acid, as in R310Q, there is no attraction between Asp[69] and Gln[310]; Asp[69] may not be positioned to interact with His[68] and the pK_2, representing the ionization of His[68], decreases to 7.33. These data indicate that His[68] must be maintained as a positively charged species for the enzyme to be fully active. In D69N and R310Q, the pK_2 is likely decreased because of their effects on His[68] ionization.

The right-hand limb of the pH-V_{max} profile has been suggested to reflect the ionization of *both* His[68] and His[89] (Brosius and Colman 2000). When His[68] is replaced by a neutral amino acid, as in H68Q, the pH-V_{max} profile is determined by the ionization of His[89] (Lee et al. 1999). For H68Q, although the specific activity is <1% that of

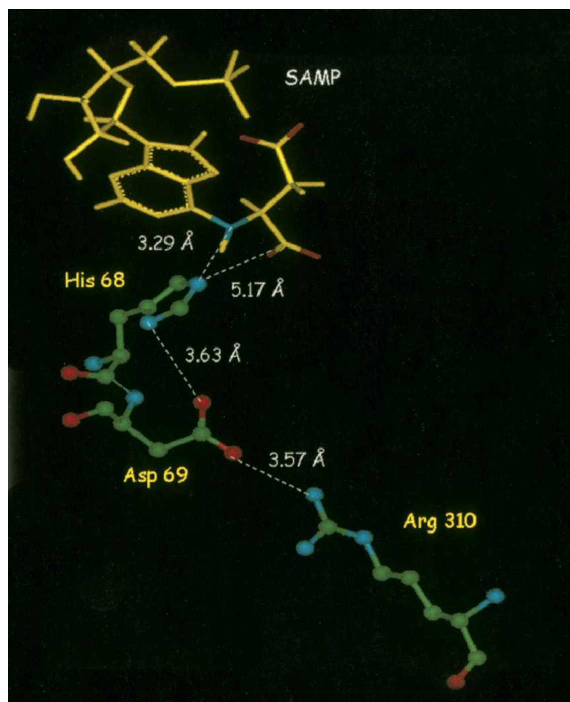

Figure 4. Homology model of *B. subtilis* adenylosuccinate lyase SAMP (yellow). Relevant active-site amino acids are colored according to the atom (green = carbon; red = oxygen; blue = nitrogen) and shown in ball and stick format.

wild-type enzyme (Lee et al. 1998), the shape of the pH-V_{max} curve is similar to that of the normal enzyme (Lee et al. 1999); this result can be interpreted as indicating that the ionization of His[89] is unperturbed in the His[68] mutant. Reciprocally, when His[89] is replaced by a neutral acid, as in H89Q, the pH-V_{max} curve reflects the ionization of only His[68] (Brosius and Colman 2000). For H89Q, the specific activity is also only 1% that of wild-type enzyme; but, (in contrast to H68Q) pK_2 of H89Q is decreased appreciably (Brosius and Colman 2000). His[89] is considered to be involved in binding the AMP moiety of the substrate and in orienting the substrate for catalysis. If the binding of substrate is altered within the active site, the ionization of His[68] in the enzyme–substrate complex may be modified *indirectly*.

The wild-type ASL enzyme pH-rate profile in the reverse reaction, using AMP and fumarate as substrates, gives pK_1 and pK_2 values of 6.53 ± 0.05 and 9.19 ± 0.07, respectively (Palenchar 2003). Heats of ionization studies of the wild-type ASL enzyme show that the pK_1 value reflects the ionization in the enzyme–substrate complex of a neutral acid such as a carboxyl group or phosphoric acid, due to the $\triangle$Hi value of 0 kcal/mol (Brosius and Colman 2000). Therefore, to determine whether the pK_1 is reflecting the ionization of the phosphate group of the substrate, adenosine 5′-O-thiomonophosphate (AMPS) with a pK of ~5.4 was used as substrate (Frey 1989). Since the pK value of a typical phosphate group is ~6.8, if the ionization of a phosphoryl group is represented in pK_1, a substantial shift in pK_1 will be observed when using AMPS as substrate. The pK_1 and pK_2 values with AMPS and fumarate are 6.58 ± 0.07 and 8.91 ± 0.08, respectively (Palenchar 2003). Since there was no significant difference in the pK_1 value when using AMP or AMPS as substrates, the pK_1 of the pH-rate profile cannot be reflective of the dissociation of a proton from the substrate's phosphoryl moiety. Instead, the pK_1 value may represent the deprotonation of COO^- groups from the succinyl portion of enzyme-bound SAMP and the *increase* in pK_1 in D69E and R310Q may reflect changes in the orientation of SAMP as a result of the mutations introduced.

Recently we reported that in the S94A mutant of ASL, perturbation of the interaction of Ser[94] with His[89] causes a decrease in pK_2 of the pH-V_{max} profile (Segall et al. 2007). In addition, we have preliminary evidence that replacement of another amino acid close to His[89] results in a decrease in pK_2. Glu[27] is only 4.1 Å from the side chain of His[89], while it is 12.1 Å and 16.3 Å from His[68] and His[141], respectively. The E27Q mutant, in which the neutral Gln replaces the negatively charged Glu, exhibits a pK_2 of the pH-V_{max} curve that is 0.4 *lower* than in the wild-type enzyme. In the present study we focused on Asp[69], which is 3.6 Å from His[68], 9.3 Å from His[89], and 10.7 Å from His[141]. Thus, the changes seen in the pH-V_{max} profile due to the mutations introduced at Asp[69] and Arg[310] provide the first *experimental* demonstration that His[68] is also a residue reflected in pK_2 of the pH-V_{max} profile.

In conclusion, this study indicates that mutations introduced at positions 69 and 310 of the *B. subtilis* ASL enzyme affect the catalytic activity and affinity for substrate, especially when the charge is changed. Also, the shift seen in the pK_2 of the pH-V_{max} profile with these mutations indicates that pK_2 reflects the ionization of His[68], since Asp[69] is close to His[68] (but not to His[89] or His[141]) in the structure. His[68] has been proposed to act as the general acid in the catalytic reaction and it may also be involved in an electrostatic interaction with a carboxylate of the adenylosuccinate to facilitate the binding of substrate.

Materials and Methods

Materials

SAMP, HEPES, 2-(*N*-morpholino)ethanesulfonic acid (MES), *N*-tris-(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS), and imidazole were purchased from Sigma. Ni-NTA resin was purchased from Qiagen. Oligonucleotides for both sequencing and site-directed mutagenesis were obtained from Biosynthesis. All other chemicals were of reagent grade.

Site-directed mutagenesis

Mutations to the pBHis plasmid (a gift from Dr. Jack E. Dixon, University of California, San Diego) that encodes ASL of *B. subtilis* were constructed using the Stratagene QuikChange mutagenesis kit. The following oligonucleotides and their complements were used to generate the Asp[69] mutant enzymes: CACGCGCCATAACGTTGTCGCTTTTAC (D69N) and CACGCGCCATGAAGTTGTCGCTTTTAC (D69E). The Arg[310] mutant enzymes were constructed using the following oligonucleotides and their complements: CTTCAGCAGAAAAAATTATTCTTCCGGATG (R310K) and CTTCAGCAGAACAGATTATTCTTCCGG (R310Q). In each case, the mutated codon is underlined. The mutations were confirmed by DNA sequencing carried out at the University of Delaware Center for Agricultural Biotechnology using an ABI Prism model 377 DNA sequencer (PE Biosystems).

The pBHis plasmid, which encodes a His_6 tag on the N terminus of ASL, was expressed in *E. coli* strain BL21 (DE3), and the enzymes were purified to homogeneity using Qiagen nickel nitrilotriacetic acid–agarose (Redinbo et al. 1996; Lee et al. 1997). The purity of the wild-type and mutant enzymes was evaluated by 12% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (Laemmli 1970), as well as by N-terminal sequencing. The protein concentrations were determined by the absorbance at 280 nm using $E^{1\%}_{280\ nm} = 10.6$ (Lee et al. 1997). The purified enzymes were stored at −80°C in 20 mM potassium phosphate containing 20 mM sodium chloride at pH 7.

Kinetics of B. subtilis *ASL*

The wild-type and mutant enzyme activity toward SAMP was measured by the time-dependent decrease in absorbance at 282 nm using the difference extinction coefficient of 10,000/M/cm

between SAMP and AMP. The enzymes were incubated at a minimum concentration of 0.4 mg/mL in 20 mM potassium phosphate containing 20 mM sodium chloride at pH 7 for 30 min at 25°C before activity measurement (Palenchar and Colman 2003). Standard assay conditions of 50 mM HEPES at pH 7.0 at 25°C with 60 μM SAMP were used for the determination of specific activity, expressed as μmol of SAMP converted/min/mg of enzyme used. The K_m for the wild-type and mutant enzymes was determined by varying the substrate concentration under the same conditions. The data were analyzed by ν versus [S] with standard error estimates obtained from the Sigma Plot software (SPSS Inc.).

The pH dependence of V_{max} was determined for wild-type and mutant enzymes using 300 μM SAMP, from pH 6.0–pH 9.3, at 25°C using the buffers MES (pH 6.0–6.9), HEPES (pH 6.8 – pH 8.0), and TAPS (pH 7.9–9.3) with a constant ionic strength of 0.03 M in all buffers. The rates were measured at 290 nm for these experiments, using a difference extinction coefficient of 4050/M/cm to calculate the rates. The pH of each assay mixture was measured after the rate determination and the data were analyzed by Sigma Plot software. The spectra of SAMP and AMP do not change over this pH range (Hampton 1962) so the same difference extinction coefficient could be used to calculate the rates.

The pH dependence of V_{max} was also determined in the direction of SAMP formation for wild-type ASL enzyme using 1 mM AMP and 10 mM fumarate as substrates. The same MES, HEPES, and TAPS buffers were used from pH 6.0–pH 8.5 at 25°C, and the rates were measured at 290 nm because of the high absorbance at 282 nm. The difference extinction coefficient of 4050/M/cm was used to calculate the rates. The wild-type ASL enzyme pH-V_{max} profile in the direction of SAMP formation was also determined using 1 mM AMPS, under the same conditions as for AMP.

CD spectroscopy

A Jasco J-710 spectropolarimeter was used to measure ellipticity as a function of wavelength from 250 to 200 nm in 0.2-nm increments using a 0.1-cm cylindrical quartz cuvette. The enzymes were incubated at 0.4 mg/mL for 30 min at 25°C before measuring the spectra. The samples were scanned five times and averaged, and the spectrum of the buffer, containing 20 mM potassium phosphate and 20 mM sodium chloride at pH 7, was subtracted. The final protein concentration was determined by a dye-binding assay (Bio-Rad protein assay), based on the method of Bradford (1976), using a Bio-Rad 2550 RIA plate reader with a 600-nm filter. Wild-type ASL was used as the protein standard. The mean residue ellipticity [θ] (deg cm^2/dmol) was calculated from the equation $[\theta] = \theta/10\ nCl$, where θ is the measured ellipticity in millidegrees, C is the molar concentration of the enzyme subunits, l is the path length in centimeters, and n is the number of residues per subunit (437, including the His_6 tag).

Molecular weight determination by light scattering

A minDAWN laser photometer (Wyatt Technology Corp.) set at a laser wavelength of 690 nm was used to determine the wild-type and mutant enzyme molecular weights. Molecular weight was measured at a protein concentration range of 0.1–0.4 mg/mL. The enzyme was preincubated at 25°C in 20 mM potassium phosphate containing 20 mM sodium chloride at pH 7, and filtered through a 0.20-μm filter before being used. The data were collected and analyzed using ASTRA software for windows (Wyatt 1993). The protein concentration was determined using the Bio-Rad protein assay (Bradford 1976).

Molecular weight determination of R310Q by native polyacrylamide gel electrophoresis

For wild-type and R310Q mutant enzyme, the molecular weight was determined by native polyacrylamide gel electrophoresis, by the method of Hedrick and Smith (1968). Gels were prepared consisting of 20 mM potassium phosphate at pH 7, 0.12% (v/v) N,N,N′,N′-tetramethylethylenediamine, 0.2 mg/mL ammonium persulfate, and appropriate volumes of distilled water and 37.5:1 acrylamide/bisacrylamide mixture to yield final acrylamide concentrations of 5%–9%. All samples and standards were prepared in potassium phosphate at pH 7 and were run at 25°C and 25 mA; the running buffer was also 20 mM potassium phosphate at pH 7.0.

Homology model of B. subtilis *ASL*

A homology model of the *B. subtilis* ASL, based on the crystal structure of the *T. maritima* enzyme (Toth and Yeates 2000), has been constructed (Brosius and Colman 2002; Segall and Colman 2004). The homology model was used to study the amino acid substitutions with energy minimization computational results obtained using software programs from Biosym Technologies (dynamics calculations from *Discover* and graphical display using *Insight II*) on an Indigo 2 workstation from Silicon Graphics.

Electronic supplemental material

The Electronic supplemental material contains two figures. Supplemental Figure S1 illustrates the single band of representative purified enzymes on SDS-PAGE. Supplemental Figure S2 shows the circular dichroism spectra of wild-type and mutant enzymes.

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