Affinity Labeling of the Active Site of Rabbit Muscle Adenylosuccinate Lyase by 2-[(4-Bromo-2,3-Dioxobutyl)Thio]Adenosine 5′-Monophosphate†

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ABSTRACT: Rabbit muscle adenylosuccinate lyase upon incubation with 7.5−50 μM 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-monophosphate (2-BDB-TAMP) in 0.05 M PIPES buffer, pH 7.0 and 10 °C, gives a time dependent biphasic inactivation. The rate of inactivation exhibits a nonlinear dependence on the concentration of 2-BDB-TAMP, which can be described by reversible binding of reagent to the enzyme (K_I = 8.5 μM, 5.2 μM) prior to the irreversible reaction, with maximum rate constants of 0.319 and 0.027 min^{-1} for the fast and slow phases, respectively. The enzyme is a tetramer, with subunits of 50 000 Da. When the enzyme was 90% inactivated, 0.84 mol of reagent/mol of subunit was incorporated as measured by protein-bound phosphate analysis; similar results were obtained using 2-BDB-[^{14}C]TAMP. Complete protection against inactivation and incorporation was afforded by 1 mM 5′-AMP and by 0.1 mM 5′-AMP + 5 mM fumarate (the natural products of adenylosuccinate hydrolysis) but not by 0.1 mM 5′-AMP alone, 5 mM fumarate alone, or 0.1 mM 5′-AMP + 5 mM maleate or 5 mM succinate. These studies suggest that 2-BDB-TAMP inactivates adenylosuccinate lyase by specific reaction at the substrate binding site, with negative cooperativity between subunits accounting for the appearance of two phases of inactivation. Cleavage of 2-BDB-TAMP-modified enzyme with cyanogen bromide and subsequent separation of peptides by reverse phase HPLC gave only one radioactive peak. This radioactive peptide was further digested with papain and the target site of the 2-BDB-TAMP reaction was identified as Arg^{112}. We conclude that Arg^{112} is located in the substrate binding site of rabbit muscle adenylosuccinate lyase.

Adenylosuccinate lyase [EC 4.3.2.2] catalyzes the conversion of adenylosuccinate to 5′-AMP and fumarate, a critical reaction in the purine biosynthesis pathway (Miller *et al.*, 1957; Ratner, 1973). Deficiency of adenylosuccinate lyase activity is associated with severe mental retardation and secondary autistic features in a number of human patients (Jaeken & Van den Berghe, 1984; Van den Berghe & Jaeken *et al.*, 1986; Jaeken *et al.*, 1988, 1992; Barshop *et al.*, 1989; Van den Berghe *et al.*, 1991). In one family, the deficiency was traced to a single mutation (Ser413Pro) which results in structural instability of the enzyme rather than a change in catalysis (Stone *et al.*, 1992).

Kinetic studies have been conducted on the enzyme from a variety of sources, including yeast (Cohen & Bridger, 1964; Bridger & Cohen, 1968, 1969), rat (Casey & Lowenstein, 1987), chicken (Aimi *et al.*, 1990), and human (Stone *et al.*, 1993). Adenylosuccinate lyases from several species are homotetramers with subunits of about 50 000 Da (Casey & Lowenstein, 1987; Stone *et al.*, 1993). The cDNAs of adenylosuccinate lyase from *Bacillus subtilis* (Ebbole & Zalkin, 1987), chicken (Aimi *et al.*, 1990), and human (Stone *et al.*, 1993) have been cloned, and their amino acid sequences have been derived from their corresponding cDNA sequences. All three exhibit considerable resemblance, and the amino acid sequences of the enzymes from chicken and human show a high degree of identity (85%) plus similarity (5%). Although Stone *et al.* (1993) have postulated a mechanism involving general acid and base catalysis in the cleavage of adenylosuccinate, no enzymatic amino acids have been directly implicated in either catalysis or substrate binding.

Affinity labeling provides a powerful approach to identify amino acid residues within specific binding sites in enzymes (Colman, 1983, 1989, 1990, 1995). In the present study, 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-monophosphate (2-BDB-TAMP)[1] has been selected as a potential affinity label for adenylosuccinate lyase because of its structural similarity to the substrate, adenylosuccinate (Figure 1). Reaction of 2-BDB-TAMP can potentially occur with the side chains of several amino acids of proteins, including Lys, Arg, Cys, His, Glu, Asp, Tyr, and Met (Colman, 1989, 1990, 1995; Kapetanovic *et al.*, 1985). Such reactions can occur at the carbonyl groups or by nucleophilic displacement of the bromide from $-CH_2Br$. Thus, once the nucleotide derivative is bound at a specific site, it is likely to have an accessible amino acid target. In addition, 2-BDB-TAMP is soluble in water, eliminating the need for addition of organic solvent in the reaction mixture. Previously, in this laboratory, bromodioxobutyl nucleotides have been shown to function as affinity labels of several enzymes including pyruvate kinase (Kapetanovic *et al.*, 1985; DeCamp *et al.*, 1988), isocitrate dehydrogenase (Huang & Colman, 1984) and glutamate dehydrogenase (Batra & Colman, 1984, 1986).

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[1] Abbreviations: 2-BDB-TAMP, 2-[(4-bromo-2,3-dioxobutyl)thio]-adenosine 5′-monophosphate; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; NEM, *N*-ethylmaleimide; TFA, trifluoroacetic acid; PIPES, piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; FPLC, fast protein liquid chromatography; MES, 2-[*N*-morpholino]ethanesulfonic acid.

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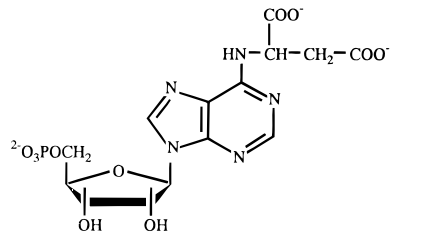
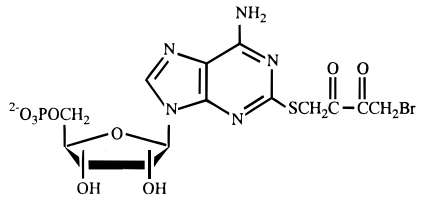


Adenylosuccinate



2-BDB-TAMP

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

We describe here the specific modification of rabbit muscle adenylosuccinate lyase by the nucleotide affinity label 2-BDB-TAMP. A preliminary version of this work has been presented (Gite & Colman, 1995).

EXPERIMENTAL PROCEDURES

Materials. Rabbit muscle (mature) was purchased from Pel-Freez Biologicals and stored frozen at −80 °C until further use. Adenylosuccinate, fumarate, succinate, maleate, 5′-AMP, dithiothreitol (DTT), *N*-ethylmaleimide (NEM), Sephadex G-10, papain, and phosphate standard solution were all obtained from Sigma Chemical Co. Carbon disulfide, chloroperoxybenzoic acid, and 1,4-dibromobutanedione were from Aldrich Chemical Co. Prior to use, 1,4-dibromobutanedione was recrystallized from petroleum ether. DEAE-cellulose (DE-52) was supplied by Whatman, Matrex Blue by Amicon, and molecular weight marker kits by Pharmacia Fine Chemicals. Cyanogen bromide was obtained from Fluka, and ammonium sulfate (ultrapure) and protease from *Staphylococcus aureus*, strain V8, were purchased from ICN Biochemicals. The Bio-Rad protein assay dye reagent was supplied by Bio-Rad. 5′-[^{14}C]AMP (generally labeled) and [^{3}H]$NaBH_4$ were obtained from New England Nuclear−DuPont. All other chemicals used were the highest purity reagent grade.

The commercially available papain, obtained from Sigma Chemical Co., exhibited numerous peptide peaks when subjected to HPLC on a C_{18} column. Thus, the papain preparation requires additional purification prior to use. Papain (20 mg) was purified by HPLC on a Varian model 5000 HPLC system equipped with a reverse phase Vydac C_{18} column (0.46 × 25 cm). The proteolytic enzyme was dissolved in 1 mL of 0.02 M ammonium acetate buffer (pH 6.0), centrifuged at 14 000 rpm for 5 min, and injected onto the column equilibrated with solvent A (0.02 M ammonium acetate, pH 6.0). The column was eluted with solvent A for 10 min, followed by a linear gradient from solvent A to 100% solvent B (0.02 M ammonium acetate, pH 6.0, in 50% acetonitrile) between 10 and 110 min. The flow rate was 1 mL/min. The effluent was continuously monitored at 280 nm, and fractions of 1 mL were collected. Fractions 54−57 were pooled and lyophilized; this purified papain preparation was used to digest adenylosuccinate lyase fragments.

Preparation of 2-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 5′-Monophosphate. 2-BDB-TAMP was synthesized from 5′-AMP according to the procedure of Kapetanovic *et al.* (1985). The concentration of the final compound was calculated from its UV spectrum in 0.1 M MES buffer, pH 6.0, using $\epsilon_{246\ nm} = 35\ 200$ and $\epsilon_{270\ nm} = 11\ 500\ M^{-1}\ cm^{-1}$. The synthesis of 2-BDB-[^{14}C]TAMP was conducted by the same method using as starting material 110 μCi of generally labeled 5′-AMP plus 0.74 mmol of non-radioactive 5′-AMP. The overall yield of 2-BDB-TAMP was 10%−20%. The specific radioactivity of 2-BDB-[^{14}C]TAMP was about 3.2 × 10^{11} cpm/mol.

Enzyme Assay and Protein Determination. The adenylosuccinate lyase activity of the enzyme was measured spectrophotometrically using a double-beam Perkin-Elmer spectrophotometer equipped with a recorder set to 0.1 absorbance full scale. The assay was performed at 25 °C in a total volume of 1 mL containing 30 μM adenylosuccinate in 10 mM Tris-HCl buffer (pH 7.4), 0.15 M NaCl, and appropriately diluted enzyme (1−5 μg). The hydrolysis of adenylosuccinate was followed by the decrease in absorbance at 282 nm using the difference extinction coefficient of 10 000 $M^{-1}\ cm^{-1}$ between the substrate, adenylosuccinate, and the product, 5′-AMP (Tornheim & Lowenstein, 1972). One unit of enzyme activity is defined as the amount of enzyme that converts one micromole of adenylosuccinate to 5′-AMP and fumarate per minute at 25 °C, and the specific activity of the enzyme is taken as the enzyme units per milligram of protein.

For the purified enzyme, the protein concentration was determined according to the method of Groves *et al.* (1968) from the difference between $A_{224\ nm}$ and $A_{233\ nm}$ using crystalline bovine serum albumin as a standard, since this method is less dependent on the amino acid composition of the particular protein. On the basis of this measurement, the purified adenylosuccinate lyase exhibits $\epsilon_{280}^{1\%} = 7.6$.

Purification of Rabbit Muscle Adenylosuccinate Lyase. Frozen rabbit muscle was thawed at 37 °C for 1 h and cut into small pieces, and the visible fat was removed. The muscle tissue (one box, approximately 800 g) was then homogenized in a Waring blender for 2 min at each speed (slow, medium, and fast) using, for each g of tissue, 4 mL of homogenization buffer [10 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, 0.2 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride (dissolved in 1 mL *N*,*N*-dimethylformamide), and 10% glycerol]. The homogenate was centrifuged at 8000 rpm for 1 h, and the supernatant was decanted through eight layers of cheesecloth. The supernatant was brought to 50% ammonium sulfate saturation and was maintained at 4 °C for 6 h. The suspension was centrifuged at 8000 rpm for 1 h, and the resulting supernatant was adjusted to 65% saturation of ammonium sulfate and stood overnight at 4 °C. The precipitate was collected by centrifugation (8000 rpm, 1 h) and dissolved in a minimum volume (∼200 mL) of 10 mM Tris-HCl buffer, pH 7.7, containing 1 mM EDTA, 0.2 mM DTT, and 20% glycerol. The enzyme was then dialyzed extensively against the same buffer.

All subsequent purification steps were conducted at room temperature. The dialysate (300 mL) was applied to a

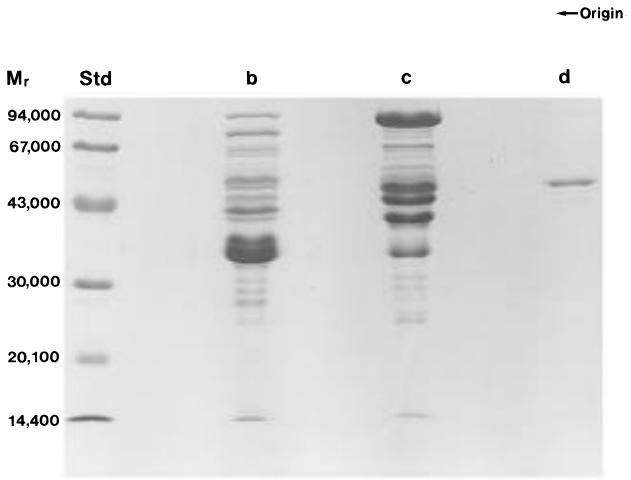


FIGURE 2: Electrophoresis of rabbit muscle adenylosuccinate lyase samples in polyacrylamide gels containing SDS after various purification steps. The letters above the lanes refer to the samples (b) after DEAE-cellulose chromatography, (c) after Matrex Blue chromatography, and (d) after FPLC Superose-12 gel filtration.

column of DEAE-cellulose (3 × 40 cm), which was equilibrated with the above buffer. The column was eluted with the same buffer, at a rate of 60 mL/h, until the $A_{280\ nm}$ of the eluate was <0.1 (~500 mL). We then applied to the column a linear gradient formed from 500 mL of the starting buffer and 500 mL of the same buffer containing 0.1 M KCl. Fractions of 6 mL were collected. The adenylosuccinate lyase eluted between 210 and 280 mL after the start of the linear gradient. The fractions showing relatively high specific activity were pooled and applied directly to a Matrex Blue column (1.5 × 25 cm) equilibrated with 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, 0.2 mM DTT, and 20% glycerol. The column was eluted with the same buffer at 30 mL/h until the unbound protein was removed. The bound enzyme was then eluted with a linear gradient composed of 200 mL of the initial buffer and 200 mL of the same buffer containing 2 mM 5′-AMP + 5 mM fumarate. Fractions of 6 mL were collected. Most enzyme activity eluted between 60 and 120 mL after the start of the gradient. Fractions showing high specific activity were pooled and concentrated by Amicon ultrafiltration unit fitted with a PM-10 membrane. The concentrated enzyme was subjected to FPLC (Pharmacia) gel filtration using a Superose-12 column (1 × 30 cm) equilibrated with 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, 0.2 mM DTT, and 10% glycerol. For each run, 0.3−0.4 mL of concentrated enzyme sample was applied to the column at a flow rate of 0.3 mL/min. Fractions (0.5 mL) were collected, and the enzyme activity was measured. The purity of individual fractions was evaluated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) prior to pooling (Laemmli, 1970). The pooled enzyme was dialyzed against 50 mM PIPES buffer, pH 7.0, containing 20% glycerol and stored frozen at −80 °C until further use. No loss of enzyme activity was observed for at least 2 months under these conditions.

The final enzyme preparation has a specific activity of 11.7 units/mg of protein and has been purified approximately 2000-fold as compared to the initial extract, with an overall recovery of about 37%. From 800 g of rabbit muscle, 3.5 mg of purified enzyme was obtained. Figure 2 illustrates the electrophoretic analysis of enzyme samples at various stages of the purification procedure. After gel filtration, the enzyme preparation exhibited a single band on polyacrylamide gel electrophoresis in the presence of SDS (Figure 2, lane d), indicating the purity of the preparation and indicating that it contains polypeptide chains of the same molecular weight. Comparison of the mobility of the enzyme with that of protein standards yields a subunit M_r of about 50 000. The M_r of adenylosuccinate lyase under non-denaturing conditions was estimated as 200 000 by FPLC gel filtration using standard molecular weight markers. These results indicate that the native enzyme is a tetramer, as are the enzymes from rat (Casey & Lowenstein, 1987) and human (Stone *et al.*, 1993). No PTH-amino acids were detected by N-terminal sequencing by the Edman method. However, four internal peptides derived from cyanogen bromide cleavage were isolated and sequenced. As compared to the sequence of the human enzyme (Stone *et al.*, 1992), the rabbit muscle adenylosuccinate lyase has the same sequence for amino acids 65−81, 201−217, 365−381, and 157−173 except for the two substitutions L217T and Q165R. The rabbit muscle enzyme thus appears to be a tetramer of identical subunits which are substantially similar to the human enzyme.

Reaction of 2-BDB-TAMP with Rabbit Muscle Adenylosuccinate Lyase. Rabbit muscle adenylosuccinate lyase (0.125 mg/mL, 2.5 μM subunit) was incubated with various concentrations (7.5−50 μM) of 2-BDB-TAMP at 10 °C in 0.05 M PIPES buffer, pH 7.0. Control samples were incubated under the same conditions except for the absence of 2-BDB-TAMP. When the protecting ligands were included, the enzyme was preincubated with them for 10 min before the reagent was added. At timed intervals, aliquots of the reaction mixture were removed and assayed for the residual enzymatic activity. The rate of reaction of enzyme with 2-BDB-TAMP was determined by using the following equation for biphasic kinetics:

$$E_t/E_o = (1 - F)e^{-k_{fast}t} + (F)e^{-k_{slow}t} \qquad (1)$$

where E_o represents the initial enzyme activity at time zero, E_t represents the residual activity at any given time, and F represents the fractional residual activity of the partially active enzyme at the end of first phase. Data analysis was performed by an IBM computer program developed by Dr. Joseph H. Noggle of the University of Delaware for estimation of nonlinear parameters; this program minimizes the differences between experimental and predicted values of E_t/E_o by adjusting the values of k_{fast} and k_{slow} in eq 1.

Measurement of Incorporation of 2-BDB-TAMP into Enzyme. Adenylosuccinate lyase (0.5 mg/mL) was incubated with 20 μM 2-BDB-TAMP, and the reaction proceeded for 60 min. Unmodified enzyme, at the same protein concentration and in the same buffer, was used as a blank. In certain experiments, various ligands were added as protectants. After the reaction, the excess reagent was removed by extensive dialysis against 0.05 M HEPES buffer, pH 7.0, and the enzyme concentration was determined by the Bio-Rad protein assay, which is based on the dye-binding method of Bradford (1976) using the Bio-Rad 2550 RIA Reader (600 nm filter). The standard curve was generated using various concentrations of purified rabbit muscle adenylosuccinate lyase in the same buffer.

The amount of reagent incorporated into the enzyme was determined from the moles of organic phosphate bound to the modified enzyme using a procedure developed by Hess and Derr (1975) and Lanzetta *et al.* (1979), as modified by Bailey and Colman (1987). For the organic phosphorus determination, the unmodified and modified samples (0.2 mL each), as obtained above, were first hydrolyzed with H_2SO_4 at 190 °C for 2 h and then bleached with 20 μL of 30% (v/v) hydrogen peroxide with heating at 190 °C for 1 h. The hydrogen peroxide treatment was repeated 2−3 times until the samples were colorless. The dry residue was then dissolved in 100 μL of HPLC grade water, and, after the addition of 300 μL of a mixture of three parts of Malachite green base and one part of ammonium molybdate solution, $A_{660\ nm}$ was determined using a total volume of 400 μL. The calibration curve was established with a standard phosphate solution treated under the identical conditions as the experimental samples.

The reagent incorporation was also measured radiochemically using 2-BDB-[^{14}C]TAMP. The amount of radioactive reagent incorporated was determined by counting aliquots of modified enzyme in 5 mL of liquid scintillation cocktail (Liquiscint, National Diagnostic) using a Packard Tricarb model 3300 liquid scintillation counter.

Treatment of 2-BDB-TAMP-Modified Enzyme with [^{3}H]-$NaBH_4$. The dioxo groups of several bromodioxobutyl nucleotide-modified enzymes have previously been reduced by [^{3}H]$NaBH_4$, thereby introducing a radioactive tag into modified enzyme (Batra *et al.*; DeCamp & Colman, 1989; Vollmer & Colman, 1990). Accordingly, we attempted to use this approach for 2-BDB-TAMP-modified adenylosuccinate lyase. Immediately following the modification of the enzyme (0.5 mg/mL, 10 μM subunit) by 40 μM 2-BDB-TAMP, the reaction mixture was placed on ice and [^{3}H]-$NaBH_4$ (dissolved in 0.02 M NaOH) was added to the reaction mixture to give a final concentration of 1 mM. After 10 min, another aliquot of [^{3}H]$NaBH_4$ was added to the reaction mixture to yield a final concentration of 2 mM, and incubation was continued for an additional 10 min. The specific radioactivity of [^{3}H]$NaBH_4$ used for reduction was 1.5×10^{12} cpm/mol of hydrogen. A control enzyme sample was also treated under identical conditions except for the absence of 2-BDB-TAMP. The excess reagent and [^{3}H]-$NaBH_4$ were removed by extensive dialysis, and the tritium incorporated into enzyme was evaluated.

Chemical Cleavage of 2-BDB-[^{14}C]TAMP-Modified Enzyme. Rabbit muscle adenylosuccinate lyase (0.5 mg/mL, 10 μM subunit) was incubated with 40 μM 2-BDB-[^{14}C]-TAMP, as described above, for 1 h after which the reaction mixture was placed on ice and treated with non-radioactive $NaBH_4$ dissolved in 0.02 M NaOH (two additions, 10 min apart, to yield a final concentration of 2 mM). *N*-Ethylmaleimide was then added to the reaction mixture to yield 10 mM final concentration, and the sample was incubated at 30 °C for 30 min to block all free −SH groups. The enzyme was then dialyzed extensively against 0.1% TFA in water. After the removal of excess reagents, the dialysate was lyophilized and dissolved in 1 mL of 70% formic acid. Cyanogen bromide (l0 mg/mg of protein) was added, and the reaction mixture was incubated for 20 h in a dark hood at room temperature. The reaction mixture was then diluted 5-fold with HPLC grade water and lyophilized.

Separation of 2-BDB-[^{14}C]TAMP-Labeled Peptide(s) by HPLC. The radioactively labeled peptide(s) were separated on a Varian model 5000 HPLC system equipped with a reverse phase Vydac C_{18} column (0.46 cm × 25 cm). The cyanogen bromide digest was dissolved in 1 mL of 0.1% TFA in water, centrifuged at 14 000 rpm, and injected onto the column equilibrated with solvent A (0.1% TFA in water). The elution rate was 1 mL/min. The column was eluted with solvent A from 0 to 10 min followed by a linear gradient from solvent A (i.e., 0% solvent B) to 60% solvent B (0.075% TFA in acetonitrile) between 10 and 130 min. The effluent was continuously monitored at 220 nm, and fractions of 1 mL were collected. Aliquots were assayed for radioactivity. Chromatography of radioactive 2-BDB-TAMP alone was conducted using the same elution protocol.

Proteolytic Cleavage of Radioactive Cyanogen Bromide Peptide(s) and Their Further Purification. The radioactive peak obtained from the first HPLC run was lyophilized. The sample was dissolved in 1 mL of 0.05 M sodium citrate−phosphate buffer, pH 4.0 (Gomori, 1955), and digested with 10% (w/w) protease from *Staphylococcus aureus*, strain V8, added in two aliquots over a total of 2 h at 37 °C. The resultant peptides were separated by HPLC using the same solvent system as that for the original cyanogen bromide peptides. The radioactive peak was pooled, lyophilized, and then further proteolyzed with 1% (w/w) purified papain for 2 h at 25 °C in 0.05 M sodium acetate buffer, pH 4.0. The digest was fractionated by HPLC using a C_{18} column equilibrated with 0.1% TFA (solvent A). After elution with solvent A for 10 min, a linear gradient was established from solvent A to 20% solvent B (0.075% TFA in acetonitrile) in 210 min. Fractions of 1 mol were collected and assayed for radioactivity.

Peptide Analysis. The amino acid sequences of isolated 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 900A. Approximately 60−200 pmol of isolated peptides was applied to the sequencer.

RESULTS

Inactivation of Rabbit Muscle Adenylosuccinate Lyase by 2-BDB-TAMP. Incubation of 2.5 μM subunit of rabbit muscle adenylosuccinate lyase with 10 μM 2-BDB-TAMP, at pH 7.0 and 10 °C, resulted in a rapid time dependent loss in the activity of the enzyme (Figure 3). In contrast, control enzyme, incubated under identical conditions but in the absence of reagent, exhibited constant activity during this time period. Biphasic inactivation kinetics were observed, which could be described in terms of a fast phase of inactivation resulting in a partially active enzyme (∼50% residual activity) followed by a slow phase of inactivation yielding completely inactivated enzyme. Reagent decomposition might be considered as one possible explanation for the biphasic inactivation. However, the rate constant for decomposition of 2-BDB-TAMP has been determined to be 0.0115 min^{-1} ($t_{1/2}$ = 60 min), as measured either by the change in absorbance at 270 or 310 nm or by the release of free bromide, at pH 7.0 and 25 °C (Kapetanovic *et al.*, 1985); the decomposition rate should be more than 2.5-fold slower at 10 °C, the temperature used for the modification reaction in the present studies. In contrast, the second phase of

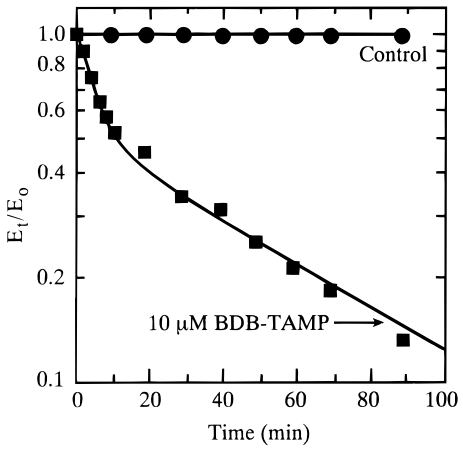


FIGURE 3: Inactivation of rabbit muscle adenylosuccinate lyase by 2-BDB-TAMP. Enzyme (0.125 mg/mL) was incubated with (■) or without (●) 10 μM 2-BDB-TAMP at pH 7.0 and 10 °C. At the times indicated, aliquots were withdrawn and assayed for enzymatic activity. Residual activity was measured as described under Experimental Procedures. E_t represents the enzyme activity at any given time t, and E_o represents the enzyme activity at time $t = 0$. The solid line is the theoretical line based on the computer fit to eq 1, with $k_{fast} = 0.16$ min^{-1} and $k_{slow} = 0.015$ min^{-1}.

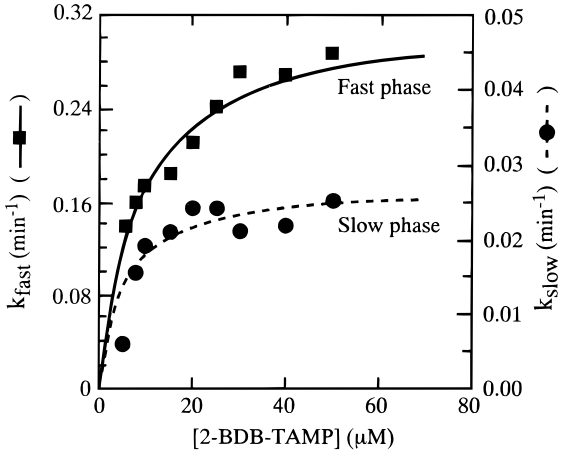


FIGURE 4: Dependence of k_{obs} on the concentration of 2-BDB-TAMP. Rabbit muscle adenylosuccinate lyase (0.125 mg/mL) was incubated with various concentrations of 2-BDB-TAMP (7.5−50 μM) in 0.05 M PIPES buffer, pH 7.0 and at 10 °C, and the rate constants were calculated as illustrated in Figure 3: (■) fast phase and (●) slow phase of inactivation.

inactivation predominates by 10 min of incubation of adenylosuccinate lyase in the reaction mixture (Figure 3). Thus, the relatively slow decomposition of the reagent can be excluded as an explanation for the biphasic kinetics.

Rate of Reaction of Adenylosuccinate Lyase as a Function of 2-BDB-TAMP Concentration. The dependence of the rate constants on the reagent concentration was determined from 7.5−50 μM 2-BDB-TAMP. At all concentrations tested, biphasic kinetics were observed (data not shown), and the rate constants and F values were calculated using eq 1. The value of F was usually about 0.52, but in single experiments ranged from 0.49 to 0.57. For comparison of the rate constants at various reagent concentrations, F was taken as 0.5. For both of the phases, a nonlinear dependence of k_{obs} on the reagent concentration was observed (Figure 4). This data indicates the initial formation of a reversible enzyme−reagent complex prior to the irreversible modification reaction, as expected for affinity labeling of enzymes. The observed rate constant (k_{obs}) at a particular concentration of

Table 1: Effect of Dicarboxylic Acids and 5′-AMP on Inactivation of Enzyme by 2-BDB-TAMP[a]

incubation	fast phase k_{+L}/k_{-L}	slow phase k_{+L}/k_{-L}
none	1.0	1.0
A. fumarate (5 mM)	0.94	0.37
maleate (5 mM)	0.84	0.57
succinate (5 mM)	1.0	0.61
B. 5′-AMP (1 mM)	0.04	0.04
5′-AMP (0.1 mM)	0.39	0.23
C. 5′-AMP (0.1 mM)		
+ fumarate (5 mM)	0.05	0.03
+ maleate (5 mM)	0.33	0.38
+ succinate (5 mM)	0.23	0.35

[a] Rabbit muscle adenylosuccinate lyase (2.5 μM enzyme subunit) was incubated with 10 μM 2-BDB-TAMP in 0.05 M PIPES buffer, pH 7.0 at 10 °C, in the presence of various ligands. The rate constants were measured as described in the Experimental Procedures and as illustrated in Figure 3. The indicated ligand(s) were added either at $t = 0$ min or after 10 min of incubation to test their effects, respectively, on the fast phase or slow phase of inactivation.

2-BDB-TAMP can be described by the equation

$$k_{obs} = \frac{k_{max}[R]}{K_I + [R]} \quad (2)$$

where [R] = the concentration of 2-BDB-TAMP, k_{max} = maximum k_{obs} at saturating concentrations of the reagent, and $K_I = (k_{-1} + k_{max})/(k_1)$, which represents the concentration of the reagent that gives half of the maximal inactivation rate (Huang & Colman, 1984). From plots of $1/k_{obs}$ versus 1/[R], values of $K_I = 8.5$ μM and $k_{max} = 0.319$ min^{-1} were calculated for the fast phase, and $K_I = 5.2$ μM and $k_{max} = 0.027$ min^{-1} were calculated for the slow phase. Although the K_I values for 2-BDB-TAMP are similar for the two phases of the reaction, the k_{max} is about 10-fold lower for the slow phase.

Effect of Dicarboxylic Acids and 5′-AMP on the Rate of Inactivation by 2-BDB-TAMP. 5′-AMP, fumarate, maleate, and succinate as well as their combinations were tested for their ability to protect the enzyme against inactivation by 2-BDB-TAMP (Table 1). Fumarate and 5′-AMP were included in the incubation mixtures at concentrations high relative to the K_i values reported for the adenylosuccinate cleavage reaction of adenylosuccinate lyase (~0.6 mM for fumarate, ~12 μM for AMP) from various species (Ratner, 1973; Casey & Lowenstein, 1987; Stone *et al.*, 1993; Pinto *et al.*, 1983; Woodward & Braymer, 1966). The protecting ligands were either added prior to the addition of the reagent or after 10 min of incubation of enzyme and reagent, to test their effect on the fast and slow phases of inactivation, respectively. The dicarboxylic acid compounds have little effect on the reaction when added by themselves, although fumarate has a somewhat greater effect on the slow phase than on the fast phase. A saturating concentration of 5′-AMP (1 mM) decreases k_{obs} 25-fold. However, 0.1 mM 5′-AMP is less effective. A combination of 0.1 mM 5′-AMP plus 5 mM fumarate affords complete protection, indicating that fumarate enhances the binding of 5′-AMP to the enzyme. In contrast, maleate or succinate (dicarboxylic acid analogs of fumarate which are not known to be substrates for the enzyme) does not enhance the protective effect of 0.1 mM 5′-AMP; these results are consistent with the specificity of the enzyme for fumarate. Both phases of inactivation are

Table 2: Incorporation of 2-BDB-TAMP into Rabbit Muscle Adenylosuccinate Lyase[a]

enzyme	% inactivation	mol of P_i/mol of enzyme subunit	mol of 2-BDB-[^{14}C]TAMP/ mol of enzyme subunit
control	0	0	0
modified enzyme	90	0.84	0.90
protected enzyme (1 mM 5′-AMP)	3	0	0.04
protected enzyme (0.1 mM 5′-AMP + 5 mM fumarate)	5	0.05	0.04

[a] Rabbit muscle adenylosuccinate lyase (0.5 mg/mL) was incubated with 20 μM 2-BDB-TAMP in 0.05 M PIPES buffer, pH 7.0 and 10 °C, in the absence and presence of added protectants. Incorporation was measured as described in Experimental Procedures.

affected similarly by 5′-AMP alone and 5′-AMP + fumarate, indicating that, despite the 10-fold difference in the rates of these two phases, they both represent reaction at the active site.

Incorporation of 2-BDB-TAMP into Rabbit Muscle Adenylosuccinate Lyase. The amount of reagent incorporated into adenylosuccinate lyase (Table 2) was determined initially by quantification of the organic phosphate bound to the modified enzyme as described in Experimental Procedures. The average reagent incorporated was 0.84 mol of the reagent/mol of enzyme subunit when the enzyme was 90% inactivated. When the enzyme was modified with 2-BDB-TAMP under the same conditions but in the presence of protecting ligands (either 0.1 mM 5′-AMP + 5 mM fumarate or 1 mM 5′-AMP alone), the measured incorporation in the active enzyme was decreased to 0−0.05 mol of reagent/mol of enzyme subunit. In addition, 2-BDB-[^{14}C]TAMP was synthesized in order to measure, radiochemically, the amount of reagent incorporated into the enzyme and to facilitate the monitoring of modified peptides after chemical cleavage and proteolytic digestion of the modified enzyme. An average value of 0.9 mol of 2-BDB-TAMP/mol of enzyme subunit was determined by this method when the enzyme was 90% inactivated. Moreover, the amount of radioactivity incorporated strikingly decreased (almost to zero) when the modification was carried out in presence of protectants using either 1 mM 5′-AMP or 0.1 mM 5′-AMP + 5 mM fumarate (Table 2). To evaluate the stability of linkage between reagent and enzyme, after the modification reaction, the modified enzyme was dialyzed against either 0.1% trifluoroacetic acid in water, pH 1.9, or 0.05 M PIPES buffer, pH 7.0, and subsequently the incorporation was determined. Similar results were obtained under these conditions.

The dioxo groups of several dioxobutyl nucleotide-modified enzymes have been successfully reduced by [^{3}H]-$NaBH_4$, thereby introducing a radioactive tag into the modified enzyme (Batra *et al.*, 1989; De Camp & Colman, 1989; Vollmer & Colman, 1990). This reduction has been accomplished effectively by exposing the modified enzyme to [^{3}H]$NaBH_4$. Introduction of approximately 2 mol of tritium is expected for each dioxobutyl group. In this study, however, treatment of 2-BDB-TAMP-modified adenylosuccinate lyase with [^{3}H]$NaBH_4$ led to a measured net incorporation of only 0.1−0.2 mol of tritium/mol of subunit enzyme. These results suggest that in the present case, the dioxo groups of 2-BDB-TAMP were unavailable for reduction by [^{3}H]$NaBH_4$, and therefore the reaction of adenylosuccinate lyase with 2-BDB-TAMP may directly involve the reagent's two carbonyl groups.

Isolation of Peptides after Chemical Cleavage and Proteolytic Digestion of 2-BDB[^{14}C]-TAMP-Modified Adenylosuccinate Lyase. Enzyme was reacted with 2-BDB-[^{14}C]-TAMP as described in Experimental Procedures. Following dialysis to remove the excess reagents, trypsin digestion was tried first. The inactivated enzyme was incubated with trypsin for 4 h, at 37 °C, either in 0.05 M ammonium bicarbonate (pH 7.8) or 0.05 M PIPES buffer (pH 7.0). However, reverse phase HPLC yielded most of the radioactivity in the void volume or in the region close to the elution position of free 2-BDB-[^{14}C]TAMP and its decomposition products; no appreciable amount of radioactively labeled peptide(s) was observed. These results suggested that the bond between the peptide and reagent is unstable during or after the digestion of modified adenylosuccinate lyase at neutral or slightly basic pH, even though the intact, modified enzyme was stable under similar conditions.

Alternatively, the modified enzyme was cleaved chemically using cyanogen bromide. The elution pattern of radioactively labeled peptide(s) is illustrated in Figure 5. Most of the radioactivity elutes in a single peak (peak I) around 46%−48% of solvent B. Since peak I was found to be a mixture of three large peptides (Ala^{65}−Met^{156}, Asp^{157}−Met^{200}, and Val^{365}−Met^{451}), these fractions were pooled, lyophilized, and subjected to SV8 protease digestion in 0.05 M sodium citrate−phosphate buffer, pH 4.0. The SV8 protease digest was subjected to HPLC, and the radioactivity again eluted at the same position as peak I in Figure 5. However, this time the radioactive peak contained only one large peptide, peptide I. This result indicates that peptide I is resistant to SV8 protease digestion, while the nonlabeled peptides were cleaved. The gas phase sequencing data for the first 17 amino acids of peptide I are shown in Table 3; these indicate that peptide I is Ala^{65}−Met^{156} (on the basis of the human adenylosuccinate lyase amino acid sequence).

Further digestion of this 92 amino acid cyanogen bromide peptide I was accomplished by papain at pH 4.0. This treatment yielded the HPLC pattern shown in Figure 6, with a region of radioactivity (labeled II) centered around 10% acetonitrile and a peak of radioactivity (labeled III) together with an $A_{220\ nm}$ peak at about 13% acetonitrile. Only radioactive peak III was associated with a peptide. Peak II did not contain recognizable peptide material: when 650 pmol (based on the specific radioactivity) was applied to a gas phase sequencer, no more than 97 pmol of any amino acid was found after the first cycle. Furthermore, when 2-BDB-TAMP was incubated under the same conditions as the cyanogen bromide peptide, it eluted as multiple peaks in the same region as II. Therefore, II appears to be decomposed reagent released from the unstable modified peptide. Although the immediate product of 2-BDB-TAMP and adenylosuccinate lyase is stable in the intact enzyme and in the large CNBr fragment, it has very limited stability when contained in a small peptide.

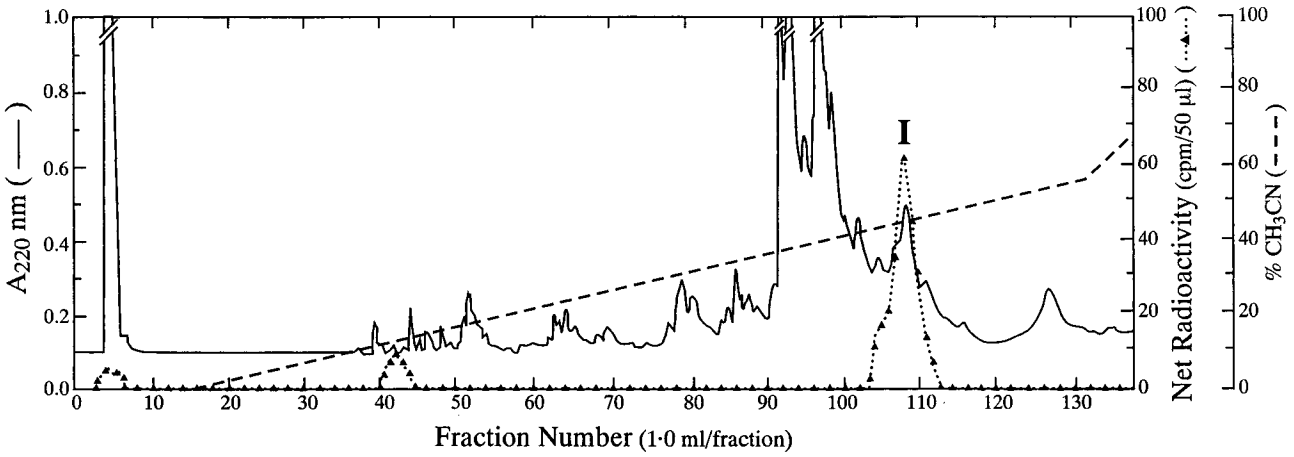


FIGURE 5: Separation of cyanogen bromide peptides of 2-BDB-TAMP-inactivated enzyme by reverse phase HPLC. The radioactive peptides were separated by elution with 0.1% TFA, pH 1.9, followed by a linear gradient of 0.075% TFA in acetonitrile as described in Experimental Procedures. A 50 μL aliquot of each fraction was assayed for radioactivity. The dotted line (···▲···) indicates the radioactivity, while the dashed line (---) shows the elution gradient. The roman numeral I designates the radioactive peak containing the 2-BDB-TAMP-modified peptide of rabbit muscle adenylosuccinate lyase.

Table 3: Amino Acid Sequence of Radioactive Peptides[a]

cycle	peptide I amino acid (pmol)	peptide III amino acid (pmol)
1	Ala (58)	Leu (71)
2	His (22)	Ala (57)
3	Val (45)	Arg (26)
4	His (18)	Val (39)
5	Thr (20)	Ile (11)
6	Phe (30)	Ser (12)
7	Gly (21)	Arg (56)[b]
8	His (12)	
9	NEM-Cys	
10	NEM-Cys	
11	Pro (17)	
12	Lys (14)	
13	Ala (17)	
14	Ala (25)	
15	Gly (11)	
16	Ile (15)	
17	Ile (21)[c]	

[a] Cyanogen bromide peak I (Figure 5), after treatment with SV8 protease and further purification, and papain digest peak III (Figure 6) were subjected to gas phase sequencing. Representative sequences are shown. [b] Peptide III is a heptapeptide, and sequencing does not continue after this residue. [c] Peptide I was sequenced for only 17 cycles.

The amino acid sequence for papain peptide III (Figure 6) is shown in Table 3. For the example shown, 72 pmol of peptide (based on the amount of radioactive reagent) was applied to the sequencer; this value is consistent with the yields of PTH-amino acids as measured by the Edman reaction. The sequence obtained is

$$Leu^{110}\text{-Ala-Arg-Val-Ile-Ser-}Arg^{116}$$

with the residues being numbered by comparison with the human adenylosuccinate lyase sequence (Stone *et al.*, 1992). It is likely that the third amino acid in the sequence (Arg^{112}) is the target of 2-BDB-TAMP: the amount of PTH-Arg observed is low at this cycle relative to that at cycle 7, and the free arginine detected probably arises by regeneration from modified Arg^{112} during the gas phase sequencing.

DISCUSSION

We have here demonstrated that 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-monophosphate (2-BDB-TAMP) reacts specifically with rabbit muscle adenylosuccinate lyase, exhibiting the characteristics of an affinity label. The 2-BDB-TAMP is a reactive analog of the substrate, adenylosuccinate, and of the product, 5′-AMP. It reacts covalently at only one site on the enzyme causing irreversible inactivation. The rate constants for the reaction of 2-BDB-TAMP with adenylosuccinate lyase exhibit a nonlinear dependence on the reagent concentration for both phases of inactivation. This finding indicates the reversible formation of enzyme−reagent complex prior to the irreversible inactivation, as expected for an affinity label. Furthermore, the inactivation was completely prevented by including in the incubation mixture a saturating concentration of 5′-AMP (1 mM) or a nonsaturating concentration of 5′-AMP (0.1 mM) plus 5 mM fumarate, although fumarate alone had little effect on the reaction. Thus, the binding of 5′-AMP appears to be enhanced in the presence of fumarate. It has been reported that, in the case of yeast adenylosuccinate lyase, fumarate binds not to the free enzyme but rather to the enzyme−AMP complex (Cohen & Bridger, 1964); the earlier report is consistent with our present results, although it is possible that fumarate may bind weakly to the free enzyme and more tightly to the enzyme−AMP complex. We have also demonstrated structural specificity for protection against inactivation by 2-BDB-TAMP: other dicarboxylic acids not known to function as substrates for the enzyme, such as maleate or succinate, do not enhance the protective effect of 5′-AMP. Moreover, no significant incorporation of the reagent was detected when the incubation with enzyme was carried out in the presence of 1 mM 5′-AMP as well as 0.1 mM 5′-AMP + 5 mM fumarate. These studies all show that 2-BDB-TAMP acts as an affinity label for the active site of rabbit muscle adenylosuccinate lyase.

The kinetics of inactivation of rabbit muscle adenylosuccinate lyase by 2-BDB-TAMP reveal that the reaction follows biphasic kinetics, although only 1 mol of 2-BDB-TAMP/mol of enzyme subunit (or 4 mol of reagent per enzyme tetramer) was incorporated upon complete inactivation. The enzyme is a homotetramer. Furthermore, we have evidence for only one modified peptide in the inactivated adenylosuccinate lyase. In addition, the compounds that provide protection against inactivation have the same effect for both

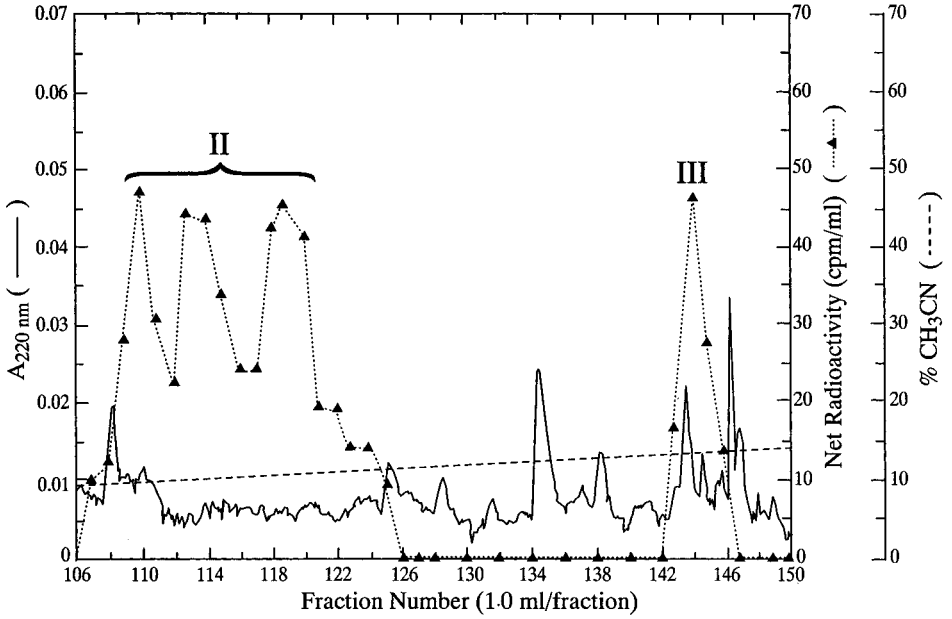


FIGURE 6: Separation of papain digest of purified peptide I by reverse phase HPLC. The radioactive peptides were separated by elution with 0.1% TFA, pH 1.9, followed by a linear gradient of 0.075% TFA in acetonitrile as described in Experimental Procedures. The solid line (—) represents the $A_{220\ nm}$. The dotted line (···▲···) indicates the radioactivity in each fraction, while the dashed line (---) shows the elution gradient. The roman numeral II designates the free radioactive compound and its degradation products, and III designates the radioactive peak containing the 2-BDB-TAMP-modified peptide of rabbit muscle adenylosuccinate lyase.

phases of inactivation although there is about a 10-fold difference in the maximum rate constants for the two phases. These observations imply that the same type of site is modified by 2-BDB-TAMP throughout the reaction. Assuming that the active sites are all initially identical, the best explanation for these results may be that reaction of 2-BDB-TAMP at the active sites of two subunits has an indirect effect on the conformation of the other two subunits, which reduces the reaction rate at the active sites of the second set of subunits, i.e., negative cooperativity among subunits is responsible for the apparent biphasic inactivation kinetics. Since the measured affinity (K_I) of the enzyme for 2-BDB-TAMP is similar in both phases of the reaction, the negative cooperativity is exhibited as a 10-fold lower maximum rate constant for the slow phase.

All the evidence presented in this paper is consistent with an arginine as the target site of 2-BDB-TAMP on the rabbit muscle adenylosuccinate lyase. The 2-BDB-TAMP-modified peptide was sufficiently stable to permit isolation only after chemical cleavage or proteolytic digestion under acidic conditions, not at neutral pH. The instability of the reaction product of 2-BDB-TAMP with the enzyme during proteolysis by trypsin in ammonium bicarbonate, pH 7.8, or in PIPES buffer, pH 7.0, is characteristic of the product of reaction of arginine with dioxo compounds. These observations are similar to those described by Patthy and Smith (1975) in their model study of modification of arginine residue by cyclohexanedione. They are also similar to the pH−stability behavior we encountered during the affinity labeling of Arg^{459} in a regulatory site of bovine glutamate dehydrogenase by adenosine 5′-*O*-[S-(4-bromo-2,3-dioxobutyl)thiophosphate] (Wrzeszczynski & Colman, 1994). In all of these studies, the arginine−reagent complex is more stable under acidic conditions while regeneration of free arginine occurs readily under neutral or slightly basic conditions.

Strong support for the reaction of 2-BDB-TAMP with arginine residue in rabbit muscle adenylosuccinate lyase comes from the lack of significant incorporation of tritium upon reduction of modified enzyme with [^{3}H]$NaBH_4$. If the reaction of 2-BDB-TAMP with enzyme had occurred by displacement of bromide, then the dioxo groups of the reagent would be available for reduction by [^{3}H]$NaBH_4$. Effective application of this borohydride reduction strategy has been described in examples of modification of several enzymes by bromodioxobutyl-containing reagents (Batra *et al.*, 1989; Vollmer & Colman, 1990). In contrast, if the dioxo group of reagent is directly involved in the reaction with the enzyme, as it is in the model reaction of cyclohexanedione with arginine (Patthy & Smith, 1975), then the carbonyl groups would not be available for reduction by [^{3}H]-$NaBH_4$ and no significant incorporation would be observed. We could not detect appreciable tritium incorporation into glutamate dehydrogenase following [^{3}H]$NaBH_4$ treatment of the enzyme with Arg^{459} modified by the nucleotide affinity label (Wrzeszczynski & Colman, 1994).

In the present study of rabbit muscle adenylosuccinate lyase, we have isolated, after cyanogen bromide cleavage under acidic conditions and subsequent proteolysis at pH 4.0, a peptide containing covalently bound radioactive 2-BDB-TAMP. The modified peptide has the sequence Leu-Ala-Arg-Val-Ile-Ser-Arg, which corresponds to residues 110−116 in the human adenylosuccinate lyase amino acid sequence (Stone *et al.*, 1992). Two arginines are included in this peptide: Arg^{112} and Arg^{116}. However, the most probable target of 2-BDB-TAMP is Arg^{112}. Papain is known to preferentially hydrolyze Arg-X or Lys-X peptides (Glazer & Smith, 1971). Since papain digestion of the modified cyanogen bromide hydrolyzed the Arg^{116}−Leu^{117} bond but did not cleave Arg^{112}−Val^{113}, it is likely that Arg^{112} was modified during the incubation with papain, thereby preventing the proteolytic digestion at that sequence position. Upon gas phase sequencing of the isolated peptide, we did detect arginine at the position corresponding to residue 112; this result is consistent with our previous experience that free

arginine is at least partly regenerated during Edman degradation (Wrzeszczynski & Colman, 1994). The sequence of the modified peptide isolated from rabbit muscle is compared below with the corresponding region of the adenylosuccinate lyases from human (Stone *et al.*, 1992), chicken (Aimi *et al.*, 1990), and *B. subtilis* (Ebbole & Zalkin, 1987):

Rabbit	L - A - R - V - I - S - R
Human	L - A - R^{112} - V - I - S - R
Chicken	L - A - R^{112} - V - I - S - R
B. subtilis	L - E - R^{119} - F - V - D - I

Significantly, Arg^{112} (numbering taken from human enzyme) is conserved in the adenylosuccinate lyases from human, chicken, and *B. subtilis* while the amino acid corresponding to position 116 is distinctly different in the bacterial enzyme. The conservation of Arg^{112} argues for the importance of *this* basic residue in the active site of the enzyme.

Two possible roles may be considered for an arginine residue in contributing to the binding of substrate by adenylosuccinate lyase. Either the arginine can interact with one or both of the carboxylates in the succinyl portion of the adenylosuccinate or it can participate in an electrostatic interaction with the negatively charged phosphate group of the AMP moiety of the substrate. Purine nucleotides in solution exist in a syn−anti equilibrium (Saenger, 1984); often one conformation is preferentially selected for binding by an enzyme. With 2-[(4-bromo-2,3-dioxobutyl)thio]-adenosine 5′-monophosphate in the syn-conformation, the dioxo group is close to the phosphate, where it is well positioned for covalent reaction with an arginine involved in an electrostatic interaction with the 5′-phosphate group. Alternatively, if the enzyme selects the anti-conformation of 2-BDB-TAMP, the dioxo group would be better positioned to react with an arginine which interacts with one of the carboxylates of adenylosuccinate.

Arginine residues of a variety of enzymes have been shown to be involved in the binding of nucleotidyl phosphate as well as of other phosphate groups. In the case of nucleoside diphosphate kinase, Arg^{92} and Arg^{109} have been shown to contribute to the binding of the β-phosphate (Morera *et al.*, 1994), and the cyclic AMP receptor protein shows an interaction between the charged phosphate of cAMP and Arg^{82} (Weber & Steitz, 1987; Belduz *et al.*, 1993). Recently, Jia *et al.* (1995), while studying the basis for phosphotyrosine peptide recognition by protein tyrosine phosphatase 1B, demonstrated that Arg^{221} forms a complex with the phosphate portion of the phosphotyrosine peptide. Thus, there is ample precedence for the involvement of an arginine residue in the binding of various phosphate group-(s). However, there are also documented examples of arginine residues interacting with a carboxylate substrate, as in the case of the binding of isocitrate by the *Escherichia coli* isocitrate dehydrogenase (Hurley *et al.*, 1991). Indeed, Clarke *et al.* (1989) have suggested that arginine is uniquely suited to interact with carboxylate groups.

This study describes the evidence that 2-BDB-TAMP functions as an affinity label of the active site of rabbit muscle adenylosuccinate lyase. We conclude that the Arg^{112} target residue is part of the substrate binding site where it is likely to participate in an electrostatic interaction with either the 5′-phosphate or one of the carboxylates of adenylosuccinate.

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