2-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 5′-Monophosphate, a New Nucleotide Analogue That Acts as an Affinity Label of Pyruvate Kinase†

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ABSTRACT: A new reactive adenine nucleotide has been synthesized: 2-[(4-bromo-2,3-dioxobutyl)thio]-adenosine 5′-monophosphate (2-BDB-TAMP). Adenosine 5′-monophosphate 1-oxide was synthesized by reaction of AMP with *m*-chloroperoxybenzoic acid. Treatment with NaOH followed by reaction with carbon disulfide yielded 2-thioadenosine 5′-monophosphate (TAMP). The final product was generated by reaction of TAMP with 1,4-dibromobutanedione. The structure of 2-BDB-TAMP was determined by UV, ^{1}H NMR, and ^{13}C NMR spectroscopy as well as by bromide and phosphorus analysis. Rabbit muscle pyruvate kinase is inactivated by 2-BDB-TAMP at pH 7.0 and 25 °C. The inactivation rate exhibits a nonlinear dependence on the reagent concentration with K_I = 0.57 mM. Protection against inactivation is provided by ADP and ATP, in the presence of Mn^{2+}, as well as by phosphoenolpyruvate, in the presence of K^+; in addition, partial protection is provided by AMP plus Mn^{2+}. Incubation of pyruvate kinase with 0.075 mM 2-BDB-TAMP for 70 min in the absence of protective ligands leads to incorporation of 1.55 mol of reagent/mol of enzyme subunit when the enzyme is 53% inactive. In the presence of ADP and Mn^{2+}, only 0.96 mol of reagent/mol of subunit is incorporated at 70 min, while the enzyme retains 100% activity. Similar results were obtained in the presence of ATP plus Mn^{2+}. Assuming that the groups modified in the absence of ligands include those modified in the presence of the nucleotides, the 53% inactivation can be attributed to the modification of 0.59 (1.55 − 0.96) group per enzyme subunit. These results indicate that 2-BDB-TAMP acts as an affinity label of the active site of rabbit muscle pyruvate kinase. This new compound may have widespread applications in the labeling of purine nucleotide sites in other proteins.

Pyruvate kinase is a tetrameric enzyme that catalyzes the transfer of a phosphoryl group from phosphoenolpyruvate (PEP)[1] to ADP in the presence of both divalent and monovalent cations. Considerable information is now available on the structure of pyruvate kinase. The crystal structure of the cat muscle enzyme has been described at 2.6-Å resolution (Stuart et al., 1979); the geometry of the active site and the role of cations in the catalytic activity of the enzyme have been extensively studied by X-ray crystallography and computer graphics (Muirhead & Clayden, 1984). In addition, nuclear magnetic resonance studies have yielded insights regarding the arrangement of substrates at the active site (Gupta & Benovic, 1978; Rao et al., 1979; Meshitsuba et al., 1981). The amino acid sequence is known for the chicken muscle, cat muscle, and yeast enzymes (Muirhead & Clayden, 1984; Burke et al., 1983; Fothergill, 1985). Although extensive chemical modification studies have been performed with pyruvate kinase, relatively little has been learned concerning the particular amino acid residues involved in the nucleotide binding site. The technique of affinity labeling, using nucleotide analogues with reactive functional groups, has the potential to yield more specific chemical modification than is usually obtained with group-specific reagents and should allow the identification of critical amino acid residues within the nucleotide binding site.

Affinity labeling of pyruvate kinase has previously been conducted with a series of purine nucleotide analogues that have the same reactive (fluorosulfonyl)benzoyl moiety in a position structurally related to the phosphoryl groups of the natural nucleotides but that differ in the purine moiety. The reaction of the enzyme with 5′-[*p*-(fluorosulfonyl)benzoyl]-adenosine (Annamalai & Colman, 1981) and with 5′-[*p*-(fluorosulfonyl)benzoyl]guanosine (Tomich et al., 1981) indicated that tyrosine and cysteine residues, respectively, were the locus of attack within the active site. The fluorescent nucleotide analogue 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine was shown to modify a histidine as well as a cysteine residue (Tomich & Colman, 1985).

In this paper, we describe the synthesis and characterization of a new nucleotide analogue, 2-[(4-bromo-2,3-dioxobutyl)-thio]adenosine 5′-monophosphate (2-BDB-TAMP), which is shown as structure V in Figure 1. Because of the location of the reactive bromodioxobutyl group adjacent to the 2-position of the adenosine ring, this compound might be expected to react with the amino acid residues in the purine region of the nucleotide binding site of the enzyme. The bromoketo group can potentially react with most nucleophiles found in proteins (Hartman, 1977), and the dioxo group adds the possibility of reaction with arginine residues (Yankeelov, 1970; Riordan, 1973). This compound should be complementary in use as an affinity label to another analogue we have previously described: 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-monophosphate (Colman et al., 1984). This paper presents evidence that 2-[(4-bromo-2,3-dioxo-

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[1] Abbreviations: 2-BDB-TAMP, 2-[(4-bromo-2,3-dioxobutyl)thio]-adenosine 5′-monophosphate; AMP 1-oxide, adenosine 5′-monophosphate 1-oxide; TAMP, 2-thioadenosine 5′-monophosphate; MES, 2-(*N*-morpholino)ethanesulfonic acid; NMR, nuclear magnetic resonance; PEP, phosphoenolpyruvate; DTT, dithiothreitol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; FSBG, 5′-[(*p*-fluorosulfonyl)benzoyl]guanosine; FSBεA, 5′-[(*p*-fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine; 6-BDB-TAMP, 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-monophosphate; 6-BDB-TADP, 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate.

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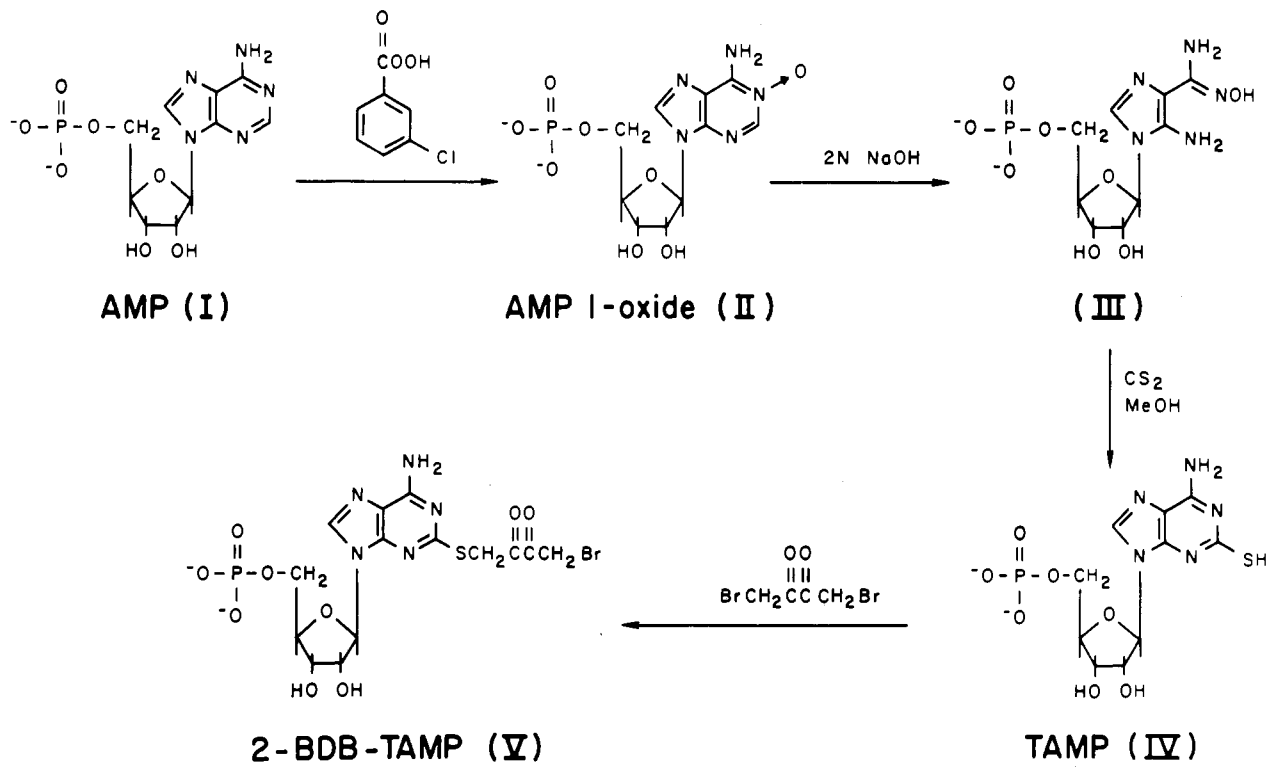

FIGURE 1: Synthetic scheme for preparation of 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-monophosphate.

butyl)thio]adenosine 5′-monophosphate reacts in a limited and specific manner in the region of the active site of rabbit muscle pyruvate kinase. A preliminary version of this work has been presented (Bailey et al., 1985).

Experimental Procedures

Materials and Methods. The nucleotides AMP, ADP, ATP, and NADH as well as phosphoenolpyruvate (PEP), dithiothreitol (DTT), phosphorus standard solution, Malachite Green base, Sephadex G-50–80, mercuric thiocyanate, and most of the buffer salts were supplied by Sigma Chemical Co. 1,4-Dibromobutanedione was obtained from Aldrich Chemical Co. and was recrystallized from petroleum ether before use. *m*-Chloroperoxybenzoic acid was purchased from Aldrich Chemical Co. Ammonium molybdate and hydrogen peroxide were supplied by Fisher Scientific Co., protein dye concentrate was from Bio-Rad, and ultrapure guanidine hydrochloride was from Schwarz/Mann.

Rabbit muscle pyruvate kinase was purchased from Boehringer Mannheim Corp. as a crystalline suspension in ammonium sulfate solution. The enzyme was dialyzed for 18 h at 4 °C against 50 mM HEPES buffer, pH 7.0, with one change of dialyzate. Any insoluble material was removed by centrifugation for 10 min at 13000 rpm. The enzyme concentration (7–8 mg/mL) was determined by using $\overset{0.1\%}{}_{280nm} = 0.54$ (Bucher & Pfleiderer, 1955) and a molecular weight of 237 000 for the tetrameric enzyme (Cottam et al., 1969) or 60 000 per enzyme subunit. The specific activity was about 240 units/mg, and this commerical enzyme was equivalent in properties to the crystalline homogeneous enzyme described in the literature (Cottam et al., 1969). The enzyme was stored in aliquots at −80 °C and was used without further purification. Lactate dehydrogenase from hog muscle was also purchased from Boehringer Mannheim Corp. as a solution (10 mg/mL) in 50% glycerol and was used without further purification.

Thin-layer chromatography (TLC) was performed on cellulose aluminum-backed sheets (EM Reagents, 0.1-mm thickness). Isobutyric acid–concentrated NH_4OH–H_2O (66:1:33) was used as the solvent system. Ultraviolet absorption spectra were obtained on a Perkin-Elmer 553 UV/vis spectrophotometer equipped with a Perkin-Elmer R100 chart recorder.

NMR spectra were obtained with a Bruker WM 250-MHz spectrometer at room temperature in deuterium oxide or methanol-d_4 for ^1H NMR and in D_2O for ^{13}C NMR spectra. Dioxane (3.71 ppm) was used as an internal standard for ^1H spectra, and triethylamine (46.7 and 8.3 ppm) was used in ^{13}C spectra.

Synthesis of 2-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 5′-Monophosphate. The overall synthetic scheme is shown in Figure 1, which also gives the numbers by which the several intermediates are designated. AMP 1-oxide (II) was synthesized by reaction of AMP (I) with *m*-chloroperoxybenzoic acid. Treatment of II with NaOH followed by reaction with carbon disulfide yielded 2-thioadenosine 5′-monophosphate (TAMP, IV). Condensation of TAMP with 1,4-dibromobutanedione gave the final product, 2-BDB-TAMP (V).

Preparation of Adenosine 5′-Monophosphate 1-Oxide (II). Adenosine 5′-monophosphate 1-oxide (II) was synthesized by analogy to the procedure used for the synthesis of adenosine cyclic 3′,5′-phosphate 1-oxide (Meyer et al., 1973). A biphasic mixture containing 3 g of AMP, 25 mL of 1 N sodium acetate, 25 mL of 1 N acetic acid, 45 mL of ethyl acetate, and 6 g of *m*-chloroperoxybenzoic acid was stirred for 48 h at room temperature. After this period, the aqueous layer was separated, chloroform (15 mL) and 25 mL of 1 N HCl were added to this layer, and the mixture was stirred at room temperature for 2 h. The aqueous layer was recovered, isopropyl alcohol (1200 mL) was added to it, and the cloudy white solution was maintained overnight at 4 °C. The product (II) was collected by filtration and dried under vacuum. The ultraviolet absorption spectrum for AMP 1-oxide exhibited an A_{260nm}/A_{233nm} of 0.22, whereas the A_{260nm}/A_{233nm} for the starting material (AMP) was 5.0. TLC showed a single UV-absorbing spot at R_f 0.45 for AMP 1-oxide compared to R_f 0.50 for AMP.

Adenosine 5′-monophosphate 1-oxide was obtained as a white powder in 92% yield.

Preparation of 5-Amino-1-β-D-ribofuranosylimidazole-4-carboxamidoxime 5′-Monophosphate (III). AMP 1-oxide was treated with 2 N NaOH by further analogy to the procedure of Meyer et al. (1973). Adenosine 5′-monophosphate 1-oxide (2 g) was dissolved in 3 mL of 2 N NaOH and poured into a refluxing solution of 20 mL of 2 N NaOH. After being refluxed for an additional 10 min, the reaction flask was cooled on ice, and the pH adjusted to 11 with AG 50W-X4 (H^+ form). The resin was filtered and washed with H_2O until the absorbance at 260 nm of the washings was less than 0.5. The filtrate and washings were applied to a 1.5 × 36 cm column of AG 1-X2 (formate form, 100–200 mesh). The column was washed with water until the absorbance at 260 nm was less than 0.02. The column was then eluted with a linear gradient (300 mL of water and 300 mL of 1 N formic acid). Fractions (2.2 mL) were collected starting with the initiation of the gradient and were monitored at 260 nm. The product was the first of two peaks to be eluted and was located between fractions 78 and 110. The fractions were pooled and concentrated under vacuum to a small volume (10 mL). The resultant sample was diluted with isopropyl alcohol (500 mL) and was maintained overnight at 4 °C. Compound III was obtained by filtration as a pure white powder in 38% yield. Ultraviolet absorption spectra showed a λ_{max} at 282 nm at pH 1 and a λ_{max} at 262 nm at pH 11. TLC exhibited a single ultraviolet-absorbing spot at R_f 0.24. NMR (D_2O) showed peaks at δ 7.65 (H_8) and 5.66 ($H_{1'}$).

Preparation of 2-Thioadenosine 5′-Monophosphate (IV). To III was added 30 mL of methanol; triethylamine was added dropwise to form the soluble triethylammonium salt. Pyridine (20 mL) and carbon disulfide (15 mL) were added, after which the mixture was refluxed for 6 h. The yellow mixture was allowed to cool to room temperature, and the solvents were removed under vacuum. The residue was dissolved in water (3 mL) and centrifuged to remove any insoluble material. The supernatant was applied to a 1.5 × 39 cm column of Sephadex G-10 and eluted with water. Elution was monitored by absorbance at 290 nm. A broad yellow band was obtained and concentrated under vacuum to 10 mL and then applied to a 1.5 × 35 cm column of AG 50W-X4 (H^+), which was eluted with distilled water. Elution was monitored by absorbance at 290 nm. The product (IV) precipitated upon concentration under vacuum and was collected by centrifugation. The yellowish white powder was washed with methanol until the supernatant was clear and was then washed with diethyl ether and dried under nitrogen. 2-Thioadenosine 5′-monophosphate was obtained as a white powder in 40% yield. The ultraviolet absorption spectrum exhibited a λ_{max} at 290 nm in water. TLC showed a single ultraviolet-absorbing spot at R_f 0.20. NMR (methanol-d_4) showed peaks at δ 8.12 (H_8) and 5.78 ($H_{1'}$). For comparison, Kikugawa et al. (1973) obtained values at δ 8.36 (H_8) and 5.88 ($H_{1'}$) for 2-thioadenosine in MeSO-d_6.

Preparation of 2-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 5′-Monophosphate (V). TAMP (IV, 10 mg) was dissolved in 1 mL of methanol by addition of triethylamine dropwise to form the soluble triethylammonium salt. Sufficient triethylamine was added to adjust the pH to approximately 5.3 as estimated by pH paper. 1,4-Dibromobutanedione (0.10 g, recrystallized) was dissolved in 0.5 mL of methanol (820 mM). This solution was added (with rapid mixing) to 0.5 mL of the TAMP solution (26 mM). Reaction occurred immediately and could be assessed spectrophotometrically from the decrease in absorbance at 290 nm. The reaction mixture was placed on ice within 2 min, and the product was precipitated by addition of 10 mL of diethyl ether. The precipitate was collected by centrifugation, redissolved in 0.5 mL of methanol, and again precipitated with diethyl ether. The resultant precipitate was washed with diethyl ether and dried under nitrogen to give a white powder. 2-BDB-TAMP was stored dry and desiccated at −80 °C, yield 70%.

Solutions of 2-BDB-TAMP used for kinetics and incorporation studies were prepared in 0.03 M MES buffer, pH 5.0, and stored at −80 °C for periods up to 1 month. These solutions were kept on ice during use and found to be stable to repeated freezing and thawing.

Determination of the Decomposition Rate of 2-BDB-TAMP. The rate of loss of bromide from 2-BDB-TAMP was measured by incubating 3 mM BDB-TAMP in 50 mM HEPES buffer, pH 7.0, at 25 °C and measuring the release of bromide by withdrawing 50-μL aliquots at different time intervals until there was no further increase in the amount of bromide in the mixture, indicating the end point of the reaction. Free bromide was measured by a procedure modified from that of Zall et al. (1956), which has been previously described by Colman et al. (1984). The loss of bromide from 2-BDB-TAMP is accompanied by an increase in absorbance at both 310 and 270 nm. 2-BDB-TAMP (0.02 mM) was incubated under the same conditions as above in a sealed quartz cuvette, and the absorbance spectrum was monitored for 6 h at timed intervals in a Perkin-Elmer 553 spectrophotometer. The decomposition rate for 2-BDB-TAMP was calculated from a semilogarithmic plot of $(A_\infty - A_t)/(A_\infty - A_0)$ vs. time where A_∞ and A_0 represent the final and initial absorbances, respectively, while A_t is the absorbance at various times. The change was measured at both 310 and 270 nm. In the case of the bromide assay, the final, initial and time-dependent free bromide concentrations were used in the same equation.

Determination of Enzyme Activity. Pyruvate kinase activity was measured spectrophotometrically at 340 nm by means of a coupled assay with lactate dehydrogenase (Likos et al., 1980). The enzyme activity was monitored at 25 °C in 50 mM Tris-HCl buffer, pH 7.5, containing 100 mM KCl, 10 mM $MgSO_4$, 0.5 mM PEP, 3 mM ADP, 0.25 mM NADH, and lactate dehydrogenase at a concentration of 0.1 mg/mL. The volume of the assay solution was 1 mL.

Reaction of Rabbit Muscle Pyruvate Kinase with 2-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 5′-Monophosphate. Rabbit muscle pyruvate kinase (0.33 mg/mL) was incubated with 2-BDB-TAMP (0.05–0.8 mM) in 50 mM HEPES buffer, pH 7.0, at 25 °C. During the course of the reaction, 10-μL aliquots were withdrawn at different time intervals, diluted 5000-fold, and assayed for enzymatic activity as described above. The rate of inactivation of pyruvate kinase by the nucleotide analogue was determined from a semilogarithmic plot of E/E_0 as a function of time, where E represents the enzymatic activity at given times and E_0 represents the initial activity.

The effect of different ligands on the rate of reaction of pyruvate kinase with 2-BDB-TAMP was tested by incubating the enzyme under the same conditions as outlined above and adding the ligands prior to the addition of the reagent. In all cases, a control was run under identical conditions as in the experimental sample, but with no 2-BDB-TAMP present.

Incorporation of 2-BDB-TAMP by Rabbit Muscle Pyruvate Kinase. The enzyme (1 mg/mL) was incubated with 0.075 mM 2-BDB-TAMP under the same conditions as described above, and the rate of inactivation was followed for 2 h. At

given time intervals, 1-mL aliquots were withdrawn, and the reaction was stopped by the addition of DTT to a final concentration of 0.2 M. After 1 min, solid guanidine hydrochloride was added to a final concentration of 5 M. In the case where the effect of different ligands on incorporation of 2-BDB-TAMP by pyruvate kinase was tested, the ligands were added to the reaction mixture prior to the addition of the reagent, and the enzyme was treated in the same way as outlined above. The excess reagent was separated from the modified enzyme by a column centrifugation procedure (Penefsky, 1979) using two consecutive Sephadex G-50–80 columns (5 mL) equilibrated with 50 mM HEPES buffer, pH 7.0, containing 5 M guanidine hydrochloride. The protein concentration in the eluate was determined with the Bio-Rad protein assay based on the method of Bradford (1976). A 10-μL aliquot was dissolved in 90 μL of 50 mM HEPES buffer, pH 7.0, and 1 mL of the diluted protein dye (1:4 in H_2O) was added. Standard protein solutions were prepared with native pyruvate kinase dissolved in the same buffer as above and also containing 10 μL of 5 M guanidine hydrochloride.

The incorporation of 2-BDB-TAMP into pyruvate kinase was measured by determination of the moles of organic phosphorus per mole of enzyme subunit by a modification of the procedures of Hess & Derr (1975) and Lanzetta et al. (1979). A 200-μL sample of the modified protein, containing organic phosphorus, was mixed with 20 μL of 10 N H_2SO_4 and digested at 190 °C for 2 h in tubes sealed with aluminum foil. The brown residues were redissolved in 50 μL of 30% H_2O_2 and incubated for 1 h at 190 °C in open tubes. The yellow residues, after the second incubation, were dissolved in 200 μL of H_2O, and 800 μL of a fresh mixture (3:1) of Malachite Green (0.045% in 0.33 N HCl) and ammonium molybdate (4.2% in 3 N HCl) was added. The absorbance at 660 nm was measured after 5 min. Standard phosphorus solutions were prepared in 50 mM HEPES buffer, pH 7.0, containing 5 M guanidine hydrochloride and were treated in the same way as the samples being analyzed. The incorporation of 2-BDB-TAMP was calculated on the basis of the molar concentration of enzyme subunits.

Results

Synthesis and Characterization of 2-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 5'-Monophosphate. The overall synthetic scheme for preparation of 2-BDB-TAMP is shown in Figure 1. The starting material, AMP, was first treated with *m*-chloroperoxybenzoic acid to yield the *N*-oxide derivative. Treatment of AMP 1-oxide with 2 N NaOH removed the carbon at position 2. Reaction of III with carbon disulfide simultaneously closed the ring and reduced the oxide function. The final product, 2-BDB-TAMP, was obtained by reaction of TAMP with 1,4-dibromobutanedione in an overall yield of 10%.

Loss of the *N*-oxide function was previously observed by Kikugawa & Suehiro (1975) during the synthesis of 2-thioadenosine. These authors proposed that the reduction of the *N*-oxide occurred at the elevated temperature in the presence of hydrogen sulfide liberated in the ring-closure reaction (Figure 1, III → IV). Loss of the oxide was readily verified by comparison of the ultraviolet absorption spectrum of TAMP (IV) with that from 2-thioadenosine (Kikugawa et al., 1973) and 2-thioadenosine 1-oxide (Cresswell & Brown, 1963). For example, TAMP exhibited an absorbance ratio of 236 nm/290 nm = 0.93 at pH 1.0. 2-Thioadenosine exhibited an absorbance ratio 236 nm/295 nm = 0.82 at pH 1.0, whereas 2-thioadenosine 1-oxide exhibited an absorbance ratio of 242 nm/281 nm = 1.4 at pH 1.1.

The purity of the product, 2-BDB-TAMP, was assessed by thin-layer chromatography as described under Experimental Procedures. A single ultraviolet-absorbing spot was observed with an R_f of 0.38, whereas the precursor, TAMP, and 1,4-dibromobutanedione exhibit UV-absorbing spots with R_f values of 0.20 and 0.78, respectively.

The elemental analysis is in agreement with the structure shown in Figure 1, structure V, for 2-BDB-TAMP, assuming that the compound was the triethylammonium salt and had cocrystallized with methanol. Resonances corresponding to triethylamine and methanol were observed in the NMR spectra (see below). Anal. Calcd for $C_{14}H_{15}N_5O_9SPBr$, 0.2 mol of triethylamine, and 3 mol of methanol: C, 33.26; H, 4.63; N, 11.08. Found: C, 32.51; H, 4.83; N, 10.60.

The ultraviolet absorption spectrum of 2-BDB-TAMP exhibits a peak at 246 nm ($\epsilon = 35.2 \times 10^3$ M^{-1} cm^{-1}) with a shoulder at 270 nm ($\epsilon = 11.5 \times 10^3$ M^{-1} cm^{-1}) when measured in 0.10 M MES buffer, pH 6.0. This property allows the reaction between TAMP and 1,4-dibromobutanedione to be conveniently followed spectrophotometrically, as TAMP exhibits absorption maxima at 290 nm (15×10^3 M^{-1} cm^{-1}) and 256 nm (21×10^3 M^{-1} cm^{-1}) when measured in 0.10 M MES buffer, pH 6.0. Reaction of TAMP with 1,4-dibromobutanedione causes a decrease in the extinction coefficient at 290 nm (5.8×10^3 M^{-1} cm^{-1} for 2-BDB-TAMP). There is a shift in λ_{max} from 256 nm (TAMP) to 246 nm (2-BDB-TAMP) and 290 nm (TAMP) to a shoulder at 270 nm (2-BDB-TAMP) when measured in the above buffer.

The bromide and phosphorus content of 2-BDB-TAMP was measured as previously described (Colman et al., 1984). The ratio of organic phosphorus to spectrophotometrically determined 2-BDB-TAMP was 0.99:1.00. The ratio of hydrolyzable bromide to 2-BDB-TAMP was 1.01:1.00.

The proton NMR spectrum of 2-BDB-TAMP (triethylammonium salt) in D_2O had peaks centered at δ 1.30 (t, $-CH_3$ of triethylamine), 3.24 (q, $-CH_2N$ of triethylamine), 3.35 ($-CH_3$ of methanol), 3.75–3.80 (m, $-CH_2Br$), 4.05–4.15 (m, $H_{4'}$ and $H_{5'}$ of ribose), 4.32 (m, $H_{3'}$ of ribose), 4.43 (m, $H_{2'}$ of ribose), 4.66–4.73 (m, $-CH_2S-$), 6.17 (d, $H_{1'}$ of ribose), and 8.39 (s, H_8). For comparison 2-thioadenosine 5'-monophosphate (TAMP), in methanol-d_4, had peaks centered at δ 3.82 (m, $H_{5'}$ of ribose), 3.96 (m, $H_{4'}$ of ribose), 4.14 (m, $H_{3'}$ of ribose), 4.36 (m, $H_{2'}$ of ribose), 5.78 (d, $H_{1'}$ of ribose), and 8.12 (s, H_8). Assignments of the ribose protons were made by comparison with the proton NMR spectrum of 5'-AMP (Davies & Danyluk, 1974). 1,4-Dibromobutanedione exhibits a single resonance peak in deuterated chloroform (δ 4.34) and in deuterated Me_2SO (δ 4.65), but in deuterated methanol a multiplet is seen at δ 3.53–3.66 in addition to the singlet at δ 4.53. It is likely that in methanol 1,4-dibromobutanedione may exist as a methanolic hemiacetal. This same explanation may apply to the bromodioxobutyl moiety of 2-BDB-TAMP. In aqueous solutions, the diketo group may exist at least partially as a carbonyl hydrate, and multiple resonances would be expected, similar to 1,4-dibromobutanedione in methanol. Resonances at δ 3.75–3.80 and δ 4.66–4.73 were therefore assigned to the $-CH_2Br$ and $-SCH_2-$ moieties of 2-BDB-TAMP, respectively. Thus, although 2-BDB-TAMP is pictured in structure V, Figure 1, as an acyclic 1,2-diketone, it probably exists at least partially in enolate or hydrated forms. The value for $-CH_2Br$ is comparable to the δ 3.36 resonance observed for the $-CH_2Br$ protons of 2'-(2-bromoethyl)-AMP (Bednar & Colman, 1982). The resonances for $-CH_2Br$ and

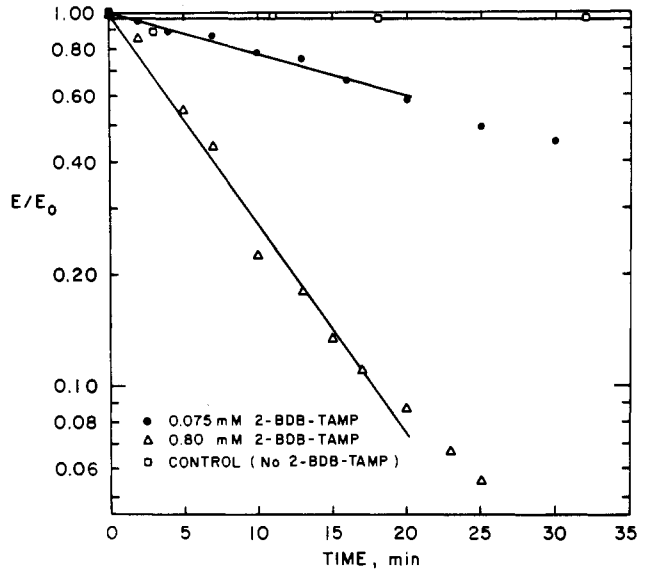

FIGURE 2: Inactivation of pyruvate kinase by 2-BDB-TAMP. Rabbit muscle pyruvate kinase (0.33 mg/mL) was incubated with various concentrations of 2-BDB-TAMP at 25 °C in 50 mM HEPES buffer, pH 7.0. The control consisted of enzyme incubated under the same conditions in the absence of the reagent. At the times indicated, aliquots were withdrawn and assayed for enzymatic activity as described under Experimental Procedures. E and E_0 represent the activities at the given time and at zero time, respectively.

$-SCH_2-$ in 2-BDB-TAMP are similar to those assigned to the corresponding hydrogens in 6-BDB-TAMP (Colman et al., 1984).

The proton-decoupled ^{13}C NMR spectra of 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-monophosphate had signals arising from triethylamine centered at δ 8.3 ($-CH_3$) and 46.7 ($-CH_2N$), from methanol at δ 48.9, from the reactive side chain at δ 34.94, 36.60 ($-CH_2Br$ and $-SCH_2-$), 94.98 [carbonyl hydrate carbon, $-C(OH)_2CH_2Br$], and 202.16 [$-C(O)-$], from the ribose ring at δ 64.49 ($C_{5'}$), 70.32 ($C_{3'}$), 74.14 ($C_{2'}$), 83.88 ($C_{4'}$), and 87.78 ($C_{1'}$), and from the purine ring at δ 115.84 (C_5), 141.22 (C_8), 144.10 (C_4), 148.47 (C_6), and 157.17 (C_2). For comparison, 2-thioadenosine 5′-monophosphate had signals arising from the ribose at δ 64.06 ($C_{5'}$), 70.48 ($C_{3'}$), 74.31 ($C_{2'}$), 84.08 ($C_{4'}$), and 86.24 ($C_{1'}$) and from the purine ring at δ 113.57 (C_5), 139.51 (C_8), 143.39 (C_4), 147.44 (C_6), and 170.61 (C_2). Resonance peak assignments were made primarily by reference to Schleich et al. (1975) and by comparison to 6-mercaptopurine and 6-(methylthio)-purine (Breitmaier & Voelter, 1974). The assignment of δ 94.98 for the carbonyl hydrate carbon was by reference to values observed for $R-C(OH)_2CH_2Cl$ (Malthouse et al., 1983). The $-CH_2Br$ of 2′-(2-bromoethyl)-AMP exhibits a resonance at δ 31.7 (Bednar & Colman, 1982). Thus, all of the spectral and analytical data are consistent with the structure of 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-monophosphate as shown in Figure 1.

Inactivation of Rabbit Muscle Pyruvate Kinase by 2-BDB-TAMP. Incubation of pyruvate kinase (0.33 mg/mL) with 2-BDB-TAMP at pH 7.0 and 25 °C leads to a progressive loss of enzymatic activity, whereas the control enzyme (in the absence of the reagent) is stable under these conditions (Figure 2). The activity of pyruvate kinase decreases to 0.7% of its initial activity at reaction times longer than shown in Figure 2 (up to 100 min), indicating that 2-BDB-TAMP is capable of totally inactivating the enzyme. The semilogarithmic plots of E/E_0 vs. time, used for calculating the inactivation rate constants, are linear for at least 20 min; at longer times the points systematically deviate from the line, suggesting decomposition of reagent. The rate constant for decomposition of 2-BDB-TAMP under these conditions was determined as 0.0115 min^{-1} ($t_{1/2}$ = 60 min) by using either the release of free bromide or the change in absorbance at 270 or at 310 nm, as described under Experimental Procedures. The pseudo-first-order rate constants for the inactivation of pyruvate kinase by 2-BDB-TAMP were calculated from data obtained during the first 20 min of the reaction in order to minimize the effect of reagent decomposition. The decomposed reagent has no significant effect on pyruvate kinase as shown by an experiment in which 0.3 mM 2-BDB-TAMP was incubated under the reaction conditions for 4 h prior to the addition of enzyme (0.33 mg/mL). At that time the reagent, which had lost 94% of the bromide ($t_{1/2}$ = 60 min), inactivated pyruvate kinase with an observed rate constant of 0.0033 min^{-1}, 2.9% of the inactivation rate constant of 0.114 min^{-1} caused by freshly dissolved reagent at the same concentration. This result also suggests that the bromoketo moiety, rather than the dioxo group, is responsible for inactivation.

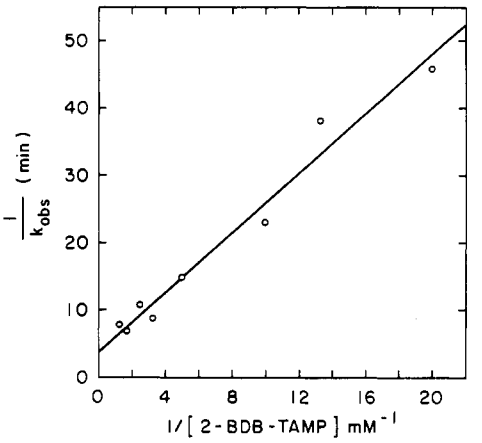

FIGURE 3: Dependence of the pseudo-first-order rate constant (k_{obsd}) for inactivation of pyruvate kinase on the concentration of 2-BDB-TAMP. Pyruvate kinase was incubated with 2-BDB-TAMP (0.05–8.0 mM) under the conditions described in Figure 2. Rate constants were determined as described under Experimental Procedures and are presented as $1/k_{obsd}$ vs. $1/[2\text{-BDB-TAMP}]$.

The enzyme was incubated with different concentrations of 2-BDB-TAMP (0.05–0.8 mM). A nonlinear dependence of the rate constant of inactivation on the reagent concentration was observed. Such behavior is consistent with the initial formation of a reversible enzyme–reagent complex prior to irreversible modification, as is expected for an affinity label, and can be expressed as

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [EI] \xrightarrow{k_{max}} [EI]^* \qquad (1)$$

where E represents the free enzyme, EI the reversible enzyme–inhibitor complex, and EI* the modified, inactive enzyme. The observed rate constant, k_{obsd}, at a particular concentration of 2-BDB-TAMP can be described by the equation:

$$\frac{1}{k_{obsd}} = \frac{K_I}{k_{max}} \frac{1}{[I]} + \frac{1}{k_{max}} \qquad (2)$$

where $K_I = (k_{-1} + k_{max})/k_1$ and represents the concentration of the reagent giving half the maximal inactivation rate (Huang & Colman, 1984). From the plot of $1/k_{obsd}$ vs. $1/[2\text{-BDB-TAMP}]$ (Figure 3), values for K_I = 0.57 mM and k_{max} = 0.26 min^{-1} were calculated.

Effect of Ligands on the Rate of Inactivation of Pyruvate Kinase by 2-BDB-TAMP. The ability of natural ligands to protect pyruvate kinase against inactivation by 2-BDB-TAMP

Table I: Effect of Ligands on Inactivation Rate of Pyruvate Kinase by 2-BDB-TAMP[a]

additions to the reaction mixture	k_{obsd} ($\times 10^2$ min^{-1})	k_{obsd}(ligand added)/ k_{obsd}(no additions)
none	2.81	1.00
$MnCl_2$ (2 mM)	1.42	0.51
ADP (1.5 mM)	1.17	0.42
ADP (1.5 mM) + $MnCl_2$ (2 mM)	0.32	0.11
ATP (1.5 mM)	1.11	0.40
ATP (1.5 mM) + $MnCl_2$ (2 mM)	0.00	0.00
AMP (1.5 mM)	2.32	0.83
AMP (1.5 mM) + $MnCl_2$ (2 mM)	0.66	0.23
KCl (100 mM)	1.36	0.48
PEP (5 mM)	0.33	0.12
PEP (5 mM) + KCl (100 mM)	0.00	0.00

[a] Pyruvate kinase (0.33 mg/mL) was incubated with 2-BDB-TAMP (0.075 mM) at 25 °C in 50 mM HEPES buffer, pH 7.0. Various ligands were added prior to the addition of 2-BDB-TAMP. The rate constants were calculated for the first 20 min of the reaction as described under Experimental Procedures.

is evaluated in Table I. The concentrations of the added ligands Mn^{2+}, ADP, ATP, and PEP exceed their respective dissociation constants for the enzyme–ligand complex severalfold. The reported dissocation constant for Mn^{2+} is 59 ± 16 μM, and for ADP alone it is 150 ± 15 μM, whereas for the Mn–ADP complex the value is 40 ± 14 μM; PEP binds to the enzyme with a dissociation constant of 100 ± 30 μM (Flashner et al., 1973). Mildvan & Cohn (1966) reported a K_d for the Mn–ATP complex of 31 ± 14 μM.

The inclusion of Mn^{2+} in the reaction mixture reduced the inactivation rate constant to half of that observed with no ligands present. ADP alone, when added at a concentration that is 10-fold its binding constant, also provided only partial protection against inactivation, whereas ADP in the presence of Mn^{2+} reduced the inactivation rate constant to 11% of the initial value. Similarly, ATP alone did not have a striking effect on the inactivation rate constant, while in the presence of Mn^{2+} it provided total protection against inactivation by 2-BDB-TAMP. This evidence suggests that the nucleotides protect the enzyme when bound in the form of a complex with Mn^{2+}. Since 2-BDB-TAMP is a derivative of AMP, the ability of AMP to protect against inactivation of pyruvate kinase was also tested. The effect of 1.5 mM AMP alone was small, but in the presence of 2 mM Mn^{2+} a 4-fold reduction of the inactivation rate constant was achieved. Thus, AMP (which is not known to saturate the enzyme at a concentration of 1.5 mM) provides substantial protection against inactivation, which is similar to that observed in the cases of ADP and ATP. When 100 mM K^+ was included in the reaction mixture, partial protection of the enzyme was observed; 5 mM PEP decreased the inactivation rate constant to 12% of that with no ligands present, while PEP + KCl provided total protection against inactivation. These results are in agreement with the report that potassium ion facilitates the binding of PEP to the enzyme (Kayne, 1973).

Incorporation of 2-BDB-TAMP by Pyruvate Kinase. The incorporation of 2-BDB-TAMP by pyruvate kinase was measured at various times during the incubation of pyruvate kinase with 0.075 mM 2-BDB-TAMP as described under Experimental Procedures. Over 100 min, the activity of pyruvate kinase dropped to 42% of the initial activity. (Measurements beyond this time were not used because of the reagent decomposition.) A plot of the residual activity of the enzyme against the number of moles of reagent incorporated per mole of enzyme subunit (Figure 4) indicates an initial

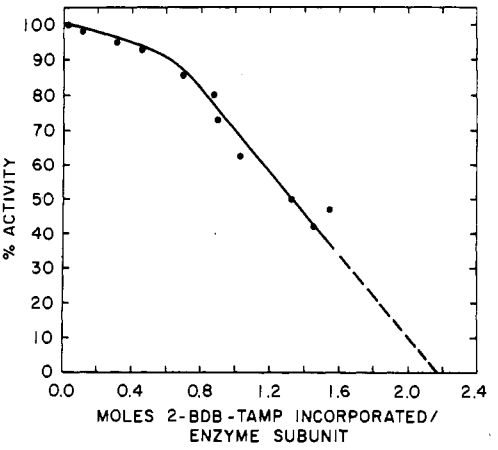

FIGURE 4: Relation between the loss of enzymatic activity of pyruvate kinase and the number of moles of 2-BDB-TAMP incorporated. Pyruvate kinase (1 mg/mL) was incubated with 2-BDB-TAMP (0.075 mM) at 25 °C in 50 mM HEPES buffer. At various times, which correspond to indicated activities, 1-mL aliquots of the reaction mixture were taken, the reaction was stopped by the addition of DTT (0.2 M), and the incorporation of 2-BDB-TAMP by pyruvate kinase was determined as described under Experimental Procedures.

Table II: Effect of Ligands on Incorporation of 2-BDB-TAMP by Pyruvate Kinase[a]

additions to the reaction mixture	residual enzymatic activity (%)	mol of 2-BDB-TAMP/ mol of enzyme subunit[b]
none	47	1.55 ± 0.12
ATP (1.5 mM) + $MnCl_2$ (2 mM)	100	1.10 ± 0.09
ADP (1.5 mM) + $MnCl_2$ (2 mM)	100	0.96 ± 0.10
PEP (5 mM) + KCl (100 mM)	100	0.72 ± 0.14

[a] Pyruvate kinase (1 mg/mL) was incubated with 2-BDB-TAMP (0.075 mM) for 70 min under the same conditions as described in Figure 4. Various ligands were added prior to the addition of 2-BDB-TAMP. At 70 min the reaction was stopped by addition of DTT (0.2 M), and the incorporation of 2-BDB-TAMP by pyruvate kinase was determined as described under Experimental Procedures. [b] The values for incorporation represent averages of three to four separate experiments.

reaction of about 0.7 mol of reagent incorporated per mole of enzyme subunit with little inactivation. This was followed by a reaction involving incorporation of additional reagent, which occurs concomitant with extensive inactivation. Extrapolation to zero activity suggests that 2 (2.16) mol of 2-BDB-TAMP would be bound per mole subunit.

The effect of different ligands on the incorporation of reagent was tested after incubation with 0.075 mM 2-BDB-TAMP for 70 min, with the results shown in Table II. In the absence of any ligands, the residual activity of pyruvate kinase was 47%, and there were 1.55 ± 0.12 mol of 2-BDB-TAMP incorporated per mole of subunit. In the presence of either of the three substrates, ATP + Mn^{2+}, ADP + Mn^{2+}, or PEP + KCl, no activity was lost, yet appreciable incorporation was observed. If it is assumed that groups modified in the absence of ligands include those modified in the presence of ligands, comparison between the extent of modification in the absence and presence of ligands gives an approach to esimating the number of groups whose modification actually gives rise to inactivation of the enzyme. With ATP + Mn^{2+} present in the reaction mixture, there were 1.10 ± 0.09 mol of reagent incorporated per mole of subunit. The difference between the incorporation in the absence of ligands (1.55) and in the presence of ATP + Mn^{2+} (1.10) was 0.45 mol/mol of subunit; this incorporation may be responsible for 53% inactivation of pyruvate kinase. In the presence of ADP + Mn^{2+},

an incorporation of 0.96 ± 0.10 mol of reagent/mol of subunit was observed; in this case, the 53% inactivation could be ascribed to the modification of 0.59 (1.55–0.96) group per subunit. These results suggest that the modification of one group per enzyme subunit could be responsible for total activity loss. When the enzyme was incubated with 5 mM PEP and 0.10 M KCl, incorporation of 0.72 ± 0.14 mol of reagent/mol of subunit was observed, without inactivation. In the presence of PEP and KCl more groups are protected than when the nucleotides ADP or ATP are included in the reaction mixture.

Discussion

The evidence presented here demonstrates that 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-monophosphate reacts with rabbit muscle pyruvate kinase and exhibits characteristics expected for an affinity label. It reacts covalently with a limited number of sites on the enzyme causing complete inactivation; a maximum incorporation of 2.16 mol of 2-BDB-TAMP/mol of subunit is obtained upon extrapolation to zero activity. The rate constants for reaction of 2-BDB-TAMP with pyruvate kinase exhibit a nonlinear dependence on reagent concentration, indicating the formation of a reversible enzyme–reagent complex prior to irreversible modification. Protection against inactivation by 2-BDB-TAMP is provided by the natural ligands ADP, ATP, and PEP.

Although total protection against inactivation is provided by ATP + Mn^{2+}, and substantial protection is provided by ADP + Mn^{2+}, an incorporation of 1 mol of reagent/mol of enzyme subunit is observed in the presence of each ligand. These results, taken together with a maximum extrapolated incorporation of about 2 mol of reagent/mol of subunit, indicate that two sites per subunit are being modified by 2-BDB-TAMP. A model can be proposed in which the natural nucleotides protect the specific binding site responsible for catalytic activity, while the 1 mol of reagent that is incorporated in the presence of these nucleotides binds to a nonessential site that has little effect on enzymatic activity. This postulate is consistent with the kinetics of inactivation being described by a single pseudo-first-order rate constant (i.e., it does not follow biphasic kinetics).

The substrate, phosphoenolpyruvate in the presence of K^+, provides complete protection of pyruvate kinase against inactivation by 2-BDB-TAMP, although an incorporation of 0.7 mol/enzyme subunit is observed. Protection by PEP could result from an overlap between the PEP and the nucleotide binding sites. Such an overlap has been suggested as occurring in the region of the third phosphoryl group of ATP (Mildvan et al., 1976). Since 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-monophosphate has only an α-phosphate, it is unlikely that it would occupy the site common to PEP and ATP; hence, the observed protection by PEP cannot readily be explained in terms of the postulated overlap between the PEP and the nucleotide binding sites. It is known that pyruvate kinase undergoes conformational changes upon binding PEP (Nowak, 1978) and that these changes may cause the nucleotide binding site to become masked and unavailable for reaction with 2-BDB-TAMP. If this is the case, PEP could provide protection against inactivation by an indirect mechanism, rather than by competition for reagent binding as in the case of ADP and ATP. Such an explanation is consistent with the observation that the measured incorporation in the presence of PEP is less than that obtained in the presence of the natural nucleotides, implying a different mechanism of protection by PEP than by the nucleotides ADP and ATP.

It is notable that under the conditions used AMP appears to bind to pyruvate kinase, protecting it against inactivation, although under different conditions AMP has been reported not to act as a competitive inhibitor of the pyruvate kinase reaction (Plowman & Krall, 1965; Reynard et al., 1961). Since the protection and incorporation patterns for the reaction of 2-BDB-TAMP with pyruvate kinase are very similar in the presence of any of the natural adenine nucleotides, it is suggested that AMP, ADP, and ATP can occupy a common binding site in pyruvate kinase.

Pyruvate kinase has previously been studied with (fluorosulfonyl)benzoyl derivatives of nucleosides as affinity labels: 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (FSBA) (Annalmalai & Colman, 1981), 5′-[*p*-(fluorosulfonyl)benzoyl]guanosine (FSBG) (Tomich et al., 1981), and 5′-[*p*-(fluorosulfonyl)-benzoyl]-1,N^6-ethenoadenosine (FSBεA) (Tomich & Colman, 1985; Likos & Colman, 1981). With all of these derivatives, reaction has been shown to occur at the nucleotide binding site, although the exact mode of interaction seems to vary with the compound. FSBA appears to inactivate pyruvate kinase by modification of cysteine and tyrosine residues (Annamalai & Colman, 1981), while FSBG inactivates by modification of two sulfhydryl groups (Tomich et al., 1981). FSBεA modifies a histidine residue, as well as causing the formation of a disulfide from two cysteines (Tomich & Colman, 1985). The differences in interaction among these derivatives can possibly be attributed to differences in the solution conformation of these three (fluorosulfonyl)benzoyl nucleoside analogues. If these derivatives exist in an extended conformation when bound to pyruvate kinase, it is likely that the (fluorosulfonyl)benzoyl moiety will react with the enzyme in the phosphoryl binding region of the nucleotide binding site, whereas, if the analogue exists predominantly in a stacked conformation, the reactive amino acid could be one that is near the purine ring binding site. Differences in the solution conformation of these nucleosides have been evaluated (Jacobson & Colman, 1984). Since FSBεA shows a high degree of stacking in solution, it is possible that the histidine modified by this analogue is in the purine binding region. Recent NMR evidence indicates that a histidyl residue is close to the 2-position of the adenine ring of the bound Mg–ATP (Mildvan et al., 1981). The position of the reactive group in our new analogue, adjacent to the 2-position of the purine ring, may allow this histidine to be modified by 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-monophosphate. Further work is in progress to identify the amino acid residue(s) modified by 2-BDB-TAMP.

Two other nucleotide analogues that are related to the one described here have been synthesized in this laboratory and tested as affinity labels on pyruvate kinase: 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-monophosphate (6-BDB-TAMP) (Colman et al., 1984) and 5′-diphosphate (6-BDB-TADP).[2] These compounds exhibit several characteristics of affinity labels for pyruvate kinase. They inactivate the enzyme with a nonlinear dependence of the inactivation rate constant on reagent concentration, and substantial protection against inactivation by the monophosphate derivative is provided by PEP plus K^+ or by ADP plus Mg^{2+}; however, neither the natural nucleotides nor the substrate provide total protection against inactivation by 6-BDB-TADP. Furthermore, if compared to 2-BDB-TAMP, for the same percent of inactivation of the enzyme, the incorporation of 6-BDB-TADP is higher: extrapolation to zero residual activity leads to an estimate of 3.4 mol of 6-BDB-TADP/mol of enzyme subunit.[2] These observations lead to the conclusion that

[2] E. Kapetanovic and R. F. Colman, unpublished experiments.

the nucleotide derivative with the reactive group adjacent to the 2-position of the purine ring is more effective as an affinity label of pyruvate kinase than is the one with the reactive group adjacent to the 6-position of the purine.

The new nucleotide analogue 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-monophosphate exhibits many desirable characteristics as an affinity label for pyruvate kinase. The bromoketo group of 2-BDB-TAMP has the ability to react covalently with the side chains of several amino acids including cysteine, lysine, histidine, glutamate, and aspartate (Hartman, 1977) and the dioxo group is capable of reacting with arginine residues (Yankeelov, 1970; Riordan, 1973). Additionally, 2-BDB-TAMP, like the natural nucleotides, is negatively charged at neutral pH and therefore soluble in water, eliminating the need for addition of organic solvents in reaction with enzyme. 2-BDB-TAMP, as contrasted with 6-BDB-TAMP and 6-BDB-TADP, still has the 6-amino group of the purine ring, thus more closely resembling the natural nucleotides. This factor may be important in specifically directing reaction within the nucleotide binding sites of some enzymes.

It is proposed that 2-[(4-bromo-2,3-dioxobutyl)thio]-adenosine 5′-monophosphate will have general applicability as an affinity label of adenosine nucleotide binding enzymes and will prove complementary to existing nucleotide analogues that either have reactive functional groups in place of the phosphoryl groups [e.g., (fluorosulfonyl)benzoyl derivatives] or have a modified ribose ring (e.g., periodate-oxidized nucleotides). The increased variety of suitable reactive analogues should allow a systematic probing of the amino acid residues in the region of the nucleotide binding sites of enzymes.

Registry No. 2-BDB-TAMP, 99098-32-5; TAMP, 59924-57-1; AMP-1-oxide, 4061-78-3; AMP, 61-19-8; ADP, 58-64-0; ATP, 56-65-5; PEP, 138-08-9; *m*-$ClC_6H_4OCO_2H$, 937-14-4; CS_2, 75-15-0; $BrCH_2(CO)_2CH_2Br$, 6305-43-7; Mn, 7439-96-5; K, 7440-09-7; pyruvate kinase, 9001-59-6.

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