Identification of His\textsuperscript{141} in the Active Site of Bacillus subtilis Adenylosuccinate Lyase by Affinity Labeling with 6-(4-Bromo-2,3-dioxobutyl)thioadenosine 5’-Monophosphate* 

(Received for publication, October 3, 1996, and in revised form, October 21, 1996)

Tom T. Lee‡, Carolyn Worby§, Jack E. Dixon§, and Roberta F. Colman‡¶

From the ‡Department of Chemistry and Biochemistry, University of Delaware, Newark, Delaware 19716 and the ¶Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, Michigan 48109

Adenylosuccinate lyase of Bacillus subtilis is inactivated by 25–400 \( \mu \)M 6-(4-bromo-2,3-dioxobutyl)thioadenosine 5’-monophosphate (6-BDB-TAMP) at pH 7.0 and 25 °C. The initial inactivation rate constant exhibits nonlinear dependence on the concentration of 6-BDB-TAMP, implying there is reversible formation of enzyme-reagent complex (\( K_i = 50 \pm 4 \mu \)M) prior to irreversible modification (\( k_{\text{obs}} = 0.139 \pm 0.005 \text{ min}^{-1} \)). The tetrameric enzyme incorporates about 1 mol of 6-BDB-TAMP per mol of enzyme subunit concomitantly with complete inactivation. Protection against inactivation and incorporation of \([^{32}\text{P}]\)reagent is provided by adenylosuccinate or a combination of AMP and fumarate, whereas either AMP or fumarate alone is much less effective. These observations suggest that 6-BDB-TAMP targets the adenylosuccinate-binding site. Hydrolyzed 6-BDB-TAMP is a competitive inhibitor with respect to adenylosuccinate in the catalytic reaction and also decreases the rate of inactivation by 6-BDB-TAMP. These results account for the decrease in the inactivation rate as the reaction of 6-BDB-TAMP with the enzyme proceeds. Purification by chromatography on dihydroxyboryl-agarose and high performance liquid chromatography of the tryptic digest of inactivated enzyme yields a single radioactive peptid, Thr\textsuperscript{340}Phe\textsuperscript{359}, as determined by gas-phase sequencing. Modified His\textsuperscript{141} is the reaction product of 6-BDB-TAMP and adenylosuccinate lyase. We conclude that 6-BDB-TAMP functions as a reactive adenylosuccinate analog in modifying His\textsuperscript{141} in the substrate-binding site of adenylosuccinate lyase, where it may serve as a general base accepting a proton from the succinyl group during catalysis.

Adenylosuccinate lyase (EC 4.3.2.2) catalyzes two distinct reactions in purine biosynthesis, one of which involves the cleavage of adenylosuccinate to form AMP and fumarate (1). The enzyme has been isolated from a variety of sources including yeast (2), Neurospora (3), wheat germ (4), Artemia embryos (5), avian liver (6), rat skeletal muscle (7), rabbit muscle (8), and human erythrocytes (9). The amino acid sequences have now been determined for the enzymes from Bacillus subtilis (10), Haemophilus influenzae (12), Methanococcus jannaschii (13), chicken (14), murine (15), and human (16). The metabolic importance of the enzyme is indicated by observations that adenylosuccinate lyase deficiency in humans is associated with mental retardation, secondary autistic behavior, and muscle wasting (16, 17). A point mutation, S413P, found in the family of one patient with adenylosuccinate lyase deficiency (16), generates a structural change in the protein which leads to an unstable but catalytically competent enzyme (18).

Kinetic studies (7, 14, 18–20) show that catalysis follows an ordered uni-bi mechanism with fumarate leaving the enzyme before AMP. The binding of AMP or the AMP portion of adenylosuccinate appears to induce a conformational change which exposes the site involved in binding fumarate or the succinate moiety of adenylosuccinate. The mechanism proposed for the action of adenylosuccinate lyase involves the attack of an enzymic general base on the \( \beta\)-H and elimination of the amino group facilitated by protonation of the ring nitrogen by an amino acid of the enzyme functioning as a general acid (1, 18). However, little information is available on the role of enzymic amino acids in catalysis. Only Arg\textsuperscript{112} of the rabbit muscle enzyme (numbering based on the human enzyme) has been identified within the substrate-binding site (8).

The sequence of B. subtilis adenylosuccinate lyase shows 25% identity plus 18% similarity to the human enzyme. We have now expressed the B. subtilis enzyme in E. coli and purified it in order to locate by affinity labeling amino acid residues in the active site of the enzyme. 6-(4-Bromo-2,3-dioxobutyl)thioadenosine 5’-monophosphate (6-BDB-TAMP) has been used as a reactive nucleotide analog to modify nucleotide-binding sites in enzymes (21). It is strikingly similar in structure to adenylosuccinate, and therefore would be predicted to bind to the adenylosuccinate site of adenylosuccinate lyase. The bromodioxobutyl group of 6-BDB-TAMP is likely to occupy the succinyl subsite, where the key catalytic steps should occur. Nucleophilic attack of the carbonyl(s) or methylene bromide group by the side chains of Lys, Arg, Cys, His, or other amino acids can result in covalent bond formation (22). In this paper, we describe the specific inactivation of B. subtilis adenylosuccinate lyase by 6-BDB-TAMP. Our results indicate that His\textsuperscript{141} is the target of 6-BDB-TAMP and that it is located in the active site of adenylosuccinate lyase. A preliminary version of this work has been presented (23).
**Affinity Labeling of Adenylosuccinate Lyase**

459

The cell lysate was centrifuged at 5000 g to harvest the cell debris. The supernatant was transferred to a small column and flushed with 250 ml of lysis buffer, followed by 250 ml of wash buffer, 50 ml sodium phosphate buffer, pH 6.0, containing 300 mM NaCl, 10% glycerol, and 0.05% sodium azide. The bound protein was eluted from the resin with a 100-ml gradient of wash buffer and elution buffer (wash buffer plus 0.5 M imidazole). The fractions containing essentially a single band of protein were then concentrated in a Centriprep-30 (Amicon). The concentrated, pure protein was lyophilized overnight against approximately 300 ml of dialysis buffer (20 mM sodium phosphate, pH 7.0, containing 20 mM NaCl, and 0.5% sodium azide) at 4°C.

The protein concentration of purified adenylosuccinate lyase was first determined according to the method of Groves et al. (25) from the difference in absorbance at 224 and 233 nm, using bovine serum albumin as standard. Based on the protein concentration determined by this method and its $A_{280}$ nm, B. subtilis adenylosuccinate lyase exhibits $\varepsilon_{280}$ nm $= 10.6$. This value was used subsequently to measure the enzyme concentration.

Preparation of 6-4-Bromo-2,3-dioxobutylthioadenosine 5'-monophosphate—6-BDB-TAMP was synthesized by coupling 1,4-dibromo-2,3-butanedione with 6-mercaptopurine riboside 5'-phosphate as described previously (21). The final product was dissolved in 30 mM MES buffer (pH 4.5) and stored at -80°C for further studies. The concentration of 6-BDB-TAMP was determined from its absorbance at 284 nm using $\varepsilon_{280}$ nm $= 16.0 \times 10^{3}$ M$^{-1}$ cm$^{-1}$.

For the synthesis of 6-BDB-[32P]TAMP, POCl$_3$ (30 $\mu$l, 0.3 mmol) was added to [32P]HPO$_4$ (1 mM), previously dried in a desicator for 24 h. The mixture was incubated at 110°C for 24 h, conditions which have been shown to allow equilibration of the phosphorus in radioactive phosphoric acid with the phosphorus in phosphorus oxychloride (28). The phosphorylation of the nucleoside was carried out according to a modification of the procedure of Ozturk et al. (27). Fifteen microliters of the incubation mixture containing [32P]POCl$_3$ were added to 0.0284 g (0.1 mmol) of 6-mercaptopurine riboside, suspended in 0.5 ml of triethyl phosphate; 60 min later, a second addition of 15 $\mu$l of the [32P]POCl$_3$ preparation was made. The reaction mixture was stirred at room temperature for 2 h and monitored by thin-layer chromatography on a Kodak TLC plate (cellulose type, with fluorescent indicator) using a solvent system of acetonitrile, 1 M lithium chloride, H$_2$O (60:10:30). The $R_F$ values were 0.70 and 0.46 for the starting material and the phosphorylation product, respectively. Barium acetate (1 ml, 0.4 M) was added following the addition of 4 ml of ethanol containing 200 $\mu$g of triethylamine. The supernatant was discarded after centrifugation for 5 min. The precipitate was washed extensively using 70% ethanol before being dissolved in 1 ml of 1 M acetic acid.

The crude phosphorylation product was applied to an AG W50-X4 column ($1 \times 7.5$ cm, H$^+$ form) and eluted with water in order to convert the 6-mercaptopurine riboside 5'-[32P]phosphate to the free acid form, as well as to separate it from inorganic phosphate. The fractions with high absorbance at 282 nm and constant specific radioactivity were collected and dried by rotary evaporation.

The coupling step with 1,4-dibromo-2,3-butanedione was identical to the synthesis of the non-radioactive 6-BDB-TAMP. The 6-mercaptopurine riboside 5'-phosphate (-40 $\mu$mol) was dissolved in 0.5 ml of methanol. The apparent pH was adjusted to 5.5 using triethylamine. Recrystallized 1,4-dibromo-2,3-butanedione (0.29 g, 1.2 mmol), dissolved in 0.5 ml of methanol, was added. The reaction was stirred for 2 h at room temperature and the conjugation of 6-mercaptopurine riboside 5'-phosphate to 6-BDB-TAMP was followed spectrophotometrically from the decrease in absorbance at 322 nm and the increase in absorbance at 284 nm. The product was precipitated by 8 ml of diethyl ether. The precipitate was collected after centrifugation and washed twice with diethyl ether. The overall yield of 6-BDB-[32P]TAMP from 6-mercaptopurine riboside was 20%. The specific radioactivity initially was $5.0 \times 10^{3}$ cpm/mol compound.

**Enzymatic Assay**—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. The assay was conducted at 25°C in 1 ml of 50 mM HEPES buffer (pH 7.0) containing 60 $\mu$g adenylosuccinate. The homogeneous B. subtilis adenylosuccinate lyase exhibits a specific activity of 2.0 $\mu$mol/min/mg under these conditions. In the $K_a$ studies, the adenylosuccinate concentration was varied from 2 to 32 $\mu$M. To determine the $K_a$ for “hydrolyzed” 6-BDB-TAMP, the $K_a$ for adenylosuccinate was measured in the presence of 1 or 2 $\mu$M hydrolyzed 6-BDB-TAMP, prepared by incubating fresh 6-BDB-TAMP for 24 h at room temperature in 10 mM potassium phosphate buffer (pH 8.0) containing 10 mM NaCl.

**Section of 6-BDB-TAMP with B. subtilis Adenylosuccinate Lyase**—Enzyme (0.2 mg/ml, 4 $\mu$l subunit) was incubated with 25–400 $\mu$M 6-BDB-TAMP at 25°C in 10 mM potassium phosphate buffer (pH 7.0) containing 10 mM NaCl (buffer A) in a total volume of 0.4 ml. The volume of 30 mM MES buffer (pH 4.5, containing 6-BDB-TAMP) added was maintained constant at 50 $\mu$l. The 6-BDB-TAMP was added last, after preincubating the enzyme for 30 min. At various times, 20-$\mu$l
Affinity Labeling of Adenylosuccinate Lyase

Aliquots of reaction mixture were withdrawn and assayed for residual activity. The reaction was measured for 70 min, during which period no activity loss was found in the control enzyme incubated under the same conditions but in the absence of 6-BDB-TAMP. To study the effect of substrate analogs on the reaction rate with 50 μM 6-BDB-TAMP, a series of ligands were included, as described under "Results." Measurement of Incorporation of 6-BDB-TAMP into B. subtilis Adenylosuccinate Lyase—Adenylosuccinate lyase (0.2 mg/ml) was incubated with 50 μM 6-BDB-[32P]TAMP in buffer A at 25°C. In order to reduce the carbonyl groups of 6-BDB-TAMP thereby decreasing markedly its reactivity, 0.2 mM NaBH₄ (dissolved in 0.02 M NaOH) was added at 5-min intervals to reach a final concentration of 2 mM. The reduction proceeded for 5 min. To study the incorporation at zero time, 50 μM 6-BDB-[32P]TAMP was incubated with 2 mM NaBH₄ for 5 min before being added to the preincubated enzyme; this sample was found to maintain full enzymatic activity. The enzyme was then separated from excess reagent by gel filtration on a Sephadex G-50 minicolumn (5 ml) equilibrated with buffer A containing 10% glycerol using a column centrifugation technique (25). The filtered protein was assayed immediately for enzymatic activity and protein concentration. The protein concentration was determined using the Bio-Rad protein assay, based on the dye-binding method of Bradford (29). Purified B. subtilis adenylsuccinate lyase was used as the standard. The incorporation of 6-BDB-[32P]TAMP was measured by counting aliquots of modified protein using a liquid Scintitron Tricarb (Model 4640) liquid scintillation counter.

Proteolysis of 6-BDB-[32P]TAMP-modified Enzyme—Adenylosuccinate lyase (0.5 mg/ml) was incubated with 50 μM 6-BDB-[32P]TAMP in the absence and presence of 1 mM AMP and 5 mM fumarate at 25°C for 70 min. Following the addition of 2 mM NaBH₄ for 5 min, 0.1 M N-ethylmaleimide was added to give a final concentration of 10 mM. After 10 min, the excess reagent and protecting ligands were removed by column centrifugation using Sephadex G-50 (equilibrated with 50 mM potassium phosphate containing 50 mM KCl, pH 7.8). The modified enzyme was then digested at 37°C by 2 successive additions (at 2 h intervals) of 5% (w/v) TPCK-treated trypsin, for a total of 4 h.

The radioactively labeled tryptic digest (~1 mg) was separated on a Varioan (Model 5000) HPLC system using a reverse phase Vydac C₄ column (0.46 × 25 cm). The digest was lyophilized, redissolved in 0.8 ml of 0.1% trifluoroacetic acid and filtered through a 0.45-μm membrane filter (Millipore) before injection. Separation was conducted at the elution rate of 1 ml/min using 0.1% trifluoroacetic acid (Solvent A) for the first 10 min, followed by a linear gradient from solvent A to 20% solvent B (0.09% trifluoroacetic acid in acetonitrile) in 100 min, a linear gradient from 20% solvent B to 40% solvent B in 40 min, a linear gradient from 40% solvent B to 100% solvent B in 5 min, and solvent B for 5 min, successively. The effluent was monitored at 220 nm. Fractions of 1 ml were collected, from which 0.4 μl was assayed for radioactivity. Hydrolyzed 6-BDB-[32P]TAMP (prepared by incubating fresh 6-BDB-[32P]TAMP in buffer A for 24 h) was applied to the same column in a separate run.

Purification and Determination of the Sequence of Modified Peptides—In order to obtain the homogeneous modified peptide, the tryptic digest was initially applied to a phenylboronate-agarose column (PBA-30) equilibrated with 50 mM potassium phosphate containing 50 mM KCl (pH 7.8) at 4°C. The unbound peptides were eluted with the same buffer. The bound peptides, which include the nucleotidyl peptide, were eluted with water. Fractions of 2 ml were collected and monitored at 220 nm. Aliquots (50 μl) were counted for radioactivity. The water effluent fractions with high radioactivity were pooled and lyophilized. This sample was further purified by HPLC using the same system described above. The fractions with high absorbance and radioactivity were lyophilized for sequencing. The sequences of the purified peptides 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 909A). The molecular weights of the labeled peptides were analyzed by electrospray mass spectrometry using a Bruker BioApex mass spectrometer equipped with an Xmass data-solving program.

RESULTS

Inactivation of B. subtilis Adenylosuccinate Lyase—Incubation of 0.2 mg/ml B. subtilis adenylosuccinate lyase (4 μM subunit) with 50 μM 6-BDB-TAMP at 25°C and pH 7.0 in buffer A resulted in time-dependent loss of enzymatic activity (Fig. 2). In contrast, the control enzyme, incubated under the same conditions, but without 6-BDB-TAMP, showed constant activity (data not shown). The semilogarithmic plot of residual activity (E/E₀) versus time of incubation with 6-BDB-TAMP was linear for the first 12 min. Data taken from this initial period were used to calculate a pseudo-first order rate constant, of 0.089 min⁻¹. At longer time periods, enzyme inactivation continued, reaching about 10% residual activity by 50 min, as shown in Fig. 2. With higher initial concentrations of 6-BDB-TAMP, the residual activity decreased below 5%.

At times greater than 12 min (Fig. 2), the points deviate progressively from the line, indicating that the rate of inactivation decreases as the reaction continues. One explanation for the line curvature is that the reagent decomposes causing a decrease in the apparent pseudo-first order rate constant. The reactive bromodioxobutyl group is known to undergo decomposition in aqueous buffers with release of free bromide; the rate of decomposition of a similar compound, 6-(4-bromo-2,3-dihydroxybutyl)iodoadenosine 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 (t₁/₂ = 61 min) (30). The decomposition results in a decreased concentration of reactive 6-BDB-TAMP. However, this effect would only cause a 2-fold decrease, at 61 min, in the apparent rate constant. Another possible explanation for the greater curvature is that the hydrolyzed 6-BDB-TAMP competes with the intact 6-BDB-TAMP for binding to the enzyme at the adenylosuccinate site.

Accordingly, the hydrolyzed 6-BDB-TAMP was tested as a competitive inhibitor with respect to the substrate in the reaction catalyzed by adenylosuccinate lyase. The B. subtilis enzyme was found to have a Kᵣ of 2.6 μM for adenylosuccinate (similar to the values of 1–3.2 μM reported for the enzymes from other species (5, 7, 9, 18, 19)) and this Kᵣ is increased by the hydrolyzed 6-BDB-TAMP, without affecting Vₘₐₓ. The Kᵣ for the hydrolyzed 6-BDB-TAMP was determined as 1.1 μM. Since about 6.5 μM of the hydrolyzed compound would be generated in 12 min from the 50 μM 6-BDB-TAMP of Fig. 2, the curvature is understandable, provided that the two compounds compete for binding at the same site and the intact 6-BDB-TAMP has a lower affinity for the enzyme than does the decomposition product.

Rate of Inactivation of Adenylosuccinate Lyase as a Function of 6-BDB-TAMP Concentration—Adenylosuccinate lyase (0.2 mg/ml) was incubated with 25–400 μM 6-BDB-TAMP. In each case, the initial rate constant of inactivation was measured...
Affinity Labeling of Adenylosuccinate Lyase

Fig. 3. Dependence of initial rate constant of inactivation of adenylosuccinate lyase on the concentration of 6-BDB-TAMP.

The enzyme was incubated with 25–400 μM 6-BDB-TAMP in pH 7.0 buffer at 25 °C. The rate constants were measured from the first 12 min of the reaction. Inset, double-reciprocal plot of 1/k_{obs} against 1/[6-BDB-TAMP].

Table I

| Ligands added | k_{obs} (min⁻¹) | k_{50} / k_{a} |
|---------------|----------------|----------------|
| a) None       | 0.089          | 1.00           |
| b) AMP (1 mM) | 0.060          | 0.68           |
| c) Fumarate (5 mM) | 0.075 | 0.84 |
| d) Fumarate (10 mM) | 0.065 | 0.73 |
| e) Adenylosuccinate (1 mM) | 0.019 | 0.20 |
| f) Adenylosuccinate (5 mM) | 0.009 | 0.10 |
| g) AMP (1 mM) + fumarate (5 mM) | 0.010 | 0.11 |
| h) AMP (1 mM) + fumarate (10 mM) | 0.014 | 0.16 |
| i) Hydrolyzed 6-BDB-TAMP (50 μM) | 0.024 | 0.27 |

"The ratio of the initial inactivation rate constant in the presence and the absence of 6-BDB-TAMP.

decreasing the rate of inactivation. The hydrolyzed 6-BDB-TAMP (Table I, i) also provides marked protection against inactivation by 6-BDB-TAMP. These results suggest that 6-BDB-TAMP targets the active site of the enzyme in the region occupied by both the AMP and succinyl moieties.

Incorporation of 6-BDB-TAMP into B. subtilis Adenylosuccinate Lyase—Incubation of adenylosuccinate lyase (0.2 mg/ml) with 50 μM 6-BDB-[32P]TAMP at pH 7.0 results in a time-dependent incorporation of reagent (Fig. 4). The addition of NaBH₄ to 6-BDB-[32P]TAMP prior to addition to enzyme prevents reagent incorporation, and addition of NaBH₄ to an incubation mixture of enzyme and reagent stops the reaction. As shown in Fig. 4, there is a strong correlation between incorporation and inactivation. Extrapolation to 0% residual activity yields an estimate of 1 mol of 6-BDB-[32P]TAMP incorporated per mol of enzyme subunit.

Peptide Mapping of 6-BDB-[32P]TAMP-modified Adenylosuccinate Lyase—The enzyme (0.5 mg/ml) was incubated with 50 μM 6-BDB-[32P]TAMP in the absence and presence of protecting ligands (1 mM AMP + 5 mM fumarate) for 70 min. The modified enzymes were digested by trypsin at 37 °C for 4 h, as described under “Experimental Procedures.” Fig. 5A shows that on reverse phase HPLC of the tryptic digest, most of the peptides elute between 80 and 150 min (14 and 40% solvent B). Three major radioactive peaks I, II, and III were observed (Fig. 5B), all of which are decreased in the enzyme sample prepared in the presence of 1 mM AMP + 5 mM fumarate (Fig. 5C). Peak III is decreased slightly more than the others. When the hydrolyzed 6-BDB-[32P]TAMP was applied to the same column, radioactivity was released at the same elution volume as peaks I and II, indicating that the amino acid derivative is unstable and releases the decomposed reagent, probably during digestion. Peak III is the only radioactive peak associated with modified peptide.

Purification and Analysis of Modified Peptide—One HPLC run is inadequate to completely purify the modified peptide. To identify the peptide of interest, the tryptic digest was first fractionated by chromatography on a phenyl boronate-agarose column (PBA-30). And subsequently the modified peptide was purified to homogeneity by HPLC using a C₁₈ column: PBA-30 is capable of binding molecules with a cis-diol (such as ribose) under neutral and basic conditions. It has previously been used to separate nucleotide-bound peptides (31–33). For the adenylosuccinate lyase digest, we found that the column binds 6-BDB-TAMP-modified peptide in potassium phosphate buffer (pH 7.8) at 4 °C (Fig. 6). The bound peptides are easily eluted with water as the ionic strength is decreased. HPLC of the radio-
active fractions eluted from the PBA-30 column yields a pure modified peptide eluting at the same position as peak III of Fig. 5.

Identification of the Modified Amino Acid—The amino acid sequence of the purified peptide was determined using an automated gas-phase sequencer by Edman degradation. Based on the radioactivity, approximately 230 pmol of peptide was applied to the sequencer. As shown in Table II, this peptide contains a single 11-amino acid peptide, assigned as Thr^{140}-Phe^{150} of *B. subtilis* adenylosuccinate lyase (10). Table II records the yield of each PTH-derivative; as expected, the yield decreases as the cycle number increases. The yield for PTH-His is characteristically low in the sequencing of standard proteins and peptides (usually 20–30% recovery); thus, the 63 pmol observed in cycle 5 for His^{144} is considered a normal yield. In contrast, the 15 pmol observed for His^{141} in cycle 2 is unusually low, even as compared with the later His^{144}. This result indicates that His^{141} is the target amino acid. The small amount of histidine observed in cycle 2 was probably regenerated during the sequencing run. No radioactivity was detected in any of the 11 cycles after organic solvent extraction; instead, most of it was retained on the sample filter paper of the sequencer. Moreover, no abnormal PTH-derivative peak was observed in that particular cycle. These results are consistent with previous reports of nucleotide analog-modified peptides (34, 35), and may be explained by the fact that the PTH-derivatives of nucleotide-modified peptides are too hydrophilic to be extracted by the solvent used in the sequencer.

Electrospray mass spectrometry was performed to further characterize the modified peptide. Analysis of peak III revealed two major species with masses of 1196.2 and 1646.4 atomic mass units, which correspond to the predicted masses of the unmodified peptide, Thr^{140}-Phe^{150} (1196.6), and 6-BDB-TAMP-modified peptide in which the bromide of 6-BDB-TAMP was displaced by a His (1646.6). The intensity ratio of the two masses was about 1:1. Although the 6-BDB-TAMP-peptide derivative was not entirely stable to electrospray mass spectrometry, sufficient product survived to demonstrate the chemical modification of the peptide. An alternate structure that might be considered for the 6-BDB-TAMP-modified peptide is one that results from addition of His^{141} to the carbon of one of the carbonyl groups of the dioxobutyl group; in that case the predicted mass of the peptide product would be 1662.6 atomic mass units if the bromide were replaced by an -OH group. Since we did not observe a species with such a high mass, it is unlikely that His^{141} reacts at the carbonyl group.

**DISCUSSION**

We have demonstrated that 6-(4-bromo-2,3-dioxobutyl)thioadenosine 5'-monophosphate acts as an affinity label of *B. subtilis* adenylosuccinate lyase. The 6-BDB-TAMP is a close structural analog of the substrate adenylosuccinate, with the reactive bromodioxobutyl group at a position equivalent to the

![Fig. 4. Time-dependent incorporation of 6-BDB-[32P]TAMP into adenylosuccinate lyase.](http://www.jbc.org/) The enzyme was incubated with 50 μM 6-BDB-[32P]TAMP in pH 7.0 buffer at 25 °C. NaBH₄ (2 mM) was added at 5, 10, 20, 30, 50, and 70 min to stop the incorporation. The excess reagent was removed by a Sephadex G-50 column. Aliquots were removed to measure the protein concentration and radioactivity. Inset, the relationship between incorporation and the residual enzymatic activity. The residual activity of the modified enzyme was determined after the Sephadex G-50 column. Enzyme treated with the same procedure but without 6-BDB-[32P]TAMP in the incubation mixture was used as a control. The data was extrapolated to 0% residual activity to estimate the incorporation associated with complete inactivation.

![Fig. 5. Peptide mapping of the modified enzyme.](http://www.jbc.org/) Tryptic digest of 6-BDB-[32P]TAMP-modified adenylosuccinate lyase was separated by a HPLC C₁₈ column under the conditions described under “Experimental Procedures.” A, the sample was incubated with the reagent in the absence of protecting ligands. The solid line represents the absorbance at 220 nm, while the dotted line is the gradient of acetonitrile. B, the radioactivity pattern of the sample shown in A. C, the radioactivity pattern of a sample prepared in the presence of 1 mM AMP and 5 mM fumarate. I, II, and III represent the major radioactive peaks.
Affinity Labeling of Adenylosuccinate Lyase

Sucinate moiety of the natural compound. The nonlinear dependence on reagent concentration of the initial rate constant for inactivation by 6-BDB-TAMP suggests the formation of a reagent-enzyme complex prior to irreversible covalent modification, which is responsible for the specificity of an affinity label. The apparent $K_i$ value ($30 \mu M$) is about 10-fold higher than the $K_m$ value of adenylosuccinate (2.6 $\mu M$), indicating the reagent binds more weakly to the enzyme than the substrate, presumably due to the somewhat different size and electrophilic characteristics of the BDB group as compared to the succinyl group. Modification by 6-BDB-TAMP results in loss of enzyme activity concomitant with incorporation of only 1 mol of reagent per 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.

Evidence was presented that decomposition of 6-BDB-TAMP by hydrolysis of the bromide yields a compound which binds tightly to the enzyme, but is not capable of covalent modification or inactivation. Furthermore, the hydrolyzed 6-BDB-TAMP is a competitive inhibitor with respect to adenylosuccinate. It binds to the enzyme with a $K_i$ of 1.1 $\mu M$, a value considerably lower than the apparent $K_i$ of 30 $\mu M$ for 6-BDB-TAMP. Replacement of the bromide by the smaller -OH implies the affinity of the nucleotide for the enzyme. The generation of hydrolyzed 6-BDB-TAMP during the modification of the enzyme by 6-BDB-TAMP can account for the decreased rate of inactivation after 12 min (i.e. the curvature in plots of lnE/E_0 versus time, as in Fig. 2). This postulate is supported by the observation that hydrolyzed 6-BDB-TAMP, when added at the start of the reaction, decreases the rate of inactivation by 6-BDB-TAMP (Table I).

Further insight into the reaction site of 6-BDB-TAMP on adenylosuccinate lyase can be gained by analyzing the ligands which protect against inactivation. The inclusion of fumarate (5 mM and 10 mM) has little effect on the inactivation; however, fumarate is a noncompetitive inhibitor (5, 9, 18, 19) and binds to the enzyme only in the presence of AMP. AMP, a competitive inhibitor of the catalytic adenylosuccinate lyase reaction, also does not protect well against inactivation, when added by itself. This is understandable since 6-BDB-TAMP (as compared with AMP) carries a BDB group which is the portion of the reagent which undergoes modification. The reagent is more similar structurally to the substrate adenylosuccinate and binds to the enzyme more tightly than does AMP. In fact, adenylosuccinate, or AMP added in conjunction with fumarate, decreases the inactivation rate constant as much as 10-fold. These results suggest that both the AMP and fumarate (equivalent to the succinyl group of adenylosuccinate) sites are occupied by 6-BDB-TAMP upon inactivation: the 6-thio-AMP moiety is expected to bind to the AMP site, while the BDB group is likely to occupy the fumarate site. Both sites must be blocked in order to maximally prevent inactivation. This conclusion is consistent with the observation that effective protection is afforded by the hydrolyzed 6-BDB-TAMP, which is capable of occupying both sites.

Histidine has been identified as the target residue of 6-BDB-TAMP in adenylosuccinate lyase. Only one modified peptide has been isolated from the tryptic digest of 6-BDB-TAMP-inactivated enzyme; and its adsorption on a phenyl boronate-agarose column is consistent with its designation as a nucleotidyl peptide. Gas-phase sequencing identified the 11-membered peptide as Thr-His-Phe, with the low yield of PT-His in cycle 2 indicating that His was the modified residue. Histidine can react with 6-BDB-TAMP by nucleophilic displacement of the bromide. The molecular weight of the peptide detected by mass spectrometry corresponds to the displacement product. However, the release of free reagent during HPLC and the small amount of histidine regenerated during sequencing indicate the limited stability of the histidine product.

The amino acid sequences of adenylosuccinate lyase are known for 7 organisms, including three bacterial (B. subtilis (10), E. coli (11), and H. influenzae (12)), one archaeal (M. jannaschii (13)), one avian (chicken (14)), and two mammalian (murine (15) and human (16)) enzymes. Alignment among these sequences exhibits 7% identity plus 23% similarity. His of B. subtilis is one of the well conserved amino acids. In fact, as shown in Fig. 7, the sequences flanking His are relatively well conserved, consistent with this region of the enzyme playing an important role in catalysis.

Adenylosuccinate lyase bears sequence homology to argininosuccinate lyase (EC 4.3.2.1), aspartase (EC 4.3.1.1), class II fumarases (EC 4.2.1.2), and cys-crystallin (34–38). This is a group of functionally related enzymes which generate fumarate by cleaving different substrates. cys-Crystallin, a major structural protein component in the avian and reptilian lens, is considered to have evolved from the enzyme argininosuccinate lyase (39–41). Moreover, all of these enzymes have been characterized as tetrameric enzymes (36, 42) having similar catalytic mechanisms (37). Chemical modification (43, 44) and pH dependence (7, 18, 45) studies have indicated an essential

**Table II**

| Cycle No. | Amino acid | Position in sequence | Amount$^a$ | pmol |
|-----------|------------|----------------------|------------|------|
| 1         | Thr        | 140                  | 256        |      |
| 2         | His        | 141                  | 15         |      |
| 3         | Gly        | 142                  | 201        |      |
| 4         | Val        | 143                  | 266        |      |
| 5         | His        | 144                  | 63         |      |
| 6         | Ala        | 145                  | 223        |      |
| 7         | Glu        | 146                  | 160        |      |
| 8         | Pro        | 147                  | 134        |      |
| 9         | Thr        | 148                  | 74         |      |
| 10        | Thr        | 149                  | 49         |      |
| 11        | Phe        | 150                  | 28         |      |

$^a$ Approximately 230 pmol of peptide was applied to the sequencer based on radioactivity.

---

**Fig. 6. Initial purification of the modified peptide on the PBA-30 column.** The tryptic digest was applied to a PBA-30 column equilibrated with 50 mM potassium phosphate containing 50 mM KCl (pH 7.8) at 4°C. The bound peptides were eluted with water. The open squares represent the elution profile as measured by absorbance at 220 nm, while the shaded bars represent the elution profile as measured by radioactivity.
histidine residue in the active site of some members in this family. Sequence comparison showed that the region displayed in Fig. 7 is of two regions of high homology among this superfamily (36–38). His141 is conserved in almost all of these enzymes. In a site-directed mutagenesis study on duck δ-crystallin in which the corresponding histidine (i.e. His162) was converted to asparagine, the protein completely lost enzymatic activity (46).

Crystal structures of two enzymes from this family have been solved. In fumarase C of E. coli, the corresponding histidine (i.e. His200) is positioned near the center of the active site cleft formed by three of the four subunits, and is proposed to form a "charge relay system" with Glu331 from another subunit which is responsible for removal of the proton in a catalytic cycle (37). A similar cleft considered as the putative active site has been described in turkey δ-crystallin, in which His160 and Glu294 (positioned close to each other) are proposed as strong candidates for general base and general acid functions of the protein (42). Crystallization of the B. subtilis adenylosuccinate lyase has recently been reported (24), but the structure has not yet been determined.

The catalytic reaction of adenylosuccinate lyase is postulated to undergo a β-elimination mechanism (1, 18, 47), in which the deprotonation of the C6 and the cleavage of N-C5 occurs in a concerted manner. The other substrate in purine biosynthesis cleaved by adenylosuccinate lyase is 5-amino-4-imidazole-N-ribotide, which closely resembles adenylosuccinate, and also has a succinyl group attached. A similar succinocarboxamideribotide, which closely resembles adenylosuccinate, cleaved by adenylosuccinate lyase is 5-amino-4-imidazole-N-ribotide. This is suggestive of a general base reflected in the pH profile, since it is among the conserved amino acid residues (14, 18). Our studies show His141 of the B. subtilis enzyme is an effective nucleophile in attacking the bromodioxobutyl group of 6-BDB-TAMP. In accordance with the structural similarity between adenylosuccinate and 6-BDB-TAMP, the succinyl moiety of the substrate may occupy the same position as the reactive BDB group of the reagent; therefore His141 may be well positioned to remove a methylene proton from the succinyl part of adenylosuccinate. Our results strongly suggest that His141 acts as a general base accepting the proton from C6 in the catalysis. Mutagenesis of His141 provides an opportunity to evaluate the proposed role of this amino acid; these studies are in progress.

Acknowledgments—We appreciate the help of Dr. Yu-Chu Huang in determining the sequence of the modified peptide and Dr. Gordon Nicol in obtaining the results from mass spectrometry. We also thank Jin Zhou for technical assistance and Dr. Howard Zalkin for the original clone encoding B. subtilis adenylosuccinate lyase.

REFERENCES
1. Ratner, S. (1972) in The Enzymes (Boyer, P. D., ed) 3rd Ed., Vol. 7, pp. 167–197, Academic Press, New York
2. Carter, C. E., and Cohen, L. H. (1956) J. Biochem. 222, 17–30
3. Woodward, D. O., and Brayner, H. K. (1968) J. Biol. Chem. 241, 580–587
4. Hatch, M. D. (1966)
5. Pinto, R. M., Aranda, A., Fernandez, A., Canales, J., Siliero, A., and Siliero, M. A. G. (1983) J. Biochem. 238, 12513–12519
6. Miller, R. W., Lukens, L. N., and Buchanan, J. M. (1955) J. Biol. Chem. 234, 1806–1811
7. Casey, P. J., and Lowenstein, J. M. (1987) Biochem. J. 246, 263–269
8. Gite, S. U., and Colman, R. F. (1990) Biochemistry 29, 2658–2667
9. Barnes, L. N., and Bishop, S. H. (1975) Int. J. Biochem. 6, 497–503
10. Ebbole, D. J., and Zalkin, H. (1987) J. Biol. Chem. 262, 8274–8287
11. He, B., Smith, J. M., and Zalkin, H. (1992) J. Bacteriol. 174, 130–136
12. Freachers, D. R., Adams, M. D., White, O., Clayton, R. A., Kirkness, E. F., Kerlavage, A. R., Bult, C. J., Tomb, J. F., Dougherty, B. A., Merrick, J. M., Mckenney, K. S., Sutton, G. F., Fitzgerald, W., Fields, C. A., Gocayne, J. D., Scott, J. D., and Shiz, L. (1995) Science 269, 496–502
13. Bult, C. J., White, O., Olsen, G. J., Zhou, L., Freachers, D. R., Sutton, G. G., Blake, J. A., Fitzgerald, L. M., Clayton, R. A., Gocayne, J. D., Kerlavage, A. R., Lefrere, J. P., Lefrere, B. A., Tomb, J. F., Adams, M. D., Reich, C. I., Overbeek, R., Kirkness, E. F., Weinstock, K. G., Merrick, J. M., Godel, A., Scott, J. L., Geoghagen, F. M., Weisman, J. F., Furhman, J. L., Nguyen, D., Utterback, T. R., Kelley, J. M., Peterson, J. D., Sadow, P. W., Hanna, M. C., Cotty, M. D., Roberts, K. M., Hurst, M. A., Kaine, B. P., Borodovsky, M., Klenk, H. P., Fraser, C. M., Smith, M. H., Weiste, C. R., and Venter, J. C. (1996) Science 275, 1058–1073
14. Aimi, J., Badyak, J., Williams, J., Chen, Z., Zalkin, H., and Dixon, J. E. (1990) J. Biol. Chem. 265, 9011–9014
15. Wong, L.-C. J., and O'Brien, W. E. (1995) Genomics 25, 130–136
16. Stone, R. L., Aimi, J., Barshop, B. A., Jaeken, J., Van den Bergh, G., Zalkin, H., and Dixon, J. E. (1992) J. Biol. Chem. 267, 19710–19716
17. Jaeken, J., and Van den Bergh, G. (1984) Lancet 2, 1058–1061
18. Stone, R. L., Zalkin, H., and Dixon, J. E. (1992) Nat. Genet. 5, 59–63
19. Bridger, W. A., and Cohen, L. H. (1968) J. Biol. Chem. 243, 644–650
20. Bridger, W. A., and Cohen, L. H. (1969) Can. J. Biochem. 47, 665–672
21. Colman, R. F., Huang, Y.-C., King, M. M., and Erh, M. (1984) Biochemistry 23, 3281–3286
22. Colman, R. F. (1990) in The Enzymes (Sigman, D. S., and Boyer, P. D., ed) 3rd Ed., Vol. 19, pp. 283–321, Academic Press, New York
23. Lee, T., Dixon, J. E., and Colman, R. F. (1996) FEBS J. 10, A1101
24. Edbo, D. J., and Zalkin, H. (1987) J. Biol. Chem. 262, 8274–8287
25. Groves, W. E., Davis, J. F., and Sells, B. H. (1968) Anal. Biochem. 22, 195–210
26. Keenan, R. W., Martinez, R. A., and Williams, R. F. (1982) J. Biol. Chem. 257, 14817–14820
27. Osturk, D. H., Park, I., and Colman, R. F. (1992) Biochemistry 31, 10544–10555
28. Penevski, H. S. (1979) Methods Enzymol. 56, 327–330
29. Cohen, L. H. (1980) In The Enzymes (Sigman, D. S., and Boyer, P. D., ed) 3rd Ed., Vol. 19, pp. 283–321, Academic Press, New York
30. Groves, W. E., Davis, J. F., and Sells, B. H. (1968) Anal. Biochem. 22, 195–210
31. Annamalai, A. E., Pal, P. K., and Colman, R. F. (1979) Anal. Biochem. 99, 85–91
32. Colman, R. F. (1990) in The Enzymes (Sigman, D. S., and Boyer, P. D., ed) 3rd Ed., Vol. 19, pp. 283–321, Academic Press, New York
33. Lee, T. T., Dixon, J. E., and Colman, R. F. (1996) FEBS J. 10, A1101
34. Redinbo, M. R., Eide, S. M., Stone, R. L., Dixon, J. E., and Yeates, T. O. (1996) Protein Sci. 5, 776–786
35. Groves, W. E., Davis, J. F., and Sells, B. H. (1968) Anal. Biochem. 22, 195–210
32. Batra, S. P., and Colman, R. F. (1986) *Biochemistry* **25**, 3508–3515
33. DeCamp, D. L., and Colman, R. F. (1989) *J. Biol. Chem.* **264**, 8430–8441
34. Vollmer, S. A., Walner, M. B., Tarbell, K. V., and Colman, R. F. (1994) *J. Biol. Chem.* **269**, 8082–8090
35. Moe, O. A., Baker-Malcom, J. F., Wang, W., Kang, C., Fromm, H. J., and Colman, R. F. (1996) *Biochemistry* **35**, 9024–9033
36. Williams, S. E., Woolridge, E. M., Ransom, S. C., Landro, J. A., Babbitt, P. C., and Kozarich, J. W. (1992) *Biochemistry* **31**, 9768–9776
37. Weaver, T. M., Levitt, D. G., Donnelly, M. I., Stevens, P. P., and Banaszak, L. J. (1995) *Nat. Struct. Biol.* **2**, 654–662
38. Wistow, G. J., and Piatigorsky, J. (1990) *Gene (Amst.)* **96**, 263–270
39. Chiou, S.-H., Lee, H.-J., Chu, H., Lai, T.-A., and Chang, G.-G. (1991) *Biochem. Int.* **25**, 705–713
40. Lee, H.-J., Chiou, S.-H., and Chang, G.-G. (1992) *Biochem. J.* **283**, 597–603
41. Wistow, G., and Piatigorsky, J. (1987) *Science* **236**, 1554–1556
42. Simpson, A., Bateman, O., Driessen, H., Lindley, P., Moss, D., Mylvaganam, S., Narebor, E., and Slingsby, C. (1994) *Nat. Struct. Biol.* **1**, 724–734
43. Ida, N., and Tokushige, M. (1984) *J. Biochem.** **96**, 1315–1321
44. Lee, H.-J., Chiou, S.-H., and Chang, G.-G. (1993) *Biochem. J.* **293**, 537–544
45. Karsten, W. E., and Viola, R. E. (1991) *Arch. Biochem. Biophys.* **287**, 60–67
46. Patejunas, G., Barbosa, P., Lacombe, M., and O’Brien, W. E. (1995) *Exp. Eye. Res.* **61**, 151–154
47. Havir, E., and Hansen, K. (1973) in *The Enzymes* (Boyer, P. D., ed) 3rd Ed., Vol. 7, pp. 75–166, Academic Press, New York
Identification of His\textsuperscript{141} in the Active Site of \textit{Bacillus subtilis} Adenylosuccinate Lyase by Affinity Labeling with 6-(4-Bromo2,3-dioxobutyl)thioadenosine 5\textquoteright-Monophosphate

Tom T. Lee, Carolyn Worby, Jack E. Dixon and Roberta F. Colman

\textit{J. Biol. Chem.} 1997, \textbf{272}:458-465.
doi: 10.1074/jbc.272.1.458

Access the most updated version of this article at http://www.jbc.org/content/272/1/458

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 47 references, 19 of which can be accessed free at http://www.jbc.org/content/272/1/458.full.html#ref-list-1