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Reaction of Pyruvate Kinase with the New Nucleotide Affinity Labels 8-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 5′-Diphosphate and 5′-Triphosphate†

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ABSTRACT: Two new reactive nucleotides have been synthesized and characterized: 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-diphosphate and 5′-triphosphate (8-BDB-TADP and 8-BDB-TATP). ADP or ATP was converted to 8-thio-ADP (-ATP) via 8-bromo-ADP (-ATP), followed by condensation with 1,4-dibromobutanedione. Rabbit muscle pyruvate kinase is inactivated by both reagents in a biphasic manner with an initial rapid loss of 75% activity, followed by a slow total inactivation. The initial fast reaction with both compounds exhibits nonlinear dependence on reagent concentration, indicating formation of a reversible enzyme–reagent complex prior to covalent attachment. The presence of the γ-phosphoryl group improves the performance of the affinity label: K_I values for the fast phase are similar (about 100 μM), whereas k_{max} for 8-BDB-TATP is about three times greater than that of 8-BDB-TADP (0.286 min^{-1} vs 0.0835 min^{-1}). After an 80-min incubation with 175 μM of either reagent, about 2 mol/mol of subunit is incorporated with 76% inactivation caused by 8-BDB-TADP and 97% inactivation by 8-BDB-TATP. Loss of activity is prevented by substrates, with the best protection afforded by a combination of ATP, Mn^{2+}, K^+, and phosphoenolpyruvate. Reaction of pyruvate kinase with either compound in the presence of protecting ligands leads to incorporation of about 1 mol of reagent/mol of subunit with only about 15% loss of activity. These results suggest that 8-BDB-TADP and 8-BDB-TATP react with two groups on the enzyme, one of which is at or near the active site. The two new bromodioxobutyl derivatives may have general application as affinity labels of purine nucleotide sites in other proteins.

Pyruvate kinase is a key glycolytic enzyme which catalyzes the formation of ATP from ADP and phosphoenolpyruvate. Considerable information is now available on the structure of pyruvate kinase. NMR experiments have led to predictions about the spatial arrangement of substrates, essential metal ions, and the structural changes that occur upon substrate binding and catalysis (Mildvan & Cohn, 1966; Gupta et al., 1976; Mildvan et al., 1976; Rao et al., 1979; Dunaway-Mariano et al., 1979; Rosevear et al., 1987). Complete amino acid sequences are known for the muscle (Lonberg & Gilbert, 1983; Muirhead et al., 1986), liver (Lone et al., 1986; Inoue et al., 1986), and erythrocyte isozymes (Noguchi et al., 1987), as well as the yeast enzyme (Burke et al., 1983). The amino acid sequence of cat muscle pyruvate kinase has been fitted to the 2.6-Å resolution electron density map (Muirhead et al., 1986), and residues that participate in substrate binding and are near the active site have been identified. Affinity labeling offers a complementary technique for investigating the active site as it exists when the enzyme is in solution.

In this paper, we describe the synthesis and characterization of the new nucleotide analogues, 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-diphosphate (8-BDB-TADP)[1] and 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-triphosphate (8-BDB-TATP). The structures are shown in Figure 1. The placement of the bromoketo group adjacent to the 8-position of the adenine ring may allow the reagents to react in a different manner from 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-monophosphate (Colman et al., 1984) and 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-monophosphate (Kapetanovic et al., 1985), which have been shown to modify rabbit muscle pyruvate kinase. Furthermore, di- and triphosphate moieties of the new reagents are identical with those of the natural substrate and product, ADP and ATP. This paper presents evidence that 8-BDB-TADP and 8-BDB-TATP

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[1] Abbreviations: 8-BDB-TADP, 8-[(4-bromo-2,3-dioxobutyl)thio]-adenosine 5′-diphosphate; 8-BDB-TATP, 8-[(4-bromo-2,3-dioxobutyl)-thio]adenosine 5′-triphosphate; 8-TADP, 8-thioadenosine 5′-diphosphate; 8-TATP, 8-thioadenosine 5′-triphosphate; PEP, phosphoenolpyruvate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)-aminomethane; FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; FSBεA, 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine; 6-BDB-TAMP, 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-monophosphate; 2-BDB-TAMP, 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-monophosphate.

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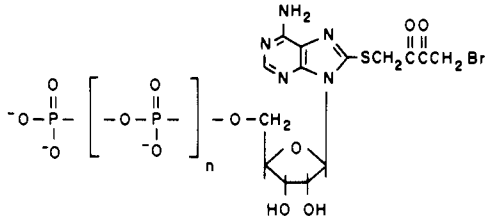

FIGURE 1: Structure of 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-diphosphate (n = 1) and 5′-triphosphate (n = 2).

react in a specific and limited manner with rabbit muscle pyruvate kinase. A preliminary version of this paper has been presented (DeCamp et al., 1988).

Experimental Procedures

Materials and Methods. Rabbit muscle pyruvate kinase was purchased from Boehringer Mannheim Biochemicals as a crystalline suspension. The enzyme was dialyzed overnight at 4 °C against 0.05 M HEPES buffer, pH 7.0, centrifuged for 10 min at 13 000 rpm, and stored at −75 °C. The enzyme concentration was determined by using $E^{0.1\%}_{280nm}$ = 0.54 (Bücher & Pfleiderer, 1955) and a M_r of 237 000 per tetramer (Cottam et al., 1969). Pyruvate kinase activity was measured spectrophotometrically at 340 nm by means of a coupled assay with lactate dehydrogenase. The enzymatic activity was monitored at 30 °C in 0.05 M Tris-HCl buffer, pH 7.5, containing 100 mM KCl, 10 mM $MgSO_4$, 0.5 mM phosphoenolpyruvate, 3 mM ADP, 0.25 mM NADH, and lactate dehydrogenase at a concentration of 0.1 mg/mL.

Malachite Green base, mercuric thiocyanate, nucleotides, phosphoenolpyruvate, dithiothreitol, buffer salts, and phosphorus standard solution were all obtained from Sigma Chemical Co. 1,4-Dibromobutanedione was purchased from Aldrich Chemical Co. and was recrystallized from petroleum ether before use. Ferric perchlorate was supplied by Fluka AG. Lactate dehydrogenase (hog muscle) in 50% glycerol was purchased from Boehringer Mannheim Biochemicals and used without further purification. Ammonium molybdate and hydrogen peroxide were supplied by Fisher Scientific Co., protein dye concentrate and AG50W-X4(H^+) were from Bio-Rad Laboratories, and Ultrapure guanidine hydrochloride was purchased from Schwarz/Mann.

NMR spectra were obtained with a Bruker WM 250-MHz spectrometer at room temperature. ^1H NMR samples were dissolved in either dimethyl sulfoxide-d_6 containing 1% tetramethylsilane as an internal standard or D_2O containing dioxane (3.71 ppm) as an internal standard. H_3PO_4 (85%) was used as an external standard for ^{31}P NMR chemical shifts.

Synthesis of 8-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 5′-Diphosphate. The overall synthetic scheme is shown in Figure 2. 8-ThioADP was prepared by the method of Ginsburg and Maurizi (1986) with minor modifications. ADP (I) was brominated to give 8-bromoADP (II), which was then reacted with aqueous LiHS to give 8-thioADP (III). Condensation of 8-TADP with 1,4-dibromobutanedione gave the final product, 8-BDB-TADP (IV).

Preparation of 8-Bromoadenosine 5′-Diphosphate (II). ADP (1 g) was dissolved in 25 mL of H_2O, and 325 μL of Br_2 was added dropwise with vigorous stirring. The mixture was stirred at room temperature in the dark for 2 h, and then 0.175 g of sodium metabisulfate was added. The solution was dried by rotary evaporation and coevaporated three times with ethanol. The solid material (II) was dissolved in 100 mL of H_2O and applied to a 1.5 × 25 cm column of DEAE-Sephadex equilibrated with 0.01 M NH_4HCO_3 at 4 °C. The column was eluted with a linear gradient formed from 1.5 L of 0.01

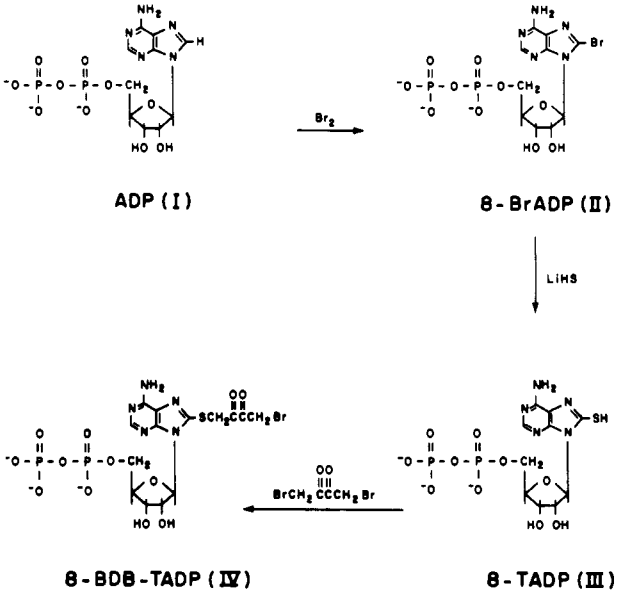

FIGURE 2: Synthetic scheme for preparation of 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-diphosphate.

M NH_4HCO_3 and 1.5 L of 0.5 M NH_4HCO_3. Fractions of 10 mL were collected, and those containing an absorbance maximum at 260 nm (fractions 52–73) were pooled and dried on a rotary evaporator. 8-BrADP (II) was obtained as a white powder in 80% yield.

Preparation of 8-Thioadenosine 5′-Diphosphate (III). Conversion of II to III was accomplished by dissolving 100 mg of 8-BrADP in a solution (2.5 mL) of 1 M LiHS, which was made by bubbling H_2S into 1 M LiOH until the pH was lowered to 9.0. The reaction solution was left at room temperature overnight, diluted with approximately ten volumes of water, and purified on a DEAE-Sephadex column as described above. Fractions containing an absorbance maximum at 295 nm (fractions 119–143) were pooled and dried on a rotary evaporator. 8-TADP (III) was redissolved in water and lyophilized repeatedly to give a white powder. ^1H NMR (D_2O) showed peaks at 8.10 (H_2) and 6.38 ppm (H_1'). Yield was 60%.

Preparation of 8-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 5′-Diphosphate (IV). To generate the final product, 8-TADP (50 mg) was dissolved in 2 mL of H_2O, applied to a 1.8 × 50 cm column of AG50W-X4(H^+), and eluted with distilled H_2O at 4 °C. Fractions (10 mL) were monitored for absorbance at 308 nm. Nucleotide-containing fractions 4–10 were pooled, dried, and coevaporated with methanol three times on a rotary evaporator.

8-TADP was coupled with 1,4-dibromobutanedione as described for the reaction of 2-thioadenosine 5′-monophosphate with 1,4-dibromobutanedione (Kapetanovic et al., 1985). Reaction occurred immediately and could be assessed spectrophotometrically by the decrease in A_{308nm} and the appearance of a peak at 278 nm. The product (IV) was obtained as a white powder in 50–60% yield from III. 8-BDB-TADP was stored dried and dessicated at −75 °C.

Synthesis of 8-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 5′-Triphosphate. 8-BDB-TATP was synthesized by procedures similar to those used for 8-BDB-TADP. The only difference was in the conversion of 8-TATP to 8-BDB-TATP. 8-TATP (35 mg) was dissolved in 1 mL of H_2O and applied to a 1.8 × 50 cm column of AG50W-X4 (pyridinium). The column was eluted with 0.05 M pyridinium acetate, pH 7.0, at room temperature. Fractions (10 mL) were collected and monitored for absorbance at 295 nm. Fractions 4–10 were pooled, and

30 μL of triethylamine was added. The pool was dried under vacuum and coevaporated three times with methanol. The residue was dissolved in methanol, and the pH was adjusted to 5 (estimated by pH paper) by the addition of triethylamine. The concentration of 8-TATP was 26 mM. 1,4-Dibromobutanedione (0.1 g, recrystallized) was dissolved in 0.5 mL of methanol (820 mM) and rapidly mixed with 0.5 mL of the 8-TATP solution. Reaction occurred within 12 min and could be assessed spectrophotometrically by the decrease in absorbance at 306 nm and the increase in absorbance at 278 nm when the reaction mixture was diluted into 0.05 M MES buffer, pH 6. The product was precipitated by the addition of 10 mL of diethyl ether, as described previously (Kapetanovic et al., 1985), and was a white powder. Yields for the various synthetic steps were similar to those for 8-BDB-TADP.

Reaction of 8-BDB-TADP and 8-BDB-TATP with Pyruvate Kinase. Rabbit muscle pyruvate kinase (0.33 mg/mL) was incubated with varying concentrations of 8-BDB-TADP and 8-BDB-TATP at 25 °C in 0.05 M HEPES buffer, pH 7.0, for measurement of the kinetics of the reaction. When protecting ligands were included, enzyme was preincubated with them for 10 min before the reagent was added. Control samples were incubated under the same conditions without affinity label. At timed intervals, aliquots of the reaction mixture were withdrawn, diluted with 0.05 M HEPES buffer, pH 7.0, at 0 °C, and assayed for residual pyruvate kinase activity. The rate of oxidation of NADH was measured by the loss of absorbance at 340 nm on a Cary 219 spectrophotometer. The rate of reaction of pyruvate kinase with the reagents was determined from a semilogarithmic plot of E/E_0 as a function of time, where E_0 represents the initial activity of the enzyme at time zero and E represents the activity at a given time.

Rate constants for the reaction were calculated from the equation

$$E/E_0 = (1 - F)e^{-k_{fast}t} + (F)e^{-k_{slow}t} \quad (1)$$

where F represents the fractional residual activity of the partially active enzyme intermediate. Analysis was conducted by using the Fortran IV computer program of Marquardt for estimation of nonlinear parameters (IBM Share Library, Distribution No. 3094, March 1964) as based on Marquardt's algorithm (Marquardt, 1963). This program minimizes the differences between the experimental and predicted values of E/E_0 by adjusting the values of k_{fast} and k_{slow} in eq 1.

Measurement of Incorporation of 8-BDB-TADP and 8-BDB-TATP into Pyruvate Kinase. The enzyme (0.66 mg/mL) was incubated with 175 μM reagent under the conditions described above. At various times a 0.5-mL aliquot of the reaction mixture was withdrawn, and 10 μL of a 1 M dithiothreitol solution was added to decompose the reagent. After 1 min, 0.24 g of solid guanidine hydrochloride was added to denature the enzyme. The modified enzyme was rapidly separated from excess reagent by the column centrifugation method of Penefsky (1979), using two consecutive Sephadex G-50-80 columns (5 mL) equilibrated with 0.05 M MES buffer, pH 6, and 5 M guanidine hydrochloride. The protein concentration in the filtrate was determined by using the Bio-Rad protein assay based on the method of Bradford (1976). The incorporation of 8-BDB-TADP and 8-BDB-TATP into pyruvate kinase was measured by determination of the moles of organic phosphorus per mole of enzyme subunit as described in Bailey and Colman (1987).

Determination of the Decomposition Rate of 8-BDB-TADP. The rate of loss of bromide was measured by incubating 3 mM 8-BDB-TADP in 0.05 M HEPES buffer, pH 7.0, at 25 °C. At various times, 50-μL aliquots (150 nmol of reagent) were withdrawn and the concentration of free bromide was measured as previously described by Colman et al. (1984). The rate constant for decomposition of the reagent was calculated from a semilogarithmic plot of $(C_\infty - C_t)/(C_\infty - C_0)$ vs time, where C_∞, C_0, and C_t represent, respectively, the final, initial, and time-dependent free bromide concentration.

RESULTS

Characterization of 8-[(4-Bromo-2,3-dioxobutyl)thio]-adenosine 5'-Diphosphate. The overall yield of the reaction (Figure 2, I–IV) was approximately 24%. The purity of the product was assessed by thin-layer chromatography on cellulose-backed sheets with isobutyric acid–concentrated NH_4OH–H_2O (66:1:33) as the solvent system. A single spot was observed with an R_f of 0.39, whereas the precursor, 8-TADP, and 1,4-dibromobutanedione exhibited spots at 0.22 and 0.78, respectively.

The extinction coefficient of 8-BDB-TADP is 1.9×10^4 M^{-1} cm^{-1} when measured at 278 nm in 0.05 M MES buffer, pH 6.0. This is in good agreement with the extinction coefficients reported for various 8-thioalkyl derivatives of cAMP (Muneyama et al., 1971).

The bromide and phosphorus contents of 8-BDB-TADP were measured as previously described (Colman et al., 1984). The ratio of organic phosphorus to spectrophotometrically determined 8-BDB-TADP is 2.08:1.0. The ratio of hydrolyzable bromide to reagent is 0.98:1.00.

The proton NMR spectrum of 8-BDB-TADP (triethylammonium salt) in dimethyl sulfoxide had peaks centered at 1.17 (t, $-CH_3$ of triethylamine), 3.08 (q, $-CH_2N$ of triethylamine), 4.04 (m, $H_{5'}$ of ribose), 4.24 (m, $H_{4'}$ of ribose), 4.65 (m, $-CH_2S-$, $-CH_2Br$), 4.70 (m, $H_{3'}$ of ribose), 4.96 (m, $H_{2'}$ of ribose), 5.86 (d, $H_{1'}$ of ribose), 7.37 (s, $-NH_2$), and 8.09 ppm (s, H_2). Assignments of the protons were made by comparison with the proton NMR spectra taken in dimethyl sulfoxide of adenosine, 1,4-dibromobutanedione (Huang et al., 1986), and 2-thioadenosine (Kikugawa et al., 1973).

The proton-decoupled ^{31}P NMR spectrum of 8-BDB-TADP was recorded at 25 °C on a 2-mL sample volume, pH 7.5, containing 20% D_2O as an internal field frequency lock, with ethylenediaminetetraacetate added to eliminate any line broadening due to paramagnetic impurities. The spectrum showed doublets at −5.26 and −5.48 ppm (β) and at −9.37 and −9.59 ppm (α), indicating a pyrophosphate moiety.

Characterization of 8-[(4-Bromo-2,3-dioxobutyl)thio]-adenosine 5'-Triphosphate. 8-BDB-TATP was subjected to thin-layer chromatography as described for 8-BDB-TADP. A single UV-absorbing spot was observed with an R_f of 0.38. The extinction coefficient of 8-BDB-TATP is 1.9×10^4 cm^{-1} M^{-1} when measured at 278 nm in 0.05 M MES buffer, pH 6.0.

The bromide and phosphorus contents of 8-BDB-TATP were measured as previously described (Colman et al., 1984). The ratio of organic phosphorus to spectrophotometrically determined 8-BDB-TATP is 3.1:1.0. The ratio of hydrolyzable bromide to 8-BDB-TATP is 1.1:1.0.

The proton NMR spectrum of 8-BDB-TATP was identical with the spectrum recorded for 8-BDB-TADP. The proton-decoupled ^{31}P NMR spectrum showed a doublet at −5.41 and −5.60 ppm, a doublet at −9.86 and −10.02 ppm, and a triplet centered at −20.48 ppm. For comparison, the spectrum of ATP has a doublet at −5.51 and −5.71 ppm (γ), a doublet at −10.24 and −10.43 (α), and a triplet centered at −20.90 ppm (β).

Test of 8-BDB-TADP as a Substrate for Pyruvate Kinase. In order to determine whether 8-BDB-TADP was a substrate

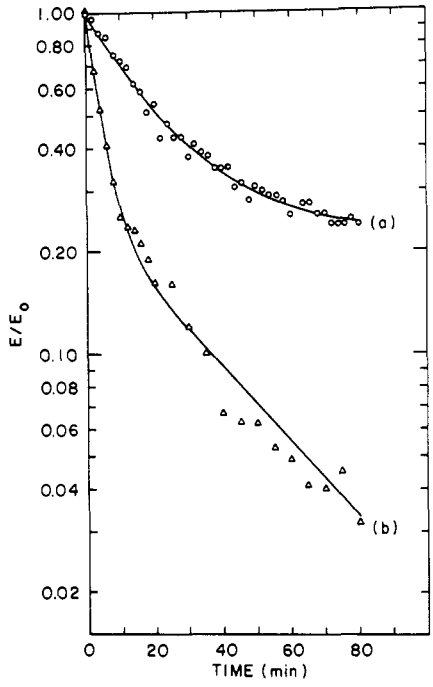

FIGURE 3: Inactivation of pyruvate kinase by 8-BDB-TADP and 8-BDB-TATP. Rabbit muscle pyruvate kinase (0.33 mg/mL) was incubated with 175 μM 8-BDB-TADP (○) and 175 μM 8-BDB-TATP (△) at 25 °C in 50 mM HEPES buffer, pH 7.0. Residual activity, E/E_0, was measured as described under Experimental Procedures. The solid lines are theoretical lines generated from a computer fit to eq 1. Values of $F = 0.25$, $k_{fast} = 0.052$ min^{-1}, and $k_{slow} = 0$ min^{-1} were used to calculate the inactivation rate of 8-BDB-TADP. For the inactivation with 8-BDB-TATP, $F = 0.25$, $k_{fast} = 0.23$ min^{-1}, and $k_{slow} = 0.025$ min^{-1} were used.

for pyruvate kinase, assay solution was prepared containing either 0.5 mM ADP or 0.5 mM 8-thioADP at pH 7.5, with concentrations of other compounds the same as in the standard assay. Enzyme assayed with ADP had a specific activity of 100 μmol/(min·mg), while enzyme assayed with 8-thioADP had a specific activity of 6.6 μmol/(min·mg). The slow reaction rate made it impractical to use the pyruvate kinase catalytic reaction to generate 8-thioATP as a precursor for 8-BDB-TATP.

Inactivation of Pyruvate Kinase by 8-BDB-TADP and 8-BDB-TATP. Incubation of pyruvate kinase with 175 μM 8-BDB-TADP and 8-BDB-TATP at pH 7.0 and 25 °C resulted in a time-dependent inactivation of the enzyme as shown in Figure 3. Control enzyme showed constant activity during this period. Biphasic inactivation kinetics were observed that could be described in terms of a fast initial phase of inactivation resulting in partially active enzyme ($F = 0.25$) followed by a slower phase. The solid lines in Figure 3 are theoretical lines based on a computer fit to eq 1 as described under Experimental Procedures, while the points are experimental.

At low concentrations of 8-BDB-TADP, the fast phase does not reach 25% residual activity in the period shown because of the time-dependent decomposition of the reagent, and the slow phase is not observed (Figure 3, line a). The rate of bromide release from 8-BDB-TADP is 0.0135 min^{-1}, giving a half-life of 50 min. Once the bromide has been released, the decomposed reagent does not inactivate the enzyme, suggesting the importance of the bromoketo moiety in the inactivation. In the case of 8-BDB-TATP, shown in Figure 3, line b, the slow phase of inactivation predominates by 20 min, indicating that reagent decomposition is not responsible for the observed biphasic kinetics. The reagent decomposition does, however, cause a slight underestimation of the rate constant of the slow phase of inactivation at the lower reagent concentrations.

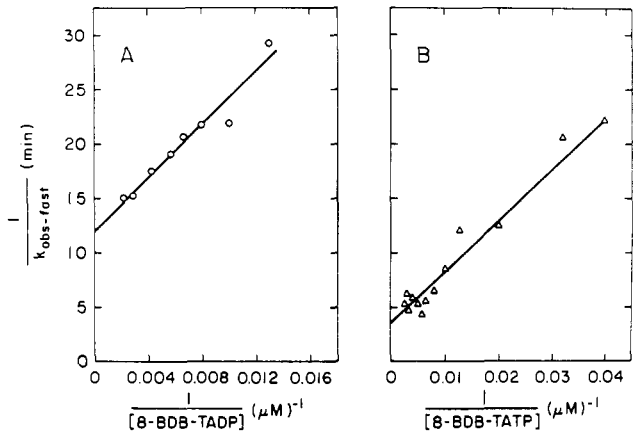

FIGURE 4: Dependence of the pseudo-first-order rate constant ($k_{obsd,fast}$) for inactivation of pyruvate kinase on the concentration of (A) 8-BDB-TADP and (B) 8-BDB-TATP. Data are shown for the fast initial phase of inactivation. Pyruvate kinase was incubated with various concentrations of reagent under the conditions described in Figure 3. Rate constants were determined by computer fit to eq 1, using a value of $F = 0.25$. The graphs are the double-reciprocal plots used to calculate k_{max} and K_I according to eq 2.

Concentration Dependence of Reaction of Pyruvate Kinase with 8-BDB-TADP and 8-BDB-TATP. Pyruvate kinase was incubated with varying concentrations of 8-BDB-TADP or 8-BDB-TATP (50–450 μM) in order to determine the dependence of the rate of inactivation on the reagent concentration. At high concentrations of 8-BDB-TADP (230–450 μM), both phases of inactivation can be observed, but the reagent decomposition still causes an underestimation of the rate constant for the slow phase of the inactivation; thus, only the fast phase was analyzed quantitatively. At all concentrations of 8-BDB-TATP used, biphasic kinetics were observed.

A constant ordinate intercept, F, of $E/E_0 = 0.25$ was used to calculate all rate constants in accordance with eq 1 and gave a satisfactory fit over the entire reagent concentration range studied. A constant value of F, independent of the reagent concentration, excludes enzyme-catalyzed decomposition of the reagent as an explanation for the biphasic kinetics.

The fast phase of inactivation by 8-BDB-TADP exhibits a nonlinear dependence on the reagent concentration. This indicates the initial formation of a reversible enzyme–reagent complex prior to irreversible modification, as is characteristic of an affinity label. The observed rate constant (k) at a particular concentration of 8-BDB-TADP (R) is described by the equation

$$1/k = (K_I/k_{max})(R) + 1/k_{max} \quad (2)$$

where $K_I = (k_{-1} + k_{max})/k_1$ and represents the concentration of reagent giving half of the maximal inactivation rate (Huang & Colman, 1984). The double-reciprocal plot shown in Figure 4A was used to calculate values of $K_I = 99$ μM and $k_{max} = 0.0835$ min^{-1} for the fast phase of inactivation by 8-BDB-TADP. Both the fast and slow phases of inactivation by 8-BDB-TATP exhibit a nonlinear dependence on the reagent concentration. A double-reciprocal plot of the rate constants for the fast phase of inactivation by 8-BDB-TATP is shown in Figure 4B, from which values of $K_I = 133$ μM and $k_{max} = 0.286$ min^{-1} were calculated. The slow phase (not shown) had values of $K_I = 117$ μM and $k_{max} = 0.0326$ min^{-1}.

Incorporation of 8-BDB-TADP and 8-BDB-TATP by Pyruvate Kinase. Pyruvate kinase was incubated with 175 μM 8-BDB-TADP or 8-BDB-TATP as described under Experimental Procedures, and the incorporation of reagent into the

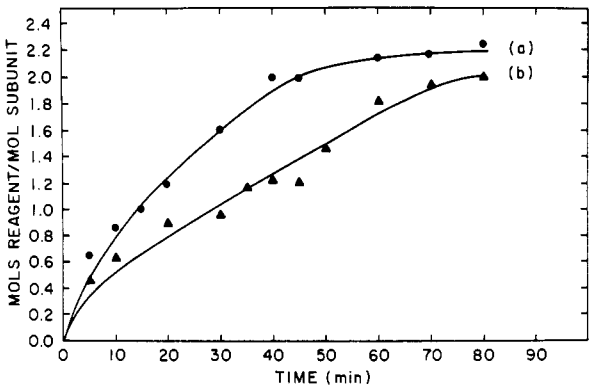

FIGURE 5: Incorporation of (a) 8-BDB-TADP (●) and (b) 8-BDB-TATP (▲) per mole of subunit of pyruvate kinase as a function of time. Pyruvate kinase (0.66 mg/mL) was incubated with 175 μM reagent. Incorporation was determined at the indicated time points as described under Experimental Procedures.

enzyme was measured at various times during the incubation by quantitation or organic phosphorus. Addition of dithiothreitol rapidly quenches the reaction between reagent and pyruvate kinase. When dithiothreitol was added to the enzyme solution before addition of reagent to prevent inactivation, no reagent incorporation was observed; these results indicate that free reagent is removed by the column centrifugation procedure. All measured incorporation must be due to covalently bound affinity label.

The time-dependent incorporation of reagent into pyruvate kinase is shown in Figure 5. The incorporation of 8-BDB-TADP (Figure 5, line a) is higher at all times compared to that of 8-BDB-TATP (Figure 5, line b). At 40 min, 1.9 mol of 8-BDB-TADP/mol of subunit is incorporated while only 1.2 mol of 8-BDB-TATP/mol of subunit is incorporated. In addition, a lower incorporation of 8-BDB-TATP produces more inactivation. Enzyme incubated with 8-BDB-TADP is 34% active at 40 min, while enzyme incubated with 8-BDB-TATP is 9% active. At 80 min, incorporation of 8-BDB-TADP is 2.24 mol/mol of enzyme subunit, with 24% activity remaining; in contrast, 2.05 mol of 8-BDB-TATP/mol of subunit is incorporated when pyruvate kinase has 3% activity remaining.

Effect of Ligands on the Inactivation of Pyruvate Kinase by 8-BDB-TADP and 8-BDB-TATP. The effect of natural ligands on the reaction of 8-BDB-TADP and 8-BDB-TATP with pyruvate kinase was investigated. The concentrations of the added ligands exceeded their respective dissociation constants for the enzyme–ligand complex severalfold (Kapetanovic et al., 1985). Protection against inactivation by two combinations of substrates is illustrated in Figure 6.

Both phosphoenolpyruvate (PEP) + KCl or a combination of PEP, KCl, ATP, and Mn^{2+} markedly decrease the rate of inactivation by 8-BDB-TADP and 8-BDB-TATP. When pyruvate kinase is incubated with 175 μM 8-BDB-TADP in the presence of 5 mM PEP + 100 mM KCl, the enzyme retains 78% activity (Figure 6A, line b), compared to 24% in the absence of ligands (Figure 6A, line a). The best protection is afforded by a combination of PEP, KCl, ATP, and Mn^{2+}, resulting in 90% residual activity (Figure 6A, line c).

In the absence of protecting ligands, pyruvate kinase is almost completely inactivated by 175 μM 8-BDB-TATP after 80 min (Figure 6B, line a). In the presence of PEP + KCl, 56% of the original activity is retained (Figure 6B, line b). As in the case of 8-BDB-TADP, the best protection is seen with a combination of substrates. When PEP + KCl + ATP + Mn^{2+} are included in the incubation mixture, the enzyme

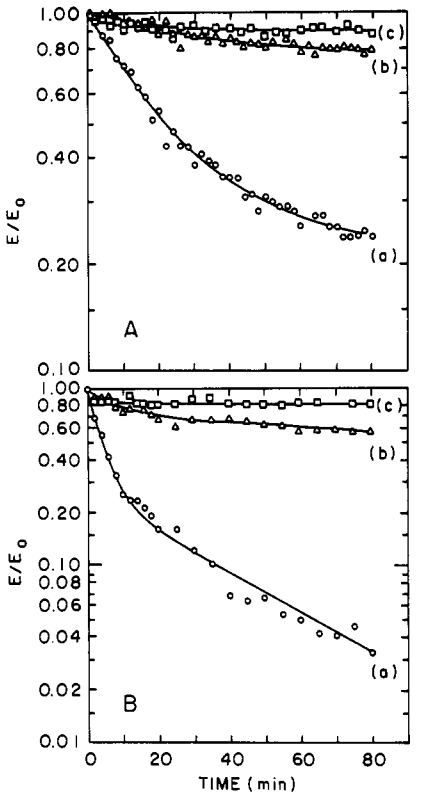

FIGURE 6: Effect of ligands on the inactivation of pyruvate kinase by (A) 8-BDB-TADP and (B) 8-BDB-TATP. Pyruvate kinase was incubated with 175 μM reagent (a) in the absence of protecting ligands (○), (b) in the presence of 5 mM PEP and 100 mM KCl (△), and (c) in the presence of 5 mM PEP, 100 mM KCl, and 2 mM $MnSO_4$ (□). At the indicated times, aliquots were withdrawn and assayed as described under Experimental Procedures.

retains 80% of its original activity after 80 min.

Effect of Ligands on the Incorporation of 8-BDB-TADP and 8-BDB-TATP by Pyruvate Kinase. The effect of different ligands on the extent of inactivation and the incorporation of reagents by pyruvate kinase was examined. Residual activity and incorporation of 8-BDB-TADP and 8-BDB-TATP were measured after an 80-min reaction under the conditions described in Table I. In the absence of any ligands, 8-BDB-TADP is incorporated to the extent of 2.24 mol/mol of enzyme subunit when the enzyme is 76% inactivated. The presence of Mn^{2+} and either ADP or ATP decreases the reaction rate so that the enzyme is only 45% inactivated, 31% less than in the absence of ligands. A combination of PEP, KCl, and Mn^{2+} gives the best protection against loss of activity, with a reduction in the incorporation of about 1 mol/mol of subunit. Since the enzyme is only 10–12% inactivated, the presence of PEP and the cations must protect a specific binding site that is necessary for catalytic activity.

Protecting ligands have a similar effect on the inactivation of pyruvate kinase by 8-BDB-TATP. The presence of Mn^{2+} and either ADP or ATP decreases the inactivation by 31%, while the incorporation of reagent is decreased by about 0.6 mol/mol of enzyme subunit. This suggests that metal–nucleotide can partially prevent the modification of an essential group. PEP + KCl + Mn^{2+} reduces the incorporation to 1 mol/mol of subunit and the extent of inactivation to 20%. The addition of ATP to PEP + KCl + Mn^{2+} does not augment the protecting effect when pyruvate kinase is incubated with either reagent.

In addition to the catalytic site, pyruvate kinase is known to have a second, weaker nucleotide binding site with no known function (Mildvan & Cohn, 1966; Stuart et al., 1979). Both

Table I: Effect of Ligands on Incorporation of 8-BDB-TADP and 8-BDB-TATP by Pyruvate Kinase[a]

ligands added to incubation mixture	8-BDB-TADP inactivation (%)	8-BDB-TADP incorporation (mol/mol of subunit)	8-BDB-TATP inactivation (%)	8-BDB-TATP incorporation (mol/mol of subunit)
no ligands	76	2.24	97	2.05
1.5 mM ADP + 2 mM $MnSO_4$	45	1.64	66	1.45
1.5 mM ATP + 2 mM $MnSO_4$	45	2.03	67	1.49
5 mM PEP + 100 mM KCl	22	0.99	44	1.07
5 mM PEP, 100 mM KCl, + 2 mM $MnSO_4$	12	1.21	20	1.00
1.5 mM ATP, 2 mM $MnSO_4$, 5 mM PEP, + 100 mM KCl	10	1.16	20	0.97

[a] Pyruvate kinase (0.66 mg/mL) was incubated in 0.05 M HEPES buffer, pH 7.0, at 25 °C with 175 μM reagent in the presence and absence of ligands. At 80 min, enzyme activity was assayed and incorporation of reagent was measured by phosphorus assay, as described under Experimental Procedures. Percent inactivation = $(1 - E/E_0) \times 100$.

sites are occupied when enzyme is exposed to high concentrations of ATP and Mg^{2+} (Rosevear et al., 1987). Pyruvate kinase was incubated with 175 μM 8-BDB-TATP in the presence of 10 mM ATP, 12 mM $MnSO_4$, and 100 mM KCl to see whether a high concentration of ATP could protect against modification of this weaker nucleotide binding site. Incorporation was not decreased under these conditions, indicating that 8-BDB-TATP is not binding at the previously detected noncatalytic site.

Discussion

8-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 5′-diphosphate and the related triphosphate exhibit the characteristics of affinity labels in their reactions with pyruvate kinase. Both compounds react covalently with a limited number of sites on the enzyme, causing substantial loss of activity. The rate constants for the fast phase of inactivation exhibit a nonlinear dependence on reagent concentration, indicating the formation of a reversible enzyme–reagent complex prior to irreversible modification. The natural ligands PEP, KCl, and Mn^{2+} provide good protection against inactivation. The K_I values for the fast phase are similar for the two reagents (about 100 μM), whereas k_{max} for 8-BDB-TATP is about three times greater than that of 8-BDB-TADP (0.286 min^{-1} vs 0.0835 min^{-1}). 8-BDB-TATP is more effective since it inactivates the enzyme rapidly with a relatively low amount of incorporation. Since the reactive group is in the same relative position on each compound, the difference in reactivity must be due to the presence of the γ-phosphoryl group. This suggests that 8-BDB-TADP and 8-BDB-TATP bind somewhat differently to the enzyme.

Although substrates are able to substantially prevent inactivation, about 1 mol of 8-BDB-TADP or 8-BDB-TATP is incorporated under the best protecting conditions. If it is assumed that the groups modified in the absence of ligands include those modified in the presence of ligands, 8-BDB-TADP must react with about three residues, since the incorporation is 2.24 mol/mol of subunit when the enzyme is 76% inactivated. If modification of one residue is responsible for most of the inactivation, an excess incorporation of about 1.5 mol/mol of subunit remains. 8-BDB-TATP reacts more selectively with pyruvate kinase in that 2.05 mol/mol of subunit is incorporated when the enzyme is about 100% inactive. The results of the protection experiments can be explained by a model in which 8-BDB-TATP reacts with one residue, causing inactivation, and a second residue which has little effect on enzyme activity. This model, however, does not account for the biphasic kinetics of inactivation. The biphasic kinetics may be explained by subunit–subunit interaction in which the modification of a critical residue on one subunit indirectly decreases catalytic activity and/or reduces the reaction rate with 8-BDB-TATP of the same residue on other subunits.

Because 8-BDB-TADP was considered to be a nucleotide analogue, the results of the protection experiments were unexpected in that PEP, KCl, and Mn^{2+} are more effective in preventing inactivation than is ADP or ATP. Pyruvate kinase requires a monovalent and a divalent cation for activity, in addition to the nucleotide-bound cation (Mildvan et al., 1976). The enzyme-bound M^{2+} orients the phosphoryl groups of PEP and ADP prior to phosphoryl transfer (Muirhead et al., 1986), and the affinity of the enzyme–Mn^{2+} complex for PEP is raised by K^+. Nowak and Mildvan (1972) have suggested that K^+ coordinates PEP at the carboxyl group. Covalent reaction of an affinity label at the binding site of Mn^{2+}, K^+, and/or PEP would be expected to cause enzyme inactivation.

It is possible that 8-BDB-TADP and 8-BDB-TATP are not acting as analogues of nucleotides but rather as PEP analogues. The bromodioxobutyl moiety probably exists partly as an enolate in solution (Kapetanovic et al., 1985); this form bears a resemblance to PEP. Thus the bromodioxobutyl group may direct the binding of 8-BDB-TADP and 8-BDB-TATP to the PEP site, followed by covalent reaction at an amino acid in the vicinity. This postulate would account for the marked protection against inactivation afforded by PEP.

Alternatively, 8-BDB-TADP and 8-BDB-TATP may react at the nucleotide binding site. Mg^{2+} ATP appears to bind to the active site of pyruvate kinase in multiple adenine–ribose conformations (Rosevear et al., 1987), and the purine binding site has been described as a "large hydrophobic hole", explaining the lack of specificity of pyruvate kinase for nucleotide substrates (Muirhead et al., 1986; Plowman & Krall, 1965). Since 8-TADP is a poor substrate, the compound may be binding to the enzyme somewhat differently from ADP or ATP, and perhaps 8-BDB-TADP and ADP can bind simultaneously. Substitution at the 8-position of the adenine ring probably constrains the molecule to the syn conformation (Dudyez et al., 1979), while substitution at the 2-position may favor the anti adenine–ribose conformation (Sheit, 1980). 2-ThioADP derivatives are acceptable substrates for pyruvate kinase. For example, 2-thioADP is quantitatively converted to 2-thioATP when incubated overnight with enzyme, PEP, Mg^{2+}, and KCl (Bailey, 1987), whereas little 8-thioATP is detected upon overnight incubation of enzyme with 8-thioADP under similar conditions. Assay solution containing 0.6 mM 2-thio-1,N^6-ethenoadenosine 5′-diphosphate gives 70% of the enzymatic activity found with 0.6 mM ADP (DeCamp and Colman, unpublished results). These results are consistent with the finding that the anti adenine–ribose conformation is favored when Mg^{2+}ATP is bound by pyruvate kinase (Rosevear et al., 1987).

Two other nucleotide analogues that are related to the ones described here have been synthesized in our laboratory and

tested as affinity labels of pyruvate kinase: 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-monophosphate (6-BDB-TAMP) and 2-[(4-bromo-2,3-dioxobutyl)thio]-adenosine 5′-monophosphate (2-BDB-TAMP). 6-BDB-TAMP inactivates the enzyme at a slow rate with kinetic constants (k_{max} = 0.067 min^{-1}, K_I = 91 μM) similar to those found with 8-BDB-TADP (Colman et al., 1984). 2-BDB-TAMP reacts faster (k_{max} = 0.26 min^{-1}) but has a relatively high K_I of 570 μM (Kapetanovic et al., 1985). The 2- and 6-bromodioxobutyl derivatives show a pattern of substrate protection that differs from the results shown in Table I. In contrast to the 8-derivatives, inactivation of pyruvate kinase by 2-BDB-TAMP and 6-BDB-TAMP is largely prevented by the presence of metal–nucleotide. The inclusion of 10 mM ADP and 20 mM Mg^{2+} decreases the rate of inactivation by 6-BDB-TAMP to 5% of its original value (Colman et al., 1984), while 1.5 mM ATP and 2 mM $MnCl_2$ totally protect against inactivation by 2-BDB-TAMP (Kapetanovic et al., 1985). These results suggest that the placement of the reactive group at different positions on the adenine ring allows the affinity label to react in different regions of the active site.

Preliminary experiments suggest that the nonessential residue modified by 8-BDB-TADP and 8-BDB-TATP may be a cysteine. The number of free cysteines per subunit [determined by reaction with 5,5′-dithiobis(2-nitrobenzoate)] appears to decrease after enzyme modification in the absence or presence of 5 mM PEP, 2 mM Mn^{2+}, and 100 mM KCl. Pyruvate kinase has previously been modified by the nucleotide analogues 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (FSBA) and 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine (FSBϵA). FSBA catalyzes the formation of two cystine bridges, which partially inactivates the enzyme, in addition to reacting with tyrosine and lysine residues which have been identified (Annamalai & Colman, 1981; DeCamp & Colman, 1986). FSBϵA catalyzes the formation of one cystine bridge and also reacts with histidine (Tomich & Colman, 1985). The bromodioxobutyl group reacts readily with free cysteine, although the product would probably not undergo the displacement reaction postulated for FSBA which leads to disulfide formation. 6-[(4-Bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate has been shown to react with cysteine-319 in bovine liver glutamate dehydrogenase (Batra & Colman, 1986). The identity of the essential residue attacked by 8-BDB-TATP is currently under investigation.

The new nucleotide analogues 8-BDB-TADP and 8-BDB-TATP exhibit desirable characteristics as affinity labels. Placement of the reactive group adjacent to the 8-position of the adenine ring allows the preservation of the negatively charged phosphoryl groups and the 6-amino group, which may be important for nucleotide binding in some enzymes. The bromoketo moiety is capable of reacting with several amino acids, including cysteine (Batra & Colman, 1986), glutamate (Bailey & Colman, 1987), aspartate, lysine, and histidine (Hartman, 1977). 8-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 5′-diphosphate and 5′-triphosphate are complementary to existing nucleotide analogues and should have general applicability as affinity labels of adenine nucleotide binding enzymes.

Registry No. I, 58-64-0; II, 23600-16-0; III, 59924-55-9; IV, 115678-78-9; ATP, 56-65-5; 8-TATP, 41106-66-5; 8-BDB-TATP, 115678-79-0; pyruvate kinase, 9001-59-6; 1,4-dibromobutanedione, 6305-43-7.

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Recombinant Rat Liver Guanidinoacetate Methyltransferase: Reactivity and Function of Sulfhydryl Groups

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ABSTRACT: Rat liver guanidinoacetate methyltransferase, produced in *Escherichia coli* by recombinant DNA technique, possesses five cysteine residues per molecule. No disulfide bond is present. Analysis of the chymotryptic peptides derived from the iodo[^{14}C]acetate-modified enzyme shows that Cys-90, Cys-15, Cys-219, and Cys-207 are alkylated by the reagent in order of decreasing reactivity. Incubation of the enzyme with excess 5,5′-dithiobis(2-nitrobenzoate) (DTNB) in the absence and presence of cystamine [2,2′-dithiobis(ethylamine)] causes the appearance of 4 and 5 mol of 2-nitro-5-mercaptobenzoate/mol of enzyme, respectively. Reaction of the methyltransferase with an equimolar amount of DTNB results in an almost quantitative disulfide cross-linking of Cys-15 and Cys-90 with loss of a large portion of the activity. The methyltransferase is completely inactivated by iodoacetate following nonlinear kinetics. Comparison of the extent of inactivation with that of modification of cysteine residues and the experiment with the enzyme whose Cys-15 and Cys-90 are cross-linked suggest that alkylation of Cys-15 and Cys-90 results in a partially active enzyme and that carboxymethylation of Cys-219 completely eliminates enzyme activity. The inactivation of guanidinoacetate methyltransferase by iodoacetate or DTNB is not protected by substrates. Furthermore, disulfide cross-linking of Cys-15 and Cys-90 or carboxymethylation of Cys-219 does not impair the enzyme's capacity to bind *S*-adenosylmethionine. Thus, these cysteine residues appear to occur outside the active-site region, but their integrity is crucial for the expression of enzyme activity.

Guanidinoacetate methyltransferase (*S*-adenosyl-L-methionine:guanidinoacetate *N*-methyltransferase, EC 2.1.1.2), which catalyzes the transfer of the methyl group of *S*-adenosylmethionine (AdoMet)[1] to guanidinoacetate to form creatine, was purified to homogeneity from the livers of pig (Im et al., 1979) and rat (Ogawa et al., 1983). The enzymes from both sources are monomeric proteins with a relatively small molecular size. A cDNA clone encoding rat liver guanidinoacetate methyltransferase has recently been obtained, and the complete amino acid sequence of the enzyme is deduced from its nucleotide sequence. Also, by introducing the cDNA into plasmid pUC118, a recombinant plasmid that expresses guanidinoacetate methyltransferase in *Escherichia coli* has been constructed. The recombinant enzyme, which occupies about 5% of bacterial soluble proteins, exhibits kinetic properties indistinguishable from those of the rat liver enzyme and appears to be structurally identical with the liver enzyme except for the absence of the N-terminal blocking group (Ogawa et al., 1988).

It was reported earlier that guanidinoacetate methyltransferase from pig liver was progressively inactivated during purification and that this inactivation was reversed by a thiol (Cantoni & Vignos, 1954). A more recent investigation in this laboratory has shown that the rat liver enzyme also undergoes this type of inactivation and possesses multiple SH groups, the integrity of which appears crucial for activity (Ogawa et al., 1983). However, detailed studies of their reactivity and functional role have been hampered by limited supply of the enzyme because of its relatively low abundance in the liver and the difficulty of purifying it in good yield. As sufficient quantities of guanidinoacetate methyltransferase amenable to mechanistic study are now available by the recombinant DNA procedure, we extended the study on the function and reactivity of SH groups in guanidinoacetate methyltransferase. The present paper reports the results obtained by using several disulfides and iodoacetate as sulfhydryl modification reagents.

Experimental Procedures

Materials. Guanidinoacetate methyltransferase used in the present study was produced in *E. coli* MV1304 transformed with plasmid pUCGAT9-1, which contained the cDNA for rat liver guanidinoacetate methyltransferase linked to the *lac* promoter (Ogawa et al., 1988). The enzyme was purified by ammonium sulfate fractionation, Sephadex G-100 gel filtration, and DEAE-cellulose chromatography as described previously (Ogawa et al., 1988). The purified enzyme gave a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Prior to experiments, the enzyme was exhaustively dialyzed against 20 mM potassium phosphate, pH 7.2/1 mM EDTA to remove the dithiothreitol added during purification. Molar concentrations of the enzyme were calculated on the basis of M_r = 26 200 (Ogawa et al., 1983, 1988). Protein concentration was determined by the method of Lowry

[1] Abbreviations: AdoMet, *S*-adenosyl-L-methionine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); 2-PDS, 2,2′-dithiodipyridine; 4-PDS, 4,4′-dithiodipyridine; TNB, 2-nitro-5-mercaptobenzoic acid; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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