Implication of Arginine-131 and Arginine-303 in the Substrate Site of Adenylosuccinate Synthetase of *Escherichia coli* by Affinity Labeling with 6-(4-Bromo-2,3-dioxobutyl)thioadenosine 5′-Monophosphate†

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ABSTRACT: Adenylosuccinate synthetase from *Escherichia coli* is inactivated in a biphasic reaction by 6-(4-bromo-2,3-dioxobutyl)thioadenosine 5′-monophosphate (6-BDB-TAMP) at pH 7.0 and 25 °C. The initial fast-phase inactivation is not affected by the presence of active-site ligands and can be completely eliminated by blocking Cys^{291} of the enzyme with *N*-ethylmaleimide (NEM). Reaction of the NEM-treated enzyme with 6-BDB-[^{32}P]TAMP results in 2 mol of reagent incorporated/mol of enzyme subunit. The inactivation kinetics of the slow-phase exhibit an apparent K_I of 40.6 μM and k_{max} of 0.0228 min^{-1}. Active-site ligands, either adenylosuccinate or IMP and GTP, completely prevent inactivation of the enzyme by 6-BDB-TAMP, whereas IMP or IMP and aspartate is much less effective in protection. 6-BDB-TAMP-inactivated enzyme has a 3-fold increase in K_m for aspartate with no change in K_m for IMP or GTP. Protease digestion of 6-BDB-[^{32}P]TAMP inactivated enzyme reveals that both Arg^{131} and Arg^{303} are modified by the affinity-labeling reagent. The crystal structure [Poland, B. W., Fromm, H. J., and Honzatko, R. B. (1996) *J. Mol. Biol. 264*, 1013−1027] and site-directed mutagenesis [Kang, C., Sun, N., Poland, B. W., Gorrell, A., and Fromm, H. J. (1997) *J. Biol. Chem. 272*, 11881−11885] of *E. coli* adenylosuccinate synthetase show that Arg^{303} interacts with the carboxyl group of aspartate and the 2′-OH of the ribose of IMP and Arg^{131} is involved in stabilizing aspartate in the active site of the enzyme. We conclude that 6-BDB-TAMP functions as a reactive adenylosuccinate analogue in modifying both Arg^{131} and Arg^{303} in the active site of adenylosuccinate synthetase.

Adenylosuccinate synthetase [IMP:L-aspartate ligase (GDP forming), EC 6.3.4.4] catalyzes the first step in the conversion of IMP to AMP in de novo purine nucleotide metabolism:

$$\text{IMP} + \text{GTP} + \text{L-aspartate} \overset{Mg^{2+}}{\rightleftharpoons} \text{adenylosuccinate} + \text{GDP} + P_i$$

Adenylosuccinate is then cleaved by adenylosuccinate lyase to produce fumarate and AMP. Three alternative mechanisms of catalysis for adenylosuccinate synthetase have been proposed (*1*). The favored mechanism involves an initial nucleophilic attack by the C-6 oxygen of IMP on the γ-phosphorus atom of GTP to yield 6-phosphoryl IMP. In the next step, nucleophilic attack by the nitrogen of aspartate occurs on the C-6 of 6-phosphoryl IMP, with the displacement of inorganic phosphate and production of adenylosuccinate.

Adenylosuccinate synthetase has been isolated from many sources including *Escherichia coli* (*2*−*4*), *Bacillus subtilis* (*5*), *Azotobacter vinelandii* (*6*), *Schizosaccharomyces pombe* (*7*), *Dictyostelium discoideum* (*8*), rat liver (*9*, *10*), and rat muscle (*11*, *12*). *E. coli* adenylosuccinate synthetase is a homodimer, each subunit consisting of 431 amino acids and exhibiting a molecular mass of 47 kDa (*13*, *14*). The *purA* gene encoding for *E. coli* adenylosuccinate synthetase was isolated and cloned into an overexpression vector by Bass et al. (*13*) and Wolfe and Smith (*15*). The active site of the enzyme has been studied by chemical modification (*16*−*20*), site-directed mutagenesis (*21*−*24*), and X-ray crystallography (*25*−*29*). Since adenylosuccinate synthetase is a target of a natural herbicide (*30*) and of a drug used to treat pediatric leukemia (*31*), further studies may enhance understanding of their mechanism of action and improve design of new drugs.

Affinity labeling previously done in this laboratory using a nucleotide analogue, guanosine 5′-*O*-[*S*-(4-bromo-2,3-

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[1] Abbreviations: NEM, *N*-ethylmaleimide; 6-BDB-TAMP, 6-(4-bromo-2,3-dioxobutyl)thioadenosine 5′-monophosphate; 6-HDB-TAMP, 6-(4-hydroxy-2,3-dioxobutyl)thioadenosine 5′-monophosphate; DBBD, 1,4-dibromo-2,3-butanedione; GTP, guanosine 5′-triphosphate; IMP, inosine 5′-monophosphate; $NaBH_4$, sodium borohydride; $POCl_3$, phosphorus oxychloride; GMPS−BDB, guanosine 5′-*O*-[*S*-(4-bromo-2,3-dioxobutyl)thio]phosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Mes, 2-(*N*-morpholino)ethanesulfonic acid; HPLC, high-performance liquid chromatography; AMPSBDB, adenosine 5′-*O*-[*S*-(4-bromo-2,3-dioxobutyl)thio]phosphate.

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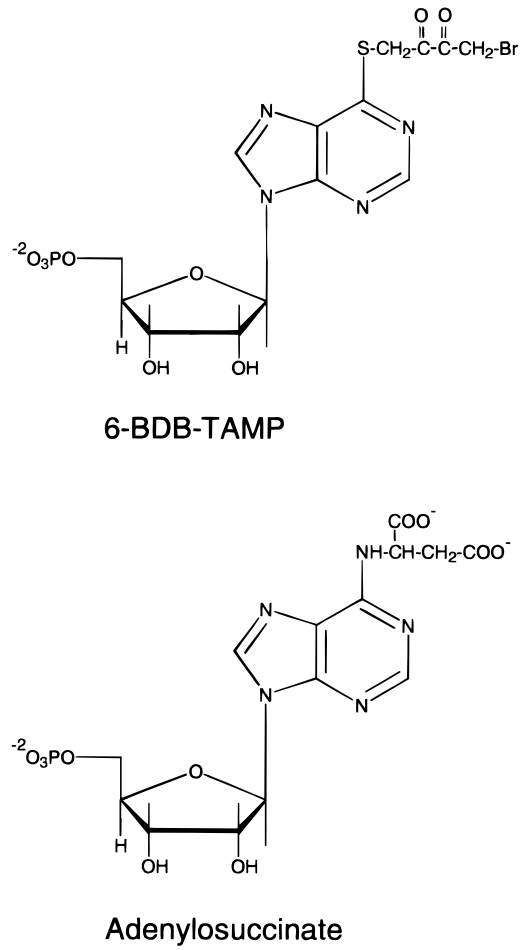

FIGURE 1: Schematic structures of 6-(4-bromo-2,3-dioxobutyl)-thioadenosine 5′-monophosphate (6-BDB-TAMP) and adenylosuccinate.

dioxobutyl)thio]phosphate (GMPS−BDB),[1] revealed that Arg[143] is the target site of the compound (*20*). Arg[143] from one subunit interacts with the 5′-phosphate of IMP in the other subunit. To better understand the nature of the enzyme-ligand interactions in solution, a different nucleotide affinity label, 6-(4-bromo-2,3-dioxobutyl)thioadenosine 5′-monophosphate (6-BDB-TAMP), was used in this study.

6-BDB-TAMP is a reactive nucleotide analogue first synthesized in this laboratory (*32*), which is structurally similar to adenylosuccinate (*33*), as shown in Figure 1. The 6-BDB-TAMP contains a reactive bromodioxobutyl group at a position equivalent to that of the succinyl moiety of adenylosuccinate. The bromoketo group of 6-BDB-TAMP can potentially react with most nucleophilic amino acid residues such as Lys, Tyr, Cys, and His, and the dioxo group is most likely to react with Arg. Our results indicate that 6-BDB-TAMP modifies both Arg[131] and Arg[303], residues which are involved in aspartate binding as well as catalysis (*23*, *24*). A preliminary version of this work has been presented (*34*).

EXPERIMENTAL PROCEDURES

Materials. *N*-Ethylmaleimide, dithiothreitol, triethylamine, 6-mercaptopurine riboside, 6-mercaptopurine 5′-phosphate (sodium salt), adenylosuccinate, L-aspartic acid, guanosine 5′-triphosphate, inosine 5′-monophosphate, bovine serum albumin, sodium borohydride, Sephadex G-50, Pipes, Mes, Hepes, *Staphylococcus* protease from *Staphylococcus aureus* (strain V8), papain, and pepsin were purchased from Sigma Chemical Co. Commercially obtained papain was further purified by reversed-phase chromatography (Vydac C_4 column) according to the method described by Gite and Colman (*35*). 1,4-Dibromo-2,3-butanedione, phosphorus oxychloride, and trifluoroacetic acid were obtained from Aldrich Chemical Co. 1,4-Dibromo-2,3-butanedione was recrystallized from petroleum ether prior to use. [^{32}P]-Phosphoric acid was from New England Nuclear. Acetonitrile, glacial acetic acid, ammonium acetate, potassium phosphate were from Fisher Scientific. AG 50W-X4 cation-exchange resin and Coomassie Blue concentrated solution were obtained from Bio-Rad Laboratories. *E. coli* adenylosuccinate synthetase was isolated and purified by the methods previously described (*13*, *36*). The protein concentration was determined spectrophotometrically using $E^{0.1\%}_{280nm} = 1.05$ (*20*); i.e., the absorbance at 280 nm was divided by 1.05 to obtain the adenylosuccinate synthetase concentration in mg/mL.

Synthesis of 6-(4-Bromo-2,3-dioxobutyl)thioadenosine 5′-Monophosphate. 6-BDB-TAMP was synthesized by the method of Colman et al. (*32*). Briefly, the sodium salt of 6-mercaptopurine ribonucleoside 5′-monophosphate (40 μmol) was dissolved in 0.5 mL of methanol. Recrystallized 1,4-dibromo-2,3-butanedione (DBBD, 1.2 mmol), which was dissolved in 0.5 mL of methanol was added to the above 6-mercaptopurine ribonucleoside 5′-monophosphate solution. The reaction was allowed to proceed at room temperature for 1 h. Periodically, the pH of the reaction mixture was checked by pH paper and adjusted to 5.5 by either triethylamine or acetic acid. The progress of the coupling reaction was monitored by measuring the decrease in absorbance at 322 nm and increase in absorbance at 284 nm. As described previously (*32*), the 6-mercaptopurine ribonucleoside 5′-monophosphate exhibits a UV peak at 322 nm; during the reaction, this 322 nm peak decreases and is replaced by the UV peak at 284 nm of 6-BDB-TAMP. At the end of the reaction, 6-BDB-TAMP was obtained by diethyl ether precipitation and was dissolved in 10 mM Mes buffer, pH 4.5. The concentration of 6-BDB-TAMP was determined spectrophotometrically using an extinction coefficient of 1.6×10^4 M^{-1} cm^{-1} at 284 nm (*32*).

Synthesis of Radioactive 6-BDB-[^{32}P]TAMP. Radioactive 6-BDB-TAMP was synthesized by the method described by Lee et al. (*33*). To produce 6-BDB-[^{32}P]TAMP with high specific radioactivity, 5 mCi of aqueous [^{32}P]phosphoric acid was dried in a desiccator under vacuum overnight. Radioactive phosphorus was introduced into phosphorus oxychloride ($POCl_3$) by the method of Keenan et al. (*37*). [^{32}P]$POCl_3$ (20 μL) was added to 6-mercaptopurine riboside (0.1 mmol) to produce radioactive 6-mercaptopurine ribonucleoside 5′-monophosphate. The reaction of DBBD with the radioactive phosphorylated ribonucleoside was carried out using the same procedures described above for the nonradioactive 6-BDB-TAMP.

Reaction of E. coli Adenylosuccinate Synthetase with N-Ethylmaleimide. Previous studies showed that cysteine[291] of adenylosuccinate synthetase, which is not involved in catalysis, covalently reacts with a nucleotide analogue causing rapid partial loss of the enzyme activity (*20*).

Cysteine^{291} is a surface cysteine and is the only cysteine residue which reacts with *N*-ethylmaleimide (NEM) under nondenaturing conditions (*17*); the NEM-treated enzyme retains most of its original activity (~85%). To prevent the interaction of Cys^{291} with 6-BDB-TAMP, adenylosuccinate synthetase in this study was treated with NEM prior to 6-BDB-TAMP inactivation. The enzyme was dialyzed against 50 mM Pipes buffer, pH 7.0, containing 100 μM EDTA prior to use. Adenylosuccinate synthetase (2 mg/mL, 42.6 μM subunit) was incubated with 4 mM NEM in 50 mM Pipes buffer, pH 7.0, at 25 °C for an hour. An aliquot (2 μL) of enzyme was withdrawn at various times and was assayed for enzyme activity. Dithiothreitol was added to the mixture to yield a final concentration of 50 mM in order to stop the NEM reaction. The NEM-treated enzyme was dialyzed at 4 °C against 50 mM Pipes, pH 7.0, containing 100 μM EDTA.

Enzyme Assay. The enzyme activity was determined at 25 °C by measuring the increase in absorbance at 280 nm which results from the conversion of IMP to adenylosuccinate ($\epsilon_{280} = 1.17 \times 10^4$ M^{-1} cm^{-1}) (*4*). The standard assay solution used to monitor enzyme inactivation contained 10 mM $MgCl_2$, 5 mM aspartate, 300 μM IMP, and 150 μM GTP in 20 mM Hepes, pH 7.7, in a total volume of 1 mL. To determine the K_m values for GTP of NEM-modified and 6-BDB-TAMP-reacted enzymes, aspartate, and IMP concentrations were fixed at 5 mM and 450 μM, respectively. To determine the K_m values for aspartate, GTP was fixed at 300 μM and IMP at 450 μM. The K_m values for IMP were determined using an assay solution containing 300 μM GTP and 5 mM aspartate. The concentrations of $MgCl_2$ and Hepes were maintained at the same level as in the standard enzyme activity assay.

Inactivation of E. coli Adenylosuccinate Synthetase by 6-BDB-TAMP. Adenylosuccinate synthetase (0.15 mg/mL, 3.2 μM subunit) was preincubated in 50 mM Pipes buffer, pH 7.0, at 25 °C for 30 min prior to the addition of 25−600 μM 6-BDB-TAMP. At various times, 20 μL aliquots of the inactivation mixture were withdrawn and added to 1 mL of substrate mixture to assay for residual enzyme activity. An enzyme control in the absence of 6-BDB-TAMP was set up and assayed under the same conditions as for the enzyme incubated with 6-BDB-TAMP. To study the ability of ligands to protect the NEM-treated adenylosuccinate synthetase against inactivation by 50 μM 6-BDB-TAMP, the enzyme was incubated with ligands for 30 min in 50 mM Pipes buffer, pH 7.0, prior to initiation of the reaction by addition of 6-BDB-TAMP.

Incorporation of 6-BDB-[^{32}P]TAMP into E. coli Adenylosuccinate Synthetase. Adenylosuccinate synthetase (0.15 mg/mL, 3.2 μM subunit) was incubated with 50 μM 6-BDB-[^{32}P]TAMP in 50 mM Pipes buffer, pH 7.0, at 25 °C. The reaction of the enzyme with 6-BDB-[^{32}P]TAMP was stopped at various times by adding to the reaction mixture 0.2 M $NaBH_4$ (dissolved in 0.02 M NaOH) to yield a final concentration of 2 mM. The $NaBH_4$ reduces the dioxo group of 6-BDB-TAMP, thereby greatly decreasing the reactivity of the compound toward the enzyme. Five minutes after the addition of $NaBH_4$, the free 6-BDB-[^{32}P]TAMP was separated from the enzyme by column centrifugation (*38*) using a 5 mL column of Sephadex G-50 which had been equilibrated with 50 mM Pipes, pH 7.0, containing 5% glycerol. To determine the incorporation of 6-BDB-TAMP at time 0, 50 μM 6-BDB-TAMP was first incubated with $NaBH_4$ for 5 min prior to addition of enzyme. After the Sephadex G-50 column centrifugation, the enzyme concentration was determined by a dye-binding Bradford method (*39*). The NEM-treated enzyme, the concentration of which had been determined spectrophotometrically, was used to generate a protein standard curve. The incorporation of 6-BDB-[^{32}P]-TAMP was determined by measuring the radioactivity of enzyme aliquots using a Packard Tri-Carb liquid scintillation counter (model 4640). In order to evaluate the effects of active-site ligands on the incorporation of 6-BDB-TAMP, the enzyme was incubated in the presence or absence of ligands for 30 min before the addition of radioactive 6-BDB-TAMP. The incorporation of 6-BDB-[^{32}P]TAMP and protein concentration were measured as described above.

Proteolytic Digestion of E. coli Adenylosuccinate Synthetase. Adenylosuccinate synthetase (0.5 mg/mL, 10.6 μM subunit) was incubated with 100 μM 6-BDB-[^{32}P]TAMP as described above. The free 6-BDB-[^{32}P]TAMP was separated from the enzyme by one Sephadex G-50 column centrifugation after an hour inactivation at 25 °C. The enzyme was then dialyzed overnight at 4 °C against 50 mM potassium phosphate buffer, pH 7.8, for V8 protease digestion overnight at 4 °C. The protease from *Staphylococcus aureus* strain V8 (V8 protease) was used directly from the commercial source without further purification. The V8 protease digestion (15% protease based on the quantity of the enzyme) was conducted at 37 °C for 5 h.

Fractionation of 6-BDB-[^{32}P]TAMP-labeled Peptides by Reversed-Phase HPLC. The adenylosuccinate synthetase digest was centrifuged at 14 000 rpm for 2 min to remove any precipitate, and the supernatant was filtered through a 0.45 μM Millipore membrane filter disk prior to analysis by reversed-phase high-performance liquid chromatography (Varian model 5000) equipped with a Vydac C_{18} column (0.46 × 25 cm). The chromatography was conducted at a flow rate of 1 mL/min of 0.1% TFA in water (solvent A) for 10 min, followed by a linear gradient from 0 to 30% of 0.075% TFA in acetonitrile (solvent B) for 120 min and, finally, a linear gradient from 30 to 100% of solvent B for 30 min. The effluent was monitored at 220 nm and fractions were measured for radioactivity using a liquid scintillation counter. HPLC fractions with high radioactivity were pooled and lyophilized for amino acid sequence determination or papain digestion. The lyophilized radioactive fractions were redissolved in 20 mM ammonium acetate, pH 4.0, and were digested by the purified papain. A total of 3% of purified papain, based on the original quantity of the inactivated enzyme, was added to the digestion mixture. The papain digestion was conducted at 28 °C for 2 h. The digested peptides were separated by HPLC under the same conditions used for the V8 protease digest. Amino acid sequence analyses were performed on an Applied Biosystems gas-phase protein sequences, model 470, equipped with an on-line phenylthiohydantoin (PTH) analyzer model 120 and computer model 900A.

RESULTS

Inactivation of Adenylosuccinate Synthetase by 6-BDB-TAMP. Adenylosuccinate synthetase (0.15 mg/mL, 3.2 μM

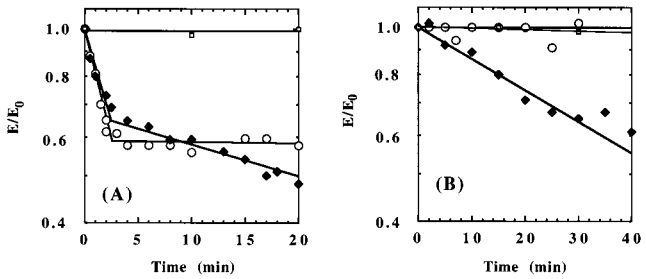

FIGURE 2: Inactivation of *E. coli* adenylosuccinate synthetase by 6-BDB-TAMP. The enzyme was incubated in 50 mM Pipes, pH 7.0, at 25 °C. At the indicated times, aliquots were withdrawn and assayed as described in the Experimental Procedures. (A) Native enzyme (0.15 mg/mL) incubated in the absence of 6-BDB-TAMP (□) or in the presence of 50 μM 6-BDB-TAMP (◆) or 50 μM 6-BDB-TAMP + 500 μM adenylosuccinate + 10 mM $MgCl_2$ (○). (B) NEM-treated enzyme (0.15 mg/mL) incubated under the same conditions as in panel A.

subunit) is inactivated as a function of time by 50 μM 6-BDB-TAMP in 50 mM Pipes, pH 7.0, and 25 °C. Activity loss of native enzyme follows a biphasic, time-dependent decay, as shown in Figure 2A. Native enzyme, incubated under the same conditions but in the absence of 6-BDB-TAMP, shows no activity loss during the entire period. Incubation of the enzyme with 500 μM adenylosuccinate prevents the second inactivation phase by 6-BDB-TAMP, but has no effect on the first phase inactivation.

Each subunit of adenylosuccinate synthetase contains four cysteine residues. However, Dong et al. (*17*) have shown that only Cys^{291} is modified by NEM under nondenaturing conditions and that the blocking of Cys^{291} by NEM does not cause significant activity loss (the enzyme still retains ~85% of its original activity).[2] Accordingly, the enzyme was incubated with NEM to block Cys^{291} prior to addition of 6-BDB-TAMP. Incubation of 6-BDB-TAMP with the NEM-treated enzyme gives a linear plot of $\ln(E/E_o)$ versus time (Figure 2B). This NEM-treated enzyme was used for all subsequent experiments. Adenylosuccinate protects completely against inactivation of the NEM-treated enzyme by 6-BDB-TAMP. After about 30 min, the rate of inactivation of the enzyme by 6-BDB-TAMP decreases. The decrease in the inactivation rate is due to two factors. First, 6-BDB-TAMP is known to undergo decomposition in aqueous buffers with release of free bromide to yield 6-(4-hydroxy-2,3-dioxobutyl)thioadenosine 5′-monophosphate (6-HDB-TAMP). The rate of decomposition of a similar compound, 6-(4-bromo-2,3-dioxobutyl)thioadenosine 5′-diphosphate, in 50 mM potassium phosphate buffer, pH 7.1, containing 10% methanol, at 25 °C, has been determined as 0.0114 min^{-1} ($t_{1/2}$ = 61 min) (*40*). No loss of activity is observed when adenylosuccinate synthetase is incubated with 6-HDB-TAMP. The decomposition of 6-BDB-TAMP results in a decreased concentration of reactive compound and, thus, in a decrease of the inactivation rate. The second factor contributing to the decrease in the rate of inactivation of adenylosuccinate synthetase by 6-BDB-TAMP, is the competition between the hydrolyzed compound and the reactive 6-BDB-TAMP for binding to the enzyme at the adenylosuccinate site. Table 1 (last line) shows that hydrolyzed 6-HDB-TAMP protects the enzyme against 6-BDB-TAMP inactivation. The 6-HDB-TAMP has also been shown to protect adenylosuccinate lyase against inactivation by 6-BDB-TAMP (*33*). Thus, the curvature in the Figure 2 plot can be attributed both to loss of the original 6-BDB-TAMP by hydrolysis and to the action of the hydrolyzed compound in preventing further inactivation. The residual activity decreased further (to 23%) after removal of 6-HDB-TAMP from the inactivation mixture by Sephadex G-50 spin column chromatography and the addition of fresh 6-BDB-TAMP (50 μM) to the enzyme.

Table 1: Effect of Active-Site Ligands on Inactivation of Adenylosuccinate Synthetase (NEM-Modified) by 50 μM 6-BDB-TAMP[a]

ligands added to incubation mixture	k_{obs} (min^{-1})	k_+/k_-
none	0.0141	1.00
10 mM $MgCl_2$	0.0150	1.06
10 mM $MgCl_2$ + 1 mM GTP	0.0124	0.88
10 mM $MgCl_2$ + 5 mM aspartate	0.0149	1.06
10 mM $MgCl_2$ + 1 mM GTP + 5 mM aspartate	0.0103	0.73
10 mM $MgCl_2$ + 5 mM IMP	0.0046	0.33
10 mM $MgCl_2$ + 1 mM IMP + 5 mM aspartate	0.0058	0.41
10 mM $MgCl_2$ + 1 mM IMP + 1 mM GTP	0.0000	0.00
10 mM $MgCl_2$ + 500 μM adenylosuccinate	0.0000	0.00
300 μM 6-HDB-TAMP	0.0020	0.14

[a] Reaction was conducted in 50 mM Pipes buffer, pH 7.0, at 25 °C, as described in the Experimental Procedures. The value k_+/k_- is the ratio of the rate constant for inactivation in the presence of an active site ligand to that in its absence.

Rate of Inactivation of Adenylosuccinate Synthetase as a Function of 6-BDB-TAMP Concentration. NEM-treated adenylosuccinate synthetase (0.15 mg/mL, 3.2 μM subunit) was incubated with 25−600 μM 6-BDB-TAMP. The apparent rate constant (k_{obs}) of inactivation was calculated from the slope of $\ln(E/E_o)$ versus time, in which E is the enzyme activity at time t and E_o is the activity at time zero. A nonlinear relationship is observed when the initial inactivation rate constant is plotted versus the concentration of 6-BDB-TAMP (Figure 3). At high concentrations of 6-BDB-TAMP, the enzyme becomes saturated with the reagent and the rate constant approaches a maximum (k_{max}). This observation supports the reversible binding of 6-BDB-TAMP with adenylosuccinate synthetase before inactivation and can be described by the equation, $1/k_{obs} = 1/k_{max} + K_I/k_{max}$ (1/[6-BDB-TAMP]). The maximum rate of inactivation (k_{max}) and apparent dissociation constant (K_I) were determined as 0.0228 min^{-1} and 40.6 μM, respectively.

Effect of Ligands on the Inactivation of Adenylosuccinate Synthetase by 6-BDB-TAMP. The ability of various ligands to protect the NEM-treated adenylosuccinate synthetase (0.15 mg/mL, 3.2 μM subunit) against inactivation by 50 μM 6-BDB-TAMP is listed in Table 1. The concentrations of adenylosuccinate, GTP, and IMP used in the studies were high relative to the K_m values reported previously (K_m = 1.8 μM for adenylosuccinate, K_m = 37.7 μM for IMP, and K_m = 23.7 μM for GTP) (*18*). $MgCl_2$ alone or combined with either GTP or aspartate does not protect adenylosuccinate synthetase against inactivation by 6-BDB-TAMP. The substrate IMP or IMP plus aspartate only partially protects the enzyme against inactivation. In contrast, either IMP plus GTP or adenylosuccinate completely protects the enzyme

[2] It has previously been shown that modification of Cys^{291} of *E. coli* adenylosuccinate synthetase by GMPS−BDB, which is a considerably larger compound than NEM, causes loss of about 50% enzymatic activity (*20*).

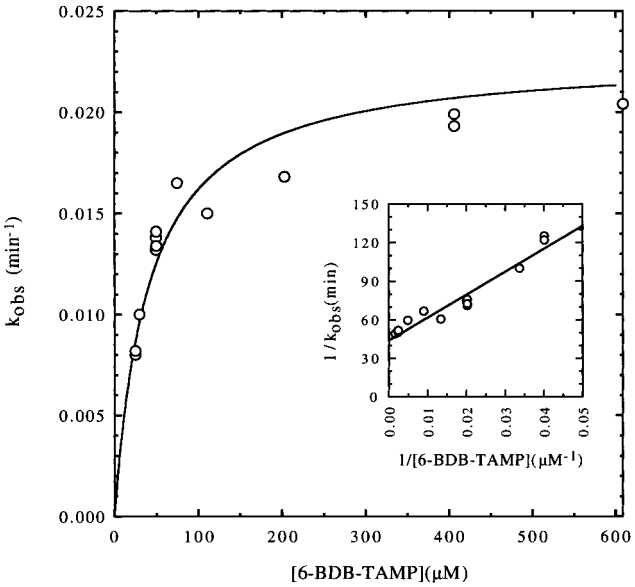

FIGURE 3: Dependence of initial rate constant of inactivation of adenylosuccinate synthetase on the concentration of 6-BDB-TAMP. NEM-modified adenylosuccinate synthetase (0.15 mg/mL) was incubated with 25−600 μM 6-BDB-TAMP in 50 mM Pipes, pH 7.0. The rate constants were measured from the first 30 min of the reaction, as illustrated in Figure 2B. (Inset) Double-reciprocal plot of $1/k_{obs}$ against 1/[6-BDB-TAMP].

against 6-BDB-TAMP inactivation. These results suggest that reaction occurs in the region of the active site best occupied by the product adenylosuccinate.

The hydrolyzed compound, 6-HDB-TAMP (lacking Br^-), does not inactivate adenylosuccinate synthetase; however, it does protect the enzyme against inactivation by fresh 6-BDB-TAMP (Table 1, last line). This observation suggests that 6-HDB-TAMP occupies the active site of the enzyme but does not form a covalent linkage to the enzyme as does fresh 6-BDB-TAMP.

Incorporation of 6-BDB-[^{32}P]TAMP into Adenylosuccinate Synthetase. Adenylosuccinate synthetase (0.15 mg/mL, 3.2 μM subunit) was incubated with 50 μM 6-BDB-[^{32}P]TAMP in 50 mM Pipes, pH 7.0, and 25 °C. The inactivation was stopped at various times by adding $NaBH_4$, after which the incorporated 6-BDB-[^{32}P]TAMP was measured as described in the Experimental Procedures. A time-dependent incorporation of 6-BDB-[^{32}P]TAMP was observed concomitant with the decrease in enzyme activity. Figure 4 shows a plot of the percentage of residual activity versus incorporation; extrapolation to 0% residual activity yields 1.88 mol of 6-BDB-[^{32}P]TAMP incorporated per mol of adenylosuccinate synthetase subunit.

The effects of active-site ligands on the 6-BDB-[^{32}P]-TAMP incorporation at a single time were tested with the results shown in Table 2. In the absence of any ligands, 0.85 mol of 6-BDB-[^{32}P]TAMP/mol subunit was incorporated into the NEM-treated enzyme, while the enzyme retained 55% of its activity. Enzyme incubated with ligands such as $MgCl_2$ or $MgCl_2$ plus GTP or aspartate had similar 6-BDB-[^{32}P]-TAMP incorporation and residual activity as in the absence of ligands. In the presence of $MgCl_2$ plus either adenylosuccinate or IMP plus GTP, the 6-BDB-[^{32}P]TAMP incorporation was reduced, respectively, to 0.11 and 0.17 mol/mol subunit, while the enzyme retained 94% of its activity. These results show that the product adenylosuccinate or IMP plus GTP decreases the reagent incorporation as well as the inactivation.

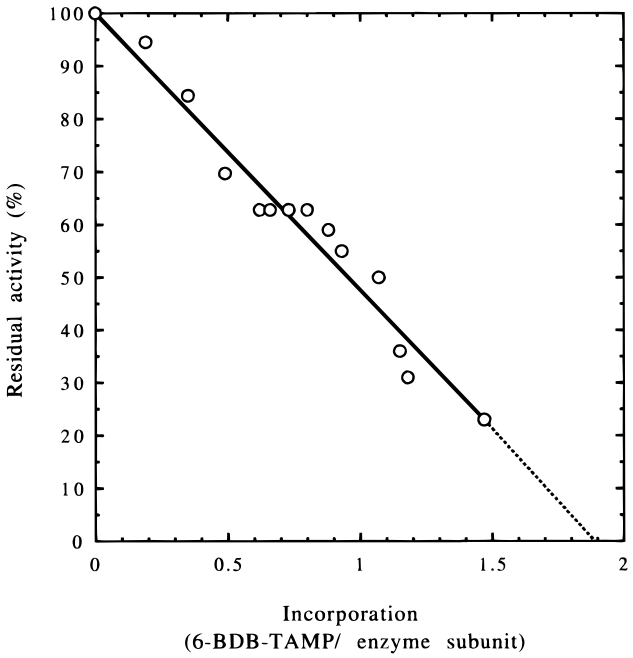

FIGURE 4: The incorporation of 6-BDB-[^{32}P]TAMP into the NEM-treated enzyme as a function of the percentage of residual activity. Extrapolation to complete inactivation of the enzyme indicates that 1.88 mol of reagent is incorporated per mol of enzyme subunit.

Table 2: Effect of Active-Site Ligands on Incorporation of 50 μM 6-BDB-TAMP and Residual Activity of Adenylosuccinate Synthetase[a]

ligands added to incubation mixture	incorporation (6-BDB/TAMP/enzyme subunit)	residual activity (%)
none	0.85	55
10 mM $MgCl_2$	0.84	59
10 mM $MgCl_2$ + 1 mM GTP	0.62	53
10 mM $MgCl_2$ + 5 mM aspartate	0.72	50
10 mM $MgCl_2$ + 1 mM GTP + 5 mM aspartate	0.60	61
10 mM $MgCl_2$ + 5 mM IMP	0.42	65
10 mM $MgCl_2$ + 1 mM IMP + 5 mM aspartate	0.38	63
10 mM $MgCl_2$ + 1 mM IMP + 1 mM GTP	0.17	94
10 mM $MgCl_2$ + 500 μM adenylosuccinate	0.11	94

[a] Reactions were conducted in 50 mM Pipes buffer, pH 7.0, at 25 °C as described in the Experimental Procedures. After 1 h of reaction with a single addition of 50 μM 6-BDB-TAMP, the residual activity was measured and expressed as percentage of the original activity. The radioactivity was measured by a Packard Tricarb model 4640 liquid scintillation counter.

Kinetic Studies of NEM-Treated and 6-BDB-TAMP-Inactivated Adenylosuccinate Synthetase. To evaluate whether covalent reaction with the nucleotide analogue produced an enzyme with altered kinetic characteristics, NEM-treated adenylosuccinate synthetase was incubated with 50 μM 6-BDB-TAMP at pH 7.0 for 1 h. The enzyme lost about 50% of its activity (i.e., as measured in the standard assay) and contained on average about 1 mol of reagent/mol of enzyme subunit after 6-BDB-TAMP treatment. For 50% active enzyme, one must consider the possibility that the

Table 3: K_m Values of NEM-Modified and 6-BDB-TAMP-Modified NEM-Treated Adenylosuccinate Synthetase[a]

enzyme	K_m^{GTP} (μM)	K_m^{IMP} (μM)	K_m^{ASP} (μM)
NEM modified	15	10	518
6-BDB-TAMP modified	18	12	1650

[a] Kinetic studies were carried out at 25 °C in 20 mM Hepes, pH 7.7, as described in the Experimental Procedures. 6-BDB-TAMP-modified enzyme retained 50% of the original activity.

enzyme sample is a mixture of fully active enzyme and completely inactive enzyme with 2 mol of 6-BDB-TAMP incorporated/mol of enzyme subunit. Alternatively, one target site of 6-BDB-TAMP may react much faster than the second site, so that at 50% residual activity, one modified site/subunit predominates with almost no unmodified enzyme and relatively little doubly modified enzyme subunits. We consider that the alternate model best describes the reaction of adenylosuccinate synthetase with 6-BDB-TAMP.

The K_m values for GTP, IMP, and aspartate of NEM-modified or 6-BDB-TAMP-inactivated NEM enzyme (~50% residual activity) are listed in Table 3. The 6-BDB-TAMP-inactivated enzyme showed a 3-fold increase in the K_m for aspartate. This result argues against the presence of appreciable amounts of unmodified enzyme, since that active enzyme would exhibit the same kinetic parameters as native, unmodified enzyme. In contrast, neither the K_m for IMP nor GTP was significantly altered by 6-BDB-TAMP modification. These data suggest that 6-BDB-TAMP might react at or near the aspartate active site of adenylosuccinate synthetase; however, since the standard assay contains 5 mM aspartate, the effect on K_m for aspartate does not completely account for the inactivation observed.

Peptide Mapping of 6-BDB-[^{32}P]TAMP-Modified Adenylosuccinate Synthetase. The modified enzyme was digested by V8 protease in 50 mM potassium phosphate, pH 7.8, at 37 °C for 5 h. Figure 5 illustrates the radioactive peak distribution of V8-digested peptides separated by HPLC on a C_{18} column. Two major radioactive peptide peaks were observed (peaks I and II). Protective ligands, such as adenylosuccinate, decrease both radioactive peptide peaks (data not shown). The 6-BDB-TAMP incubated under the same conditions but in the absence of adenylosuccinate synthetase yields radioactive peaks which are eluted before 72 mL. Thus, the radioactive peak centered at 70 mL in Figure 5 is attributed to decomposed, free 6-BDB-[^{32}P]TAMP which presumably dissociated from modified enzyme during the digestion procedure at pH 7.8.

Table 4 shows the amino acid sequences of two samples of peak II (Figure 5), revealing the peptide Phe^{297}−Asp^{311} from the known sequence of adenylosuccinate synthetase. The yield of PTH-amino acid generally decreases as the number of Edman degradation cycles increases, as is expected. Arginine is the most likely reaction target within that sequence. It has previously been found that, upon sequencing, products of arginine and bromodioxobutyl nucleotides are not eluted at the normal position of any PTH-amino acid and are associated with a decreased yield of PTH−Arg (*20*, *41*). In this case, Arg^{303} is considered to be the most likely residue modified by 6-BDB-TAMP because of the lower yield of PTH derivative in that cycle. In fact, the yield is lower for PTH−Arg^{303} than it is for arginines found at later positions in the peptide.

Peak I contains the peptide Lys^{119}−Glu^{138} (data not shown). Since this peptide contains both Arg^{121} and Arg^{131}, peak I from the V8 digest (Figure 5) was further cleaved by papain in 20 mM ammonium acetate, pH 4.0, and rechromatographed in the same HPLC system. One radioactive peak (centered at 73 min) was observed after papain redigestion (Figure 6). The sequence of the peptide isolated after redigestion by papain reveals peptide Arg^{131}−Glu^{138} (Table 4). Arg^{131} is designated the amino acid modified by 6-BDB-[^{32}P]TAMP because of the low yield of PTH-amino acid. Liquid scintillation counting of the fractions from each sequencing cycle, and the application filter, indicates that the radioactivity does not elute with any PTH derivative but largely remains on the application filter. This observation suggests that the PTH derivatives of these nucleotide-modified peptides are too hydrophilic to be extracted by the organic solvent used in the sequencer. Similar results have been observed for peptides isolated from GMPS−BDB-modified adenylosuccinate synthetase (*20*) and 6-BDB-TAMP-modified adenylosuccinate lyase (*33*). The PTH−Arg seen is probably due to partial regeneration of the modified residue under the strongly acidic conditions to which it is exposed during gas-phase sequencing.

As an alternate method of identifying the target amino acids, modified enzyme was digested with pepsin at pH 2.0, conditions under which arginine derivatives are more stable (*41*). Pepsin is a relatively nonspecific protease, and numerous radioactive peaks were observed from the HPLC pattern of pepsin digest (data not shown). However, all the sequences of radioactive peptides found in the pepsin digest correspond to peptides containing either Arg^{131} or Arg^{303}. Both proteolysis procedures thus indicate that the two amino acids of adenylosuccinate synthetase modified by 6-BDB-TAMP are Arg^{131} and Arg^{303}.

DISCUSSION

The reactive compound 6-(4-bromo-2,3-dioxobutyl)thioadenosine 5′-monophosphate is structurally very similar to adenylosuccinate, and the results presented here show that it functions as an affinity label of that substrate site of adenylosuccinate synthetase. Indeed, 6-BDB-TAMP has recently been demonstrated to react at the substrate site of the next enzyme in the metabolic sequence leading to AMP formation: adenylosucccinate lyase (*33*). Initially, our analysis of the specific reaction of adenylosuccinate synthetase with 6-BDB-TAMP was complicated by rapid partial inactivation, which was not affected by the presence of substrates, as expected for reaction at the active site. This complication was eliminated by prior reaction of Cys^{291} with *N*-ethylmaleimide, which is considerably smaller in size than 6-BDB-TAMP. The crystal structure of adenylosuccinate synthetase (*14*) indicates that Cys^{291} is located at an external surface of each subunit, far from the active site, where it is accessible to a variety of reactive compounds, including nucleotide analogues (*20*). Our subsequent experiments with the catalytically active NEM-treated adenylosuccinate synthetase show that 6-BDB-TAMP binds and reacts only at the active site of the enzyme.

The nonlinear relationship between the initial inactivation rate constant and the 6-BDB-TAMP concentration (i.e., saturation kinetics) reflects the reversible binding of 6-BDB-

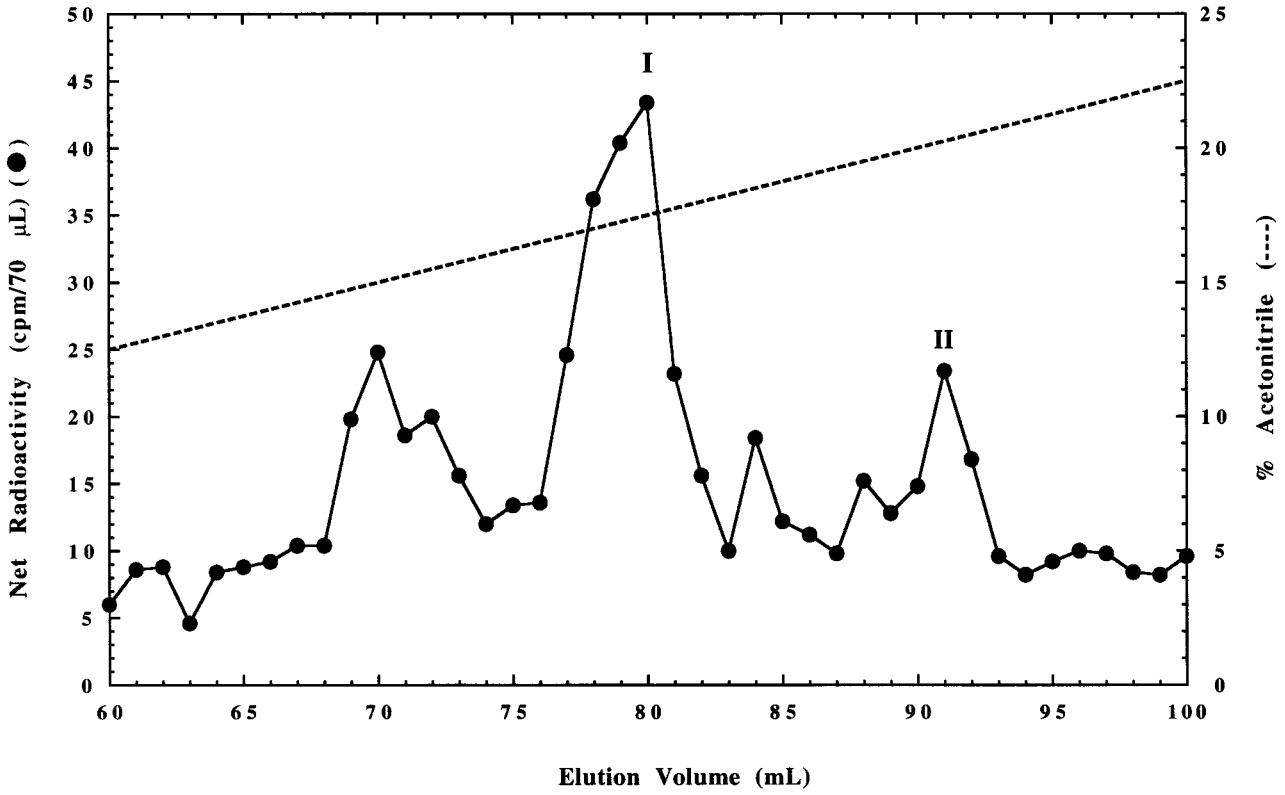

FIGURE 5: Separation of radioactive V8-digested peptides of 6-BDB-[^{32}P]TAMP-labeled adenylosuccinate synthetase by reversed-phase HPLC. The 6-BDB-[^{32}P]TAMP-labeled enzyme was digested by V8 protease in 50 mM phosphate buffer, pH 7.8. The peptides were separated by HPLC on a C_{18} column, equilibrated with 0.1% trifluoroacetic acid using a gradient in acetonitrile, as a described in the Experimental Procedures. Aliquots (70 μL) were withdrawn from each fraction for radioactivity measurement using a Packard Tricarb liquid scintillation counter (●). The dashed line represents the percentage of acetonitrile.

Table 4: Amino Acid Sequence of Peptides from Proteolysis[a]

		peak II from Figure 5[b] (pmol)			peak I from
cycle no.	amino acid	sample 1	sample 2	amino acid	Figure 6[b] (pmol)
1	Phe-297	337	58	Arg-131	80[c]
2	Gly-298	207	24	Gly-132	184
3	Ala-299	150	29	Ile-133	391
4	Thr-300	137	14	Gly-134	225
5	Thr-301	143	15	Pro-135	296
6	Gly-302	60	16	Ala-136	298
7	Arg-303	67[c]	4[c]	Tyr-137	221
8	Arg-304	117	6	Glu-138	95[d]
9	Arg-305	103	13		
10	Arg-306	136	7		
11	Thr-307	57	7		
12	Gly-308	91	10		
13	Trp-309	29	3		
14	Leu-310	67	8		
15	Asp-311	26[d]	7[d]		

[a] Amino acid sequencing was performed on an Applied Biosystems gas-phase sequencer, as described in the Experimental Procedures. [b] These are representative sequences and do not come from the same run. Therefore, they do not reflect the relative magnitude of each of the peaks. [c] Products of arginine and bromodioxobutyl nucleotides have previously been detected by a decreased yield of PTH−Arg; however, the modified arginine is partially regenerated during gas-phase sequencing (*41*). [d] End of peptide.

TAMP to adenylosuccinate synthetase before inactivation. The K_I value for 6-BDB-TAMP (40.6 μM) is comparable in magnitude to the K_m values for IMP (10 μM) and GTP (15 μM) of the NEM-treated enzyme, as well as to the previously reported K_d values for IMP (37.7 μM) and GTP (23.7 μM) of wild-type enzyme (*18*), suggesting that the enzyme has similar affinity for the reagent as for the natural substrates.

Examination of the ligands which protect against inactivation by 6-BDB-TAMP provides insight into the functional site which is targeted. The complete protection by adenylosuccinate shows that 6-BDB-TAMP occupies the adenylosuccinate site of *E. coli* adenylosuccinate synthetase, as might be expected from the striking similarity in structure between the substrate and reagent. In contrast, IMP causes only a limited reduction in the inactivation of the enzyme by 6-BDB-TAMP. The X-ray crystal structure of *E. coli* adenylosuccinate synthetase reveals that the IMP site is in the vicinity of the aspartate and GTP sites (*25*−*29*). An occupied IMP site on the enzyme could hinder the binding of 6-BDB-TAMP to either the aspartate or GTP site and is consistent with the partial protection by IMP against 6-BDB-TAMP inactivation. The mechanism proposed for adenylosuccinate synthetase involves the formation of 6-phosphoryl-IMP in the first step by transfer of the γ-phosphate from GTP (*1*), and Poland et al. (*28*) observed the presence of 6-thiophosphoryl-IMP in the active site of the enzyme by X-ray crystallography. The 6-BDB-TAMP must react with enzymatic amino acids located in the region of its C-6 moiety, which is normally occupied by the phosphoryl group of 6-phosphoryl-IMP. Since IMP + GTP would also occupy this region, it is understandable that this combination of nucleotides completely protects the enzyme against 6-BDB-TAMP inactivation. The lack of protection by aspartate is likely due to the inability of aspartate to bind to the enzyme in the absence of the 6-phosphoryl-IMP intermediate (*18*).

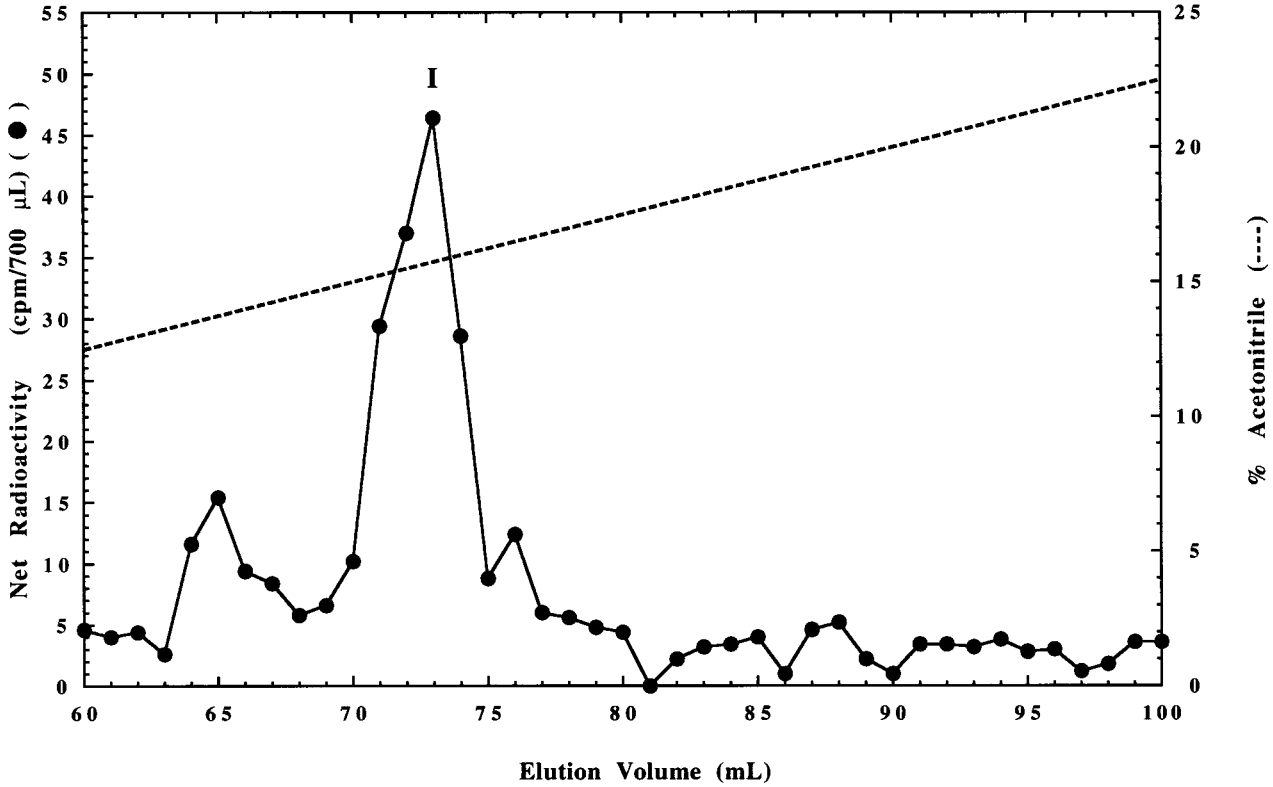

FIGURE 6: Papain digest of peak I isolated from V8 protease digest. Radioactive peak I from V8 protease digest was collected and lyophilized. The dried fraction was redissolved in 20 mM ammonium acetate, pH 4.0, and subjected to papain digestion as described in the Experimental Procedures. The digest was separated by gradient elution under the same conditions as in Figure 5. Aliquots (700 μL) were withdrawn from each fraction and assayed for radioactivity (●). The dashed line represents the percentage of acetonitrile.

Escherichia coli	^{126}I-G-T-T-G-**R**^{131}-G-I-G-P^{135}--------^{298}G-A-T-T-G-**R**^{303}-R-R-R-T-G-W^{309}
Haemophilus influenzae	^{126}I-G-T-T-G-**R**^{131}-G-I-G-P^{135}--------^{298}G-A-V-T-G-**R**^{303}-P-R-R-C-G-W^{309}
Vibrio parahaemolyticus	^{127}I-G-T-T-G-**R**^{132}-G-I-G-P^{136}--------^{305}G-A-T-T-G-**R**^{310}-L-R-R-T-G-W^{316}
Thiobacillus ferrooxidans	^{127}I-G-T-T-G-**R**^{132}-G-I-G-P^{136}--------^{299}G-A-T-T-G-**R**^{304}-A-R-R-C-G-W^{310}
Brucella abortus	^{126}I-G-T-T-K-**R**^{131}-G-I-G-P^{135}--------^{296}G-V-V-T-G-**R**^{301}-K-R-R-C-G-W^{307}
Synechocystis sp. strain PCC6803	^{126}I-G-T-T-G-**R**^{131}-G-I-G-P^{135}--------^{298}G-T-T-T-G-**R**^{303}-R-R-R-C-G-W^{309}
Aquifex aeolicus	^{123}I-G-T-T-L-**R**^{128}-G-I-G-P^{132}--------^{295}G-S-T-T-G-**R**^{300}-P-R-R-C-G-W^{306}
Bacillus subtilis	^{125}I-G-T-T-K-**K**^{130}-G-I-G-P^{134}--------^{297}G-T-T-T-G-**R**^{302}-P-P-R-V-G-W^{308}
Spiroplasma citri	^{132}I-G-T-T-K-**K**^{137}-G-I-G-P^{141}--------^{304}G-T-V-S-G-**R**^{309}-A-R-W-I-G-W^{315}
Helicobacter pylori	^{118}I-G-T-T-K-**K**^{123}-G-I-G-P^{127}--------^{289}G-T-T-T-K-**R**^{294}-P-R-R-C-G-W^{300}
Mouse non-muscle isozyme	^{159}L-G-T-T-K-**K**^{164}-G-I-R-P^{168}--------^{329}G-V-T-T-G-**R**^{334}-K-R-R-C-G-W^{340}
Human	^{158}L-G-T-T-K-**K**^{163}-G-I-G-P^{167}--------^{328}G-V-T-T-G-**R**^{333}-K-R-R-C-G-W^{339}
Mouse muscle isozyme	^{160}I-G-T-T-K-**K**^{165}-G-I-G-P^{169}--------^{330}G-V-T-T-G-**R**^{335}-K-R-R-C-G-W^{341}
Schizosaccharomyces pombe	^{139}I-G-T-T-G-**K**^{144}-G-I-G-P^{148}--------^{307}G-V-T-T-G-**R**^{312}-K-R-R-C-G-W^{318}
Saccharomyces cerevisiae	^{130}I-G-R-R-G-**K**^{135}-G-I-G-P^{139}--------^{303}G-V-T-T-G-**R**^{308}-K-R-R-C-G-W^{314}
Caenorhabditis elegans	^{158}I-G-T-T-N-**R**^{163}-G-I-G-P^{167}--------^{329}G-V-T-T-G-**R**^{334}-K-R-R-C-G-W^{340}
Arabidopsis thaliana	^{192}I-G-T-T-K-**R**^{197}-G-I-G-P^{201}--------^{363}G-T-T-T-G-**R**^{368}-P-R-R-C-G-W^{374}
Dictyostelium discoideum	^{129}I-G-T-T-K-**R**^{134}-G-I-G-P^{138}--------^{297}G-T-T-T-G-**R**^{302}-P-R-R-I-G-W^{308}

FIGURE 7: Amino acid sequence alignment of adenylosuccinate synthetase from 18 species. Multiple sequence alignment was performed by the CLUSTAL program.

During digestion of the 6-BDB-TAMP-modified adenylosuccinate synthetase, we noted that the reaction product was less stable at pH 7.8 (used for V8 protease treatment) than at pH 2.0 (used for pepsin treatment). In a model study of the reaction of arginine with the dioxo compound cyclohexanedione, Patthy and Smith (*42*) showed that the product formed is N^7,N^8-(1,2-dihydroxy-cyclohex-1,2-ylene)-L-arginine, which is stable under mildly acidic conditions, but regenerates free arginine in neutral or slightly basic conditions. This characteristic pH-dependent instability of

arginine derivatives was also observed for the product of adenosine 5′-*O*-[*S*-(4-bromo-2,3-dioxobutyl)thiophosphate] with Arg^{459} of glutamate dehydrogenase (*41*). Thus, the pH-dependent instability of 6-BDB-TAMP-modified adenylosuccinate synthetase is consistent with attack of arginine residues. Since the reaction of arginine with dioxo compounds involves attack of the carbonyl groups (*41*, *42*), one might expect that, even after hydrolysis of 6-BDB-TAMP to 6-(4-hydroxy-2,3-dioxobutyl)thioadenosine 5′-monophosphate, the reagent would attack arginines. However, the proximity of the $-CH_2Br$ to the carbonyls of 6-BDB-TAMP enhances the polarization of the carbonyls and hence their reactivity, thereby accounting for the much greater reaction rate of 6-BDB-TAMP than of 6-HDB-TAMP with adenylosuccinate synthetase.

Isolation of modified peptides indicates that both Arg^{131} and Arg^{303} of adenylosuccinate synthetase are targeted by 6-BDB-TAMP, which is consistent with 2 mol of 6-BDB-[^{32}P]TAMP incorporated/mol of enzyme subunit when the enzyme is completely inactivated. Arg^{131} and Arg^{303} labeled by 6-BDB-TAMP are clearly distinct from the amino acid residue (Arg^{143}) modified by another nucleotide affinity label, guanosine 5′-*O*-[*S*−(4-bromo-2,3-dioxobutyl)thio]phosphate (*20*). These results demonstrate the specificity of the nucleotide affinity labels and the importance played by the position of the reactive group in determining the amino acid residue which is modified.

In 6-BDB-TAMP-modified enzyme with 50% residual activity and about 1 mol of reagent/enzyme subunit, we consider that the predominant enzyme species is enzyme with one site modified per enzyme subunit. If, instead, this enzyme sample contained an equal mixture of fully active, unmodified enzyme and completely inactive, doubly modified enzyme, one would expect an equal distribution between peak I (Arg^{131}) and peak II (Arg^{303}) in the HPLC separation of the V8 protease digest shown in Figure 5. In reality, the higher amount of radioactivity seen in peak I as compared with peak II (Figure 5) suggests that Arg^{131} reacts more rapidly than Arg^{303} and that, at 50% residual activity, the major enzyme species is that with only Arg^{131} modified/subunit. This enzyme retains catalytic activity but exhibits a 3-fold increase in the K_m value for aspartate with no significant change in the K_m values for IMP or GTP.

X-ray crystallographic studies reveal that the guanidinium group of Arg^{303} forms hydrogen bonds with both the 2′-oxygen of IMP and β-carboxyl group of aspartate (*26*). The β-carboxyl group of aspartate also interacts with the side chain of Arg^{131} (*25*). Replacement of the Arg^{303} by leucine greatly increased the K_m for aspartate, without affecting the K_ms for IMP or GTP (*23*) while replacement of Arg^{131} with leucine resulted in a 4-fold increase in the K_m for aspartate without affecting the affinities for IMP or GTP (*24*). The k_{cat} value for the R303L mutant was decreased 10-fold as compared to wild-type enzyme, but no significant decrease in k_{cat} was observed for the R131L mutant enzyme. These mutagenesis results indicate that Arg^{303} may participate in both aspartate binding and catalysis; however, Arg^{131} may contribute only to aspartate binding (*23*, *24*). Our incorporation measurements suggest that the active site of adenylosuccinate synthetase must be sufficiently flexible to accommodate two molecules of 6-BDB-TAMP in each subunit; modification of only one of the arginines perturbs the binding of aspartate, but does not eliminate catalytic activity.

Complete amino acid sequences have been determined for adenylosuccinate synthetases from 18 sources. The sequence identity between two sources ranges from 34% for *Spiroplasma citri* and mammals, to 96% for human and mouse. However, sequence alignment in the region of Arg^{303} and Arg^{131} of adenylosuccinate synthetases from 18 species (shown in Figure 7) indicates that these sequences are more highly conserved. Arg^{303} is completely conserved throughout the adenylosuccinate synthetase family. Either arginine or lysine occupies position 131 in all adenylosuccinate synthetase sequences, indicating the importance of the positive charge at this position to interact with the β-carboxylate group of aspartate (*25*).

This paper demonstrates that 6-(4-bromo-2,3-dioxobutyl)-thioadenosine 5′-monophosphate functions as a reactive adenylosuccinate analogue in modifying both Arg^{131} and Arg^{303} in the active site of adenylosuccinate synthetase. The results of affinity labeling of the enzyme in solution, which are consistent with mutagenesis and X-ray crystallographic studies, indicate that these two arginine residues are important in the normal functioning of the enzyme.

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