# Implication of His[68] in the Substrate Site of *Bacillus subtilis* Adenylosuccinate Lyase by Mutagenesis and Affinity Labeling with 2-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 5′-monophosphate[†]

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ABSTRACT: Adenylosuccinate lyase of *Bacillus subtilis* is inactivated by 2-[(4-bromo-2,3-dioxobutyl)thio]-adenosine 5′-monophosphate (2-BDB-TAMP) at pH 7.0. As the reagent concentration is increased, a maximum rate constant is approached, indicative of reversible enzyme-reagent complex formation ($K_R$ = 68 ± 9 $\mu$M) prior to irreversible modification ($k_{max}$ = 0.081 ± 0.004 min$^{-1}$). Complete inactivation occurs concomitant with about 1 mol of 2-BDB-[$^{14}$C]TAMP incorporated/mol of enzyme subunit. Adenylosuccinate, or a combination of AMP and fumarate, decreases the inactivation rate and reduces incorporation of [$^{14}$C] reagent, whereas either AMP or fumarate alone is much less effective. These observations suggest that 2-BDB-TAMP attacks the adenylosuccinate binding site. Proteolytic digestion of inactivated enzyme, followed by purification of the digest by HPLC, yields the radioactive peptide Ile[62]-Ala[72], in which Arg[67] and His[68] are the most likely targets. Thus 2-BDB-TAMP reacts with adenylosuccinate lyase at a site distinct from the His[141] attacked by 6-BDB-TAMP (Lee, Worby, Dixon, and Colman (1997) *J. Biol. Chem. 272,* 458−465). Site-directed mutagenesis was used to construct mutant enzymes with replacements for both Arg[67] and His[68], and either Arg[67] or His[68]. The R67M mutant enzyme has almost the same specific activity as the wild-type enzyme under standard assay conditions, whereas the single mutant H68Q and double mutant R67M−H68Q enzymes exhibit specific activities that are decreased more than 100-fold. These results indicate that while Arg[67] and His[68] may both be in the region of the substrate site, only His[68] is important for the catalytic activity of *B. subtilis* adenylosuccinate lyase. A role is proposed for His[68] as a general acid−base catalyst.

Adenylosuccinate lyase (EC 4.3.2.2), an essential enzyme in purine biosynthesis, catalyzes both the cleavage of 5-amino-4-imidazole-*N*-succinocarboxamide ribotide to form 5-amino-4-imidazolecarboxamide ribotide and fumarate, and the cleavage of adenylosuccinate to form AMP and fumarate (*1*). In addition, adenylosuccinate lyase participates in the purine nucleotide cycle, which has an important metabolic role in muscle, kidney, and brain (*2*, *3*). The enzyme has been isolated from a variety of sources (*4*−*11*). The amino acid sequences have been deduced from nucleotide sequences obtained by either cDNA cloning or genome sequencing and subsequent gene assignment (*12*−*26*). Clinical and biochemical data have shown that adenylosuccinate lyase deficiency is associated with mental retardation and autistic features, and that the patients have an accumulated level in their body fluids of nucleosides corresponding to the two substrates (*27*, *28*). In one case of adenylosuccinate lyase deficiency, the substituted amino acid has been identified (*26*).

Kinetic studies suggest that the cleavage reaction catalyzed by adenylosuccinate lyase is achieved by $\beta$-elimination through a general base−general acid mechanism which involves the attack on the $\beta$-H by a general base and the protonation of the ring nitrogen by a general acid of the enzyme (*1*, *29*). The kinetic mechanism is ordered, with fumarate leaving the enzyme before AMP (*9*, *24*, *29*−*31*).

Previously we have expressed the *Bacillus subtilis* enzyme and identified His[141] as a potential general base in the cleavage reaction by affinity labeling with 6-(4-bromo-2,3-dioxobutyl)thioadenosine 5′-monophosphate (6-BDB-TAMP)[1] (*32*). However, little is known about other amino acid residues involved in catalysis or substrate binding.

2-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 5′-monophosphate (2-BDB-TAMP) (*33*, *34*) was designed as an affinity label for nucleotide binding sites in proteins. As shown in Figure 1, it also resembles adenylosuccinate structurally and might be expected to occupy the adenylosuccinate site of adenylosuccinate lyase. The difference in the position of

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[1] Abbreviations: 2-BDB-TAMP, 2-[(4-bromo-2,3-dioxobutyl)thio]-adenosine 5′-monophosphate; 6-BDB-TAMP, 6-(4-bromo-2,3-dioxobutyl)thioadenosine 5-monophosphate; MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethane sulfonic acid; HPLC, high-performance liquid chromatography; TPCK, tosyl-L-phenylalanine chloromethyl ketone; PTH, phenylthiohydantoin.

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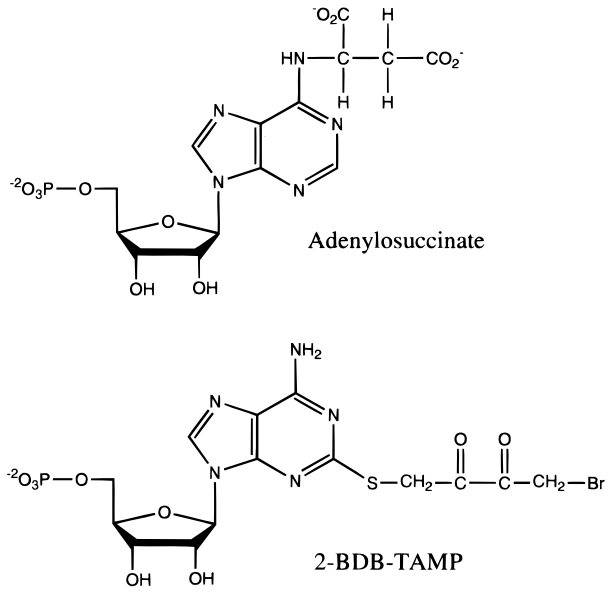


FIGURE 1: Schematic comparison of the structure of adenylosuccinate and 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-monophosphate (2-BDB-TAMP).

the reactive bromodioxobutyl group between 2-BDB-TAMP and 6-BDB-TAMP provides an opportunity to label an amino acid residue in the active site other than His[141]. The nucleophilic side chains of several amino acids (including Lys, His, Glu, Asp, and Cys) can attack the carbonyl(s) or methylene bromide group to yield a covalently labeled enzyme (*35*). Here, we describe the specific inactivation of *B. subtilis* adenylosuccinate lyase by 2-BDB-TAMP, isolation of the labeled peptide, and construction of mutant enzymes with replacements of two of the amino acid residues of the peptide targeted by 2-BDB-TAMP. A preliminary version of this work has been presented (*36*).

## EXPERIMENTAL PROCEDURES

*Materials*. Adenylosuccinate, adenosine 5′-monophosphate, fumarate, Sephadex G-15, Sephadex G-50, *N*-ethylmaleimide, TPCK-treated trypsin, pepsin, MES, and HEPES were purchased from Sigma. 1,4-Dibromo-2,3-butanedione, chloroperoxybenzoic acid, and carbon disulfide were from Aldrich Chemical Co. 1,4-Dibromo-2,3-butanedione was recrystallized from petroleum ether before use. [$^{14}$C]Adenosine 5′-monophosphate (generally labeled) was supplied by DuPont NEN. AG W50-X4 and Bio-Rad protein assay concentrate were from Bio-Rad. Trifluoroacetic acid and protease from *Staphylococcus aureus*, strain V8, were from ICN Biochemicals. HPLC-grade acetonitrile was supplied by Fisher Scientific. All other chemicals were reagent grade.

*Kinetics of B. subtilis Adenylosuccinate Lyase*. The homogeneous enzyme was prepared as reported previously (*32*). The adenylosuccinate lyase activity was measured from the decrease in absorbance at 282 nm using the difference extinction coefficient of 10 000 $M^{-1}$ $cm^{-1}$ between adenylosuccinate and AMP, as described earlier (*32*). The enzyme was preincubated at 25 °C for 30 min before measurements and yielded a stable specific activity of 2.0 $\mu$mol/min/mg. To determine the $K_I$ for fresh and hydrolyzed 2-BDB-TAMP, the $K_m$ for adenylosuccinate was measured in the absence and presence of 5 $\mu$M or 10 $\mu$M fresh 2-BDB-TAMP, and of 2.5 $\mu$M or 5 $\mu$M hydrolyzed 2-BDB-TAMP, prepared by incubating fresh 2-BDB-TAMP for 24 h at room temperature in 50 mM HEPES (pH 7.0). To determine the $K_I$ for AMP, 200 $\mu$M or 400 $\mu$M AMP was included in the assay when the $K_m$ for adenylosuccinate was determined. The adenylosuccinate concentration was varied from 2 to 100 $\mu$M.

*Synthesis of 2-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 5′-monophosphate*. 2-BDB-TAMP was prepared from AMP according to the procedure of Kapetanovic et al. (*33*). 2-BDB-[$^{14}$C]TAMP was synthesized according to the procedure of Gite and Colman (*10*). The final product was dissolved in 30 mM MES buffer (pH 4.5) and stored at −80 °C for further studies. The concentration of 2-BDB-TAMP was determined from its absorbance using $\epsilon_{246\ nm} = 35.2 \times 10^3$ $M^{-1}$ $cm^{-1}$ and $\epsilon_{270\ nm} = 11.5 \times 10^3$ $M^{-1}$ $cm^{-1}$. The specific radioactivity of 2-BDB-[$^{14}$C]TAMP was $2.6 \times 10^{11}$ cpm/mol of compound.

*Reaction of B. subtilis Adenylosuccinate Lyase with 2-BDB-TAMP*. Enzyme (0.2 mg/mL, 4 $\mu$M subunit) was incubated with 2-BDB-TAMP (30−400 $\mu$M) at 25 °C in 10 mM potassium phosphate buffer containing 10 mM NaCl (buffer A). The volume of 30 mM MES buffer (pH 4.5, containing 2-BDB-TAMP) added was maintained constant such that the final pH was 7.0. The enzyme was preincubated for 30 min at 25 °C before 2-BDB-TAMP was added. To compare with the inactivation by 100 $\mu$M 2-BDB-TAMP, "hydrolyzed" 2-BDB-TAMP (prepared by incubating 2-BDB-TAMP in the reaction mixture without enzyme at 25 °C for 24 h) was added to the preincubated enzyme to reach 100 $\mu$M. At various times, 20-$\mu$L aliquots of reaction mixture were withdrawn and assayed for residual activity. The reaction was measured for 60 min, during which period no activity loss was found in the control enzyme incubated under the same conditions but in the absence of 2-BDB-TAMP. The effects of substrate analogues on the reaction rate with 100 $\mu$M 2-BDB-TAMP were tested by including these ligands with enzyme together with the reagent, as described in Results.

To determine the amount of reagent incorporation into adenylosuccinate lyase, enzyme was incubated with 100 $\mu$M 2-BDB-[$^{14}$C]TAMP in buffer A in the absence and presence of 5 mM AMP and 10 mM fumarate. At various times of incubation, 200 mM $NaBH_4$ (dissolved in 20 mM NaOH) was added to reach a final concentration of 2 mM. The carbonyl groups of 2-BDB-TAMP were reduced by $NaBH_4$, thus markedly decreasing the reactivity of the compound. The $NaBH_4$ treatment was conducted for 5 min. The excess reagent was removed by column centrifugation (*37*), as described previously (*32*). The filtered protein was assayed immediately for enzymatic activity and protein concentration. The protein concentration was determined using the Bio-Rad protein assay (*38*), using purified *B. subtilis* adenylosuccinate lyase as the protein standard. The incorporation of 2-BDB-[$^{14}$C]TAMP was determined by measuring the radioactivity of modified protein using a Packard Tri-Carb liquid scintillation analyzer (Model 1500).

*Enzymatic Digestion of 2-BDB-[$^{14}$C]TAMP-Modified Enzyme*. Adenylosuccinate lyase (0.5 mg/mL) was incubated with 100 $\mu$M 2-BDB-[$^{14}$C]TAMP at 25 °C for 60 min. The incubation mixture was treated with 2 mM $NaBH_4$ for 5 min, followed by reaction with 10 mM *N*-ethylmaleimide for 10 min. After removal of the excess reagent by column

centrifugation (using Sephadex G-50 equilibrated with 40 mM ammonium acetate, pH 4.0), the modified enzyme was digested at 37 °C by 2 successive additions (at 2-h intervals) of 5% (w/w) protease from *S. aureus*, strain V8, for a total of 4 h.

The radioactively labeled enzyme digest (~2 mg) was lyophilized, redissolved in 0.8 mL of 0.1% trifluoroacetic acid, and applied to a Varian (Model 5000) HPLC system using a reverse-phase Vydac $C_{18}$ column (0.46 × 25 cm). Separation was conducted at the elution rate of 1 mL/min using solvent A (0.1% trifluoroacetic acid) for the first 10 min, followed by a linear gradient from solvent A to 60% solvent B (0.09% trifluoroacetic acid in acetonitrile) in 120 min, a linear gradient from 60% solvent B to 100% solvent B in 20 min, and solvent B for 10 min, successively. The effluent was monitored on-line at 220 nm. Fractions of 1 mL were collected, from which 100 $\mu$L was assayed for radioactivity. Hydrolyzed 2-BDB-[$^{14}$C]TAMP (prepared by incubating fresh 2-BDB-[$^{14}$C]TAMP in buffer A for 24 h) was applied to the same column in a separate run.

*Purification and Characterization of Modified Peptide.* To obtain the homogeneous modified peptide, the radioactive peak obtained from the first HPLC run was lyophilized, redissolved in 0.8 mL of 0.1% trifluoroacetic acid, and digested at 37 °C by 20 $\mu$g of pepsin for 2 h. The peptic digest was fractionated by HPLC using the same system described above. For better separation, elution was conducted at a shallower gradient using solvent A for the first 10 min, followed by a linear gradient from solvent A to 5% solvent B in 10 min, a linear gradient from 5% solvent B to 15% solvent B in 100 min, a linear gradient from 15% solvent B to 100% solvent B in 30 min, and solvent B for 10 min. Fractions of 1 mL were collected, from which 100 $\mu$L was assayed for radioactivity. The radioactive fractions were pooled, lyophilized, redissolved in 0.4 mL 0.1% trifluoroacetic acid, and applied to a Sephadex G-15 column (1 × 50 cm, equilibrated with 0.1% trifluoroacetic acid) at room temperature. The elution rate was 0.18 mL/min. Fractions of 0.9 mL were collected, from which 400 $\mu$L was assayed for radioactivity. The sequences of the peptides in the radioactive peaks (after lyophilization) were determined using an automated gas-phase protein/peptide sequence analyzer from Applied Biosystems (Model 470A), equipped with an on-line PTH analyzer (Model 120) and computer (Model 900A).

*Site-Directed Mutagenesis.* Site-directed mutagenesis of pBHis was performed using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The oligonucleotides used were GGACACGCGCGCAGACGTTGTCGC (H68A), AGGACACGCGCAAGGACGTTGTCGC (H68K), GGACACGCGCCAGGACGTTGTCGC (H68Q), and CGAAAAGGACACGATGCATGACGTTGTCG (R67M) and their complements. For the double mutant, R67M−H68Q, the His to Gln change was made in the previous R67M mutant using the oligonucleotide AGGACACGATGCAGGACGTTGTCGCT and its complement. All mutants were confirmed by nucleotide sequence analysis.

The plasmid was expressed in *Escherichia coli* strain BL21 (DE3) and the mutant enzymes were purified as described previously (*32*, *39*). The purity of the mutant proteins was assessed by 12% SDS−polyacrylamide gel electrophoresis.

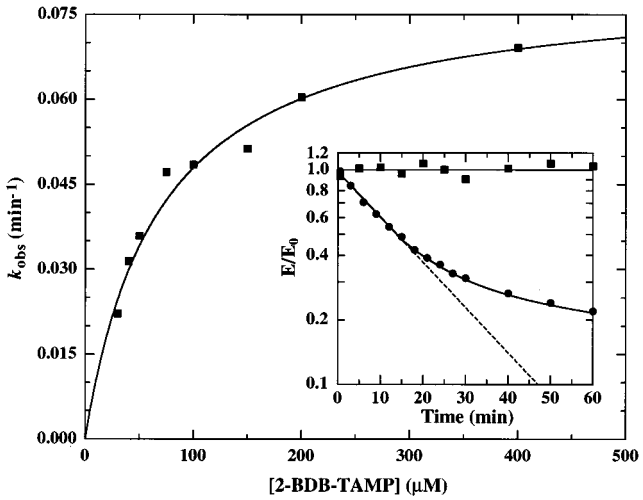


FIGURE 2: Dependence of initial rate constant of inactivation of adenylosuccinate lyase on the concentration of 2-BDB-TAMP. The enzyme was incubated with 30−400 $\mu$M 2-BDB-TAMP in buffer A (pH 7.0) at 25 °C. The rate constants were measured from the first 12 min of the reaction. The saturation behavior of $k_{obs}$ against [2-BDB-TAMP] is shown. Inset: Time-dependent inactivation of *B. subtilis* adenylosuccinate lyase by 100 $\mu$M 2-BDB-TAMP. The preincubated enzyme was mixed with 100 $\mu$M fresh (●) or hydrolyzed (■) 2-BDB-TAMP in buffer A (pH 7.0) at 25 °C. At the specified times, 20-$\mu$L aliquots were withdrawn to assay the residual activity as described in Experimental Procedures. The solid lines represent the curves that fit the actual data over the entire 60-min period, while the dashed line is a linear regression for data taken from the initial 12 min in the reaction with fresh 2-BDB-TAMP.

## RESULTS

*Inactivation of B. subtilis Adenylosuccinate Lyase.* A time-dependent inactivation of the *B. subtilis* adenylosuccinate lyase was observed when 0.2 mg/mL enzyme (4 $\mu$M subunit) was incubated with 100 $\mu$M 2-BDB-TAMP at 25 °C and pH 7.0 in buffer A (Figure 2, inset). The control enzyme showed constant activity over this time period when incubated under the same conditions but in the absence of reagent (data not shown). The semilogarithmic plot of residual activity ($E/E_0$) versus time of incubation with 2-BDB-TAMP exhibits linearity for the first 12 min. A pseudo-first-order rate constant of 0.0485 min$^{-1}$ was calculated using data taken from this initial period. The enzyme inactivation continued as the incubation proceeded, reaching about 21% residual activity by 60 min, as shown in the inset of Figure 2. By gel filtration of the enzyme and subsequent addition of fresh compound, the enzyme activity can be reduced to less than 5% of the original activity.

Progressive deviation from the first-order kinetics was observed beyond 12 min (Figure 2, inset), indicating that the rate of inactivation decreases as the reaction continues. Similar results were observed in the reaction of adenylosuccinate lyase and 6-BDB-TAMP (*32*). Two factors are responsible for this behavior. First, 2-BDB-TAMP is known to undergo decomposition in aqueous buffers with release of free bromide; the rate of decomposition has been determined as 0.0115 min$^{-1}$ ($t_{1/2}$ = 61 min) at pH 7.0 and 25 °C (*33*). No loss of activity was observed when the enzyme was incubated with previously hydrolyzed 2-BDB-TAMP (100 $\mu$M) (Figure 2, inset). The decomposition of the reagent results in a decreased concentration of reactive

Table 1: Effect of Substrate and Other Ligands on Inactivation of Adenylosuccinate Lyase by 2-BDB-TAMP[a]

| ligands added | $k_{obs}$ ($min^{-1}$) | $k_+/k_-$[b] |
|---|---|---|
| a) none | 0.0485 | 1.00 |
| b) AMP (1 mM) | 0.0304 | 0.63 |
| c) AMP (5 mM) | 0.0149 | 0.31 |
| d) fumarate (10 mM) | 0.0344 | 0.71 |
| e) Adenylosuccinate (5 mM) | 0.0081 | 0.17 |
| f) AMP (1 mM) + fumarate (10 mM) | 0.0091 | 0.19 |
| g) AMP (5 mM) + fumarate (5 mM) | 0.0077 | 0.16 |
| h) AMP (5 mM) + fumarate (10 mM) | 0.0063 | 0.13 |
| i) hydrolyzed 2-BDB-TAMP (100 $\mu$M) | 0.0232 | 0.48 |

[a] The enzyme was incubated with 100 $\mu$M 2-BDB-TAMP in buffer A at 25 °C in the presence of ligands, as described in Experimental Procedures. To test for protection by AMP and fumarate, the ligands were preincubated together with the enzyme; in testing for protection by adenylosuccinate and the hydrolyzed 2-BDB-TAMP, the ligands were added to the incubation mixture immediately before the addition of 2-BDB-TAMP. [b] The ratio of the initial inactivation rate constant in the presence and the absence of 2-BDB-TAMP.

2-BDB-TAMP and, consequently, a decrease in the apparent pseudo-first-order rate constant. Second, the hydrolyzed 2-BDB-TAMP competes with the intact 2-BDB-TAMP for binding to the enzyme at the adenylosuccinate site (see below). This explanation for the decreased inactivation rate after 12 min is supported by the fact that the initial rate of inactivation is decreased by addition of the hydrolyzed 2-BDB-TAMP to the reaction mixture (Table 1, line i).

The fresh and hydrolyzed 2-BDB-TAMP inhibit the cleavage reaction catalyzed by adenylosuccinate lyase. The *B. subtilis* enzyme has a $K_m$ of 2.6 $\mu$M for adenylosuccinate (*32*). The presence of fresh and hydrolyzed 2-BDB-TAMP increases this $K_m$ without affecting $V_{max}$. The $K_I$ values for the fresh and hydrolyzed 2-BDB-TAMP were determined to be 1.3 and 0.6 $\mu$M, respectively. These results suggest that both of the compounds are competitive inhibitors with respect to adenylosuccinate. The intact 2-BDB-TAMP has a somewhat lower affinity for the enzyme than does the decomposition product.

*Concentration Dependence of the Rate of Inactivation of Adenylosuccinate Lyase by 2-BDB-TAMP*. Adenylosuccinate lyase was incubated with various concentrations of 2-BDB-TAMP (30−400 $\mu$M). The initial rate constant of inactivation was determined from the first 12 min in each experiment. As shown in Figure 2, the apparent rate constant exhibits nonlinear dependence on reagent concentration. Saturation of the enzyme is achieved at high reagent concentrations. This result is indicative of the initial reversible binding of 2-BDB-TAMP prior to the irreversible inactivation. The observed rate constant ($k_{obs}$) is expressed by

$$k_{obs} = \frac{k_{max}[R]}{K_R + [R]} \quad (1)$$

where [R] is the reagent concentration, $k_{max}$ is the maximum rate constant at saturating concentrations of 2-BDB-TAMP, and $K_R = (k_{-1} + k_{max})/k_1$, the apparent dissociation constant of enzyme-reagent complex. The data were analyzed using GraphPad software InPlot for least-squares parameter evaluation. The calculated $k_{max}$ is 0.081 ± 0.004 $min^{-1}$, with $K_R$ = 68 ± 9 $\mu$M.

*Effects of Substrate and Other Ligands on the Rate of Inactivation by 2-BDB-TAMP*. The ability of various ligands to decrease the rate of inactivation by 100 $\mu$M 2-BDB-TAMP was tested to indicate the target site. The adenylosuccinate concentration (5 mM) was high relative to its $K_m$ (2.6 $\mu$M) (*32*) and the AMP concentration (5 mM) was high relative to its $K_I$ value of 128 $\mu$M (determined as described in Experimental Procedures), respectively. The fumarate concentrations (5 and 10 mM) were high relative to its $K_I$ values reported from various species (0.16-2.4 mM) (*7*, *11*, *29*, *30*). Adenylosuccinate alone (Table 1, line e), or a combination of AMP and fumarate (Table 1, lines f−h), is most effective in decreasing the rate against inactivation by 2-BDB-TAMP, whereas either AMP or fumarate alone is much less protective (Table 1, lines b−d). The hydrolyzed 2-BDB-TAMP, albeit at a lower concentration (Table 1, line i), also provides considerable protection against inactivation by 2-BDB-TAMP. These results suggest that 2-BDB-TAMP targets the active site of the enzyme in the region occupied by both the AMP and succinyl moieties.

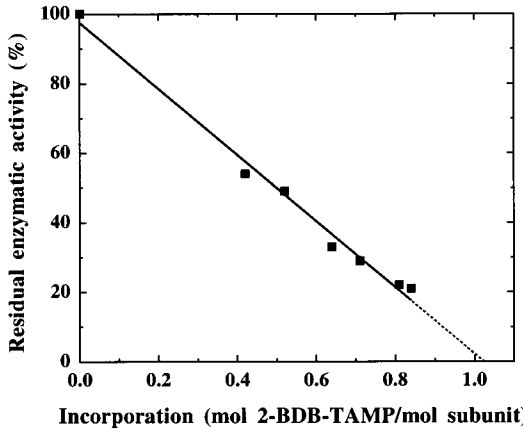


FIGURE 3: Relationship between inactivation and incorporation of 2-BDB-[$^{14}$C]TAMP into adenylosuccinate lyase. The enzyme was incubated with 100 $\mu$M 2-BDB-[$^{14}$C]TAMP in pH 7.0 buffer at 25 °C. The incorporation was stopped by addition of $NaBH_4$ (2 mM) at 10, 15, 25, 35, 55, and 65 min. The excess reagent and its hydrolyzed product were removed by two consecutive Sephadex G-50 columns. Aliquots were removed from the filtrate to measure the protein concentration and radioactivity. The residual activity of the enzyme was determined after 2-BDB-TAMP removal. Enzyme treated with the same procedure but without 2-BDB-[$^{14}$C]TAMP in the incubation mixture was used as a control for activity. The data were extrapolated to 0% residual activity to estimate the incorporation associated with complete inactivation.

*Incorporation of 2-BDB-TAMP into B. subtilis Adenylosuccinate Lyase*. Adenylosuccinate lyase was incubated with 2-BDB-[$^{14}$C]TAMP, as described in Experimental Procedures. The addition of $NaBH_4$ at various times to an incubation mixture of enzyme and reagent stops the reaction by reducing the carbonyl groups of the free reagent. Figure 3 shows the correlation between enzyme inactivation and incorporation of 2-BDB-[$^{14}$C]TAMP. The decrease in residual activity is linearly related to the increase in reagent incorporation. Extrapolation to 0% residual activity yields an incorporation of 1 mol of 2-BDB-[$^{14}$C]TAMP/mol of enzyme subunit. Inclusion of 5 mM AMP and 10 mM fumarate reduces the incorporation at 65 min to 0.28 mol of 2-BDB-[$^{14}$C]TAMP incorporated/mol of enzyme subunit while the enzyme retains 80% activity; for comparison, at 65 min, in the absence of substrate, the enzyme incorporates 0.84 mol of reagent/mol of enzyme subunit.

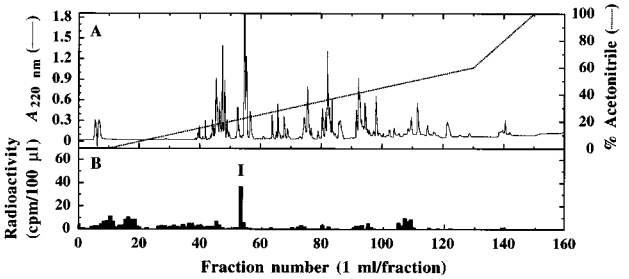


FIGURE 4: Peptide mapping of the modified enzyme. V8 protease-digested 2-BDB-[$^{14}$C]TAMP-modified adenylosuccinate lyase was separated by a HPLC $C_{18}$ column under the conditions described in Experimental Procedures. Panel A shows the $A_{220\ nm}$ profile (the solid line) and the gradient of acetonitrile (the dotted line), while panel B shows the radioactivity pattern of the same chromatograph.

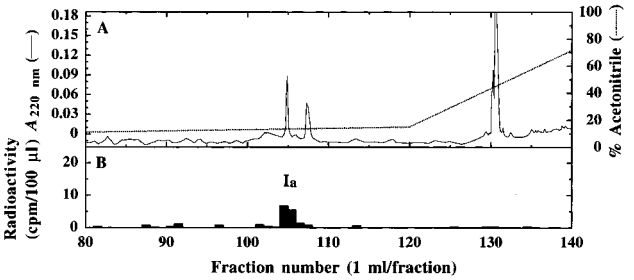


FIGURE 5: Separation of the peptic redigest of peak **I**. Peak **I** (Figure 4) was digested by pepsin before application to the same column that separated the initial V8 protease digest. Panel A shows the $A_{220\ nm}$ profile (the solid line) and the gradient of acetonitrile (the dotted line), while panel B shows the radioactivity pattern of the same chromatograph.

*Peptide Mapping of 2-BDB-[$^{14}$C]TAMP-Modified Adenylosuccinate Lyase*. In initial attempts to isolate the labeled peptides, the modified enzyme was passed through a Sephadex G-50 column equilibrated with 10 mM potassium phosphate buffer (pH 7.0, containing 10 mM NaCl and 10% glycerol) and digested by (10% w/w) TPCK-treated trypsin at pH 7.0 for 4 h. However, isolation of the radioactively labeled peptides by HPLC was unsuccessful: no distinct radioactive peak was observed except for those eluted in a region of decomposed 2-BDB-TAMP. These results indicate that the bond between the reagent and the target amino acid is much less stable at pH 7.0 once the enzyme is digested, even though the intact modified enzyme was relatively stable under similar conditions.

As an alternate approach, the modified enzymes were digested by V8 protease at pH 4.0 and 37 °C for 4 h, as described in Experimental Procedures. Figure 4A shows that, on reverse-phase HPLC of the enzyme digest, most of the peptides elute between 40 and 110 min (15% and 50% solvent B). When the hydrolyzed 2-BDB-[$^{14}$C]TAMP was applied to the same column, radioactivity was eluted before 45 mL, accounting for the early peaks observed in the enzyme digest. Thus the amino acid derivative is unstable and the decomposed reagent is partially released, probably during digestion at pH 4.0. The major radioactive peak (Figure 4B, peak **I**), which contains the modified peptide, is a mixture of at least five peptides, Ile$^{62}$-Glu$^{79}$, Asn$^{294}$-Glu$^{309}$, Leu$^{37}$-Glu$^{50}$, and Met$^{10}$-Glu$^{17}$ of *B. subtilis* adenylosuccinate lyase (*12*), and Ala$^{225}$-Glu$^{243}$ of V8 protease (*40*) (data not shown).

*Isolation of Modified Peptide*. To identify the modified peptide and localize the target sequence region, peak **I** was treated with pepsin at pH 2.0 and purified by a $C_{18}$ column (Figure 5). The radioactive peak $\mathbf{I_a}$ was separated from the rest of the peptide peaks. It contains two peptides, assigned as Ile$^{62}$-Ala$^{72}$ and Phe$^{73}$-Glu$^{79}$ of *B. subtilis* adenylosuccinate lyase, as determined using an automated gas-phase sequencer by Edman degradation (Table 2). Based on the radioactivity, the sample that was applied to the sequencer contained approximately 60 pmol of labeled peptide, assuming 1:1 peptide−reagent binding. This estimate makes Ile$^{62}$-Ala$^{72}$ the more likely candidate for modification.

To test this hypothesis, the modified peptide was purified to homogeneity by applying peak $\mathbf{I_a}$ to a Sephadex G-15 column at pH 2.0. The radioactive peak $\mathbf{I_b}$ was eluted close to the void volume, 13.5 mL, as calibrated using bovine serum albumin. The molecular mass cutoff of Sephadex G-15 is 1500 Da. The masses of Ile$^{62}$-Ala$^{72}$, Phe$^{73}$-Glu$^{79}$, and the free hydrolyzed 2-BDB-TAMP are 1282, 808, and 480 Da, respectively. Only Ile$^{62}$-Ala$^{72}$ labeled with 2-BDB-TAMP would have a mass higher than 1500 Da, and therefore be eluted at the void volume, suggesting that Ile$^{62}$-Ala$^{72}$ is the modified peptide. In fact, the gas-phase sequencing result shows that peak $\mathbf{I_b}$ contains only one peptide, Ile$^{62}$-Ala$^{72}$. Table 2 reports the amino acid sequences of peptides in peaks $\mathbf{I_a}$ and $\mathbf{I_b}$; the most likely target amino acids are Arg or His. Nucleotidyl derivatives of arginine or histidine have, in several cases, been recognized by their low yields of PTH-amino acid upon sequencing (*32*, *41*, *42*), although arginine and histidine can be partially regenerated. For the peptide of peak $\mathbf{I_b}$, there are low yields for both Arg$^{67}$ and His$^{68}$. The instability during tryptic digestion at neutral pH and relative stability under acidic conditions that we observed during the purification of the 2-BDB-TAMP-peptide product is characteristic of derivatives of arginine and dioxo compounds (*41*−*43*), and differs from our previous experience with a BDB-histidine derivative (*32*). These results indicate that Arg$^{67}$ is the most likely reaction target of 2-BDB-TAMP. However, no direct evidence was found to exclude the possibilities of modification of both Arg$^{67}$ and His$^{68}$ or His$^{68}$ alone.

Table 2: Amino Acid Sequences of Peak $\mathbf{I_a}$ and $\mathbf{I_b}$[a]

| | $\mathbf{I_a}$ (from Figure 5) | | | | $\mathbf{I_b}$ (from Sephadex G-15 column)[b] | |
|---|---|---|---|---|---|---|
| cycle no. | amino acid in sequence | amount (pmol) | amino acid in sequence | amount (pmol) | amino acid in sequence | amount (pmol) |
| 1 | Ile-62 | 95 | Phe-73 | 225 | Ile-62 | 133 |
| 2 | Glu-63 | 56 | Thr-74 | 116 | Glu-63 | 32 |
| 3 | Lys-64 | 67 | Arg-75 | 69 | Lys-64 | 73 |
| 4 | Asp-65 | 48 | Ala-76 | 222 | Asp-65 | 15 |
| 5 | Thr-66 | 41 | Val-77 | 215 | Thr-66 | 32 |
| 6 | Arg-67 | 24 | Ser-78 | 51 | Arg-67 | 1 |
| 7 | His-68 | 20 | Glu-79 | 34 | His-68 | 3 |
| 8 | Asp-69 | 35 | | | Asp-69 | 15 |
| 9 | Val-70 | 31 | | | Val-70 | 40 |
| 10 | Val-71 | 20 | | | Val-71 | 43 |
| 11 | Ala-72 | 9 | | | Ala-72 | 10 |

[a] Peak **I** (Figure 4) from HPLC of the V8 protease digest of 2-BDB-[$^{14}$C]TAMP modified enzyme was treated with pepsin. Peak $\mathbf{I_a}$ (Figure 5) was isolated by HPLC from the peptic digest. Peak $\mathbf{I_b}$ was obtained when peak $\mathbf{I_a}$ was applied to a Sephadex G-15 column. Peaks $\mathbf{I_a}$ and $\mathbf{I_b}$ were subjected to gas-phase sequencing. This table is representative of sequences from several samples of modified enzymes. [b] The data shown for peak $\mathbf{I_b}$ was from a different sample of modified enzyme than that for peak $\mathbf{I_a}$.

Table 3: Kinetic Parameters of Wild-Type and Mutant Enzymes[a]

| | specific activity ($\mu$mol min$^{-1}$ mg$^{-1}$) | $k_{cat}$ ($s^{-1}$) | $K_m$ ($\mu$M) | $k_{cat}/K_m$ ($\mu M^{-1}$ $s^{-1}$) |
|---|---|---|---|---|
| wild-type | 2.0 | $1.7 \pm 0.1$ | $2.6 \pm 0.4$ | 0.65 |
| R67M−H68Q | 0.0088 | $0.011 \pm 0.001$ | $27. \pm 5$ | 0.00041 |
| R67M | 2.3 | $3.1 \pm 0.1$ | $11. \pm 1$ | 0.27 |
| H68Q | 0.016 | $0.015 \pm 0.001$ | $9.1 \pm 1.0$ | 0.0016 |
| H68A | <0.00021[b] | nd[c] | nd | nd |
| H68K | <0.00024[b] | nd | nd | nd |

[a] The specific activities were measured under standard assay conditions, as described in Experimental Procedures. The $k_{cat}$ and $K_m$ constants were determined by varying the concentration of adenylosuccinate and fitting the data into the Michaelis−Menten equation.[b] The specific activities of these two mutants are below the detection limit. [c] Not determined.

*Characteristics of Mutant Enzymes with replacements for Arg[67] and His[68].* Since we could not definitively identify the amino acid of peptide **$I_b$** modified by 2-BDB-TAMP, mutant enzymes with substitutions for Arg[67] and His[68] were constructed, expressed, and purified. The purity of the mutant enzymes was ascertained by SDS−PAGE and the N-terminal sequences were determined for the mutant enzymes. Met-His-His-His-His-His-His-Ile-Glu-Arg-Tyr-Ser-Arg-Pro-Glu-Met was found as the sequence of the first 16 residues of each mutant, matching the *B. subtilis* enzyme sequence with an inserted $His_6$ tag (*12*, *32*). Mutant and wild-type enzymes exhibit very similar CD spectra, indicating that no major structural changes have resulted from the amino acid substitution (data not shown). Table 3 presents the kinetic characteristics of these purified enzymes, with the specific activities under standard assay conditions shown in column 1. The double mutant, R67M−H68Q, was evaluated in order to test the importance for activity of the local region which includes both residues. The observed specific activity of the double mutant is less than 0.5% that of the wild-type enzyme, consistent with a functional role for one or both of the original two amino acids. Single mutations of His[68] dramatically decrease the activity, whereas the single mutation of Arg[67] has little effect. These results indicate that His[68], rather than Arg[67], is important for enzyme activity. Table 3 also reports the $k_{cat}$ and $K_m$ of the mutants. The $K_m$ for adenylosuccinate is increased about 4-fold in the R67M and H68Q enzymes as compared to wild-type enzyme. However, replacements of His[68] result in more striking decreases in $k_{cat}$, reflecting its involvement in catalysis.

To evaluate the effect of mutations on the reaction with 2-BDB-TAMP, inactivation of the mutant enzymes was tested. H68Q, R67M, R67M−H68Q, and wild-type enzymes (0.7 mg/mL) were incubated with 200 $\mu$M 2-BDB-TAMP under the same reaction conditions described previously. Compared to the wild-type enzyme, all three mutants exhibit slower inactivation. The ratio of the initial rate constants is 1.00:0.76:0.55:0.34 in the order of wild type, H68Q, R67M, and R67M−H68Q. These results suggest that either amino acid residue can react with 2-BDB-TAMP, although reaction at Arg[67] or His[68] must be mutually exclusive (i.e., in a given enzyme molecule, 2-BDB-TAMP can react with only one amino acid residue). Since the reaction rate is faster in the case of the H68Q mutant as compared to R67M, we consider that Arg[67] probably reacts more rapidly than His[68]. The slow inactivation of the double mutant may be caused by reaction with one or more unknown residues when neither Arg[67] nor His[68] is present.

## DISCUSSION

The results of this paper demonstrate that 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-monophosphate reacts specifically and irreversibly as an affinity label of *B. subtilis* adenylosuccinate lyase. Modification by 2-BDB-TAMP results in loss of enzyme activity concomitant with incorporation of only 1 mol of reagent/mol of enzyme subunit. Furthermore, both inactivation and incorporation are decreased by addition of substrate or products, indicating the reagent targets the active site of the enzyme.

In the standard assay of the catalytic activity of adenylosuccinate lyase, both the fresh and hydrolyzed 2-BDB-TAMP are competitive inhibitors with respect to adenylosuccinate. The hydrolyzed 2-BDB-TAMP binds to the enzyme with a $K_I$ of 0.6 $\mu$M, a little more tightly than does the fresh 2-BDB-TAMP ($K_I$ of 1.3 $\mu$M), presumably due to the replacement of the bulky bromide with the smaller hydroxyl.

The nonlinear dependence on reagent concentration of the initial rate constant for inactivation of adenylosuccinate lyase by 2-BDB-TAMP suggests the formation of a reagent−enzyme complex prior to irreversible covalent modification, which is characteristic of an affinity label. The apparent $K_R$ value (68 $\mu$M) is about 50-fold higher than the $K_I$ value (1.3 $\mu$M) determined by the competition with adenylosuccinate in the assay. According to its definition described in Results, $K_R$ is not a simple dissociation constant; rather, it contains an extra kinetic constant which may account for part of the discrepancy between $K_I$ and $K_R$. In addition, it is notable that the inactivation buffer is phosphate, whereas the assay buffer is HEPES. It has been observed that phosphate not only stabilizes the enzyme in the incubation mixture but also protects the enzyme against inactivation (data not shown). In fact, phosphate inhibits the cleavage reaction catalyzed by the enzymes from rat skeletal muscle (*9*) and human erythrocytes (*11*). In the case of the human enzyme, phosphate is a competitive inhibitor with a $K_I$ value of 0.48 mM (*11*).

The 2-BDB-TAMP is structurally similar to the substrate adenylosuccinate and therefore was expected to target the active site of the enzyme. Studies on protection against inactivation were conducted in order to gain more information on the reaction site of 2-BDB-TAMP on adenylosuccinate lyase. Ligands were included in the reaction mixture to analyze their abilities to reduce the rate of inactivation. Both AMP and fumarate, when added by themselves individually, have a limited effect on the rate of inactivation. Much more effective protection is provided by adenylosuccinate or by a combination of AMP and fumarate. Similar results have been reported for the inactivation by 6-BDB-TAMP, where the label occupies both the AMP and fumarate sites (*32*). 2-BDB-TAMP can assume a structure with the reactive bromodioxobutyl group oriented at a position close to that of the succinate moiety of the natural substrate. Therefore it is also capable of targeting the same site as does 6-BDB-TAMP. The 2-thio-AMP moiety is likely to bind to the AMP site, whereas the bromodioxobutyl group may bind to a region overlapping the fumarate (equivalent to the succinyl group of adenylosuccinate) site. Both sites must be blocked in order to maximally prevent inactivation.

Purification of the V8 protease digest of the 2-BDB-TAMP-inactivated enzyme by HPLC yields only one radioactive peak associated with the modified peptide. This peptide was digested by pepsin and purified to homogeneity. Gas-phase sequencing identified the 11-membered peptide as Ile$^{62}$-Ala$^{72}$, which contains Arg$^{67}$ and His$^{68}$. In a model study of the reaction of arginine with cyclohexanedione, Patthy and Smith (*43*) showed that the reaction is initiated by addition to the diketo moiety. The product is much less stable under neutral and basic conditions than at acidic pH, matching the observation of the present study. Thus, Arg$^{67}$ is the most likely target of 2-BDB-TAMP. However, the possibility still exists that His$^{68}$ may be attacked by the reagent as well. The stoichiometry of incorporation suggests that two reactions must be mutually exclusive within a single active site. The PTH-Arg$^{67}$ and PTH-His$^{68}$ observed during the sequencing of peptide 62−72 are probably due to the partial regeneration of arginine and histidine under the solvent conditions used in the gas-phase sequencer, as has been observed previously for bromodioxo nucleotide products of arginine and histidine in enzymes (*32*, *41*, *42*).[2]

The complete amino acid sequences of adenylosuccinate lyase have been determined for 15 species. Comparison between any two sequences yields 20−95% identity. Alignment of all 15 sequences indicates that 21 positions (4.1%) are identical and 51 positions (9.9%) are similar among all the sequences. Arg$^{67}$His$^{68}$ is located in a well-conserved region. The sequence alignment of this region is shown in Figure 6. Although Arg$^{67}$ is replaced by lysine and asparagine in some cases, several residues around it, including His$^{68}$, are completely conserved throughout the 15 species, indicating the importance of this region.

The reactive bromodioxobutyl group is likely to target a residue at or near the fumarate binding site on the basis of the structural similarity and the results of protection studies. Fumarate, or the succinyl moiety of adenylosuccinate, carries two negative charges. The positive charge presented by Arg$^{67}$ or His$^{68}$ (provided it is protonated in the native structure) therefore may stabilize the enzyme−substrate complex. In fact, if the AMP moiety of adenylosuccinate and the 2-thio-AMP moiety of 2-BDB-TAMP were superimposed, the diketo group of 2-BDB-TAMP would be only ~3.7 Å away from the α-carboxyl, and ~4.6 Å away from the $\beta$-carboxyl, which makes it possible that the same amino acid residue could have a favorable electrostatic interaction with both carboxylate groups.

His$^{68}$ may also be involved in catalysis. Adenylosuccinate lyase belongs to the fumarase superfamily which catalyzes the cleavage of carbon−nitrogen bonds of various substrates to produce fumarate (*44*−*46*). All members of the family follow general base−general acid mechanisms (*45*). The pH dependence of $k_{cat}/K_m$ of the human enzyme suggests the participation of two enzymic groups with p$K_a$ values of 6.4 and 7.5 for the unprotonated and protonated forms, respectively (*29*). Similar results were obtained for the rat muscle enzyme, in which the unprotonated group has a p$K_a$ of 6.4 and the protonated group a p$K_a$ of 8.2 (*9*). Thus, the conservation of His$^{68}$ raises the possibility of its role as a general acid or general base.

| | |
|---|---|
| *B. subtilis* | I$^{62}$EKDT**R**$^{67}$**H**$^{68}$DVVA$^{72}$ |
| *M. tuberculosis* | R$^{70}$ERVL**R**$^{75}$**H**$^{76}$DVKA$^{80}$ |
| *M. leprae* | R$^{85}$ERVL**R**$^{90}$**H**$^{91}$DVKA$^{95}$ |
| *Synechocystis sp.* | I$^{62}$EAEV**R**$^{67}$**H**$^{68}$DVIA$^{72}$ |
| *H. pylori* | I$^{62}$EKTT**K**$^{67}$**H**$^{68}$DLIA$^{72}$ |
| *E. coli* | I$^{84}$ERTT**N**$^{89}$**H**$^{90}$DVKA$^{94}$ |
| *H. influenzae* | I$^{84}$ERTT**N**$^{89}$**H**$^{90}$DVKA$^{94}$ |
| *P. falciparum* | I$^{84}$EEET**N**$^{89}$**H**$^{90}$DVKA$^{94}$ |
| *M. thermoautotrophicum* | I$^{70}$ERDT**K**$^{75}$**H**$^{76}$DIAS$^{80}$ |
| *M. jannaschii* | I$^{82}$EKQT**K**$^{87}$**H**$^{88}$DVVA$^{92}$ |
| *S. cerevisiae* | Q$^{77}$EAIV**R**$^{82}$**H**$^{83}$DVMA$^{87}$ |
| *C. elegans* | E$^{76}$ERKL**K**$^{81}$**H**$^{82}$DVMA$^{86}$ |
| Chicken | E$^{55}$EKKL**R**$^{60}$**H**$^{61}$DVMA$^{65}$ |
| Murine | E$^{80}$EKRL**R**$^{85}$**H**$^{86}$DVMA$^{90}$ |
| Human | E$^{55}$EKRL**R**$^{60}$**H**$^{61}$DVMA$^{65}$ |
| | * • * *• • |

FIGURE 6: Amino acid sequence alignment of adenylosuccinate lyases from 15 species (partial). The sequences are taken from *Bacillus subtilis* (*12*), *Mycobacterium tuberculosis* (*13*), *Mycobacterium leprae* (*14*), *Synechocystis sp.* (*15*), *Helicobacter pylori* (*16*), *Escherichia coli* (*17*), *Haemophilus influenzae* (*18*), *Plasmodium falciparum* (*19*), *Methanobacterium thermoautotrophicum* (*20*), *Methanococcus jannaschii* (*21*), *Saccharomyces cerevisiae* (*22*), *Caenorhabditis elegans* (*23*), chicken (*24*), murine (*25*), and human (*26*). The sequences were analyzed by PC/GENE (IntelliGenetics Inc. and Genofit). Multiple sequence alignment was performed using the program CLUSTAL: *, positions that are perfectly conserved; ●, positions that are well conserved.

To investigate the functional importance of the two residues, site-directed mutant enzymes were characterized. The positively charged arginine was replaced by methionine, a neutral, hydrophobic amino acid of similar size, while histidine was initially replaced by glutamine, which is close to the wild-type amino acid in size but lacks its ability to act as a general acid−base catalyst. The single mutant enzyme R67M has almost the same specific activity as the wild-type enzyme, indicating that this arginine is not essential for activity. In contrast, substitutions for His$^{68}$ reduce the specific activity at least 100-fold, indicating the functional importance of His$^{68}$ in the catalytic reaction.

Further studies on the kinetic constants of the catalytic reaction reveal that the R67M mutant raises the $K_m$ for adenylosuccinate about 4-fold. Even though the $k_{cat}$ is actually increased 2-fold, the overall catalytic efficiency ($k_{cat}/K_m$) drops more than 2-fold. Thus, although Arg$^{67}$ is not critical for the activity, it appears to contribute to substrate binding, presumably through electrostatic interaction. In fact, all but 2 of the 15 known sequences have either arginine or lysine at this position (Figure 6).

Replacement of His$^{68}$ by glutamine also results in almost a 4-fold increase in $K_m$. However, a much more dramatic decrease was found (100-fold) in $k_{cat}$. The double mutant exhibits a combined effect, consistent with the results obtained from the single mutants. These results demonstrate that the major function of His$^{68}$ is catalysis. Histidine is

[2] In the studies of the rabbit muscle enzyme, 2-BDB-TAMP targets Arg$^{112}$ (numbering based on the human enzyme), equivalent to Arg$^{119}$ of the *Bacillus subtilis* enzyme (*10*). However, arginine at this position is less frequent than at positions corresponding to Arg$^{67}$. This difference in the reaction product of 2-BDB-TAMP with the mammalian and bacterial enzymes may reflect an actual difference in the binding site of the enzymes from these distinct species. It is notable that although the bacterial and mammalian enzymes catalyze the same reaction, the sequence of *B. subtilis* adenylosuccinate lyase exhibits only 25% identity plus 18% similarity when compared with the human enzyme.

similar in size, hydrophilicity, and hydrogen-bonding ability to glutamine, but is also capable of functioning as a proton donor−acceptor, consistent with the hypothesis that $His^{68}$ acts as a general base or a general acid. The complete abolition of activity observed in the H68K mutant provides additional evidence that $His^{68}$ is not functioning solely because it is positively charged.

$His^{141}$ has been identified in the active site of the *B. subtilis* enzyme (*32*). The equivalent histidines in other members of the superfamily have been shown to be important in catalysis. Most papers on the related enzymes propose a role as general base catalyst for the equivalent histidine, although there is still no direct evidence. If $His^{141}$ and $His^{68}$ are indeed the catalytic pair in adenylosuccinate lyase, the former is more likely to be the general base since it is conserved throughout the enzymes of the fumarase superfamily even though the substrates are structurally diverse. Although the general base abstracts the same $\beta$-H in the substrates of every enzyme of the fumarase family, the enzymic general acid may have different targets. In most cases, the general acid would protonate the α-nitrogen of the substrate to make it a better leaving group. For the reaction catalyzed by adenylosuccinate lyase, the protonation of the substrate may occur at the ring nitrogen (1-N), as proposed by Hanson and Havir (*47*). The difference in the substrates may result in a change in the enzymic general acid among the members of the fumarase family. In fact, in contrast to $His^{141}$, the sequence flanking $His^{68}$ is not conserved across the family, suggesting its specific role in the adenylosuccinate lyases. Thus, $His^{68}$ may be a good candidate for a proton donor in the catalytic reaction.

Overall, our results suggest that $Arg^{67}$ and $His^{68}$ reside in the active site of *B. subtilis* adenylosuccinate lyase. $Arg^{67}$ is not required for the activity, although it may enhance substrate binding through electrostatic interactions with the carboxylates of the substrate. $His^{68}$ clearly plays a role in catalysis and could act either as a general acid in donating a proton to the ring nitrogen or as a general base in abstracting the $\beta$-H. Crystallization of the *B. subtilis* adenylosuccinate lyase has been reported (*39*). The structural studies will allow comparisons to be made with our solution studies on this bacterial enzyme.

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