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Affinity Labeling of $NADP^+$-Specific Isocitrate Dehydrogenase by a New Fluorescent Nucleotide Analogue, 2-[(4-Bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-Bisphosphate†

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ABSTRACT: A new reactive fluorescent adenine nucleotide analogue has been synthesized and characterized: 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate (BDB-TεADP). This compound reacts irreversibly with $NADP^+$-specific isocitrate dehydrogenase. Biphasic kinetics of inactivation are observed that can be described in terms of a fast initial phase of inactivation resulting in partially active enzyme of 8–10% residual activity, followed by a slower phase leading to total inactivation. NADPH protects completely against the fast phase of the reaction, indicating that modification occurs at the coenzyme binding site, whereas isocitrate with $MnSO_4$ protects totally against the slow phase of reaction. The inactivation rate constants for both phases exhibit nonlinear dependence on BDB-TεADP concentration, consistent with the formation of a reversible complex with the enzyme prior to irreversible modification. Covalent incorporation of BDB-TεADP is limited and specific; only 0.99 mol of reagent/mol of subunit is incorporated when the enzyme is 98% inactivated in the absence of ligands. A maximum incorporation of 0.5 mol of reagent/mol of subunit is obtained in the presence of isocitrate and $MnSO_4$, while incorporation in the presence of NADPH equals the difference between the incorporation in the absence of ligands and that measured in the presence of isocitrate and $MnSO_4$. It appears that 0.5 mol of reagent/mol of subunit is responsible for the fast phase of inactivation and the remaining incorporation causes the slow phase. Under all conditions used in this study, isocitrate dehydrogenase has been shown to exist as a dimer by analytical ultracentrifugation and by cross-linking with dimethyl suberimidate followed by analysis on polyacrylamide gels in the presence of sodium dodecyl sulfate. It is proposed that, in the fast phase of inactivation, 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate reacts at the coenzyme binding site of one subunit of dimeric isocitrate dehydrogenase, causing complete inactivation of the modified subunit and substantial inactivation of the other subunit. This new reagent is likely to have general applicability as an affinity label for other $NADP^+$ binding enzymes.

Chemical modification studies have previously been performed on pig heart $NADP^+$-specific isocitrate dehydrogenase [threo-D_s-isocitrate:$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.42] using a variety of group-specific reagents. Cysteinyl, glutamyl, arginyl, lysyl, methionyl, and histidyl residues have all been implicated as essential groups [see Colman (1983a) for a recent review]. Of these, only a histidyl residue has clearly been shown to be in the coenzyme binding site (Ehrlich & Colman, 1978). Affinity labeling using purine nucleotide analogues that have reactive functional groups has the potential to yield more specific chemical modification than is usually achieved with group-specific reagents (Colman, 1983b). The presence of a 2′-phosphate has been demonstrated to be essential for binding of nucleotides to $NADP^+$-specific isocitrate dehydrogenase (Ehrlich & Colman, 1978; Mas & Colman, 1984), so it is critical that any potential affinity label for this enzyme has such a 2′-phosphate. Few purine nucleotide affinity labels with a 2′-phosphate have been described. We here report the synthesis of such an analogue: 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate (BDB-TεADP)[1] (shown in Figure 1 as structure VI). This new reagent has many characteristics desirable for a purine nucleotide affinity label including solubility in water, negative charge at neutral pH, reasonable stability in the pH range generally optimal for enzymes, and high reactivity of the bromodioxobutyl moiety enabling potential covalent reaction with a variety of amino acids. The fluorescent properties of this new reagent offer a convenient means of introducing a fluorescent probe into the nucleotide binding site of an enzyme. Because of the location of the functional group adjacent

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[1] Abbreviations: BDB-TεADP, 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate; PADPR, 2′-phosphoadenosine 5′-diphosphoribose; εPADPR, 2′-phospho-1,N^6-ethenoadenosine 5′-diphosphoribose; TεADP, 2-thio-1,N^6-ethenoadenosine 2′,5′-bisphosphate; MES, 2-(*N*-morpholino)ethanesulfonic acid; SDS, sodium dodecyl sulfate; NMR, nuclear magnetic resonance; Me_2SO, dimethyl sulfoxide.

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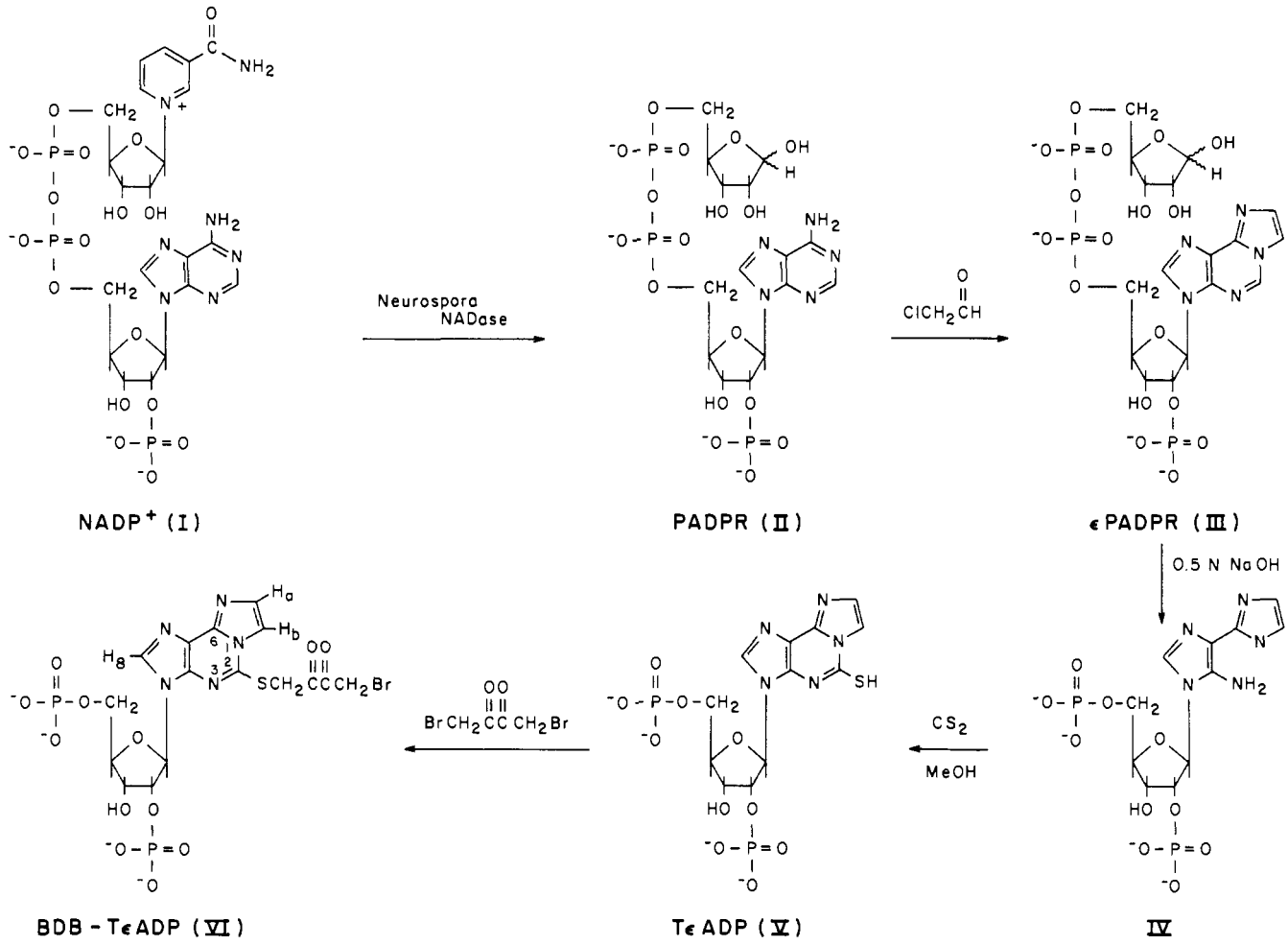

FIGURE 1: Synthetic scheme for preparation of 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate.

to the 2-position, the compound might be expected to react with amino acid residues in the purine region of the nucleotide binding sites of proteins. The analogous *S*-(4-bromo-2,3-dioxobutyl) coenzyme A has previously been prepared and has been found to inactivate several enzymes that bind acetyl-CoA (Owens & Barden, 1978; Clements et al., 1979; Katiyar et al., 1982). Additionally, 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-monophosphate and 5′-diphosphate have been synthesized in this laboratory and found to react covalently at nucleotide binding sites of several enzymes (Colman et al., 1984; Batra & Colman, 1984; Huang & Colman, 1984). In this paper, we present evidence that 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate acts an an affinity label for pig heart $NADP^+$-specific isocitrate dehydrogenase. A preliminary version of this work has been presented (Bailey & Colman, 1984).

Experimental Procedures

Materials and Methods. Porcine heart $NADP^+$-specific isocitrate dehydrogenase was purified as described by Bacon et al. (1981). The specific activity of the enzyme used in this study was 37–42 units/mg. Enzyme concentrations (in mg/mL) were determined from $E^{0.1\%}_{280nm} = 1.08$ (Johanson & Colman, 1981). A subunit molecular weight of 58 000 (Colman et al., 1970) was used to calculate the concentration of enzyme subunits.

Neurospora NADase (crude preparation), Malachite Green base, mercuric thiocyanate, coenzymes, dithiothreitol, buffer salts, DL-isocitrate, and protein molecular weight standards (used in gel electrophoresis) were all obtained from Sigma Chemical Co. Chloroacetaldehyde was purchased as a 50% aqueous solution from Pfaltz & Bauer and was distilled immediately prior to use. 1,4-Dibromobutanedione was obtained from Aldrich Chemical Co. and was recrystallized from petroleum ether before use. Ammonium molybdate was from Mallinckrodt. Ultrapure guanidine hydrochloride was supplied by Schwarz/Mann, and dimethyl suberimidate was obtained from Pierce.

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 with D_2O for both ^1H NMR and ^{13}C NMR spectra. Dioxane (3.71 ppm) was used as an internal standard for ^1H spectra, and triethylamine (46.8 and 8.3 ppm) was used in ^{13}C spectra. H_3PO_4 (85%) was used as an external standard for ^{31}P NMR chemical shifts. Chemical shifts downfield from the H_3PO_4 resonance were given a positive sign.

The determination of the phosphorus content of BDB-TϵADP was performed by a modification of the procedures of Hess & Derr (1975) and Lanzetta et al. (1979), as described by Colman et al. (1984). Free bromide was measured by a procedure modified from that of Zall et al. (1956) and was described in detail by Colman et al. (1984).

Determination of Quantum Yield. Corrected emission spectra were obtained on a Perkin-Elmer MPF-44B spectrofluorometer equipped with a Hitachi 057 recorder by using 1-cm path-length quartz cells at 25 °C. The quantum yield of BDB-TεADP was determined relative to a value of 0.70 for quinine sulfate in 0.1 N H_2SO_4 (Scott et al., 1970) by using the comparative relationship of Parker & Reese (1960)

$$Q_2/Q_1 = (F_2A_1)/(F_1A_2) \quad (1)$$

where Q_2 is the calculated quantum yield, F_2 is the area of the corrected emission spectrum, and A_2 is the absorbance at the exciting wavelength (302 nm) of BDB-TεADP. Q_1, F_1, and A_1 are the corresponding values for the standard, quinine sulfate. The absorbance at 302 nm was measured on a Perkin-Elmer 553 UV/vis spectrophotometer.

Synthesis of 2-[(4-Bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-Bisphosphate. The overall synthetic scheme is shown in Figure 1. Starting with $NADP^+$ (I), PADPR (II) was generated enzymatically and was converted to εPADPR (III) by reaction with chloroacetaldehyde. Treatment with NaOH followed by reaction with carbon disulfide yielded 2-thio-1,N^6-ethenoadenosine 2′,5′-bisphosphate [TεADP (V)]. Condensation of TεADP with 1,4-dibromobutanedione gave the final product BDB-TεADP (VI).

Preparation of 2′-Phosphoadenosine 5′-Diphosphoribose (II). $NADP^+$ (800 mg) was dissolved in 6 mL of 100 mM potassium phosphate buffer, pH 7.6, and the pH was adjusted to 7.5 with 1 N KOH. Three units of crude *Neurospora* NADase, dissolved in 1.5 mL of 100 mM potassium phosphate buffer, pH 7.6 was added to the $NADP^+$ solution, and the reaction mixture was incubated for 20 h at 35 °C. Progress of the reaction was followed by analytical TLC as described above: unreacted $NADP^+$ has an R_f of 0.29 as compared to R_f values of 0.15 and 0.75 for PADPR and nicotinamide, respectively. The reaction mixture was then applied to a 2 × 31 cm column of DE-52 (Whatman) equilibrated with 0.01 M ammonium bicarbonate at room temperature. The column was eluted with a linear gradient (800 mL of 0.01 M ammonium bicarbonate and 800 mL of 0.4 M ammonium bicarbonate). Fractions (2.2 mL) were monitored at 260 nm. Nicotinamide was eluted between fractions 50 and 70, while unreacted $NADP^+$ and PADPR were eluted between fraction 260 and 275 and fraction 290 and 360, respectively. Fractions containing PADPR were pooled and lyophilized. The sample was redissolved in water and lyophilized repeatedly until all the ammonium bicarbonate had been removed. The yield was 85–90%: NMR δ 8.55 (H_8), 8.3 (H_2), and 6.25 (adenosine $H_{1'}$).

Preparation of 2′-Phospho-1,N^6-ethenoadenosine 5′-Diphosphoribose (III). PADPR (700 mg) was reacted with chloroacetaldehyde according to the procedure of Secrist et al. (1972). The reaction mixture was incubated for 24 h at 37 °C and the pH maintained at 4.5 by periodic adjustments with 1 N NaOH. At completion of the reaction, the ultraviolet absorbance at 265 and 275 nm was equal at pH 7.0 (for comparison, A_{275nm}/A_{265nm} for PADPR is 0.45). The product (III) was obtained as a white powder in 90–95% yield. TLC showed two fluorescent spots: a major one at R_f 0.10 (compound III) and a minor one at R_f 0.22; NMR δ 9.45 (H_2), 8.82 (H_8), 8.25 (H_b), 7.90 (H_a), and 6.45 (adenosine $H_{1'}$). Numbering and lettering are in accordance with Figure 1. For comparison, values obtained in Secrist et al. (1972) for ethenoadenosine in the same solvent were δ 9.25 (H_2), 8.58 (H_8), 8.17 (H_b), and 7.82 (H_a).

Preparation of 3-β-D-(2′,5′-Bisphosphoribofuranosyl)-4-amino-5-(imidazol-2-yl)imidazole (IV). εPADPR (600 mg) from the previous step was dissolved in 16 mL of 0.5 N NaOH, according to the method of Yip & Tsou (1973), and maintained at room temperature for 24 h. An UV absorption spectrum with λ_{max} at 276 nm (pH 7.0) indicates completion of the conversion of III to IV. The reaction mixture was reduced to 1 mL under vacuum at room temperature and then neutralized with 1 N acetic acid. The resultant mixture was then applied to a 1 × 21 cm column of Ag50W-X4 (H^+) and eluted with distilled water at 4 °C. Fractions (2 mL) were collected with monitoring of absorbance at 280 nm. A minor peak appearing at fractions 5–20 was discarded. Fractions 25–100 contained the expected product (IV) and were evaporated under vacuum to dryness. A clear glass was obtained in 55% yield. TLC showed a single UV absorbing spot at R_f 0.35: NMR δ 8.45 (H_8), 7.45 (H_a and H_b), and 6.0 ($H_{1'}$). For comparison, Tsou et al. (1974) obtained the values δ 7.55 (H_8), 7.10 (H_a and H_b), and 5.65 ($H_{1'}$) for 3-β-D-ribofuranosyl-4-amino-5-(imidazol-2-yl)imidazole in Me_2SO-d_6 and D_2O.

Preparation of 2-Thio-1,N^6-ethenoadenosine 2′,5′-Bisphosphate (V). TεADP was prepared by condensation of IV with CS_2 following the procedure of Tsou et al. (1974) with minor modifications. To IV was added 15 mL of methanol; triethylamine was added dropwise to form the soluble triethylammonium salt. Pyridine (15 mL) and carbon disulfide (15 mL) were added, and the mixture was refluxed for 3 h. The yellow colored mixture was allowed to cool to room temperature, and solvent was removed under vacuum. The residue was coevaporated under vacuum 3 times with ethanol and water to remove any residual pyridine. The residue was dissolved in water and centrifuged to remove any insoluble material, and the supernatant was evaporated to dryness. TLC showed a single UV-absorbing spot with an R_f of 0.17. The UV absorption spectrum has a λ_{max} of 302 nm in aqueous solution at pH 6.0: yield 90%; NMR δ 8.60 (H_8), 8.35 (H_b), 7.72 (H_a), and 6.45 ($H_{1'}$). For comparison, Tsou et al. (1974) obtained the values δ 8.43 (H_8), 8.40 (H_b), 7.83 (H_a), and 6.04 ($H_{1'}$) for 2-thio-1,N^6-ethenoadenosine in Me_2SO-d_6.

Preparation of 2-[(4-Bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-Bisphosphate (VI). TεADP (V) was dissolved in a mixture of methanol and 1 N acetic acid and evaporated to dryness. Methanol and 1 N acetic acid coevaporation was repeated until the pH of TεADP in methanol was estimated (by pH paper) to be 5.0. 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 a 52 mM TεADP solution in methanol at room temperature. Reaction occurred immediately and could be assessed spectrophotometrically from the decrease in absorbance at 302 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 light tan colored powder. BDB-TεADP was stored dry and desiccated at −80 °C: yield 60% for V to VI.

The overall yield of BDB-TεADP via the synthetic scheme shown in Figure 1 was 24%. The selection of $NADP^+$ (rather than PADPR or 2′,5′-ADP) as a starting material for the synthesis of BDB-TεADP was dictated by the financial feasibility of the synthetic scheme. $NADP^+$ was the least expensive starting material, and since the reactions involving the transformation of I to IV all proceeded in high yield, the coenzyme was the starting nucleotide of choice.

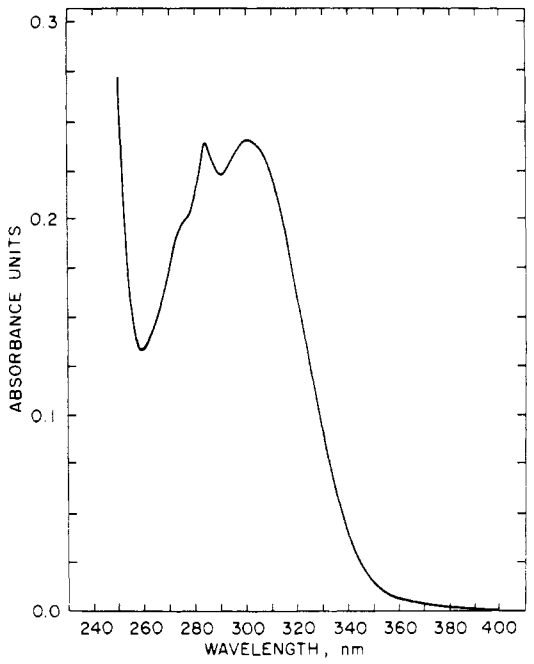

FIGURE 2: Ultraviolet absorption spectrum of 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate in 0.05 M MES buffer, pH 6.0.

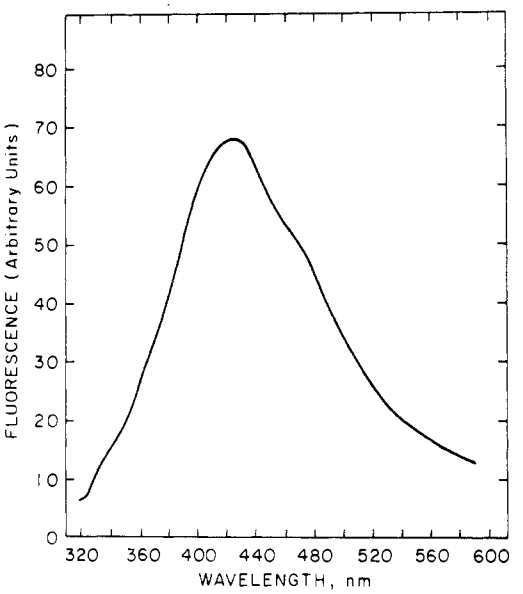

FIGURE 3: Corrected fluorescence emission spectrum of 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate in 0.05 M MES buffer, pH 6.0 (excitation at 302 nm).

Solutions of BDB-TεADP used for the inactivation and incorporation experiments were prepared in 0.03 M MES buffer, pH 5.0, and stored at −20 °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.

Characterization of 2-[(4-Bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-Bisphosphate. The purity of the product BDB-TεADP was assessed by thin-layer chromatography as discussed under Experimental Procedures. A single fluorescent, ultraviolet-absorbing spot was observed with an R_f of 0.20, whereas the precursor, TεADP, and 1,4-dibromobutanedione exhibited nonfluorescent, UV-absorbing spots with R_f values of 0.17 and 0.78, respectively.

The elemental analysis is in agreement with the structure shown in Figure 1 for BDB-TεADP, assuming that the compound was the triethylammonium salt and had cocrystallized with methanol. The molecular weight was calculated as 875.6. Anal. Cacld for $C_{26}H_{49}N_6O_{16}SP_2Br$: C, 35.66; H, 5.64; N, 9.60. Found: C, 36.56; H, 5.76; N, 9.65.

The ultraviolet absorption spectrum of BDB-TεADP is shown in Figure 2. Two peaks of equal intensity are observed at 302 and 284 nm with a shoulder at 275 nm when measured in 0.05 M MES buffer, pH 6.0. This property allows the reaction between TεADP and 1,4-dibromobutanedione to be conveniently followed spectrophotometrically as TεADP exhibits an absorption maximum at 312 nm (from methanolic solution) with an extinction coefficient of 15.2×10^3 M^{-1} cm^{-1} when measured in 0.05 M MES buffer, pH 6.0. Reaction of TεADP with 1,4-dibromotutanedione causes a shift in λ_{max} from 312 (TεADP) to 302 nm (BDB–TεADP) along with a concomitant decrease in the extinction coefficient at 312 nm to 8.1×10^3 M^{-1} cm^{-1}. BDB-TεADP has an extinction coefficient at 302 nm of 8.96×10^3 M^{-1} cm^{-1} when measured in the above buffer.

The corrected fluorescence emission spectrum of BDB-TεADP in 0.05 M MES buffer, pH 6.0, is shown in Figure 3. A fluorescence emission maximum was observed at 428 nm (302-nm excitation), and a maximum at 302 nm was observed in the corrected excitation spectrum. The shape of the emission spectrum was found to be independent of excitation wavelength, thus implying the presence of a single fluorophore. The quantum yield of BDB-TεADP in 0.05 M MES buffer, pH 6.0, was determined to be 0.065 relative to a value of 0.7 for quinine sulfate in 0.1 N H_2SO_4 (Scott et al., 1970). Compound V, TεADP, was found to be nonfluorescent under the same conditions. The fluorescence quantum yield found for BDB-TεADP compares to 0.008 observed for 5-(*p*-fluorosulfonylbenzoyl)-1,N^6-ethenoadenosine (Likos & Colman, 1981) and 0.54 for ethenoadenosine (Secrist et al., 1972).

The ratio of organic phosphorus to spectrophotometrically determined BDB-TεADP was 2.04:1.00. The bromide content was determined after hydrolysis in NaOH, yielding a ratio of hydrolyzable bromide to BDB-TεADP of 0.98:1.00.

The proton NMR spectrum of BDB-TεADP (triethylammonium salt) 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), 3.52–3.61 and 3.82 (m, $-CH_2Br$), 4.13–4.21 (m, $H_{4'}$ and $H_{5'}$ of ribose), 4.44 (m, $H_{3'}$ of ribose), 4.61–4.67 (m, $-CH_2S-$), 5.15 (m, $H_{2'}$ of ribose), 6.45 (d, $H_{1'}$ of ribose), 7.73 (d, H_a), 7.93 (d, H_b), and 8.63 (s, H_8). For comparison 2-thio-N^6-ethenoadenosine 2′,5′-bisphosphate (TεADP) had peaks centered at δ 4.25 (m, $H_{5'}$ of ribose), 4.54 (m, $H_{4'}$ of ribose), 4.76 (m, $H_{3'}$ of ribose), 5.25 (m, $H_{2'}$ of ribose), 6.45 (d, $H_{1'}$ of ribose), 7.72 (d, H_a), 8.35 (d, H_b), and 8.60 (s, H_8). Assignments of the ribose protons were made by comparison with the proton NMR spectrum of 2′-AMP (Davies & Danyluk, 1975). Assignments of the purine ring protons were made by comparison to those of ethenoadenosine (Secrist et al., 1972). 1,4-Dibromobutanedione exhibits a single resonance peak in deuterated chloroform, but in deuterated methanol, multiplets are seen at δ 3.57–3.59 and at δ 4.51 and 4.57 (Colman et al., 1984). It is likely that, in more polar protic solvents, such as methanol or D_2O, 1,4-dibromobutanedione may exist in several enolate forms, each exhibiting different resonances. This same explanation would apply to the bromodioxobutyl moiety of BDB-TεADP. Resonances at 3.52–3.61 and 3.82 were therefore assigned to the $-CH_2Br$ moiety of BDB-TεADP, and the 4.61–4.67 resonance was assigned to the $-SCH_2-$ moiety. The value for $-CH_2Br$ is comparable to the 3.36 resonance observed for the $-CH_2Br$

protons of 2′-(2-bromoethyl)-AMP (Bednar & Colman, 1982). The resonances for $-CH_2Br$ and $-SCH_2-$ in BDB-TεADP 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]-1,N^6-ethenoadenosine 2′,5′-bisphosphate had signals arising from triethylamine centered at δ 8.3 ($-CH_3$) and 46.8 ($-CH_2N$), methanol at δ 49.0, the reactive side chain at δ 37.5 ($-CH_2Br$ and $-CH_2S-$), the ribose ring at δ 64.4 ($C_{5'}$), 70.1 ($C_{3'}$), 77.2 ($C_{2'}$), 83.6 ($C_{4'}$), and 86.5 ($C_{1'}$), and those from the purine ring at δ 112.8 (2), 116.5, 123.4, 137.8, 141.9, and 148.0. For comparison, 2-thio-1,N^6-ethenoadenosine 2′,5′-bisphosphate had signals arising from the ribose at δ 64.64 ($C_{5'}$), 70.53 ($C_{3'}$), 77.11 ($C_{2'}$), 84.06 ($C_{4'}$), and 85.98 ($C_{1'}$), and those from the purine ring at δ 113.86, 116.26, 120.54, 137.24, 139.76, 144.56, and 163.67. Resonance peak assignments were assigned primarily by reference to Schleich et al. (1975). The $-CH_2Br$ of 2′-(2-bromoethyl)-AMP exhibits a resonance at δ 31.7 (Bednar & Colman, 1982).

The proton-decoupled ^{31}P NMR spectrum of 2-thio-1,N^6-ethenoadenosine 2′,5′-bisphosphate (V) was performed at 25 °C in a 2-mL sample volume, pH 6.5, containing 20% D_2O as an internal field frequency lock and 0.1 mM ethylenediaminetetraacetic acid to eliminate any line broadening due to paramagnetic impurities. Two resonances of equal intensity were observed at 2.55 and 2.77 ppm, consistent with expectations for a 2′- and 5′-phosphate at this pH (Mas & Colman, 1984). Had the pyrophosphate linkage not been cleaved in compound V, a resonance from the pyrophosphate moiety would have been expected at approximately −10 ppm (Mas & Colman, 1984); no such signal was observed. Thus, all of the spectral and analytical data are consistent with the structure of 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate as shown in Figure 1 (VI).

Enzyme Assays. Enzyme assays were performed at 25 °C by using the rate of appearance of NADPH from the absorbance at 340 nm on a Gilford Model 240 spectrophotometer with the scale set to 0.1 *A* units full scale (Colman, 1968). The initial rate measurements were performed in a 1-mL solution containing 30 mM triethanolamine hydrochloride buffer, pH 7.4, 0.1 mM $NADP^+$, 4 mM DL-isocitrate, and 2 mM $MnSO_4$.

Reaction of BDB-TεADP with Isocitrate Dehydrogenase. Isocitrate dehydrogenase (0.12 mg/mL) was incubated with varying concentrations of BDB-TεADP at 25 °C in 0.10 M triethanolamine hydrochloride buffer, pH 7.0, in a total volume of 1 mL. $NADP^+$, NADPH, and NAD^+ were included, as indicated. Control samples were incubated under the same conditions except for the absence of BDB-TεADP. At timed intervals, aliquots of the reaction mixture were assayed for residual activity as described above. The rate of reaction of isocitrate dehydrogenase with BDB-TεADP 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 reactions exhibiting biphasic kinetics were calculated from the equation

$$E/E_0 = (1 - F)e^{-k_{fast}t} + (F)e^{-k_{slow}t} \quad (2)$$

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 2. In all cases, the iteration process was continued until Student's t = 3.0.

Incorporation of BDB-TεADP into Isocitrate Dehydrogenase. To determine the stoichiometry of the reaction, isocitrate dehydrogenase (0.3 mg/mL) was incubated with 75 μM BDB-TεADP under the same conditions described above. At various times a 0.5-mL aliquot of the reaction mixture was withdrawn, and 20 μL of a 5 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 procedure of Penefsky (1977) using two consecutive Sephadex G-50-80 columns (5 mL) equilibrated with 50 mM MES buffer, pH 6.0, 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). Standard protein solutions were prepared with unmodified isocitrate dehydrogenase in the same buffer.

Incorporation of BDB-TεADP into isocitrate dehydrogenase was measured from the fluorescence intensity of modified enzyme on a Perkin-Elmer Hitachi MPF-3 spectrofluorometer with a thermostated cell. Fluorescence intensity was measured at 420 nm with an excitation wavelength of 302 nm. Unmodified isocitrate dehydrogenase at the same protein concentration and in the same buffer as the samples was used as a blank. The fluorescence of modified enzyme was corrected for its blank and compared to the fluorescence of BDB-TεADP standards measured under identical conditions (i.e., in 50 mM MES buffer, pH 6.0, containing 5 M guanidine hydrochloride).

Calculation of the Rate of Decomposition of BDB-TεADP. Calculation of the rate constant for loss of bromide from BDB-TεADP. Calculation of the rate constant for loss of bromide from BDB-TεADP made use of a convenient spectal change that occurs concomitant with hydrolysis of the reagent. The extinction coefficient of BDB-TεADP at 302 nm, as measured at pH 7.0, is 9.0×10^3 M^{-1} cm^{-1}; however, loss of bromide causes an increase in ϵ_{302} to 11.9×10^3 M^{-1} cm^{-1}. Thus, the rate of decomposition can be followed by monitoring the change in A_{302} nm as a function of time. The absorption spectrum of a 1-mL solution of BDB-TεADP (31 μM) at 25 °C in 0.10 M triethanolamine hydrochloride buffer, pH 7.0, in a sealed quartz cuvette, was monitored for 30 h at timed intervals on a Perkin-Elmer 553 spectrophotometer. The rate constant for loss of bromide from BDB-TεADP was calculated from a semilogarithimic plot of $(A_\infty - A_t)/(A_\infty - A_0)$ vs. time, where A_∞ and A_0 are the final and initial absorbances, respectively, at 302 nm, and A_t is the absorbance at the same wavelength at various times.

Molecular Weight Determination by Sedimentation Equilibrium. Sedimentation equilibrium measurements were made at 26000 and 34000 rpm in a Beckman Model E analytical ultracentrifuge with interference optics. The molecular weight was determined by the meniscus depletion method of Yphantis (1964). A six-channel cell was used to centrifuge simultaneously three extensively dialyzed protein/buffer pairs. The identify of fringe displacements on photographs taken at 18 and 24 h indicated that equilibrium had been achieved during this time period at 25 °C. After equilibrium had been achieved, the speed of the centrifuge was changed, and photographs were again taken until equilibrium was reestablished.

Measurements of the photographic plates were made on a Nikon comparator. The measurements of fringe displacements were made on five fringes, and an average value was used in

all calculations. The slopes of ln C vs. r^2 (C is measured by fringe displacement and r is the radial distance in the rotor) were obtained from least-squares analyses of the data. A partial specific volume, $\bar{v}$, of 0.737 was calculated from the amino acid composition (Johanson & Colman, 1981) and partial specific volumes for the individual amino acids given by Lee & Timasheff (1979).

Cross-Linking of Isocitrate Dehydrogenase and Electrophoresis on SDS Gels. Isocitrate dehydrogenase (0.3 mg/mL) was labeled with 75 μM BDB-TϵADP as described above. At various times the reaction was quenched by addition of dithiothreitol to 2 mM, and the pH of the reaction mixture was adjusted to 8.0 by the addition of a small volume of triethanolamine. By use of 5 μL of a concentrated solution of dimethyl suberimidate in 0.1 M triethanolamine hydrochloride buffer, pH 8.5, the modified enzyme solution was made 20 mM in dimethyl suberimidate. The final pH of the reaction mixture was 7.8. The reaction mixture was incubated at 25 °C for 2 h. Native enzyme, not labeled with BDB-TϵADP, was cross-linked with dimethyl suberimidate following the same procedure.

Gel electrophoresis was conducted in the presence of sodium dodecyl sulfate on polyacrylamide gels (7.5%) by the method of Weber & Osborn (1969). Both cross-linked and uncross-linked isocitrate dehydrogenase was incubated at 40 °C for 2 h in 2% SDS before application to tube gels (7.5 cm). After being stained and destained the gels were scanned at 630 nm by using a Gilford 240 spectrophotometer equipped with a Model 2410S linear transport attachment.

Results

Inactivation of Isocitrate Dehydrogenase by BDB-TϵADP. Incubation of isocitrate dehydrogenase with 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate resulted in a time-dependent inactivation of the enzyme as shown in Figure 4. In contrast, no change in activity was observed over this time period in control enzyme, in the absence of BDB-TϵADP. Biphasic inactivation kinetics were observed that could be described in terms of a fast initial phase of inactivation resulting in partially active enzyme of 8–10% residual activity followed by a slower phase leading to total inactivation. The solid line in Figure 4 is a theoretical line based on a computer fit of eq 2 as described under Experimental Procedures, while the points are experimental.

One possible explanation for the observed biphasic kinetics might be reagent decomposition. Since the bromodioxobutyl group of 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate is highly reactive and the related compound 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate has been observed to undergo loss of bromide in aqueous buffers (Batra & Colman, 1984), it was of interest to determine the rate of loss of bromide from BDB-TϵADP under the conditions used for the modification of isocitrate dehydrogenase. By use of a convenient spectral change, described in detail under Experimental Procedures, a half-life of 138 min was found for the decomposition rate of BDB-TϵADP. Since the second phase of inactivation predominates by 20 min (Figure 4), the relatively slow reagent decomposition can be excluded as an explanation for the biphasic kinetics. The reagent decomposition will, however, cause a slight underestimation of the rate constant of the slow phase of inactivation at the lower reagent concentrations.

Another possible explanation for the observed biphasic kinetics might be that the bromoketo and dioxo moieties of BDB-TϵADP, both possible sites of reaction with enzymes, are reacting at different rates with isocitrate dehydrogenase. This possibility was ruled out in a separate experiment in which BDB-TϵADP (75 μM) was incubated at pH 7.0 for 25 h (to hydrolyze the Br^-) prior to addition of enzyme. The rate constant of inactivation was only 0.0016 min^{-1}, 0.40% and 15% of k_{fast} and k_{slow}, respectively, as observed with a freshly prepared solution of 75 μM BDB-TϵADP. Thus, the bromoketo group, rather than the dioxo group, is primarily responsible for both phases of inactivation of isocitrate dehydrogenase.

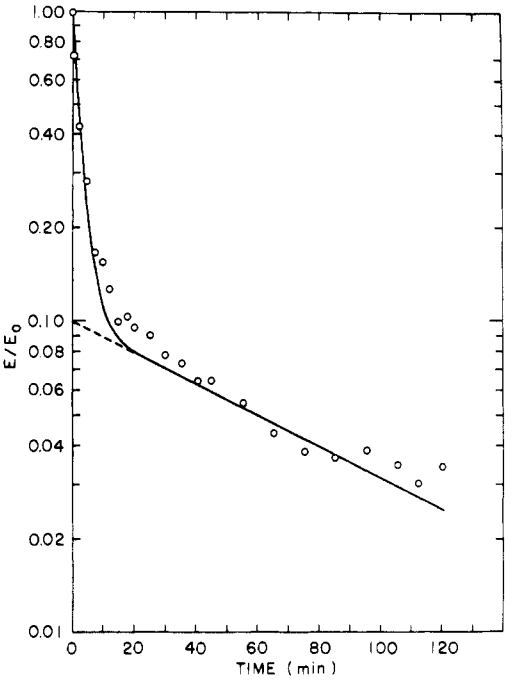

FIGURE 4: Reaction of 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate with $NADP^+$-specific isocitrate dehydrogenase. Isocitrate dehydrogenase (0.12 mg/mL) was incubated with 100 μM BDB-TϵADP at 25 °C in 0.10 M triethanolamine hydrochloride buffer, pH 7.0, as described under Experimental Procedures. At the indicated times, aliquots were withdrawn and assayed as described under Experimental Procedures. The open circles are experimental data, while the solid line is a theoretical line, generated from a computer fit to eq 2 by using values of $F = 0.10$, $k_{fast} = 0.37$ min^{-1}, and $k_{slow} = 0.0115$ min^{-1}.

In order to further characterize the reaction of BDB-TϵADP with isocitrate dehydrogenase, the dependence of the rates of inactivation on the concentration of BDB-TϵADP was determined. At all concentrations of BDB-TϵADP tested (15–400 μM), biphasic kinetics were observed, and the rate constants could be calculated by computer fit to eq 2 with a constant ordinate intercept, F, of $E/E_0 = 0.10$. A constant value of F, independent of the concentration of BDB-TϵADP, eliminates enzyme-catalyzed decomposition of reagent as an explanation for the observed biphasic kinetics. For both phases, a nonlinear dependence of the rate constant of inactivation on the reagent concentration was observed. These data indicate the initial formation of a reversible enzyme–reagent complex prior to irreversible modification, as is expected for an affinity label. The observed rate constant (k_{obsd}) at a particular concentration of BDB-TϵADP (I) is described by the equation

$$\frac{1}{k_{obsd}} = \frac{K_I}{k_{max}[I]} + \frac{1}{k_{max}} \quad (3)$$

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). From the double-reciprocal plots shown in Figure 5, values of $K_I = 56$ μM and $k_{max} = 0.55$ min^{-1} were

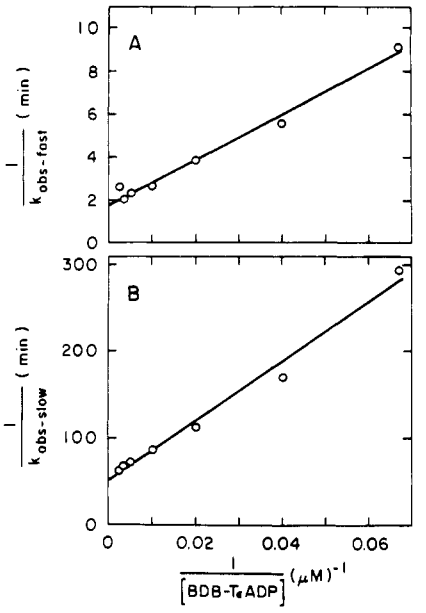

FIGURE 5: Dependence of the pseudo-first-order rate constant (k_{obsd}) for inactivation of NADP$^+$-specific isocitrate dehydrogenase on the concentration of BDB-TϵADP. Isocitrate dehydrogenase was incubated with BDB-TϵADP (15–400 μM) under the conditions described in Figure 4. Rate constants were determined by computer fit to eq 2 by using a value of $F = 0.10$ as described under Experimental Procedures. (A) Fast phase; (B) slow phase of inactivation. The graphs are the double-reciprocal plots used to calculate k_{max} and K_I for both the fast and slow phases, according to eq 3.

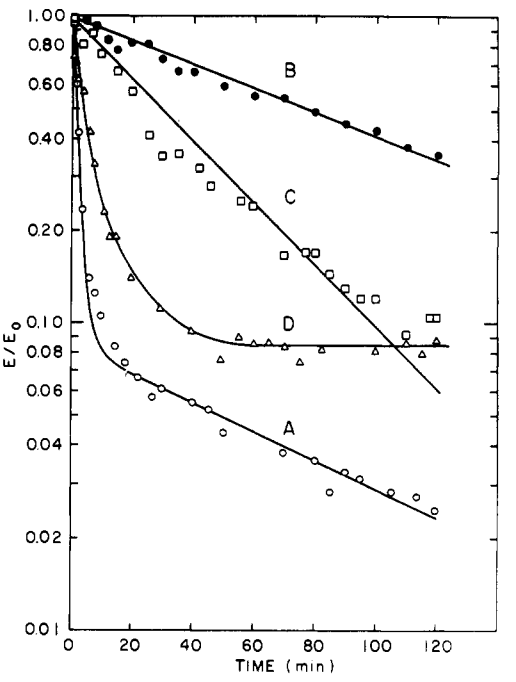

FIGURE 6: Effect of ligands on the inactivation of isocitrate dehydrogenase by BDB-TϵADP. Isocitrate dehydrogenase (0.12 mg/mL) was incubated at 25 °C in 0.10 M triethanolamine hydrochloride buffer, pH 7.0, containing 75 μM BDB-TϵADP in the absence of protecting ligands (line A) (○) and in the presence of 200 μM NADPH (line B) (●), 600 μM NADP$^+$ (line C) (□), or 4 mM DL-isocitrate and 2 mM $MnSO_4$ (line D) (△). At the indicated times aliquots were withdrawn and assayed as described under Experimental Procedures. Rate constants $k_{fast} = 0.41$ min^{-1} and $k_{slow} = 0.0106$ min^{-1} in the absence of ligands (line A) were calculated as described in the text by using a value of $F = 0.085$. Rate constants in the presence of 200 μM NADPH (●) and 600 μM NADP$^+$ (□) were calculated to be 0.0095 min^{-1} and 0.023 min^{-1}, respectively, by assuming a single phase leading to totally inactive enzyme. The rate constant of inactivation in the presence of isocitrate and $MnSO_4$ (△) was calculated as described in the text and found to be 0.14 min^{-1}.

calculated for the fast phase, with values of $K_I = 58$ μM and $k_{max} = 0.019$ min^{-1} for the slow phase.

Effect of Ligands on the Inactivation of Isocitrate Dehydrogenase by BDB-TϵADP. NADP$^+$, NADPH, and isocitrate–Mn^{2+} were tested for their abilities to protect against inactivation by BDB-TϵADP (Figure 6) by including them in the incubation mixture at concentrations much higher than their known binding constants (Ehrlich & Colman, 1978; Mas & Colman, 1985). Line A (Figure 6) shows the biphasic inactivation of isocitrate dehydrogenase in the absence of ligands. In the presence of 200 μM NADPH (Figure 6, line B), complete protection is observed against the fast phase, while the slow phase appears to be unaffected. A pseudo-first-order rate constant for inactivation of 0.0095 min^{-1} was calculated in the presence of NADPH, which is identical with that observed for the slow phase, in the absence of ligands (Figure 6, line A). Although NADP$^+$ (Figure 6, line C) does not provide as much protection against inactivation by BDB-TϵADP as does NADPH, it causes a drastic decrease in the rate of inactivation as compared to line A (Figure 6). In the presence of 4 mM isocitrate and 2 mM $MnSO_4$ (line D), complete protection against the slow phase is observed, with the rate of inactivation leveling off at 8.5% residual activity which is the same as the value observed in this experiment for the partially active enzyme ($F = 0.085$) in the absence of ligands (Figure 6, line A). The rate constant in the presence of isocitrate and Mn^{2+} was calculated from a semilogarithmic plot of $[(E/E_0)_t - F]/(1 - F)$ as a function of time, where F represents the fractional residual activity of the partially active enzyme (0.085). A rate constant of 0.14 min^{-1} was calculated, which is 35% of that observed for k_{fast} in the absence of ligands (Figure 6, line A).

To test for specificity among the ligands that protect against inactivation by BDB-TϵADP, NAD$^+$ was evaluated for its effect on the reaction. The addition of 600 μM NAD$^+$ to the reaction mixture had no effect on the rate of inactivation (data not shown), which is consistent with the evidence that NAD$^+$ is neither a coenzyme nor an effective inhibitor for this NADP$^+$-dependent isocitrate dehydrogenase (Ehrlich & Colman, 1978). Only nucleotides with a 2′-phosphate appear to bind to the site modified by BDB-TϵADP.

Stoichiometry of Reaction of Isocitrate Dehydrogenase with BDB-TϵADP. The incorporation of BDB-TϵADP per mole of subunit, in the absence and presence of ligands, was determined by comparison of the fluorescence of the modified enzyme to that of BDB-TϵADP standards. The enzyme was denatured with 5 M guanidine hydrochloride prior to fluorescence measurements to minimize any protein environmental effects on the fluorescence of covalently linked BDB-TϵADP. The assumption was made that the fluorescence of covalently reacted reagent on the denatured enzyme is the same as that of free reagent. It was important to determine if the loss of bromide resulting from nucleophilic attack on the bromoketo group would affect the fluorescence of BDB-TϵADP. This potential problem was excluded since free BDB-TϵADP standards treated with a 6-fold excess of β-mercaptoethanol showed no change in fluorescence intensity when compared to untreated BDB-TϵADP.

Dithiothreitol (0.2 M) was added to rapidly quench the reaction of BDB-TϵADP with enzyme so that incorporation could be reliably related to the measured residual activity. To test for complete quenching of reaction, isocitrate dehydrogenase was incubated with 75 μM BDB-TϵADP; dithiothreitol (0.2 M) was added after 2 min and enzymatic

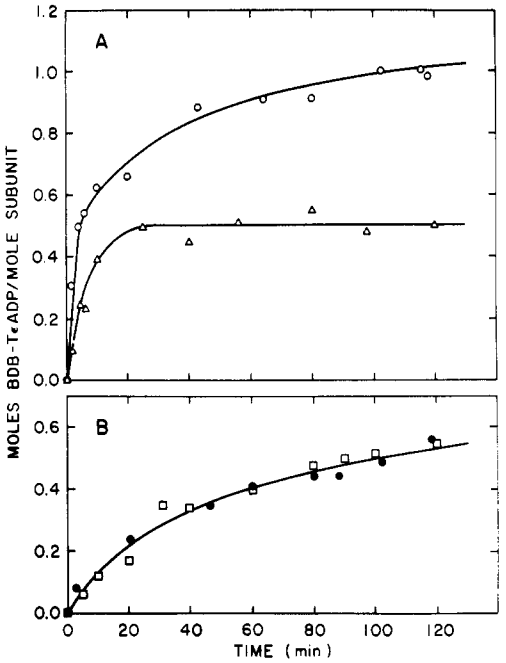

FIGURE 7: Incorporation of BDB-TεADP per mole of subunit of isocitrate dehydrogenase as a function of time. Isocitrate dehydrogenase (0.3 mg/mL) was incubated at 25 °C in 0.10 M triethanolamine hydrochloride buffer, pH 7.0, with 75 μM BDB-TεADP in the absence and presence of ligands as described in Figure 6. Incorporation at the indicated time points was determined as described under Experimental Procedures. (A) Time course of incorporation in the absence of protecting ligands (○) and in the presence of 4 mM DL-isocitrate and 2 mM $MnSO_4$ (△). (B) Time course of incorporation in the presence of 600 μM $NADP^+$ (□) and 200 μM NADPH (●).

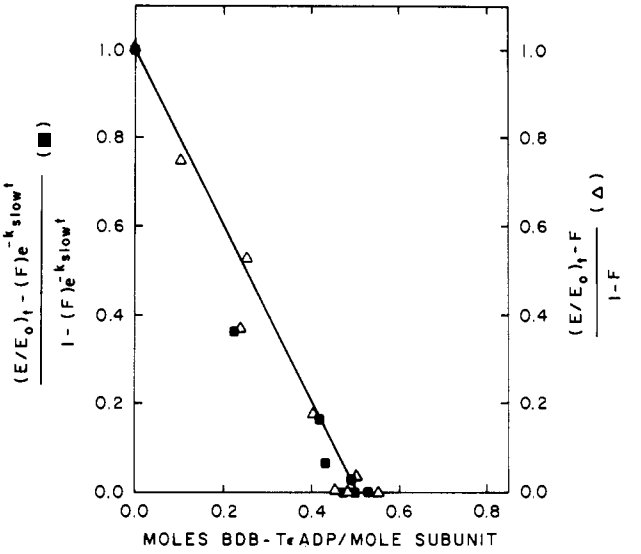

FIGURE 8: Relationship between inactivation of isocitrate dehydrogenase and incorporation of BDB-TεADP. The fraction of the maximum inactivation of isocitrate dehydrogenase in the presence of 4 mM isocitrate, 2 mM $MnSO_4$, and 75 μM BDB-TεADP (as calculated from Figure 6, line D) is plotted vs. incorporation (shown in Figure 7A) in the presence of the same concentration of ligands and BDB-TεADP (△). The fraction of maximum inactivation due to the fast phase of inactivation in the absence of protecting ligands and in the presence of 75 μM BDB-TεADP (based on eq 2 and the data of Figure 6, line A) is plotted vs. the difference (calculated from Figure 7A,B) in incorporation, at the same concentration of BDB-TεADP in the absence of protecting ligands and presence of 200 μM NADPH (■).

activity monitored. After addition of dithiothreitol, residual activity remained constant at 55% for the next 118 min, whereas when dithiothreitol was not added, residual activity fell to 2.5% over the same time period. These results demonstrate that dithiothreitol completely quenches the reaction between reagent and enzyme and that dithiothreitol does not reactivate the enzyme. No reagent was incorporated if dithiothreitol was added before addition of BDB-TεADP, thus ruling out the possibility of nonspecific modification caused by denaturation of the enzyme in the presence of quenched reagent.

Incorporation (moles of BDB-TεADP per mole of enzyme subunit) in the absence and presence of ligands is shown in Figure 7. In the absence of ligands (Figure 7A, open circles) 0.99 mol of reagent/mol of subunit is incorporated with only 2.5% activity remaining. Thus, it is apparent that there is a limited extent of incorporation of BDB-TεADP into isocitrate dehydrogenase, which is an essential criterion for an affinity label.

The incorporation of reagent into the enzyme is reduced in the presence of 4 mM isocitrate and 2 mM $MnSO_4$ as is also shown in Figure 7A (open triangles). A maximum of 0.5 mol of BDB-TεADP/mol of enzyme subunit is incorporated when the enzyme has only 8.5% of its activity remaining. Since isocitrate and $MnSO_4$ protect only against the slow phase of inactivation (Figure 6, line D), these results suggest that the fast phase of inactivation may be caused by incorporation of 0.5 mol of reagent/mol of enzyme subunit. Figure 7B shows the incorporation of reagent in the presence of 600 μM $NADP^+$ and 200 μM NADPH. Although kinetically the rates of inactivation in the presence of these two coenzyme ligands are different, the rates of incorporation are identical. This result implies modification, in the presence of these two coenzymes, of distinct amino acids, each differing in relative importance to catalysis. Even though the reagent may be binding in the same general region on the enzyme, subtle differences in the orientation of BDB-TεADP may result in the presence of each ligand.

When the incorporation at a particular time in the presence of isocitrate and Mn^{2+} is added to the incorporation at the same time in the presence of NADPH, they sum to give the observed incorporation in the absence of any added ligands. This result suggests that the two phases for the rate of inactivation in the absence of ligands can be separated in the presence of various ligands.

A direct correlation between activity loss and incorporation of reagent can also provide an indication of the specificity of the reagent for a specific site. Since NADPH protects against the fast phase, the difference between incorporation measured in the absence of ligands (Figure 7A, open circles) and incorporation measured in the presence of 200 μM NADPH (Figure 7B) would yield the incorporation corresponding to the fast phase of inactivation. When activity loss due only to the fast phase (in the absence of any added ligands) was plotted vs. this difference in incorporation (Figure 8, filled box), a maximum incorporation of 0.5 mol of reagent/mol of subunit was observed. This result suggests that an incorporation of 0.5 mol of BDB-TεADP/mol of subunit is responsible for the fast phase of inactivation (in the absence of ligands) and that the remaining incorporation corresponds to the slow phase of inactivation. When the fraction of maximum activity loss in the presence of isocitrate and Mn^{2+} was plotted vs. the incorporation measured in the presence of the same ligand (Figure 8 open triangle), a striking correlation was seen. Modification in the presence of isocitrate and Mn^{2+} also reaches a maximum of 0.5 mol of reagent/mol of subunit when the enzymatic activity is reduced to 8.5%. These results imply that the site modified in the presence of isocitrate and Mn^{2+} is the same site modified in the fast phase in the absence of

ligands and that an incorporation of 0.5 mol of reagent/mol of subunit causes this phase of inactivation. These results also suggest that the site of modification in the presence of NADPH and the site of modification corresponding to the slow phase of inactivation (when no ligands are present) may be the same.

Molecular Weight of BDB-TεADP-Modified and Unmodified Isocitrate Dehydrogenase. $NADP^+$-specific isocitrate dehydrogenase from pig heart has been reported to exist under various conditions as a monomer or as a dimer of subunits of M_r 58 000 (Colman, 1972; Kelly & Plaut, 1981). It was of interest to investigate the molecular weight of isocitrate dehydrogenase under the conditions used in this report. Sedimentation equilibrium of isocitrate dehydrogenase was performed as described under Experimental Procedures. Isocitrate dehydrogenase (0.3 mg/mL) in 0.1 M triethanolamine hydrochloride buffer, pH 7.0, was centrifuged until equilibrium was reached at 25 °C. An apparent molecular weight of 116 900 and 117 200 was obtained at 26 000 and 34 000 rpm, respectively. The independence on the observed molecular weights on rotor speed indicates that any errors due to a finite concentration at the meniscus are not significant. The plots of ln C vs. r^2 are linear with a correlation coefficient of 0.99, indicating homogeneity of the enzyme preparation. Other samples run under identical conditions in the presence of 4 mM isocitrate and 2 mM $MnSO_4$ or 200 μM NADPH gave similar molecular weights, indicating a dimeric structure for the enzyme both in the absence and in the presence of the substrates tested. Similar results were obtained when enzyme samples containing no ligands or isocitrate and Mn^{2+} were centrifuged at 9 °C.

Cross-linking of multisubunit enzymes and subsequent analysis on polyacrylamide gels containing sodium dodecyl sulfate are another method to evaluate the existence of a multimeric structure in a complete enzyme. When subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and in the absence of dimethyl suberimidate, isocitrate dehydrogenase exhibits a single band, indicative of homogeneity, with a molecular weight, of 55 000 when compared to protein standards. In contrast, after cross-linking with dimethyl suberimidate, isocitrate dehydrogenase exhibits two bands with molecular weights of approximately 48 000 (minor band) and 120 000 (major band), indicative of a dimeric structure (Figure 9). In contrast, chymotrypsinogen (a known monomer) gave a single band of M_r 20 000 after reaction with dimethyl suberimidate under the same conditions, and lactate dehydrogenase (a known tetramer) yielded four bands after incubation with dimethyl suberimidate but only one band without treatment with the cross-linking agent. These results demonstrate the reliability of this technique in evaluating the number of subunits that are associated to form a whole enzyme.

Since the biphasic kinetics observed during the reaction of BDB-TεADP with isocitrate dehydrogenase could be attributed to dissociation of enzyme dimer to monomer, the subunit composition of isocitrate dehydrogenase modified by BDB-TεADP under various conditions was examined by cross-linking and subsequent analysis on gels containing sodium dodecyl sulfate. Isocitrate dehydrogenase in the absence of any added ligands was incubated in the absence or presence of 75 μM BDB-TεADP for 6 or 90 min, corresponding to 0.55 and 0.90 mol of BDB-TεADP incorporated/mol of subunit, respectively. Both of these samples when subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate exhibited the same percentage of dimer as did unmodified enzyme (Table I, lines 1–4), indicating that there is no change in the dimeric structure of isocitrate de-

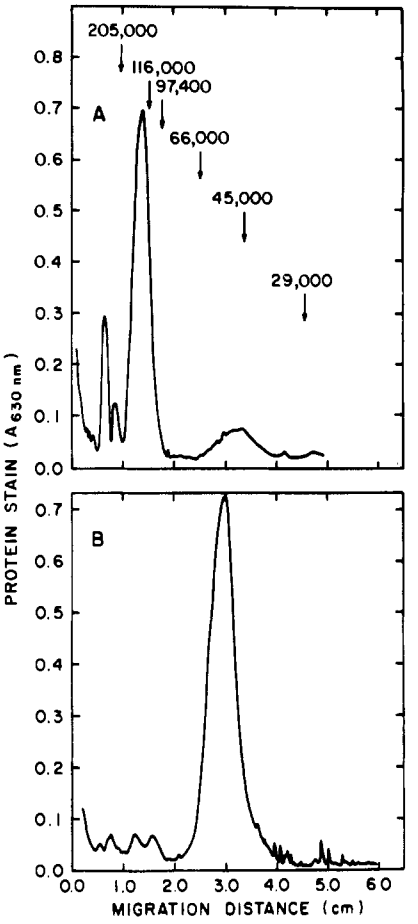

FIGURE 9: Absorbance scan of polyacrylamide gels with samples of native and cross-linked $NADP^+$-specific isocitrate dehydrogenase. (A) Isocitrate dehydrogenase was cross-linked with dimethyl suberimidate and subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate as described under Experimental Procedures. Gels were stained with Coomassie Blue and then scanned at 630 nm. Molecular weight standards are indicated by the arrows. (B) Control isocitrate dehydrogenase, not treated with dimethyl suberimidate.

Table I: Effect of Added Substrate, Coenzyme, or Reaction with BDB-TεADP on Percent Dimer of Isocitrate Dehydrogenase Detected by Cross-Linking[a]

	additions to incubation mixture			
line	ligands	BDB-TεADP (μM)	time of incubation (min)	% dimer[b]
1			6	86
2		75	6	88
3			90	79
4		75	90	84
5	isocitrate (4 mM) + $MnSO_4$ (2 mM)		90	80
6	isocitrate (4 mM) + $MnSO_4$ (2 mM)	75	90	76
7	NADPH (200 μM)		90	82
8	NADPH (200 μM)	75	90	79

[a] Isocitrate dehydrogenase (0.3 mg/mL) was incubated at 25 °C in 0.1 M triethanolamine hydrochloride buffer, pH 7.0, alone or with 75 μM BDB-TεADP, in the absence or presence of ligands, for the time indicated. At the end of the incubation period, dithiothreitol was added to quench the reaction, the pH was adjusted to 8.0, and the enzyme was cross-linked with dimethyl suberimidate as described under Experimental Procedures. [b] The several enzyme samples were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and scanned as illustrated in Figure 9. The areas under the two major peaks were used to estimate the percentage dimer and monomer.

hydrogenase upon modification with BDB-TεADP. Enzyme incubated either with isocitrate and Mn^{2+} or with NADPH without exposure to BDB-TεADP showed almost the same percentage dimer as did enzyme incubated without added substrates (compare line 5 and 7 with lines 1 and 3 of Table I), indicating that these ligands do not change the state of aggregation of the enzyme. Enzyme modified with BDB-TεADP in the presence of either isocitrate and Mn^{2+} (Table I, line 6) or NADPH (Table I, line 8) also exhibited no change in dimeric structure. Therefore, under all conditions used, native isocitrate dehydrogenase exists as a dimeric enzyme, and this dimeric structure is unchanged by modification with BDB-TεADP.

Discussion

The new fluorescent nucleotide analogue 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate reacts with pig heart $NADP^+$-specific isocitrate dehydrogenase, exhibiting characteristics expected for an affinity label. It reacts covalently with a limited number of sites on the enzyme: only 0.99 mol of reagent/mol of subunit is incoporated when the enzyme is 98% inactive. The rate constants for reaction of BDB-TεADP with isocitrate dehydrogenase exhibit a nonlinear dependence on reagent concentration, indicating the formation of a reversible enzyme–reagent complex prior to irreversible modification.

The reaction of BDB-TεADP with isocitrate dehydrogenase, in the absence of ligands, exhibits biphasic kinetics of inactivation; this can be described by rapid modification of one group to yield a partially active enzyme and modification of another group to yield completely inactive enzyme. Since different natural ligands protect against the two phases of the reaction, the two groups appear to be distinct and distinguishable. In the presence of NADPH or isocitrate and $MnSO_4$, these two groups can be examined separately. Covalent incorporation of BDB-TεADP responsible for the fast phase of inactivation in the absence of ligands appears to occur at the same site as does modification by BDB-TεADP in the presence of isocitrate and $MnSO_4$, both reaching a maximum incorporation of 0.5 mol of reagent per peptide chain and producing enzyme which exhibits only 8–10% residual activity. In contrast, incorporation occurring in the presence of NADPH is likely to correspond to that correlated with the slow phase of inactivation in the absence of ligands. Incorporation of reagent in the presence of isocitrate and $MnSO_4$, when added to the incorporation in the presence of NADPH, yields the measured incorporation in the absence of protecting ligands; this result supports the postulate that these two distinguishable reaction sites account for the total reaction of BDB-TεADP with the enzyme.

$NADP^+$-specific isocitrate dehydrogenase from pig heart has been reported to exist under various conditions as a monomer or as a dimer with subunits of 58 000 daltons (Colman, 1972; Kelly & Plaut, 1981). The enzyme has been shown in the present paper to exist as a dimer under all the conditions used in this study both by sedimentation equilibrium measurements and by cross-linking with dimethyl suberimidate followed by analysis on polyacrylamide gels in the presence of sodium dodecyl sulfate. No evidence for dissociation of the enzyme to a monomeric form could be found under any of the conditions tested. The results found in this report in 0.10 M triethanolamine hydrochloride buffer, pH 7.0, are different from those reported by Kelly & Plaut (1981), who found the enzyme to exist as a monomer under similar conditions. However, the results presented in this study are in good agreement with sedimentation equilibrium experiments conducted under similar conditions by Reynolds et al. (1978) for the enzyme from bovine heart. Bovine heart isocitrate dehydrogenase in 0.05 M triethanolamine hydrochloride buffer, pH 7.0, was shown to exist in a dimeric form with an apparent molecular weight of 93 000–96 000.

Given the dimeric structure of pig heart isocitrate dehydrogenase under the conditions used for the present investigation, the maximum incorporation of 0.5 mol of BDB-TεADP/mol of peptide chain in each of the two distinguishable phases of the reaction can be interpreted as modification of one subunit per enzyme dimer. That modification of one of the two subunits corresponds to 8–10% activity remaining rather than the expected 50% residual activity implies the existence of interactions between the two subunits of isocitrate dehydrogenase. Modification and inactivation of the active site on one subunit must indirectly decrease substantially the catalytic function of the active site on the second subunit. Unequal effects of binding to one of two coenzyme binding sites per dimer of the pig heart (Mas & Colman 1985) and bovine heart enzymes (Reynolds, et al., 1978) have previously been indicated by studies of enhancement of protein fluorescence upon coenzyme binding. Mas & Colman (1985) found two binding sites per enzyme dimer for $NADP^+$, NADPH, and PADPR by UV difference spectral titrations and ultrafiltration experiments; however, enhancement of protein fluorescence gave an apparent stoichiometry of only one site per dimer for $NADP^+$ and PADPR. Since the maximum fluorescence enhancement was obtained upon saturation of half of the $NADP^+$ binding sites, it was suggested that the binding of the first molecule of ligand to an enzyme dimer was responsible for the total change observed. That enhancement of protein fluorescence, rather than quenching, was observed upon coenzyme binding suggests that the observed effect is the result of conformational changes of the enzyme. Similar results were found by Reynolds et al. (1978) for the bovine heart enzyme.

NADPH protects completely against the fast phase of inactivation of isocitrate dehydrogenase by BDB-TεADP, yielding a rate of inactivation that is the same as that of the slow phase of inactivation observed in the absence of ligands. These results indicate that the most reactive site attacked by BDB-TεADP is the natural coenzyme site of the enzyme. $NADP^+$ also provides significant protection against inactivation by BDB-TεADP. Although the residual rates of inactivation are different in the presence of NADPH and $NADP^+$, the rates of incorporation are identical. Both coenzymes may protect completely against modification of the most reactive site; however, modification of distinct groups at the second site may occur in the presence of NADPH or $NADP^+$, each differing in relative importance to catalysis. Differences in the binding or orientation of BDB-TεADP may be due to subtle differences in enzyme conformation in the presence of each coenzyme. It may be relevant that Ehrlich & Colman (1984) have observed differences in the conformation of $NADP^+$ and NADPH when bound to isocitrate dehydrogenase by examining intramolecular ^1H–^1H nuclear Overhauser effects.

These results can be explained by a model in which BDB-TεADP, in the absence of ligands, reacts rapidly at the coenzyme binding site on one of two subunits of the dimeric enzyme, causing a conformational change, which facilitates modification by BDB-TεADP on the second subunit at a site other than the coenzyme binding site. The protection by isocitrate and Mn^{2+} against the second site of modification may occur because substrate stabilizes the initial conformation

of the enzyme, which does not allow BDB-TεADP access to the second site. This postulate is consistent with the fact that no enhancement of protein fluorescence is observed upon nucleotide binding in the presence of isocitrate and $MnSO_4$ (Mas & Colman, 1985), indicating that no detectable change in protein conformation occurs upon nucleotide binding when isocitrate–Mn^{2+} is present. Alternatively, it may be that BDB-TεADP is actually modifying the isocitrate binding site on the second subunit, and hence, protection by isocitrate and $MnSO_4$ would be expected. Thus, the biphasic kinetics of inactivation of isocitrate dehydrogenase by BDB-TεADP might best be explained as the fast phase resulting from rapid modification of the coenzyme binding site on one of two subunits, with a concomitant conformational change, followed by a slower rate of modification at another site on the other subunit, which may or may not be the isocitrate binding site on that subunit.

The new nucleotide analogue 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate exhibits many desirable characteristics as an affinity label for $NADP^+$-specific isocitrate dehydrogenase. The fluorescence of BDB-TεADP allows a convenient means of measuring the incorporation of reagent. Additionally, the fluorescent properties of this reagent offer a convenient means of introducing a covalent fluorescent probe into a nucleotide binding site of an enzyme. BDB-TεADP, like the natural coenzymes, is negatively charged at neutral pH and therefore soluble in water, eliminating the need for addition of organic solvents in reaction with enzyme. The presence of an ionized 2′-phosphate has been shown to be necessary for binding of coenzymes and nucleotides to this enzyme (Ehrlich & Colman, 1978; Mas & Colman, 1984). To date, few purine nucleotide affinity labels have been synthesized with the 2′-phosphate which is necessary for binding to many $NADP^+$ enzymes. Periodate-oxidized derivatives of $NADP^+$ and NADPH have been synthesized (Rippa et al., 1975; Mas & Colman, 1983), but these derivatives are limited in the number of amino acid side chains with which they can react. The bromoketo group of BDB-TεADP 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 allows the possibility of reaction with arginine residues (Yankeelov, 1970; Riordan, 1973). Further work is in progress to identify the amino acid residue(s) of isocitrate dehydrogenase modified by BDB-TεADP. It is proposed that 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate will have general applicability as an affinity label of $NADP^+$ binding enzymes and provide a convenient means of introducing a fluorescent probe into the $NADP^+$ binding sites of these enzymes.

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