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6-[(4-Bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-Monophosphate and 5′-Diphosphate: New Affinity Labels for Purine Nucleotide Sites in Proteins†

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ABSTRACT: Two new adenine nucleotide analogues have been synthesized and characterized: 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-monophosphate and 5′-diphosphate. The bromoketo and dioxobutyl moieties have the ability to react with the nucleophilic side chains of several amino acids, as well as with arginine. 6-[(4-Bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-monophosphate reacts irreversibly with rabbit muscle pyruvate kinase, causing inactivation. Addition of ADP to the reaction mixture (in the presence of Mg^{2+}) markedly decreases the rate of inactivation. Pig heart NAD-dependent isocitrate dehydrogenase is allosterically activated by ADP, which reduces the K_m for isocitrate. 6-[(4-Bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate reacts irreversibly with isocitrate dehydrogenase, causing, rapidly, a loss of the ability of ADP to increase the initial velocity of assays conducted at low isocitrate concentrations and, more slowly, inactivation. Addition of ADP to the reaction mixture (in the presence of Mn^{2+}) protects this enzyme against the loss of allosteric activation. It is proposed that the 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenine nucleotides react at the active site of pyruvate kinase and at the ADP activating site of isocitrate dehydrogenase and that these compounds may have general applicability as affinity labels of catalytic and regulatory adenine nucleotide sites in proteins.

Purine nucleotide analogues that have reactive functional groups at particular positions of the purine or ribose ring have proved effective in the affinity labeling of a variety of proteins (Colman, 1983). Desirable features of a purine nucleotide affinity label include structural similarity to the normal nucleotides (i.e., the presence of purine, ribose, and phosphate moieties), water solubility, reasonable stability in the pH range generally optimal for reaction with enzymes, and relatively high reactivity with many different types of amino acids since the participating residues in a given binding site will frequently not have been identified. Many of the known nucleotide affinity labels are limited in their usefulness because of their poor solubility, their low reactivity, or the bulkiness of their functional groups. We have synthesized two new adenosine nucleotide analogues, 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-monophosphate and 5′-diphosphate, and have determined their structures. These compounds are closely related to the adenine nucleotides, are water soluble, and are negatively charged at neutral pH. The bromoketo group is potentially reactive with most nucleophiles found in proteins (Hartman, 1977), and the dioxo group lends the possibility of reaction with arginine residues (Yankeelov, 1970; Riordan, 1973). Because of the location of the functional groups adjacent to the 6-position, the compounds might be expected to react with amino acid residues in the purine region of the adenine nucleotide binding sites of proteins. The analogous 5-(4-bromo-2,3-dioxobutyl) coenzyme A has previously been synthesized (although it was not fully characterized) and has been found to inactivate several enzymes that bind acetyl-CoA (Owens & Barden, 1978; Clements et al., 1979; Katiyar et al., 1982). In this paper, we present evidence suggesting that the new 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenine nucleotides react covalently at the catalytic site of rabbit muscle pyruvate kinase and at the ADP activating site of pig heart NAD-dependent isocitrate dehydrogenase.

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Experimental Procedures

Materials. The barium salt of 6-mercaptopurine ribonucleoside 5′-phosphate was purchased from P-L Biochemicals. The 1,4-dibromobutanedione was obtained from Aldrich Chemical Co. and was recrystallized from petroleum ether prior to use. Malachite Green base, mercuric thiocyanate, 1,1′-carbonyldiimidazole, isocitrate, ADP, NAD, NADH, and phosphoenolpyruvate were all supplied by Sigma Chemical Co. Ferric perchlorate was purchased from Gallard Schlesinger Chemical Corp. and ammonium molybdate from Mallinckrodt. The pig heart NAD-dependent isocitrate dehydrogenase was purified as described by Ehrlich et al. (1981). Rabbit muscle pyruvate kinase was purchased from Boehringer Mannheim Corp. as an ammonium sulfate suspension and was dialyzed and stored as described previously (Tomich et al., 1981). Pig muscle lactate dehydrogenase was obtained from Boehringer Mannheim Corp. as a solution in 50% glycerol and was used without further purification. All other chemicals were reagent grade.

Preparation of 6-[(4-Bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-Monophosphate. The barium salt of 6-mercaptopurine ribonucleoside 5′-phosphate (79 μmol), dissolved in 0.5 mL of 1 M acetic acid, was applied to a 5-mL column of AG 50-X4 (H^+ form, 100–200 mesh) (Bio-Rad Laboratories) and eluted with water in order to convert the nucleotide to the free acid form. After initial evaporation of water, the sample was dried by repeated evaporation from methanol before being dissolved in 7.5 mL of methanol. The apparent pH was adjusted to approximately 4 (pH paper) by the addition of triethylamine. Recrystallized 1,4-dibromobutanedione (759 μmol) was added, and the reaction was allowed to proceed at room temperature. The pH was readjusted to about 4 by addition of small volumes of triethylamine at 1-h intervals, and the progress of the reaction was monitored spectrophotometrically from the decrease in absorbance at 322 nm and the increase in absorbance at 282 nm of aliquots diluted in 0.05 M sodium acetate buffer, pH 4.5. After 6 h, when the reaction was complete, the product was precipitated by a 15-fold dilution of the reaction mixture with cold diethyl ether. The precipitate was collected by centrifugation and washed twice with diethyl ether. The resultant 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-monophosphate (BDB-TAMP)[1] could be dissolved in methanol and stored at −80 °C.

Preparation of 6-[(4-Bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-Diphosphate. The synthesis of BDB-TADP involved the initial phosphorylation of 6-mercaptopurine ribonucleoside 5′-monophosphate to the corresponding diphosphate derivative and purification of 6-mercaptopurine ribonucleoside 5′-diphosphate by column chromatography on DEAE-cellulose, followed by condensation with 1,4-dibromobutanedione. The barium salt of 6-mercaptopurine ribonucleoside 5′-phosphate (100 μmol), dissolved in 1 mL of 1 M acetic acid, was applied to a 10-mL column of AG 50-X4 (pyridinium form, 100–200 mesh) and eluted with 50 mM pyridinium acetate, pH 7.0. The nucleotide-containing fractions were pooled, tributylamine (200 μmol) was added, and the mixture was evaporated under vacuum at about 25 °C. The oily residue was repeatedly (3 times) dissolved in 10 mL of pyridine and evaporated, followed by the addition and evaporation of distilled dimethylformamide in order to remove residual water. The resultant tributylammonium salt of 6-mercaptopurine ribonucleoside 5′-phosphate was dissolved in 5 mL of dimethylformamide to yield a 20 mM solution. Tributylammonium phosphate was prepared by passing Na_2HPO_4 (500 μmol), dissolved in 1 mL of 0.05 M pyridinium acetate, through a column of AG 50-X4 (pyridinium form) and following the same procedure as for tributylammonium 6-mercaptopurine ribonucleoside 5′-phosphate. The final residue was dissolved in 5 mL of dimethylformamide.

The phosphorylation was conducted by the method of Kozarich et al. (1973). With vigorous stirring, a solution of 200 μmol of 1,1′-carbonyldiimidazole in 1.5 mL of dimethylformamide was added to 100 μmol of tributylammonium 6-mercaptopurine ribonucleoside 5′-phosphate in 5 mL of dimethylformamide. The solution was stirred at room temperature for 45 min. Methanol (400 μmol) was added and the reaction mixture was stirred for an additional 30 min. Tributylammonium phosphate (250 μmol in 2.5 mL of dimethylformamide) was added with stirring, and the reaction mixture was maintained at room temperature for 20 h. The reaction was terminated by the addition of 9 mL of methanol, followed by evaporation. The crude product was dissolved in 2 mL of water and stored at −80 °C if not purified immediately.

The 6-mercaptopurine ribonucleoside 5′-diphosphate was isolated by application of 50–100 μmol of the crude nucleotide products to a 1.2 × 28 cm column of DE-52 (Whatman) equilibrated with 0.01 M triethylammonium acetate buffer, pH 4.5. A linear gradient (500 mL of 0.15 M triethylammonium acetate, pH 4.5, and 500 mL of 0.25 M triethylammonium acetate, pH 4.5) was used to elute the column at about 30 mL/h, and 5-mL fractions were collected. The 6-mercaptopurine ribonucleoside 5′-monophosphate eluted between fractions 23 and 36, while the desired product 6-mercaptopurine ribonucleoside 5′-diphosphate was collected between fractions 87 and 139. The concentration was calculated from A_{322nm} (Figure 2), and the pooled fractions were repeatedly evaporated to dryness to remove most of the salt. The residue was dissolved in 2 mL of water, passed through a 5-mL column of AG 50-X4 (H^+ form, 100–200 mesh), and evaporated to dryness.

The white crystalline residue was dissolved in methanol, with the addition of small volumes of diluted triethylamine to give a solution of about 10 mM. Recrystallized 1,4-dibromobutanedione was added at room temperature to yield a concentration of 100 mM, and the pH was adjusted periodically to about 3–4 (pH paper) with small volumes of triethylamine. The reaction was monitored spectrophotometrically until the A_{322nm} of aliquots of the reaction mixture had decreased to approximately 5% of its initial value (about 3 h). A 6-fold dilution of the reaction mixture with cold diethyl ether terminated the reaction and precipitated the product. The precipitate was collected by centrifugation, redissolved in 0.5 mL of methanol, and reprecipitated with ether to remove all the excess 1,4-dibromobutanedione. The product 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate was dissolved in methanol with the addition of 10% triethylamine (in methanol) to yield the soluble triethylammonium salt and was stored at −80 °C.

Bromide Analysis. Free bromide was measured by a procedure modified from that of Zall et al. (1956), in which bromide displaces thiocyanate from mercuric thiocyanate and the liberated thiocyanate reacts with ferric ion to form a

[1] Abbreviations: BDB-TAMP, 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-monophosphate; BDB-TADP, 6-[(4-bromo-2,3-dioxobutyl)thio]-6-adenosine 5′-diphosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PIPES, 2,2′-piperazine-1,4-diylbis[ethanesulfonic acid]; HPLC, high-performance liquid chromatography; CoA, coenzyme A; Tris, tris(hydroxymethyl)aminomethane.

colored complex, which is then measured spectrophotometrically. An aliquot (10–50 μL) containing 10–200 nmol of bromide was mixed with 100 μL of 60% perchloric acid. To this solution were added 120 μL of 0.07% mercuric thiocyanate (saturated water solution) and 240 μL of 0.17 M ferric perchlorate in 4 N perchloric acid; methanol was added to bring the total volume to 1.0 mL. The absorbance at 460 nm was measured for standard bromide solutions and unknown samples. Samples containing covalently bound bromide were hydrolyzed with 0.2 M NaOH at room temperature for at least 1 h before addition of test reagents.

Phosphorus Analysis. The inorganic phosphorus determination was a modification of the procedures of Hess & Derr (1975) and Lanzetta et al. (1979). A 150–200-μL sample containing inorganic phosphate was mixed with 20 μL of 10 N H_2SO_4 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). The A_{660nm} was measured after 5 min. Samples containing organic phosphorus were digested prior to analysis by incubating up to 10 nmol of sample (dry or in up to 200 μL) with 20 μL of 10 N H_2SO_4 at 190 °C for 2 h in a tube closed with aluminum foil. If the residue was colored at this point, 50 μL of 30% H_2O_2 was added and the sample was incubated again at 190 °C for 1 h in an open tube.

Reaction of Rabbit Muscle Pyruvate Kinase with 6-[(4-Bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5'-Monophosphate. Pyruvate kinase (0.33 mg/mL) was incubated at 25 °C with BDB-TAMP (0.04–0.67 mM) in 0.05 M HEPES buffer, pH 7.0, containing 6% methanol. A control consisted of incubating the enzyme under the same conditions (i.e., containing 6% methanol) but in the absence of BDB-TAMP. At various time intervals during the reaction, aliquots were withdrawn, diluted 4000-fold, and assayed for pyruvate kinase activity spectrophotometrically at 340 nm by means of a coupled assay with lactate dehydrogenase, as previously described (Tomich et al., 1981). The rate of reaction of pyruvate kinase with the nucleotide analogue was determined from a semilogarithmic plot of E/E_0 as a function of time for the first 30 min of the reaction, where E represents the enzymatic activity at a given time for the experimental or control reaction and E_0 represents the initial activity.

Reaction of Pig Heart NAD-Dependent Isocitrate Dehydrogenase with 6-[(4-Bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5'-Diphosphate. Isocitrate dehydrogenase (0.45 mg/mL) was preincubated for 3 h at 25 °C in 0.05 M PIPES buffer, pH 6.1, containing 20% glycerol and 0.2 mM $MnSO_4$. The BDB-TADP in methanol was then added to yield concentrations of 0.035–0.21 mM and 10% methanol. Controls consisted of incubating the enzyme under the same conditions but in the absence of BDB-TADP. During the course of the reaction, aliquots were withdrawn, diluted approximately 500-fold, and assayed spectrophotometrically from the increase in absorbance of NADH at 340 nm at 25 °C in Tris–33 mM acetate buffer, pH 7.2, with three different assays in a total volume of 1.0 mL: (1) "high isocitrate" contained 20 mM DL-isocitrate, 1 mM $MnSO_4$, and 1 mM NAD, (2) "low isocitrate" contained 0.4 mM DL-isocitrate, 1 mM $MnSO_4$, and 1 mM NAD, and (3) "low isocitrate + ADP" contained 1 mM ADP in addition to 0.4 mM DL-isocitrate, 1 mM $MnSO_4$, and 1 mM NAD. In assay 1, the enzyme is saturated with isocitrate and the measured rate represents V_{max}. A comparison between assay 2 and assay 3 gives a measure of the activation by the allosteric activator ADP, which lowers the K_m for isocitrate. The reaction rates of enzyme with BDB-TADP were calculated from a semilogarithmic plot of

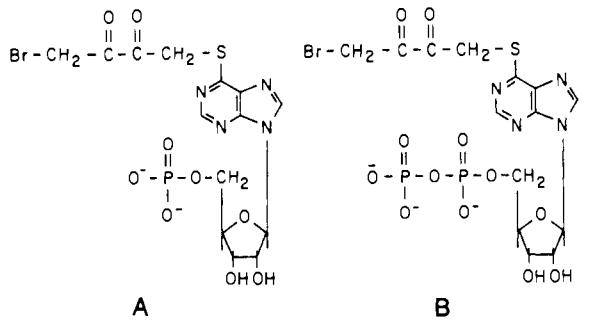

FIGURE 1: Structures of adenine nucleotide analogues: (A) 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5'-monophosphate and (B) 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5'-diphosphate.

E/E_0 vs. time, where E represents the enzymatic activity at a given time in one of the three assays as specified and E_0 represents the initial activity in the high isocitrate assay. Since the reagent was diluted approximately 500-fold in the assay, it was considered that almost no further reaction occurred between BDB-TADP and isocitrate dehydrogenase during the 1–2 min required for the assay.

Results and Discussion

Characterization of 6-[(4-Bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5'-Monophosphate. The purity of the product BDB-TAMP can be assessed by thin-layer chromatography on cellulose-coated aluminum plates (EM Reagents, 0.1-mm thickness) using butanol–acetic acid–water (5:1:2) as the solvent system. A single ultraviolet absorbing spot was observed with $R_f = 0.45$. For comparison, the starting materials 6-mercaptopurine ribonucleoside 5'-phosphate and 1,4-dibromobutanedione exhibit R_f values of 0.17 and 0.91, respectively. The purity was also evaluated by high-performance liquid chromatography (HPLC) using an anion-exchange Varian Micropak SAX-10 column (4 × 300 mm) at room temperature on a Varian Model 5000 liquid chromatograph. The solvents used were (A) 0.007 M potassium phosphate buffer, pH 4.0, and (B) 0.25 M potassium phosphate buffer, pH 4.56, containing 0.5 M KCl. With a linear gradient from 5% to 30% solvent B in 15 min at a flow rate of 2.0 mL/min, one ultraviolet absorbing peak was observed at 15.6% solvent B.

The elemental analysis is in agreement with the structure given in Figure 1 for BDB-TAMP, assuming that the compound was the triethylamine salt. The molecular weight was taken as 628.4. Anal. Calcd for $C_{20}H_{31}N_5O_9SPBr$: C, 38.23; H, 4.97; N, 11.14. Found: C, 36.80; H, 5.16; N, 10.53.

The ultraviolet absorption spectrum of the parent compound 6-mercaptopurine ribonucleoside 5'-monophosphate exhibits a maximum at 322 nm with an extinction coefficient (ϵ) of 25.9×10^3 M^{-1} cm^{-1} when measured in 0.05 M sodium acetate buffer, pH 4.5. During the course of the reaction with 1,4-dibromobutanedione, the absorbance at 322 nm decreases and that at 280 nm increases, with an isosbestic point at 295 nm. As illustrated in Figure 2, the product BDB-TAMP has an extinction coefficient of 16.0×10^3 M^{-1} cm^{-1} when measured at pH 4.5. In methanol, BDB-TAMP exhibits a peak at 278 nm ($\epsilon = 17.9 \times 10^3$ M^{-1} cm^{-1}) with a shoulder at 284 nm as compared to a maximum at 322 nm ($\epsilon = 23.5 \times 10^3$ M^{-1} cm^{-1}) for 6-mercaptopurine ribonucleoside 5'-monophosphate.

The bromide content was determined after hydrolysis in 0.2 N NaOH, as described under Experimental Procedures. The ratio of hydrolyzable bromide to spectrophotometrically determined BDB-TAMP was 1.04:1.00. The organic phosphorus was measured after acid hydrolysis as detailed under Exper-

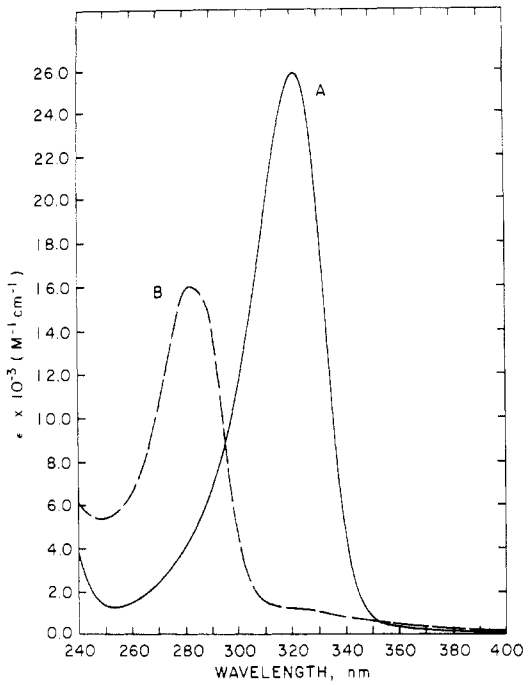

FIGURE 2: Spectra of 6-mercaptopurine ribonucleoside 5′-phosphate (A) and 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-phosphate (B) in 0.05 M sodium acetate buffer, pH 4.5.

imental Procedures, yielding a ratio of 1.02:1.00 of organic phosphorus to BDB-TAMP.

The NMR spectra were obtained with a Bruker WM-250 MHz spectrometer at room temperature using methanol-d_4 (Aldrich) for the proton NMR spectra and D_2O (Stohler Isotope chemicals) for the ^{13}C NMR spectra. The proton NMR spectrum of BDB-TAMP (triethylamine salt) had peaks centered at δ 1.29 (t, $-CH_3$ of triethylamine), 3.24 (q, $-CH_2N$ of triethylamine), 3.67–3.81 (m, $-CH_2Br$), 4.21–4.29 (m, $H_{4'}$, $H_{5'}$ of ribose), 4.41 (m, $H_{3'}$ of ribose), 4.58–4.61 (m, $-CH_2S-$), 4.71 (m, $H_{2'}$ of ribose), 6.17 (d, $H_{1'}$ of ribose), and 8.59–8.72 (m, H_2, H_8). Tetramethylsilane was used as an internal standard. For comparison, the parent compound 6-mercaptopurine ribonucleoside 5′-monophosphate had peaks centered at δ 4.21–4.29 (m, $H_{4'}$, $H_{5'}$ of ribose), 4.37 (m, $H_{3'}$ of ribose), 4.62 (m, $H_{2'}$ of ribose), 6.10 (d, $H_{1'}$ of ribose), 8.19 (s, H_2), and 8.70 (s, H_8). Assignments of the nucleotide protons were made by comparison with the proton NMR spectrum of 5′-AMP (Davies & Danyluk, 1974). The compound 1,4-dibromobutanedione in deuterated methanol exhibits multiplets at δ 3.57–3.59 and at δ 4.51 and 4.57, although in deuterated chloroform it has only a single peak. It is possible that the several enolate forms, which can exist in a relatively polar solvent such as methanol, may account for the more complex spectrum in that solvent and that this same explanation may apply to those peaks attributed to the protons of the $-CH_2C(=O)C(=O)CH_2Br$ moiety of BDB-TAMP. A δ 4.44 has been assigned to the $-CH_2Br$ protons of 3′-(2-bromoethyl)-AMP and δ 3.36 to the corresponding protons of 2′-(2-bromoethyl)-AMP (Bednar & Colman, 1982).

The resonance peaks of the ^{13}C NMR spectra were assigned primarily by reference to those of standard adenosine phosphates (Schleich et al., 1975). For 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-monophosphate, the signals arising from the trimethylamine were centered at δ 8.3 ($-CH_3$) and 46.8 ($-CH_2N$), that from the reactive side chain at δ 35.8 ($-CH_2Br$), those from the ribose at δ 64.5 ($C_{5'}$), 70.4 ($C_{3'}$), 74.6 ($C_{2'}$), 84.4 ($C_{4'}$), and 88.1 ($C_{1'}$), and those from the purine ring at δ 130.7, 143.2, 145.3, 149.5, and 152.0. For comparison, 6-mercaptopurine ribonucleoside 5′-phosphate exhibits signals from the ribose at δ 63.4 ($C_{5'}$), 69.7 ($C_{3'}$), 73.7 ($C_{2'}$), 83.2 ($C_{4'}$), and 87.1 ($C_{1'}$) and those from the purine ring at δ 133.9, 141.7, 143.8, 146.3, and 174.7. The $-CH_2Br$ of 3′-(2-bromoethyl)-AMP exhibits a resonance at δ 32.6 (Bednar & Colman, 1982). All of the analytical and spectral data are thus consistent with the structure of 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-monophosphate shown in Figure 1.

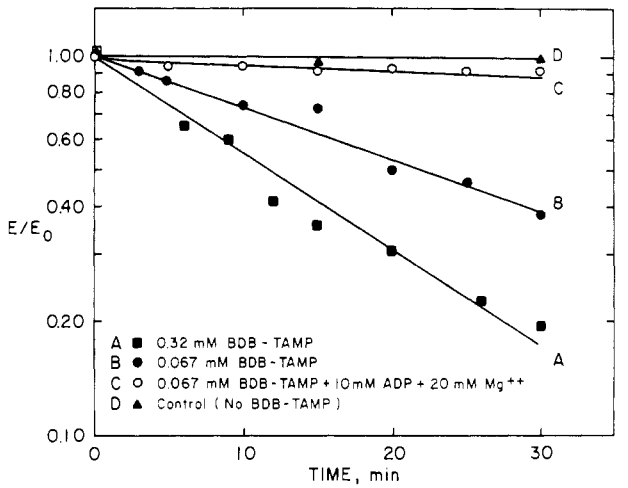

FIGURE 3: Reaction of 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-monophosphate with rabbit muscle pyruvate kinase. Pyruvate kinase was incubated with BDB-TAMP at 25 °C in 0.05 M HEPES buffer, pH 7.0, as described in the text. At each indicated time, an aliquot was withdrawn and assayed for maximal enzyme activity, as indicated under Experimental Procedures. (A) 0.32 mM BDB-TAMP, k = 0.0584 min^{-1}; (B) 0.067 mM BDB-TAMP, k = 0.0315 min^{-1}; (C) 0.067 mM BDB-TAMP + 10 mM ADP + 20 mM Mg^{2+}; (D) control, no BDB-TAMP present.

Characterization of 6-Mercaptopurine Ribonucleoside 5′-Diphosphate and 6-[(4-Bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-Diphosphate. The pooled fractions from the DEAE-cellulose column containing 6-mercaptopurine ribonucleoside 5′-diphosphate exhibited one peak at 31% solvent B upon analysis by HPLC using the system described above (Micropak SAX-10 column). In contrast, the crude 6-mercaptopurine ribonucleoside 5′-diphosphate, prior to purification by chromatography on DEAE-cellulose, exhibited multiple peaks in the same HPLC system, the most prominent occurring at 16.5% and 30.1% solvent B. After reaction of 6-mercaptopurine ribonucleoside 5′-diphosphate with 1,4-dibromobutanedione, the isolated 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate has an R_f of 0.34, as compared to an R_f of 0.13 for the parent nucleotide when subjected to the same thin-layer chromatography system used for BDB-TAMP. The ultraviolet absorption spectrum of BDB-TADP is the same as that of BDB-TAMP. On the basis of an extinction coefficient of 16.0×10^3 M^{-1} cm^{-1} at 280 nm as measured in buffer at pH 4.5, the isolated product contains 0.98 mol of hydrolyzable bromide and 1.94 mol of organic phosphorus per mole of spectrophotometrically determined 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate.

Reaction of BDB-TAMP with Rabbit Muscle Pyruvate Kinase. Muscle pyruvate kinase is an enzyme that requires a nucleotide as coenzyme for its catalytic reaction but is relatively tolerant of structural variation of the purine moiety (Plowman & Krall, 1965). Incubation of the enzyme with 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-monophosphate at pH 7.0 leads to a progressive loss of pyruvate kinase activity, as shown in Figure 3A,B whereas the

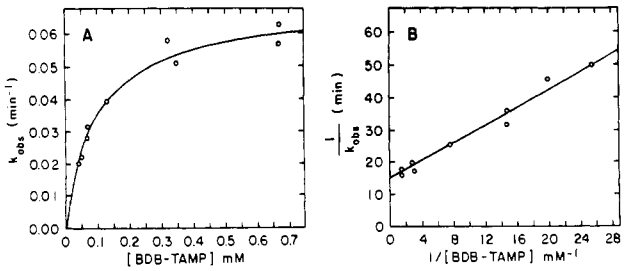

FIGURE 4: Dependence of the pseudo-first-order rate constant (k_{obsd}) for inactivation of pyruvate kinase on the concentration of BDB-TAMP. Rabbit muscle pyruvate kinase was incubated with BDB-TAMP (0.04–0.67 mM) under the conditions described in Figure 3. Rate constants were determined as described under Experimental Procedures. (A) Direct plot; (B) double reciprocal plot in accordance with the equation $1/k_{obsd} = 1/k_{max} + K/k_{max}(1/[BDB\text{-}TAMP])$.

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

additions to reaction mixture	k_{obsd}(+ligand)/ k_{obsd}(−ligand)
none	1.00
10 mM ADP + 20 mM Mg^{2+}	0.05
10 mM ADP	0.41
20 mM Mg^{2+}	0.31
1 mM phosphoenolpyruvate	0.38
10 mM ATP + 10 mM Mg^{2+}	0.31

[a] Rabbit muscle pyruvate kinase (0.33 mg/mL) was incubated at 25 °C with 0.067 mM BDB-TAMP in 0.05 M HEPES buffer, pH 7.0, containing 6% methanol. Various ligands were added before the addition of the BDB-TAMP. Rate constants were measured as described under Experimental Procedures and are here expressed as relative rate constants compared to that measured at 0.067 mM BDB-TAMP in the absence of ligands.

control enzyme (in the absence of reagent) is stable under these conditions. The time-dependent inactivation follows pseudo-first-order kinetics over the first 30 min. The rate constant exhibits a nonlinear dependence on the reagent concentration as indicated by an increase of only 1.9-fold in k_{obsd} as the BDB-TAMP concentration is increased 4.8-fold (Figure 3A vs. Figure 3B). The rate constant (k_{obsd}) was measured over the BDB-TAMP concentration range from 0.04 to 0.67 mM, as shown in Figure 4A. From a double-reciprocal plot of $1/k_{obsd}$ vs. $1/[BDB\text{-}TAMP]$, shown in Figure 4B, a value of $K = 0.091$ mM was estimated for the dissociation constant of a reversible enzyme–reagent complex that forms prior to the irreversible inactivation; the calculated maximum rate constant for inactivation (k_{max}) is 0.067 min^{-1} at saturating concentrations of BDB-TAMP. Such kinetic behavior is normally expected for an affinity label. Inactivation is not reversed by dilution, dialysis, or gel filtration, indicating that an irreversible process has occurred.

Specificity of a protein chemical modification reaction can be indicated by the ability of natural ligands to protect against inactivation. Figure 3C demonstrates that ADP in the presence of metal ion strikingly decreases the rate of inactivation by BDB-TAMP. Table I compares the ability of various ligands to decrease the rate of inactivation. The substrate, phosphoenolpyruvate provides only partial protection, even when included at a concentration high relative to its known binding constant. Similarly, ADP alone and Mg^{2+} alone cause a relatively small decrease in the inactivation rate constant. In contrast, 10 mM ADP together with 20 mM Mg^{2+} decreases the rate constant about 20-fold. As pronounced a decrease in the rate constant is also provided by 1 mM ADP plus 1.5 mM Mg^{2+}, which is consistent with the measured K_m of 0.19 mM for ADP in HEPES buffer, pH 7.0. In the

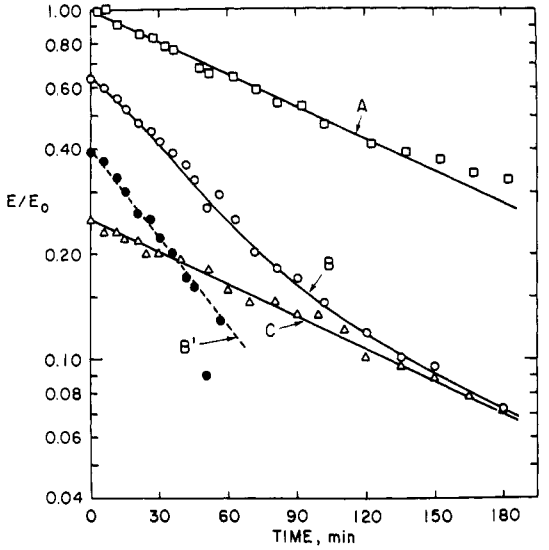

FIGURE 5: Effect of 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deamino-adenosine 5′-diphosphate on enzymatic activity and on ADP activation of pig heart NAD-specific isocitrate dehydrogenase. Isocitrate dehydrogenase was incubated with 0.14 mM BDB-TADP at 25 °C in 0.5 M PIPES buffer, pH 6.1, as described in the text. At the indicated times, aliquots were withdrawn and assayed either at 20 mM isocitrate (line A), at 0.4 mM isocitrate (line C), or at 0.4 mM isocitrate plus 1 mM ADP (line B). Line B′ was generated by subtracting E/E_0 for line C from E/E_0 for line B at each time point. The pseudo-first-order rate constants are 0.007 min^{-1} for lines A and C and 0.021 min^{-1} for line B′.

presence of Mg, ATP provides only about a 3-fold reduction in the rate of inactivation, indicating that the Mg nucleoside diphosphate and Mg nucleoside triphosphate sites are not identical. A similar distinction between the effects of the two nucleotides has been observed before in affinity labeling of pyruvate kinase (Tomich et al., 1981). The pattern of protection by ligands suggests that the Mg-ADP site may be the locus of attack of pyruvate kinase by BDB-TAMP.

Reaction of BDB-TADP with Pig Heart NAD-Dependent Isocitrate Dehydrogenase. Pig heart NAD-specific isocitrate dehydrogenase is allosterically activated by ADP, which lowers the K_m for isocitrate, but has no effect on the maximum velocity (Cohen & Colman, 1972). Figure 5 shows the effect on maximum enzymatic activity (line A) and on ADP activation of incubating isocitrate dehydrogenase with 0.14 mM BDB-TADP at pH 6.1. There is a progressive, relatively slow inactivation reflected in the parallel lines A and C (assayed at saturating or low concentration of isocitrate, respectively), yielding a pseudo-first-order rate constant of 0.007 min^{-1}. No change in enzymatic activity or ADP activation was observed when the enzyme was incubated in the absence of reagent under otherwise identical conditions (not shown). Since reaction with BDB-TADP results in some loss in enzymatic activity, any effect of the reagent on regulation by ADP would be observed only if the loss in ADP activation occurs more rapidly than overall inactivation. The effect of ADP is conveniently monitored as an increase in initial velocity caused when the nucleotide is added to assay solutions containing nonsaturating concentrations of isocitrate. In Tris–acetate buffer, pH 7.2, the addition of 1 mM ADP produces an increase of about 2.7-fold in the initial velocity of the reaction catalyzed by native enzyme with 0.4 mM isocitrate, as represented by the difference in the zero time points of Figure 5, lines B and C. As a function of time of incubation with BDB-TADP, line B approaches line C, and by 100 min, ADP has no effect on the initial velocity of assays at low isocitrate concentration; i.e., the ability of ADP to activate the enzyme

by lowering the K_m for isocitrate has been eliminated. The "end point" of loss of ADP activation is an assay rate for 0.4 mM isocitrate plus ADP, identical with the assay rate for 0.4 mM isocitrate alone. Therefore, the ADP rate data of line B are corrected by subtracting that fraction of measured activity attributable to the unactivated reaction; that is, line C is subtracted from line B to generate line B′, which yields the corrected pseudo-first-order rate constant for loss of ADP activation, 0.021 min^{-1}. At 0.14 mM BDB-TADP, the loss of ADP activation occurs at more than three times the rate of inactivation of the enzyme. Addition of 0.1 mM ADP to the reaction mixture containing 0.14 mM BDB-TADP and 0.2 mM $MnSO_4$ leads to almost complete protection against loss of ADP activation but only about a 2-fold decrease in the rate of overall inactivation. These results suggest that BDB-TADP reacts most rapidly at the high-affinity allosteric ADP site of isocitrate dehydrogenase.

The reaction rates of isocitrate dehydrogenase with BDB-TADP were measured from 0.035 to 0.21 mM reagent, and a rate saturation effect was observed in which the rate constants are dependent on the BDB-TADP concentration until the enzyme becomes saturated with reagent; but further increases in the BDB-TADP concentration do not enhance the rate constants. These observations constitute kinetic evidence for the initial formation of a reversible enzyme–reagent complex prior to the irreversible modification, as expected for an affinity label. For reaction at the site leading to loss of ADP activation, the dissociation constant for the reversible enzyme–reagent complex is estimated at 0.069 mM, with the maximum rate constant equal to 0.031 min^{-1}, while for reaction at the site causing overall inactivation, K_d for the initial reversible enzyme–reagent complex is 0.018 mM, with k_{max} = 0.0083 min^{-1}. Reaction of BDB-TADP with NAD-dependent isocitrate dehydrogenase appears to be irreversible, since neither the ADP activation nor the activation of the enzyme is regained after dilution, dialysis, or gel filtration. It is proposed that the new 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenine nucleotides react at the active site of pyruvate kinase and at the ADP activating site of isocitrate dehydrogenase and that these compounds may have general applicability as affinity labels of catalytic and regulatory purine nucleotide sites in proteins.

Registry No. BDB-TAMP, 90269-31-1; BDB-TADP, 90269-32-2; 6-mercaptopurine ribonucleoside 5′-phosphate barium salt, 90269-33-3; 6-mercaptopurine ribonucleoside 5′-phosphate, 53-83-8; 1,4-dibromobutanedione, 6305-43-7; 6-mercaptopurine ribonucleoside 5′-phosphate tributylammonium salt, 90269-34-4; 6-mercaptopurine ribonucleoside 5′-diphosphate, 805-63-0; pyruvate kinase, 9001-59-6; NAD-dependent isocitrate dehydrogenase, 9001-58-5.

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