# Activation of Bovine Liver Glutamate Dehydrogenase by Covalent Reaction of Adenosine 5′-*O*-[*S*-(4-Bromo-2,3-dioxobutyl)thiophosphate] with Arginine-459 at an ADP Regulatory Site†

Kazimierz O. Wrzeszczynski and Roberta F. Colman*

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

*Received April 29, 1994; Revised Manuscript Received July 27, 1994*®

ABSTRACT: Bovine liver glutamate dehydrogenase is an allosteric enzyme which is activated by ADP. The affinity label adenosine 5′-*O*-[*S*-(4-bromo-2,3-dioxobutyl)thiophosphate] (AMPSBDB), a new ADP analog featuring a reactive group at a position equivalent to that of the pyrophosphate, reacts with this glutamate dehydrogenase to yield enzyme containing about 0.9 mol/mol of enzyme subunit. The reaction results in a time-dependent irreversible activation of the enzyme. Glutamate dehydrogenase (8.9 μM subunit) modified with 10–60 μM AMPSBDB is about 3.2-fold more active than native enzyme. The modified enzyme is still inhibited by GTP and by high concentrations of NADH, but is no longer activated by ADP. The addition to the reaction mixture of (a) NADH or α-ketoglutarate; (b) GTP + NADH; or (c) α-ketoglutarate + NADH has little effect on the functional changes produced by AMPSBDB; whereas, the reaction is prevented by ADP. Purification of labeled peptide from proteolytic and chemical digests of [2-$^3$H]AMPSBDB-modified enzyme leads to identification of Arg$^{459}$ as the target amino acid. We conclude that AMPSBDB functions as an ADP mimic covalently bound to Arg$^{459}$ within the ADP activator site of the allosteric bovine liver glutamate dehydrogenase.

Bovine liver glutamate dehydrogenase [L-glutamate:NAD(P)$^+$ oxidoreductase (deaminating), EC 1.4.1.3] is an allosteric enzyme, composed in its active form of six identical subunits, each of which has multiple binding sites for purine nucleotides. The enzyme is activated by ADP and is inhibited by GTP and high concentrations of NADH. The allosteric activator ADP, and the coenzyme NADH, each bind at two sites per subunit (Batra & Colman, 1986; Colman, 1991). The allosteric inhibitor GTP occupies two sites per enzyme subunit in the presence of NADH but only one site in the absence of reduced coenzyme (Pal & Colman, 1979).

The amino acid sequence of bovine liver glutamate dehydrogenase is known (Julliard & Smith, 1979); however, no crystal structure of the mammalian enzyme is yet available. Affinity labeling, using purine nucleotide derivatives, has provided considerable information on the amino acids present in the regulatory sites of glutamate dehydrogenase (Colman, 1983, 1991). The GTP-dependent NADH inhibitory site has been modified at Cys$^{319}$ by the nucleotide analog 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-triphosphate (Ozturk *et al.*, 1990; Ozturk & Colman, 1991). Similarly, guanosine 5′-*O*-[*S*-(3-bromo-2-oxopropyl)thiophosphate] reacts at the GTP-dependent NADH inhibitory site, labeling Met$^{169}$ and Tyr$^{262}$ (Ozturk *et al.*, 1992). The compound 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine is incorporated into glutamate dehydrogenase at the NADH inhibitory site, labeling Lys$^{420}$ and Tyr$^{190}$ (Pal *et al.*, 1975; Saradambal *et al.*, 1981; Schmidt & Colman, 1984). The fluorescent nucleotide analog 5′-[*p*-(fluorosulfonyl)benzoyl]-1,$N^6$-ethenoadenosine reacts covalently at a GTP inhibitory site (Jacobson & Colman, 1982), while the adenine nucleotide analog 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-monophosphate modifies an ADP regulatory site upon reacting with His$^{82}$ (Batra & Colman, 1986; Batra *et al.*, 1989). Each of these affinity labels reacts covalently, blocking a particular regulatory site; however, none of these has mimicked the activating or inhibitory effects of the natural, reversibly binding nucleotides, ADP or GTP.

Adenosine 5′-*O*-[*S*-(4-bromo-2,3-dioxobutyl)thiophosphate] is a new ADP analog recently synthesized in this laboratory (Vollmer *et al.*, 1994), which features a reactive bromodioxobutyl group at a position equivalent to that of pyrophosphate as pictured in Figure 2 of that paper. We now demonstrate that AMPSBDB[1] reacts covalently with bovine liver glutamate dehydrogenase to produce an irreversibly activated enzyme, thus mimicking the normal action of ADP. A preliminary version of this work has been presented (Wrzeszczysnki & Colman, 1994).

## 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 for 20 h. The dialyzed enzyme was centrifuged at 15 000 rpm for 20 min at 4 °C to remove any precipitated protein. The enzyme concentration in the supernatant was determined spectrophotometrically by using $\epsilon^{1\%}_{280nm}$ = 9.7 (Olson & Anfinsen, 1952), and the ratio of $A_{280nm}/A_{260nm}$ was 1.9. A molecular mass of 56 100 Da for each identical subunit was used (Smith *et al.*, 1970). The enzyme was stored in aliquots at −80 °C.

Coenzymes, nucleotides, EDTA, Hepes, Pipes, Tris, Malachite Green base, and Sephadex G50–80 were purchased from Sigma. [2-$^3$H]Adenosine was obtained from Amersham Life Sciences. All other chemicals were reagent grade.

† This research was supported by National Science Foundation Grant DMB 9105116.

* Author to whom correspondence should be addressed.

® Abstract published in *Advance ACS Abstracts,* September 1, 1994.

[1] Abbreviations: AMPSBDB, adenosine 5′-*O*-[*S*-(4-bromo-2,3-dioxobutyl)thiophosphate]; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, disodium ethylenediaminetetraacetate; HPLC, high-performance liquid chromatography.

0006-2960/94/0433-11544$04.50/0 

Adenosine 5′-*O*-[*S*-(4-bromo-2,3-dioxobutyl)thiophosphate] (AMPSBDB) was synthesized according to the procedure of Vollmer *et al.* (1994). In the case of [2-$^3$H]AMPSBDB, various proportions of [2-$^3$H]adenosine to adenosine were used; however, typically about 5 mCi of [2-$^3$H]adenosine was added to 2 mmol of adenosine, and the remainder of the procedures were the same as those reported in Vollmer *et al.* (1994). The yield of both the nonradioactive product and [2-$^3$H]AMPSBDB was 25–35%. The reagent, dissolved in water, was stored at −80 °C.

*Enzyme Assays.* Glutamate dehydrogenase activity was monitored, using a Gilford Model 240 spectrophotometer with an expanded scale (0.0–0.1), by the oxidation of NADH ($\epsilon_{340nm} = 6.22 \times 10^3$ M$^{-1}$ cm$^{-1}$) at 25 °C in Tris–0.01 M acetate buffer, pH 8.0, containing 10 μM EDTA. The assay solution (with a total volume of 1 mL) was 100 μM in NADH, 5 mM in α-ketoglutarate, and 50 mM in ammonium chloride. For assays "in the presence of ADP", the concentration of the allosteric activator was 200 μM.

The conditions and the substrate concentrations used in determining the effects of varying concentrations of regulatory nucleotides were the same as above. In the case of NADH, assays at concentrations of 0.2 mM and above were monitored at 375 nm ($\epsilon = 1.85 \times 10^3$ M$^{-1}$ cm$^{-1}$).

*Reaction of AMPSBDB with Bovine Liver Glutamate Dehydrogenase.* The enzyme (0.5 mg/mL, 8.9 μM subunit) was incubated at 25 °C with varying concentrations of the nucleotide analog in 50 mM Pipes buffer, pH 7.0; a few experiments, as indicated in Results, were conducted at 5 °C. At various times, 20 μL aliquots were withdrawn from the reaction mixture and diluted 50-fold in 0.1 M potassium phosphate, pH 7.1, at 0 °C. Enzyme activity was measured using 20 μL of this diluted solution.

In order to determine the effect of substrates or allosteric regulators on the reaction of AMPSBDB with bovine liver glutamate dehydrogenase, the enzyme was first incubated at 25 °C in 50 mM Pipes buffer, pH 7.0, with a specified concentration of desired substrate or allosteric ligand (see Results) for 15 min, after which 20 μM AMPSBDB was added to the incubation mixture. This incubation mixture was then assayed in the absence and presence of ADP, as described above; in each case, the average specific activity was determined after 20 min of incubation with AMPSBDB. A control enzyme was incubated under the same conditions without AMPSBDB.

*Preparation of Bovine Liver Glutamate Dehydrogenase Modified by AMPSBDB in the Absence and Presence of Allosteric Ligands.* Enzyme (2.0 mg/mL, 35.6 μM subunit) was incubated with 80 μM AMPSBDB, for 45 min, under the conditions described above. In certain experiments, various concentrations of ligands and substrates were added as protectants. The excess reagent was separated from modified and protected enzymes at 4 °C by the column centrifugation procedure described by Penefsky (1979). Control enzyme was subjected to the same procedure. Columns were prepared in 5 mL disposable syringes filled with Sephadex G50–80 mesh, supported by glass wool, and equilibrated with 0.1 M Hepes buffer, pH 7.0. Each column was loaded with 0.5 mL of the reaction mixture. Two successive centrifugations were necessary to remove the excess reagent completely. After elution, the enzyme concentration was determined by the Bio-Rad protein assay, which is based on the Bradford dye-binding method (Bradford, 1976). The standard curve was generated by using various concentrations of unmodified bovine liver glutamate dehydrogenase in the same buffer. A control enzyme was subjected to the same conditions, but in the absence of AMPSBDB, and was isolated in the same manner. The kinetic properties of these isolated enzymes were determined.

*Incorporation of Nonradioactive Reagent and [2-$^3$H]-AMPSBDB into Bovine Liver Glutamate Dehydrogenase.* The amount of reagent incorporated was determined from the 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). Aliquots (0.2–0.4 mL) of modified and unmodified enzyme samples were first hydrolyzed with $H_2SO_4$ at 190 °C for 2 h and then bleached with 20 μL of 30% hydrogen peroxide solution with heating at 190 °C for 1 h. The hydrogen peroxide treatment was repeated 2–3 times until the samples were clear. After addition of the Malachite Green base–ammonium molybdate solution [3:1 (v/v)], $A_{660nm}$ was determined using a total volume of 1.0 mL. The standard curve was established with aliquots of a 100 μM standard phosphate solution purchased from Sigma Diagnostics. The volume of 0.1 M Hepes, pH 7.0, was kept constant in all assays.

Enzyme (2.0 mg/mL) was modified with 80 μM [2-$^3$H]-AMPSBDB, as described above. Removal of excess reagent was accomplished by dialyzing against two changes of 0.1 M Hepes buffer, pH 7.0, for 20 h. The amount of reagent incorporated was determined from the amount of radioactivity per 100 μL of modified enzyme in 5 mL of Liquiscint scintillation cocktail using a Packard Tricarb Model 3300 liquid scintillation counter. The specific radioactivities of representative different preparations of [2-$^3$H]AMPSBDB were $5.7 \times 10^{10}$ and $1.3 \times 10^{12}$ cpm/mol.

*Treatment of AMPSBDB-Modified Glutamate Dehydrogenase with [$^3$H]NaBH$_4$.* Reduction of the dioxo groups of AMPSBDB by [$^3$H]NaBH$_4$ would allow the incorporation of a radioactive tag into the AMPSBDB-modified enzyme. The reduction reaction was attempted on enzyme (35.6 μM subunit) samples, maintained on ice, with two additions (0.5 h apart) of 192 mM [$^3$H]NaBH$_4$ (in 0.02 M NaOH) to yield 2.5 mM [$^3$H]NaBH$_4$ in the incubation mixture after each addition. The specific radioactivity of the [$^3$H]NaBH$_4$ was $1 \times 10^{12}$ cpm/mol of hydrogen. A control enzyme was prepared and treated with [$^3$H]NaBH$_4$ under the same conditions. The incubation with [$^3$H]NaBH$_4$ was followed by two successive column centrifugations under the conditions described above. Tritium incorporation into the enzyme was determined from the counts in 50 μL aliquots.

*Proteolytic Digestion and Chemical Cleavage of [2-$^3$H]-AMPSBDB-Modified Bovine Liver Glutamate Dehydrogenase.* Enzyme (4.0 mg/mL, 71.2 μM subunit) was incubated with 480 μM [2-$^3$H]AMPSBDB, as described above, after which the modified enzyme was dialyzed against two changes of 0.1 M Hepes buffer, pH 7.0, for 20 h. In order to covalently block free -SH groups, the modified enzyme was denatured by the addition of solid urea to yield 6 M and maintained on ice for 15 min, followed by the addition of *N*-ethylmaleimide (NEM) to yield 10 mM. The enzyme was then dialyzed against an appropriate buffer for each particular protease (see Results). In the case of V8 protease, dialysis was against 20 mM ammonium acetate, pH 4.0, and digestion was conducted for 4 h at 37 °C with a total of 5.0% (w/w) V8 protease as compared with glutamate dehydrogenase. Digests were separated by reverse-phase HPLC on the same day. The same procedure was performed on glutamate dehydrogenase incubated with [2-$^3$H]AMPSBDB in the presence of protecting ligand.

Chemical cleavage of the modified and protected enzyme was accomplished using cyanogen bromide. Modification of the enzyme was performed as above, with a final dialysis against two changes of 20 mM ammonium acetate, pH 4.0, for 20 h at 4 °C. After lyophilization, the modified enzyme was dissolved in 1 mL of 70% formic acid; to it was added 7% by weight of cyanogen bromide per milligram of glutamate dehydrogenase. The reaction mixture was stirred for 24 h at 25 °C in the dark, after which 3–5 mL of HPLC grade water was added, and the sample was lyophilized. Cleavage of HPLC-purified peptide peaks was performed in the same manner after lyophilization of the sample. When the reagent alone was treated under chemical cleavage conditions, 2–3 mg of cyanogen bromide in 1 mL of 70% formic acid was used.

*Separation of [2-$^3$H]AMPSBDB-Labeled Peptides by High-Performance Liquid Chromatography.* The radioactively labeled peptides were separated on a Varian Model 5000 HPLC system equipped with a reverse-phase Vydac $C_{18}$ column (0.46 × 25 cm). Each 1.5 mL digest was divided into two equal portions, centrifuged for 2–3 min at 14 000 rpm, and filtered through 0.45 μm Millipore membrane filters prior to injection. Separation of the V8 protease digest was carried out at an elution rate of 1 mL/min in 0.1% trifluoroacetic acid, pH 1.9 (solvent A), from 0 to 10 min, then by a linear gradient from solvent A to 30% solvent B (0.07% trifluoroacetic acid in acetonitrile) between 10 and 70 min, followed by a linear gradient to 35% solvent B between 70 and 95 min, and finally by a linear gradient to 40% solvent B between 95 and 170 min. Fractions of 1 mL were collected, the effluents were monitored at 220 nm, and 150 μL from the eluted fractions was assayed for radioactivity as described above. In some cases, the radioactive peptide peak was further cleaved by cyanogen bromide, as described above, and the resultant solution was fractionated by reverse-phase HPLC using the elution scheme described.

The cyanogen bromide-treated peptides were dissolved in 0.8 mL of 0.1% trifluoroacetic acid, pH 1.9, and separated on a $C_{18}$ column at an elution rate of 1 mL/min in solvent A from 0 to 10 min, followed by a linear gradient from solvent A to 10% solvent B between 10 and 30 min, by a linear gradient to 20% Solvent B from 30 to 130 min, and by a linear gradient to 40% from 130 to 190 min. Fractions of 1 mL were collected, and an aliquot of 150 μL was assayed for radioactivity. Chromatography of radioactive compound alone was carried out with the same elution protocols used for the V8 protease digest and cyanogen bromide cleavages, respectively.

*Analysis of Radioactively Labeled Peptides.* The amino acid sequences of purified peptides were determined using 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. Approximately 100–600 pmol of the isolated peptides was applied to the sequencer.

## RESULTS

*Activation of Bovine Liver Glutamate Dehydrogenase by AMPSBDB.* Incubation of 8.9 μM subunit bovine liver glutamate dehydrogenase with 20 μM AMPSBDB, at pH 7.0 and 25 °C, results in a rapid increase in the activity of the enzyme, as assayed subsequently (after dilution) in a standard assay (Figure 1A). In contrast, when the enzyme is incubated in the absence of reagent, constant activity is observed. The time dependence of this increase in enzymatic activity upon reaction with AMPSBDB is more readily observed when the

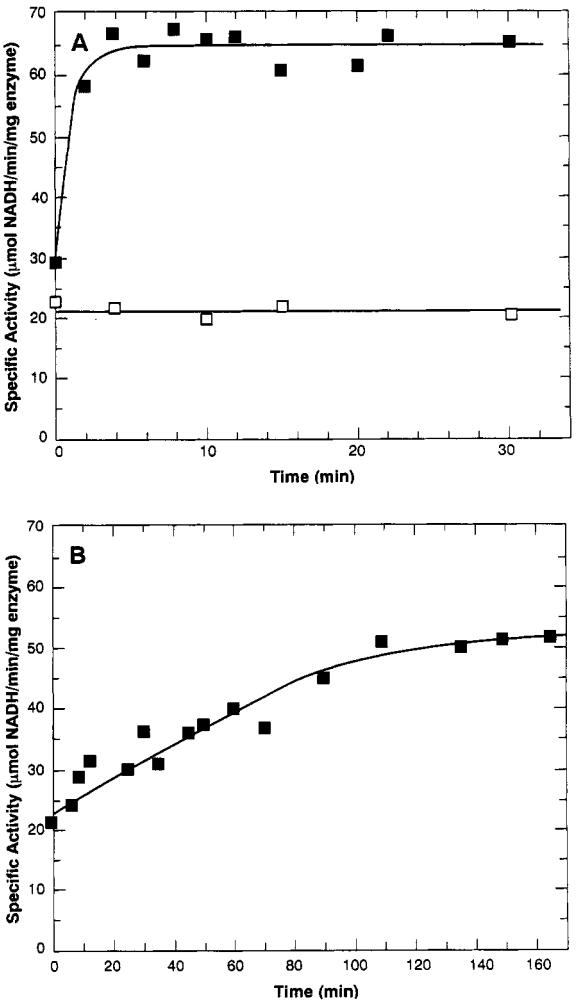


FIGURE 1: Effect of AMPSBDB on the activity of bovine liver glutamate dehydrogenase. The enzyme (0.5 mg/mL, 8.9 μM subunit) was incubated with 20 μM AMPSBDB in 0.05 M Pipes buffer, pH 7.0, at (A) 25 °C or (B) 5 °C. At the indicated times aliquots of the incubation mixture were assayed as described under Experimental Procedures. (■) represents the specific activity of GDH in the presence of AMPSBDB, and (□) is the specific activity of control enzyme incubated under the same conditions in the absence of reagent.

reaction is conducted at 5 °C (Figure 1B) than at 25 °C (Figure 1A); apparently, the reaction rate of AMPSBDB with enzyme is considerably faster at the higher temperature. The activity of bovine liver glutamate dehydrogenase after 20 min of incubation with 1–60 μM AMPSBDB at 25 °C was measured. Maximum increase in activity (about 3.2-fold) upon reaction with AMPSBDB is reached at reagent/enzyme ratios of slightly greater than 1, as shown in Figure 2.

*Effect of Substrates and Allosteric Ligands on Reaction of AMPSBDB with Bovine Liver Glutamate Dehydrogenase As Monitored by Change in ADP Activation.* The enzyme was incubated with 20 μM AMPSBDB at 25 °C, and substrates or allosteric regulators were included as indicated in Table 1. The maximum velocities in the presence and absence of ADP were measured using dilutions of enzyme after 20 min of incubation with AMPSBDB. When assayed in the presence of 200 μM ADP, in the standard assay, native bovine liver glutamate dehydrogenase exhibits an activation of 1.97. In contrast, after the enzyme is incubated with AMPSBDB, no activation is observed when ADP is added to the assay; thus the ratio of $V_{max}$ in the presence of 200 μM ADP to $V_{max}$ in the standard assay is 1.05 (Table 1, row 1). Any protective effect of a particular ligand on the reaction of glutamate dehydrogenase with AMPSBDB would result in a

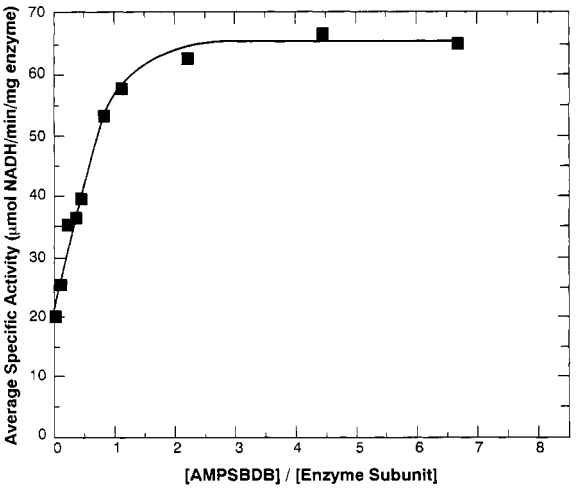


FIGURE 2: Dependence on AMPSBDB concentration of the maximal activity of bovine liver glutamate dehydrogenase. The enzyme (0.5 mg/mL, 8.9 μM subunit) was incubated with various concentrations of AMPSBDB ranging from 1 to 60 μM at 25 °C in 0.05 M Pipes buffer, pH 7.0. After 20 min, the incubation mixture was assayed as described under Experimental Procedures. The average maximal specific activity was determined from a series of assays after 20–40 min of incubation. The ratio of [AMPSBDB] (μM) to [enzyme] (8.9 μM subunit) is used to express the varying concentrations.

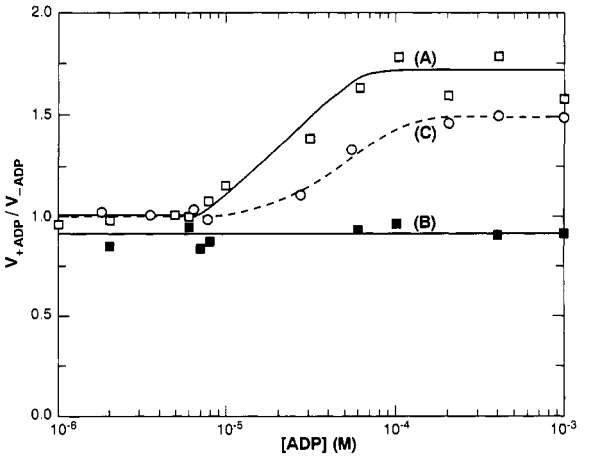


FIGURE 3: Effect of ADP on the maximum velocity of native glutamate dehydrogenase (A, □), enzyme modified with AMPSBDB in the absence of ligands (B, ■), and enzyme modified with AMPSBDB in the presence of 1 mM ADP (C, ○). The coenzyme was 100 μM NADH in the assay solutions. The $K_a$ was determined from the graph as described by Frieden (1963), who showed that the $K_a$ equals the concentration of ADP yielding $(V_{+ADP} + V_{-ADP})/2$, where, $V_{+ADP}$ is the maximum velocity measured at saturating concentrations of ADP and $V_{-ADP}$ is the maximum velocity measured with no ADP present.

Table 1: Effect of Substrates and Allosteric Ligands on the Maximum Velocity of Bovine Liver Glutamate Dehydrogenase after Incubation with 20 μM AMPSBDB[a]

| ligands | $V_{+ADP}/V_{-ADP}$ |
|---|---|
| 1. none | 1.05 |
| 2. 0.2 mM ADP | 1.82 |
| 3. 1.0 mM ADP | 1.78 |
| 4. 1.0 mM ADP + 0.1 mM NADH | 1.70 |
| 5. 0.025 mM GTP + 0.1 mM NADH | 1.11 |
| 6. 0.1 mM NADH | 1.05 |
| 7. 1.0 mM NADH | 1.30 |
| 8. 5.0 mM NADH | 1.67 |
| 9. 10 mM α-ketoglutarate | 1.18 |
| 10. 10 mM α-ketoglutarate + 0.1 mM NADH | 0.94 |
| 11. 10 mM α-ketoglutarate + 1.0 mM NADH | 1.45 |

[a] Assays were conducted at 25 °C in the presence and absence of 200 μM ADP. The average maximum velocity after 20 min of incubation time was determined for both assays and reported as the ratio of $V_{+ADP}/V_{-ADP}$. The ratio of $V_{+ADP}/V_{-ADP}$ for unmodified glutamate dehydrogenase is 1.97.

ratio of $V_{+ADP}/V_{-ADP}$ that is closer to that of native enzyme (i.e., $V_{+ADP}/V_{-ADP}$ of 1.97). The allosteric regulator ADP shows the greatest extent of protection against the reaction between AMPSBDB and bovine liver glutamate dehydrogenase. The $V_{+ADP}/V_{-ADP}$ ratio for two different concentrations of ADP (Table 1, rows 2–4) is similar to that of native enzyme, indicating significant protection of bovine liver glutamate dehydrogenase from AMPSBDB. In contrast, the allosteric inhibitor GTP (Table 1, row 5) shows no difference in the $V_{+ADP}/V_{-ADP}$ ratio as compared to enzyme and reagent alone (row 1). The coenzyme NADH, at a concentration sufficiently high to occupy only the catalytic site (0.1 mM), shows little effect on the reaction of bovine liver glutamate dehydrogenase with AMPSBDB (Table 1, row 6), as indicated by $V_{+ADP}/V_{-ADP} = 1.05$. At higher concentrations of NADH (Table 1, rows 7 and 8), the reduced coenzyme decreases the extent of reaction with AMPSBDB, as indicated by higher ratios of $V_{+ADP}/V_{-ADP}$; this effect may reflect the ability of high concentrations of NADH to compete for binding to the ADP site (Batra & Colman, 1986). The substrate α-ketoglutarate, added either alone or together with low concentrations of NADH, has no effect on the $V_{+ADP}/V_{-ADP}$ ratio (Table 1, rows 9 and 10). The higher value of the $V_{+ADP}/V_{-ADP}$ ratio for 10 mM α-ketoglutarate + 1.0 mM NADH (Table 1, row 11) is probably caused by the high concentration of NADH competing for binding at the ADP site.

*Kinetic Properties of Enzyme Modified with AMPSBDB.* In order to evaluate any change in the affinity for allosteric regulators, we compared the kinetic properties of enzyme incubated with AMPSBDB in the absence of any ligands or in the presence of protectant 1 mM ADP, with those of native enzyme. (These enzymes were isolated from free reagent and ligands as described in the Experimental Procedures.) Figure 3 shows the effect of increasing concentrations of ADP (in the assay) on the activity of AMPSBDB-modified, ADP-protected, and control enzymes. Concentrations of ADP as high as 1 mM had no effect on the modified enzyme (Figure 3, line B). In contrast, native enzyme (line A) is activated 1.7-fold and ADP-protected enzyme (line C) is activated 1.5-fold in this experiment. The activation constants, $K_a$, are 15 μM for native enzyme and 30 μM for the ADP-protected enzyme, calculated from Figure 3 as described by Frieden (1963). These results indicate almost complete protection by ADP from modification.

Since covalent modification by AMPSBDB causes activation of the enzyme upon subsequent assay, we wondered if the reagent would function as a reversible activator of native enzyme when added directly to the assay. The effect of AMPSBDB concentration on the assay of native and modified enzyme is shown in Figure 4. In fact, native enzyme is activated 2-fold by added AMPSBDB, with a $K_a$ of about 3 μM, as estimated from Figure 4; this value is about 5-fold lower than that for ADP. (We should point out that in the time-dependent incubation mixtures of enzyme and AMPSBDB, as in Figure 1, aliquots are diluted to yield ~30 nM AMPSBDB in the assay, at which concentration AMPSBDB has no effect on the assay of native enzyme, as seen in Figure 4, line A.) In contrast to native enzyme, isolated AMPSBDB-modified enzyme exhibited no activation at any concentration of free AMPSBDB added in the assay (Figure 4, line B).

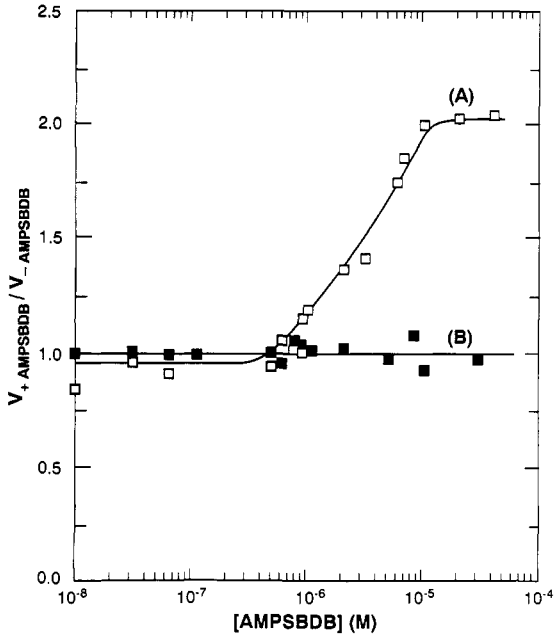


FIGURE 4: Effect of AMPSBDB on the maximum velocity of native glutamate dehydrogenase (A, □), and enzyme modified with AMPSBDB in the absence of ligands (B, ■). The coenzyme concentration was 100 μM NADH in the assay solutions.

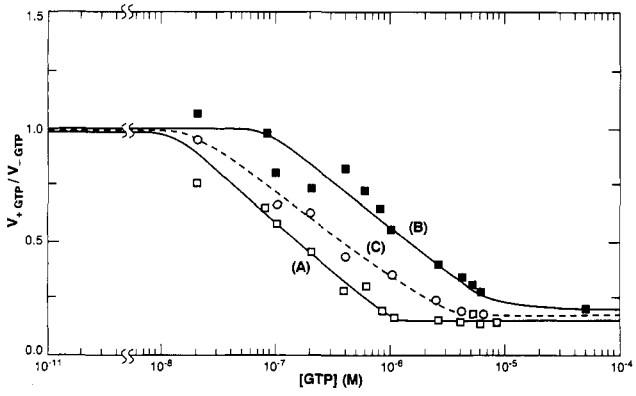


FIGURE 5: Effect of GTP on the maximum velocity of native glutamate dehydrogenase (A, □), enzyme modified with AMPSBDB in the absence of ligands (B, ■), and enzyme modified with AMPSBDB in the presence of 1 mM ADP (C, ○). The coenzyme was 100 μM NADH in the assay solutions. The $K_i$ values, calculated from the graph, equal the concentration of GTP yielding $(V_{-GTP} + V_{+GTP})/2$, where $V_{-GTP}$ is the maximum velocity in the absence of GTP and $V_{+GTP}$ is the maximum velocity measured at saturating concentrations of GTP (Frieden, 1963).

Figure 5 shows the concentration dependence of GTP inhibition for the three isolated enzymes. At saturating GTP concentrations, modified enzyme was inhibited maximally 80% while ADP-protected enzyme and native enzyme were both inhibited approximately 88%. The inhibition constants, $K_i$, for the GTP–enzyme complex, for modified, ADP-protected, and native enzyme, were 0.90 μM, 0.30 μM, and 0.12 μM, respectively, as calculated from Figure 5 in accordance with Frieden (1963). The extent of inhibition was therefore similar, although the affinity for GTP was somewhat affected by modification. These results indicate that no appreciable alterations of the GTP binding site occur upon modification of the enzyme by AMPSBDB.

Figure 6 compares the dependence of velocity on NADH concentration for the modified, ADP-protected, and control enzymes. Inhibition of native and ADP-protected glutamate dehydrogenase is exhibited at NADH concentrations greater than 100 μM, which is consistent with the well-known ability

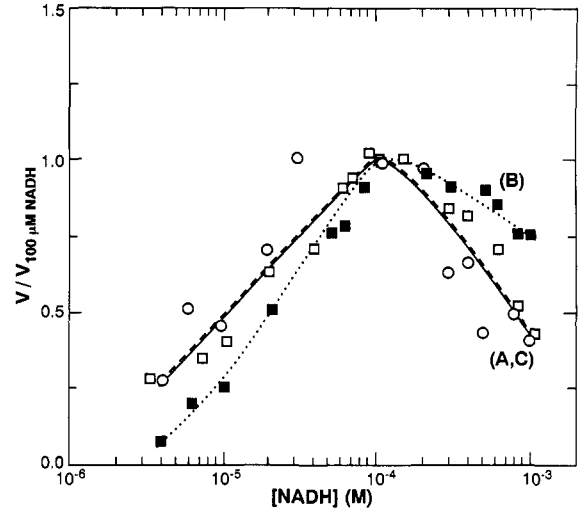


FIGURE 6: Initial velocity as a function of [NADH] for native glutamate dehydrogenase (A, □), enzyme modified with AMPSBDB in the presence of 1 mM ADP (C, ○), and enzyme modified with AMPSBDB in the absence of ligands (B, ■). Up to 0.2 mM NADH, the velocities (calculated in terms of μmol of NADH/min) were measured at 340 nm as described under Experimental Procedures. Velocity measurements at higher NADH concentrations were determined at 375 nm. The extinction coefficients for NADH are $6.22 \times 10^3$ $M^{-1}$ $cm^{-1}$ and $1.85 \times 10^3$ $M^{-1}$ $cm^{-1}$ at 340 and 375 nm, respectively.

of NADH to bind to an inhibitory site as well as the catalytic site (Colman, 1991). The AMPSBDB-modified enzyme behaves similarly, also exhibiting coenzyme inhibition, although the curve is shifted to higher NADH concentrations. These results indicate that AMPSBDB does not react directly at an NADH site, but may cause slight weakening of the affinity of enzyme for NADH.

*Incorporation of AMPSBDB into Bovine Liver Glutamate Dehydrogenase.* The amount of reagent incorporated into glutamate dehydrogenase was determined by quantification of the organic phosphate bound to the isolated, modified enzyme, as described in the Experimental Procedures. The average reagent incorporation was 0.83 mol of AMPSBDB/mol of enzyme subunit when the enzyme was maximally activated. After the enzyme was incubated with AMPSBDB under the same conditions, but in the presence of protecting agent (1 mM ADP), the average incorporation was decreased to 0.27 mol of reagent/mol of enzyme subunit.

The dioxo groups of several (bromodioxobutyl)nucleotide-modified enzymes have been reduced by [$^3$H]$NaBH_4$, thereby introducing a radioactive tag into the modified enzyme (Batra *et al.*, 1989; DeCamp & Colman, 1989; Vollmer & Colman, 1990). The reduction can be accomplished effectively by exposing the modified enzyme to [$^3$H]$NaBH_4$ in the presence of a denaturant and is expected to introduce 2 mol of tritium for each mole of dioxobutyl group incorporated. The incorporation calculated from the tritium results was expected to be comparable to values determined from the moles of organic phosphate bound to the enzyme. However, treatment of AMPSBDB-modified glutamate dehydrogenase with [$^3$H]-$NaBH_4$ led to a measured incorporation of only 0.25 mol of tritium/mol of enzyme subunit; while control enzyme, not exposed to AMPSBDB but treated with [$^3$H]$NaBH_4$, resulted in 0.22 mol of tritium/mol of enzyme subunit. These results suggest that the dioxo groups were not available for reduction by [$^3$H]$NaBH_4$, and therefore covalent reaction of glutamate dehydrogenase with AMPSBDB may directly involve the reagent's two carbonyl groups.

[2-$^3$H]Adenosine 5′-*O*-[*S*-(4-bromo-2,3-dioxobutyl)thiophosphate] was synthesized in order to measure radiochemically the amount of reagent incorporated into the enzyme and to facilitate the monitoring of modified peptides after proteolytic digestion and chemical cleavage of the reacted enzyme. An average value of incorporation of 0.90 mol of [2-$^3$H]AMPSBDB/mol of enzyme subunit was determined by this method. To evaluate the stability of the linkage between reagent and enzyme, the modified enzyme was dialyzed against 0.1% trifluoroacetic acid, pH 1.9, or 50 mM ammonium bicarbonate, pH 7.8, containing 6 M urea, and the incorporation was redetermined; 0.93 and 0.89 mol of [2-$^3$H]AMPSBDB/mol of enzyme subunit, respectively, were measured after these treatments, indicating the stability of the modified enzyme under these conditions.

*Isolation of Peptides after Proteolysis of [2-$^3$H]AMPSBDB-Modified Bovine Liver Glutamate Dehydrogenase.* Enzyme was reacted with [2-$^3$H]AMPSBDB in the absence and presence of 1 mM ADP, as described in the Experimental Procedures. Following dialysis, the modified enzyme was incubated with selected proteolytic enzymes. Trypsin digestion (5.0% w/w) for 4 h, at 37 °C in 50 mM ammonium bicarbonate, pH 7.8, was tried first. However, isolation of radioactively labeled peptides by reverse-phase HPLC was unsuccessful: no radioactively labeled peptides were found after HPLC, and most of the radioactivity eluted in a region close to that of AMPSBDB. Similar results were obtained when the modified enzyme was digested with thermolysin (5.0% w/w) for 4 h, at 37 °C in 50 mM ammonium bicarbonate at pH 7.8. These results suggest that the bond between peptide and reagent is unstable during digestion of glutamate dehydrogenase at neutral pH, even though the intact modified enzyme had been stable during dialysis under similar conditions.

The modified enzyme was then digested under acidic conditions, either with pepsin (5.0% w/w) for 4 h at 37 °C in 0.1% trifluoroacetic acid in water, pH 1.9, or with V8 protease (5.0% w/w) for a total of 4 h, at 37 °C in 20 mM ammonium acetate, pH 4.0. Digestion at these lower pH values allowed the isolation of radioactively labeled peptides by reverse-phase HPLC. Protease V8-digested modified protein yielded a less complex chromatogram than that produced by pepsin; thus, subsequent work was performed using protease V8.

Panels A and B of Figure 7 illustrate the distribution of radioactivity in representative chromatograms of protease V8 digests of enzyme modified in the absence and presence of ADP, respectively; while Figure 7C shows the distribution of radioactivity in an HPLC run of reagent alone which had been treated under the conditions used for treatment with V8 protease. Two major radioactive peptide peaks are observed in Figure 7A: peak I and peak II representing, respectively, 20% and 24% of the total radioactivity recovered. Peaks I and II are greatly decreased in the digest of ADP-protected modified enzyme, Figure 7B. For [2-$^3$H]AMPSBDB, no radioactivity was detected in the region of peaks I and II, Figure 7C. Fraction 142 of peak I was isolated for sequence analysis. Peak II was isolated, redigested with V8 protease under the same conditions, and subjected to HPLC again. The redigested peak exhibited a radioactivity distribution pattern similar to that of Figure 7A except that no radioactivity was detected in the region of peak II. We conclude that peak II is an incompletely digested peptide which contains peak I.

*Isolation of Peptides from Chemical Cleavage of [2-$^3$H]AMPSBDB-Modified Bovine Liver Glutamate Dehydrogenase.* Peak I of Figure 7A obtained from another V8 protease digest was further cleaved chemically with cyanogen bromide. The distribution pattern of radioactivity (data not shown) exhibited a single peptide peak at approximately 13–14% solvent B corresponding to fractions 65–67 of Figure 8. These fractions were isolated for sequence analysis.

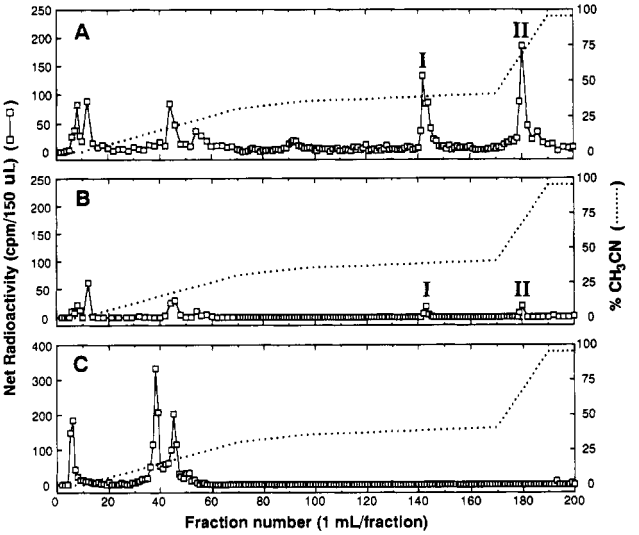


FIGURE 7: Separation of V8 protease-digested peptides by reverse-phase HPLC on a $C_{18}$ Vydac column. The radioactive peptides were separated by elution with 0.1% trifluoroacetic acid, pH 1.9, followed by a linear gradient of 0.07% TFA in acetonitrile as described under Experimental Procedures. A 150 μL aliquot of each fraction was assayed for radioactivity. The dotted line (···) indicates the elution gradient, and the Roman numeral designates radioactive peaks containing [2-$^3$H]AMPSBDB]-modified peptides from a digest of enzyme modified with [2-$^3$H]AMPSBDB in the absence of ligands (A), and enzyme modified with [2-$^3$H]AMPSBDB in the presence of 1 mM ADP protecting ligand (B). The reverse-phase HPLC radioactivity pattern of [2-$^3$H]AMPSBDB incubated at 37 °C in 0.05 M ammonium acetate, pH 4.0, for 4 h is illustrated in (C).

In a separate experiment, complete enzyme modified with [2-$^3$H]AMPSBDB, in the absence and presence of 1 mM ADP, was chemically cleaved with cyanogen bromide. Panels A and B of Figure 8 represent respectively the distribution of radioactivity after cyanogen bromide cleavage of comparable amounts of enzyme modified in the absence and the presence of ADP. Figure 8C shows the radioactivity pattern of reagent alone which had been treated under the cleavage conditions used for CNBr cleavage. Figure 8A shows one distinct peptide peak (I, maximum at fraction 67), representing 30% of the radioactivity recovered. As shown in Figure 8B, this same peak is observed in the ADP-protected enzyme, but in reduced amounts. The chromatogram of [2-$^3$H]AMPSBDB and its decomposition products, shown in Figure 8C, exhibits radioactivity close to peak I, but the maximum occurs slightly earlier at fractions 64–65. HPLC fractions 66–67 and 68–69 from peak I, Figure 8A, were applied to the gas-phase sequencer to evaluate whether peak I contained modified peptide.

*Characterization of Isolated Peptides from Proteolysis and Chemical Cleavage of Modified Bovine Liver Glutamate Dehydrogenase.* Table 2 reports the amino acid sequences of representative peptides resulting from cleavage of modified glutamate dehydrogenase by V8 protease or cyanogen bromide. The first peptide reported in Table 2A is that from a V8 protease digest analogous to peak I (fraction 142) of Figure 7A, which was only sequenced for 17 cycles. These first 17 amino acids match the sequence of peptide Arg$^{459}$–Leu$^{475}$ of bovine liver glutamate dehydrogenase, as reported by Julliard and Smith (1979). Fraction 142 from another V8 protease digest was analyzed for 29 cycles and yielded the amino acid

Table 2: Amino Acid Sequences of Peptides Modified by AMPSBDB

| | (A) V8 protease—peak I | | | | (B) CNBr—peak $I_{V8}$ | | | | (C) CNBr-modified enzyme—peak I | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | sample 1 | | sample 2 | | sample 1 | | sample 2 | | sample 1 | | sample 2 | |
| cycle | amino acid | pmol | amino acid | pmol | amino acid | pmol | amino acid | pmol | amino acid | pmol | amino acid | pmol |
| 1 | Arg | 57 | Arg | 28 | Arg | 120 | Arg | 14 | Glu | 1006 | Glu | 695 |
| 2 | Ser | 70 | Ser | 73 | Ser | 57 | Ser | 24 | Arg[b] | 235 | Arg | 85 |
| 3 | Ala | 413 | Ala | 167 | Ala | 475 | Ala | 220 | Ser | 208 | Ser | 98 |
| 4 | Arg | 115 | Arg | 50 | Arg | 82 | Arg | 49 | Ala | 717 | Ala | 653 |
| 5 | Gln | 201 | Gln | 132 | Gln | 295 | Gln | 179 | Arg | 333 | Arg | 109 |
| 6 | Ile | 278 | Ile | 478 | Ile | 100 | Ile | 81 | Gln | 448 | Gln | 421 |
| 7 | Met | 276 | Met | 273 | Met[a] | 10 | Met[a] | 7 | Ile | 290 | Ile | 174 |
| 8 | Arg | 108 | Arg | 97 | | | | | Met[a] | 70 | Met[a] | 21 |
| 9 | Thr | 42 | Thr | 44 | | | | | | | | |
| 10 | Ala | 276 | Ala | 181 | | | | | | | | |
| 11 | Met | 245 | Met | 208 | | | | | | | | |
| 12 | Lys | 175 | Lys | 231 | | | | | | | | |
| 13 | Tyr | 164 | Tyr | 160 | | | | | | | | |
| 14 | Asn | 150 | Asn | 71 | | | | | | | | |
| 15 | Leu | 209 | Leu | 269 | | | | | | | | |
| 16 | Gly | 62 | Gly | 78 | | | | | | | | |
| 17 | Leu | 181 | Leu | 248 | | | | | | | | |
| 18 | *c* | | Asp | 60 | | | | | | | | |
| 19 | | | Leu | 234 | | | | | | | | |
| 20 | | | Arg | 59 | | | | | | | | |
| 21 | | | Thr | 19 | | | | | | | | |
| 22 | | | Ala | 77 | | | | | | | | |
| 23 | | | Ala | 45 | | | | | | | | |
| 24 | | | Tyr | 114 | | | | | | | | |
| 25 | | | Val | 98 | | | | | | | | |
| 26 | | | Asn | 19 | | | | | | | | |
| 27 | | | Ala | 25 | | | | | | | | |
| 28 | | | Ile | 89 | | | | | | | | |
| 29 | | | *d* | | | | | | | | | |

[a] Detected as homoserine lactone eluting in the region of Thr. [b] Radioactivity (15 cpm) observed in the cycle. [c] Sample was sequenced for only 17 cycles. [d] Glu is expected from the known sequence, but the amount was too low to detect reliably.

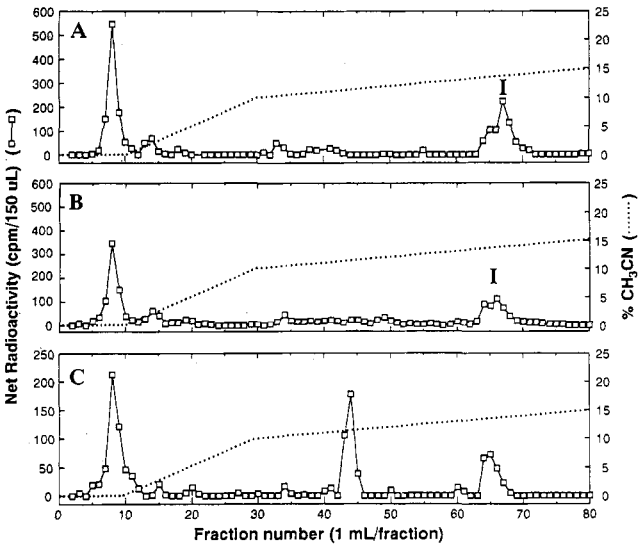


FIGURE 8: Separation of peptides, cleaved by cyanogen bromide, by reverse-phase HPLC on a $C_{18}$ Vydac column. The radioactive peptides were separated by elution with 0.1% trifluoroacetic acid, pH 1.9, followed by a linear gradient of 0.07% TFA in acetonitrile as described under Experimental Procedures. A 150 μL aliquot of each fraction was assayed for radioactivity. The dotted line (···) indicates the elution gradient, and the Roman numeral designates the radioactive peak containing [2-$^3$H]AMPSBDB-modified peptides from a cyanogen bromide cleavage of enzyme modified with [2-$^3$H]AMPSBDB in the absence of ligands (A), and enzyme modified with [2-$^3$H]AMPSBDB in the presence of 1 mM ADP protecting ligands (B). The reverse-phase HPLC radioactivity pattern of [2-$^3$H]AMPSBDB incubated with cyanogen bromide, as described in Experimental Procedures, is illustrated in (C).

sequence of $Arg^{459}$–$Glu^{487}$(Arg-Ser-Ala-Arg-Gln-Ile-Met-Arg-Thr-Ala-Met-Lys-Tyr-Asn-Leu-Gly-Leu-Asp-Leu-Arg-Thr-Ala-Ala-Tyr-Val-Asn-Ala-Ile-Glu), as shown in Table 2 under V8 protease—peak I, sample 2. Since these were large peptides, with several possible targets for modification by reagent, it was desirable to further cleave the isolated peptide in order to identify a specific modified site. Therefore, peak I, Figure 7A, was treated with cyanogen bromide, and the peptide mixture was purified and sequenced. Two samples shown in Table 2 demonstrate the amino acid sequence of $Arg^{459}$–$Met^{465}$.

Additional samples for amino acid sequence determination came from cyanogen bromide treatment of whole modified enzyme, followed by fractionation by HPLC. Table 2 reports two samples derived from peak I, Figure 8A (CNBr-modified enzyme, samples 1 and 2), which yield the same peptide sequence, $Glu^{458}$-Arg-Ser-Ala-Arg-Gln-Ile-$Met^{465}$. The two most reasonable sites of modification of this peptide are $Arg^{459}$ and $Arg^{462}$. Upon amino acid sequencing of the 5th peptide of Table 2, radioactivity was detected with the second PTH-amino acid from the sequencer. Furthermore, in several amino acid sequences of these isolated peptides, $Arg^{459}$ is observed in lower quantities than any of the other possible target amino acids, including $Arg^{462}$. It appears that $Arg^{459}$ is the modified amino acid residue and that it is partially regenerated during the gas phase sequencing.

## DISCUSSION

The adenine nucleotide analog adenosine 5′-*O*-[*S*-(4-bromo-2,3-dioxobutyl)thiophosphate] reacts covalently with bovine liver glutamate dehydrogenase to produce an irreversibly activated enzyme. The functional target site of AMPSBDB is the ADP regulatory site of the enzyme, as indicated by the kinetic properties of the modified enzyme, as well as by the natural ligands that protect against the covalent reaction. The

catalytic site is not attacked by AMPSBDB, since the activity of the enzyme does not decrease when measured in the absence of regulatory compounds in the standard assay. Moreover, failure of 10 mM $\alpha$-ketoglutarate and 100 $\mu$M NADH [which are known to bind to the enzyme under similar conditions (Sund *et al.*, 1974)] to protect against the modification supports the conclusion that AMPSBDB does not react at the active site.

The GTP-regulatory site is also an unlikely target, since GTP does not provide any protection against the functional changes produced by AMPSBDB. The modified enzyme is still inhibited about 80% by GTP, and the $K_i$ for the GTP–enzyme complex is only slightly greater for the modified as compared to native enzyme. These observations suggest the ability of GTP to bind to a slightly perturbed regulatory site. At elevated concentrations of NADH, inhibition is observed for the AMPSBDB-modified enzyme, as for the native enzyme; however, somewhat higher concentrations of NADH are needed for inhibition of the modified enzyme, likely reflecting a weakening of the affinity of enzyme for NADH upon modification. The decreased affinity of modified enzyme for NADH is probably an indirect result of reaction of AMPSBDB at another site.

AMPSBDB appears to affect directly the ADP regulatory site of glutamate dehydrogenase. Most significant is the 3-fold increase in activity which occurs as a result of modification by AMPSBDB. The modified enzyme is not further activated when assayed in the presence of ADP, suggesting that the normal site for ADP is no longer available. Furthermore, ADP is the most effective protectant against the time-dependent activation by AMPSBDB. Protection against the functional change is also provided by high concentrations of NADH, which can be explained in terms of an indirect interaction between the ADP site and the NADH inhibitory site. Various binding studies indicate an apparent competition between ADP and inhibitory concentrations of NADH (Pantaloni & Dessen, 1969; Cross & Fisher, 1970; Batra & Colman, 1986; Lark & Colman, 1990), although chemical modification studies demonstrate that the ADP and NADH sites are not identical (Pal *et al.*, 1975).

Here, we have presented evidence that AMPSBDB modifies an arginine in bovine liver glutamate dehydrogenase. We were able to isolate the AMPSBDB-modified peptide after digestion or chemical cleavage at acidic pH, but not after proteolysis at neutral pH. The instability of the product of the AMPSBDB and enzyme reaction under the conditions used for proteolytic digestion by trypsin or thermolysin in ammonium bicarbonate at pH 7.8, coupled with the greater stability of the protein product under acidic conditions, suggests the characteristics of the product of reaction of arginine with a dioxo compound. These results are very similar to those reported by Patthy and Smith (1975) in their model study of the modification of arginine residues by cyclohexanedione, pictured in Figure 9A. They showed that, in the pH range 7–9, arginine residues react with cyclohexanedione to form $N^7$,$N^8$-(1,2-dihydroxycyclohex-1,2-ylene)-L-arginine (Figure 9, structure **I**), which is stable under acidic solutions and in borate buffers, but readily regenerates free arginine in neutral or weakly alkaline solutions. Furthermore, decomposition is more rapid in buffers containing species which are capable of reacting with cyclohexanedione (such as Tris or ammonium), probably because of nucleophilic catalysis of decomposition and trapping of the liberated cyclohexanedione (Patthy & Smith, 1975). Finally, Patthy and Smith reported a 10-fold decrease in the half-time of the reaction between cyclohexanedione and arginine, upon going from 25 to 40 °C, which is similar to the large enhancement we observed in the reaction rate of AMPSBDB and glutamate dehydrogenase between 5 and 25 °C (Figure 1).

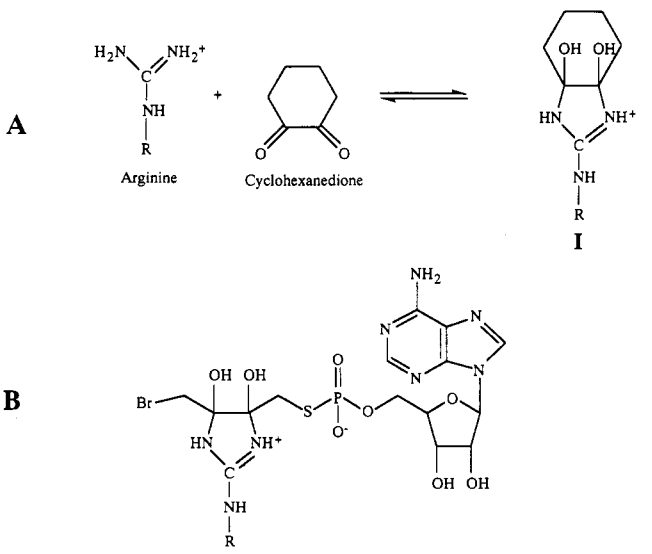


FIGURE 9: Schematic representations of (A) the reaction between arginine and cyclohexanedione resulting in the product $N^7$,$N^8$-(1,2-dihydroxycyclohex-1,2-ylene)-L-arginine (**I**), as described in Patthy and Smith (1975), and (B) the postulated product of the reaction of an arginine residue with AMPSBDB.

Strong support for reaction at AMPSBDB with arginine in glutamate dehydrogenase comes from the lack of incorporation of tritium upon addition to the modified enzyme of [$^3$H]-$NaBH_4$. If reaction with AMPSBDB occurs by displacement of the bromide, the dioxo groups would remain available for reduction by [$^3$H]$NaBH_4$. Successful application of this strategy has been described in examples of modification of several different enzymes (Batra *et al.*, 1989; Vollmer & Colman, 1990). However, if the dioxo groups are directly involved in the reaction with the enzyme, they would not be available for reduction and no incorporation of tritium would be observed, as in this reaction between glutamate dehydrogenase and AMPSBDB. Depicted in Figure 9B is the postulated product of reaction of an arginine residue of glutamate dehydrogenase with AMPSBDB.[2] Reaction between a bromodioxobutyl nucleotide and an arginine has previously been detected as a minor product (DeCamp & Colman, 1989), but this is the first time that arginine has been observed as the primary reaction site of a (bromodioxobutyl)nucleotide affinity label.

Further evidence shows that the specific site of modification by AMPSBDB is $Arg^{459}$ of bovine liver glutamate dehydrogenase. The amino acid sequence data of peptides modified by AMPSBDB, when proteolytically cleaved with V8 protease, revealed a 29-amino acid peptide with the sequence of $Arg^{459}$–$Glu^{487}$ of bovine liver glutamate dehydrogenase. This peptide has numerous potential targets for modification. Further cleavage of this 29-amino acid peptide by cyanogen bromide provided a much smaller peptide, $Arg^{459}$–$Met^{465}$ (Arg-Ser-Ala-Arg-Gln-Ile-Met) with fewer potential reaction sites. Also, chemical cleavage of whole modified enzyme with cyanogen bromide yielded the peptide $Glu^{458}$–$Met^{465}$. The two most

[2] Although the product is pictured with the bromine atom, we do not in fact know whether bromine is retained in the modified enzyme. However, the stability characteristics of the modified amino acid and the lack of incorporation into modified enzyme of tritium from [$^3$H]$NaBH_4$ would not be changed by the absence or presence of the bromine.

reasonable sites of modification of these peptides, based on the evidence summarized above, are $Arg^{459}$ and $Arg^{462}$. Upon amino acid sequencing of one of the radioactively labeled peptides obtained from the chemical cleavage of whole enzyme, radioactivity was detected with the second PTH-amino acid from the sequencer, suggesting the modification of $Arg^{459}$ by radioactive AMPSBDB. Furthermore, examination of the amino acid sequence data of several isolated peptides reveals that $Arg^{459}$ appears in lower quantities than any other possible target amino acids, including $Arg^{462}$. This observation is significant because, upon gas phase sequencing, the detected quantity of a particular amino acid generally decreases with increasing cycles. Thus, the lower yields of $Arg^{459}$, as compared to the later amino acid $Arg^{462}$, indicates that $Arg^{459}$ is modified by AMPSBDB, and that free arginine is partially regenerated under the solvent conditions in the gas phase sequencer.

The adenine nucleotide analog AMPSBDB is an affinity label which has a reactive functional group that is equivalent to the pyrophosphate group of ADP. The modification of amino acid $Arg^{459}$ of bovine liver glutamate dehydrogenase by AMPSBDB suggests that this amino acid may contribute to the reversible binding of ADP by the enzyme through an electrostatic interaction with the pyrophosphate moiety. Arginine residues of several enzymes have been shown to participate in the binding of nucleotidyl phosphate groups. For example, the crystal structure of the nucleoside diphosphate kinase–ADP–$Mg^{2+}$ complex (NDP kinase–ADP–$Mg^{2+}$) identifies $Arg^{92}$ and $Arg^{109}$ in binding the $\beta$-phosphate region of ADP (Morera *et al.*, 1994); the cyclic AMP receptor protein exhibits an interaction between the charged phosphate of cAMP and $Arg^{82}$ to form a salt bridge (Weber & Steitz, 1987; Belduz *et al.*, 1993); in rod transducin, $Arg^{174}$ interacts with the phosphates of GTP$\gamma$S (Noel *et al.*, 1993). Finally, the $NAD^+$ binding site for lactate dehydrogenase involves an electrostatic interaction between $Arg^{101}$ of the enzyme with the pyrophosphate moiety of the coenzyme (Holbrook *et al.*, 1975). Thus, there is ample precedence for the involvement of arginine in the binding of nucleotidyl phosphates.

The AMPSBDB reagent modifies bovine liver glutamate dehydrogenase in a region close to the C-terminus. Further comparison of the amino acid sequence of this region among various species of vertebrates (i.e., chicken, bovine, rat, mouse, human) reveals that this region is highly conserved, while very little conservation of sequence exists among bacterial species (Teller *et al.*, 1992). Furthermore, there is very little conservation within this region when comparing bacterial and vertebrate enzymes. Specifically, $Arg^{459}$ and the amino acids comprising $Ala^{443}$ to the C-terminus are completely identical for the vertebrate enzymes mentioned above, whereas very little similarity in amino acid sequence can be seen between bacterial species (e.g., *Clostridium symbiosum*, *Escherichia coli*, *Salmonella typhimurium*, *Neurospora crassa*) and vertebrate forms in this $\alpha$16 and $\alpha$17 region of the structure of *C. symbiosum* (Baker *et al.*, 1991). This lack of homology in amino acid sequence between bacterial forms of glutamate dehydrogenase and vertebrate forms is significant since bacterial glutamate dehydrogenases are not allosterically regulated (Smith *et al.*, 1975). The correlation between conservation of $Arg^{459}$ and response to ADP activation supports our conclusion that reaction of bovine liver glutamate dehydrogenase by the ADP analog AMPSBDB at $Arg^{459}$ represents a modification within the enzyme's ADP regulatory site. It is also of interest that $Leu^{477}$ and $Arg^{478}$ in the carboxy-terminal region of the enzyme have previously been designated as within the NADH inhibitory site on the basis of photoaffinity cross-linking by 5′-(*p*-fluorobenzoyl)-8-azidoadenosine (Dombrowski *et al.*, 1992). Thus, the ADP and NADH regulatory sites are reasonably close within the linear sequence of the enzyme.

The modification of bovine liver glutamate dehydrogenase by AMPSBDB is unusual since it causes a time-dependent *activation* of the enzyme. Affinity labeling studies of enzymes generally cause inactivation or desensitization toward allosteric regulation of the enzyme [e.g., Batra and Colman (1986), and Ozturk *et al.* (1990), and Ozturk *et al.* (1992)]; it is uncommon for an affinity label to mimic the catalytic or regulatory effect of the natural compound. Hulla and Fasold (1972) have described a 30% activation of rabbit skeletal muscle phosphorylase *b* by an adenosine 5′-monophosphate analog after prolonged incubation with the compound; and affinity labels for the estrogen receptor that function as estrogen agonists have been reported (Zablocki *et al.*, 1987). However, there are few other examples in the literature of this type of effect. Bovine liver glutamate dehydrogenase is maximally activated 3.2-fold when incubated with AMPSBDB. This is a time-dependent activation, best observed at low temperatures, to yield an irreversibly *activated* enzyme. The ability of AMPSBDB to mimic the effect of activation of the natural compound, ADP, strongly indicates not only that the compound is structurally equivalent to ADP but also that it binds to glutamate dehydrogenase in a similar manner in order to produce a comparable allosteric effect on the catalytic activity of the enzyme.

## ACKNOWLEDGMENT

The authors thank Dr. Yu-Chu Huang for her contributions in obtaining the peptide sequences.

## REFERENCES

Bailey, J. M., & Colman, R. F. (1987) *J. Biol. Chem. 262*, 12620–12626.

Baker, P. J., Britton, K. L., Engel, P. C., Farrants, G. W., Lilley, K. S., Rice, D. W. & Stillman, T. J. (1992) *Proteins 12*, 75–86.

Batra, S. P., & Colman, R. F. (1986) *J. Biol. Chem. 261*, 15565–15571.

Batra, S. P., Lark, R. H., & Colman, R. F. (1989) *Arch. Biochem. Biophys. 270*, 277–285.

Belduz, A. O., Lee, E. J., & Harman, J. G. (1993) *Nucleic Acids Res. 21*, 1827–1835.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Colman, R. F. (1983) *Annu. Rev. Biochem. 52*, 67–91.

Colman, R. F. (1991) in *A Study in Enzymes, Volume III, Mechanism of Enzyme Action* (Kuby, S. A., Ed.) Chapter 7, pp 173–192, CRC Press, Boca Raton, FL.

Cross, D. G., & Fisher, H. F. (1970) *J. Biol. Chem. 245*, 2612–2621.

DeCamp, D. L., & Colman, R. F. (1989) *J. Biol. Chem. 264*, 8430–8441.

Dombrowski, K. E., Huang, Y.-C., & Colman, R. F. (1992) *Biochemistry 31*, 3785–3793.

Frieden, C. (1963) *J. Biol. Chem. 238*, 3286–3299.

Hess, H. H., & Derr, J. E. (1975) *Anal. Biochem. 63*, 607–613.

Holbrook, J. J., Liljas, A., Steindel, S. J., & Rossman, M. G. (1975) in *The Enzymes* (Boyer, P. D., Ed.) Vol. XI, Part A, pp 191–292, Academic Press, New York.

Hulla, F. W., & Fasold, H. (1972) *Biochemistry 11*, 1056–1061.

Jacobson, M. A., & Colman, R. F. (1982) *Biochemistry 21*, 2177–2186.

Julliard, J. H., & Smith, E. L. (1979) *J. Biol. Chem. 254*, 3427–3438.

Lanzetta, P. A., Alvarez, L. J., Reinach, P. S., & Candia, O. A. (1979) *Anal. Biochem. 100*, 95–97.
Lark, R. H., & Colman, R. F. (1990) *Eur. J. Biochem. 188*, 377–383.
Morera, S., Lascu, I., Dumas, C., LeBras, G., Briozzo, P., Vernon, M., & Janin, J. (1994) *Biochemistry 33*, 459–467.
Noel, J. P., Hamm, H. E., & Sigler, P. B. (1993) *Nature 366*, 654–663.
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.
Ozturk, D. H., Park, I., & Colman, R. F. (1992) *Biochemistry 31*, 10544–10555.
Pal, P. K., & Colman, R. F. (1979) *Biochemistry 18*, 838–845.
Pal, P. K., Wechter, W. J., & Colman, R. F. (1975) *J. Biol. Chem. 250*, 8140–8147.
Pantaloni, D., & Dessen, P. (1969) *Eur. J. Biochem. 11*, 510–519.
Patthy, L., & Smith, E. L. (1975) *J. Biol. Chem. 250*, 557–564.
Penefsky, H. S. (1979) *Methods Enzymol. 56*, 527–530.
Saha, A., Huang, Y.-C., & Colman, R. F. (1989) *Biochemistry 28*, 8425–8431.
Saradambal, K. V., Bednar, R. A., & Colman, R. F. (1981) *J. Biol. Chem 256*, 11866–11872.
Schmidt, J. A., & Colman, R. F. (1984) *J. Biol. Chem. 259*, 14515–14519.
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.
Smith, E. L., Austen, B. M., Blumenthal, K. M., & Nyc, J. F. (1975) in *The Enzymes* (Boyer, P. D., Ed.) Vol. XI, Part A, pp 294–367, Academic Press, New York.
Sund, H., Markau, K., & Koberstein, R. (1975) *Biol. Macromol. 7c*, 225–287.
Teller, J. K., Smith, R. J., McPherson, M. J., Engel, P. C., & Guest, J. R. (1992) *Eur. J. Biochem. 206*, 151–159.
Vollmer, S. H., & Colman, R. F. (1990) *Biochemistry 29*, 2495–2501.
Vollmer, S. H., Walner, M. B., Tarbell, K. V., & Colman, R. F. (1994) *J. Biol. Chem. 269*, 8082–8090.
Weber, L. T., & Steitz, T. A. (1987) *J. Mol. Biol. 198*, 311–326.
Wrzeszczynski, K. O., & Colman, R. F. (1994) *FASEB J. 8* (7), A1357.
Zablocki, J. A., Katzenellenbogen, J. A., Carlson, K. E., Norman, M. J., & Katzenellenbogen, B. S. (1987) *J. Med. Chem. 30*, 829–838.