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Identification of Cysteine-319 as the Target Amino Acid of 8-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 5′-Triphosphate in Bovine Liver Glutamate Dehydrogenase†

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ABSTRACT: The affinity label 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-triphosphate (8-BDB-TA-5′-TP) has been shown to react with bovine liver glutamate dehydrogenase in the region of the GTP-dependent NADH inhibitory site with incorporation of about 1 mol of reagent/mol of subunit [Ozturk, D. H., Safer, D., & Colman, R. F. (1990) *Biochemistry 29*, 7112-7118]. The modified enzyme was shown to contain only 5 free sulfhydryl groups upon 5,5′-dithiobis(2-nitrobenzoate) titration as compared with 6 in the unmodified enzyme. In the unmodified enzyme digested with trypsin, 6 cysteinyl peptides were detected by high-performance liquid chromatography upon treatment with iodo[^{3}H]acetic acid. In contrast, only 5 (carboxymethyl)cysteinyl peptides were detected in 8-BDB-TA-5′-TP-modified enzyme. When carboxymethylated modified and unmodified enzymes were digested with thermolysin, 6 peptide sequences containing (carboxymethyl)cysteine were obtained in the unmodified enzyme, but only 5 were observed in the modified enzyme. The (carboxymethyl)cysteine which was absent in the modified enzyme was determined to be Cys-319, leading to the conclusion that 8-BDB-TA-5′-TP reacts with Cys-319, thereby preventing it from subsequent reaction with radioactive iodoacetate. It was previously reported that 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate (6-BDB-TA-5′-DP) modifies Cys-319 in this enzyme [Batra, S. P., & Colman, R. F. (1986) *Biochemistry 25*, 3508-3515]. Evidence is here presented that the reaction product is different for the two nucleotide analogues: while Cys-319 reacts at the methylene group of 6-BDB-TA-5′-DP by displacement of bromide, the same cysteine appears to attack the carbonyl group of 8-BDB-TA-5′-TP, leading to a thiohemiacetal adduct.

Bovine liver glutamate dehydrogenase [L-glutamate:NAD(P)$^+$ oxidoreductase (deaminating), EC 1.4.1.3] is an allosteric enzyme with multiple binding sites for purine nucleotides. The enzyme is inhibited by GTP and high concentrations of NADH, while ADP functions as an allosteric activator (Colman, 1990). The enzyme is composed of six identical subunits in its active form, which binds 2 mol of GTP/mol of subunit in the presence of NADH, but only 1 mol of GTP/mol of subunit in the absence of reduced coenzyme (Pal & Colman, 1979). Furthermore, the allosteric activator ADP,

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as well as the coenzyme NADH, each occupy 2 sites/subunit (Batra & Colman, 1986a; Colman, 1990).

Affinity labeling with reactive purine nucleotide analogues has led to the identification of several amino acids within regulatory sites of glutamate dehydrogenase (Colman, 1983, 1989, 1990). For example, reaction with 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine caused modification of lysine-420 and tyrosine-190 within the NAD inhibitory site (Pal et al., 1975; Saradambal et al., 1981; Schmidt & Colman, 1984), and incubation with 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate yielded labeling of cysteine-319, also in the NADH inhibitory site (Batra & Colman, 1984, 1986b).

We have recently reported that bovine liver glutamate dehydrogenase reacts covalently with 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-triphosphate (8-BDB-TA-5′-TP)[1] with incorporation of about 1 mol of reagent/mol of enzyme subunit (Ozturk et al., 1990). The modified enzyme is catalytically active, but exhibits decreased sensitivity to inhibition by GTP and by high concentrations of NADH. The presence of 200 μM GTP and 5 mM NADH in the incubation mixture of enzyme and 8-BDB-TA-5′-TP prevents these changes in the regulatory properties and decreases reagent incorporation to about 0.1 mol of reagent/mol of enzyme subunit, suggesting that 8-BDB-TA-5′-TP reacts in the region of the GTP-dependent NADH inhibitory site. However, the target amino acid was not investigated. In this paper we consider the identification of the amino acid residue of bovine liver glutamate dehydrogenase which reacts with 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-triphosphate and compare this reaction with that of other affinity labels of this enzyme.

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 any insoluble protein. The enzyme concentration in the supernatant was determined spectrophotometrically by using $E^{1\%}_{280} = 9.7$ (Olson & Anfinsen, 1952). The ratio of A_{280}/A_{260} for the enzyme was 1.8. The enzyme was stored in aliquots at −75 °C and was thawed rapidly prior to use. For calculations, 56 000 Da was used as the molecular mass for the subunit (Smith et al., 1970).

Dithiothreitol, HEPES, 5,5′-dithiobis(2-nitrobenzoate), ammonium bicarbonate, 8-bromo-ATP, Sephadex G-50–80, TPCK-treated trypsin, and thermolysin (type X) were purchased from Sigma. Ultrapure guanidine hydrochloride and urea were obtained from ICN Biochemicals. HPLC-grade acetonitrile was from Mallinckrodt, and trifluoroacetic acid was supplied by Aldrich Chemical Co. Iodo[^{3}H]acetic acid and [^{3}H]$NaBH_4$ were purchased from New England Nuclear Corp., while [^{35}S]Na_2S was supplied by Amersham. All other reagents were analytical grade.

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

Preparation of Glutamate Dehydrogenase Modified by 8-BDB-TA-5′-TP in the Presence and Absence of Protecting Ligands. Glutamate dehydrogenase (2 mg/mL) was incubated with 0.2 mM 8-BDB-TA-5′-TP at 25 °C in 0.05 M potassium phosphate buffer, pH 7.1. After 3 h, the reaction was quenched with 20 mM dithiothreitol (final concentration). The modified enzyme was isolated by the column centrifugation technique using Sephadex G-50–80 mesh, as described by Penefsky (1979). Two successive column centrifugations were carried out at 4 °C to remove the excess reagent, the first column being equilibrated with 0.1 M potassium phosphate buffer, pH 7.1, and the second column equilibrated with 0.1 M potassium phosphate buffer, pH 8.0. The "protected" enzyme was prepared under the same conditions except that 200 μM GTP + 5 mM NADH were included in the incubation mixture (Ozturk et al., 1990). A control enzyme was also prepared with neither ligands nor 8-BDB-TA-5′-TP present in the incubation mixture and was isolated in the same manner.

Incorporation of 8-BDB-TA-5′-TP into Glutamate Dehydrogenase. The amount of 8-BDB-TA-5′-TP incorporated into the modified and the protected enzyme after 3-h incubation with 0.2 mM reagent was determined from the amount of organic phosphate incorporated into these enzyme samples, as described previously (Ozturk et al., 1990).

Reaction of Hydrolyzed 8-BDB-TA-5′-TP with Glutamate Dehydrogenase. 8-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 5′-triphosphate has been shown to decompose with release of bromide, with a half-life of 50 min at pH 7.0 and 25 °C (DeCamp et al., 1988). The nucleotide analogue (0.2 mM) was incubated at 25 °C for 6 h in 0.1 M potassium phosphate buffer, pH 7.1. At the end of this period, 0.45 mg/mL glutamate dehydrogenase (final concentration) was added to the incubation mixture and the change in GTP inhibition was monitored spectrophotometrically by assaying the enzyme in the presence of 1 μM GTP. The assay conditions were the same as described by Ozturk et al. (1990). For comparison, glutamate dehydrogenase was incubated under the same conditions using freshly prepared reagent (0.2 mM).

Synthesis of 8-[(4-Bromo-2,3-dioxobutyl)[^{35}S]thio]-adenosine 5′-Triphosphate. The radioactive precursor 8-[^{35}S]thioadenosine 5′-triphosphate was synthesized by reacting 8-bromo-ATP with an aqueous solution of radioactive LiSH. To prepare the aqueous radioactive LiSH solution, H_2S gas (generated by adding 6 M HCl to crushed FeS rocks) was bubbled through a 1 M solution of LiOH until the pH was constant at 9.0. (The solution turns green when there is no further change in pH.) Solid [^{35}S]Na_2S (1 mCi) was then dissolved in 3 mL of LiSH solution. Conversion of 8-bromo-ATP to 8-[^{35}S]thioadenosine 5′-triphosphate was accomplished by dissolving 0.5 mmol of 8-bromo-ATP in this radioactive solution, adjusting the pH to 9.0 by addition of KOH, and allowing it to stand overnight at room temperature. Purification was achieved by chromatography on DEAE-cellulose as described previously (DeCamp et al., 1988). It is important that the radioactive mixture be diluted in 500 mL of 10 mM ammonium bicarbonate, pH 8.0, before loading onto the column to prevent evolution of any radioactive H_2S gas. The coupling reaction with recrystallized 1,4-dibromobutanedione to give the final radioactive product was accomplished by the procedure of DeCamp et al. (1988).

Incorporation of 8-[^{35}S]BDB-TA-5′-TP into Bovine Liver Glutamate Dehydrogenase. The enzyme (2 mg/mL) was incubated with 0.4 mM radioactive 8-BDB-TA-5′-TP for 2 h and was isolated by the same procedure as described above. The isolated enzyme was dialyzed overnight against 0.1 M

[1] Abbreviations: 8-BDB-TA-5′-TP, 8-[(4-bromo-2,3-dioxobutyl)-thio]adenosine 5′-triphosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; DTNB, 5,5′-dithiobis(2-nitrobenzoate); Cm, carboxymethylated; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone; 6-BDB-TA-5′-DP, 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate; PTH, phenylthiohydantoin.

potassium HEPES + 0.4 M KCl, pH 7.0. Four 50-μL aliquots were counted in 5 mL of Liquiscint (National Diagnostics) scintillation cocktail, and the enzyme concentration was determined by the Bio-Rad assay, which is based on the Bradford dye-binding method (Bradford, 1976). The incorporation of radioactive 8-BDB-TA-5′-TP into the enzyme was also determined after denaturation of the enzyme in 5 M urea followed by dialysis against 8 M urea for 30 h. The dialysate was then incubated at 37 °C for 2 h, followed by an overnight dialysis against 8 M urea. Incorporation was determined as described above.

Reduction of 8-BDB-TA-5′-TP-Modified Enzyme by [^3H]NaBH$_4$. Glutamate dehydrogenase was first modified with nonradioactive 8-BDB-TA-5′-TP. The modified enzyme, freed from excess reagent, was denatured in 6 M guanidine hydrochloride. To introduce a radioactive tag into the 8-BDB-TA-5′-TP–enzyme complex [^{3}H]NaBH$_4$ was used to reduce the dioxo groups of the enzyme-bound reagent. The reduction was carried out at 0 °C by two additions (1 h apart) of aliquots of 200 mM [^{3}H]NaBH$_4$ in 0.1 M NaOH, to yield 1.5 mM in the incubation mixture after each addition. The specific activity of the [^{3}H]NaBH$_4$ was (4–6) × 10^{12} cpm/mol containing four hydrogens. Samples of the protected and the unmodified enzyme were also prepared and treated with [^{3}H]NaBH$_4$ under the same conditions.

Titration of Sulfhydryl Groups with 5,5′-Dithiobis(2-nitrobenzoate). The sulfhydryl determination was conducted by adding aliquots of the unmodified and 8-BDB-TA-5′-TP-modified enzyme (0.2 mL of 1 mg/mL in 50 mM sodium phosphate buffer, pH 7.1) to 0.6 mL of 200 mM Tris-HCl buffer, pH 8.0, in a 1.0-mL cuvette. Sodium dodecyl sulfate [0.1 mL, 10% (w/v)] was used as the denaturant, and the absorbance of the solution was read at 412 nm against a blank identical with the test solution, except for the absence of protein. Freshly prepared 10 mM DTNB (0.1 mL) solution was added to each cuvette, and the A_{412nm} was measured. The concentration of free SH groups in each enzyme sample was calculated by using a molar extinction coefficient of 13 600 M^{-1} cm^{-1} at 412 nm (Ellman, 1958).

Proteolytic Digestion of [^3H]Carboxymethylated, 8-BDB-TA-5′-TP-Modified and Unmodified Glutamate Dehydrogenase. Carboxymethylation of the enzyme samples (prepared by using nonradioactive 8-BDB-TA-5′-TP and isolated as described above) was accomplished at pH 8.0 after denaturing the samples in 6 M urea. Iodo[^{3}H]acetate [specific radioactivity (2–4) × 10^{12} cpm/mol] was added to yield a final concentration of 50 mM. Incubation was carried out for 30 min at room temperature. The reaction was quenched by the addition of 0.5 M β-mercaptoethanol; incubation was continued for 10 min. After dialysis overnight against 20 mM ammonium bicarbonate, pH 8.0, the enzyme was digested at 37 °C by two successive additions of 1/20 (w/w) trypsin to glutamate dehydrogenase 1 h apart. The samples were desalted and dried by lyophilization. Digestion with thermolysin was conducted under the same conditions at 37 °C for 1 h with 1/25 (w/w), followed by a second addition of 1/100 (w/w) protease to glutamate dehydrogenase.

Separation of [^3H]Cm-cysteinyl Tryptic and Thermolysin Peptides by High-Performance Liquid Chromatography. The [^{3}H]Cm-cysteinyl tryptic peptides were separated on a Varian Model 5000 HPLC system equipped with a Vydac C_{18} reverse-phase column (0.46 × 25 cm). Each digest (about 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 by eluting at 1 mL/min with 0.1% trifluoroacetic acid, pH 2.0 (solvent A), from 0 to 10 min followed by a linear gradient from solvent A to 60% solvent B (0.07% trifluoroacetic acid in acetonitrile) over 120 min (elution system I). Fraction of 1 mL were collected. In the case of the thermolysin digest, elution was effected with a linear gradient from solvent A to 20% solvent B from 0 to 110 min (elution system II). In both cases, the effluents were continuously monitored at 220 nm and aliquots (50 μL from the eluted fractions) were assayed for radioactivity in 5 mL of Liquiscint scintillation cocktail (National Diagnostics) using a Packard Tricarb liquid scintillation counter, Model 3330.

The radioactive peaks of interest were further purified on a C_{18} column at pH 5.8. The radioactive pools were lyophilized and resuspended in 0.8 mL of 20 mM ammonium acetate, pH 5.8 (solvent C). The tryptic peptides 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 70% solvent D (20 mM ammonium acetate in 50% acetonitrile) from 10 to 150 min (elution system III). For the thermolysin peptides, purification was achieved by applying a linear gradient between solvent C and 20% solvent D over 90 min (elution system IV). In both cases, 220-nm absorbance was monitored and 100-μL aliquots of the 1-mL fractions were assayed for radioactivity.

Analysis of the Isolated [^3H]Cm-cysteinyl Peptides. The amino acid sequences of the purified peptides were determined by using an automated gas-phase protein/peptide sequence analyzer (Applied Biosystems Model 470A), equipped with an on-line PTH analyzer, Model 120, and computer, Model 900A. The amounts of isolated peptides loaded on the sequencer ranged from 300 to 6000 pmol. The picomoles of CmCys were calculated by using the PTH-Gln standard.

Results

Incorporation of 8-BDB-TA-5′-TP into Glutamate Dehydrogenase. In order to isolate the peptide modified by 8-BDB-TA-5′-TP, 2 mg/mL enzyme was incubated with the nucleotide analogue. Glutamate dehydrogenase is known to aggregate to an extent dependent on protein concentration and solvent conditions (Frieden, 1962; Fisher et al., 1965; Frieden & Colman, 1967). Since, in the previous study (Ozturk et al., 1990), reaction with 8-BDB-TA-5′-TP was conducted at a protein concentration of 0.5 mg/mL, the reagent incorporation was measured at a higher enzyme concentration (2.0 mg/mL) to evaluate the possibility that enzyme aggregation altered the extent of the reaction. The reagent incorporation determined from the moles of organic phosphate bound to the enzyme was 1.12 mol of reagent/mol of enzyme subunit after 3-h incubation with 8-BDB-TA-5′-TP when the functional change was complete. This result is similar to the value we obtained previously (Ozturk et al., 1990).

Reduction of 8-BDB-TA-5′-TP-Modified Glutamate Dehydrogenase with [^3H]NaBH$_4$. The dioxo groups of bromodioxobutyl-nucleotide-modified enzymes have been reduced by [^{3}H]NaBH$_4$, thereby introducing a radioactive tag into the modified enzyme (Batra & Colman, 1986b; DeCamp & Colman, 1989; Vollmer & Colman, 1990). Such reduction should produce an amount of tritium incorporated into the enzyme which is comparable to the reagent incorporation determined from the moles of organic phosphate bound to the enzyme. However, treatment of 8-BDB-TA-5′-TP-modified glutamate dehydrogenase with [^{3}H]NaBH$_4$ led to only about 0.025 mol of tritium incorporated/mol of subunit. This result suggested that the modification of the enzyme could be through the carbonyls, thus making them unavailable for [^{3}H]NaBH$_4$ reduction.

Incorporation of 8-[^{35}S]BDB-TA-5′-TP into Glutamate Dehydrogenase. Glutamate dehydrogenase modified by 8-[^{35}S]BDB-TA-5′-TP was tested for incorporation of the radioactive reagent, yielding about 1 mol of reagent/mol of subunit after a 3-h incubation period when the catalytically active modified enzyme was isolated in 0.1 M phosphate buffer, pH 8.0. During an attempt to isolate the peptide modified by 8-[^{35}S]BDB-TA-5′-TP, loss of the tethered radioactive reagent was observed. To minimize the possibility of time-dependent dissociation of the bound reagent, the period of incubation with the reagent was reduced to 2 h. Incorporation measured after the 2-h incubation period was 0.82 mol of reagent/mol of subunit. Following overnight dialysis against 0.1 M HEPES + 0.4 M KCl, pH 7.0, at 4 °C (conditions under which the enzyme retains its catalytic activity), the enzyme exhibited an incorporation of 0.79 mol of reagent/mol of subunit. These were the buffer conditions used for the measurement of organic phosphate incorporation previously (Ozturk et al., 1990). However, when the enzyme was denatured in urea and prepared as described under Experimental Procedures to simulate the conditions of proteolytic digestion but without the protease present, the incorporation was reduced to 0.15 mol of reagent/mol of subunit. This result indicates that the linkage of the reagent to glutamate dehydrogenase is stable when the enzyme is catalytically active but is labile when the enzyme is denatured.

Attempt To Isolate the Peptide Modified by 8-[^{35}S]BDB-TA-5′-TP. Attempts to isolate the peptide labeled by 8-[^{35}S]BDB-TA-5′-TP, either from a trypsin digest using elution system I or from a thermolysin digest using elution system II, failed to yield radioactive label clearly associated with glutamate dehydrogenase peptides. This observation, suggesting instability of the enzyme–reagent linkage, is consistent with the loss of radioactive reagent upon dialysis against 8 M urea.

During this work, a peptide was isolated which revealed an error in the amino acid sequence reported by Julliard and Smith (1979). The sequence of the peptide we isolated is as follows: Asn-Tyr-Thr-Asp-Asn-Glu-Leu-Glu-Lys. this peptide corresponds to that of residues 135–143 in the Julliard and Smith sequence except that residues 139 and 140 are Asn-Glu rather than Glu-Asp. The Asn^{139}-Glu^{140} agrees with the sequence obtained for human liver glutamate dehydrogenase cDNA (Nakatani et al., 1987; Amuro et al., 1988). Bovine and human liver glutamate dehydrogenases are about 95% homologous in their amino acid sequences.

Results of the DTNB Titrations and Isolation of Tryptic Peptides from Modified and Unmodified Glutamate Dehydrogenase. Reagents containing haloketones are known to react with cysteines in proteins (Hartman, 1977). Furthermore, 8-BDB-TA-5′-TP-modified bovine liver glutamate dehydrogenase exhibits striking similarities in kinetic characteristics to those of bovine liver glutamate dehydrogenase modified by 6-BDB-TA-5′-DP (Batra & Colman, 1984; Ozturk et al., 1990), and the reagent 6-BDB-TA-5′-DP has been shown to modify Cys-319 in glutamate dehydrogenase (Batra & Colman, 1986b). Because of these similarities, the possibility that 8-BDB-TA-5′-TP reacts with a cysteine was explored by quantifying the free cysteines in the enzyme by DTNB titration as described under Experimental Procedures. Unmodified enzyme exhibited 6.20 SH groups/enzyme subunit, while modified enzyme yielded 4.74 cysteines titrable by DTNB. This result suggested that the target of 8-BDB-TA-5′-TP is a cysteine in glutamate dehydrogenase.

This possibility was further investigated by carboxymethylating the modified and unmodified enzymes with iodo[^{3}H]acetic acid. It was reasoned that if 8-BDB-TA-5′-TP reacts with a cysteine, that residue would be protected from subsequent reaction with radioactive iodoacetic acid. Thus, reaction of 8-BDB-TA-5′-TP with a particular cysteine would be detected from the absence of a particular [^{3}H]carboxymethylated peptide from the characteristic pattern of unmodified enzyme. The carboxymethylated enzymes were digested by trypsin, and the digests were fractionated by HPLC using elution system I. The results, shown in Figure 1, illustrate the elution profile of the (carboxymethyl)cysteine-containing peptides from the unmodified (Figure 1A) and modified (Figure 1B) enzymes in terms of radioactivity. The unmodified enzyme exhibits similar amounts of radioactivity incorporated into each of peaks I–IV, while the last peak contains almost twice the amount of radioactivity; these results suggest that two (carboxymethyl)cysteines are present in this peak (designated as V + VI). In contrast, the modified enzyme yields similar amounts of radioactivity in all of its peaks (I to V + VI). Table I summarizes the amount of radioactivity in each of the peaks shown in Figure 1. These results indicate that the cysteine modified by 8-BDB-TA-5′-TP is in the last peak.

Comparable experiments were performed on the protected enzyme which exhibited a radioactive pattern close to that of

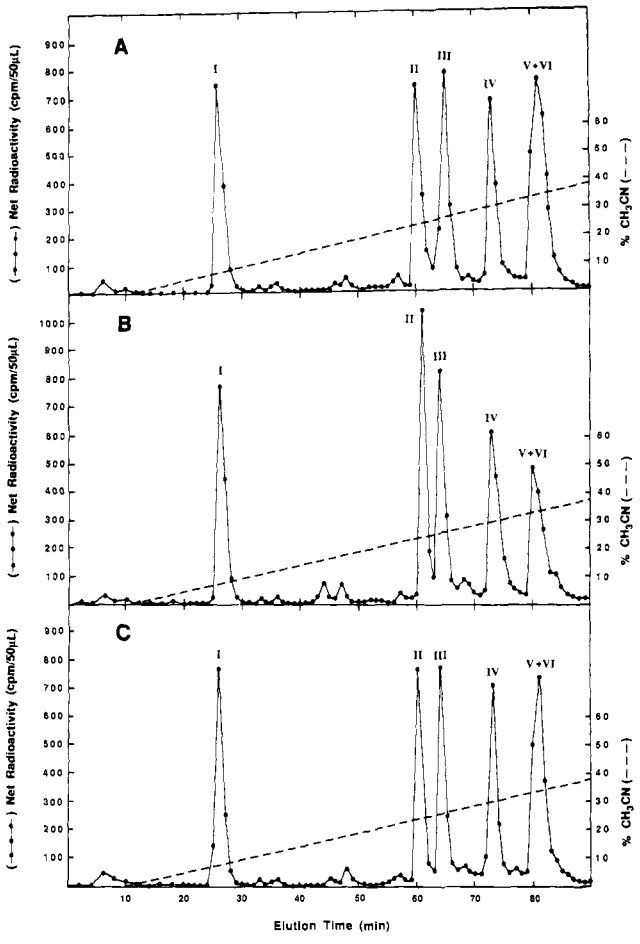


FIGURE 1: Separation of tryptic ([^{3}H]carboxymethyl)cysteinyl peptides by HPLC on a Vydac C_{18} column. The radioactive peptides were separated by elution with 0.1% TFA, pH 2.0, followed by a linear gradient of 0.07% TFA in acetonitrile as described under Experimental Procedures. A 50-μL aliquot of each fraction was assayed for radioactivity (●). The dashed line (– – –) indicates the elution gradient, and Roman numerals designate radioactive peaks containing ([^{3}H]carboxymethyl)cysteinyl peptides from a 2-mg trypsin digest each of unmodified (A), modified (B) and protected (C) enzyme samples.

Table I: Total Radioactivity Measured in CmCys-Containing Peaks of Unmodified, Modified, and Protected Enzyme Samples from Tryptic Digest after Separation on HPLC[a]

CmCys-containing peaks	unmodified enzyme[b] (total cpm)	modified enzyme[c] (total cpm)	protected enzyme[d] (total cpm)
I	1223	1316	1108
II	1236	1247	1043
III	1311	1246	1026
IV	1148	1243	1047
V + VI[e]	2295	1400	1843

[a] All enzyme samples were treated with 50 mM iodo[^{3}H]iodoacetate and digested with 10% (w/w) trypsin as described under Experimental Procedures. [b] The unmodified enzyme was incubated for 2 h at 25 °C in the absence of 8-BDB-TA-5′-TP and natural ligands. [c] The modified enzyme was incubated with 0.2 mM 8-BDB-TA-5′-TP in the absence of any natural ligands for 2 h at 25 °C. [d] The protected enzyme was prepared under the same conditions as the modified enzyme except for the addition of 5 mM NADH + 200 μM GTP in the incubation mixture. [e] This radioactive peak contains two CmCys, so it is designated as V + VI.

Table II: Representative Sequences of the Peptides Obtained from Peaks V and VI of Unmodified and Modified Enzymes from Tryptic Digest[a]

	unmodified enzyme		modified enzyme	
cycle	peak V[b] (pmol)	peak VI[c] (pmol)	peak V[b] (pmol)	peak VI[c] (pmol)
1	Ile (806)	Glu (131)	Ile (896)	Glu (110)
2	Tyr (991)	Met (169)	Tyr (1020)	Met (65)
3	Glu (687)	Ser (106)	Glu (936)	Ser (17)
4	Gly (655)	Trp (16)	Gly (967)	Trp (18)
5	Ser (647)	Ile (121)	Ser (711)	Ile (44)
6	Ile (533)	Ala (126)	Ile (603)	Ala (34)
7	Leu (612)	Asp (66)	Leu (689)	Asp (81)
8	Glu (379)	Thr (42)	Glu (453)	Thr (19)
9	Val (422)	Tyr (96)	Val (598)	Tyr (24)
10	Asp (311)	Ala (89)	Asp (313)	Ala (21)
11	CmCys^{319} (382)	Ser (54)	CmCys^{319} (496)	Ser (6)
12	Asp (270)	Thr (24)	Asp (301)	Thr (5)
13	Ile (266)	Ile (50)	Ile (287)	Ile (13)
14	Leu (303)	Gly (31)	Leu (269)	Gly (11)
15	Ile (242)	His (13)	Ile (234)	*e*
16	Pro (239)	Tyr (47)	Pro (199)	Tyr (9)
17	Ala (277)	Asp (30)	Ala (167)	Asp (24)
18	Ala[d] (305)	Ile (37)	Ala (113)	Ile (6)
19		Asn (32)	Ser (41)	Asn (4)
20		Ala (35)	Glu (31)	Ala (6)
21		His (11)	Lys (54)	*e*
22		Ala (31)		Ala (6)
23		CmCys^{197} (13)		CmCys^{197} (3)
24		Val[d] (8)		*e*

[a] The purification of peaks V + VI was achieved by HPLC, and the amino acid sequences for these peptides were obtained as explained under Experimental Procedures. These are representative sequences and were not taken from the same digests of the modified enzyme. Therefore, the amounts of the peptides from the several enzyme samples do not reflect the relative magnitude of the peaks shown in Figure 2. [b] Trypsin digestion of glutamate dehydrogenase gives a 21 amino acid fragment (residues 309–329) which contains Cys-319. This fragment is designated as peak V. [c] Peak VI represents a 37 amino acid tryptic peptide (residues 175–211) containing Cys-197. Low amounts of this peptide could be due to its low solubility in elution system III. [d] Sequencer was stopped at this cycle. [e] Amount too low to detect reliably.

the unmodified enzyme (Figure 1C; Table I); i.e., the amount of radioactivity present in the region of peak V + VI is almost twice as great as that of the other peaks. These data indicate that when the regulatory properties of the enzyme are maximally altered, as in modified enzyme, reaction with iodoacetate of one of the cysteines of peak V + VI is blocked; and conversely, when alteration of the regulatory properties is prevented, as in protected enzyme, both cysteines of peak V + VI are available to iodoacetate.

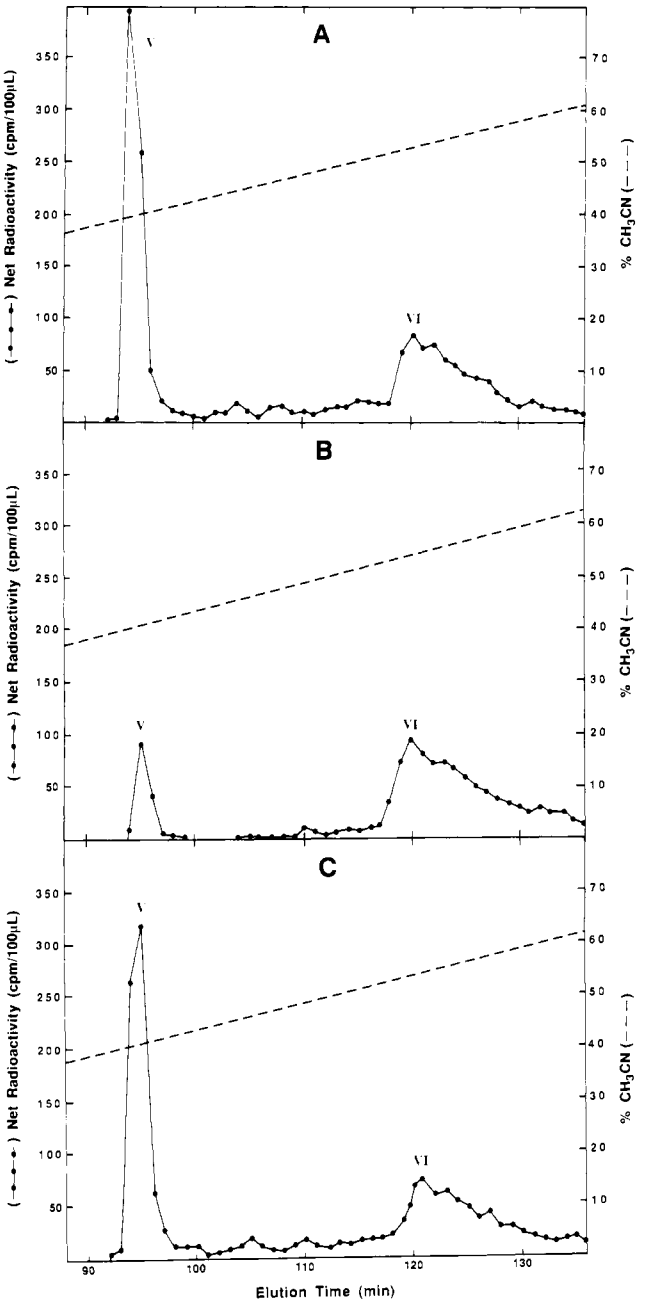


FIGURE 2: Isolation of ([^{3}H]carboxymethyl)cysteinyl peptides in peak V + VI (Figure 1). The purification was effected by HPLC on a Vydac C_{18} column using 20 mM ammonium acetate, pH 5.8, followed by a linear gradient (– – –) of 20 mM ammonium acetate in 50% acetonitrile as described under Experimental Procedures. Aliquots of 100 μL from each fraction were assayed for radioactivity (●). The letters designate the elution profile of radioactive (carboxymethyl)-cysteinyl peptides from peak V + VI of unmodified (A), modified (B) and protected (C) enzymes.

Identification of the Cysteinyl Peptide Modified by 8-BDB-TA-5′-TP from Tryptic Digest of Glutamate Dehydrogenase. Peak V + VI isolated on HPLC (Figure 1) of modified, unmodified, and protected enzyme samples was further purified by using elution system III. The elution profile, as monitored by radioactivity, exhibited two major peaks (Figure 2). The total radioactivity in peak V of the modified enzyme was drastically reduced to about 20% (Figure 2B) of that for unmodified and protected enzymes (Figure 2 panels A and C, respectively). Peak VI of each sample exhibited a similar amount of total radioactivity. Table II shows

Table III: Sequences from Thermolysin Digest of Iodo[^{3}H]acetate-Treated Unmodified Enzyme and 8-BDB-TA-5′-TP-Modified Enzyme

	unmodified enzyme[a]						
cycle	1 (pmol)	2 (pmol)	3A (pmol)	3B (pmol)	4 (pmol)	5 (pmol)	6 (pmol)
1	Val (1480)	Tyr (4670)	Ile (1000)	Ala (2550)	Ile (5390)	Phe (3890)	Ile (2090)
2	Asp (1760)	Lys (4360)	Lys (1220)	Gln (1770)	Asn (4160)	Gly (1370)	Ile (2310)
3	CmCys^{319} (982)	CmCys^{115} (1770)	Pro (834)	His (518)	Ala (6270)	Ala (1900)	Lys (2590)
4	Asp (490)		CmCys^{55} (901)	Ser (1470)	His (1070)	Lys (614)	Pro (1870)
5			Asn (811)	Gln (1120)	Ala (5820)	CmCys^{270} (201)	CmCys^{55} (1870)
6			His (21)	His (297)	CmCys^{197} (1890)		Asn (1430)
7				Arg (221)			His (193)
8				Thr (981)			
9				Pro (905)			
10				CmCys^{89} (849)			
11				Lys[c] (1080)			

	8-BDB-TA-5′-TP-modified enzyme[b]							
cycle	1 (pmol)	2 (pmol)	3A (pmol)	3B′[e] (pmol)	3B″ (pmol)	4 (pmol)	5 (pmol)	6 (pmol)
1	*d*	Tyr (2860)	Ile (896)	His (417)	Ala (253)	Ile (678)	Phe (526)	Ile (1950)
2		Lys (3200)	Lys (1280)	Ser (134)	Gln (331)	Asn (286)	Gly (115)	Ile (1300)
3		CmCys^{115} (1130)	Pro (703)	Gln (207)	His (270)	Ala (346)	Ala (93)	Lys (1210)
4			CmCys^{55} (972)	His (179)	Ser (131)	His (142)	Lys (37)	Pro (969)
5			Asn (954)	Arg (197)	Gln (178)	Ala (144)	CmCys^{270} (22)	CmCys^{55} (914)
6			His (58)	Thr (38)	His (96)	CmCys^{197} (39)		Asn (1060)
7				Pro (50)	Arg (111)			His (141)
8				CmCys^{89} (29)	Thr (41)			
9				Lys (11)	Pro (41)			
10				Gly (11)	CmCys^{89} (22)			
11				Gly (8)	Lys (10)			
12					Gly (10)			
13					Gly (8)			

[a] Glutamate dehydrogenase (2 mg/mL) was reacted with 50 mM iodo[^{3}H]acetate in the presence of 5 M urea at room temperature. Thermolysin-digested enzyme was subjected to HPLC (Vydac C_{18} column), and the (carboxymethyl)cysteine peptides were isolated by using elution system III (in Table III, numbers designate the peaks isolated in this system). Radioactive peaks were further purified in elution system IV (designated with capital letters in Table III). Representative sequences obtained from (carboxymethyl)cysteine-containing peptides after gas-phase sequencing are shown above. [b] Enzyme containing about 0.80 mol of 8-BDB-TA-5′-TP/mol of subunit was treated in the same manner as unmodified enzyme. Representative sequences of all (carboxymethyl)cysteine-containing peptides observed are tabulated in Table III. [c] Sequencer was stopped at this cycle. [d] No (carboxymethyl)cysteine peptide containing CmCys-319 was observed in 8-BDB-TA-5′-TP-modified enzyme. [e] Peptide 3B′ is a shorter form of peptide 3B″ due to additional cleavage by thermolysin. This was not observed in unmodified enzyme.

the sequences of representative samples (not the same amounts) of peak V and peak VI. The sequence of peak V in each case was found to contain Cys-319, while that of peak VI contained Cys-197 (Table II). Comparison of these sequences with the known amino acid sequence of glutamate dehydrogenase revealed that trypsin digestion would give a 21 amino acid peptide for peak V and a 37 amino acid peptide for peak VI. The theoretical p*I* for peak VI is 6.3, close to the pH of the elution system III (pH 5.8) which, together with the large size of the peak VI peptide, is probably responsible for the broad radioactivity distribution observed in this peak (Figure 2).

The incorporation of some radioactivity into peak V of the modified enzyme (Figure 2B) can be explained by the incorporation of 8-[^{35}S]BDB-TA-5′-TP into the enzyme: about 0.80 mol of reagent was incorporated/mol of subunit enzyme after 2 h of incubation, leaving about 0.2 mol of free cysteine available for the reaction with iodo[^{3}H]acetic acid. This measurement is consistent with the observation that the radioactivity in peak V of the modified enzyme is 20% of the radioactivity in peak V of the unmodified or protected enzymes.

Identification of the Modified Cysteinyl Peptide from Thermolysin Digests. The size of the peptide in peak VI and its apparent low solubility at pH 5.8 prompted us to try an alternative digestion procedure to circumvent this problem. Thermolysin-digested enzyme samples were subjected to HPLC as described under Experimental Procedures, and all the radioactive carboxymethylated cysteine peaks from both modified and unmodified enzymes were subjected to gas-phase sequencing. Table III summarizes peptide sequences from all the radioactive peaks obtained from HPLC for both unmodified and modified enzymes after thermolysin digestion. In Table III, numbers designate the radioactive peaks separated in elution system III. Further resolution of some of the numbered peaks in elution system IV led to distinct peaks, designated by letters.

Due to the broad specificity of thermolysin, different cleavage patterns were observed in the peptidase sequenced. Peaks 3A and 6 both contain CmCys-55 as alternative cleavage products of the same peptide. This was evident in both unmodified and modified enzymes. In unmodified enzyme, peaks 3A and 3B both came from the same peak in elution system III, from which peptides containing two distinct (carboxymethyl)cysteines (Cys-55 and Cys-89) were separated by using elution system IV. Similar results were obtained with peak 3 of the modified enzyme. In this case, two different cleavage products related to peptide 3B (containing Cys-89) were observed, and these were designated as 3B′ and 3B″ (Table III). All six cysteines were detected in unmodified enzyme, as expected. In contrast, modified enzyme exhibits only five of the Cm-cysteines: peak 1 (observed in unmodified enzyme) which contained CmCys-319 was missing. This analysis confirms the results obtained from the tryptic digest and indicates that the target of 8-BDB-TA-5′-TP in bovine liver glutamate dehydrogenase is Cys-319.

Kinetics of Glutamate Dehydrogenase Measured with the Hydrolyzed 8-BDB-TA-5′-TP. The lability of the linkage between the reagent and the enzyme, as well as the low incorporation of tritium upon [^{3}H]$NaBH_4$ reduction of the modified enzyme, raised the possibility of a reaction involving the carbonyl groups of the reagent. To check this possibility, enzyme incubated with the hydrolyzed reagent was assayed for activity. The hydrolyzed reagent is the corresponding

alcohol of 8-BDB-TA-5′-TP formed upon displacement of the bromide by hydroxide when the reagent is incubated in 0.1 M phosphate buffer, pH 7.1. It was reported earlier that 8-BDB-TA-5′-TP desensitizes glutamate dehydrogenase to inhibition by GTP (Ozturk et al., 1990). The rate constant for reaction of 0.2 mM 8-BDB-TA-5′-TP, as measured by this desensitization effect in assays containing 1 μM GTP, was found to be about 29.0×10^{-3} min^{-1} with a 3-fold increase in activity as measured in the presence of the inhibitor GTP. In the case of hydrolyzed compound, the reaction rate is much slower. Although a 3-fold change in activity in the presence of 1 μM GTP was also produced by the hydrolyzing reagent, the rate constant was only 5.3×10^{-3} min^{-1}. These results suggest that reaction of 8-BDB-TA-5′-TP occurs through the carbonyls and that the presence of bromide α to one carbonyl enhances the polarization of the carbonyl groups, thereby facilitating nucleophilic attack. The characteristics of this reaction are consistent with attack by Cys-319 to form a thiohemiacetal derivative of 8-BDB-TA-5′-TP.

Discussion

In our previous study, evidence was presented that the nucleotide analogue 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-triphosphate reacts with bovine liver glutamate dehydrogenase to the extent of about 1 mol/mol of subunit at the GTP-dependent inhibitory NADH site (Ozturk et al., 1990). The enzyme modified by 8-BDB-TA-5′-TP shows desensitization to inhibition by GTP, as well as to inhibition by relatively high concentrations of NADH and less extensive activation by ADP. These changes in the allosteric properties of the enzyme are prevented when the incubation of 8-BDB-TA-5′-TP with glutamate dehydrogenase is conducted in the presence of 200 μM GTP plus 5 mM NADH; in contrast, ADP does not prevent the changes in the enzyme's allosteric properties caused by 8-BDB-TA-5′-TP. These results were interpreted as indicating covalent reaction of 8-BDB-TA-5′-TP in the region of the GTP-dependent NADH inhibitory site (Ozturk et al., 1990).

In this paper, we have shown that 8-BDB-TA-5′-TP reacts with a cysteine in bovine liver glutamate dehydrogenase. Radioactive iodoacetate was used to carboxymethylate all free cysteines in either unmodified or 8-BDB-TA-5′-TP-modified glutamate dehydrogenase. Analysis of tryptic digests revealed a 21 amino acid peptide (from Ile-309 to Lys-329 and containing CmCys-319) as the only peptide exhibiting drastically reduced radioactivity in the 8-BDB-TA-5′-TP-modified enzyme as compared to the control enzyme. In an alternative approach in which the protease was thermolysin, all (carboxymethyl)cysteine-containing peptides were sequenced from both unmodified and modified enzymes: the only (carboxymethyl)cysteine peptide that was present in the unmodified enzyme and absent in 8-BDB-TA-5′-TP-modified enzyme was the tetrapeptide Val-317–Asp-320 containing CmCys-319. Affinity labeling of glutamate dehydrogenase by 8-BDB-TA-5′-TP apparently prevents subsequent formation of radioactive CmCys-319. These results indicate that the target amino acid of 8-BDB-TA-5′-TP in bovine liver glutamate dehydrogenase is Cys-319.

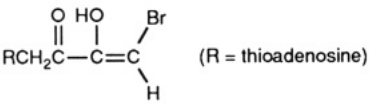
In earlier studies, the involvement of cysteine in regulation of glutamate dehydrogenase was described: the reaction of methylmercuric iodide with a cysteine in bovine liver glutamate dehydrogenase decreases the response of the enzyme to ADP activation, but the particular cysteine involved was never identified (Nishida & Yielding, 1970). Later, it was reported that the reaction of the enzyme with *p*-mercuribenzoate alters the regulatory behavior of the enzyme associated with the allosteric activator ADP. The reaction of *p*-mercuribenzoate was reported to be at Cys-319 (Cosson et al., 1976; Cosson & Pantaloni, 1976; Cosson et al., 1978). However, in none of these studies was an evaluation made of the ability of natural ligands to protect against modification of the enzyme by mercurials, nor was a test made of the ability of the enzyme to be inhibited by NADH.

More recently, Batra and Colman (1984) studied the reaction of 6-BDB-TA-5′-DP with bovine liver glutamate dehydrogenase and presented evidence for reaction of Cys-319 with 6-BDB-TA-5′-DP in the GTP-dependent NADH inhibitory site of this enzyme (Batra & Colman, 1986b). Reaction of glutamate dehydrogenase with either 6-BDB-TA-5′-DP (Batra & Colman, 1984) or with 8-BDB-TA-5′-TP (Ozturk et al., 1990) causes qualitatively similar decreases in sensitivity to GTP inhibition and to NADH inhibition (although these effects are not quantitatively identical). Furthermore, the combined addition of both GTP and NADH prevents reaction of bovine liver glutamate dehydrogenase with either 6-BDB-TA-5′-DP or 8-BDB-TA-5′-TP, suggesting these two affinity labels react at a similar site in this enzyme.

Here, we have presented evidence that 8-BDB-TA-5′-TP also labels Cys-319 in bovine liver glutamate dehydrogenase; however, the chemical characteristics of the nucleotidyl-enzyme products differ markedly for the two reagents. In the case of the 6-BDB-TA-5′-DP-modified enzyme, [^{3}H]$NaBH_4$ was used successfully to reduce the carbonyl groups of the enzyme-bound reagent and the tritium of the reduced carbonyls allowed monitoring of the modified peptide from proteolytic digests (Batra & Colman, 1986b). In contrast, we have now demonstrated that essentially no tritium is incorporated into the 8-BDB-TA-5′-TP-modified enzyme, indicating that the carbonyls are not available for reduction. Furthermore, experiments with 8-[^{35}S]BDB-TA-5′-TP suggested that the reaction between the enzyme and reagent was reversible under denaturing conditions. The most reasonable interpretation seems to be that Cys-319 of the enzyme reacts with 6-BDB-TA-5′-DP by nucleophilic attack on the methylene carbon with displacement of the bromide from the analogue, whereas the same Cys-319 reacts with 8-BDB-TA-5′-TP through a carbonyl group on the functional bromodioxobutyl moiety to form a thiohemiacetal.

In the literature, there are various examples of compounds with a dicarbonyl functional group which can react with a thiol. For example, glyoxylate thiohemiacetals are known to form readily and indeed serve as substrates for the peroxisomal enzymes, D-amino acid oxidase and L-hydroxy acid oxidase (Gunshore et al., 1985; Law & Hamilton, 1986). It has been reported that oxalyl thioesters react with nucleophiles such as aminothiols and that in such reactions the dicarbonyl moiety later is transferred to another nucleophile with a measurable rate (Law & Hamilton, 1986; Al-Arab & Hamilton, 1987). The precedent could explain our inability to clearly associate the radioactive label with 8-[^{35}S]BDB-TA-5′-TP-modified glutamate dehydrogenase peptides after trypsin or thermolysin digestion. The multiple ^{35}S peaks we observed in these attempts could be due to migration of the nucleotidyl label to a number of nearby nucleophilic amino acids. The equilibrium formation of thiohemiacetals from phenylglyoxal and glutathione or from acetaldehyde and ethanethiol (Creighton & Pourmotabbed, 1988) may be analogous to the mode of reaction of Cys-319 with the dioxo moiety of 8-BDB-TA-5′-TP. The reversible nature of such a reaction could explain the limited stability of the 8-BDB-TA-5′-TP linkage with the enzyme and the decreased reagent incorporation measured

after denaturation of the enzyme. It is possible that the native conformation of 8-BDB-TA-5′-TP-modified enzyme shields the site of modification from solvent and stabilizes the enzyme–8-BDB-TA-5′-TP adduct, thereby making feasible the isolation of enzyme with a stoichiometric incorporation of reagent. In contrast, denaturation may expose the modification site to solvent, rendering the adduct formation freely reversible and resulting in the release of the reagent. Reaction of the enzyme with a carbonyl group of 8-BDB-TA-5′-TP is also consistent with the observation that the hydrolyzed reagent (i.e., with Br^- lost) still reacts with bovine liver glutamate dehydrogenase although at a much slower rate. Furthermore, it is known that the adenosine derivatives with the dioxobutyl functional moiety can exist in the enol form in aqueous solution (DeCamp & Colman, 1989; Bailey & Colman, 1985; Kapetanovic et al., 1985):



This property could account for the observation that virtually no tritium was incorporated into the 8-BDB-TA-5′-TP-modified enzyme upon [^{3}H]$NaBH_4$ reduction: if a thiohemiacetal is formed from the remaining carbonyl, there would be no carbonyl available for reduction.

The structures of 6-BDB-TA-5′-DP and 8-BDB-TA-5′-TP are compared in Figure 3 in an attempt to explain the different mode of reaction of Cys-319 of glutamate dehydrogenase with these two reagents. Nucleotides with bulky substituents at the 8-position of the purine ring are known to exist in solution predominantly in the syn conformation (Czarnecki et al., 1979; Saenger, 1984; King et al., 1989). It is possible that 6-BDB-TA-5′-DP and 8-BDB-TA-5′-TP bind similarly so that the purine, ribose, and phosphate moieties occupy the same site of the enzyme. Although various positions of the bromodioxobutyl side chain are possible, Figure 3 shows that it is energetically permissible to arrange these compounds so that the Cys-319 at a fixed position would attack the methylene bromide of 6-BDB-TA-5′-DP, but the carbonyl of 8-BDB-TA-5′-TP.

The importance of the nucleotide structure of 8-BDB-TA-5′-TP in directing reaction with Cys-319 should be emphasized. Hucho et al. (1975) have shown that when glutamate dehydrogenase is maintained in phosphate buffer (as in the present study), none of the cysteinyl sulfhydryl groups is reactive, as tested by modification by iodoacetate. In Tris buffer, the cysteines do react with iodoacetate, but the order of reactivity is Cys-89 > Cys-55 > Cys-197 > Cys-115, Cys-270 > Cys-319 (Hucho et al., 1975). Clearly, the binding of the nucleotidyl affinity label 8-BDB-TA-5′-TP to glutamate dehydrogenase must be responsible for specific modification of Cys-319, which is inherently the least reactive of the enzyme's cysteines.

This family of nucleotide analogues with a bromodioxobutyl functional moiety tethered to distinct positions of the purine ring (2-, 6-, and 8-BDB-nucleotides) has yielded information about several amino acids located at specific functional sites of glutamate dehydrogenase as well as of other enzymes. 2-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 5′-monophosphate was shown to modify His-82 at an ADP activating site in bovine liver glutamate dehydrogenase (Batra et al., 1989), while 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 2′,5′-bisphosphate modifies Cys-283 in the coenzyme site of NADP-specific glutamate dehydrogenase of *Salmonella typhimurium* (Haeffner-Gormley et al., 1990) and 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate also react at Cys-283 at the same site in this bacterial enzyme (Haeffner-Gormley et al., 1991). For rabbit muscle pyruvate kinase, Tyr-147 in the active site is labeled when the reactive substituent is in the 2-position of the purine, as in 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 5′-diphosphate (DeCamp & Colman, 1989); however, with the dioxobutyl moiety in the 8-position, as in 8-BDB-TA-5′-TP, Cys-151 is modified in the active site of the same enzyme (Vollmer & Colman, 1990). For human platelet cAMP phosphodiesterase, 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 3′,5′-cyclic monophosphate acts reversibly as a competitive inhibitor, while 8-[(4-bromo-2,3-dioxobutyl)thio]-adenosine 3′,5′-cyclic monophosphate functions as an irreversible affinity label (Grant et al., 1990). The previous cases in which peptide–reagent products were isolated have been considered to represent reaction through the methylene group of the analogues. The work we have presented here provides the first evidence for involvement of a carbonyl group in a reaction of a member

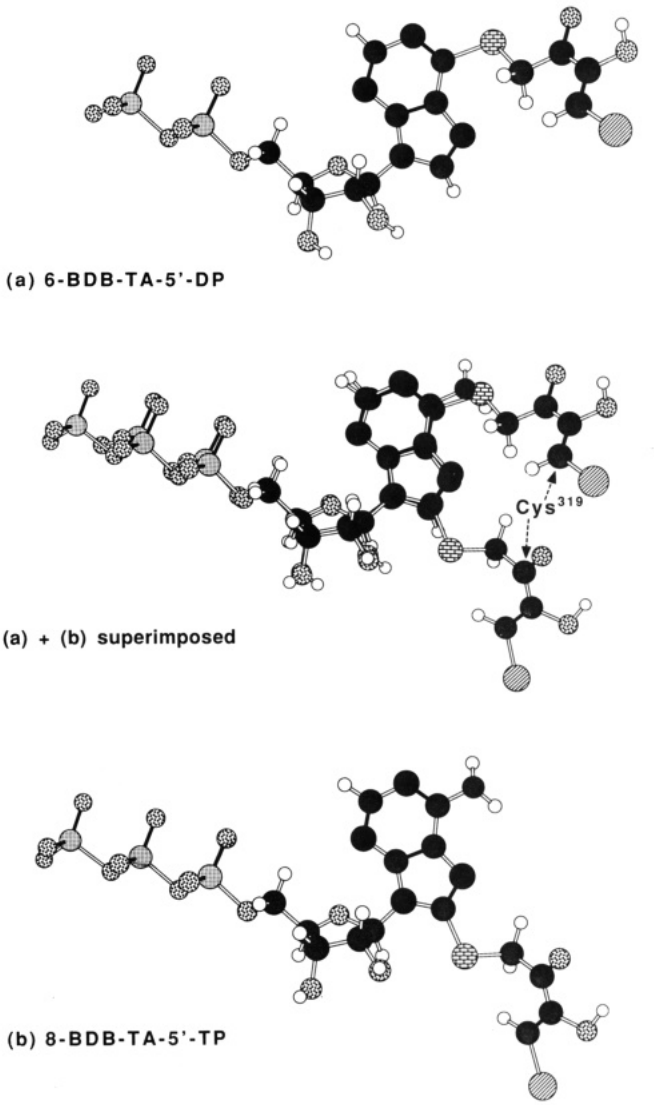


FIGURE 3: Structures of the syn conformation of the enol forms of 6-BDB-TA-5′-DP (a) and 8-BDB-TA-5′-TP (b). In the middle, the two nucleotide structures are superimposed so that the purine, ribose, and phosphates coincide (a + b). The superimposed model indicates how the sulfhydryl group of Cys-319, located at a fixed position, could react at the methylene group of 6-BDB-TA-5′-DP by displacement of bromide or at the carbonyl group of 8-BDB-TA-5′-TP. Molecular modeling was conducted by using the program Chem 3D (Cambridge Scientific Corp.).

of this class of affinity labels. It would seem advisable in evaluating affinity labeling experiments with other enzymes to consider the possibility of reaction at either the carbonyl or methylene groups of bromodioxobutyl-nucleotidyl derivatives.

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