Affinity Labeling of the Reduced Diphosphopyridine Nucleotide Inhibitory Site of Glutamate Dehydrogenase by 6-[(4-Bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-Diphosphate†

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ABSTRACT: Bovine liver glutamate dehydrogenase reacts covalently with the new adenosine analogue 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate with incorporation of about 1 mol of reagent/mol of enzyme subunit. Modified enzyme completely loses its normal ability to be inhibited by high concentrations of reduced diphosphopyridine nucleotide (DPNH) (>100 μM), which binds at a regulatory site distinct from the catalytic site; however, the modified enzyme retains its full activity when assayed at 100 μM DPNH in the absence of allosteric compounds. The enzyme is still activated by ADP, is inhibited by GTP (albeit at higher concentrations), and binds 1.5–2 mol of [^{14}C]GTP/subunit. A plot of initial velocity vs. DPNH concentration for the modified enzyme, in contrast to the native enzyme, followed Michaelis–Menten kinetics. The rate constant (k) for loss of DPNH inhibition (as measured at 0.6 mM DPNH) exhibits a nonlinear dependence on reagent concentration, suggesting a reversible binding of reagent (K_d = 0.19 mM) prior to irreversible modification. At 0.1 mM 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate, k = 0.036 min^{-1} and is not affected by α-ketoglutarate, 100 μM DPNH, or GTP alone but is decreased to 0.0094 min^{-1} by 5 mM DPNH and essentially to zero by 5 mM DPNH plus 100 μM GTP. Incorporation after incubation with 0.25 mM 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate for 2 h at pH 7.1 is 1.14 mol/mol of subunit in the absence but only 0.24 mol/mol of subunit in the presence of DPNH plus GTP. The modified enzyme prepared in the presence of DPNH plus GTP behaves like native enzyme in its dependence of velocity on DPNH concentration, in its activation by ADP, and in its inhibition by GTP. These results indicate that 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate acts as a specific affinity label of the DPNH inhibitory site of glutamate dehydrogenase.

Bovine glutamate dehydrogenase [L-glutamate:NAD(P)$^+$ oxidoreductase (deaminating), EC 1.4.1.3] is an allosteric enzyme that catalyzes the reversible oxidative deamination of L-glutamate to α-ketoglutarate and ammonia. The activity of this enzyme is affected by several purine nucleotides: ADP activates, GTP inhibits, and relatively high concentrations of DPNH[1] also inhibit by binding to a site distinct from the catalytic site (Goldin & Frieden, 1972; Eisenberg et al., 1976). Each of the six identical subunits in the catalytically functional hexamer has several purine nucleotide sites including one for ADP, two for GTP, and two for DPNH (one catalytic and one regulatory) (Goldin & Frieden, 1972; Sund et al., 1975; Pal & Colman, 1979). Several group-specific reagents have been used to chemically modify amino acid residues in the catalytic and regulatory sites of glutamate dehydrogenase (Goldin & Frieden, 1972; Eisenberg et al., 1976); in many cases, however, incorporation of considerably more than 1 mol of reagent and modification of more than one type of site have complicated interpretation of the results. Affinity labeling using purine nucleotide containing reactive functional groups has the potential for yielding specific modification of particular catalytic or regulatory sites (Colman, 1983).

The adenine nucleotide analogue 5′-[p-(fluorosulfonyl)-benzoyl]adenosine has been shown to react at the DPNH inhibitory site of glutamate dehydrogenase (Pal et al., 1975). The DPNH inhibition is eliminated upon incorporation of 3 mol of reagent/mol of hexameric enzyme and equal amounts of modified lysine and tyrosine account for all the incorporation (Saradambal et al., 1981). More recently, the modified residues have been identified as lysine-420 and tyrosine-190 (Schmidt & Colman, 1984). In 5′-[p-(fluorosulfonyl)-benzoyl]adenosine, the reactive p-(fluorosulfonyl)benzoyl group may be located in a position analogous to the ribose proximal to the nicotinamide ring of DPNH, and therefore, the amino acids modified might be expected to be situated in the region of the enzyme to which this ribose normally binds. A new adenine nucleotide analogue has recently been described: 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate (Colman et al., 1984), the structure of which is shown in Figure 1. This compound is closely related to the natural adenine nucleotide structure, is water soluble, and is negatively charged at neutral pH. Because of the location of the reactive functional groups adjacent to the 6-position, the compound might be expected to react with amino acid residues in the purine region of the adenine nucleotide binding sites of proteins and might thus be complementary to 5′-[p-(fluorosulfonyl)benzoyl]adenosine in the affinity labeling of different subsites of the purine nucleotide binding regions of dehydrogenases and kinases. This paper describes the specific reaction of 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate with glutamate dehydrogenase. A preliminary version of this work has been presented (Batra & Colman, 1984).

Experimental Procedures

Materials. A crystalline suspension of bovine liver glutamate dehydrogenase, purchased from Boehringer Mannheim Corp., was dialyzed in 20–40-mg batches for 18 h at 4 °C against two changes of 0.1 M potassium phosphate buffer, pH 7.1. The dialyzed material was centrifuged at 4 °C for 30 min at 15 000 rpm to remove any precipitated, denatured protein.

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[1] Abbreviations: BDB-TADP, 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate; DPNH, reduced diphosphopyridine nucleotide; GTP, guanosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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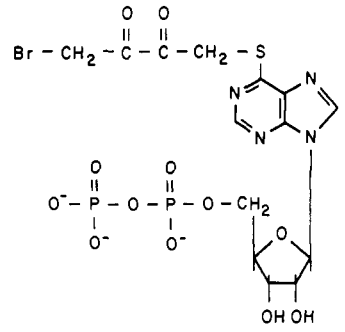

FIGURE 1: Structure of 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate.

The enzyme concentration was determined by using the value $E^{1\%}_{279} = 9.7$ (Olson & Anfinsen, 1952); the ratio of E_{280}/E_{260} was 1.9 Enzyme was stored in aliquots at −80 °C. A molecular weight of 56 100 for the identical polypeptide chains of glutamate dehydrogenase was used in the calculations (Smith et al., 1970).

DPNH, GTP, ADP, EDTA, Tris, Malachite Green Base, mercuric thiocyanate, and 1,1′-carbonyldiimidazole were obtained from Sigma. The barium salt of 6-mercaptopurine ribonucleoside 5′-phosphate was purchased from P-L Biochemicals. The 1,4-dibromobutanedione was obtained from Aldrich Chemical Co. and was recrystallized from petroleum ether prior to use. [^{32}P]Orthophosphoric acid and [U-^{14}C]GTP were supplied by New England Nuclear Corp. All other chemicals were reagent grade.

Preparation of 6-[(4-Bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-Diphosphate (BDB-TADP). The compound 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate (shown in Figure 1) was synthesized by reaction of 1,4-dibromobutanedione with 6-mercaptopurine ribonucleoside 5′-diphosphate, which was prepared by phosphorylating 6-mercaptopurine 5′-monophosphate (Colman et al., 1984).

The β-^{32}P-labeled 6-mercaptopurine ribonucleoside 5′-diphosphate was synthesized from [^{32}P]phosphoric acid. [^{32}P]Phosphoric acid (10 mCi) was added to a solution of 36 mg (250 μmol) of nonradioactive phosphate (tributyl ammonium salt) in 2.5 mL of dimethylformamide, followed by evaporation. The residue was then dissolved in 5.0 mL of dimethylformamide, and the phosphorylation of 6-mercaptopurine ribonucleoside 5′-monophosphate followed by the condensation with 1,4-dibromobutanedione was conducted in accordance with the procedures of Colman et al. (1984) described for synthesis of the unlabeled compound.

Enzymatic Assay. Bovine liver glutamate dehydrogenase activity was generally determined at 25 °C in Tris–0.01 M acetate buffer, pH 8.0, containing 10 μM EDTA by measuring the oxidation of reduced coenzyme at 375 nm ($\epsilon_{375nm} = 1.85 \times 10^3$ M^{-1} cm^{-1} for DPNH) in a Gilford Model 240 spectrophotometer equipped with an expanded scale recorder. The assay mixture in the total volume of 1.0 mL contained 5 mM α-ketoglutarate, 50 mM ammonium chloride, and 600 μM DPNH. At 600 μM, DPNH inhibits glutamate dehydrogenase by binding to a site distinct from the active site (Frieden, 1963). This inhibition is overcome on reacting with 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate, as is described under Results.

Conditions used for testing the effects of varying concentrations of regulatory compounds such as ADP or GTP are indicated under Results. For these activity measurements, the substrate concentrations used were the same as indicated above (except that of DPNH, which was 100 μM). The enzyme activity was assayed spectrophotometrically at 340 nm by measuring the oxidation of DPNH ($\epsilon_{340nm} = 6.22 \times 10^3$ M^{-1} cm^{-1}).

Reaction of Glutamate Dehydrogenase with 6-[(4-Bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-Diphosphate. Glutamate dehydrogenase (1 mg/mL) was incubated with BDB-TADP (0.05–0.30mM) at 25 °C in 0.05 M potassium phosphate buffer, pH 7.1, containing 10% methanol. Methanol had no effect on the stability or activity of the enzyme as determined from a control that consisted of enzyme incubated under the same conditions but in the absence of BDB-TADP. Aliquots of 20 μL were withdrawn from experimental and control samples at various time intervals and diluted 20-fold with Tris–0.1 M acetate buffer, pH 8.0 at 0 °C; an aliquot of 20 μL from this dilution was assayed as described above.

Determination of the Rate Constant for Decomposition of BDB-TADP. The rate constant for decomposition of BDB-TADP was measured by incubating 0.4 mM reagent, in the absence of enzyme, in 0.05 M potassium phosphate buffer, pH 7.1, containing 10% methanol, conditions similar to those used for reaction with enzyme. The decomposition of BDB-TADP was monitored at 25 °C by measuring the amount of bromide released as a function of time. Free bromide was measured by a procedure modified from that of Zall et al. (1956) in which bromide displaces thiocyanate from mercuric thiocyanate and the liberated thiocyanate reacts with ferric ion to form a colored complex, which is then measured spectrophotometrically at 460 nm. The rate constant for decomposition of BDB-TADP was calculated from the semilogarithmic plot of $[\text{BDB-TADP}]_t/[\text{BDB-TADP}]_0$ as a function of time, where $[\text{BDB-TADP}]_t$ and $[\text{BDB-TADP}]_0$ are the reagent concentrations at any indicated time and at zero time, respectively.

Incorporation of BDB-TADP into Glutamate Dehydrogenase. Two alternate methods were used to determine the number of moles of 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate incorporated into the enzyme. The first method depends on measurement of the protein-bound radioactivity after reaction of the enzyme with 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-[β-^{32}P]diphosphate. Glutamate dehydrogenase (1 mg/mL) was incubated with 0.25 mM [β-^{32}P]BDB-TADP [specific activity ranges (2–38) × 10^{12} cpm/mol] under the conditions described above. At the various time intervals the reaction mixture was cooled to 0 °C, and the reaction was quenched by addition of dithiothreitol to a final concentration of 20 mM. The modified enzyme was separated from excess reagent by the column centrifugation procedure described by Penefsky (1979). Columns were prepared in 5-mL disposable syrings barrels filled with Sephadex G-50, 80 mesh (supported by a small glass wool plug), equilibrated with 0.05 M potassium phosphate buffer, pH 7.1. The eluate was obtained by centrifugation in a clinical centrifuge. Two successive centrifuge columns followed by an overnight dialysis against 0.05 M potassium phosphate buffer, pH 7.1, were required to completely remove any noncovalently bound reagent. The amount of BDB-TADP incorporated into this dialyzed enzyme was determined by measuring the radioactivity of aliquots, in ACS scintillation liquid (Amersham) with a Packard TriCarb liquid scintillation counter, Model 3330. The protein concentration was determined from the Bio-Rad protein assay, which is based on the method of Bradford (1976). Native glutamate dehydrogenase was used to establish a standard curve.

The second method used to determine incorporation of BDB-TADP was based on the quantitation of phosphorus in

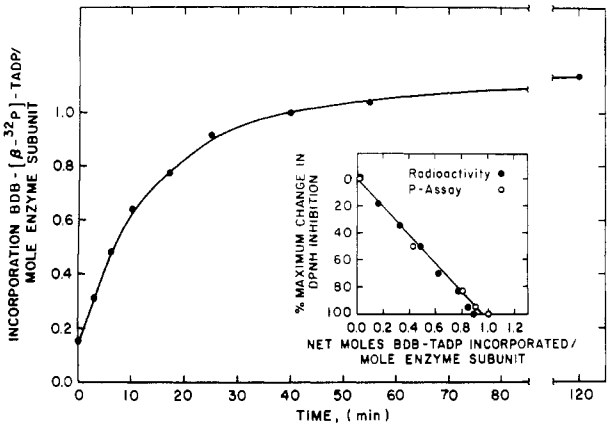

FIGURE 2: Incorporation of 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate as a function of time. Glutamate dehydrogenase (1 mg/mL) was incubated with 0.25 mM [β-^{32}P]-BDB-TADP, and enzyme was isolated by column centrifugation at various times as described under Experimental Procedures. (Inset) Relationship between percentage change in DPNH inhibition and incorporation of BDB-TADP The percentage of the maximum change in activity at a given time is defined by $(v_t - v_0)/(v_\infty - v_0) \times 100$, where v_t is the enzymatic velocity measured in the presence of 600 μM DPNH at a particular time and v_0 and v_∞ are the velocities measured under the same conditions at zero time and at complete reaction, respectively. The percent maximum change in DPNH inhibition was calculated from the inset of Figure 5, and the net incorporation (total incorporation at the given time minus the incorporation at zero time) of BDB-TADP at that same time was determined either from the covalently bound radioactivity after reaction with [β-^{32}P]BDB-TADP (●) or from assay of the phosphorus content of the enzyme modified with the nonradioactive BDB-TADT (○).

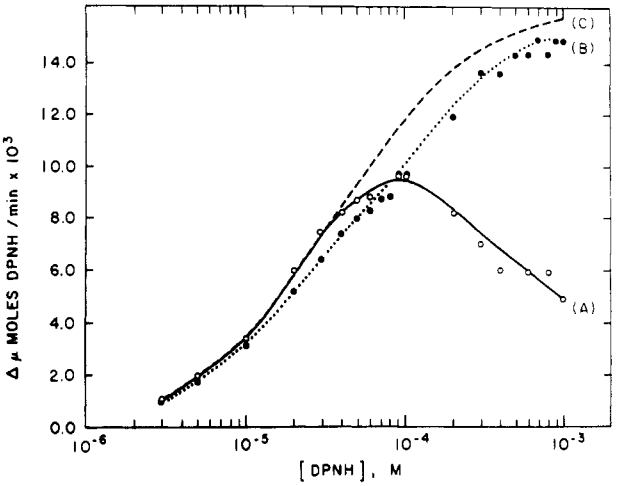

FIGURE 3: Initial velocity as a function of DPNH concentration for native enzyme (○, A) and modified enzyme (●, B) containing about 1 mol of [β-^{32}P]BDB-TADP/subunit. (C) The theoretical curve drawn for native enzyme in the absence of inhibition at higher DPNH concentrations, calculated for K_m = 38 μM and V_{max} = 0.0158 μmol of DPNH/min. At DPNH concentrations up to 2 × 10^{-4} M, velocity measurements were made at 340 nm as described under Experimental Procedures. At higher DPNH concentrations, measurements were made at 375 nm. The amount of coenzyme oxidized as calculated by using the values of ϵ_{340nm} = 6.22 × 10^3 M^{-1} cm^{-1} and ϵ_{375nm} = 1.85 × 10^3 M^{-1} cm^{-1} for DPNH.

the isolated modified enzymes. The inorganic phosphorus determination was a modification of the procedures of Hess & Derr (1975) and Lanzetta et al. (1979). Sample (200 μL) containing inorganic phosphorus was mixed with 20 μL of 10 N H_2SO_4 and 800 μL of a fresh mixture (3:1) of Malachite Green (0.045% in 0.33 N HCl) and ammonium molybdate (4.2% in 3 N HCl). The absorbance was measured after 5 min at 660 nm. Samples containing organic phosphorus were digested prior to analysis by incubating up to 5 nmol of sample with 20 μL of 10 N H_2SO_4 at 190 °C for 2 h in a tube closed with aluminum foil. If the residue was colored at this point, 50 μL of 30% H_2O_2 was added, and the sample was incubated again at 190 °C for 1 h in an open tube.

GTP Binding Studies. Binding of [U-^{14}C]GTP to native and modified enzymes was measured at 25 °C by an ultrafiltration technique using an Amicon Model 10-PA ultrafiltration cell with a PM-10 membrane to separate free ligand from enzyme-bound ligand (Pal & Colman, 1979). Initial solutions contained 0.4 mg/mL glutamate dehydrogenase, 100 μM DPNH, and varying concentrations of GTP in Tris–0.043 M acetate buffer (pH 7.1) containing 10 mM potassium phosphate and 86 μM EDTA. Both free and total ligand concentrations were determined from the specific activity of [U-^{14}C]GTP with a liquid scintillation counter. The concentration of bound ligand was calculated from the difference between the concentrations of total and free ligand.

Results

Incorporation of 6-[(4-Bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-[β-^{32}P]Diphosphate by Glutamate Dehydrogenase. Incubation of glutamate dehydrogenase (1 mg/mL) with 0.25 mM [β-^{32}P]BDB-TADP at pH 7.1 results in a time-dependent incorporation of reagent. As shown in Figure 2, there is relatively little increase in incorporation after 55 min and approximately 1 mol of [β-^{32}P]BDB-TADP/mol of enzyme subunit is incorporated at 120 min. In order to evaluate any changes in the catalytic or regulatory characteristics of glutamate dehydrogenase upon reaction with BDB-TADP, the modified enzyme was isolated by the column centrifugation method after the reaction had proceeded for 120 min.

Kinetic Properties of Modified Enzyme. The kinetic characteristics of modified glutamate dehydrogenase containing about 1 mol of BDB-TADP/mol of subunit were compared with those of native enzyme. As indicated in Figure 3, native glutamate dehydrogenase is inhibited by concentration of DPNH above 1 × 10^{-4} M. For enzyme modified with BDB-TADP, the plot of velocity vs. DPNH concentration is similar to that of native enzyme up to 1 × 10^{-4} M DPNH, implying that reaction does not occur at the catalytic site; however, no inhibition is noted at high concentrations of DPNH. The modified enzyme exhibits normal Michaelis–Menten kinetics: a linear plot of 1/V vs. 1/[DPNH] is observed with K_m = 44 μM and V_{max} = 0.0149 μmol of DPNH/min. This may be compared with 38 μM and 0.0158 μmol of DPNH/min for native enzyme as calculated from the linear portion of the Lineweaver–Burk Plot. Curve C of Figure 3 is a theoretical calculation for the native enzyme in the absence of inhibition by high concentration of DPNH, using a K_m of 38 μM. The fully modified enzyme exhibits a maximum velocity that is only 6% less than the theoretical V_{max} calculated for the native enzyme were there to be no inhibition by DPNH.

The concentration dependence of activation by ADP for native and modified enzyme as measured in the presence of 100 μM DPNH has been compared. The activation constant for the enzyme–ADP complex (K_{ADP}) can be calculated from the relationship for an uncompetitive allosteric modifier as described by Frieden (1963). The constant has been shown to be numerically equal to the concentration of ADP at which the velocity equals

$$(1/2)(V_0 + V_A) \quad (1)$$

where V_0 and V_A are the maximum velocities in the absence

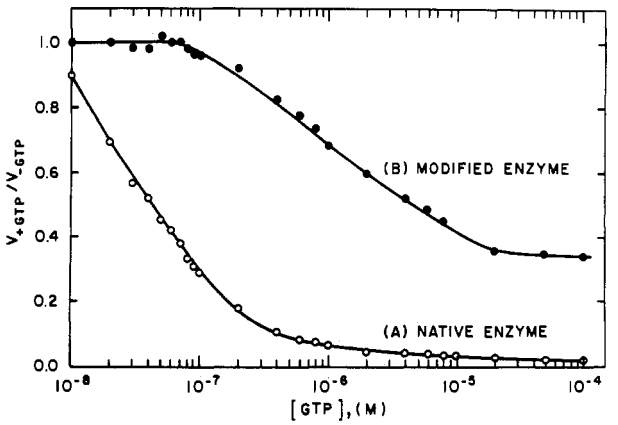

FIGURE 4: Ratio of maximum velocities in the presence and absence of GTP for native and modified enzymes. The velocities were measured as a function of GTP concentration with 100 μM DPNH as coenzyme for native (○, A) and modified (●, B) enzyme containing about 1 mol of [β-^{32}P]BDB-TADP/mol of subunit.

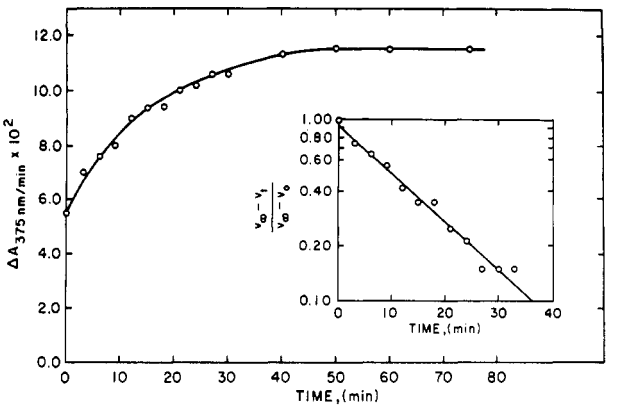

FIGURE 5: Reaction of 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate with glutamate dehydrogenase, as measured by the loss of DPNH inhibition. Glutamate dehydrogenase (1 mg/mL) was incubated with [β-^{32}P]BDB-TADP (0.25 mM) at 25 °C in 0.05 M potassium phosphate buffer, pH 7.1, containing 10% methanol. At each indicated time, a 20-μL aliquot was withdrawn, diluted 20-fold with Tris–0.1 M acetate buffer, pH 8 at 0 °C, and assayed in the presence of 1600 μM DPNH as described under Experimental Procedures. (Inset) The rate constant was measured from the semilogarithmic plot of $(v_\infty - v_t)/(v_\infty - v_0)$, where v_t and v_0 are the enzymatic velocities measured at the indicated time and zero time, respectively, and v_∞ is the constant velocity observed at the end of the reaction.

and presence of saturating concentrations of the modifier, respectively (Frieden, 1963). The data indicate that the maximum extent of activation by ADP (V_{+ADP}/V_{-ADP}) is reduced from 2.1 for the native to 1.4 for the modified enzyme. However, the dissociation constants for ADP from native (19 μM) and modified (25 μM) enzymes are not appreciably different.

The concentration dependence of inhibition by GTP for native enzyme and the modified enzyme is shown in Figure 4. The inhibition constant for the enzyme–GTP complex has been shown to be numerically equal to the concentration of GTP at which the velocity equals

$$(1/2)(V_0 + V_I) \qquad (2)$$

where V_0 and V_I are the maximum velocities in the absence and presence of GTP, respectively (Frieden, 1963). The maximum velocity of native glutamate dehydrogenase is decreased 97% at saturating concentrations of the inhibitory regulator GTP, and a dissociation constant of 0.04 μM GTP was calculated. In comparison, the maximum velocity of the modified enzyme is decreased only 66% by saturating levels of GTP, with a dissociation constant of 1.1 μM.

Kinetics of Reaction of Glutamate Dehydrogenase with 6-[(4-Bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-Diphosphate. One of the most striking differences between the native and modified enzymes is the absence of inhibition by high concentrations of DPNH in the case of modified enzyme. This is reflected in a much higher velocity for the modified enzyme than for the native enzme, when measured at 6×10^{-4} M DPNH (Figure 3). This difference has been exploited to allow measurement of the rate of the reaction of glutamate dehydrogenase with BDB-TADP. Figure 5 indicates that incubation of enzyme with 0.25 mM BDB-TADP produces a time-dependent increase in the activity as assayed in the presence of the constant concentration of 6×10^{-4} M DPNH. In contrast, no appreciable change in catalytic activity is observed if the incubated enzyme is assayed at DPNH concentrations up to 1×10^{-4} M, suggesting that the active site of glutamate dehydrogenase is not modified by BDB-TADP. The rate of reaction of BDB-TADP with the enzyme can be monitored by this time-dependent desensitization to inhibition by 6×10^{-4} M DPNH. As shown in the inset of Figure 5, a pseudo-first-order rate constant of 0.062 min^{-1} can be calculated from a semilogarithmic plot of $(v_\infty - v_t)/(v_\infty - v_0)$ vs. time where v_t and v_0 (0.055 ΔA_{375nm}/min) are the enzymatic velocities measured at the indicated time and at zero time, respectively, and v_∞ (0.115 ΔA_{375nm}/min) is the constant maximal velocity reached after long incubation times.

Since the same conditions were used for incubation of BDB-TADP with glutamate dehydrogenase when measuring the time dependence of reagent incorporation (Figure 2) and the time dependence of loss of DPNH inhibition (Figure 5), the inset of Figure 5 can be used to relate the two processes. The percentage of maximum change in DPNH inhibition is linearly proportional to the incorporation of BDB-TADP, as shown by the inset of Figure 2, and extrapolates to 0.96 mol of BDB-TADP covalently bound per subunit when the enzyme becomes completely unresponsive to DPNH inhibition.

The bromodioxobutyl moiety is highly reactive, and it might be expected that BDB-TADP would undergo decomposition in aqueous buffers. The rate constant for decomposition of BDB-TADP was measured from the time dependence of bromide release by incubation of the reagent in the absence of enzyme under similar conditions to those used for reaction with glutamate dehydrogenase. For decomposition of BDB-TADP at pH 7.1 and 25 °C, $k = 0.0114$ min^{-1}, thus indicating that the reagent has a half-life of 61 min under these conditions.

The pseudo-first-order rate constants for reaction of glutamate dehydrogenase with BDB-TADP were measured as a function of reagent concentration over the range 0.05–0.30 mM BDB-TADP. The first 30 min were used to calculate the rate constants in order to minimize the effect of reagent decomposition. The rate constants were calculated by the method illustrated in the inset of Figure 5. The experimental end point (i.e., v_∞) was the same at concentrations of BDB-TADP from 0.13 to 0.30 mM, indicating that the plateau observed after 50 min in Figure 5 is a true end point for the reaction. At lower concentrations of BDB-TADP, the true end point is not reached because of the reagent decomposition, as has been demonstrated for other unstable compounds (Jacobson & Colman, 1982); but the same value of v_∞ (0.115 ΔA_{375nm}/min) was used in calculating the rate constants for the reactions at all concentrations of BDB-TADP.

A plot of the observed rate constants vs. BDB-TADP concentration is not linear but rather exhibits saturation kinetics.

Table I: Effect of Substrates and Allosteric Ligands on Rate of Reaction of BDB-TADP with Glutamate Dehydrogenase[a]

additions to reaction mixture	k_{obsd} ($\times 10^3$ min^{-1})
none	36.0
α-ketoglutarate (20 mM)	39.6
DPNH (100 μM)	40.0
GTP (50 μM)	37.4
ADP (1 mM)	9.6
ADP (1 mM) + DPNH (100 μM)	9.6
DPNH (5 mM)	9.4
DPNH (5 mM) + GTP (10 μM)	10.6
DPNH (5 mM) + GTP (25 μM)	3.9
DPNH (5 mM) + GTP (50 μM)	2.7
DPNH (5 mM) + GTP (100 μM)	*b*

[a] Glutamate dehydrogenase (1 mg/mL) was incubated with 0.1 mM BDB-TADP at 25 °C in 0.05 M potassium phosphate buffer, pH 7.1. Ligands were added as indicated. The pseudo-first-order rate constants were determined as described in Figure 5 and under Experimental Procedures. [b] No reaction.

This observation suggests that the reagent (I) binds reversibly to the enzyme prior to irreversible covalent modification to yield modified enzyme (EI′) as follows:

$$E + I \rightleftharpoons EI \xrightarrow{k} EI' \quad (3)$$

This is a kinetic characteristic that might be expected for a reagent that is functioning as an affinity label. The observed rate constant for modification can be expressed as

$$k_{obsd} = \frac{k}{1 + K_D/[I]} \quad (4)$$

where k_{obsd} is the apparent rate constant observed at a particular concentration of BDB-TADP, K_D is the apparent dissociation constant of the enzyme–BDB-TADP complex, and k is the intrinsic rate constant for covalent modification of the enzyme observed at infinitely high concentrations of the reagent. From a plot of the reciprocal form of this equation

$$\frac{1}{k_{obsd}} = \frac{1}{k} + \frac{K_D}{k}\frac{1}{[I]} \quad (5)$$

the dissociation constant K_D = 0.19 mM and the maximum rate constant k = 0.13 min^{-1} can be calculated. The K_D for BDB-TADP is higher than that of DPNH binding to the catalytic site of glutamate dehydrogenase but lower than that of DPNH binding to the inhibitory site.

Effect of Added Substrates and Allosteric Ligands on Reaction Rate of Enzyme with BDB-TADP. The effects of added substrates and allosteric modifiers on the rate constant for the reaction of glutamate dehydrogenase with 0.10 mM BDB-TADP are shown in Table I. The substrate α-ketoglutarate when added to the reaction mixture does not affect the rate constant although it was present at a high concentration compared to its Michaelis constant (Goldin & Frieden, 1972). Furthermore, 100 μM DPNH (a concentration sufficient to bind to the catalytic site but not appreciably to the inhibitory site) also fails to reduce the reaction rate. These results indicate that modification by BDB-TADP does not occur at the active site.

The nucleotide GTP when added by itself does not affect the rate constant. In contrast, the inclusion in the reaction mixture of saturating concentrations of ADP (1 mM) or a combination of ADP (1 mM) and DPNH (100 μM) causes a 4-fold decrease in the rate constant. Concentrations of DPNH sufficiently high to occupy the inhibitory site (5 mM) causes considerable decrease in the rate of modification by BDB-TADP. The nucleotide GTP is known to tighten the binding of DPNH (Frieden, 1963; Koberstein et al., 1973; Sund et al., 1975). While the addition of 10 μM GTP together with 5 mM DPNH does not reduce the rate constant more than 5 mM DPNH alone, the combination of 5 mM DPNH with 25, 50, and 100 μM GTP causes a progressively more striking decrease in the reaction rate so that at 100 μM GTP plus 5 mM DPNH no effect of BDB-TADP on DPNH inhibition of glutamate dehydrogenase is observed.

Properties of Enzyme Protected with DPNH (5 mM) and GTP (100 μM). In order to evaluate whether DPNH and GTP would protect against the other changes produced by BDB-TADP in the kinetic characteristics of glutamate dehydrogenase (Figures 3 and 4), enzyme was incubated for 2 h with 0.25 mM [β-^{32}P]BDB-TADP in the presence of 5.0 mM DPNH and 100 μM GTP. Only 0.24 mol of BDB-TADP was incorporated per peptide chain under these protected conditions, as compared with 1.14 mol/peptide chain in the absence of ligands. The enzyme protected with DPNH and GTP exhibited a curve for velocity vs. [DPNH] that was coincident with that of native enzyme (Figure 3A). The protected enzyme was activated 2.35-fold by saturating concentrations of ADP, with a K_{ADP} of 26 μM; the corresponding values for native enzyme are 2.45-fold and 21 μM. The protected enzyme was inhibited 98% by saturating concentration of GTP with K_{GTP} = 0.04 μM; these values can be compared with 98% inhibition and K_{GTP} = 0.03 μM for native enzyme. These results suggest that DPNH in the presence of GTP completely protects against the changes in sensitivity to GTP and ADP regulation as well as against the loss of DPNH inhibition produced by reaction with BDB-TADP. Changes in all of these regulatory properties may stem from reaction at the same site, as is considered further under Discussion.

Binding of GTP by Native and BDB-Modified Glutamate Dehydrogenase. Because DPNH plus GTP protects against changes in the regulatory properties of glutamate dehydrogenase caused by BDB-TADP and because the kinetically determined inhibition constant for GTP is significantly higher for modified than for native enzyme, it might be postulated that BDB-TADP reacts at a GTP site of glutamate dehydrogenase. In order to evaluate this possibility, we measured the reversible binding of [U-^{14}C]GTP to native and modified enzyme by an ultrafiltration technique. These data have been analyzed in accordance with the Scatchard equation:

$$\frac{r}{[GTP]_{free}} = \frac{n}{K_d} - \frac{r}{K_d} \quad (6)$$

where r is the number of moles of GTP bound per mole of peptide chain, n is the number of GTP binding sites per subunit, and K_d is the dissociation constant for the enzyme–GTP complex. It has previously been shown that native glutamate dehydrogenase has two binding sites for GTP per peptide chain in the presence of 100 μM DPNH (Pal & Colman, 1979; Jacobson & Colman, 1982). At 0.4–0.5 mg/mL enzyme, binding to the high-affinity site exhibits nonlinearity of a plot of $r/[GTP]_{free}$ vs. r, with a K_d of 0.6 μM being calculated from the limiting slope for this site; and binding to the second site exhibits an estimated K_d of 40 μM (Jacobson & Colman, 1982). We have obtained similar values for native enzyme in the present study. [It has previously been observed that the directly measured binding constant for the high-affinity GTP site is 20–25 times greater than the kinetically determined inhibition constant, which has been attributed to differences in the buffer composition, pH, and enzyme concentration in the two types of experiments (Jacobson & Colman, 1982).]

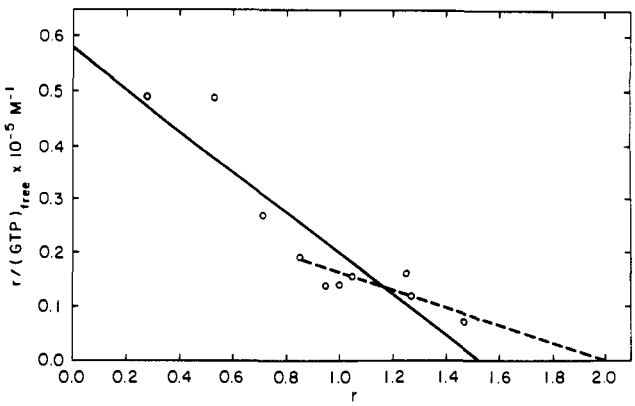

FIGURE 6: Binding of GTP to BDB-modified glutamate dehydrogenase in Tris–0.043 M acetate buffer (pH 7.1) containing 10 mM potassium phosphate and 86.0 μM EDTA. Measurements were made in the presence of 100 μM DPNH, at an enzyme concentration of 0.4 mg/mL. The solid line shows the linear regression line for all the experimental points. The dashed line is drawn for the points above $r = 0.8$.

Figure 6 shows the Scatchard plot for the binding of [U-^{14}C]GTP, in the presence of DPNH, to enzyme containing about 1 mol of BDB-TADP incorporated per peptide chain. The binding of radioactive GTP by modified enzyme is weaker than that by native enzyme. If all the data points are included, as indicated by the solid line, a value of 1.53 is obtained for the number of sites per peptide chain, with K_d = 26 μM. Alternatively, in analogy to the shape of the Scatchard plot obtained for native enzyme (Pal & Colman, 1979; Jacobson & Colman, 1982), the experimental data can be resolved into two distinct sites of differing affinities with K_d values of about 13 μM and 60 μM, as illustrated by the dashed line. In either case, it is clear that, although modification of glutamate dehydrogenase by BDB-TADP may weaken its affinity for GTP, it does not eliminate either of the two GTP sites of the native enzyme.

Discussion

The nucleotide analogue 6-[(4-bromo-2,3-dioxobutyl)-thio]-6-deaminoadenosine 5′-diphosphate exhibits several characteristics of an affinity label in its reaction with glutamate dehydrogenase. It reacts covalently in a limited, specific manner: only 1 mol of BDB-TADP is incorporated per mol of peptide chain, as measured either by radioactivity or phosphorus assay. The rate constant for reaction of BDB-TADP with glutamate dehydrogenase exhibits the characteristic nonlinear dependence on reagent concentration indicative of reversible binding of reagent prior to irreversible modification. Marked protection against modification by BDB-TADP is observed when certain of the natural nucleotides are included in the reaction mixture.

Structurally, 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate can most reasonably be viewed as an analogue of ADP or DPNH, although all of the nucleotide sites should be considered as possible targets for attack by BDB-TADP. The function of the enzymatic site that reacts can be evaluated by considering the properties of the enzyme that are altered by the covalent reaction, as well as the substrates or regulatory compounds that protect against the modification reaction. The catalytic site is not attacked by BDB-TADP because incubation with reagent produces no change in the intrinsic activity of the enzyme when measured in the absence of regulatory compounds at high α-ketoglutarate concentration and 100 μM DPNH. In addition, the Michaelis constant of the modified enzyme for DPNH is not appreciably different from that of the native enzyme when measured at low concentrations of DPNH to monitor the catalytic site. Furthermore, lack of protection against modification by the substrate α-ketoglutarate and by 100 μM DPNH, which are known to bind to the enzyme under similar conditions (Goldin & Frieden, 1972; Sund et al., 1975), supports the conclusion that BDB-TADP does not react at the active site.

One or more of the regulatory sites must then constitute the primary locus of reaction of BDB-TADP. The most striking effect of modification by BDB-TADP is the complete desensitization of the enzyme to inhibition by high concentrations of DPNH. The modified enzyme is not inhibited by DPNH concentrations above 200 μM, as is the native enzyme; indeed, the modified enzyme exhibits a V_{max} that is almost constant above 400 μM DPNH. The most reasonable explanation is that BDB-TADP reacts covalently within the DPNH regulatory site, thereby preventing high concentrations of DPNH from binding at that site and inhibiting the enzyme. It is notable that 5 mM DPNH alone markedly decreases the rate constant for modification by BDB-TADP. Furthermore, 5 mM DPNH plus 100 μM GTP decrease the reaction rate below the level of detection. Since GTP alone does not influence the reaction rate of BDB-TADP with the enzyme, this result is consistent with the reports that GTP tightens the binding of DPNH both to the catalytic and the inhibitory sites (Goldin & Frieden, 1972; Koberstein & Sund, 1973; Sund et al., 1975) and that DPNH is the ligand most directly responsible for protection.

It may also be considered that BDB-TADP reacts at one of the two GTP binding sites of the enzyme since the responsiveness of the enzyme to GTP is significantly decreased upon covalent modification. For example, the kinetically determined inhibition constant for GTP is about 25-fold higher in the modified than in the native enzyme. The following evidence suggests, however, that the change in K_d for GTP is an indirect effect of modification of the DPNH site rather than the result of a direct reaction within a GTP regulatory site. First, the maximum velocity of the modified enzyme is still inhibited 66% by saturating concentrations of GTP. Second, GTP alone does not decrease the rate of modification by BDB-TADP, and when 5 mM DPNH is combined with 10 μM GTP (a concentration that is considerably above the dissociation constant for the high-affinity GTP site), there is no further decrease in the rate constant beyond that produced by 5 mM DPNH alone. In contrast, for the reaction of glutamate dehydrogenase with 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine (5′-FSBεA), a compound that reacts specifically at the high-affinity GTP site (Jacobson & Colman, 1982), a 13-fold decrease in the reaction rate constant is produced by 100 μM DPNH plus 10 μM GTP. In the case of the BDB-TADP reaction, GTP concentrations in the range of the K_d for the low-affinity GTP site (25–100 μM) are required to enhance the protection provided by 5 mM DPNH. Finally, direct measurement of the binding of radioactive GTP to BDB-TADP-modified enzyme indicates that this altered enzyme can still bind 1.5–2 mol of GTP/mol of peptide chain. In contrast, enzyme stoichiometrically modified with 5′-FSBεA binds only 1.0 mol of radioactive GTP under the same conditions (Jacobson & Colman, 1982). It is concluded that BDB-TADP does not modify a GTP site directly but reacts instead at a different site, causing a conformational change that indirectly weakens the binding of GTP. Since there appear to be mutual interactions between the GTP and DPNH sites (Goldin & Frieden, 1972; Koberstein et al., 1973; Sund et al., 1975), it is not surprising that modification of the DPNH

regulatory site would indirectly influence binding at the GTP sites.

The nucleotide analogue 6-[(4-bromo-2,3-dioxobutyl)-thio]-6-deaminoadenosine 5′-diphosphate also does not seem to react directly with the ADP activating site. Although there is a decrease in the maximum extent of activation of the modified enzyme by ADP, there is no significant change in the kinetically measured dissociation constant for that nucleotide. It is apparent that ADP can still bind to the BDB-TADP-modified enzyme. When added to the reaction mixture at concentrations high relative to its known binding constant (Frieden & Colman, 1967), ADP causes a 4-fold decrease in the rate constant for reaction with BDB-TADP. These results can best be explained in terms of indirect interactions between the ADP site and the DPNH inhibitory sites. Various binding studies have indicated an apparent competition between ADP and inhibitory concentrations of DPNH (Pantaloni & Dessen, 1969; Cross & Fisher, 1970; Koberstein & Sund, 1973; Koberstein et al., 1973; Pantaloni & Lecuyer, 1973), although chemical modification studies [e.g., Pal et al. (1975)] demonstrate that the ADP and DPNH sites are not identical. If BDB-TADP were reacting both at an ADP and separately at a DPNH site, it might have been expected that the inclusion of ADP in the reaction mixture would have protected the enzyme specifically against the change in maximum extent of activation by ADP; however, this was not the case. Instead, glutamate dehydrogenase reacted with BDB-TADP in the presence of 5 mM DPNH plus 100 μM GTP functions kinetically like the native enzyme with respect to its inhibition by GTP, activation by ADP, and inhibition by high concentrations of DPNH. These results suggest that the primary site of attack by BDB-TADP is the DPNH regulatory site. The other changes observed in the regulatory properties of the enzyme can be explained as indirect effects resulting from the primary modification of the DPNH inhibitory site; protection against reaction at the DPNH site concomitantly prevents changes in the kinetic effects of ADP and GTP.

The bromoketo and dioxobutyl groups of the new nucleotide analogue 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate have the ability to react with the side chains of several amino acids, including cysteine, histidine, aspartic acid, glutamic acid, and arginine (Hartman, 1977; Riordan, 1973). Because of the location of the functional group adjacent to the 6-position of the thioadenine ring, it would be expected that BDB-TADP reacts with an amino acid residue of glutamate dehydrogenase that participates in the adenine subsite of the DPNH inhibitory site. Work is in progress to identify that amino acid residue. It is anticipated that 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate will be a valuable addition to the nucleotide analogues available for the affinity labeling of adenine nucleotide sites in a variety of proteins.

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