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2-[(4-Bromo-2,3-dioxobutyl)thio]- and 2-[(3-Bromo-2-oxopropyl)thio]adenosine 2′,5′-Bisphosphate: New Nucleotide Analogues That Act as Affinity Labels of Nicotinamide Adenine Dinucleotide Phosphate Specific Isocitrate Dehydrogenase†

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ABSTRACT: Two new reactive adenine nucleotide analogues have been synthesized and characterized: 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 2′,5′-bisphosphate (2-BDB-TA-2′,5′-DP) and 2-[(3-bromo-2-oxopropyl)thio]adenosine 2′,5′-bisphosphate (2-BOP-TA-2′,5′-DP). Starting with $NADP^+$, 2′-phosphoadenosine 5′-(diphosphoribose) (PADPR) was generated enzymatically and was converted to PADPR 1-oxide by reaction with *m*-chloroperoxybenzoic acid. Treatment with NaOH followed by reaction with carbon disulfide yielded 2-thioadenosine 2′,5′-bisphosphate (TA-2′,5′-DP). Condensation of TA-2′,5′-DP with 1,4-dibromobutanedione or 1,3-dibromo-2-propanone gave the final products 2-BDB-TA-2′,5′-DP and 2-BOP-TA-2′,5′-DP, respectively. The structure of these new reagents was determined by UV, ^1H NMR, ^{31}P NMR, and ^{13}C NMR spectroscopy as well as by bromide and phosphorus analysis. Both of these reagents exhibit properties expected for an affinity label of the coenzyme site of $NADP^+$-dependent isocitrate dehydrogenase. With both reagents, 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 6–7% residual activity, followed by a slower phase leading to total inactivation. The inactivation rate constants for both reagents exhibit a nonlinear dependence on reagent concentration, consistent with the formation of a reversible complex with the enzyme prior to irreversible modification. The enzyme incorporates both reagents to a limited extent and is protected against inactivation by $NADP^+$ and NADPH. The reaction of these new nucleotide analogues with isocitrate dehydrogenase is compared to the much slower inactivation caused by bromoacetone, indicating the importance of the nucleotide moiety in the functioning of the affinity labels. It is likely that 2-BDB-TA-2′,5′-DP and 2-BOP-TA-2′,5′-DP will have general applicability as affinity labels for other $NADP^+$ binding enzymes.

Pig heart $NADP^+$-dependent isocitrate dehydrogenase [*threo*-D_s-isocitrate:$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.42] catalyzes the oxidative decarboxylation of isocitrate. Extensive chemical modification studies have been performed with isocitrate dehydrogenase by using a variety of group-specific reagents [see Colman (1983a) for a review]. The technique of affinity labeling, using nucleotide analogues with reactive functional groups, can potentially result in more specific chemical modification and should allow the identification of critical amino acid residues within the nucleotide binding site (Colman, 1983b). Studies of the binding of coenzymes and coenzyme fragments to $NADP^+$-specific isocitrate dehydrogenase have demonstrated that a 2′-phosphate is essential for the enzyme–nucleotide interaction (Ehrlich & Colman, 1978; Mas & Colman, 1985), suggesting that any potential affinity label for this enzyme retain the 2′-phosphate.

Affinity labeling of the coenzyme binding site of $NADP^+$-dependent isocitrate dehydrogenase has previously

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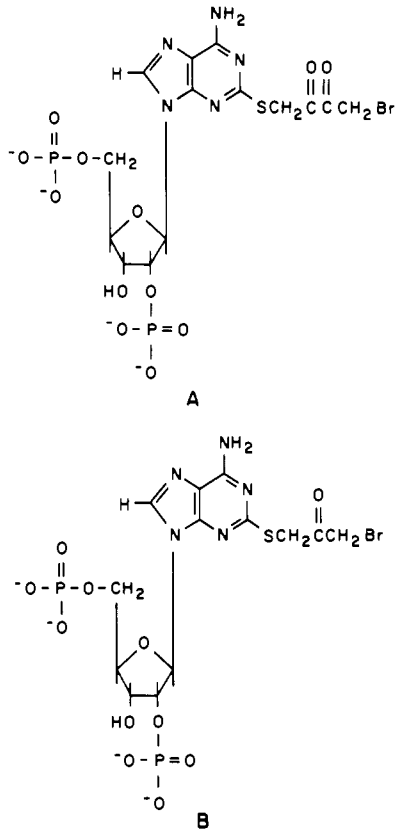

FIGURE 1: Structures of adenine nucleotide analogues: (A) 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 2′,5′-bisphosphate and (B) 2-[(3-bromo-2-oxopropyl)thio]adenosine 2′,5′-bisphosphate.

been conducted with periodate-oxidized NADPH (Mas & Colman, 1983) and with 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate (2-BDB-TεA-2′,5′-DP)[1] (Bailey & Colman, 1985). 2-BDB-TεA-2′,5′-DP specifically labeled the coenzyme binding site on one subunit of the enzyme dimer. In this paper, we describe the synthesis and characterization of two new 2′-phosphate nucleotide analogues, 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 2′,5′-bisphosphate and 2-[(3-bromo-2-oxopropyl)thio]adenosine 2′,5′-bisphosphate, which have bromoketo moieties capable of covalent reaction with the nucleophilic side chains of several amino acids. These compounds have reactive groups of different chain length, as shown in Figure 1, and are expected to be complementary to each other and to 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate, which has an additional etheno ring. We here describe the reaction of these two new compounds at the coenzyme site of isocitrate dehydrogenase.

Experimental Procedures

Materials and Methods. Porcine heart NADP+-specific isocitrate dehydrogenase was purified to homogeneity 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, 1981a,b). A subunit molecular weight of 58 000 (Colman et al., 1970) was used to calculate the concentration of enzyme subunits.

2-[(4-Bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate (2-BDB-TεA-2′,5′-DP) and 2-thioadenosine 5′-monophosphate were synthesized as previously described (Bailey & Colman, 1985; Kapetanovic et al., 1985). *Neurospora* NADase (crude preparation), Malachite Green base, mercuric thiocyanate, coenzymes, dithiothreitol, buffer salts, DL-isocitrate, and phosphorus standard solution were all obtained from Sigma Chemical Co. 1,4-Dibromobutanedione was obtained from Aldrich Chemical Co. and was recrystallized from petroleum ether before use. 1,3-Dibromo-2-propanone was from Lancaster Synthesis. *m*-Chloroperoxybenzoic acid was purchased from Aldrich Chemical Co. Ammonium molybdate and hydrogen peroxide were supplied by Fisher Scientific Co., protein dye concentrate was from Bio-Rad, and ultrapure guanidine hydrochloride was from Schwarz/Mann.

NMR spectra were obtained with a Bruker WM 250-MHz spectrometer at room temperature with D_2O for both ^1H and ^{13}C NMR spectra. Dioxane (3.71 ppm) was used as an internal standard for ^1H NMR spectra, and benzene (128 ppm) was used as an external standard for ^{13}C NMR spectra. H_3PO_4 (85%) was used as an external standard for ^{31}P NMR chemical shifts. Chemical shifts downfield from H_3PO_4 resonance were given a positive sign.

Synthesis of 2-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 2′,5′-Bisphosphate and 2-[(3-Bromo-2-oxopropyl)thio]-adenosine 2′,5′-Bisphosphate. The overall synthetic scheme is shown in Figure 2, which also gives the numbers by which the several intermediates are designated. Starting with NADP+ (I), PADPR (II) was generated enzymatically and was converted to PADPR 1-oxide (III) by reaction with *m*-chloroperoxybenzoic acid. Treatment with NaOH followed by reaction with carbon disulfide yielded 2-thioadenosine 2′,5′-bisphosphate [TA-2′,5′-DP (V)]. Condensation of TA-2′,5′-DP with 1,4-dibromobutanedione or 1,3-dibromo-2-propanone gave the final products 2-BDB-TA-2′,5′-DP (VI) and 2-BOP-TA-2′,5′-DP, respectively.

Preparation of 2′-Phosphoadenosine 5′-(Diphosphoribose) 1-Oxide (III). NADP+ (1 g) was converted to PADPR, and the latter was purified as previously described (Bailey & Colman, 1985). 2′-Phosphoadenosine 5′-(diphosphoribose) 1-oxide was synthesized by analogy to the procedure used for the synthesis of adenosine 5′-monophosphate 1-oxide (Kapetanovic et al., 1985) and adenosine cyclic 3′,5′-phosphate 1-oxide (Meyer et al., 1973). A biphasic mixture containing 760 mg of PADPR, 8 mL of 1 N sodium acetate, 8 mL of 1 N acetic acid, 15 mL of ethyl acetate, and 2 g of *m*-chloroperoxybenzoic acid was stirred for 48 h at room temperature. After this period, the aqueous (lower) layer was separated,

Table I: R_f Values of Nucleotide Analogues on Thin-Layer Chromatography[a]

compound	R_f	compound	R_f
1,4-dibromobutanedione	0.78	TAMP	0.24
2-BOP-TA-2′,5′-DP	0.60	AMP 1-oxide	0.31
2-BOP-TAMP	0.55	ADP 1-oxide	0.25
2-BDB-TA-2′,5′-DP	0.37	PADPR 1-oxide	0.11
TA-2′,5′-DP	0.14		

[a] Thin-layer chromatography was performed on cellulose aluminum backed sheets (EM Reagents, 0.1-mm thickness) using isobutyric acid/concentrated NH_4OH/H_2O (66:1:33) as the solvent.

[1] Abbreviations: 2-BDB-TεA-2′,5′-DP, 2-[(4-bromo-2,3-dioxobutyl)-thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate; 2-BDB-TA-2′,5′-DP, 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 2′,5′-bisphosphate; 2-BDB-TAMP, 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-monophosphate; 2-BOP-TA-2′,5′-DP, 2-[(3-bromo-2-oxopropyl)thio]adenosine 2′,5′-bisphosphate; 2-BOP-TAMP, 2-[(3-bromo-2-oxopropyl)thio]adenosine 5′-monophosphate; TA-2′,5′-DP, 2-thioadenosine 2′,5′-bisphosphate; TAMP, 2-thioadenosine 5′-monophosphate; PADPR, 2′-phosphoadenosine 5′-(diphosphoribose); 2′,5′-ADP, adenosine 2′,5′-bisphosphate; MES, 2-(*N*-morpholino)ethanesulfonic acid; TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid; NADase, NAD+ glycohydrolase.

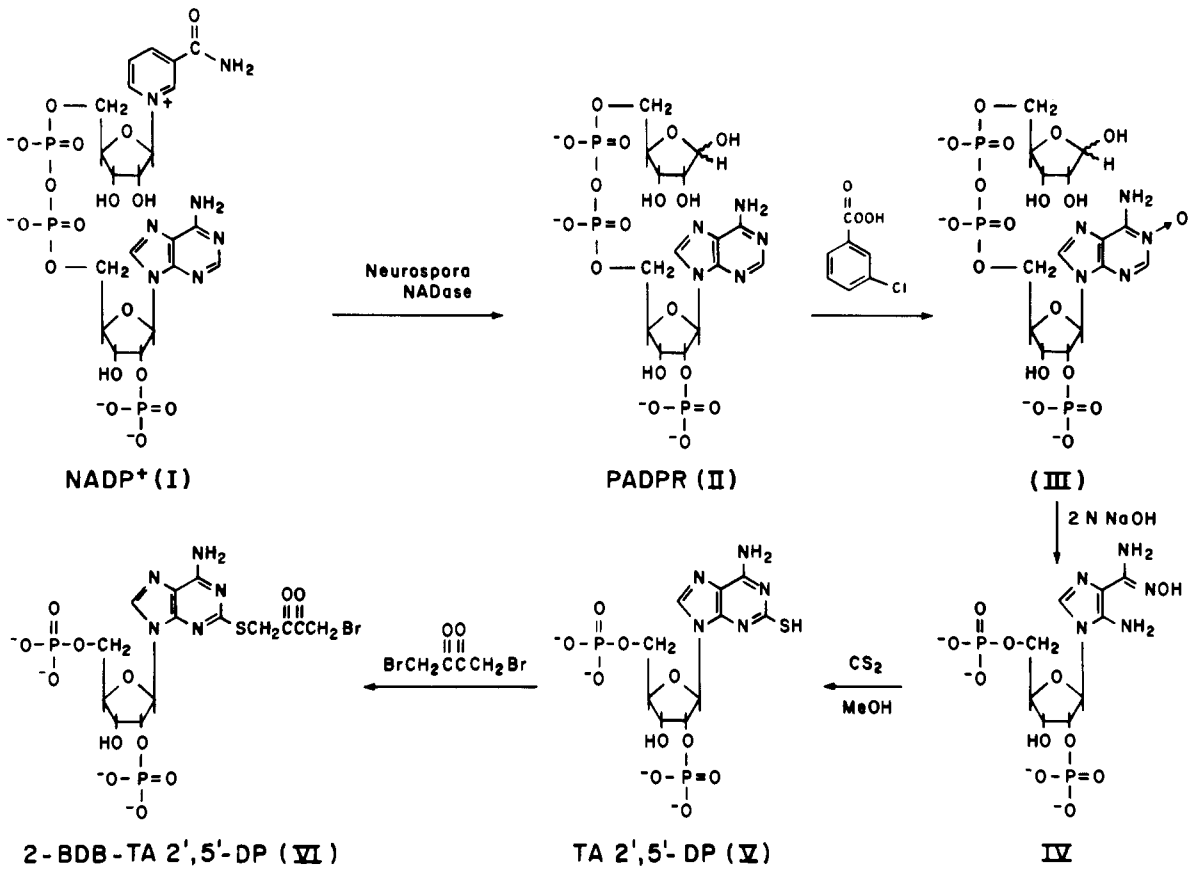

FIGURE 2: Synthetic scheme for preparation of 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 2',5'-bisphosphate.

chloroform (5 mL) and 1 N HCl (8 mL) were added to this layer, and the mixture was stirred at room temperature for 1 h. The aqueous (upper) layer was recovered, isopropyl alcohol (500 mL) was added to it, and the cloudy white solution was maintained overnight at 4 °C. The material was concentrated under vacuum and dried to a white powder. TLC exhibited a single UV-absorbing spot for this PADPR 1-oxide, as summarized in Table I. The ultraviolet absorption spectrum for PADPR 1-oxide exhibited an A_{260nm}/A_{233nm} ratio of 0.23, which is the same as that observed for AMP 1-oxide. The ratio of A_{260nm}/A_{233nm} for the starting material (PADPR) was 5.0. The yield was 90%.

Preparation of 5-Amino-1-β-D-ribofuranosylimidazole-4-carboxamide Oxime 2',5'-Bisphosphate (IV). PADPR 1-oxide was treated with NaOH in a manner analogous to the procedure described for AMP 1-oxide (Kapetanovic et al., 1985). 2'-Phosphoadenosine 5'-(diphosphoribose) 1-oxide from the previous step was dissolved in 6 mL of 2 N NaOH. The resultant yellow solution was added to 6 mL of refluxing 2 N NaOH. Ten minutes later, the reaction flask was cooled on ice and the pH adjusted to 11 with AG50W-X4 (H^+ form, 100–200 mesh). The resin was filtered and washed with H_2O until the absorbance at 260 nm of the washings was less than 0.01. The filtrate and washings were applied to a 1.5 × 45 cm column of AG1-X2 (formate form, 100–200 mesh), which was washed with distilled water until the absorbance at 260 nm was less than 0.02. The column was then eluted with a linear gradient (1 L of water and 1 L of 2 N formic acid) and monitored at 260 nm. The product was the second of three peaks to be eluted and was located between fractions 186 and 251 (4.5 mL/fraction). The fractions were pooled and concentrated under vacuum to give a clear glass. This material was dried by coevaporating twice with 10 mL of methanol plus 10 mL of isopropyl alcohol. The product was obtained as a white powder in 48% yield. Ultraviolet absorption spectra showed a λ_{max} at 285 nm at pH 1 and a λ_{max} at 260 nm at pH 11. TLC exhibited a single ultraviolet absorbing spot with R_f = 0.12, whereas the corresponding 5'-diphosphate and 5'-monophosphate analogues had R_f values of 0.13 and 0.24, respectively.

Preparation of 2-Thioadenosine 2',5'-Bisphosphate (V). The triethylammonium salt of IV was refluxed with carbon disulfide in methanol and pyridine, as described for the preparation of compound IV of Kapetanovic et al. (1985). The crude product was dissolved in water and centrifuged to remove any insoluble material. The supenatant was applied to a 2 × 35 cm column of DEAE-Sephadex (A-25) equilibrated with 0.01 M NH_4HCO_3 at 4 °C. The column was eluted with a linear gradient from 0.1 (4 L) to 0.5 M NH_4HCO_3 (4 L). The product (TA-2',5'-DP) was the second of two peaks to elute and was located in fractions 241–310 (16 mL/fraction). 2-Thioadenosine 2',5'-bisphosphate was pooled, evaporated to dryness under vacuum, and desalted by repeatedly redissolving in water and evaporating to dryness under vacuum. The product was obtained as a yellowish oil in 85% yield. The ultraviolet absorption spectrum exhibited a λ_{max} at 292 and 256 nm in 50 mM MES buffer, pH 6.0. TLC showed a single ultraviolet absorbing spot at R_f = 0.14 (Table I).

Preparation of 2-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 2',5'-Bisphosphate (VI). TA-2',5'-DP (V, 240 mg) was dissolved in 2 mL of H_2O and applied to a 1 × 26 cm column of AG50W-X4 (H^+ form, 100–200 mesh) at 4 °C and eluted with distilled water. Elution was monitored by absorbance at 240 nm. The product (free acid) was collected and evaporated to dryness under reduced pressure. 2-BDB-TA-2',5'-DP was synthesized by reaction of TA-2',5'-DP with 1,4-dibromobutanedione as described for the reaction of 2-thioadenosine 5'-monophosphate with 1,4-dibromobutanedione

(Kapetanovic et al., 1985). The yield for this step was 80%.

Preparation of 2-[(3-Bromo-2-oxopropyl)thio]adenosine 2′,5′-Bisphosphate. TA-2′,5′-DP (V, 10 mg of free acid) was dissolved in 1 mL of methanol by the addition of triethylamine dropwise to form the soluble triethylammonium salt. Sufficient triethylamine was added to adjust the pH to approximately 5.3 as estimated by pH paper. 1,3-Dibromo-2-propanone (0.05 mL) was added to 0.45 mL of methanol (930 mM). This solution was added (with rapid mixing) to 0.5 mL of the TA-2′,5′-DP solution (22 mM). Reaction occurred immediately and could be assessed spectrophotometrically from the decrease in absorbance at 290 nm. The reaction mixture was placed on ice within 2 min, and the product was precipitated by addition of 10 mL of diethyl ether. The precipitate was collected by centrifugation, redissolved in 0.5 mL of methanol, and again precipitated with diethyl ether. 2-BOP-TA-2′,5′-DP was stored dry and desiccated at −80 °C; yield 80%.

Preparation of 2-[(3-Bromo-2-oxopropyl)thio]adenosine 5′-Monophosphate and 1-Bromo-2-propanone (Bromoacetone). 2-[(3-Bromo-2-oxopropyl)thio]adenosine 5′-monophosphate (2-BOP-TAMP) was prepared as described above, except that 2-thioadenosine 5′-monophosphate (TAMP) was used for condensation with 1,3-dibromo-2-propanone instead of TA-2′,5′-DP. Bromoacetone was prepared essentially as described by Rappe (1963). One mole of acetone was mixed with 100 mL of 48% hydrobromic acid and placed in an ice–water bath. One mole of bromine was added dropwise with stirring. After addition of 200 mL of distilled water, the organic (lower) layer was separated and bromoacetone distilled under reduced pressure.

Solutions of 2-BDB-TA-2′,5′-DP and 2-BOP-TA-2′,5′-DP used for kinetics and incorporation studies were prepared in 50 mM MES buffer, pH 5.0, and stored at −80 °C for periods up to 1 month.

Characterization of 2-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 2′,5′-Bisphosphate. The overall yield of 2-BDB-TA-2′,5′-DP was 26%. NADP+ (rather than PADPR or 2′,5′-ADP) was chosen as started material for the synthesis of 2-BDB-TA-2′,5′-DP because it was the least expensive precursor and the conversion of I to IV proceeds in high yield. The purity of the product, 2-BDB-TA-2′,5′-DP, was assessed by thin-layer chromatography: a single ultraviolet-absorbing spot was observed with an R_f of 0.37 (Table I).

The difference in the ultraviolet absorption spectrum of TA-2′,5′-DP and 2-BDB-TA-2′,5′-DP allows the reaction between TA-2′,5′-DP and 1,4-dibromobutanedione to be followed spectrophotometrically in 50 mM MES buffer, pH 6.0, as described for the formation of 2-BDB-TAMP (Kapetanovic et al., 1985). Reaction of TA-2′,5′-DP with 1,4-dibromobutanedione causes a decrease in the extinction coefficient at 290 nm (5.8×10^3 M^{-1} cm^{-1} for 2-BDB-TA-2′,5′-DP). The extinction coefficient for 2-BDB-TA-2′,5′-DP calculated at 270 nm (11.5×10^3 M^{-1} cm^{-1}) is in good agreement with the value found for 2-BDB-TAMP (Kapetanovic et al., 1985).

The bromide and phosphorus contents of 2-BDB-TA-2′,5′-DP were measured as previously described (Colman et al., 1984). The ratio of organic phosphorus to spectrophotometrically determined 2-BDB-TA-2′,5′-DP was 2.05:1.00. The ratio of hydrolyzable bromide to 2-BDB-TA-2′,5′-DP was 1.01:1.00.

The proton NMR spectrum of 2-BDB-TA-2′,5′-DP (triethylammonium salt) in D_2O had the peaks indicated in Table II. Assignments of the ribose protons were made by comparison with the proton NMR spectrum of 2′-AMP (Davies & Danyluk, 1975) and those of the $-CH_2S-$ and $-CH_2Br$ peaks by comparison with the spectra of 1,4-dibromobutanedione and 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-monophosphate (Kapetanovic et al., 1985).

Table II: ^1H NMR Spectra of Adenine Nucleotides (D_2O) (δ)

assignment	2-BDB-TA-2′,5′-DP[a]	TA-2′,5′-DP	2-BOP-TA-2′,5′-DP[a]	2-BOP-TAMP[a]
$-CH_3$, triethylamine	1.17		1.15	1.26
$-CH_2N$, triethylamine	3.08		3.09	3.15
$-CH_2Br$	3.65–3.80			
H_5', ribose	4.02	4.03	4.08	4.07
H_4', ribose	4.28	4.36	4.30	4.31
H_3', ribose	4.50	4.57	4.48	4.44
$-CH_2S-$	*b*	*b*	4.58	4.56
H_2', ribose	*b*	*b*	*b*	4.66
H_1', ribose	6.23	6.12	6.19	6.03
H_8	8.35	8.34	8.44	8.40

[a] Triethylammonium salts. [b] Obscured by the HDO peak.

Table III: ^{13}C NMR Spectra of Adenine Nucleotides (D_2O) (δ)

assignment	2-BOP-TAMP[a]	TA-2′,5′-DP
$-CH_3$, triethylamine	8.3	
$-SCH_2-$, $-CH_2Br$	35.24	
	38.13	
$-CH_2N$, triethylamine	46.7	
C_5', ribose	64.31	64.22
C_3', ribose	70.30	69.03
C_2', ribose	74.42	77.45
C_4', ribose	83.87	83.24
C_1', ribose	87.34	87.25
C_5, purine	115.99	113.33
C_8, purine	140.97	139.39
C_4, purine	143.78	141.79
C_6, purine	148.28	148.79
C_2, purine	157.02	169.38
—C(=O)—	200.37	

[a] Triethylammonium salt.

The proton-decoupled ^{13}C NMR spectra of 2-thioadenosine 2′,5′-bisphosphate exhibited the signals recorded in Table III. Resonance peak assignments were made primarily by reference to Schleich et al. (1975) and Bednar and Colman (1982) and by comparison to 2-thioadenosine 5′-monophosphate (Kapetanovic et al., 1985). The ^{13}C NMR data indicates that only the 2′,5′-phosphate isomer is present. Had any of the 3′,5′-phosphate isomer been formed, a resonance of 75 ppm corresponding to the C_3' would have been expected (Schleich et al., 1975); no such signal was observed.

The proton-decoupled ^{31}P NMR spectrum of 2-thioadenosine 2′,5′-bisphosphate (V) was performed at 25 °C in a 2-mL sample volume, pH 8.4, containing 20% D_2O as an internal field frequency lock and EDTA to eliminate any line broadening due to paramagnetic impurities. Two resonances of equal intensity were observed at 4.57 and 4.86 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.

Characterization of 2-[(3-Bromo-2-oxopropyl)thio]adenosine 2′,5′-Bisphosphate. 2-[(3-Bromo-2-oxopropyl)thio]adenosine 2′,5′-bisphosphate and 2-[(3-bromo-2-oxopropyl)thio]adenosine 5′-monophosphate were synthesized by condensation of 1,3-dibromo-2-propanone with TA-2′,5′-DP and 2-thioadenosine 5′-monophosphate (TAMP), respectively. Due to the larger quantity of TAMP than TA-2′,5′-DP available, the 5′-monophosphate analogue was synthesized in

order to allow more extensive characterization of the oxopropyl group. Upon thin-layer chromatography, a single ultraviolet-absorbing spot was observed for each of 2-BOP-TA-2′,5′-DP and 2-BOP-TAMP (Table I).

The ultraviolet absorption spectrum of 2-BOP-TA-2′,5′-DP and 2-BOP-TAMP is identical with that observed for 2-BDB-TA-2′,5′-DP, and therefore the reaction between TA-2′,5′-DP and 1,3-dibromo-2-propanone could be followed spectrophotometrically from the decrease in absorbance at 290 nm as described for reaction of TAMP with 1,4-dibromobutanedione. An extinction coefficient of 11.5×10^3 M^{-1} cm^{-1} for 2-BOP-TA-2′,5′-DP and 2-BOP-TAMP at 270 nm was calculated when measured in 50 mM MES buffer, pH 6.0.

The bromide and phosphorus contents of the two compounds indicate ratios of organic phosphorus to spectrophotometrically determined 2-BOP-TA-2′,5′-DP and 2-BOP-TAMP of 1.97:1.00 and 1.00:1.00, respectively. The ratio of hydrolyzable bromide to 2-BOP-TA-2′,5′-DP and 2-BOP-TAMP was 1.02:1.00 and 0.98:1.00, respectively.

The proton NMR spectrum of 2-BOP-TA-2′,5′-DP (triethylammonium salt) and 2-BOP-TAMP in D_2O had the peaks summarized in Table II. Assignments of the ribose protons were made by comparison with the proton NMR spectrum of 2′-AMP (Davies & Danyluk, 1975) and 5′-AMP (Davies & Danyluk, 1974). 1,3-Dibromo-2-propanone exhibits a single resonance peak in deuteriated chloroform (δ 4.19) and in deuteriated methanol (δ 4.01). Therefore, it is likely that the $-CH_2Br$ resonance in 2-BOP-TA-2′,5′-DP and 2-BOP-TAMP is obscured by the H_5' of ribose.

The proton-decoupled ^{13}C NMR spectra of 2-[(3-bromo-2-oxopropyl)thio]adenosine 5′-monophosphate had the signals indicated in Table II. Resonance peak assignments were made primarily by reference to Schleich et al. (1975) and by comparison to 2-thioadenosine 5′-monophosphate and 2-BDB-TAMP (Kapetanovic et al., 1985). 1,3-Dibromo-2-propanone in $CDCl_3$ exhibits resonances at δ 30.98 ($-CH_2Br$) and at δ 193.58 [—C(=O)—]. No evidence could be found for a carbonyl hydrate as was previously seen by ^{13}C NMR for the corresponding diketo analogue, 2-BDB-TAMP (Kapetanovic et al., 1985). Thus all the spectral and analytical data are consistent with the structures of 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 2′,5′-bisphosphate and 2-[(3-bromo-2-oxopropyl)thio]adenosine 2′,5′-bisphosphate as shown in Figure 1.

Reaction of 2-BDB-TA-2′,5′-DP with Isocitrate Dehydrogenase. Isocitrate dehydrogenase (0.12 mg/mL) was incubated with varying concentrations of 2-BDB-TA-2′,5′-DP at 25 °C in 0.10 M triethanolamine chloride 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 2-BDB-TA-2′,5′-DP. At timed intervals, aliquots of the reaction mixture were assayed for residual isocitrate dehydrogenase activity on the basis of the rate of appearance of NADPH as measured by the absorbance at 340 nm on a Gilford Model 240 spectrophotometer (full scale, 0.1 *A* unit) (Colman, 1968). The rate of reaction of isocitrate dehydrogenase with 2-BDB-TA-2′,5′-DP 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

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

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).

Reaction of 2-BOP-TA-2′,5′-DP or Bromoacetone with Isocitrate Dehydrogenase. Isocitrate dehydrogenase (0.12 mg/mL) was incubated with varying concentrations of 2-BOP-TA-2′,5′-DP or bromoacetone at 25 °C in 0.10 M MES buffer, pH 6.5, in a total volume of 1 mL. Bromoacetone in methanol was added to yield concentrations of 100–500 μM and 2% methanol. The rate of reaction of 2-BOP-TA-2′,5′-DP with isocitrate dehydrogenase was analyzed as described above for 2-BDB-TA-2′,5′-DP.

Incorporation of 2-BDB-TA-2′,5′-DP and 2-BOP-TA-2′,5′-DP by Isocitrate Dehydrogenase. The enzyme (0.8 mg/mL) was incubated with either 100 μM 2-BDB-TA-2′,5′-DP or 200 μM 2-BOP-TA-2′,5′-DP under the conditions described above. At various times a 0.5-mL aliquot of the reaction mixture was withdrawn, and the reaction was stopped by the addition of dithiothreitol to a final concentration of 0.2 M. 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). A 10-μL aliquot was dissolved in 90 μL of 50 mM MES buffer, pH 6.0, and 0.9 mL of the diluted protein dye (1:4 in H_2O) was added. Standard protein solutions were prepared with native isocitrate dehydrogenase in the same buffer as above except it also contained 10 μL of 5 M guanidine hydrochloride. The incorporation of 2-BDB-TA-2′,5′-DP and 2-BOP-TA-2′,5′-DP into isocitrate dehydrogenase was measured by determination of the moles of organic phosphorus per mole of enzyme subunit by a modification of the procedures of Hess and Derr (1975) and Lanzetta et al. (1979), as described previously (Bailey & Colman, 1987a).

Incorporation of 2-BDB-TεA-2′,5′-DP into Modified Isocitrate Dehydrogenase. Incorporation of 2-BDB-TεA-2′,5′-DP into isocitrate dehydrogenase, previously modified by either 2-BDB-TA-2′,5′-DP or 2-BOP-TA-2′,5′-DP, was measured by comparison of the fluorescence intensity of modified enzyme to the fluorescence of 2-BDB-TεA-2′,5′-DP standards as described previously (Bailey & Colman, 1985).

Determination of Decomposition Rate of Reagents. The rate of loss of bromide from the bromodioxobutyl and bromooxopropyl derivatives was measured by incubating 3 mM 2-BDB-TAMP, 2-BOP-TAMP, and 2-BDB-TεA-2′,5′-DP in 0.10 M triethanolamine acetate buffer, pH 7.0, or in 0.10 M MES buffer, pH 6.5. The 5′-monophosphate analogues of the new reagents were used for this study because of the greater ease of their preparation. At various times, 50-μL aliquots (150 nmol of reagent) were withdrawn and the concentration of free bromide was measured by a procedure modified from that of Zall et al. (1956), which has been previously described by Colman et al. (1984). The rate constant for decomposition of 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 concentrations.

Preparation of 2-BDB-TA-2′,5′-DP- and 2-BOP-TA-2′,5′-DP-Modified Enzyme. Isocitrate dehydrogenase (1.0 mg/mL) was incubated at 25 °C in the presence of 4 mM isocitrate

and 2 mM $MnSO_4$ with either 100 μM 2-BDB-TA-2′,5′-DP in 0.10 M triethanolamine chloride buffer, pH 7.0, or 200 μM 2-BOP-TA-2′,5′-DP in 0.10 M MES buffer, pH 6.5, in a total volume of 5 mL. The incubation was allowed to continue for 90 min, after which the solution was made 0.2 M in dithiothreitol. The enzyme was then extensively dialyzed at 4 °C against three changes of 2 L of 50 mM triethanolamine chloride buffer, pH 7.6, containing 10% glycerol and 0.5 mM EDTA. The dialyzed enzyme was centrifuged at 4 °C for 20 min at 14000 rpm to remove any precipitated or denatured protein, and the concentration was determined by 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.

Ultrafiltration Measurements. Ultrafiltration was performed at 25 °C in 0.10 M triethanolamine chloride buffer, pH 7.6, containing 10% glycerol and 0.5 mM EDTA. Protein and NADPH solutions of known concentrations were mixed in a total of 2 mL and placed in an Amicon ultrafiltration cell, Model 3, containing a Diaflo PM-10 membrane (Amicon). About 1 mL of the filtrate was collected following an initial equilibration of the solution at room temperature for 5–10 min. The initial concentration and concentration of free NADPH in the filtrate were measured spectrophotometrically by using an extinction coefficient of 6200 M^{-1} cm^{-1} at 340 nm.

RESULTS

Inactivation of Isocitrate Dehydrogenase by 2-BDB-TA-2′,5′-DP. Incubation of isocitrate dehydrogenase with 100 μM 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 2′,5′-bisphosphate at pH 7.0 and 25 °C resulted in a time-dependent inactivation of the enzyme as shown in Figure 3 (line A). In contrast, no change in activity was observed over this same time period in control enzyme in the absence of 2-BDB-TA-2′,5′-DP. Biphasic inactivation kinetics were observed that could be described in terms of a fast initial phase of inactivation resulting in partially active enzyme of 6% residual activity followed by a much slower phase leading to total inactivation. The solid line in Figure 3 (line A) is a theoretical line based on a computer fit to eq 1 as described under Experimental Procedures, while the points are experimental.

One possible explanation for the observed biphasic kinetics might be reagent decomposition. The rate constant for decomposition of the analogous 2-BDB-TAMP, under the conditions used for incubation with isocitrate dehydrogenase, was determined as 0.0134 min^{-1} ($t_{1/2}$ = 52 min) by measuring the release of free bromide as described under Experimental Procedures. This agrees well with the rate of decomposition of 0.0139 min^{-1} ($t_{1/2}$ = 50 min) measured for 2-BDB-TεA-2′,5′-DP from the release of free bromide, indicating that the etheno ring on 2-BDB-TεA-2′,5′-DP has no effect on the rate of bromide loss. Since the second phase of inactivation predominates by 10 min into the reaction time (Figure 3, line A), 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.

Effect of Ligands on Inactivation of Isocitrate Dehydrogenase by 2-BDB-TA-2′,5′-DP. $NADP^+$, NADPH, and isocitrate–Mn^{2+} were tested for their abilities to protect against inactivation by 100 μM 2-BDB-TA-2′,5′-DP (Figure 3) by including them in the incubation mixture at concentrations much higher than their known binding constants (Ehrlich & Colman, 1978; Mas & Colman, 1985). In the presence of 200 μM NADPH (Figure 3, line B), marked protection against

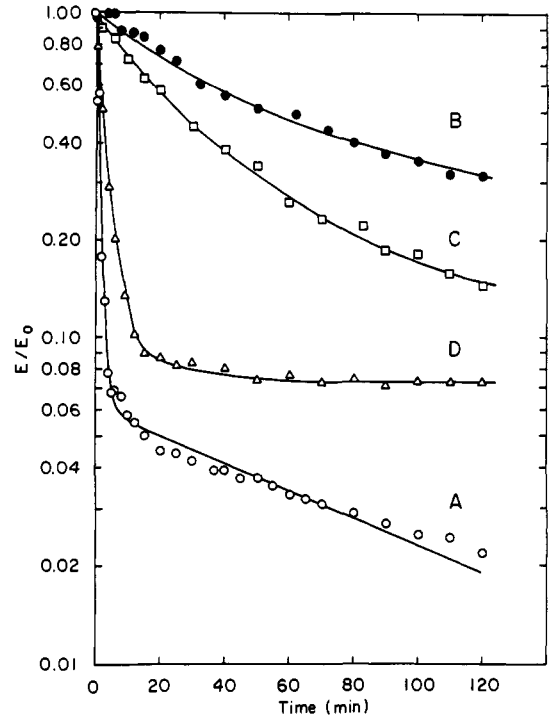

FIGURE 3: Effect of ligands on inactivation of isocitrate dehydrogenase by 2-BDB-TA-2′,5′-DP. Isocitrate dehydrogenase (0.12 mg/mL) was incubated at 25 °C in 0.10 M triethanolamine chloride buffer, pH 7.0, containing 100 μM 2-BDB-TA-2′,5′-DP in the absence of protecting ligands (line A) (○) and in the 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} = 0.90 min^{-1} and k_{slow} = 0.0098 min^{-1}, in the absence of ligands (line A) were calculated as described in the text. The open circles are experimental data, while the solid line (line A) is a theoretical line, generated from a computer fit to eq 1. The rate constant of inactivation in the presence of isocitrate and $MnSO_4$ (△) was calculated as described in the text and found to be 0.26 min^{-1}.

inactivation is observed. Although $NADP^+$ (Figure 3, line C) does not provide as much protection against inactivation by 2-BDP-TA-2′,5′-DP as does NADPH, it causes a drastic decrease in the rate of inactivation as compared to line A (Figure 3). Higher concentrations of $NADP^+$ (3 mM) caused no further decrease in the inactivation as compared with that observed in the presence of 600 μM $NADP^+$ (Figure 3, line C). In the presence of 4 mM isocitrate and 2 mM $MnSO_4$ (Figure 3, line D), complete protection against the slow phase is observed, with the rate of inactivation leveling off at 7.3% residual activity. This same effect was observed with 4 mM isocitrate alone, but not with 2 mM Mn^{2+} alone (data not shown). The rate constant in the presence of isocitrate–Mn^{2+} and isocitrate alone 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. Rate constants of 0.26 min^{-1} and 0.39 min^{-1} were calculated in the presence of isocitrate–Mn^{2+} and isocitrate alone, respectively. These rates are 29% and 43%, respectively, of that calculated for k_{fast} in the absence of ligands (Figure 3, line A).

To test for specificity among the ligands that protect against modification by 2-BDB-TA-2′,5′-DP, NAD^+ was evaluated for its effect on the reaction. The addition of 600 μM NAD^+ to the reaction mixture did not affect the inactivation rate (data not shown), which is consistent with evidence that NAD^+ is a poor coenzyme and is not an effective inhibitor for this $NADP^+$-dependent isocitrate dehydrogenase (Ehrlich & Colman, 1978). Only nucleotides with a 2′-phosphate appear

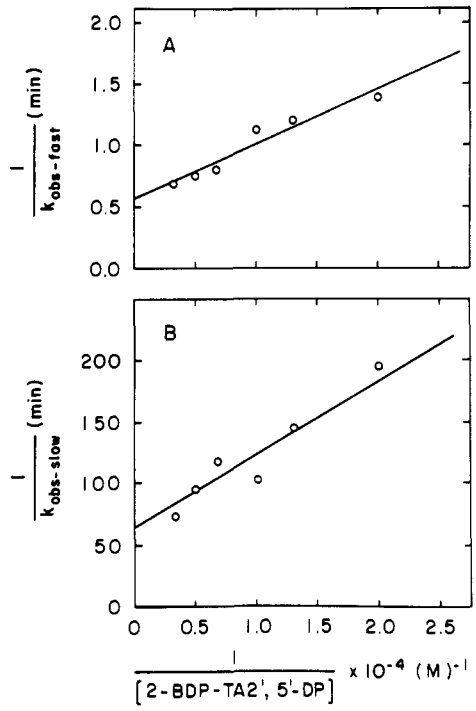

FIGURE 4: Dependence of pseudo-first-order rate constant (k_{obsd}) for inactivation of NADP$^+$-specific isocitrate dehydrogenase on concentration of 2-BDB-TA-2′,5′-DP. Isocitrate dehydrogenase (0.12 mg/mL) was incubated with 2-BDB-TA-2′,5′-DP (50–300 μM) at 25 °C in 0.10 M triethanolamine chloride buffer, pH 7.0, as described under Experimental Procedures. Rate constants were determined by computer fit to eq 1 using a value of $F = 0.06$. (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 2.

to bind to the site modified by 2-BDB-TA-2′,5′-DP.

Rate of Reaction of Isocitrate Dehydrogenase as a Function of 2-BDB-TA-2′,5′-DP Concentration. In order to characterize further the reaction of 2-BDB-TA-2′,5′-DP with isocitrate dehydrogenase, the dependence of the rates of inactivation on the concentration of 2-BDB-TA-2′,5′-DP was determined. At all concentrations of 2-BDB-TA-2′,5′-DP tested (50–300 μM), biphasic kinetics were observed, and the rate constants could be calculated by computer fit to eq 1 with a constant ordinate intercept, F, of $E/E_0 = 0.06$. (The data were evaluated by using $F = 0.05$–0.07, but the best fit for most of the data was found by using $F = 0.06$.) A constant value of F, independent of the concentration of 2-BDB-TA-2′,5′-DP, 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 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 2-BDB-TA-2′,5′-DP (I) is described by the equation

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

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). From the double-reciprocal plots shown in Figure 4, values of $K_I = 79$ μM and $k_{max} = 1.78$ min^{-1} were calculated for the fast phase, with values of $K_I = 94$ μM and $k_{max} = 0.016$ min^{-1} for the slow phase.

Inactivation of Isocitrate Dehydrogenase by 2-BOP-TA-2′,5′-DP. The kinetics of reaction of 2-BOP-TA-2′,5′-DP with isocitrate dehydrogenase were examined at pH 6.5 rather than pH 7.0 (as was done with 2-BDB-TA-2′,5′-DP) because of the

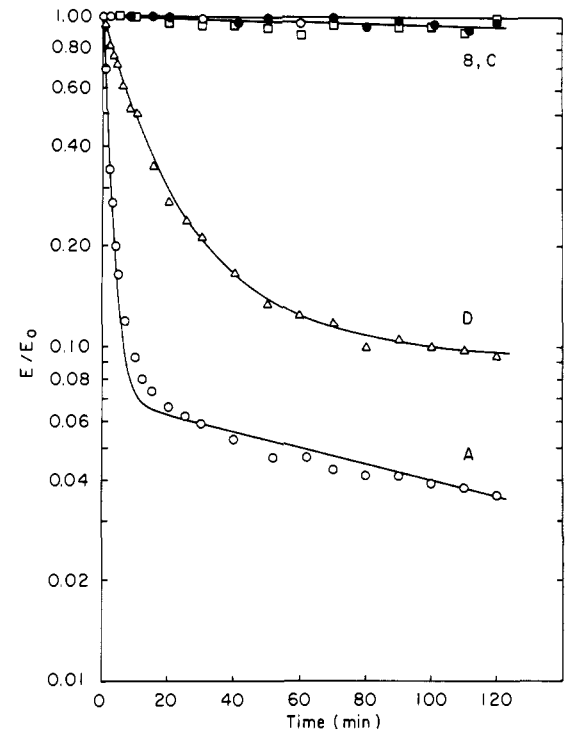

FIGURE 5: Effect of ligands on inactivation of isocitrate dehydrogenase by 2-BOP-TA-2′,5′-DP. Isocitrate dehydrogenase (0.12 mg/mL) was incubated at 25 °C in 0.10 M MES buffer, pH 6.5, containing 200 μM 2-BOP-TA-2′,5′-DP in the absence of protecting ligands (line A) (○) and in the presence of 200 μM NADPH (line B) (●), 3 mM 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 $k_{fast} = 0.53$ min^{-1} and $k_{slow} = 0.0055$ min^{-1} in the absence of ligands (line A) were calculated as described in the text. The open circles are experimental data, while the solid line (line A) is a theoretical line generated from a computer fit to eq 1, using a value of $F = 0.07$. The rate constant of inactivation (k_{fast}) in the presence of isocitrate and $MnSO_4$ (△) was calculated as described in the text and found to be 0.064 min^{-1}.

higher decomposition rate for the monoketo derivative of the nucleotide as compared with the diketo compound, 2-BDB-TA-2′,5′-DP. As determined by the release of bromide ion, the rate constants for decomposition of 2-BOP-TAMP at pH 7.0 and pH 6.5 were 0.0267 min^{-1} ($t_{1/2} = 26$ min) and 0.017 min^{-1} ($t_{1/2} = 41$ min), respectively.

Incubation of isocitrate dehydrogenase with 200 μM 2-[(3-bromo-2-oxopropyl)thio]adenosine 2′,5′-bisphosphate at pH 6.5 and 25 °C resulted in a time-dependent inactivation of the enzyme as shown in Figure 5. No change in activity was observed over this time period in control enzyme in the absence of 2-BOP-TA-2′,5′-DP. As seen with 2-BDB-TA-2′,5′,DP, biphasic kinetics of inactivation were observed with 2-BOP-TA-2′,5′-DP that could be described in terms of a fast initial phase of inactivation resulting in partially active enzyme of 7% residual activity followed by a slower phase leading to total inactivation. The solid line (line A) in Figure 5 is a theoretical line based on a computer fit of eq 1 as described under Experimental Procedures, while the points are experimental.

Effect of Ligands on Inactivation of Isocitrate Dehydrogenase by 2-BOP-TA-2′,5′-DP. NADP$^+$, NADPH, and isocitrate–Mn^{2+} were tested for their abilities to protect against inactivation by 200 μM 2-BOP-TA-2′,5′-DP (Figure 5) by including them in the incubation mixture as described for the reaction of 2-BDB-TA-2′,5′-DP with isocitrate dehydrogenase. Line A (Figure 5) shows the biphasic inactivation of isocitrate

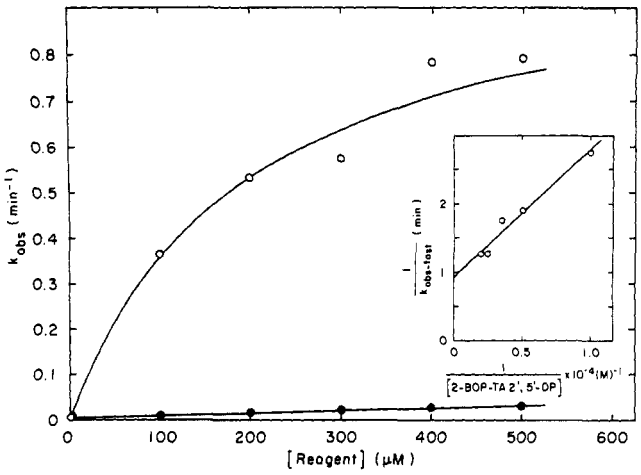

FIGURE 6: Dependence of pseudo-first-order rate constant (k_{obsd}) for inactivation of NADP+-specific isocitrate dehydrogenase on concentration of 2-BOP-TA-2',5'-DP (○) and bromoacetone (●). Isocitrate dehydrogenase (0.12 mg/mL) was incubated with 2-BOP-TA-2',5'-DP (100–500 μM) and bromoacetone (100–500 μM) at 25 °C in 0.10 M MES buffer, pH 6.5, as described under Experimental Procedures. Rate constants for 2-BOP-TA-2',5'-DP were determined by computer fit to eq 1 using a value of $F = 0.07$. Rate constants for bromoacetone were determined as described in the text. Inset: Double-reciprocal plot in accordance with eq 2 for the fast phase of inactivation by 2-BOP-TA-2',5'-DP.

dehydrogenase in the absence of ligands. In the presence of 200 μM NADPH or 3 mM NADP+ (Figure 5, lines B and C), complete protection against inactivation is observed. In the presence of 4 mM isocitrate and 2 mM $MnSO_4$ (line D), the rate of the fast phase of inactivation (with $F = 0.07$) is reduced to 0.064 min^{-1}, a value which is 12% of that observed for k_{fast} in the absence of ligands. In the presence of 4 mM isocitrate alone or 2 mM $MnSO_4$ alone, decreased rates of inactivation were observed, but unlike the reaction with 2-BDB-TA-2',5'-DP neither of these ligands protected completely against the slow phase of inactivation. Rate constants of 0.14 min^{-1} and 0.15 min^{-1}, were measured in the presence of isocitrate alone and Mn^{2+} alone, which are 27% and 28%, respectively, of k_{fast} in the absence of ligands. As with 2-BDB-TA-2',5'-DP, the addition of 600 μM NAD^+ to the reaction mixture had no effect on the rate of inactivation by 2-BOP-TA-2',5'-DP.

Rate of Reaction of Isocitrate Dehydrogenase as a Function of 2-BOP-TA-2',5'-DP Concentration. Isocitrate dehydrogenase was incubated with different concentrations of 2-BOP-TA-2',5'-DP (Figure 6, open circles) in order to determine the dependence of the rates of inactivation on the concentration of 2-BOP-TA-2',5'-DP. At all concentrations of 2-BOP-TA-2',5'-DP tested (100–500 μM), biphasic kinetics were observed, and the rate constants could be calculated by computer fit to eq 1 with a constant ordinate intercept, F, of $E/E_0 = 0.07$. For the fast phase, a nonlinear dependence of the rate constant of inactivation on reagent concentration was observed, whereas for the slow phase the rates of inactivation were too slow to obtain an accurate saturation curve. The data for the fast phase were analyzed according to eq 2, and from the double-reciprocal plot shown in the inset of Figure 6, values of $K_I = 194$ μM and $k_{max} = 1.05$ min^{-1} were calculated.

Reaction of Bromoacetone with Isocitrate Dehydrogenase. Incubation of isocitrate dehydrogenase with bromoacetone at pH 6.5 and 25 °C resulted in a time-dependent inactivation of the enzyme; the data were best fit by a model of biphasic kinetics with $F = 0.07$. Rate constants were calculated from a semilogarithmic plot of $[(E/E_0)_t - F]/(1 - F)$ as a function of time in order to compare the inactivation rate constants for bromoacetone with k_{fast} for 2-BOP-TA-2',5'-DP in the absence of ligands. NADPH and isocitrate–Mn^{2+} were tested for their abilities to protect against inactivation by 400 μM bromoacetone. In the presence of 200 μM NADPH, a pseudo-first-order rate constant for inactivation of 0.0170 min^{-1} was calculated, while in the presence of 4 mM isocitrate and 2 mM $MnSO_4$ a rate constant of 0.0025 min^{-1} was obtained; these rates are 65% and 9.6%, respectively, of the rate constant of 0.0261 min^{-1} observed in the absence of ligands.

When isocitrate dehydrogenase was incubated with different concentrations of bromoacetone (100–500 μM), a linear dependence of the rate constant of inactivation on the reagent concentration was observed (Figure 6, closed circles), indicating that, in contrast to 2-BOP-TA-2',5'-DP, a reversible enzyme–reagent complex is not detected prior to irreversible modification. At 200 μM, the rate constant for inactivation by 2-BOP-TA-2',5'-DP was 32 times greater than that for bromoacetone. A second-order rate constant of 58.3 M^{-1} min^{-1} was calculated for reaction of bromoacetone with isocitrate dehydrogenase. A comparison of the rate constants for inactivation of isocitrate dehydrogenase by bromoacetone and 2-BOP-TA-2',5'-DP shows a large rate enhancement was achieved by placing the reactive bromoacetone group on a nucleotide specific for the coenzyme binding site. This is expected for an affinity label (Colman, 1983b).

Incorporation of 2-BDB-TA-2',5'-DP and 2-BOP-TA-2',5'-DP by Isocitrate Dehydrogenase. Isocitrate dehydrogenase was incubated with either 100 μM 2-BDB-TA-2',5'-DP or 200 μM 2-BOP-TA-2',5'-DP, in the absence and presence of ligands, as described under Experimental Procedures. The incorporation of reagent into enzyme was measured at various times during the incubation by quantitation of organic phosphorus.

Addition of dithiothreitol was found to quench rapidly the reaction between reagent and enzyme. No incorporation of reagent was observed if dithiothreitol was added before the addition of 2-BDB-TA-2',5'-DP or 2-BOP-TA-2',5'-DP, indicating that all free reagent is removed by the gel filtration procedure used.

Incorporation of 2-BDB-TA-2',5'-DP (moles of reagent per mole of enzyme subunit) under two sets of conditions is shown in Figure 7A. In the absence of ligands (Figure 7A, open circles) 1.61 mol of reagent/mol of subunit is incorporated with only 2.2% activity remaining at 120 min. This incorporation of reagent into the enzyme is reduced in the presence of 4 mM isocitrate and 2 mM $MnSO_4$ (Figure 7A, closed circles): a maximum of 1.0 mol of 2-BDB-TA-2',5'-DP/mol of enzyme subunit is incorporated when the enzyme has only 7.3% of its activity remaining. Since isocitrate and $MnSO_4$ protect only against the slow phase of inactivation (Figure 3, line D), these results suggest that the fast phase of inactivation may be caused by incorporation of 1.0 mol of reagent/mol of enzyme subunit. Table IV compares the incorporation at 60 and 120 min in the absence and presence of protecting ligands. Although each of the coenzymes, NADP+ and NADPH, can protect significantly against inactivation, incorporation of 2-BDB-TA-2',5'-DP still occurs.

The incorporation of 2-BOP-TA-2',5'-DP in the absence of ligands is shown in Figure 7B (open circles); 1.78 mol of reagent are incorporated when the enzyme has only 3.6% activity remaining. As with 2-BDB-TA-2',5'-DP, the incorporation of 2-BOP-TA-2',5'-DP is reduced in the presence of 4 mM isocitrate and 2 mM $MnSO_4$. A maximum of 1.2 mol of reagent/mol of enzyme subunit is incorporated (Figure 7B, solid circles). Table V compares the incorporation of 2-

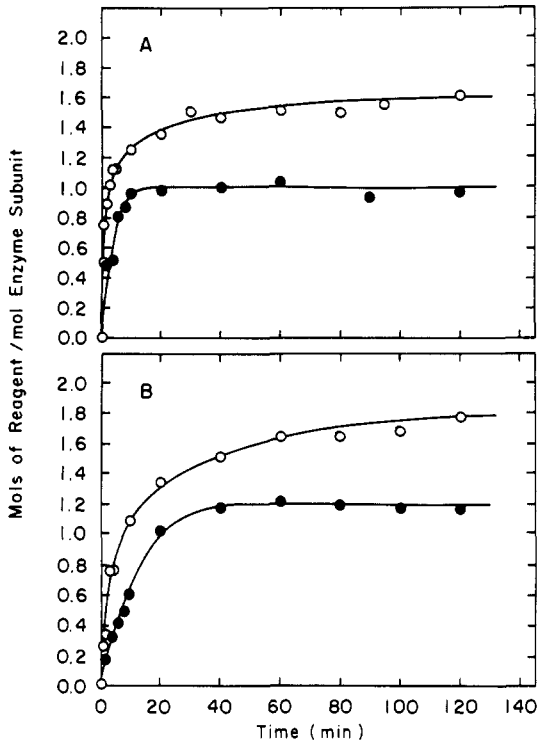

FIGURE 7: Incorporation of (A) 2-BDB-TA-2′,5′-DP and (B) 2-BOP-TA-2′,5′-DP per mole of subunit of isocitrate dehydrogenase as a function of time in the absence (○) and presence (●) of 4 mM isocitrate and 2 mM $MnSO_4$. Isocitrate dehydrogenase (0.8 mg/mL) was incubated at 25 °C in 0.10 M triethanolamine chloride buffer, pH 7.0, with 100 μM 2-BDB-TA-2′,5′-DP and in 0.10 M MES buffer, pH 6.5, with 200 μM 2-BOP-TA-2′,5′-DP. Incorporation at the indicated time points was determined as described under Experimental Procedures.

Table IV: Incorporation of 2-BDB-TA-2′,5′-DP in the Absence and Presence of Protecting Ligands[a]

	60 min		120 min	
	incorporation	% act. remaining	incorporation	% act. remaining
no ligands	1.52	3	1.61	2
4 mM isocitrate, 2 mm $MnSO_4$	1.04	8	0.97	7
600 μM $NADP^+$	1.18	26	1.66	15
200 μM NADPH	0.84	52	1.11	31

[a] Isocitrate dehydrogenase (0.8 mg/mL) was incubated with 100 μM 2-BDB-TA-2′,5′-DP in 0.10 M triethanolamine chloride buffer, pH 7.0, in the absence and presence of protecting ligands. Incorporation was determined as described under Experimental Procedures and is expressed as moles of reagent per mole of enzyme subunit.

BOP-TA-2′,5′-DP at 60 and 120 min in the absence and presence of protecting ligands. Although both $NADP^+$ and NADPH can protect completely against inactivation, measurable reagent incorporation (albeit reduced) is observed when these coenzymes are present. It is probable that although these ligands are protecting against modification by 2-BOP-TA-2′,5′-DP at a specific coenzyme site, some nonspecific modification is occurring elsewhere on the protein.

As isocitrate–Mn^{2+} can protect against the slow phase of inactivation of isocitrate dehydrogenase by 2-BDB-TA-2′,5′-DP and 2-BOP-TA-2′,5′-DP and the maximum incorporation observed by these reagents in the presence of isocitrate and Mn^{2+} is 1.0 mol of reagent/mol of subunit and 1.2 mol of reagent/mol of subunit, respectively, it is possible that modification by either of these reagents to the extent of 1 mol of reagent/mol of subunit is responsible for the fast phase of the reaction.

Table V: Incorporation of 2-BOP-TA-2′,5′-DP in the Absence and Presence of Protecting Ligands[a]

	60 min		120 min	
	incorporation	% act. remaining	incorporation	% act. remaining
no ligands	1.65	5	1.78	4
4 mM isocitrate, 2 mm $MnSO_4$	1.22	13	1.17	10
3 mM $NADP^+$	0.30	90	0.37	99
200 μM NADPH	0.46	96	0.57	97

[a] Isocitrate dehydrogenase (0.8 mg/mL) was incubated with 200 μM 2-BOP-TA-2′,5′-DP in 0.10 M MES buffer, pH 6.5, in the absence and presence of protecting ligands. Incorporation was determined as described under Experimental Procedures and is expressed as moles of reagent per mole of enzyme subunit.

Table VI: Determination of Stoichiometry of NADPH Binding to Native and 2-BDB-TA-2′,5′-DP- and 2-BOP-TA-2′,5′-DP-Modified Enzymes[a]

enzyme	enzyme subunits (μM)	total NADPH (μM)	free NADPH (μM)	mol of bound NADPH/mol of enzyme subunit
native	24.0	66.1	43.0	0.96
2-BDB-TA-2′,5′-DP modified[b]	23.6	46.8	35.3	0.49
2-BOP-TA-2′,5′-DP modified[c]	18.6	60.0	49.7	0.55

[a] The ultrafiltration experiments were performed at 25 °C in 50 mM triethanolamine chloride buffer, pH 7.6, containing 10% glycerol and 0.5 mM EDTA, as described under Experimental Procedures. [b] Isocitrate dehydrogenase (1 mg/mL) was modified by 100 μM 2-BDB-TA-2′,5′-DP in the presence of 4 mM isocitrate and 2 mM $MnSO_4$ as described under Experimental Procedures. The modified enzyme, with an incorporation of 1 mol of reagent/mol of enzyme subunit, had 11% residual activity. [c] Isocitrate dehydrogenase (1 mg/mL) was modified by 200 μM 2-BOP-TA-2′,5′-DP in the presence of 4 mM isocitrate and 2 mM $MnSO_4$ as described under Experimental Procedures. The modified enzyme, with an incorporation of 1.2 mol of reagent/mol of enzyme subunit, had 23% residual activity.

Stoichiometry of NADPH Binding to Native and Modified Isocitrate Dehydrogenase. Since the enzyme has some residual activity remaining after modification in the presence of isocitrate and Mn^{2+}, it is important to ascertain whether one or both of the coenzyme sites per enzyme dimer are blocked by modification with reagent. The stoichiometry of reversible NADPH binding to native enzyme and enzyme modified by either reagent in the presence of isocitrate and Mn^{2+} was determined by ultrafiltration and is shown in Table VI. Native isocitrate dehydrogenase binds 1 mol of NADPH/mol of subunit. This is identical with the stoichiometry previously determined by fluorescence titration (Mas & Colman, 1985). Enzyme with 1.0 mol of 2-BDB-TA-2′,5′-DP/mol of subunit was 11% residual activity and can still bind 0.5 mol of NADPH/mol of subunit or 1 mol of coenzyme/mol of enzyme dimer. This result implies that 2-BDB-TA-2′,5′-DP is specifically modifying the coenzyme site of only one subunit of the enzyme dimer. These results are similar to the previously observed maximum NADPH binding of 0.5 mol/mol of average enzyme subunit for enzyme modified by the fluorescent 2-BDB-TεA-2′,5′-DP (in the presence of isocitrate and Mn^{2+}) at one site per enzyme dimer (Bailey & Colman, 1987b). In that case, the modified enzyme exhibits a dissociation constant 1000 times higher than that of native enzyme as determined by Scatchard analysis; but K_d for the NADPH-modified enzyme complex is still as low as 0.57 μM, indicating that conditions of saturation of enzyme with coenzyme are readily attained.

Isocitrate dehydrogenase modified by 2-BOP-TA-2′,5′-DP in the presence of isocitrate and $MnSO_4$ has an incorporation

of 1.2 mol of reagent/mol of subunit with 23% residual activity and, like the 2-BDB-TA-2′,5′-DP modified enzyme, can bind 0.5 mol of NADPH/mol of enzyme subunit. These results imply that, with each reagent, only 0.5 mol of reagent/mol of subunit of the total 1.0 mol of reagent/mol of subunit and 1.2 mol of reagent/mol of subunit for 2-BDB-TA-2′,5′-DP and 2-BOP-TA-2′,5′-DP, respectively, is specific for the coenzyme binding site. The remaining incorporation of 0.5 and 0.7 mol/mol of subunit for each reagent may result from a nonspecific modification of isocitrate dehydrogenase.

Reaction of 2-BDB-TεA-2′,5′-DP with Modified Isocitrate Dehydrogenase. Reaction of isocitrate dehydrogenase with 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate (2-BDB-TεA-2′,5′-DP) resulted in enzyme with 24% residual activity and an incorporation of 0.5 mol of reagent/mol of enzyme subunit when the reaction was carried out in the presence of 4 mM isocitrate and 2 mM $MnSO_4$. This reaction was shown to occur specifically at the coenzyme binding site of one subunit of the enzyme dimer (Bailey & Colman, 1985). When isocitrate dehydrogenase was first modified in the presence of isocitrate and $MnSO_4$ by either 2-BDB-TA-2′,5′-DP or 2-BOP-TA-2′,5′-DP as described under Experimental Procedures and then reacted with 75 μM 2-BDB-TεA-2′,5′-DP in the presence of 4 mM isocitrate and 2 mm $MnSO_4$, no further inactivation by or incorporation of 2-BDB-TεA-2′,5′-DP was observed. This result indicates that modification of isocitrate dehydrogenase by 2-BDB-TA-2′,5′-DP or 2-BOP-TA-2′,5′-DP blocks modification by 2-BDB-TεA-2′,5′-DP, and suggests that 2-BDB-TA-2′,5′-DP and 2-BOP-TA-2′,5′-DP both can attack the same site on isocitrate dehydrogenase that is modified by 2-BDB-TεA-2′,5′-DP.

Discussion

The evidence presented here demonstrates that 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 2′,5′-bisphosphate and 2-[(3-bromo-2-oxopropyl)thio]adenosine 2′,5′-bisphosphate react with pig heart $NADP^+$-dependent isocitrate dehydrogenase and exhibit many characteristics expected for an affinity label. Both compounds react covalently with a limited number of sites on the enzyme: 1.61 mol of 2-BDB-TA-2′,5′-DP/mol of subunit and 1.78 mol of 2-BOP-TA-2′,5′-DP/mol of subunit are incorporated when the enzyme is 98% and 96% inactive, respectively. The rate constants for reaction 2-BDB-TA-2′,5′-DP and 2-BOP-TA-2′,5′-DP exhibit a nonlinear dependence on reagent concentration, indicating the formation of a reversible enzyme–reagent complex prior to irreversible modification.

The interaction of the fluorescent 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate (2-BDB-TεA-2′,5′-DP) with isocitrate dehydrogenase has previously been studied (Bailey & Colman, 1985). This reagent is identical with 2-BDB-TA-2′,5′-DP described here except for the additional etheno ring. A comparison of the kinetics of 2-BDB-TεA-2′,5′-DP with 2-BDB-TA-2′,5′-DP should allow an evaluation of the effect of the etheno ring on interaction with isocitrate dehydrogenase. The kinetics of inactivation of isocitrate dehydrogenase by these two reagents are qualitatively similar: both reagents produce biphasic kinetics of inactivation in the absence of protecting ligands that can be described in terms of a fast initial phase of inactivation resulting in partially active enzyme of 6–10% residual activity, followed by a much slower phase leading to total inactivation. Quantitatively, the rate constant for inactivation is faster for the rapid initial phase with 2-BDB-TA-2′,5′-DP than with 2-BDB-TεA-2′,5′-DP, whereas the rate constants for the slow phase are similar for these two reagents. With 2-BDB-TεA-2′,5′-DP a $k_{max} = 0.55\ min^{-1}$ and $k_{max} = 0.019\ min^{-1}$ for the fast and slow phases, respectively, were calculated, whereas with 2-BDB-TA-2′,5′-DP $k_{max} = 1.78\ min^{-1}$ and $k_{max} = 0.016\ min^{-1}$ for the fast and slow phases, respectively. The similarity of reaction of 2-BDB-TεA-2′,5′-DP and 2-BDB-TA-2′,5′-DP with isocitrate dehydrogenase indicates that a free 6-amino group is not essential for binding or reaction of the nucleotide analogue with this enzyme and also that the etheno ring does not appreciably interfere with or distort the binding of reagent.

The interaction of 2-BOP-TA-2′,5′-DP with isocitrate dehydrogenase can be compared with that of 2-BDB-TA-2′,5′-DP to ascertain the effect of decreasing the length of the reactive group by one carbonyl. As with 2-BDB-TA-2′,5′-DP and 2-BDB-TεA-2′,5′-DP, the reaction of 2-BOP-TA-2′,5′-DP with isocitrate dehydrogenase exhibits biphasic kinetics of inactivation. A $k_{max} = 1.05\ min^{-1}$ was calculated for the fast initial phase of inactivation; this value is somewhat slower than that observed with 2-BDB-TA-2′,5′-DP.[2] The slow phase, however, was considerably decreased as compared with that observed for 2-BDB-TA-2′,5′-DP, so an accurate measure of k_{max} could not be made. The similarity of the reaction of 2-BOP-TA-2′,5′-DP and 2-BDB-TA-2′,5′-DP with isocitrate dehydrogenase suggests that the bromooxopropyl derivative may set a maximum distance between the reacting amino acid side chain and the bromoketo group on the nucleotide analogue. The faster reaction observed with the bromodioxobutyl reagent may be due to a more optimal distance between the reactive bromoketo group and this reactive amino acid side chain.

The coenzymes NADPH and $NADP^+$ provide complete protection against inactivation by 2-BOP-TA-2′,5′-DP but only partial protection against inactivation by 2-BDB-TA-2′,5′-DP. These results indicate that 2-BOP-TA-2′,5′-DP may be more specific for the coenzyme site than is 2-BDB-TA-2′,5′-DP.

Isocitrate and Mn^{2+} decrease the rate constant of the fast phase for all three reagents, albeit not to the same extent. The rate constant for inactivation by 2-BOP-TA-2′,5′-DP in the presence of isocitrate with $MnSO_4$ is 12% of that observed for k_{fast} in the absence of ligands, whereas the rate constants for 2-BDB-TA-2′,5′-DP and 2-BDB-TεA-2′,5′-DP in the presence of isocitrate with $MnSO_4$ are respectively 29% and 35% of k_{fast} in the absence of ligands.

Isocitrate dehydrogenase is modified to a maximum extent of 0.5 mol of reagent/mol of enzyme subunit by 2-BDB-TεA-2′,5′-DP in the presence of isocitrate and $MnSO_4$ (Bailey & Colman, 1985). This reaction has been shown to occur at the coenzyme site of one subunit of the enzyme dimer. When isocitrate dehydrogenase previously modified by either 2-BDB-TA-2′,5′-DP or 2-BOP-TA-2′,5′-DP in the presence of isocitrate–Mn^{2+} was incubated with 2-BDB-TεA-2′,5′-DP in the presence of substrate, no incorporation of the fluorescent reagent was observed. This result indicates that both 2-BDB-TA-2′,5′-DP and 2-BOP-TA-2′,5′-DP block subsequent reaction of 2-BDB-TεA-2′,5′-DP, suggesting that the enzyme sites attacked by 2-BDB-TA-2′,5′-DP and 2-BOP-TA-2′,5′-DP include those that react with the fluorescent compound. Of the total of 1.0 mol of reagent/mol of subunit observed for 2-BDB-TA-2′,5′-DP and 1.2 mol of reagent/mol of subunit measured for 2-BOP-TA-2′,5′-DP in the presence of isocitrate

[2] For a more valid comparison of all three reagents, the kinetics of inactivation of isocitrate dehydrogenase by 200 μM 2-BDB-TεA-2′,5′-DP and 200 μM 2-BDB-TA-2′,5′-DP were also examined in 0.10 M MES buffer, pH 6.5. The inactivation of isocitrate dehydrogenase observed by both of these reagents at pH 6.5 was similar to that at pH 7.0. Both reagents exhibited biphasic kinetics of inactivation. The rate constants of inactivation for both phases and also for both reagents were approximately 1.5 times faster at pH 6.5 than at pH 7.0.

and Mn^{2+}, 0.5 mol may be due to reaction at the same site modified by 2-BDB-TεA-2′,5′-DP (the coenzyme site on one subunit of the enzyme dimer). This postulate is supported by the observation that enzyme separately modified by 2-BDB-TA-2′,5′-DP, 2-BOP-TA-2′,5′-DP, and 2-BDB-TεA-2′,5′-DP in the presence of isocitrate and $MnSO_4$ all bind ~0.5 mol of NADPH/mol of enzyme subunit, indicating that only one of the two coenzyme binding sites per dimeric enzyme is blocked. It is notable that the presence of the etheno ring on 2-BDB-TεA-2′,5′-DP apparently prevents the nonspecific incorporation seen with 2-BDB-TA-2′,5′-DP and 2-BOP-TA-2′,5′-DP when the enzyme is modified in the presence of isocitrate and $MnSO_4$.

The biphasic kinetics of inactivation observed with 2-BDB-TεA-2′,5′-DP have been shown to be due to the fast specific modification at the coenzyme site of one subunit of the enzyme dimer (0.5 mol of reagent/mol of subunit) and the slow nonspecific interaction of reagent with enzyme (Bailey & Colman, 1987a). A single modified triskaidekapeptide has been isolated from a proteolytic digest of enzyme labeled with the fluorescent 2-BDB-TεA-2′,5′-DP in the presence of isocitrate and Mn^{2+}, and a glutamate residue has been designated as the target of attack (Bailey & Colman, 1987a). On the basis of the similarities between the reactions with isocitrate dehydrogenase of 2-BDB-TεA-2′,5′-DP and these new reagents, it is proposed that 2-BDB-TA-2′,5′-DP and 2-BOP-TA-2′,5′-DP also interact specifically with the coenzyme site of one subunit of the enzyme dimer. Any observed incorporation of the new reagents greater than 0.5 mol of reagent/mol of enzyme subunit would likely result from reaction at additional sites.

Reaction of isocitrate dehydrogenase with bromoacetone provides a measure of the effect of linking the same reactive functional group to a nucleotide that binds to a particular site (2′,5′-ADP). Unlike 2-BOP-TA-2′,5′-DP, the rate constants for reaction of bromoacetone with isocitrate dehydrogenase exhibit a linear dependence on reagent concentration, providing no evidence for reversible enzyme–reagent complex formation prior to irreversible modification. Although NADPH protects completely against inactivation by 2-BOP-TA-2′,5′-DP, it does not appreciably decrease inactivation by bromoacetone. The rate constant for inactivation by bromoacetone of isocitrate dehydrogenase is at least 1 order of magnitude slower than that measured for 2-BOP-TA-2′,5′-DP at the same concentration. These results indicate that attachment of bromoacetone to the nucleotide enhances the reaction rate and that the nucleotide specifically directs modification at the coenzyme binding site of isocitrate dehydrogenase.

The marked protection by isocitrate–Mn^{2+} against bromoacetone may indicate that bromoacetone actually modifies a group in the isocitrate binding site. The addition of isocitrate and Mn^{2+} causes the same decrease in the extent of inactivation against bromoacetone as against 2-BOP-TA-2′,5′-DP. As the adenine ring of bound coenzyme, as well as the coenzyme fragments, 2′,5′-ADP and PADPR, is in a syn conformation (Ehrlich & Colman, 1985), it is possible that 2-BOP-TA-2′,5′-DP (and BDB-TA-2′,5′-DP) is also bound in a syn conformation. As a result, the reactive bromoketo group may be modifying an amino acid side chain in the vicinity of the bound nicotinamide ring of $NADP^+$ (and hence in the vicinity of the isocitrate site), while the nucleotide part of the reagent is located in the adenosine ribose portion of the coenzyme binding site.

These new nucleotide analogues, 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 2′,5′-bisphosphate and 2-[(3-bromo-2-oxopropyl)thio]adenosine 2′,5′-bisphosphate, exhibit characteristics of affinity labels for isocitrate dehydrogenase. 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. We have reported on the synthesis of 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate (2-BDB-TεA-2′,5′-DP) (Bailey & Colman, 1985), which has the potential to react covalently with the side chains of several amino acids including cysteine (Batra & Colman, 1986), lysine, histidine, glutamate, and aspartate (Hartman, 1977) as well as with arginine (Yankeelov, 1970; Riordan, 1973); however, some enzymes may not tolerate the additional etheno substituent. These new reagents exhibit the natural 6-amino group which may be required for specific binding to certain enzymes, while retaining the ability to react with the same range of amino acid side chains as does 2-BDB-TεA-2′,5′-DP. Furthermore, availability of related nucleotide analogues with reactive side chains of different length may allow reaction to occur with residues at different distances from the nucleotide binding site. It is anticipated that these new nucleotide analogues will be complementary to the existent $NADP^+$ analogues and will have general applicability as affinity labels of $NADP^+$ binding enzymes.

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Accuracy of Alternative Representations for Integrated Biochemical Systems†

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ABSTRACT: The Michaelis–Menten formalism often provides appropriate representations of individual enzyme-catalyzed reactions in vitro but is not well suited for the mathematical analysis of complex biochemical networks. Mathematically tractable alternatives are the linear formalism and the power-law formalism. Within the power-law formalism there are alternative ways to represent biochemical processes, depending upon the degree to which fluxes and concentrations are aggregated. Two of the most relevant variants for dealing with biochemical pathways are treated in this paper. In one variant, aggregation leads to a rate law for each enzyme-catalyzed reaction, which is then represented by a power-law function. In the other, aggregation produces a composite rate law for either net rate of increase or net rate of decrease of each system constituent; the composite rate laws are then represented by a power-law function. The first variant is the mathematical basis for a method of biochemical analysis called metabolic control, the latter for biochemical systems theory. We compare the accuracy of the linear and of the two power-law representations for networks of biochemical reactions governed by Michaelis–Menten and Hill kinetics. Michaelis–Menten kinetics are always represented more accurately by power-law than by linear functions. Hill kinetics are in most cases best modeled by power-law functions, but in some cases linear functions are best. Aggregation into composite rate laws for net increase or net decrease of each system constituent almost always improves the accuracy of the power-law representation. The improvement in accuracy is one of several factors that contribute to the wide range of validity of this power-law representation. Other contributing factors that are discussed include the nonlinear character of the power-law formalism, homeostatic regulatory mechanisms in living systems, and simplification of rate laws by regulatory mechanisms in vivo.

Biochemical systems are often difficult to understand intuitively. Even simple feedback loops can exhibit very different types of behavior: system components may quickly return to the original or a new steady-state after perturbation, they may oscillate in a stable cycle, or the entire system may become unstable. It is evident that the repertoire of behavior for a system with many components and with activating and inhibiting interactions is much wider. The central problem in understanding the function of intact biochemical systems is the large number and the complex nonlinear character of interactions among the molecular constituents (Savageau, 1976, 1985a). This problem is in general too complex to be solved intuitively and requires systematic mathematical models and analyses.

Since the turn of the century, a variety of mathematical functions have been proposed that approximate single reactions reasonably well in vitro and presumably in vivo. The most familiar are the Michaelis–Menten rate law (Michaelis & Menten, 1913) and the Hill rate law (Brown & Hill, 1923). Both equations are simple rational functions, whose properties are readily calculated as long as only one reaction or a system of very few reactions is being studied. However, under physiological conditions, each reaction is embedded in an intricate network of reactions, subject to general buffers and specific modulations, which makes mathematical analysis with Michaelis–Menten or Hill equations or more complex rational functions (Monod et al., 1965; Koshland et al., 1966; Cleland,

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