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# Affinity Labeling of Bovine Liver Glutamate Dehydrogenase with 8-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 5′-Diphosphate and 5′-Triphosphate†

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*Received January 30, 1990; Revised Manuscript Received April 11, 1990*

ABSTRACT: Bovine liver glutamate dehydrogenase reacts with 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-diphosphate (8-BDB-TA-5′-DP) and 5′-triphosphate (8-BDB-TA-5′-TP) to yield enzyme with about 1 mol of reagent incorporated/mol of enzyme subunit. The modified enzyme is catalytically active but has decreased sensitivity to inhibition by GTP, reduced extent of activation by ADP, and diminished inhibition by high concentrations of NADH. Since modified enzyme, like native glutamate dehydrogenase, reversibly binds more than 1 mol each of ADP and GTP, it is unlikely that 8-BDB-TA-5′-TP reacts directly within either the ADP or GTP regulatory sites. The rate constant for reaction of enzyme exhibits a nonlinear dependence on reagent concentration with $K_D$ = 89 μM for 8-BDB-TA-5′-TP and 240 μM for 8-BDB-TA-5′-DP. The ligands ADP and GTP alone and NADH alone produce only small decreases in the rate constant for the reaction of enzyme with 8-BDB-TA-5′-TP, but the combined addition of 5 mM NADH + 200 μM GTP reduces the reaction rate constant more than 10-fold and the reagent incorporation to about 0.1 mol/mol of enzyme subunit. These results suggest that 8-BDB-TA-5′-TP reacts as a nucleotide affinity label in the region of the GTP-dependent NADH regulatory site of bovine liver glutamate dehydrogenase.

Bovine liver glutamate dehydrogenase [L-glutamate:NAD(P)$^+$ oxidoreductase (deaminating), EC 1.4.1.3] is an allosteric enzyme that is activated by ADP and is inhibited by GTP as well as by high concentrations of NADH, both of which bind to a regulatory coenzyme site distinct from the catalytic site (Colman, 1990). The allosteric inhibitor GTP occupies two sites per subunit of the enzyme in the presence of NADH but only one site in the absence of the reduced coenzyme (Pal & Colman, 1979). The allosteric activator ADP also binds to two sites per subunit (Batra & Colman, 1986a).

Previously, group-specific reagents have been used to chemically modify the amino acids in the regulatory and catalytic sites, but in many cases, high incorporation of the reagents complicated the interpretation of the results and the identification of the critical amino acids (Goldin & Frieden, 1972; Eisenberg et al., 1976). Approaches using purine nucleotide analogues as affinity labels have yielded more specific modifications of amino acids at the regulatory and catalytic sites (Colman, 1983).

This study is part of a systematic effort using the strategy of affinity labeling to locate regions of glutamate dehydrogenase critical for its regulatory behavior. The (fluorosulfonyl)benzoyl nucleosides (Colman, 1983, 1989) have their reactive functional groups at a position structurally equivalent to the pyrophosphate region of ATP, GTP, or the nicotinamide ribose of NADH. The adenine nucleotide analogue 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine was shown to be incorporated into glutamate dehydrogenase at the NADH inhibitory site to the extent of 3 mol/mol of hexameric enzyme, labeling equal amounts of lysine-420 and tyrosine-190 (Pal et al., 1975; Saradambal et al., 1981; Schmidt & Colman, 1984). The fluorescent analogues, 5′-[*p*-(fluorosulfonyl)-benzoyl]-1,$N^6$-ethenoadenosine and 5′-[*p*-(fluorosulfonyl)-benzoyl]guanosine have been shown to react covalently at a GTP inhibitory site (Pal & Colman, 1979; Jacobson & Colman, 1982). More recently, another class of affinity labels

† This research was supported by USPHS Grant DK 37000.

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has been prepared with reactive bromodioxobutyl groups adjacent to various positions of the purine ring. 6-[(4-Bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate labels the NADH regulatory site of glutamate dehydrogenase (Batra & Colman, 1984) concomitant with covalent reaction at cysteine-319 (Batra & Colman, 1986b). 2-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 5′-monophosphate reacts at histidine-82 and blocks an ADP site (Batra & Colman, 1986a; Batra et al., 1989).

Two new nucleotide analogues have been reported: 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-triphosphate and 5′-diphosphate (DeCamp et al., 1988). This paper evaluates the effect on the regulatory behavior of glutamate dehydrogenase of covalent reaction with these novel nucleotide analogues.

## Experimental Procedures

*Materials.* Bovine liver glutamate dehydrogenase was purchased from Boehringer Mannheim Corp. as a crystalline suspension in ammonium sulfate solution. It was dialyzed against two changes of 0.1 M potassium phosphate buffer, pH 7.1 over 18 h at 4 °C. The dialysate was centrifuged at 4 °C for 20 min at 15000 rpm to remove denatured protein. The concentration of protein in the supernatant was determined spectrophotometrically by using $E^{1\%}_{280} = 9.7$ (Olson & Anfinsen, 1952). The ratio of $A_{280}/A_{260}$ for the protein was 1.8. Enzyme was stored at −75 °C in aliquots and thawed rapidly prior to use. The molecular weight used for the identical peptide chains was 56 100 (Smith et al., 1970).

Coenzymes, nucleotides, EDTA, Tris, Malachite Green base, and Sephadex G-50–80 were purchased from Sigma. HEPES[1] was from Behring Diagnostics. [8-$^{14}$C]ADP and [8-$^{14}$C]GTP were supplied by New England Nuclear Corp. All other chemicals were reagent grade.

8-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 5′-diphosphate (8-BDB-TA-5′-DP) and 5′-triphosphate (8-BDB-TA-5′-TP) were synthesized as previously described (DeCamp et al., 1988). The yield was 50–60%, and the product was stored desiccated at −75 °C.

*Enzymatic Assay.* Bovine liver glutamate dehydrogenase activity was measured at 25 °C in a Gilford Model 240 spectrophotometer with an expanded scale (0.0–0.1) by monitoring the oxidation of NADH ($\epsilon_{340nm} = 6.22 \times 10^3$ M$^{-1}$ cm$^{-1}$) in Tris–0.01 M acetate buffer, pH 8.0, containing 10 μM EDTA. The assay mixture contained 100 μM NADH, 5 mM α-ketoglutarate, and 50 mM ammonium chloride in a total volume of 1.0 mL. The GTP concentration was 1 μM when the activity was measured in the presence of this allosteric inhibitor.

The conditions and the substrate concentrations used in determining the effects of varying concentrations of regulatory nucleotides such as GTP and ADP were the same as above, except that at NADH concentrations of 0.2 mM and above assays were followed at 375 nm ($\epsilon = 1.85 \times 10^3$ M$^{-1}$ cm$^{-1}$). In order to evaluate the effect on velocity of varying the zinc concentration, the EDTA was excluded from the Tris–0.01 M acetate buffer, but all other conditions were the same.

*Reaction of 8-BDB-TA-5′-DP and 8-BDB-TA-5′-TP with Bovine Liver Glutamate Dehydrogenase.* The enzyme (0.5 mg/mL) was incubated at 25 °C with varying concentrations of the nucleotide analogue in 0.1 M potassium phosphate buffer, pH 7.1. Control enzyme was incubated under the same conditions without the nucleotide analogue. At various times, 20-μL aliquots were withdrawn from both the control and the experimental incubation mixtures and diluted 20-fold in Tris–0.1 M acetate buffer, pH 8.0, at 0 °C. From this dilution, 30 μL was used to measure the activity in the absence of GTP and 60 μL was used for assays with 1 μM GTP present.

*Incorporation of 8-BDB-TA-5′-DP and 8-BDB-TA-5′-TP into Bovine Liver Glutamate Dehydrogenase.* Enzyme (0.45 mg/mL) was incubated up to 2 h with 0.2 mM nucleotide analogue. Enzyme incubated under the same conditions but in the absence of the nucleotide analogue was used as a control. The reaction was quenched by the addition of dithiothreitol to a final concentration of 20 mM. The excess reagents were separated from the modified enzyme at 4 °C by the column centrifugation procedure described by Penefsky (1979). Columns were prepared in 5-mL disposable syringes filled with Sephadex G-50–80 mesh, supported by glass wool plugs, and equilibrated with 0.1 M HEPES buffer containing 0.4 M potassium chloride, pH 7.0. Each column was loaded with 0.5 mL of the incubation mixture. Three successive centrifugations were necessary to remove completely the excess reagents and phosphate. After elution, the enzyme concentration was determined by the Bio-Rad assay, which is based on the Bradford dye-binding method (Bradford, 1976). The amount of reagent incorporated was determined by the moles of organic phosphate in the isolated modified enzyme. The procedure of Hess and Derr (1975) and Lanzetta et al. (1979), as modified by Bailey and Colman (1987), was followed.

*Measurement of Reversible Binding of GTP and ADP by Native and 8-BDB-TA-5′-TP-Modified Enzyme.* Binding of [8-$^{14}$C]GTP and [8-$^{14}$C]ADP to native and modified enzyme was measured at 25 °C in an Amicon Model 10-PA ultrafiltration apparatus, equipped with a PM-10 membrane to separate the free ligand from the enzyme-bound ligand (Pal & Colman, 1979). The experiments on the binding of [8-$^{14}$C]GTP to native and modified enzymes were conducted by using 16 μM enzyme in the presence of 100 μM NADH with varying GTP concentrations in Tris–0.05 M acetate buffer, pH 7.1, containing 10 mM phosphate and 100 μM EDTA in a total volume of 2 mL. The modified enzyme was prepared by incubating 2 mg/mL enzyme for 3 h with 0.2 mM 8-BDB-TA-5′-TP and was isolated by column centrifugation as described above using Sephadex G-50–80 equilibrated with Tris–0.05 M acetate (pH 7.1) containing 10 mM phosphate and 100 μM EDTA in a total volume of 2 mL. (This procedure has been shown to yield modified enzyme with the same amount of reagent incorporated as obtained by the method described above.) The binding of [8-$^{14}$C]ADP was measured the same way except that no NADH was added to the solutions.

## Results

*Effect of 8-Thioadenosine 5′-Triphosphate on the Catalytic Activity of Glutamate Dehydrogenase.* Glutamate dehydrogenase is reversibly inhibited by 8-thioadenosine 5′-triphosphate (8-TA-5′-TP) as assayed in Tris–0.01 M acetate buffer, pH 8.0, in the presence of 100 μM NADH as coenzyme, at 25 °C. The activity was measured over a concentration range of 5.0 μM–1.0 mM 8-TA-5′-TP, where the extent of inhibition reaches a limiting maximum value. The inhibition constant, $K_I$, for enzyme–nucleotide complex was calculated from the relationship described by Frieden (1963) for an uncompetitive allosteric inhibitor; $K_I$ has been shown

[1] Abbreviations: 8-BDB-TA-5′-DP, 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-diphosphate; 8-BDB-TA-5′-TP, 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-triphosphate; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; 8-TA-5′-TP, 8-thioadenosine 5′-triphosphate; 6-BDB-TA-5′-DP, 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate; 2-BDB-TA-5′-MP, 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-monophosphate.

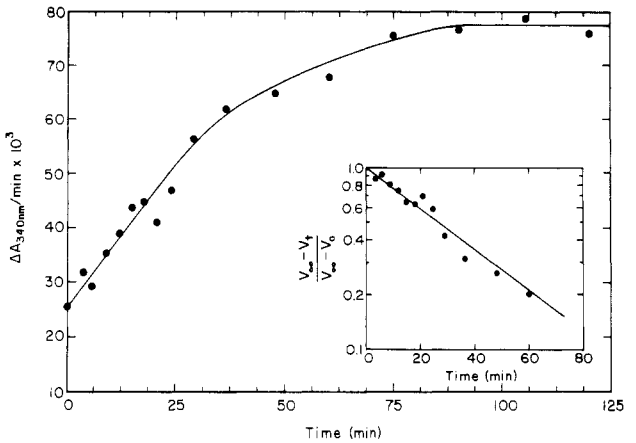


FIGURE 1: Reaction of 0.15 mM 8-[(4-bromo-2,3-dioxobutyl)thio]-adenosine 5′-triphosphate with glutamate dehydrogenase. The enzyme (0.5 mg/mL) was incubated as described under Experimental Procedures and assayed in the presence of 1.0 $\mu$M GTP. At the indicated time intervals during the 2-h incubation, 20-$\mu$L aliquots were withdrawn, diluted 20-fold in Tris–0.1 M acetate buffer, pH 8, at 0 °C, and assayed in the presence of 100 $\mu$M NADH as described under Experimental Procedures. Inset: Determination of pseudo-first-order rate constant from the increase in enzyme activity assayed in the presence of GTP. The change in enzyme activity as a function of time is expressed as $(V_\infty - V_t)/(V_\infty - V_0)$, where $V_t$ and $V_0$ are the velocities measured at various times and zero time, respectively, and $V_\infty$ is the constant velocity attained at the end of the reaction.

to be equal to the inhibitor concentration at which the velocity equals the average of the velocity in the absence of the inhibitor and the minimum velocity at saturating concentrations of the inhibitor. From this relationship, $K_I$ was calculated to be 7.6 $\times 10^{-6}$ M, with a maximum inhibition by 8-TA-5′-TP of 50%. Despite the weaker binding and less extensive inhibition of glutamate dehydrogenase by 8-TA-5′-TP, this behavior is similar to the well-known inhibition of this enzyme by GTP and ATP. In the native enzyme, 1.0 $\mu$M GTP inhibits the activity about 90%, while ATP inhibits maximally about 57% (Lascu et al., 1977).

*Kinetics of Reaction of Glutamate Dehydrogenase with 8-BDB-TA-5′-TP and 8-BDB-TA-5′-DP.* Incubation of glutamate dehydrogenase with either 8-BDB-TA-5′-TP or 8-BDB-TA-5′-DP causes no change in the maximum velocity as measured in the absence of regulatory compounds (data not shown); however, both compounds desensitize the enzyme to inhibition by GTP. Upon incubation with 0.15 mM 8-BDB-TA-5′-TP (Figure 1), the activity measured in the presence of 1.0 $\mu$M GTP exhibits a time-dependent increase, to a limit of about 3-fold after 2 h of incubation. No further increase in activity was observed when higher concentrations of the reagent were used. To test whether the end point had been reached, a second addition of the reagent was made at the end of 2 h. No further change in velocity was observed. The pseudo-first-order rate constants were calculated by the method shown in the inset of Figure 1. The bromodioxobutyl nucleotides decompose in aqueous solution with the release of bromide; under the conditions used in these experiments, the half-life of each reagent is about 50 min (DeCamp et al., 1988). To minimize the effect of reagent decomposition, the reaction rates of 8-BDB-TA-5′-DP and 8-BDB-TA-5′-TP with glutamate dehydrogenase were determined from the data in the first 40 min of incubation. For 8-BDB-TA-5′-TP, the pseudo-first-order rate constant was calculated to be 0.029 min$^{-1}$ at 0.20 mM reagent concentration, while that of 8-BDB-TA-5′-DP was determined as 0.021 min$^{-1}$.

A plot of the observed rate constants versus reagent concentration (over the range 0.05–0.5 mM) is not linear but

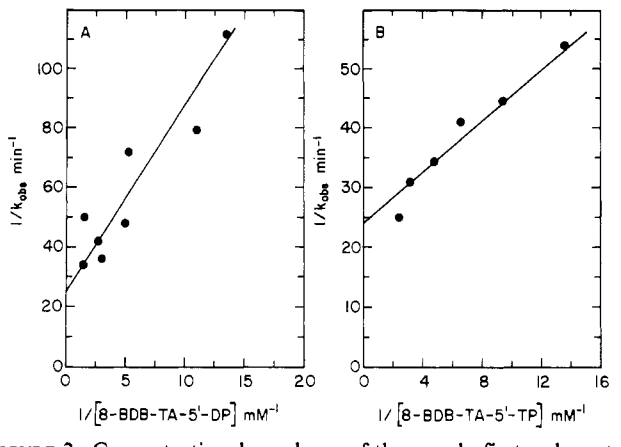


FIGURE 2: Concentration dependence of the pseudo-first-order rate constant, $k_{obs}$, on 8-BDB-TA-5′-DP (A) and 8-BDB-TA-5′-TP (B). The enzyme (0.5 mg/mL) was incubated with varying reagent concentrations (0.05–0.5 mM) as described under Experimental Procedures. Rate constants were calculated as illustrated in Figure 1.

rather shows saturation kinetics. The reagents can therefore be considered to bind reversibly to the enzyme before covalent modification occurs. This behavior is characteristic of an affinity label. The modification reaction can be described in terms of the kinetic model:

$$E + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ER \xrightarrow{k} ER' \qquad (1)$$

The observed rate constant for modification can then be expressed as

$$k_{obs} = k/(1 + K_D/[R]) \qquad (2)$$

where $k_{obs}$ is the rate constant observed at a given reagent concentration, $K_D$ is the apparent dissociation constant of the reagent–enzyme complex $[(k_{-1} + k)/k_1]$, and $k$ is the intrinsic rate constant for covalent modification of the enzyme at infinite reagent concentration. This equation can be rearranged as

$$1/k_{obs} = 1/k + K_D/k[R] \qquad (3)$$

Figure 2 shows the reciprocal plot of $k_{obs}$ versus various reagent concentrations for both reagents. This plot gives a $K_D$ of 0.24 mM and $k$ of 0.038 min$^{-1}$ for 8-BDB-TA-5′-DP, while for 8-BDB-TA-5′-TP, $K_D$ is 0.089 mM and $k$ is 0.042 min$^{-1}$. It is apparent that both compounds cause the same change in sensitivity to GTP inhibition, with similar maximum rate constants, but that the nucleoside triphosphate has a greater affinity for the enzyme. The lack of one phosphate group could be contributing to lesser affinity of 8-BDB-TA-5′-DP for glutamate dehydrogenase. This is similar to the observed lower affinity of GDP as compared to GTP when tested as an allosteric inhibitor of glutamate dehydrogenase (Frieden, 1963).

*Effect of Allosteric Ligands on the Reaction Rate of Enzyme with the Reagents.* The effect of various combinations of allosteric ligands on the rate constant of reaction of the enzyme with 0.20 mM 8-BDB-TA-5′-TP is shown in Table I. At 200 $\mu$M ADP in the incubation mixture (a concentration higher than the $K_D$ values in order to populate both the high- and the low-affinity ADP allosteric sites), the rate constant was decreased only moderately (Table I). A similar small effect was observed when ATP was used, although 8-BDB-TA-5′-TP is an ATP analogue substituted at position 8. When added in the absence of coenzyme, GTP binds to only the low-affinity site and decreases the rate constant about 2-fold. At 5.0 mM NADH (a concentration sufficiently high to bind to both catalytic and regulatory coenzyme sites), the

Table I: Effect of Ligands on Rate of Reaction of 8-BDB-TA-5′-TP with Bovine Liver Glutamate Dehydrogenase[a]

| ligands added | $10^3 k_{obs}$ ($min^{-1}$) |
|---|---|
| none | 29.0 |
| ADP (200 μM) | 18.3 |
| ATP (200 μM) | 22.3 |
| GTP (200 μM) | 13.7 |
| NADH (5.0 mM) | 21.7 |
| ATP (200 μM) + NADH (100 μM) | 26.6 |
| ADP (200 μM) + NADH (100 μM) | 14.5 |
| NADH (5.0 mM) + GTP (200 μM) | 2.7 |
| NADH (5.0 mM) + GTP (25 μM) | 8.9 |
| NADH (100 μM) + GTP (200 μM) | 13.7 |

[a] Glutamate dehydrogenase (0.5 mg/mL) was incubated in 0.1 M potassium phosphate buffer, pH 7.1, at 25 °C with 0.20 mM reagent in the absence and presence of ligands. The rate constants were calculated by the method shown in Figure 1.

Table II: Effect of Ligands on Rate of Reaction of 8-BDB-TA-5′-DP with Bovine Liver Glutamate Dehydrogenase[a]

| ligands added | $10^3 k_{obs}$ ($min^{-1}$) |
|---|---|
| none | 21.0 |
| ADP (2.0 mM) | 19.0 |
| NADH (5.0 mM) | 17.0 |
| NADH (100 μM) + GTP (11 μM) | 17.0 |
| NADH (5.0 mM) + GTP (200 μM) | 3.3 |

[a] Glutamate dehydrogenase (0.44 mg/mL) was incubated in 0.05 M potassium phosphate buffer, pH 7.1, at 25 °C with 0.23 mM 8-BDB-TA-5′-DP in the absence and presence of ligands. The rate constants were calculated by the method illustrated in Figure 1.

change in rate constant was minimal. The combined addition to the reaction mixture of 200 μM ATP + 100 μM NADH, or 200 μM ADP + 100 μM NADH, caused at most a 2-fold decrease in $k_{obs}$.

The most striking decrease in the rate constant (about 10-fold) was caused by 5.0 mM NADH + 200 μM GTP, concentrations high enough to populate both NADH and both GTP sites. At concentrations sufficient to occupy both NADH sites and only the high-affinity GTP site (5.0 mM NADH + 25 μM GTP) or only the catalytic NADH site and both GTP sites (100 μM NADH + 200 μM GTP) (Colman, 1990), the protective effects were significantly diminished. Thus, the best protection against the effect of this reagent on the enzyme was observed in the case of 5.0 mM NADH + 200 μM GTP, suggesting that 8-BDB-TA-5′-TP might be modifying at or near the "GTP-dependent NADH regulatory site".

The effect of ligands on the rate constant for reaction of glutamate dehydrogenase with 8-BDB-TA-5′-DP is shown in Table II. The results are similar to those obtained with the triphosphate reagent. The best protection is provided by 5.0 mM NADH + 200 μM GTP, suggesting that the two 8-BDB-nucleotides target the same site on the enzyme.

*Incorporation of 8-BDB-TA-5′-TP and 8-BDB-TA-5′-DP.* Glutamate dehydrogenase (0.45 mg/mL) was incubated with 0.20 mM 8-BDB-TA-5′-TP, and the incorporation was determined (as described under Experimental Procedures) by quantitation of the organic phosphate in aliquots removed at various incubation times over a period of 120 min. A plot of the moles of 8-BDB-TA-5′-TP incorporated into glutamate dehydrogenase as a function of percentage of the maximum change in GTP inhibition (Figure 3) extrapolates to 0.92 mol of reagent/mol of enzyme subunit. The incorporation was also determined after a 100-min incubation with 0.20 mM 8-BDB-TA-5′-TP in the presence of 5.0 mM NADH alone, 200 μM GTP alone, 5.0 mM NADH plus 200 μM GTP, 200 μM ADP alone, and 100 μM NADH plus 200 μM ADP. This incubation time is sufficient to give maximum reagent in-

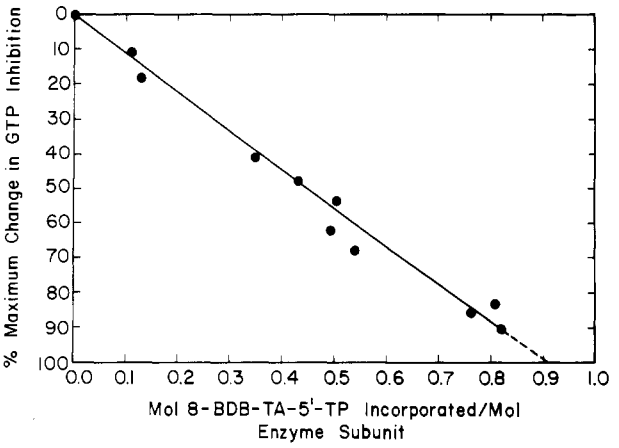


FIGURE 3: Incorporation of 8-BDB-TA-5′-TP into glutamate dehydrogenase as a function of percent maximum change in GTP inhibition. Glutamate dehydrogenase (0.45 mg/mL) was incubated with 0.20 mM 8-BDB-TA-5′-TP for 2 h. At 15-min intervals, aliquots were withdrawn and assayed in the presence of 1.0 μM GTP, and excess reagent was removed by gel filtration centrifugation as described under Experimental Procedures. The percent maximum change in GTP inhibition is expressed as $(V_t - V_0)/(V_\infty - V_0) \times 100$, where $V_t$ is the rate measured in the presence of GTP at a particular time and $V_\infty$ and $V_0$ are the velocities measured under the same conditions at the completion of the reaction and at zero time, respectively. The percent maximum change in GTP inhibition was calculated from data from 0.20 mM reagent concentration by the method illustrated in Figure 1, and the incorporation of 8-BDB-TA-5′-TP was determined from the organic phosphorus content of the modified enzyme.

Table III: Incorporation of 8-BDB-TA-5′-TP into Bovine Liver Glutamate Dehydrogenase[a]

| ligands | mol of 8-BDB-TA-5′-TP/mol of enzyme subunit |
|---|---|
| none | 1.07 |
| ADP (200 μM) | 0.32 |
| ADP (200 μM) + NADH (100 μM) | 0.42 |
| NADH (5.0 mM) | 0.49 |
| GTP (200 μM) | 0.40 |
| NADH (5.0 mM) + GTP (200 μM) | 0.10 |

[a] Glutamate dehydrogenase (0.45 mg/mL) was incubated for 100 min in 0.1 M potassium phosphate buffer, pH 7.1, at 25 °C with 0.20 mM 8-BDB-TA-5′-TP in the presence and absence of ligands. Modified enzyme was isolated by the gel filtration method, and the amount of reagent incorporated was determined from the moles of organic phosphate as described under Experimental Procedures. A correction was made for the small amount (if any) of phosphate measured in the control enzyme incubated under the same conditions but in the absence of reagent.

corporation in the absence of ligands. The results of this experiment are shown in Table III. The combined addition of 5.0 mM NADH plus 200 μM GTP caused the maximum decrease in reagent incorporation (to only 0.1 mol/mol of enzyme subunit).

The incorporation of 8-BDB-TA-5′-DP into the enzyme was measured by the same method. It was determined that 1.17 mol of reagent/mol of enzyme subunit was incorporated when the maximum change in GTP inhibition had occurred. These results suggest that both reagents label the enzyme with a stoichiometry of 1 and possibly at a single site.

*Kinetic Properties of the Enzyme Modified by 8-BDB-TA-5′-TP.* Enzyme incubated with 8-BDB-TA-5′-TP in the absence of ligands (containing about 1 mol of reagent/mol of enzyme subunit) or in the presence of the protectants 5.0 mM NADH and 200 μM GTP (containing 0.1 mol of reagent/mol of enzyme subunit) was separated from free reagent and ligands and was evaluated kinetically in comparison with the native enzyme. The concentration dependence of GTP in-

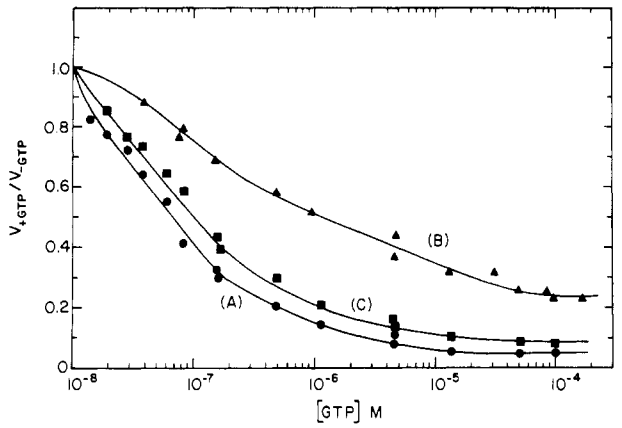


FIGURE 4: Effect of GTP on the maximum velocity of native glutamate dehydrogenase (A, ●), enzyme modified with 8-BDB-TA-5′-TP in the absence of ligands (B, ▲), and enzyme modified with 8-BDB-TA-5′-TP in the presence of 5 mM NADH and 200 μM GTP (C, ■). The coenzyme was 100 μM NADH in the assay solutions. The modified enzyme in B contained about 1 mol of 8-BDB-TA-5′-TP/mol of enzyme subunit and in C about 0.1 mol of 8-BDB-TA-5′-TP/mol of enzyme subunit.

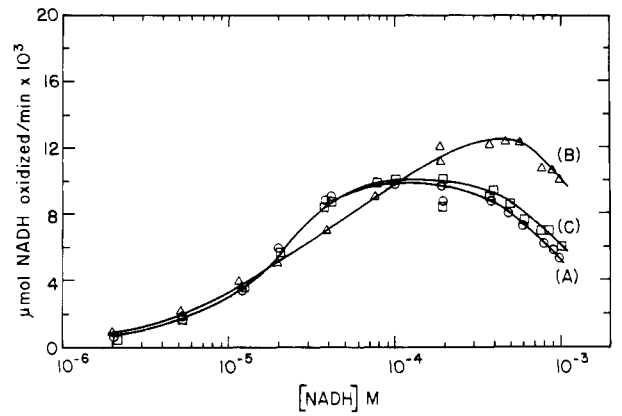


FIGURE 5: Initial velocity as a function of [NADH] for native glutamate dehydrogenase (A, ○), enzyme modified with 8-BDB-TA-5′-TP in the absence of ligands (B, △), and enzyme modified with 8-BDB-TA-5′-TP in the presence of 5 mM NADH and 200 μM GTP (C, □). The enzyme samples used are described in the legend to Figure 4. Up to 0.2 mM NADH, the rates were measured at 340 nm as described under Experimental Procedures. Measurements at higher NADH concentrations were determined at 375 nm. Extinction coefficients of $6.22 \times 10^3$ $M^{-1}$ $cm^{-1}$ and $1.85 \times 10^3$ $M^{-1}$ $cm^{-1}$ for NADH were used at 340 and 375 nm, respectively, in calculating the rate of μmol of NADH oxidized/min.

hibition was measured in the presence of 100 μM NADH, as shown in Figure 4. At saturating GTP concentrations, the modified enzyme (Figure 4, curve B) was inhibited maximally 76% while the native enzyme (Figure 4, curve A) and the protected enzyme (Figure 4, curve C) were inhibited 96% and 92%, respectively. The inhibition constant for enzyme–GTP complex [$K_I$, calculated from Figure 4 in accordance with Frieden (1963)] was 0.33 μM for modified enzyme, 0.056 μM for native enzyme, and 0.081 μM for protected enzyme. Thus the reaction of glutamate dehydrogenase with 8-BDB-TA-5′-TP results in a 6-fold increase in the dissociation constant of the enzyme–GTP complex, and this change is prevented when GTP and NADH are present during the incubation of enzyme with 8-BDB-TA-5′-TP.

In studying the effect of NADH concentration, we found that the modified enzyme (Figure 5, curve B) follows normal Michaelis–Menten kinetics up to an NADH concentration of 0.3 mM. A Lineweaver–Burk plot over the concentration range of 2–200 μM NADH gives $K_m^{NADH}$ = 26.9 μM and $V_{max}$ = 0.0125 μmol of NADH oxidized/min for the modified enzyme. In contrast, native enzyme (Figure 5, curve A) and protected enzyme (incubated with 8-BDB-TA-5′-TP in the presence of GTP and NADH; Figure 5, curve C) are inhibited at concentrations of 0.1 mM NADH or greater. Protected and native enzymes behave similarly, yielding values of $V_{max}$ = 0.0152 μmol of NADH oxidized/min and $K_m^{NADH}$ = 16.4 μM. The observation that modified enzyme is also inhibited by NADH, albeit at higher NADH concentrations, suggests that 8-BDB-TA-5′-TP does not bind directly to the NADH regulatory site but rather desensitizes the enzyme to this inhibition.

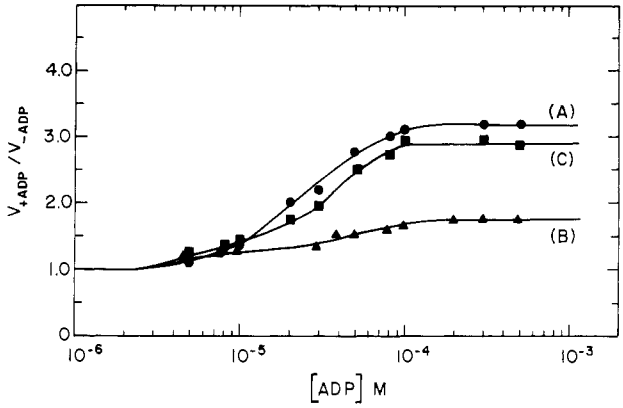


FIGURE 6: Effect of ADP on the maximum velocity of native glutamate dehydrogenase (A, ●), enzyme modified in the absence of ligands (B, ▲), and enzyme modified in the presence of NADH and GTP (C, ■). The coenzyme concentration was 100 μM NADH in the assay solutions. The enzyme samples are described in the legend to Figure 4.

The response to ADP activation of native enzyme was also compared with that of enzyme modified by 8-BDB-TA-5′-TP (Figure 6). Although the activation constants [$K_A$, in accordance with Frieden (1963)] for modified, native, and protected (GTP + NADH) enzymes were similar (30.0 μM, 27.0 μM, and 30.0 μM, respectively), the maximum extents of activation were markedly different. For native enzyme, the maximum activation by ADP was found to be 3.1-fold (curve A), in agreement with that reported earlier (Batra & Colman, 1986a), while the extent of activation for modified enzyme is only 1.7-fold (curve B). The enzyme incubated with 8-BDB-TA-5′-TP in the presence of GTP + NADH was activated 2.9-fold by ADP (curve C). The similarity of protected to native enzyme suggests that GTP + NADH protect against the effect of reagent on the maximum extent of activation by ADP.

To assess whether ADP would specifically protect against the decrease in ADP activation, enzyme was incubated with 0.20 mM 8-BDB-TA-5′-TP in the presence of 200 μM ADP or 200 μM ADP + 100 μM NADH. The isolated enzymes, which were only partially protected against the change in sensitivity to GTP inhibition, exhibited an extent of activation by ADP of 2.7 and 2.5, values intermediate between those of native and fully modified enzymes. These results imply that the modification by 8-BDB-TA-5′-TP has an indirect effect on the ADP site, since the presence of ADP in the reaction mixture does not completely prevent desensitization to either allosteric effector.

Bovine liver glutamate dehydrogenase has been shown to be inhibited by $Zn^{2+}$, similar to the inhibition by GTP (Colman & Foster, 1970). Since reaction of enzyme with 8-BDB-TA-5′-TP perturbs the enzyme's inhibition by GTP, the effect of $Zn^{2+}$ on 8-BDB-TA-5′-TP-modified and protected enzymes was investigated. The $K_I$ values, calculated in accordance with

Table IV: Direct Binding of [8-$^{14}$C]GTP and [8-$^{14}$C]ADP to Native and 8-BDB-TA-5′-Modified Glutamate Dehydrogenase[a]

| | native enzyme | | | 8-BDB-TA-5′-TP-modified enzyme | | |
|---|---|---|---|---|---|---|
| | concentration | | mol of ligand bound/mol of enzyme subunit | concentration | | mol of ligand bound/mol of enzyme subunit |
| ligand | total ligand ($\mu$M) | free ligand ($\mu$M) | | total ligand ($\mu$M) | free ligand ($\mu$M) | |
| GTP | 127 | 111 | 0.99 | 121 | 112 | 0.59 |
| | 137 | 117 | 1.26 | 141 | 119 | 1.38 |
| | 149 | 130 | 1.28 | 154 | 132 | 1.38 |
| ADP | 112 | 88 | 1.49 | 118 | 102 | 1.04 |
| | 123 | 102 | 1.33 | 129 | 113 | 0.98 |
| | 133 | 110 | 1.45 | 138 | 115 | 1.44 |

[a] The reversible binding measurements were conducted with 16 $\mu$M enzyme in Tris–0.05 M acetate buffer, pH 7.1, containing 10 mM potassium phosphate and 100 $\mu$M EDTA in a total volume of 2 mL. In addition, the solution contained 100 $\mu$M NADH in the case where the ligand used was GTP.

Frieden (1963), were about 0.03 $\mu$M with a maximum extent of inhibition of 92% for the modified, protected, and native enzymes. This result indicates that the reagent does not affect the metal-binding site and that the metal and GTP sites are therefore distinguishable.

*Binding of Radioactive GTP and ADP by 8-BDB-TA-5′-TP-Modified and Native Enzymes.* The kinetic characteristics of 8-BDB-TA-5′-TP-modified enzyme suggest that reaction may occur at a GTP and/or an ADP site. To evaluate this possibility, the number of binding sites was determined for GTP and for ADP in the modified enzyme at nucleotide concentrations sufficiently high to ensure binding to both sites for GTP and ADP. The reversible binding of [8-$^{14}$C]GTP and [8-$^{14}$C]ADP was studied by using an ultrafiltration technique, and the results are shown in Table IV. GTP binding experiments were conducted in the presence of 100 $\mu$M NADH, since NADH tightens the binding of GTP. No NADH was used in the ADP binding experiments, since NADH weakens the binding of ADP (Batra & Colman, 1986a). The results show that, as observed for native enzyme, more than 1 mol of each of GTP and ADP can still bind to the modified enzyme. These data suggest that modification by 8-BDB-TA-5′-TP does not completely block either the GTP or the ADP sites. Covalent modification may affect the binding of GTP and ADP indirectly.

## Discussion

The reaction of bovine liver glutamate dehydrogenase with 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-diphosphate or 5′-triphosphate exhibits many of the characteristics associated with affinity labeling. Reaction occurs at a limited number of sites, as indicated by the incorporation of about 1 mol of either reagent/mol of enzyme subunit. The nonlinear dependence of the rate constant on reagent concentration suggests the reversible formation of an enzyme–reagent complex prior to covalent modification. Protection by natural nucleotide ligands against the functional changes and against the covalent reagent incorporation are also indicative of specific modification by 8-BDB-TA-5′-DP and 8-BDB-TA-5′-TP. Furthermore, the parent compound, which lacks the reactive alkylating group (8-thioadenosine 5′-triphosphate), functions as a reversible allosteric inhibitor, consistent with reaction of 8-BDB-TA-5′-TP at a regulatory site.

The functional target site of 8-BDB-TA-5′-TP may be revealed by a review of the kinetic and binding properties of the modified enzyme, as well as of the natural ligands that protect against the covalent reaction. The catalytic site can be excluded since the enzyme does not appreciably change activity if assayed in the absence of regulatory agents and at NADH concentrations up to about 0.1 mM. The ADP-activating sites are unlikely targets of 8-BDB-TA-5′-TP, since ADP provides only modest protection against the functional changes produced by 8-BDB-TA-5′-TP. In addition, the modified enzyme retains its ability to be activated by ADP and to bind reversibly more than 1 mol of ADP/mol of enzyme subunit.

The best candidates for the target of 8-BDB-TA-5′-TP are the regulatory sites of GTP and/or NADH. Modification leads to changes in both the sensitivity of the enzyme to inhibition by GTP and the inhibition by high concentrations of NADH. The observation that 8-BDB-TA-5′-TP has a higher affinity for glutamate dehydrogenase ($K_D$ = 0.089 mM) than for the corresponding diphosphate reagent is in accordance with the affinity order for the guanosine nucleotides (Frieden, 1963); one might have expected that 8-BDB-TA-5′-DP would be the more effective of these two reagents if the reaction target were the NADH site with its presumed preference for a 5′-diphosphate moiety. On the other hand, modified enzyme containing about 1 mol of reagent/mol of enzyme subunit is still inhibited 76% by saturating concentrations of GTP, albeit with a higher $K_I$ than for native enzyme. Furthermore, the modified enzyme still binds more than 1 mol of radioactive GTP (in the presence of 100 $\mu$M NADH), suggesting that neither of the two GTP sites is irreversibly blocked by reaction of enzyme with 8-BDB-TA-5′-TP. Detection of inhibition by NADH requires a considerably higher concentration of the reduced coenzyme for modified enzyme than for native enzyme (Figure 5), but the observation of any inhibition of modified enzyme by NADH implies that occupation of both NADH-binding sites is still possible after modification by 8-BDB-TA-5′-TP (Koberstein & Sund, 1973; Koberstein et al., 1973). Thus, none of the normal regulatory sites seems to be completely missing in the modified enzyme, although the characteristics of several (most notably, the GTP and NADH sites) appear to be perturbed.

Only minimal protection against modification by 8-BDB-TA-5′-TP is provided by ADP alone, GTP alone, or NADH alone. However, 5 mM NADH plus 200 $\mu$M GTP, concentrations sufficiently high to occupy both the inhibitory NADH site and the low-affinity GTP site (Koberstein & Sund, 1973; Koberstein et al., 1973; Pal & Colman, 1979), produces a 10-fold decrease in the reaction rate constant of glutamate dehydrogenase with 8-BDB-TA-5′-TP. It might be postulated that 8-BDB-TA-5′-TP reacts at two separate sites (i.e., an NADH site and a GTP site) and that the striking protection provided by GTP + NADH is the result of the occupation of both sites. However, it is known that GTP tightens the binding of NADH at both of its sites (Koberstein & Sund, 1973) and that GTP binds to the enzyme with higher affinity when NADH is present (Goldin & Frieden, 1972). Mutual interaction between the GTP and NADH allosteric sites is well established, and a GTP-dependent regulatory NADH site has previously been designated (detected at 200 $\mu$M GTP + 5 mM

NADH) (Lark & Colman, 1986). Occupation of this GTP-dependent regulatory NADH site seems to be responsible for protection against modification by 8-BDB-TA-5′-TP. It is possible that the amino acid target of 8-BDB-TA-5′-TP lies in a cleft region of glutamate dehydrogenase, not directly involved in any of the regulatory sites, which becomes blocked upon simultaneous binding of NADH to its inhibitor site and of GTP to its low-affinity site. The combined presence of both nucleotides would thus be required to shield the target amino acid residue from 8-BDB-TA-5′-TP. However, once reaction with 8-BDB-TA-5′-TP was complete, the enzyme would still be able to bind GTP and NADH, albeit with weakened affinities and altered effects.

Glutamate dehydrogenase has previously been found to react with 6- and 2-(4-bromo-2,3-dioxobutyl)thionucleotides, with results different from those reported here for 8-BDB-TA-5′-TP. For example, enzyme modified by 6-BDB-TA-5′-DP exhibits *no* inhibition at high concentrations of NADH (Batra & Colman, 1984), and protection against reaction with 2-BDB-TA-5′-MP is produced by ADP alone (Batra & Colman, 1986a). 6-BDB-TA-5′-DP has been reported to react at $Cys^{319}$ within the NADH regulatory site (Batra & Colman, 1986b), whereas 2-BDB-TA-5′-MP reacts at $His^{82}$ within an ADP-activating site (Batra et al., 1989). We are currently investigating the specific amino acid(s) modified by 8-BDB-TA-5′-TP.

These three nucleotide analogues with the same reactive functional group tethered to distinct positions of the purine ring (2-, 6-, and 8-BDB-5′-nucleotides) might be expected to label different nucleotide sites on a given protein, provided that the several sites have distinguishable structural requirements for binding. Alternatively, even if the different nucleotide analogues bind essentially to the same site, the reactive group might encounter different amino acids. Thus, in the case of rabbit muscle pyruvate kinase, 2-[(4-bromo-2,3-dioxobutyl)-thio]-1,$N^6$-ethenoadenosine 5′-diphosphate reacts with $Tyr^{147}$ in the active site (DeCamp & Colman, 1989), while 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-triphosphate labels $Cys^{151}$ (Vollmer & Colman, 1990). Similarly, in the case of human platelet cAMP phosphodiesterase, 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 3′,5′-cyclic monophosphate is a competitive inhibitor but does not inactivate the enzyme, while 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 3′,5′-cyclic monophosphate functions as an irreversible affinity label (Grant et al., 1990). The 2-, 6-, and 8-substituted nucleotide analogues may be complementary in widespread applications as affinity labels of other proteins with nucleotide sites.

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