Guanosine 5′-*O*-[*S*-(3-Bromo-2-oxopropyl)]thiophosphate: A New Reactive Purine Nucleotide Analog Labeling Met-169 and Tyr-262 in Bovine Liver Glutamate Dehydrogenase†

Derya H. Ozturk, Inshik Park, and Roberta F. Colman*

Department of Chemistry and Biochemistry, University of Delaware, Newark, Delaware 19716

Received April 7, 1992; Revised Manuscript Received July 13, 1992

ABSTRACT: A new guanosine nucleotide has been synthesized and characterized: guanosine 5′-*O*-[*S*-(3-bromo-2-oxopropyl)]thiophosphate (GMPSBOP), with a reactive functional group which can be placed at a position equivalent to the pyrophosphate region of GTP. This new analog is negatively charged at neutral pH and is similar in size to GTP. GMPSBOP has been shown to react with bovine liver glutamate dehydrogenase with an incorporation of 2 mol of reagent/mol of subunit. The modification reaction desensitizes the enzyme to inhibition by GTP, activation by ADP, and inhibition by high concentrations of NADH, but does not affect the catalytic activity of the enzyme. The rate constant for reaction of GMPSBOP with the enzyme exhibits a nonlinear dependence on reagent concentration with K_D = 75 μM. The addition to the reaction mixture of α-ketoglutarate, GTP, ADP, or NADH alone results in little decrease in the rate constant, but the combined addition of 5 mM NADH with 0.4 mM GTP or with 10 mM α-ketoglutarate reduces the reaction rate ~6-fold. GMPSBOP modifies peptides containing Met-169 and Tyr-262, of which Tyr-262 is not critical for the decreased sensitivity of the enzyme toward allosteric ligands. The presence of 0.4 mM GTP plus 5 mM NADH protects the enzyme against reaction at both Met-169 and Tyr-262, but yields enzyme with 1 mol of reagent incorporated/mol of subunit which is modified at an alternate site, Met-469. In the presence of 0.2 mM GTP + 0.1 mM NADH, protection against modification of Tyr-262, but only partial protection against labeling of Met-169, is observed. In contrast, the presence of 10 mM α-ketoglutarate + 5 mM NADH protect only against reaction with Met-169. The results suggest that GMPSBOP reacts at the GTP-dependent NADH regulatory site [Lark, R. H., & Colman, R. F. (1986) *J. Biol. Chem. 261*, 10659–10666] of bovine liver glutamate dehydrogenase, which markedly affects the sensitivity of the enzyme to GTP inhibition. The reaction of GMPSBOP with Met-169 is primarily responsible for the altered allosteric properties of the enzyme.

Affinity labeling has been used successfully in elucidating information about the function–structure relationships in enzymes (Colman, 1991). In particular, purine nucleotides containing reactive groups have been effective in probing catalytic and regulatory nucleotide sites of many enzymes (Colman, 1983, 1991). Although there are a number of reactive adenosine derivatives, relatively few reactive guanosine derivatives are available. Among the most useful guanosine derivatives are 8-azidoGTP (Geahlen & Haley, 1979; Potter & Haley, 1983) and 5′-[*p*-(fluorosulfonyl)benzoyl]guanosine (Pal & Colman, 1979; Tomich et al., 1981). In 5′-[*p*-(fluorosulfonyl)benzoyl]guanosine, the reactive (fluorosulfonyl)benzoyl group is in the region normally occupied by the phosphoryl group of GTP; however, the compound lacks the phosphate group and the charge of GTP. The 8-azidoGTP derivative has the appropriate charge but the reactive azido group is adjacent to the purine ring, where it may change the predominant conformation of the nucleotide in solution.

In this paper, we report the synthesis of guanosine 5′-*O*-[*S*-(3-bromo-2-oxopropyl)]thiophosphate (GMPSBOP),[1] a new reactive guanosine derivative which represents a novel class of compounds containing a bromo keto moiety linked to the sulfur of a purine nucleotide thiophosphate. GMPSBOP, shown schematically in Figure 1a compared to GTP in Figure 1b, has the reactive bromooxopropyl group at a position which may be structurally equivalent to the pyrophosphate region of adenosine and guanosine nucleotides. In particular, the bromooxopropyl compound is similar in size to the natural triphosphate, GTP. Potentially, the bromo keto moiety can react with the nucleophilic side chains of several types of amino acids including cysteine, methionine, lysine, tyrosine, and histidine once GMPSBOP binds to a purine nucleotide site (most likely a GTP site) in an enzyme.

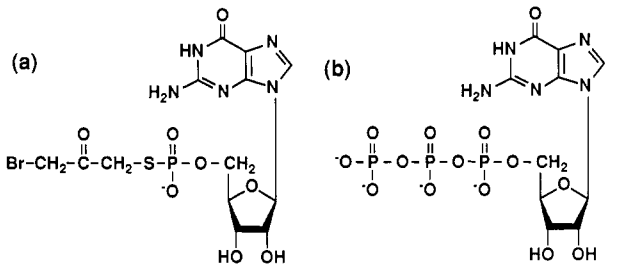

FIGURE 1: Schematic structures of (a) GMPSBOP and (b) GTP.

Bovine liver glutamate dehydrogenase [L-glutamate:NAD(P)$^+$ oxidoreductase (deaminating), EC 1.4.1.3] is an allosteric enzyme which is regulated by purine nucleotides. The enzyme

† This research was supported by USPHS Grant DK 37000 and NSF Grant DMB-9105116.

[1] Abbreviations: GMPSBOP, guanosine 5′-*O*-[*S*-(3-bromooxopropyl)]-thiophosphate; GMPS, guanosine 5′-*O*-thiophosphate; 8-BDB-TA-5′-TP, 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-triphosphate; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); FSBεA, 5′-[*p*-fluorosulfonylbenzoyl]-1,N^6-ethenoadenosine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; TFA, trifluoroacetic acid; PTH, phenylthiohydantoin; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; UV, ultraviolet.

0006-2960/92/0431-10544$03.00/0

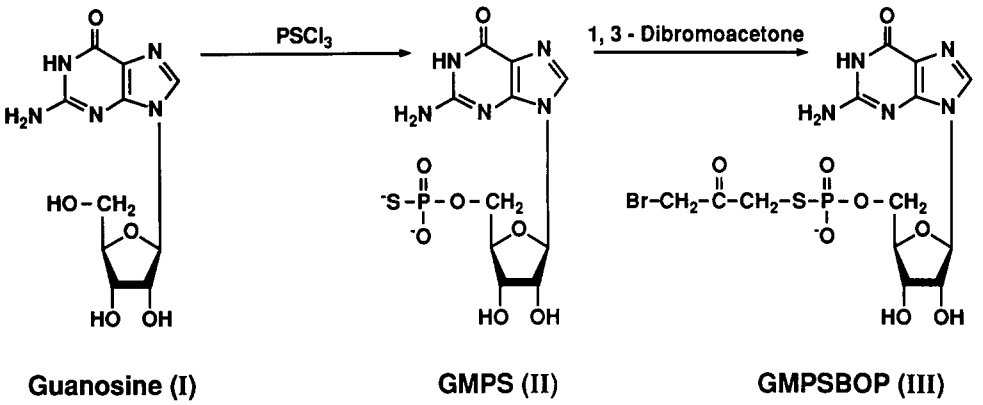

FIGURE 2: Synthetic scheme for preparation of guanosine 5′-*O*-[*S*-(3-bromo-2-oxopropyl)]thiophosphate (GMPSBOP).

responds to several purine nucleotide regulators, of which ADP activates the enzyme while GTP and high concentrations of NADH are inhibitors. All of these allosteric effectors bind to regulatory sites distinct from the catalytic coenzyme site (Colman, 1991). The enzyme is composed of six identical subunits in its active form, each of which binds 2 mol of NADH (catalytic and regulatory sites) (Colman, 1991); the inhibitor GTP also occupies two sites per subunit of enzyme in the presence of NADH but only one site in the absence of the reduced coenzyme (Pal & Colman, 1979). In addition, the allosteric activator ADP occupies two sites per subunit (Batra & Colman, 1986a).

No crystal structure is available for bovine liver glutamate dehydrogenase as yet. Studies of this enzyme using purine nucleotide analogs with reactive groups as affinity labels have provided information about several amino acid residues within regulatory sites (Colman, 1991; Schmidt & Colman, 1984; Batra et al., 1989; Dombrowski et al., 1992; Ozturk et al., 1990; Ozturk & Colman, 1991). This paper demonstrates that the new guanosine analog, GMPSBOP, reacts covalently with bovine liver glutamate dehydrogenase allowing identification within the enzyme of additional amino acids that influence the enzyme's response to regulatory compounds.

EXPERIMENTAL PROCEDURES

Materials. Bovine liver glutamate dehydrogenase was purchased from Boehringer Mannheim Corp. as an ammonium sulfate suspension. It was dialyzed against two changes of 0.1 M potassium phosphate buffer (pH 7.1) at 4 °C over 18 h. The dialysate was centrifuged at 15 000 rpm for 20 min at 4 °C to remove precipitated and denatured protein. The enzyme concentration in the supernatant was determined spectrophotometrically by using $E^{1\%}_{280nm}$ = 9.7 (Olson & Anfinsen, 1952). The ratio of A_{280nm}/A_{260nm} for the enzyme was 1.8. The enzyme was stored in aliquots at −75 °C and was thawed rapidly prior to use. For calculations, 56 100 was used as the molecular weight for the enzyme subunit (Smith et al., 1970).

Coenzymes, nucleotides, guanosine, EDTA, PIPES, dithiothreitol, HEPES, ammonium bicarbonate, Sephadex G-50–80, Malachite Green base, phosphorus standard solution, and TPCK-treated trypsin were purchased from Sigma. Ammonium molybdate and 30% hydrogen peroxide were supplied by Fisher Scientific Co. Ultrapure urea and Tris were obtained from ICN Biochemicals. DE-52 was obtained from Whatman; Dowex 50W-X4 (100–200 mesh) and protein dye concentrate were from Bio-Rad Laboratories. Thiophosphoryl chloride and trifluoroacetic acid were supplied by Aldrich Chemical Co., and 1,3-dibromoacetone was from Lancaster Synthesis Ltd. HPLC grade acetonitrile was obtained from Polysciences Inc. [^{3}H]$NaBH_4$ was purchased from New England Nuclear Corp. All other reagents were analytical grade.

Analytical Methods. NMR spectra were obtained with a Bruker WM 250-MHz spectrometer at room temperature. ^{31}P NMR samples were run in water containing 10% D_2O, and H_3PO_4 (85%) was used as an external standard for chemical shifts. ^{1}H NMR samples were run in D_2O using tetramethylsilane as an internal standard.

The rate of hydrolysis of GMPSBOP (followed as the rate of bromide release) was determined with a microprocessor pH/millivolt meter, Model 811 (Orion Research) equipped with a bromide electrode. For these determinations, 1 mM GMPSBOP was used in 5 mL of buffer solutions at room temperature.

Synthesis of Guanosine 5′-O-[S-(3-Bromo-2-oxopropyl)]-thiophosphate. The overall synthetic scheme is summarized in Figure 2. Guanosine (I) was thiophosphorylated to give guanosine 5′-*O*-thiophosphate (II), which was then coupled with 1,3-dibromoacetone to yield the final product guanosine 5′-*O*-[*S*-(3-bromo-2-oxopropyl)]thiophosphate (III).

Synthesis of Guanosine 5′-O-Thiophosphate (II). Guanosine 5′-*O*-thiophosphate (GMPS) was prepared according to a modification of the procedures of Murray and Atkinson (1968) and Connolly et al. (1982). Guanosine (2.80 g, 10 mmol) was suspended in 50 mL of anhydrous trimethyl phosphate for 1.5 h at 40 °C with continual stirring. To the resultant milky suspension was added 30 mmol (3.2 mL) of thiophosphoryl chloride (dried over molecular sieves) at room temperature. The reaction flask was covered with aluminum foil and was stirred for 4 h at room temperature. A second portion of thiophosphoryl chloride (1.6 mL) was added at the end of the 4-h period, and the reaction was allowed to proceed overnight at 4 °C. The crude nucleotides were precipitated by addition of 100 mL of 10% (w/v) barium acetate, and the mixture was maintained on ice for 0.5 h. Ice-cold ethanol (400 mL) containing 2.5% triethanolamine (v/v) was added to the flask, which was kept on ice for 1 h with occasional stirring. The supernatant was discarded after centrifugation for 10 min at 15 000 rpm, 4 °C. The precipitate was washed with 100 mL of 70% ethanol followed by centrifugation for 8 min at 8000 rpm, 4 °C. The washing step was repeated three times. The precipitated barium salt of the product GMPS was then extracted repeatedly by stirring with 100 mL of water for 15 min at room temperature followed by centrifugation; the supernatant was saved. The extraction was continued until A_{252nm} decreased to a constant value of ~2.0 in the supernatant. The pH was adjusted to 8.0 (if necessary) with 0.1 M NaOH. The total water extract (~1500 mL) was applied to a 3 × 60 cm column of DEAE-cellulose (DE-52, bicarbonate form) equilibrated with water at 4 °C. Unreacted guanosine eluted during the application of the

extract to the column. The column was eluted with a linear gradient formed from 1.8 L of H_2O and 1.8 L of 0.4 M NH_4HCO_3. Fractions (11 mL) were monitored for the characteristic UV absorption spectrum of guanosine (λ_{max} = 252 nm). The desired GMPS product (as determined by ^{31}P NMR and UV spectra) was recovered between 0.094 and 0.14 M NH_4HCO_3 (fractions 323–363) as the last peak in the elution profile, following two other minor peaks. The pool of fractions 323–363 was evaporated to dryness under vacuum. Excess salt was removed by repeated rotary evaporation (five times) with 50-mL portions of water. The dry product was dissolved in 20 mL of water and applied to a 3 × 60 cm column of Dowex W50-X4 (H^+ form) equilibrated in water. The column was washed with water, and the acid form of GMPS was eluted in tubes (11-mL fractions) 4–35. The purity of this pool was evaluated by thin-layer chromatography (TLC) and UV spectroscopy. The product was evaporated to dryness followed by coevaporation three times with dry methanol (50-mL portions), giving an overall yield of ~28%. The dry product was stored desiccated at −80 °C.

Preparation of Guanosine 5′-O-[S-(3-Bromo-2-oxopropyl)]-thiophosphate. The final product was obtained by reaction of GMPS (dissolved in water) with 1,3-dibromoacetone (dissolved in methanol). For the coupling reaction, 1 mL of 40 mM GMPS in water (pH 2.9) was used and the pH was adjusted to 5.2 using dilute NaOH. About 0.18 mL of 1,3-dibromoacetone (10.3 M) was dissolved in 1 mL of methanol, and this was mixed with the GMPS solution. The reaction mixture was incubated at 30 °C for 0.5 h. Excess 1,3-dibromoacetone was extracted by chloroform (10 mL, twice) followed by extraction with diethyl ether (12 mL, 3 times). After each extraction, the organic layer was discarded. The reaction goes to completion, and the final product was in the water layer at pH 5.2. The purity of the final product was evaluated by TLC and ^{31}P NMR and was stored frozen in water in aliquots at −80 °C. Under these conditions, GMPSBOP is stable.

Enzymatic Assay. Bovine liver glutamate dehydrogenase was assayed at 25 °C in a Gilford Model 240 spectrophotometer with an expanded scale (0.0–0.1) by monitoring the oxidation of NADH (ϵ_{340nm} = 6.22 × 10 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, 50 mM ammonium chloride, and 5 mM α-ketoglutarate in a total volume of 1.0 mL. In many cases, as indicated in Results, the activity was measured in the presence of 1 μM GTP (allosteric inhibitor). In certain experiments designed to test the response of the enzyme to the allosteric activator ADP, all components were the same in the assay solution except that 200 μM ADP was used in place of 1 μM GTP. In order to determine the effects of varying concentrations of regulatory compounds such as GTP and ADP, conditions and the substrate concentrations were the same as above except that, for NADH concentrations of 0.2 mM or higher, the activity was measured at 375 nm (ϵ = 1.85 × 10^3 M^{-1} cm^{-1}).

Reaction of GMPSBOP with Bovine Liver Glutamate Dehydrogenase. Enzyme (0.5 mg/mL, 8.9 μM enzyme subunits) was incubated with GMPSBOP (0.1–4.0 mM) at 25 °C in 50 mM PIPES buffer (pH 7.15) in a total volume of 0.5 mL. At various times, 20-μL aliquots were withdrawn and diluted 20-fold in Tris–0.1 M acetate buffer (pH 8.0) at 0 °C. A 30-μL aliquot from this dilution was assayed to measure the activity in the presence of 1 μM GTP, and a 10-μL aliquot was used for assays containing 200 μM ADP. In order to determine the effects of allosteric regulators on the reaction rate of GMPSBOP with glutamate dehydrogenase, varying concentrations and combinations of GTP, ADP, NADH, and α-ketoglutarate were included in the incubation mixture under the same conditions and the reaction rates were monitored with 1 μM GTP included in the assays. A control enzyme was also incubated under the same conditions, but without GMPSBOP, to measure catalytic activity as well as response to regulatory compounds.

Preparation of Glutamate Dehydrogenase Modified by GMPSBOP in the Absence and Presence of Allosteric Ligands. Glutamate dehydrogenase [2 mg/mL (35.6 μM enzyme subunits), 2 mL total] was incubated with 0.75 mM GMPSBOP (in the absence or presence of ligands) at 25 °C for 2 h, in 50 mM PIPES buffer (pH 7.15). These were the general conditions used in preparing the modified enzyme samples. In certain experiments, as indicated in Results, substrates and/or nucleotides were added as protectants. The modified and protected enzyme samples were isolated by the column centrifugation technique using Sephadex G-50–80 mesh, as described by Penefsky (1979). The columns were prepared in 5-mL disposable syringes, and the Sephadex G-50–80 equilibrated in 50 mM PIPES buffer (pH 7.15) was supported in columns by glass wool plugs. Each column was loaded with 0.5 mL of the incubation mixture. Two successive column centrifugations were used at 4 °C to remove the excess reagent and ligands. After elution, the protein concentration was determined by the Bio-Rad protein assay (Bradford dye-binding method) (Bradford, 1976). The standard curve was established using different concentrations of unmodified bovine liver glutamate dehydrogenase under the same buffer conditions. A control enzyme was incubated under the same conditions, but in the absence of GMPSBOP or other ligands, and was isolated in the same manner.

Incorporation of GMPSBOP into Bovine Liver Glutamate Dehydrogenase. Incorporation of GMPSBOP into the enzyme obtained by column centrifugation was determined by quantitation of moles of organic phosphorus using a procedure described by Hess and Derr (1975) and Lanzetta et al. (1979), as modified by Bailey and Colman (1987). For this measurement, aliquots of unmodified (control) and modified enzyme samples (0.2–0.4 mL) were first hydrolyzed with H_2SO_4 and then bleached with 30% hydrogen peroxide solution until clear (three or four times with 0.15-mL portions followed by heating for 1 h at 190 °C after each addition). After addition of the Malachite Green base–ammonium molybdate solution [3:1 (v/v)], the A_{660nm} was determined using a total volume of 1.0 mL. The standard curve was established by using aliquots of a 100 μM standard phosphate solution. The volume of 50 mM PIPES buffer (pH 7.15) was maintained constant in all assays whether they contained enzyme or phosphate standards.

It has been shown that, in some cases, dithiothreitol (DTT) can displace a reagent which is covalently bound to enzyme, resulting in restoration of the normal enzymatic activity (Likos & Colman, 1981; Annamalai & Colman, 1981; Tomich et al., 1981). Modified enzyme was prepared as described above, except that at the end of the incubation period 20 mM DTT (final concentration) was added to the incubation mixture. The enzymatic activity was followed in assays containing 1 μM GTP for an additional 1 h. In order to evaluate whether DTT affects the reagent incorporation, the reaction was quenched with the addition of 20 mM DTT (final concentration) prior to the isolation of the GMPSBOP-modified enzyme. To determine the time dependence of incorporation, aliquots were withdrawn at various incubation times over a period of 2 h and 20 mM (final concentration) DTT was used to quench the reaction. The moles of organic phosphorus in

the enzyme sample prepared in this manner were determined as described above.

Reduction of GMPSBOP-Modified Enzyme by [^3H]-$NaBH_4$. A radioactive label was introduced into the GMPS-BOP-modified enzyme by reducing the oxo group of the enzyme-bound reagent with [^{3}H]$NaBH_4$. The reduction reaction was carried out on enzyme samples, which were maintained on ice, with two additions (0.5 h apart) of 100 mM [^{3}H]$NaBH_4$ (in 0.1 M NaOH) to yield 1.5 mM [^{3}H]-$NaBH_4$ in the incubation mixture after each addition. The specific activity of the [^{3}H]$NaBH_4$ was (3–6) $\times 10^{12}$ cpm/mol of hydrogen. A control enzyme was also prepared and treated with [^{3}H]$NaBH_4$ under the same conditions. The reduction reaction was followed by two successive column centrifugations under the conditions described above. Tritium incorporation into the enzyme samples was determined from the counts in 50-μL aliquots.

Preparation of GMPSBOP-Modified Enzyme for Trypsin Digestion. Enzyme samples reduced by [^{3}H]$NaBH_4$ were denatured in 5 M urea and subjected to carboxymethylation by addition of 50 mM iodoacetate (final concentration) at room temperature. After 20 min, iodoacetate was decomposed by incubation with β-mercaptoethanol (0.5 M final concentration) for an additional 10 min at room temperature. Samples were then dialyzed against 20 mM ammonium bicarbonate (pH 8.0) in a fume hood (room temperature) for 4 h. This was followed by exhaustive dialysis at 4 °C against 4 L of 20 mM ammonium bicarbonate (pH 8.0) overnight and finally against 2 L of the same buffer for an additional 6 h. Each enzyme sample was dialyzed in individual flasks. After dialysis, enzyme samples were digested at 37 °C by two successive additions of 3% (w/w) trypsin to glutamate dehydrogenase 1 h apart. Samples were desalted and dried by lyophilization.

Separation of ^3H-Labeled Tryptic Peptides by High-Performance Liquid Chromatography. The ^{3}H-labeled tryptic peptides were separated on a Varian Model 5000 HPLC system equipped with a reverse-phase Vydac C_{18} column (0.46 × 25 cm). Each digest (~1.5 mg of enzyme) was dissolved in 0.8 mL of 0.1% trifluoroacetic acid and filtered through 0.45-μm membrane filters (Millipore) prior to injection. Separation of peptides was carried out at an elution rate of 1 mL/min in 0.1% trifluoroacetic acid, pH 2.0 (solvent A) from 0 to 10 min, followed by a linear gradient from solvent A to 10% solvent B (0.07% trifluoroacetic acid in acetonitrile) between 10 and 50 min and then by a linear gradient to 40% solvent B between 50 and 300 min (elution system I). Fractions of 1 mL were collected. The effluents were monitored at 220 nm, and aliquots (80 μL from the eluted fractions) were assayed for radioactivity in 5 mL of Liquiscint scintillation cocktail (National Diagnostics) using a Packard Tricarb Model 3330 liquid scintillation counter. Further purification of radioactive peaks was achieved on a reverse-phase C_{18} column at pH 5.8. The radioactive pools of interest obtained from elution system I were lyophilized and redissolved in 0.8 mL of 20 mM ammonium acetate, pH 5.8 (solvent C). The tryptic peptides which had eluted between 15% and 40% solvent B (in elution system I) were purified by elution at 1 mL/min from 0 to 10 min with solvent C, followed by a linear gradient from solvent C to 20% Solvent D (20 mM ammonium acetate in 50% acetonitrile) between 10 and 90 min, and then to 40% solvent D between 90 and 290 min (elution system II). Alternatively, the radioactive peaks which were obtained from elution system I between 0% and 15% solvent B were redissolved in solvent C. Purification of these peptides was achieved by elution for 10 min in solvent C followed by a linear gradient to 30% solvent D between 10 and 300 min (elution system III). The elution rate was 1 mL/min. Absorbance at 220 nm was monitored on-line, and aliquots were assayed for radioactivity.

Analysis of ^3H-Labeled Tryptic Peptides. An automated gas-phase protein/peptide sequence analyzer from Applied Biosystems (Model 470A), equipped with an on-line PTH analyzer, Model 120, and computer Model 900A was employed in determining the amino acid sequences of the purified tryptic peptides. Amounts ranging from 50 to 200 pmol of the isolated peptides were loaded on the sequencer.

RESULTS

Characterization of Guanosine 5′-O-Thiophosphate and Guanosine 5′-O-[S-(3-Bromo-2-oxopropyl)]thiophosphate. The synthesis of guanosine 5′-*O*-[*S*-(3-bromo-2-oxopropyl)]-thiophosphate was conducted by the steps outlined in Figure 2. The yield of guanosine 5′-*O*-thiophosphate from guanosine was 28% and the reaction with 1,3-dibromoacetone to give the final product went to completion (100% conversion of GMPS into the monosodium salt of GMPSBOP); the product was stored frozen in water, as described in Experimental Procedures. The purity of GMPS was demonstrated by thin-layer chromatography on microcrystalline cellulose adsorbent with fluorescent indicator (160 μm) coated on flexible ESTAR Base sheets (Eastman Kodak Co.), using isobutyric acid–concentrated ammonium hydroxide–water (66:1:33) as the solvent system. In this solvent system, guanosine (R_f = 0.63) is readily distinguished from GMPS (R_f = 0.31). The presence of the thiophosphate moiety was assessed by spraying the dried TLC plates, after development, with a solution of 0.015% *N*-ethylmaleimide (NEM) in ethanol, followed by a solution of 1 M KOH in ethanol. This test was positive (color changes to pink) only for the spot with R_f = 0.31.

Substitution for an oxygen by a sulfur in a particular phosphoryl group of a purine nucleotide causes a large downfield shift in the ^{31}P NMR spectrum; typically, chemical shifts of 40–45 ppm have been reported for purine α-monophosphorothioates as compared to chemical shifts of 0–5 ppm of purine α-monophosphates (Eckstein & Goody, 1976; Jaffe & Cohn, 1978; Connolly & Eckstein, 1982). ^{31}P NMR analysis of the GMPS, obtained in this study by thiophosphorylation of guanosine, gives a single peak with a shift at 41 ppm, as expected for the guanosine 5′-*O*-thiophosphate.

The final product GMPSBOP was tested for purity in acetonitrile–1.0 M LiCl–water (60:10:30) as solvent system. In this system, R_f values of 0.51, 0.20, 0.28, and 0.60 are obtained, respectively, for guanosine, GMP, GMPS, and GMPSBOP (exhibiting a single spot as visualized under UV light), while 1,3-dibromoacetone migrates with the solvent front on the TLC plate. Furthermore, the NEM test on the spot with R_f = 0.28 was positive (GMPS), but was negative on the spot with R_f = 0.60 (GMPSBOP).

The ultraviolet absorption spectrum of GMPSBOP (shown in Figure 3) is identical with that of GMP (λ_{max} = 252 nm; $\epsilon = 13.7 \times 10^3$ M^{-1} cm^{-1}). The λ_{max} and extinction coefficient values are also the same as those of GMPS reported earlier by Connolly et al. (1982). The UV absorption spectrum of GMPSBOP indicates that the purine ring remains unalkylated in this product, since substitution of alkyl groups in the purine ring of guanosine would be accompanied by shifts in λ_{max}, as exemplified by λ_{max} = 300 nm for 7-methylguanosine (Banik & Roy, 1990).

The organic phosphorus content of GMPSBOP, determined by the procedure of Hess and Derr (1975) and Lanzetta et al. (1979), was 1.06 mol/mol of GMPSBOP, with the concentration of the nucleotide product determined from its

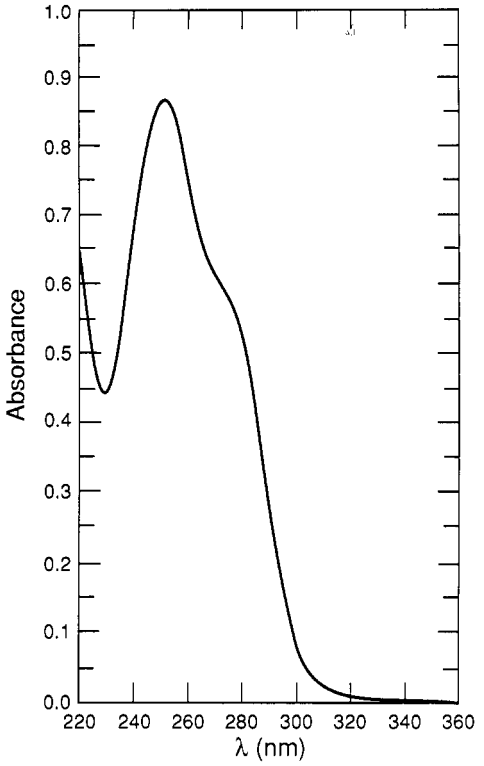

FIGURE 3: Ultraviolet absorption spectrum of GMPSBOP (63.5 μM) in water (pH 5.2) at room temperature.

A_{252nm}. For various samples, the hydrolyzable bromide content was 1.1–1.3 mol/mol of GMPSBOP with the nucleotide product concentration based on A_{252nm}. The approximate ratio of 1 between the hydrolyzable bromide and organic phosphorus content of GMPSBOP eliminates the possibility that the product contains any appreciable amount of a bis(thioether) derivative.

The ^{31}P NMR spectrum of the product GMPSBOP exhibits a chemical shift of 21.7 ppm, similar to the 22.9 ppm reported for *S*-methyl-substituted AMPS by Connolly and Eckstein (1982) and markedly different from the 41 ppm of the parent compound GMPS. The ^{1}H NMR spectrum of GMPSBOP exhibits chemical shifts at 8.45 (s, H-8) and 6.0 ppm (d, H-1′), comparable to those of GMP with 8.15 (s, H-8) and 5.9 ppm (d, H-1′). The ^{1}H NMR spectrum also indicates that the purine ring in GMPSBOP is not alkylated.

Bromo keto derivatives of purine nucleotides have been shown to decompose in aqueous solutions with the release of bromide (DeCamp et al., 1988). Hydrolyzed GMPSBOP exhibits an R_f value of 0.37 on TLC using the acetonitrile–1.0 M LiCl–water (60:10:30) solvent system. After hydrolysis of GMPSBOP with release of bromide, the corresponding alcohol gives a distinguishable ^{31}P NMR chemical shift at 25.0 ppm.

The stability of GMPSBOP in different buffers and pH's was evaluated electrochemically by bromide release, as described in Experimental Procedures. At a given pH, GMPSBOP is more stable in PIPES buffer than in HEPES buffer: at pH 7.15, the first-order rate constant for decomposition was calculated to be 1.3×10^{-2} min^{-1} in 50 mM PIPES buffer ($t_{1/2}$ = 53 min) and 6.4×10^{-2} min^{-1} ($t_{1/2}$ = 12 min) in 50 mM HEPES buffer (pH 7.0). However, the stability decreases as the PIPES concentration increases; e.g., in 0.18 M PIPES buffer (pH 7.15), $k = 3.6 \times 10^{-2}$ min^{-1} and $t_{1/2}$ = 19 min. All the evidence obtained from the analyses of the final product is consistent with the structure shown in Figure 1a.

Effects of GMPSBOP on the Activity of Bovine Liver Glutamate Dehydrogenase. Kinetics of GMPSBOP in Reaction with the Enzyme. Incubation of glutamate dehydrogenase with 2.0 mM GMPSBOP has no effect on the maximum velocity, as measured in the absence of allosteric ligands (Figure 4). Since there is no inactivation, this result indicates that GMPSBOP does not react at the active site of the enzyme.

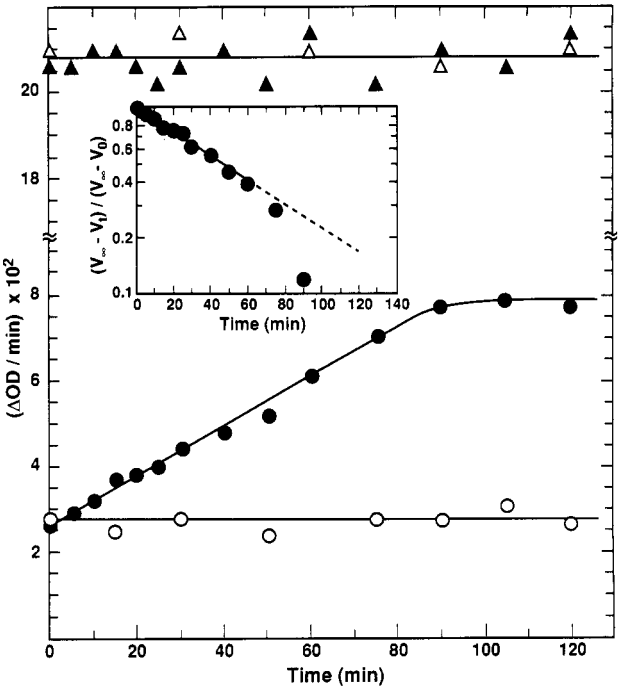

FIGURE 4: Reaction of guanosine 5′-*O*-[*S*-(3-bromo-2-oxopropyl)]-thiophosphate with glutamate dehydrogenase. The enzyme (0.5 mg/mL) was incubated with 2.0 mM GMPSBOP and assayed in the absence (▲) and presence (●) of 1 μM GTP, as described in Experimental Procedures. At various times, during 2 h of incubation, 20-μL aliquots were withdrawn, diluted 20-fold in Tris–0.1 M acetate buffer (pH 8.0) at 0 °C, and assayed using 100 μM NADH as coenzyme. Control enzyme was incubated under the same conditions but without GMPSBOP and assayed in the absence (△) and presence (○) of GTP. Inset: Semilogarithmic plot used to calculate the rate constant for the change in activity as measured in the presence of GTP (see text).

In contrast, the reagent causes desensitization of the enzyme to inhibition by GTP, as evidenced by a time-dependent increase in activity as measured in assays containing 1.0 μM GTP (Figure 4). The maximum increase observed after 90 min was 3.0-fold. A second addition of the reagent at the end of 2 h resulted in no further increase in activity when measured in the presence of 1.0 μM GTP. Furthermore, incubation with higher reagent concentrations yielded the same maximum increase of 3.0-fold in the presence of 1 μM GTP, indicating that this is indeed the maximum change caused by GMPSBOP. The pseudo-first-order rate constants were calculated from the relationship $\ln (V_\infty - V_t)/(V_\infty - V_0) = k_{obs}t$, where V_t and V_0 are the velocities measured at various times and zero time, respectively, and V_∞ is the constant velocity attained when the maximum functional change is complete. Values of V_0 = 0.025ΔOD/min and V_∞ = 0.076ΔOD/min were used in all calculations of rate constants. The inset to Figure 4 illustrates the replot of the data used in these calculations of k_{obs}. Under the experimental conditions used (50 mM PIPES, pH 7.15), GMPSBOP decomposes with $t_{1/2}$ = 53 min, as indicated above. The reaction rates of GMPSBOP with the enzyme were determined from the data obtained during the first 30 min of incubation in order to minimize the effect of reagent decomposition. The pseudo-first-order rate constant at 2.0 mM reagent concentration, as in Figure 4, was calculated to be 0.0148 min^{-1}. The reaction appears to require the bromo keto moiety of GMPSBOP, since incubation of enzyme for

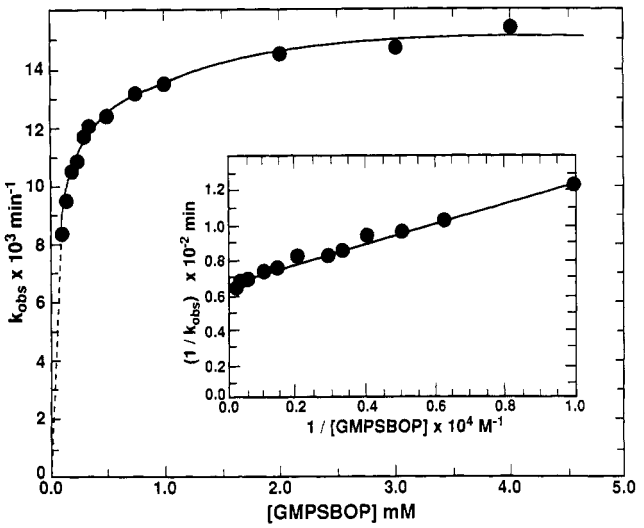

FIGURE 5: Pseudo-first-order rate constant, k_{obs}, as a function of GMPSBOP concentration. The enzyme (0.5 mg/mL) was incubated with varying reagent concentrations (0.1–4.0 mM) as described under Experimental Procedures. Rate constants were calculated as illustrated in the inset of Figure 4. Inset: $1/k_{obs}$ versus $1/[GMPSBOP]$.

120 min under the same conditions with 2.0 mM of hydrolyzed GMPSBOP[2] produced no change in enzymatic activity as assayed in the presence of 1 μM GTP.

The observation that a plot of rate constant versus reagent concentration over a range of 0.1–4.0 mM exhibits saturation kinetics (nonlinear) (Figure 5) suggests that GMPSBOP binds reversibly to the enzyme prior to covalent modification. This reaction mode is characteristic of an affinity label. The kinetic model depicting such a reaction is given by

$$E + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ER \overset{k}{\rightarrow} ER^* \quad (1)$$

The observed rate constant for this model can be expressed as

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

where k_{obs} represents the rate constant observed at a given reagent concentration, K_D is the apparent dissociation constant of the enzyme–reagent complex defined by $(k_{-1} + k)/k_1$, and k is the maximum rate constant at infinite reagent concentration. Rearrangement of this relationship gives

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

A reciprocal plot of $1/k_{obs}$ versus $1/[R]$ in the case of GMPSBOP reaction with glutamate dehydrogenase (Figure 5, inset) yields K_D = 74.7 μM and k = 0.0149 min^{-1}.

Reaction Rates of Enzyme with GMPSBOP in the Presence of Substrates and Allosteric Ligands. The rate constants of the reaction of enzyme with 0.75 mM GMPSBOP in the presence of various combinations and concentrations of allosteric ligands and substrates are shown in Table I. The substrate α-ketoglutarate or NADH alone, at a concentration high enough to bind to both the catalytic and regulatory sites (Colman, 1991), does not change the rate constant appreciably (entries 2 and 3). Similarly, oxidized coenzyme NAD^+ causes only a small decrease of the rate constant (entry 4). The activator ADP, when added either alone or together with NADH, causes less than a 2-fold decrease in the rate constant

[2] Hydrolyzed GMPSBOP (the product obtained upon release of free bromide) was prepared by incubation of GMPSBOP in 50 mM PIPES, pH 7.15 for 16 h at room temperature prior to initiation of the incubation with enzyme.

Table I: Effect of Ligands on Rate of Reaction of GMPSBOP with Bovine Liver Glutamate Dehydrogenase[a]

	ligands added	$10^3 k_{obs}$ (min^{-1})
(1)	no ligands	13.5 ± 0.38
(2)	10 mM α-ketoglutarate	13.5 ± 0.99
(3)	5.0 mM NADH	11.4 ± 0.40
(4)	5.0 mM NAD^+	11.7 ± 0.48
(5)	0.2 mM ADP	7.8 ± 0.48
(6)	0.2 mM ADP+ 0.1 mM NADH	9.4 ± 0.95
(7)	0.4 mM GTP	9.2 ± 0.30
(8)	0.01 mM GTP+ 5.0 mM NADH	8.7 ± 0.36
(9)	0.4 mM GTP+ 0.1 mM NADH	6.3 ± 0.54
(10)	0.4 mM GTP+ 5.0 mM NADH	2.5 ± 0.26
(11)	10 mM α-ketoglutarate + 1 mM NADH	4.4 ± 0.41
(12)	10 mM α-ketoglutarate + 5 mM NADH	2.8 ± 0.26
(13)	10 mM α-ketoglutarate + 5 mM NAD^+	5.6 ± 0.30

[a] Glutamate dehydrogenase (0.5 mg/mL) was incubated in 50 mM PIPES buffer (pH 7.15) at 25 °C with 0.75 mM reagent in the absence and presence of ligands. The rate constants were calculated by the method illustrated in Figure 4, inset.

(entries 5 and 6). Since 200 μM is high relative to the dissociation constants for both ADP sites (Batra & Colman, 1986a), this result indicates that GMPSBOP is not reacting at the ADP sites. The inhibitor GTP, when added alone, also causes a small reduction in k_{obs} (entry 7). Since, when added in the absence of coenzyme NADH, GTP binds only at its low-affinity site (Colman, 1991), it is apparent that this GTP site cannot be the reaction target. In contrast, the rate constant was markedly reduced (about 5–6-fold) when 5.0 mM NADH + 0.4 mM GTP (entry 10) were included in the incubation mixture. Since a smaller decrease in the rate constant is caused by a low concentration of GTP together with a high concentration of NADH (entry 8) or a high concentration of GTP along with a low concentration of NADH (entry 9), we conclude that occupation of both GTP and both NADH sites is required to provide maximum protection.

Surprisingly, we found that the combined addition of α-ketoglutarate together with NADH (entries 11 and 12) provided an alternative way of protecting against decreased sensitivity to GTP inhibition. The oxidized coenzyme NAD^+ has a similar effect when combined with α-ketoglutarate (entry 13). Thus, the ligands which best protect against the effects of GMPSBOP on the enzyme are 0.4 mM GTP + 5.0 mM NADH or 10 mM α-ketoglutarate + 5.0 mM NADH (entries 10 and 12). These results suggest that the reagent might be reacting at two distinct sites: the GTP-dependent NADH regulatory site (Lark & Colman, 1986) and the catalytic site. However, the latter possibility can be excluded since the reagent GMPSBOP does not inactivate the enzyme.

Incorporation of GMPSBOP into Enzyme. The incorporation of the reagent was determined by quantitation of the enzyme-bound organic phosphate as a function of time of incubation of enzyme with 0.75 mM GMPSBOP. Addition of DTT (to yield 20 mM) after 2 h of incubation causes no change in the inhibition by GTP nor does it change the incorporation of the reagent into the enzyme; therefore, 20 mM DTT was used to quench the reaction in aliquots withdrawn from the incubation mixture at various times. The results, shown in Figure 6, indicate that the incorporation of reagent into the enzyme is limited: incorporation of ~1 mol of reagent/mol of subunit is observed within the first 20 min, with the incorporation increasing by 120 min to ~2 mol of GMPSBOP/mol of subunit. A plot of the percent maximum change in GTP inhibition as a function of moles of reagent incorporated/mol of subunit (Figure 6, inset) indicates that incorporation of the first mole of reagent occurs with relatively

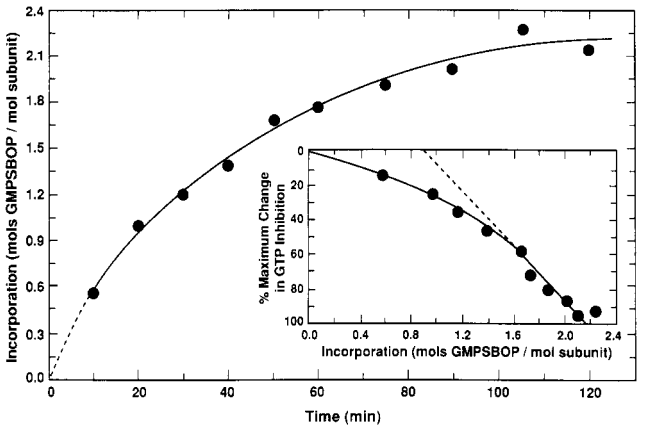

FIGURE 6: Time dependence of incorporation of GMPSBOP into glutamate dehydrogenase. The enzyme (2 mg/mL) was incubated with 0.75 mM GMPSBOP for 2 h, and incorporation of GMPSBOP was determined from the organic phosphorus content of the modified enzyme as described in Experimental Procedures.[3] Inset: GMPSBOP incorporation as a function of the percent maximum change in GTP inhibition at the same reagent concentration. The percent maximum change in GTP inhibition was determined as illustrated in Figure 4, inset.

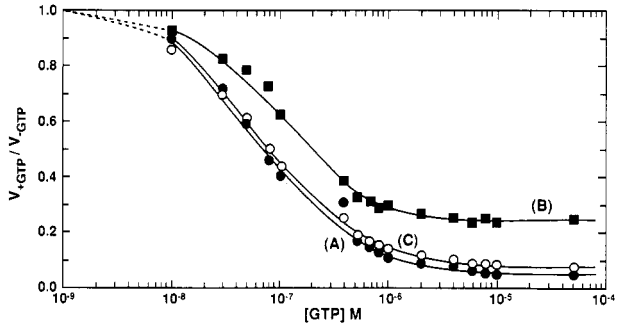

FIGURE 7: Effect of GTP on the maximum velocity of unmodified glutamate dehydrogenase (A, ●), GMPSBOP-modified enzyme prepared in the absence of ligands (B, ■), and enzyme modified with GMPSBOP in the presence of 0.4 mM GTP and 5 mM NADH (C, ○). Assay solutions contained 100 μM NADH. The modified enzyme in curve B contained ~2 mol of GMPSBOP/mol of subunit and in (C) ~1 mol of GMPSBOP/mol of subunit. The inhibition constant, K_I, for the GTP–enzyme complex was calculated from Figure 7, according to a relationship described by Frieden (1963): an uncompetitive allosteric inhibitor has an inhibition constant equal to the inhibitor concentration corresponding to the average of the velocity measured in the absence of the inhibitor and the minimum velocity at saturating concentrations of the inhibitor.

little change in the sensitivity to GTP inhibition (~25%), while the maximum change occurs at an extrapolated value of ~2 mol of reagent/mol of subunit. Incorporation determined after the 2-h incubation period in the presence of the protectants 0.4 mM GTP + 5.0 mM NADH (the time at which the functional change in GTP inhibition is complete in the absence of allosteric ligands) was decreased to 0.79 mol of reagent/mol of subunit. These results suggest that GMPSBOP might be reacting at two different sites, of which only one site is responsible for desensitization of the enzyme to GTP inhibition.

Kinetic Properties of GMPSBOP-Modified Enzyme. Modified enzymes prepared in the absence or presence of protecting ligands (0.4 mM GTP + 5.0 mM NADH) were compared with unmodified glutamate dehydrogenase in their response to allosteric regulators. Figure 7 shows the concentration dependence of GTP inhibition measured over a range of 10 nM–0.5 mM in the presence of 100 μM NADH. At saturating GTP concentrations, GMPSBOP-modified enzyme exhibits a maximum inhibition of 78% (Figure 7, curve B) with a K_I of 110 nM. In contrast, unmodified enzyme (Figure 7, curve A) and "protected enzyme" (curve C) exhibit maximum inhibition of 92–95% with K_I values of ~62 nM. The 2-fold increase in the dissociation constant of GTP–enzyme complex in GMPSBOP-modified enzyme is prevented when 0.4 mM GTP + 5.0 mM NADH are present during incubation of the enzyme with GMPSBOP.

[3] The rate of change in GTP inhibition was found to be the same at glutamate dehydrogenase concentrations of 0.5 mg/mL (8.9 μM enzyme subunits) and 2.0 mg/mL (35.6 μM enzyme subunits).

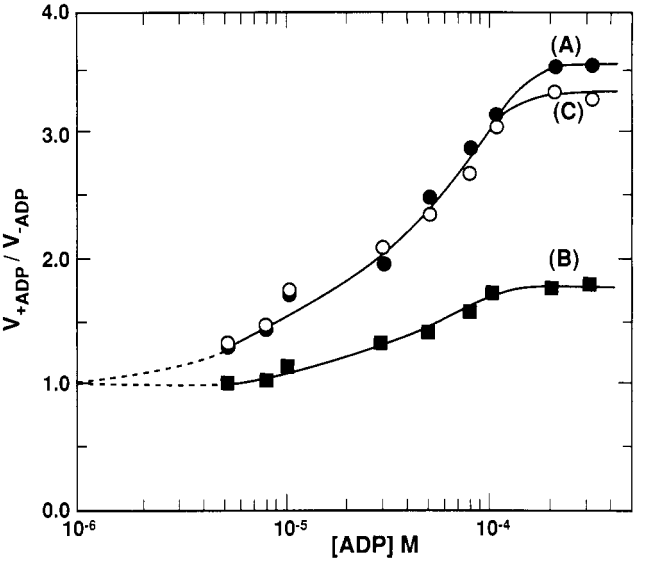

FIGURE 8: Response to ADP activation in unmodified glutamate dehydrogenase (A, ●), enzyme modified in the absence of ligands (B, ■) and enzyme modified in the presence of 0.4 mM GTP and 5 mM NADH (C, ○). The coenzyme concentration was 100 μM NADH in the assays. Enzyme samples used are described in the legend of Figure 7. The dissociation constant, K_A, for the ADP–enzyme complex was calculated from Figure 8, according to the relationship described by Frieden (1963): for an uncompetitive allosteric activator, K_A is equal to the activator concentration at which the velocity equals the average of the velocity in the absence of the activator and the maximum velocity at saturating concentrations of the activator.

The effect of GMPSBOP modification of glutamate dehydrogenase on activation by ADP is shown in Figure 8. Over the concentration range of 5 μM–0.3 mM ADP, the extent of activation in unmodified enzyme was ~3.5-fold (Figure 8, curve A), while that of 0.4 mM GTP + 5.0 mM NADH protected enzyme was ~3.3-fold (curve C). In contrast, modified enzyme with 2 mol of reagent/mol of subunit, was activated only 1.8-fold (Figure 8, curve B). The K_A values were obtained for unmodified, modified, and protected enzymes: 31, 45, and 36 μM, respectively.

The observation that reaction of GMPSBOP with glutamate dehydrogenase decreased its sensitivity to ADP activation suggested that reaction might occur at two separate sites: one an ADP and the other a GTP site. This possibility was further evaluated by monitoring the reaction of glutamate dehydrogenase with GMPSBOP by assaying in the presence of a constant amount of ADP (200 μM); covalent reaction with GMPSBOP is accompanied by a time-dependent decrease in the velocity measured in the presence of 200 μM ADP. The rate constant for reaction of 0.75 mM GMPSBOP with enzyme was (12.0 ± 0.57) × 10^{-3} min^{-1} when measured from the decrease in ADP activation, very similar to the k_{obs} determined by monitoring the change in GTP inhibition (Table I, entry 1). Enzyme incubated with the reagent in the presence of 200 μM ADP also shows a decrease in the extent of activation by ADP (data not shown), indicating that ADP does not protect

appreciably against the decreased sensitivity to ADP activation, and k_{obs} in the presence of 200 μM ADP [(8.1 ± 0.17) × 10^{-3} min^{-1}] measured from the loss in ADP activation was also very similar to the rate constant determined by monitoring the change in GTP inhibition (Table I, entry 5). The similarities in the rate constants suggest that GMPSBOP is not reacting at a distinct ADP site but rather that both the change in ADP activation and the change in GTP inhibition monitor the rate of a single reaction. When the enzyme is incubated with GMPSBOP in the presence of 0.4 mM GTP + 5 mM NADH, protection is provided against the decreased sensitivities to both ADP activation and GTP inhibition.

Bovine liver glutamate dehydrogenase is known to be inhibited by the reduced coenzyme NADH upon binding at the regulatory coenzyme site (at concentrations greater than 0.2 mM NADH) (Colman, 1991). The observation that protection against the reaction of GMPSBOP with enzyme is provided in the presence of 0.4 mM GTP + 5 mM NADH prompted us to investigate the possibility of GMPSBOP reacting at the regulatory NADH site. The effect of NADH concentration on glutamate dehydrogenase activity was studied at NADH concentrations of 2 μM-1.0 mM. The modified enzyme follows normal Michaelis–Menten kinetics up to 0.2 mM NADH, while unmodified and protected enzymes start to deviate from normal Michaelis–Menten kinetics at ~0.07 mM NADH (data not shown). The fact that modified enzyme is still inhibited by NADH, albeit at higher NADH concentrations than those required to inhibit unmodified and protected enzymes, suggests that the regulatory NADH site is perturbed but is still available for nucleotide binding.

Incorporation of Tritium into GMPSBOP-Modified Enzyme. In previous studies, it has been shown that the nucleotide analogs containing keto groups can be reduced with [^{3}H]-$NaBH_4$ in order to introduce a radioactive tag into the modified enzyme (Batra & Colman, 1986b; DeCamp & Colman, 1989; Vollmer & Colman, 1990). GMPSBOP-modified and protected enzyme samples were treated with [^{3}H]$NaBH_4$ and isolated as described under Experimental Procedures, and the amount of tritium incorporated into the enzyme samples was determined. Modified enzyme was found to contain 0.92 mol of tritium/mol of subunit while protected enzyme contained 0.39 mol of tritium/mol of subunit. Pasto and Lepeska (1976) have previously studied the kinetic isotope effect observed upon reduction of carbonyl groups by [^{3}H]$NaBH_4$ in various ketones. The magnitude of the kinetic isotope effect depends on the steric hindrance to attack at the carbonyl group, and actual values of 1–3 have been found during the studies with (4-bromo-2,3-dioxobutyl)thio derivatives of nucleotides (Bailey & Colman, 1987; DeCamp & Colman, 1989). Hence, assuming a kinetic isotope effect of ~2 for GMPSBOP-modified and protected enzyme samples, the results of tritium incorporation are consistent with the results determined from quantitation of organic phosphate.

Identification of the Target Amino Acids. Figure 9 shows the elution profile of ^{3}H-labeled tryptic peptides fractionated by HPLC in elution system I. Modified enzyme containing 2 mol of reagent/mol of subunit exhibited two major radioactive peaks (peak I, eluting at ~13% solvent B, and peak III, eluting at ~18.5 solvent B) present in approximately equal amounts, along with two minor peaks (peaks II and IV) (Figure 9A). Further purification of peak I in elution system III yielded a single radioactive peak which eluted at ~12% solvent D. Sequence analysis of this peak (Table II) gave a tetrapeptide with the following sequence: Tyr-Leu-His-Arg, corresponding to amino acids 262–265 in the linear sequence of bovine liver glutamate dehydrogenase (Julliard & Smith, 1979). No radioactivity was detected in fractions collected after each sequencing cycle; rather, all the counts were recovered on the filter of the sample-load chamber of the sequencer. Therefore, the amino acid modified by GMPSBOP in this tetrapeptide must have been regenerated by the acid conditions used during sequencing reactions. Possible targets would be tyrosine, histidine, and arginine.

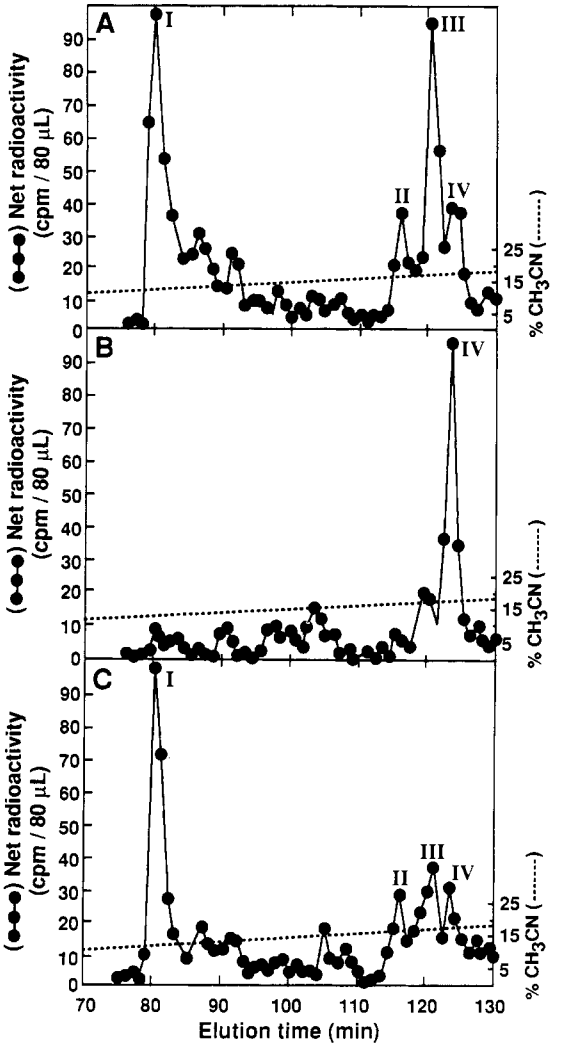

FIGURE 9: Separation of ^{3}H-labeled tryptic peptides by HPLC on a C_{18} column. The radioctive peptides were fractionated by elution with 0.1% TFA (pH 2.0), followed by a linear gradient in acetonitrile using elution system I, as described in Experimental Procedures. An aliquot of each fraction was assayed for radioactivity. Roman numerals designate radioactive peaks containing ^{3}H-labeled peptides from the tryptic digest: (A) after 2-h reaction time of enzyme with GMPSBOP in the absence of ligands; (B) after 2-h reaction time of enzyme with GMPSBOP in the presence of 0.4 mM GTP and 5 mM NADH; and (C) after 20-min reaction time of enzyme with GMPSBOP in the absence of ligands. In chromatograms A–C, the recovery of the total radioactivity applied to the HPLC column was 81%, 84%, and 86%, respectively.

Purification of peak III in elution system II yielded a single radioactive peak eluting at 27% solvent D. Sequence analysis of this tryptic peptide gave a 19-amino acid fragment with the following sequence: Gly-Phe-Ile-Gly-Pro-Gly-Val-Asp-Val-Pro-Ala-Pro-Asp-Met-Ser-Thr-Gly-Glu-Arg (Table II), which corresponds to amino acids from 156 to 175 in the linear sequence of bovine liver glutamate dehydrogenase (Julliard & Smith, 1979). The picomole amount of the PTH derivative dropped considerably in cycle 14 (Table II); furthermore, radioactivity was detected in this cycle, indicating that the target amino acid of GMPSBOP must be in this cycle. The

Table II: Representative Sequences of the Peptides from Tryptic Digests of [^{3}H]$NaBH_4$-Treated Enzymes Modified by GMPSBOP in the Absence and Presence of Protecting Ligands

	modified enzyme[a]		0.4 mM GTP + 5 mM NADH protected enzyme[b]	10 mM α-KG + 5 mM NADP protected enzyme[c]
cycle	I (pmol)	III (pmol)	IV (pmol)	I (pmol)
1	Tyr (132)	Gly (78)	Thr (20)	Tyr (38)
2	Leu (127)	Phe (83)	Ala (51)	Leu (27)
3	His (29)	Ile (50)	Met (<1)[e]	His (8)
4	Arg (23)	Gly (52)	Lys (19)	Arg (2)
5		Pro (49)	Tyr (29)	
6		Gly (52)	Asn (33)	
7		Val (37)	Leu (29)	
8		Asp (41)	Gly (20)	
9		Val (37)	Leu (26)	
10		Pro (39)	Asp (11)	
11		Ala (47)	Leu (21)	
12		Pro (36)	Arg (3)	
13		Asp (29)		
14		Met (2)[d]		
15		Ser (6)		
16		Thr (13)		
17		Gly (15)		
18		Glu (8)		
19		Arg (8)		

[a] GMPSBOP-modified glutamate dehydrogenase containing ~2 mol of GMPSBOP/mol of subunit was prepared and reduced with 3 mM [^{3}H]$NaBH_4$ as described under Experimental Procedures. The trypsin digest was subjected to a Vydac C_{18} HPLC column, and ^{3}H-labeled peptides were fractionated using elution system I. Radioactive peaks were designated as peaks I–IV (as in Figure 9A). Further purification was achieved in elution systems II and III. Representative sequences are shown for peaks I and III from Figure 9A, determined by gas-phase sequencing. [b] Protected enzyme was prepared and treated the same way as was modified enzyme except that 0.4 mM GTP + 5 mM NADH was included in the incubation mixture. Peak IV (Figure 9B) was subjected to gas-phase sequencing. [c] Alternatively, protected enzyme was prepared by including 10 mM α-ketoglutarate (α-KG) + 5 mM NADH in the incubation mixture. The enzyme sample was treated by the same procedures used for modified enzyme. Peak I from Figure 10A was sequenced. [d] Total radioactivity detected in this cycle (14) was 30 cpm. In cycle 13, counts were 17 cpm while, in cycles 15, 16, and 17 counts were 22, 19, and 17 cpm, respectively. A blank gives a background of ~17 cpm. [e] In cycle 3, a total of 26 cpm was detected. In contrast, in cycle 2, total cpm detected was 14. Total cpm in cycles 4, 5, and 6 were 23, 20, and 18, respectively, while the blank was ~16 cpm.

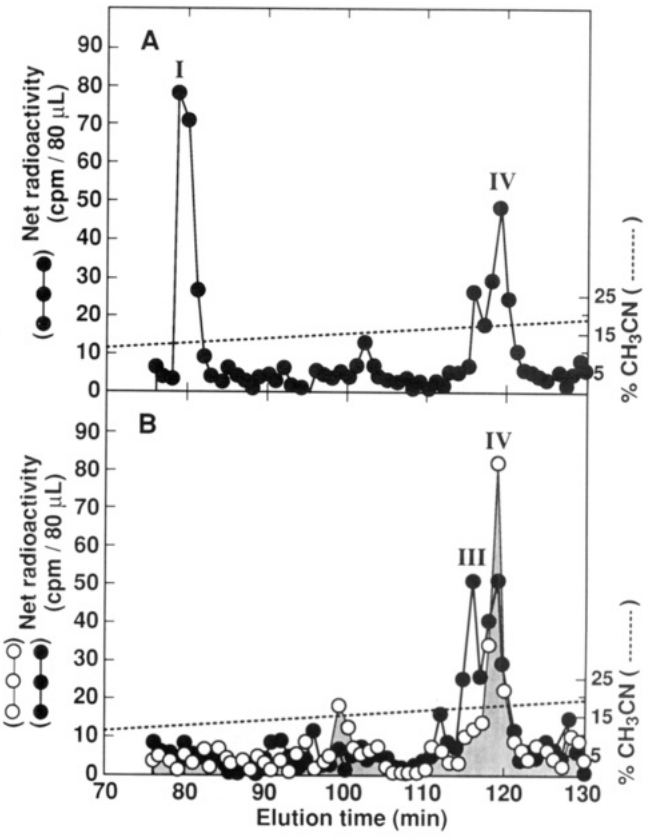

FIGURE 10: Tryptic digests of glutamate dehydrogenase modified by GMPSBOP in the presence of various protectants. Enzyme was modified by GMPSBOP (A) in the presence of 10 mM α-ketoglutarate and 5 mM NADH and (B) in the presence of 0.2 mM GTP and 0.1 mM NADH (●) and of 0.4 mM GTP and 5 mM NADH (○). Solvents and elution gradient are the same as described in the legend of Figure 9, but the column age was different in the experiments shown in Figure 9. The chromatograms were obtained in paired experiments, and the designation of the peaks was confirmed by amino acid sequencing. In chromatograms A and B, the recovery of the total radioactivity applied to the HPLC column was 76% and 74%, respectively. In the case of 0.4 mM GTP + 5 mM NADH protected enzyme, the recovery was 73%.

amino acid in this cycle is Met-169 of bovine liver glutamate dehydrogenase.

Separation of modified peptides from the enzyme samples prepared in the presence of 0.4 mM GTP + 5.0 mM NADH and in the presence of 10 mM α-ketoglutarate + 5.0 mM NADH (both samples containing ~1 mol of reagent/mol of subunit) was also carried out in elution system I. In the case of protectants GTP and NADH (Figure 9B), both peak I and peak III of the modified enzyme were markedly decreased and a large increase of a different radioactive peak was observed; this is designated peak IV and was the only prominent radioactive peak present. Purification of peak IV in elution system II gave a single radioactive peak eluting at 29% solvent D, which yielded a sequence (shown in Table II) corresponding to the tryptic peptide Thr-467–Arg-478 of this enzyme (Julliard & Smith, 1979). In this case, a different methionine was modified, as indicated by the low picomole content and the radioactivity found in the third cycle of the sequencer corresponding to Met-469. Modification of Met-469 was supported by the fact that trypsin did not cleave the peptide after Lys-470, which is the adjacent residue; methionine modification must have hindered the cleavage at Lys-470. Met-469 probably becomes accessible to GMPSBOP when the protectants GTP and NADH are bound to the enzyme.

To test whether modification of the tyrosyl peptide (peak I) and Met-169 (peak III) are both important for the functional change observed in GTP inhibition, glutamate dehydrogenase was incubated with GMPSBOP for a period of only 20 min, after which the enzyme was isolated, reduced with [^{3}H]-$NaBH_4$, and digested by trypsin. After 20 min of incubation with GMPSBOP, the enzyme contains ~1 mol of reagent/mol of subunit and the percent maximum change in GTP inhibition is only 25% (Figure 6). HPLC separation of the tryptic peptides (Figure 9C) showed that radioactive peaks I, II, and IV were all present in amounts comparable to those obtained after the 2-h incubation period; however, peak III was reduced ~65%. These results imply that modification of peaks I, II, and IV has little effect on the functional change and the 35% incorporation of the reagent into Met-169 approximately correlates with the 25% maximum change in GTP inhibition after 20 min of incubation period. In addition, peak I accounts for the major part of the first mole of reagent incorporated in the 20-min reaction period since the radioactivity in this peak did not differ appreciably between the digests prepared from the 20-min as compared to 2-h incubation period.

Analysis of the radioactively labeled tryptic peptides when 10 mM α-ketoglutarate + 5.0 mM NADH were used as protectants (Figure 10A) showed a marked decrease in peak III (the Met-169 peptide); however, peak I (the tyrosyl tetrapeptide) and peak IV (the Met-469 peptide) were still present (Figure 10A). Since, in bovine liver glutamate dehydrogenase, GMPSBOP alters the allosteric effects of

inhibitor GTP the most, enzyme was prepared in the presence of protectants 0.2 mM GTP + 0.1 mM NADH, conditions which saturate both GTP sites but allow binding of NADH only to the catalytic site. The resultant HPLC for the digest (Figure 10B, ●) reveals a complete loss of peak I; in contrast, peak III was protected only partially and there was some labeling of peak IV. Apparently, occupation of both GTP sites is sufficient to prevent reaction of GMPSBOP with peptide I (the tyrosyl peptide) but not with peptide III (the Met-169 peptide). These results contrast with the total protection against labeling of peptides I and III observed (Figure 10B, ○) at the higher concentration of 5 mM NADH together with GTP.

DISCUSSION

In this paper, we have reported the synthesis and characterization of guanosine 5′-*O*-[*S*-(3-bromo-2-oxopropyl)]-thiophosphate as a new affinity label. The synthesis of GMPSBOP from GMPS is straightforward, and the reasonable stability of GMPSBOP at pH 7.15 in 50 mM PIPES buffer ($t_{1/2}$ = 53 min, from the rate of release of bromide) makes it suitable for evaluation as an affinity label for a variety of other enzymes. It is noteworthy that the corresponding adenosine 5′-*O*-[*S*-3-bromo-2-oxopropyl)]thiophosphate (AMPSBOP) is much less stable under similar buffer conditions [Walner and Colman (1990); Vollmer and Colman, unpublished data]. A critical structural feature of GMPSBOP (which is absent in AMPSBOP) is the 2-amino of the guanine ring; although other conformations of GMPSBOP are energetically permissible, this 2-amino group may form a hydrogen bond with one of the phosphoryl oxygens of the α-thiophosphate which imparts stability to GMPSBOP while free in solution, preventing the internal nucleophilic displacement of bromide by the phosphoryl oxygen.

The reactive group in GMPSBOP is similar to the 4-bromo-2,3-dioxobutyl and 3-bromooxopropyl moieties of the other purine nucleotide derivatives which were reported earlier (Colman, 1989); in these analogs the reactive group is a substituent of the purine ring itself. In contrast, the 3-bromo-2-oxopropyl reactive moiety in GMPSBOP is directly tethered to the α-thiophosphate group adjacent to the 5′-position. An affinity label with a reactive group at this location can be used to probe the phosphate-binding regions of nucleotide sites of enzymes. Molecular modeling of GMPSBOP with energy minimization (Chem 3D, Cambridge Scientific Corp.) gives a size estimate of the compound which is comparable to that of GTP. Superimposition of the molecules around the purine and the ribose rings shows that the two molecules are almost identical in size: an energetically permissible extended conformation for the reactive arm of GMPSBOP gives a distance estimate of 8.4 Å between the 5′-carbon of the ribose ring and the CH_2Br carbon. A comparable distance can be estimated for GTP: 8.2 Å between the 5′-carbon of the ribose ring and the γ-phosphorus atom. Hence, GMPSBOP may function as an affinity label of GTP sites in other proteins, such as the G-proteins, tubulin, and the elongation factors. Alternatively, since GMPSBOP has a single negative charge at neutral pH, it might be considered as a GMP analog. However, in the case of glutamate dehydrogenase, this distinction may not be important since GMP is also known to function as an allosteric inhibitor, albeit at higher concentrations than GTP (Frieden, 1963).

Guanosine 5′-*O*-[*S*-(3-bromo-2-oxopropyl)]thiophosphate exhibits many of the characteristics of an affinity label in its reaction with bovine liver glutamate dehydrogenase (Colman, 1983, 1989, 1990). The reagent reacts covalently with a limited number of sites on the enzyme as revealed by the incorporation of ~2 mol of reagent/mol of subunit. The dependence of the observed reaction rate constant on reagent concentration is nonlinear, indicating the formation of a reversible enzyme–reagent complex prior to covalent modification. Several natural ligands decrease markedly the rate of functional change produced by GMPSBOP and decrease the reagent incorporation, consistent with specificity for the reaction.

In order to discern the target site(s) of GMPSBOP, a study of the kinetic properties of the modified enzyme was conducted, along with an evaluation of the ligands which decrease the reaction rate constant. Incubation of glutamate dehydrogenase with GMPSBOP does not decrease the enzyme activity as measured in the absence of allosteric ligands. Thus, the possibility of modification at the catalytic site can be excluded.

Modified enzyme containing ~2 mol of reagent/mol of subunit exhibits decreased sensitivity to inhibition by GTP, to inhibition at high concentrations of NADH, and to activation by ADP. Therefore, the target amino acids of GMPSBOP may lie in the GTP sites, the regulatory NADH site, and/or the ADP sites. Although the K_I for the GTP–enzyme complex is 2-fold higher than for the unmodified enzyme, the modified enzyme still retains its ability to be inhibited by GTP to an extent of 78% at saturating concentrations of GTP (Figure 7). At saturating NADH concentrations, inhibition is observed in the modified enzyme as in the case of the unmodified enzyme, but somewhat higher concentrations of NADH are needed to achieve inhibition. When the modified enzyme is assayed in the presence of the activator ADP, the magnitude of velocity enhancement is decreased, although the enzyme can still be activated by ADP. Thus, all of the regulatory sites still seem to be available for ligand binding and the altered kinetic properties of the enzyme may be due to perturbation of the regulatory sites caused by the modification reaction.

Among the natural regulatory compounds of glutamate dehydrogenase, GTP alone, ADP, or NADH alone causes less than a 2-fold decrease in the rate constant for reaction of GMPSBOP with the enzyme. Since the concentrations of GTP, ADP, and NADH used in these experiments were at least 10 times the dissociation constants reported for these ligands (Pal & Colman, 1979; Batra & Colman, 1986a; Colman & Frieden, 1966; Krause et al., 1974), these results eliminate the ADP sites, NADH sites, and one of the GTP sites as the *direct* reaction targets of GMPSBOP. The best protection by allosteric ligands against GMPSBOP modification (5–6-fold decrease in the rate constant) is provided by 0.4 mM GTP + 5.0 mM NADH, concentrations sufficiently high to populate all GTP and NADH sites (Colman, 1991). It is well-known that GTP enhances the affinity of glutamate dehydrogenase for NADH and, reciprocally, that NADH enhances the enzyme's affinity for GTP (Goldin & Frieden, 1972; Koberstein & Sund, 1973; Colman, 1991). The mutual interaction between the low-affinity GTP and NADH sites provides the best explanation for the functional effect of GMPSBOP on this enzyme. The target amino acids may lie in a cleft region close to (but not directly within) these regulatory sites, which become blocked upon simultaneous binding of NADH to its inhibitory site and of GTP to its low-affinity site. Both NADH and GTP would thus be required to shield the target amino acids, but once reaction with GMPSBOP had occurred, the enzyme would still be able to bind GTP and NADH, but with altered affinities. Covalent reaction of an amino acid within such a cleft region of glutamate dehydrogenase has been postulated previously (Ozturk et al., 1990).

For enzyme with 2 mol of reagent/mol of subunit, analyses of the ^{3}H-labeled tryptic peptides show that Met-169 and a tetrapeptide Tyr^{262}-Leu-His-Arg^{265} are modified. In the latter, the target amino acid could not be identified directly. Modification of arginine can be excluded since trypsin cleaved after this amino acid, leaving tyrosine and histidine as the possible reaction targets. It has been shown that a histidine or tyrosine modified with a bromo keto compound can be regenerated during acid hydrolysis (DeCamp & Colman, 1989; Batra et al., 1989). Previously, Tyr-262 was reported to be modified by the nucleoside analog 5′-[*p*-(fluorosulfonyl)-benzoyl]-1,N^6-ethenoadenosine in the high-affinity GTP site (Jacobson & Colman, 1984a). Saturation at the high-affinity GTP site was sufficient to completely block the reaction of 5′-FSBεA with glutamate dehydrogenase (Jacobson & Colman, 1982). In the present case of GMPSBOP, occupation of both GTP sites prevented labeling of peptide I (Figure 10B, ●), which is consistent with the reaction of GMPSBOP at the same Tyr-262 as is modified by 5′-FSBεA. However, occupation of both GTP sites by the addition of 0.4 mM GTP + 0.1 mM NADH to the reaction mixture of enzyme with GMPSBOP decreases the rate of functional change only ~2-fold (Table I, entry 9). The results suggest that, although both reagents modify the same peptide, once bound they have different effects. This difference may be due to a distinction in the conformation of the enzyme-bound reagents; evidence has been presented indicating that 5′-SBεA is arranged with the benzoyl moiety stacked on the purine group so that the target amino acid is located close to the purine ring (Jacobson & Colman, 1984b). In contrast, GMPSBOP may be arranged on the enzyme in an extended conformation with only the phosphoryl part of the reagent close to the target amino acid and the purine group located further away. In any case, modification of the tyrosyl peptide by GMPSBOP seems to be noncritical for the changes in regulatory behavior of this enzyme since only 25% of the maximum change in inhibition by GTP is observed when the incorporation of the first mole of reagent is complete after 20-min reaction time (Figure 6). This reaction time is sufficient for the tyrosyl peptide to be fully modified, but for only ~35% of labeling of peak III (containing Met-169) (Figure 9C). Reaction of GMPSBOP with Met-169 is correlated with the changes in allosteric properties.

Involvement of a methionyl residue critical for activity and regulation of this enzyme had been reported earlier (Rosen et al., 1973), but the particular methionine was not identified. David et al. (1977) have shown that iodoacetate inactivates bovine liver glutamate dehydrogenase concomitant with modification of Met-169 and this inactivation was prevented by saturating concentrations of α-ketoglutarate and NADH. These authors thus postulated that Met-169 lies in the catalytic site. However, they also reported that substantial protection against the inactivation by iodoacetate was provided by 1.0 mM NADH + 0.1 mM GTP (conditions similar to the protection conditions used against the modification reaction by GMPSBOP). In the present study, we have shown that Met-169 is labeled by GMPSBOP, but the effects of modification alter only the regulatory behavior of the enzyme and not its catalytic activity. Although two peptides were labeled, modification of Met-169 seems to be responsible for the changes in the kinetic properties of GMPSBOP-modified enzyme. It might be noted that GMPSBOP reacts much more rapidly with Met-169 than does iodoacetate: the rate constant measured for 0.75 mM GMPSBOP with glutamate dehydrogenase [(13.5 ± 0.38) × 10^3 min^{-1}] may best be compared with the rate constant calculated for 0.75 mM iodoacetate (4.5 × 10^{-4} min^{-1}) from the data of David et al. (1977). Compounds with reactive bromo keto moieties have been shown to react readily with methionine in enzymes, as exemplified by the reactions of phenacyl bromides with Met-192 of chymotrypsin (Sigman et al., 1969).

Recently, Baker et al. (1992) have described the crystal structure of the nonallosteric bacterial NAD^+-dependent glutamate dehydrogenase from *Clostridium symbiosum*. This enzyme exists as a hexamer, similar to bovine liver glutamate dehydrogenase, although the subunit size is ~10% smaller. Based on the electron density map, the dimensions of the cylindrical hexameric enzyme are very similar to those estimated for bovine liver glutamate dehydrogenase (Fiskin et al., 1971; Rice et al., 1987). Moreover, comparison of the known sequences from several glutamate dehydrogenases shows the highest sequence homology with several conserved residues in the substrate- and coenzyme-binding domain (Baker et al., 1992). Although Met-169 is not a conserved residue, sequence alignment of *C. symbiosum* and bovine liver glutamate dehydrogenase suggests that the bovine Met-169 is located close to the substrate-binding site proposed by Baker et al. (1992) for the *C. symbiosum* enzyme. Baker et al. (1992) also postulated that a conformational change (perhaps triggered by substrate binding) occurs in the catalytic site to bring the substrate into proximity with the nicotinamide region of the coenzyme. An analogous conformational change in bovine liver glutamate dehydrogenase may account for our observation that both α-ketoglutarate and NADH (rather than either α-ketoglutarate or NADH alone) are needed to protect Met-169 against reaction with GMPSBOP. The inactivation of the enzyme observed when Met-169 is modified by iodoacetate (David et al., 1977) is probably due to the negative charge on this reagent, which may weaken binding of the substrate by electrostatic repulsions. When Met-169 is labeled by GMPSBOP, the enzyme retains catalytic activity, suggesting that Met-169 is not critical for catalysis. In this case, the negative charge of the phosphoryl group may be positioned further from the substrate site so that it does not interfere with substrate binding. The covalently bound GMPSBOP does, however, affect the allosteric properties of the enzyme.

In the studies of Baker et al. (1992), it was observed that the sequence homology between nonvertebrate and vertebrate glutamate dehydrogenases is very poor toward the C-terminal. In addition, there are ~50 additional residues in the vertebrate enzyme in the C-terminal region. Baker et al. (1992) have suggested that these additional residues may form a binding pocket for regulatory ligands and provide communication between subunits in controlling the complex allosteric behavior of the vertebrate glutamate dehydrogenases. It is known that the occupation of both NADH and GTP sites causes a conformational change (Goldin & Frieden, 1972, Koberstein et al., 1973, Hucho et al., 1975), which may bring the tyrosyl peptide and Met-169 close to the postulated location of the regulatory sites. The cleft model proposed earlier (Ozturk et al., 1990) may best describe the location (designated as the GTP-dependent NADH regulatory site) of amino acid targets of GMPSBOP. On the basis of this model, the critical target residue of GMPSBOP is most likely in the vicinity of (but not directly within) the regulatory GTP and NADH sites and the simultaneous binding of NADH to its regulatory site and of GTP to its low-affinity site are necessary to block the reaction of GMPSBOP with Met-169 in the enzyme.

In enzyme protected by 0.4 mM GTP + 5.0 mM NADH, incorporation of 1 mol of reagent/mol of subunit was detected. However, the amino acid modified under these protecting

conditions was Met-469. Interestingly, this methionine is close to the C-terminal of the enzyme, and according to the model of Baker et al. (1992), it is buried in the subunit interface. The GMPSBOP labeling experiments suggest that the C-terminal is not normally accessible, but becomes exposed to the solvent when GTP and NADH bind to the enzyme and initiate a conformational change. This conformational change not only blocks the GTP-dependent NADH regulatory site containing Met-169 but also makes Met-469 available for reaction with GMPSBOP.

Previously, we have reported that, 8-BDB-TA-5′-TP modifies the GTP-dependent NADH regulatory site in bovine liver glutamate dehydrogenase (Ozturk et al., 1990) upon reaction at Cys-319 (Ozturk & Colman, 1991). The effect of GMPSBOP on the regulatory behavior of this enzyme is qualitatively similar to the effects observed with 8-BDB-TA-5′-TP in that both result in desensitization of glutamate dehydrogenase to GTP inhibition. The other kinetic properties of GMPSBOP-modified glutamate dehydrogenase and the protection conditions against the modification reaction are also very similar to that of the reaction of 8-BDB-TA-5′-TP with this enzyme. The reaction of GMPSBOP results in a stable covalent linkage with the enzyme by displacement of bromide from the bromo keto moiety; in contrast, the reaction of 8-BDB-TA-5′-TP with the enzyme occurs through a carbonyl group of the reactive moiety and most likely forms a reversible thiohemiketal (Ozturk & Colman, 1991). The parent molecule in each analog is similar, but the position of the reactive moiety is very different. Probably both analogs bind in a similar fashion at the GTP-dependent NADH regulatory site, but because of the position of the reactive arm, they modify amino acids located at different regions of this site.

ACKNOWLEDGMENT

We thank Dr. Yu-Chu Huang for her help in determining the peptide sequences, Dr. Robert S. Ehrlich for his help in obtaining the NMR spectra, and Dr. Sara H. Vollmer for her help in evaluating the structure of GMPSBOP by Chem 3D.

REFERENCES

Annamalai, A. E., & Colman, R. F. (1981) *J. Biol. Chem. 256*, 10276–10283.
Bailey, J. M., & Colman, R. F. (1987) *J. Biol. Chem. 262*, 12620–12626.
Baker, P. J., Britton, L. K., Engel, P. C., Farrants, G. W., Lilley, K. S., Rice, D. W., & Stillman, T. J. (1992) *Proteins 12*, 75–86.
Banik, U., & Roy, S. (1990) *Biochem. J. 266*, 611–614.
Batra, S. P., & Colman, R. F. (1986a) *J. Biol. Chem. 261*, 15565–15571.
Batra, S. P., & Colman, R. F. (1986b) *Biochemistry 25*, 3508–3515.
Batra, S. P., Lark, R. H., & Colman, R. F. (1989) *Arch. Biochem. Biophys. 270*, 277–285.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Colman, R. F. (1983) *Annu. Rev. Biochem. 52*, 67–91.
Colman, R. F. (1989) in *Protein Function: A Practical Approach* (Creighton, T. E., Ed.) Chapter 4, pp 77–99, IRL Press, New York.
Colman, R. F. (1990) *Enzymes (3rd Ed.) 19*, 283–321.
Colman, R. F. (1991) in *A Study of Enzymes, Vol III, Mechanism of Enzyme Action* (Kuby, S. A., Ed.) pp 173–192, CRC Press, Boca Raton, FL.
Colman, R. F., & Frieden, C. (1966) *J. Biol. Chem. 241*, 3652–3660.
Connolly, B. A., & Eckstein, F. (1982) *Biochemistry 21*, 6158–6167.
Connolly, B. A., Romaniuk, P. J., & Eckstein, F. (1982) *Biochemistry 21*, 1983–1989.
David, M., Rasched, I. R., & Sund, H. (1977) *Eur. J. Biochem. 74*, 379–385.
DeCamp, D. L., & Colman, R. F. (1989) *J. Biol. Chem. 264*, 8430–8441.
DeCamp, D. L., Lim, S., & Colman, R. F. (1988) *Biochemistry 27*, 7651–7658.
Dombrowski, K. E., Huang, Y. C., & Colman, R. F. (1992) *Biochemistry 31*, 3785–3793.
Eckstein, F., & Goody, R. (1976) *Biochemistry 15*, 1685–1691.
Fiskin, A. M., van Bruggen, E. F. J., & Fisher, H. F. (1971) *Biochemistry 10*, 2396–2408.
Frieden, C. (1963) *J. Biol. Chem. 238*, 3286–3299.
Geahlen, R., & Haley, B. E. (1979) *J. Biol. Chem. 254*, 11982–11987.
Goldin, B. R., & Frieden, C. (1972) *Curr. Top. Cell. Regul. 4*, 77–117.
Hess, H. H., & Derr, J. E. (1975) *Anal. Biochem. 63*, 607–613.
Hucho, F., Rasched, I., & Sund, H. (1975) *Eur. J. Biochem. 52*, 221–230.
Jacobson, M. A., & Colman, R. F. (1982) *Biochemistry 21*, 2177–2186.
Jacobson, M. A., & Colman, R. F. (1984a) *Biochemistry 23*, 6377–6382.
Jacobson, M. A., & Colman, R. F. (1984b) *J. Biol. Chem. 259*, 1454–1460.
Jaffe, E. K., & Cohn, M. (1978) *Biochemistry 17*, 652–657.
Julliard, J. H., & Smith, E. L. (1979) *J. Biol. Chem. 254*, 3427–3438.
Koberstein, R., & Sund, H. (1973) *Eur. J. Biochem. 36*, 545–552.
Koberstein, R., Krause, J., & Sund, H. (1973) *Eur. J. Biochem. 40*, 543–548.
Krause, J., Bühner, M., & Sund, H. (1974) *Eur. J. Biochem. 41*, 593–602.
Lanzetta, P. A., Alvarez, L. J., Reinach, P. S., & Candia, O. A. (1979) *Anal. Biochem. 100*, 95–97.
Lark, R. H., & Colman, R. F. (1986) *J. Biol. Chem. 261*, 10659–10666.
Likos, J. J., & Colman, R. F. (1981) *Biochemistry 20*, 491–499.
Murray, A. W., & Atkinson, M. R. (1968) *Biochemistry 7*, 4023–4029.
Olson, J. A., & Anfinsen, C. B. (1952) *J. Biol. Chem. 197*, 67–79.
Ozturk, D. H., & Colman, R. F. (1991) *Biochemistry 30*, 7126–7134.
Ozturk, D. H., Safer, D., & Colman, R. F. (1990) *Biochemistry 29*, 7112–7118.
Pal, P. K., & Colman, R. F. (1979) *Biochemistry 18*, 838–845.
Pasto, D. J., & Lepeska, B. (1976) *J. Am. Chem. Soc. 98*, 1091–1095.
Penefsky, H. S. (1979) *Methods Enzymol. 56*, 527–530.
Potter, R. L., & Haley, B. E. (1983) *Methods Enzymol. 91*, 613–633.
Rice, D. W., Baker, P. J., Farrants, G. W., & Hornby, D. P. (1987) *Biochem. J. 242*, 789–795.
Rosen, N. L., Bishop, L., Burnett, J. B., Bishop, M., & Colman, R. F. (1973) *J. Biol. Chem. 248*, 7359–7369.
Schmidt, J. A., & Colman, R. F. (1984) *J. Biol. Chem. 259*, 14515–14519.
Sigman, D. S., Torchia, D. A., & Blout, E. R. (1969) *Biochemistry 8*, 4560–4566.
Smith, E. L., Landon, M., Piszkiewicz, D., Brattin, W. J., Langley, T. J., & Melamed, M. D. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 724–730.
Tomich, J. M., Marti, C., & Colman, R. F. (1981) *Biochemistry 20*, 6711–6720.
Vollmer, S. H., & Colman, R. F. (1990) *Biochemistry 29*, 2495–2501.
Walner, M. B., & Colman, R. F. (1990) *Biochemistry 29*, 2202.