Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463.
Shapiro, M. B., & Senepathy, P. (1987) *Nucleic Acids Res. 15*, 7155.
Shen, N. L. L., & Edgington, T. S. (1991) *Arterioscler. Thromb. 11*, 1543a (Abstract).
Shen, N. L. L., Altieri, D. C., & Edgington, T. S. (1990) *FASEB J. 4*, A2275 (Abstract).
Smith, M. W. (1988) *J. Mol. Evol. 27*, 45.
Southern, E. M. (1975) *J. Mol. Biol. 98*, 503.
Stubbs, J. D., Lekutis, C., Singer, K. L., Bui, A., Yuzuki, D., Srinivasan, U., & Parry, G. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8417.
Toole, J. J., Knopf, J. L., Wozney, J. M., Sultzman, L. A., Buecker, J. L., Pittman, D. D., Kaufman, R. J., Brown, E., Shoemaker, C., Orr, E. C., Amphlett, G. W., Foster, W. B., Coe, M. L., Knutson, G. J., Fass, D. N., & Hewick, R. M. (1984) *Nature 312*, 342.
Toole, J. J., Pittman, D. D., Orr,E. C., Murtha, P., Wasley, L. C., & Kaufman, R. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5939.
Toribara, N. W., Gum, J. R., Culhane, P. J., Lagace, R. E., Hicks, J. W., Petersen, G. M., & Kim, Y. S. (1991) *J. Clin. Invest. 88*, 1005.
Tracy, P. B., Eide, L. L., Bowie, E. J., & Mann, K. G. (1982) *Blood 60*, 59.
Vehar, G. A., Keyt, B., Eaton, D. L., Rodriguez, H., O'Brien, D. P., Rotblat, F., Oppermann, H., Keck, R., Wood, W. I., Harkins, R. N., Tuddenham, E. G. D., Lawn, R. M., & Capon, D. J. (1984) *Nature 312*, 337.
Wang, H., Riddell, D. C., Guinto, E. R., & MacGillivray, R. T. A. (1988) *Genomics 2*, 234.
Watson, M. L., Kingsmore, S. F., Johnston, G. I., Siegelman, M. H., Le Beau, M. M., Lemons, R. S., Bora, N. S., Howard, T. A., Weissman, I. L., McEver, R. P., & Seldin, M. F. (1990) *J. Exp. Med. 172*, 263.
Wilson, D. B., Salem, H. H., Mruk, J. S., Maruyama, I., & Majerus, P. W. (1984) *J. Clin. Invest. 73*, 654.

Identification of Amino Acids Modified by the Bifunctional Affinity Label 5′-(*p*-Fluorosulfonyl)benzoyl)-8-azidoadenosine in the Reduced Coenzyme Regulatory Site of Bovine Liver Glutamate Dehydrogenase†

Kenneth E. Dombrowski,‡ Yu-Chu Huang, and Roberta F. Colman*
Department of Chemistry and Biochemistry, University of Delaware, Newark, Delaware 19716
Received December 24, 1991

ABSTRACT: Bovine liver glutamate dehydrogenase reacts with the bifunctional affinity label 5′-(*p*-(fluorosulfonyl)benzoyl)-8-azidoadenosine (5′-FSBAzA) in a two-step process: a dark reaction yielding about 0.5 mol of -SBAzA/mol of subunit by reaction through the fluorosulfonyl moiety, followed by photoactivation of the azido group whereby covalently bound -SBAzA becomes cross-linked to the enzyme [Dombrowski, K. E., & Colman, R. F. (1989) *Arch. Biochem. Biophys. 275*, 302–308]. We now report that the rate constant for the dark reaction is not reduced by ADP or GTP, but it is decreased 7-fold by 2 mM NADH and 40-fold by 2 mM NADH + 0.2 mM GTP, suggesting that 5′-FSBAzA reacts at the GTP-dependent NADH inhibitory site. The amino acid residues modified in each phase of the reaction have been identified. Modified enzyme was isolated after each reaction phase, carboxymethylated, and digested with trypsin, chymotrypsin, or thermolysin. The digests were fractionated by chromatography on a phenylboronate agarose column followed by HPLC. Gas-phase sequencing of the labeled peptides identified Tyr^{190} as the major amino acid which reacts with the fluorosulfonyl group; Lys^{143} was also modified but to a lesser extent. The predominant cross-link formed during photolysis is between modified Tyr^{190} and the peptide Leu^{475}-Asp^{476}-Leu^{477}-Arg^{478}, which is located near the C-terminus of the enzyme. Thus, 5′-FSBAzA is effective in identifying critical residues distant in the linear sequence, but close within the regulatory nucleotide site of glutamate dehydrogenase.

Bovine liver glutamate dehydrogenase [L-glutamate:NAD(P)$^+$ oxidoreductase (deaminating), EC 1.4.1.3] is an allosteric enzyme that is activated by ADP, but inhibited by GTP and high concentrations of NADH. The enzyme in its smallest, active form is a hexamer of six identical subunits (Goldin & Frieden, 1972; Julliard & Smith, 1979). For each subunit there are six binding sites for catalytic and regulatory purine nucleotides: one catalytic site that binds either NAD(H) or NAD(P)H (Goldin & Frieden, 1972), one additional coenzyme site which is regulatory (Krause et al., 1974), two ADP sites (Batra & Colman, 1986a), and two GTP binding sites as measured in the presence of NADH (one of high affinity and one of low affinity) (Pal & Colman, 1979).

Considerable information regarding the amino acid residues within the catalytic and regulatory sites of bovine liver glutatmate dehydrogenase has come from affinity labeling ex-

† This work was supported by USPHS Grant DK 37000 (to R.F.C.), NSF Grant DMB-9105116 (to R.F.C.), and NIH Postdoctoral Fellowship GM12936 (to K.E.D.).
‡ Present address: Department of Internal Medicine, Texas Tech University Health Sciences Center, 1400 Wallace Blvd., Amarillo, TX 79124.

0006-2960/92/0431-3785$03.00/0

periments [for a review, see Colman (1991)]. For example, 5′-(*p*-(fluorosulfonyl)benzoyl)adenosine (5′-FSBA),[1] a compound which can be considered an analogue of NADH, has been used successfully in the identification of an NADH inhibitory site. The compound is related to the natural cofactor by having both the adenine and the ribose moieties and a carbonyl group adjacent to the 5′-position which is structurally similar to the first phosphoryl group of natural purine nucleotides. In addition, if the molecule is arranged in an extended conformation, the fluorosulfonyl group can be located in a position equivalent to that of the ribose proximal to the nicotinamide of NADH. The sulfonylfluoride can act as an electrophile in covalent reactions with several amino acids. 5′-FSBA reacted with glutamate dehydrogenase yielding an incorporation of up to 1 mol of reagent/mol of subunit (Pal et al., 1975; Saradambal et al., 1979), although all functional changes in the enzyme were complete when only 0.5 mol of reagent were incorporated per average subunit. Strikingly, enzyme modified with the (sulfonylbenzoyl)adenosine group exhibited complete loss of the allosteric inhibition by high concentrations of NADH but retained full catalytic activity (Pal et al., 1975). The modified enzyme was still inhibited by GTP and activated by ADP. Additionally, the pseudo-first-order rate constant for modification of the enzyme was drastically reduced when the reaction was performed in the presence of high NADH concentrations, and the modification rate was decreased to zero in the presence of high GTP and NADH concentrations (Pal et al., 1975; Saradambal et al., 1979). The loss of NADH inhibition and protection against modification by that ligand strongly implicated the NADH inhibitory site as the site of reaction with 5′-FSBA. Further work indicated that two residues, Tyr^{190} and Lys^{420}, were specifically labeled in equal amounts by 5′-FSBA (Schmidt & Colman, 1984).

More recently, the new bifunctional affinity label 5′-(*p*-fluorosulfonyl)benzoyl)-8-azidoadenosine (5′-FSBAzA) was shown to react with bovine liver glutamate dehydrogenase (Dombrowski & Colman, 1989). This compound contains both an electrophilic (fluorosulfonyl)benzoyl group and a photoactivatable azido group. 5′-FSBAzA reacts with glutamate dehydrogenase in a two-step process: a dark reaction through the fluorosulfonyl moiety, followed by photoactivation of the azido group in which covalently bound -SBAzA becomes cross-linked to the enzyme. The affinity label is incorporated to a level of 0.5 mol of -SBAzA/mol of enzyme subunit through the fluorosulfonyl group. UV activation of the azido group of the tethered molecule yields a minimum efficiency of photoincorporation of 25%. 5′-FSBAzA-modified glutamate dehydrogenase, both before and after photolysis, retains full catalytic activity but is less sensitive to allosteric inhibition by GTP, to activation by ADP, and to inhibition by 1 mM NADH.

This paper describes the identification of residues in the regulatory nucleotide binding site which are modified by reaction with 5′-FSBAzA. Photoactivation of the covalently bound reagent allows the identification of amino acids distant in the amino acid sequence but close to each other in the purine nucleotide binding site of glutamate dehydrogenase. A preliminary version of this study has been presented (Dombrowski et al., 1991).

[1] Abbreviations: 5′-FSBA, 5′-(*p*-(fluorosulfonyl)benzoyl)adenosine; 5′-FSBAzA, 5′-(*p*-(fluorosulfonyl)benzoyl)-8-azidoadenosine; -SBAzA, (sulfonylbenzoyl)-8-azidoadenosine; DTT, dithiothreitol; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; PBA, phenylboronate agarose; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin.

Materials and Methods

Materials. Bovine liver glutamate dehydrogenase was purchased as an ammonium sulfate suspension from Boehringer Mannheim Corp. and was dialyzed against two changes of 0.1 M potassium phosphate buffer (pH 7.1) at 4 °C. The dialysate was centrifuged at 4 °C for 20 min at 15 000 rpm, and the protein concentration of the supernatant was determined by measuring the absorbance at 280 nm and using $E^{1\%}_{280nm} = 9.7$ (Olson & Anfinsen, 1951); the A_{280nm}:A_{260nm} ratio was 1.9. A molecular weight of 56 100 was used to calculate the subunit concentration (Goldfin & Freiden, 1972). The dialyzed enzyme was stored in aliquots at −75 °C and thawed immediately prior to use.

Nucleotides, coenzymes, α-ketoglutarate, 8-azidoadenosine, *p*-(fluorosulfonyl)benzoyl chloride, thermolysin, TPCK-treated trypsin, chymotrypsin, and dithiothreitol were purchased from Sigma. Phenylboronate agarose-30 was purchased from Amicon. [2-^{3}H]Adenosine, Protosol, and Econofluor were purchased from New England Nuclear. All other chemicals were of reagent or HPLC grade purity.

Synthesis of 5′-FSBAzA. 5′-FSBAzA was prepared by reaction of 8-azidoadenosine with *p*-(fluorosulfonyl)benzoyl chloride and purified by silica gel chromatography as described previously (Dombrowski & Colman, 1989). [2-^{3}H]-5′-FSBAzA was prepared from [2-^{3}H]adenosine in a three-step procedure: bromination of [2-^{3}H]adenosine by a modification of the method of Ikehara and Kaneko (1970) to give [2-^{3}H]-8-bromoadenosine, reaction of [2-^{3}H]-8-bromoadenosine with sodium azide as described by Holmes and Robins (1965), and coupling of [2-^{3}H]-8-azidoadenosine with *p*-(fluorosulfonyl)benzoyl chloride as described previously (Dombrowski & Colman, 1989).

In a typical preparation, [2-^{3}H]adenosine (5 mCi in 5 mL of 70% ethanol) was diluted with nonradioactive adenosine (3.1 mmol) and dissolved in 15 mL of sodium acetate, pH 4. To the solution was added 350 μL of Br_2, and the mixture was stirred for 2–3 h. The reaction was quenched by the addition of $NaHSO_3$ with stirring. The solution was adjusted to pH 7 with 50% NaOH and stirred for 1 h at 4 °C. [2-^{3}H]-8-Bromoadenosine was collected by filtration, after which the solid was washed with water and acetone and air dried.

[2-^{3}H]-8-Azidoadenosine was prepared by incubation of [2-^{3}H]-8-bromoadenosine (0.78 mmol) with NaN_3 (1.8 mmol) in 5 mL of DMSO in a sealed tube at 75 °C for 16–20 h. [2-^{3}H]-8-Azidoadenosine was precipitated by its dropwise addition into 250 mL of CH_2Cl_2 at 4 °C with vigorous stirring. The suspension was stirred for 3 h, and the product was collected by filtration, washed with water and then acetone and air dried. [2-^{3}H]-5′-FSBAzA was prepared from 8-azidoadenosine as described previously (Dombrowski & Colman, 1989). The specific radioactivity of various preparations of [2-^{3}H]-5′-FSBAzA was 4.5×10^{11} to 1.5×10^{12} cpm/mol.

Enzyme Assay. Bovine liver glutamate dehydrogenase activity was determined by measuring the oxidation of NADH as the reduced coenzyme using a Cary 219 spectrophotometer with a full-scale absorbance of 0.1 to monitor the reaction. The standard assay contained Tris-0.01 M acetate buffer (pH 8.0), 10 μM EDTA, 50 mM NH_4Cl, 5 mM α-ketoglutarate, 55 μM NADH, and 3 μg of enzyme in a final volume of 1 mL. The reaction was monitored at 340 nm ($\epsilon = 6.22 \times 10^3$ M^{-1} cm^{-1}).

Reaction of 5′-FSBAzA with Glutamate Dehydrogenase. Glutamate dehydrogenase (2 mg/mL) was incubated with 0.3 mM 5′-FSBAzA (dissolved in ethanol) at 30 °C in a 10 mM

sodium barbital buffer, pH 8.0, containing 0.4 mM KCl. The final ethanol concentration was maintained at 10%. Other ligands were added as specified in the Results. Following the incubation, unreacted 5′-FSBAzA was removed from the modified enzyme by gel filtration centrifuge columns (Penefsky, 1977). The columns were prepared in 5-mL disposable syringes containing Sephadex G-50-80 equilibrated with 0.1 M potassium phosphate buffer, pH 7.1. Enzyme that was incubated under similar conditions except for the absence of 5′-FSBAzA was found to be stable. The average incorporation of the affinity label was 0.47 mol of SBAzA/mol of enzyme subunit.

Photolysis of SBAzA-Modified Glutamate Dehydrogenase. SBAzA-modified glutamate dehydrogenase was photolyzed with a 60-W Spectroline UV lamp, Model ENF-24, that was equipped with a filter to cut off irradiation below 300 nm to avoid damage to the protein. Aliquots of 1 mL were photolyzed for 15 min on porcelain serology plates on ice at an enzyme concentration of 1–2 mg/mL with the lamp positioned 1 cm above the plate. Following photolysis, DTT was added to a final concentration of 10 mM as a free radical scavenger to block indirect photoinsertion through any long-lived intermediates (Cartwright et al., 1976; Wower et al., 1989). To samples that were not photolyzed, DTT was added to a final concentration of 10 mM to reduce the azido moiety, thereby ensuring the absence of nonspecific photolytic products.

Carboxymethylation and Proteolytic Digestion of SBAzA-Modified Glutamate Dehydrogenase. The pH of the modified enzyme preparations was adjusted to 8.0, and urea was added to a final concentration of 6 M. Iodoacetic acid was then added in an amount 2.2 times the number of moles of dithiothreitol added previously, followed 30 min later by the addition of β-mercaptoethanol at 10 times the amount of that of iodoacetate. After an additional incubation of 20 min, the modified enzyme was dialyzed overnight at 4 °C against 50 mM ammonium bicarbonate, pH 8.0 (4 L, with two changes). Modified enzyme was digested with TPCK-treated trypsin, thermolysin, or chymotrypsin, 2.5:100 (w/w) with respect to glutamate dehydrogenase, at 37 °C for 1.5 h in 50 mM ammonium bicarbonate, pH 8.0, followed by incubation for another 1.5 h with a second addition of the same amount of proteolytic enzyme.

Purification and Analysis of Modified Peptides. Nucleotidyl peptides were initially purified on a phenylboronate agarose (PBA-30) column, 1 × 20 cm, equilibrated with 0.1 M potassium phosphate, pH 7.1. The digest (typically obtained from about 30 mg of glutamate dehydrogenase) was dissolved in about 10 mL of 50 mM NH_4HCO_3, pH 8.0, and applied to the column, equilibrated with the same buffer. The column was washed with approximately 75 mL of the equilibration buffer until the absorbance of the eluate at 220 nm was <0.1. The bound nucleotidyl peptides were eluted by washing the column with deionized water. Fractions containing radioactivity were pooled and lyophilized.

The peptides from the water pool were further separated on a Vydac C_{18} column (0.46 × 25 cm) using a Varian Model 5000 HPLC system equipped with a Varichrom absorbance monitor. The samples were applied to the column equilibrated with 20 mM ammonium acetate buffer, pH 5.8, in water (solvent A). After elution with solvent A for 10 min, a linear gradient was run to 60% solvent B (20 mm ammonium acetate in 50% acetonitrile) in 130 min, followed by a linear gradient to 100% solvent B in the next 30 min. The flow rate was 1 mL/min. The effluent was continuously monitored for absorbance at 220 nm, and fractions of 1 mL were collected.

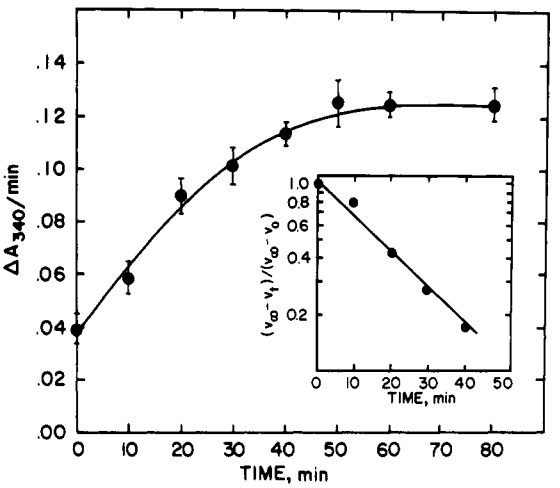

FIGURE 1: Reaction of 5′-FSBAzA with glutamate dehydrogenase. Glutamate dehydrogenase (2 mg/mL) was incubated with 5′-FSBAzA (0.3 mM) at pH 8.0 as described in Materials and Methods. At each indicated time, an aliquot (10 μL) was removed and diluted 20-fold with Tris–0.1 M acetate buffer, pH 8.0, and a 30-μL aliquot was assayed in the presence of 1 μM GTP and 55 μM NADH as described in Materials and Methods. Inset: Determination of the pseudo-first-order rate constant from the increase in observed velocity. V_t and V_0 (0.039 ΔA/min) are the enzymic velocities at a given time and initial velocity, respectively, and V_∞ (0.125 ΔA/min) is the velocity of the enzyme at the end point of the reaction. The rate constant was calculated to be 0.0416 min^{-1} from the slope of this semilogarithmic plot.

Aliquots (400 μL) of fractions were mixed with 5 mL of Liquiscint (National Diagnostics) and counted using a Packard Tri-Carb liquid scintillation counter (Model 1500).

Fractions containing radioativity were lyophilized. Automated sequence analysis was performed on an Applied Biosystems gas-phase sequencer, Model 470A, equipped with an on-line PTH analyzer, Model 120, and computer, Model 900A.

Results

Reaction of 5′-FSBAzA with Glutamate Dehydrogenase. Glutamate dehydrogenase is normally inhibited about 90% by 1 μM GTP (Frieden, 1963). The effect of 1 μM GTP is decreased upon reacting the enzyme with 5′-FSBAzA, reflecting a 3.6-fold increase in the K_I for GTP (Dombrowski & Colman, 1989). The reaction of 5′-FSBAzA with glutamate dehydrogenase was monitored using an assay system containing the constant concentration of 1 μM GTP; under these conditions, a time-dependent increase in activity is observed as the affinity of the enzyme for GTP is decreased. Figure 1 illustrates this time-dependent increase in activity upon reaction with 0.3 mM 5′-FSBAzA, with a maximum increase in activity of about 3-fold occurring after about 1 h of incubation. No further increase in activity was observed upon prolonging the incubation time to 80 min or upon using higher concentrations of 5′-FSBAzA; therefore, this was taken as the end point of the reaction. The reaction exhibited a pseudo-first-order rate constant of 0.0416 min^{-1} with 0.3 mM 5′-FSBAzA (Figure 1, inset). The rate constant was linearly dependent on the reagent concentration from 0.15 to 0.50 mM 5′-FSBAzA with a second-order rate constant of 0.136 min^{-1} mM^{-1}.

Effect of Ligands on the Rate Constant for the Reaction of 5′-FSBAzA with Glutamate Dehydrogenase. Measurement of the effect of ligands on the reaction rate of glutamate dehydrogenase with 5′-FSBAzA can yield information on the target site of the reagent. When ADP [at a concentration at least 10-fold higher than its K_D (Batra & Colman, 1986a)]

Table I: Rate of Reaction of 5′-FSBAzA with Glutamate Dehydrogenase in the Presence and Absence of Catalytic and Regulatory Nucleotides[a]

	nucleotides added	k (min^{-1}) × 10^3
1	none	41.6
2	ADP (1 mM)	43.3
3	GTP (200 μM)	37.5
4	NADH (0.2 mM)	38.9
5	NADH (2 mM)	6.0
6	GTP (2 μM) + NADH (2 mM)	4.6
7	GTP (20 μM) + NADH (2 mM)	5.7
8	GTP (200 μM) + NADH (2 mM)	1.2

[a] Glutamate dehydrogenase was incubated with 0.3 mM 5′-FSBAzA under the conditions described in Figure 1. The pseudo-first-order rate constants (k) were calculated from the time-dependent decrease of the inhibition by GTP as illustrated in Figure 1. Values represent the average of three determinations.

was present in the reaction mixture, no change in the rate of reaction of 5′-FSBAzA with glutamate dehydrogenase was observed (Table I, line 2). Similarly, when 200 μM GTP was present by itself in the reaction mixture, no protection against the loss of GTP inhibition was observed (Table I, line 3). These results suggest that 5′-FSBAzA does not modify the ADP sites or the single site occupied by GTP in the absence of NADH (Pal & Colman, 1979).

When 0.2 mM NADH was added to the reaction mixture, a concentration at which there is appreciable binding only to the catalytic site, the rate constant remained unchanged (Table I, line 4). In contrast, 2 mM NADH, which allows occupation of the NADH regulatory site as well, resulted in a 7-fold decrease in the reaction rate (Table I, line 5). These data suggest that the NADH regulatory site is modified by 5′-FSBAzA.

The binding of NADH to its regulatory site is tightened by the binding of GTP to the enzyme (Koberstein & Sund, 1973), and the incubation of both nucleotides with glutamate dehydrogenase had a greater effect on the reaction rate of 5′-FSBAzA with the enzyme than did either nucleotide alone. At GTP concentrations below 20 μM (which saturates only the high-affinity binding site) plus 2 mM NADH (to occupy the regulatory site), no protection against the loss of GTP inhibition was observed beyond that provided by 2 mM NADH alone (Table I, lines 6 and 7). However, in the presence of 2 mM NADH, a further 10-fold increase in GTP concentration (to populate the low-affinity binding site) slowed the reaction of 5′-FSBAzA with glutamate dehydrogenase about 40-fold (Table I, line 8). These results suggest that the affinity label reacts at or near the GTP-dependent NADH regulatory site.

Amino Acids Modified through the Fluorosulfonyl Moiety of 5′-FSBAzA in the Dark Reaction. Glutamate dehydrogenase which had been modified to the extent of 0.5 mol of -SBAzA/mol of subunit was carboxymethylated and digested with proteolytic enzymes as described in Materials and Methods. The initial purification of the radioactive nucleotidyl peptides from a thermolysin digest was achieved by phenylboronate agarose column chromatography (Figure 2). Most of the unmodified peptides were eluted from the column in a 0.1 M potassium phosphate buffer, pH 7.1, along with <10% of the radioactivity. The majority of the radioactively labeled peptides (>90%) were eluted with deionized water (Figure 2, peak II).

The radioactively labeled nucleotidyl peptides were further fractionated by HPLC on a C_{18} reverse-phase column equilibrated with ammonium acetate, pH 5.8, using an acetonitrile gradient as described in Materials and Methods. Figure 3 shows three major peaks of radioactivity. Sequence analysis of peak I yielded no recognizable amino acids, and the elution position was close to one found for decomposed affinity label (prepared by incubation of 5′-FSBAzA in ammonium bicarbonate at 37 °C prior to HPLC). It was therefore concluded that peak I is a decomposition product of 5′-FSBAzA.

Peak II (Figure 3) contained about 30% of the radioactively labeled peptides from a thermolysin digest of modified enzyme. Analysis of its amino acid sequence yielded Leu-Glu (Table II, column 1). This sequence occurs five times in glutamate dehydrogenase and therefore does not uniquely determine the site of labeling by 5′-FSBAzA. Digestion by trypsin of nonphotolyzed, modified enzyme, followed by PBA chromatography and HPLC, yielded an octapeptide containing radioactivity and ending in the sequence Leu-Glu (Table II, column 3). Upon comparison with the known amino acid sequence of glutamate dehydrogenase (Julliard & Smith, 1979), the octapeptide was identified as Asn^{135} to Glu^{142}. Glutamate is not a common tryptic cleavage site; however, Lys^{143} is the next amino acid in the sequence (Julliard & Smith, 1979). Since modification did not yield a recognizable amino acid in cycle 9 (Table II, column 3), we concluded that Lys^{143} was modified by 5′-FSBAzA. Peak II of Figure 3 was therefore designated as the tripeptide Leu^{141}-Glu^{142}-Lys^{143}.

Peak III (Figure 3) was the major radioactive peak obtained from HPLC fractionation of a thermolysin digest of nonphotolyzed, modified enzyme, and it contained about 70% of

Table II: Amino Acid Sequences of -SBAzA-Containing Peptides from Nonphotolyzed Modified Enzyme

	amount of amino acid (pmol)							
	thermolysin peptides							
cycle no.	peak II[a]		peak III[b]		trypsin peptide[c]		chymotrypsin peptide[d]	
1	Leu	(168)	Ile^{187}	(305)	Asn^{137}	(193)	Ala^{184}	(456)
2	Glu	(123)	Gly	(233)	Tyr	(159)	Ser	(464)
3	X		His	(65)	Thr	(174)	Thr	(151)
4			X		Asp	(121)	Ile	(293)
5			Asp^{191}	(50)	Asn	(125)	Gly	(202)
6					Glu	(90)	His	(116)
7					Leu	(127)	X	
8					Glu^{142}	(85)	Asp	(266)
9					X		Ile	(233)
10							Asn	(298)
11							Ala	(271)
12							His^{195}	(30)

[a] Peak II from the thermolysin digest of -SBAzA-modified glutamate dehydrogenase shown in Figure 3. [b] Peak III from the thermolysin digest of -SBAzA-modified glutamate dehydrogenase shown in Figure 3. [c] Obtained from a tryptic digest of -SBAzA-modified glutamate dehydrogenase following PBA chromatography and HPLC as described in Materials and Methods. [d] Peak I from the chymotryptic digest of -SBAzA-modified glutamate dehydrogenase shown in Figure 4A.

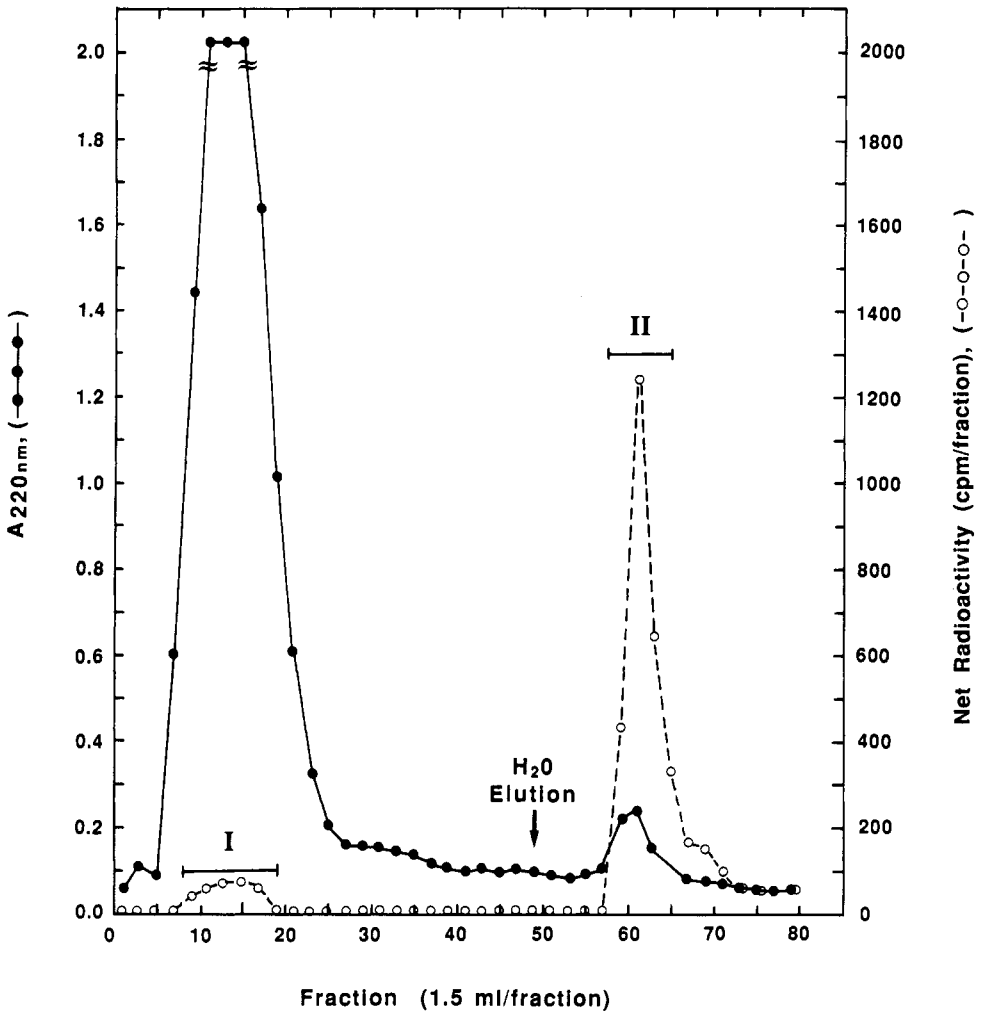

FIGURE 2: Separation of nucleotidyl peptides by phenylboronate agarose chromatography. Nonphotolyzed -SBAzA-modified glutamate dehydrogenase (10 mg) was digested with thermolysin as described in Materials and Methods. The digest was applied to a phenylboronate agarose-30 column equilibrated with 0.1 M potassium phosphate, pH 7.1. The column was washed with the equilibration buffer, and bound nucleotidyl peptides were eluted with distilled water. Fractions of 1.5 mL were collected.

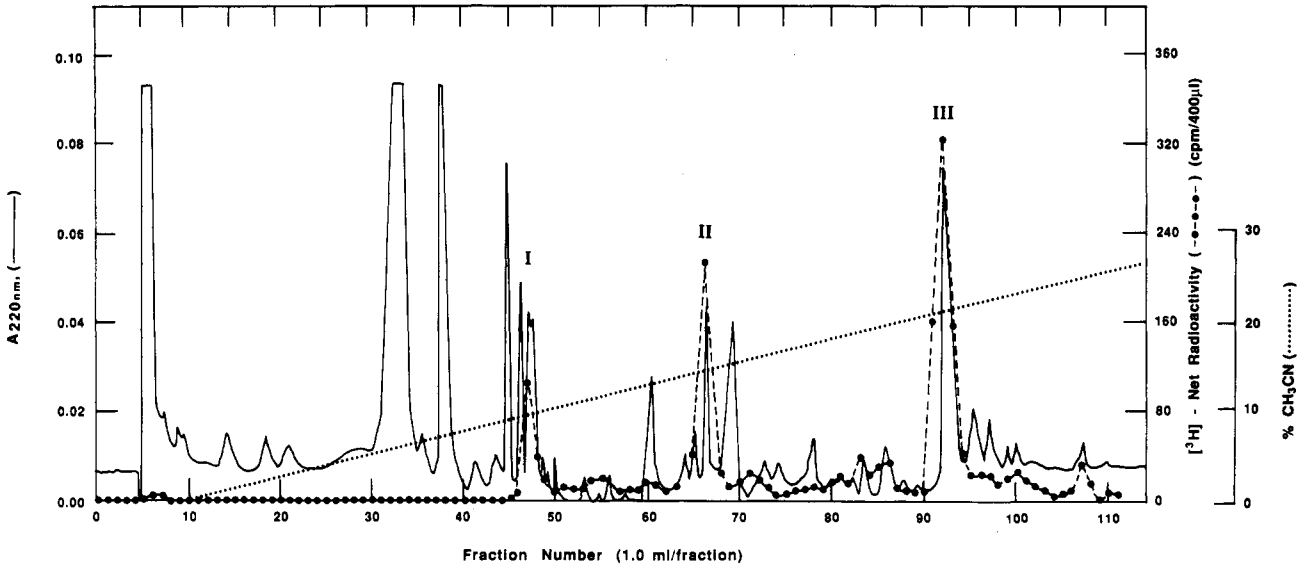

FIGURE 3: Fractionation by HPLC of nucleotidyl peptides from a thermolysin digest. A lyophilized peak II pool of nucleotidyl peptides from a thermolysin digest of nonphotolyzed -SBAzA-modified glutamate dehydrogenase (Figure 2, peak II) was dissolved in 1 mL of 20 mM ammonium acetate, pH 5.8, in water (solvent A) and applied to Vydac C_{18} reverse-phase column equilibrated with the same solvent. The gradient in acetonitrile (···) is described more fully in Materials and Methods. Fractions of 1 mL were collected, and A_{220} nm was monitored. Aliquots of 0.4 mL were withdrawn and counted for radioactivity.

the radioactivity. Sequence analysis yielded the pentapeptide Ile^{187}–Asp^{191} (Table II, column 2). No recognizable amino acid derivative appeared in the fourth cycle, a position corresponding to Tyr^{190} in the known sequence of the enzyme

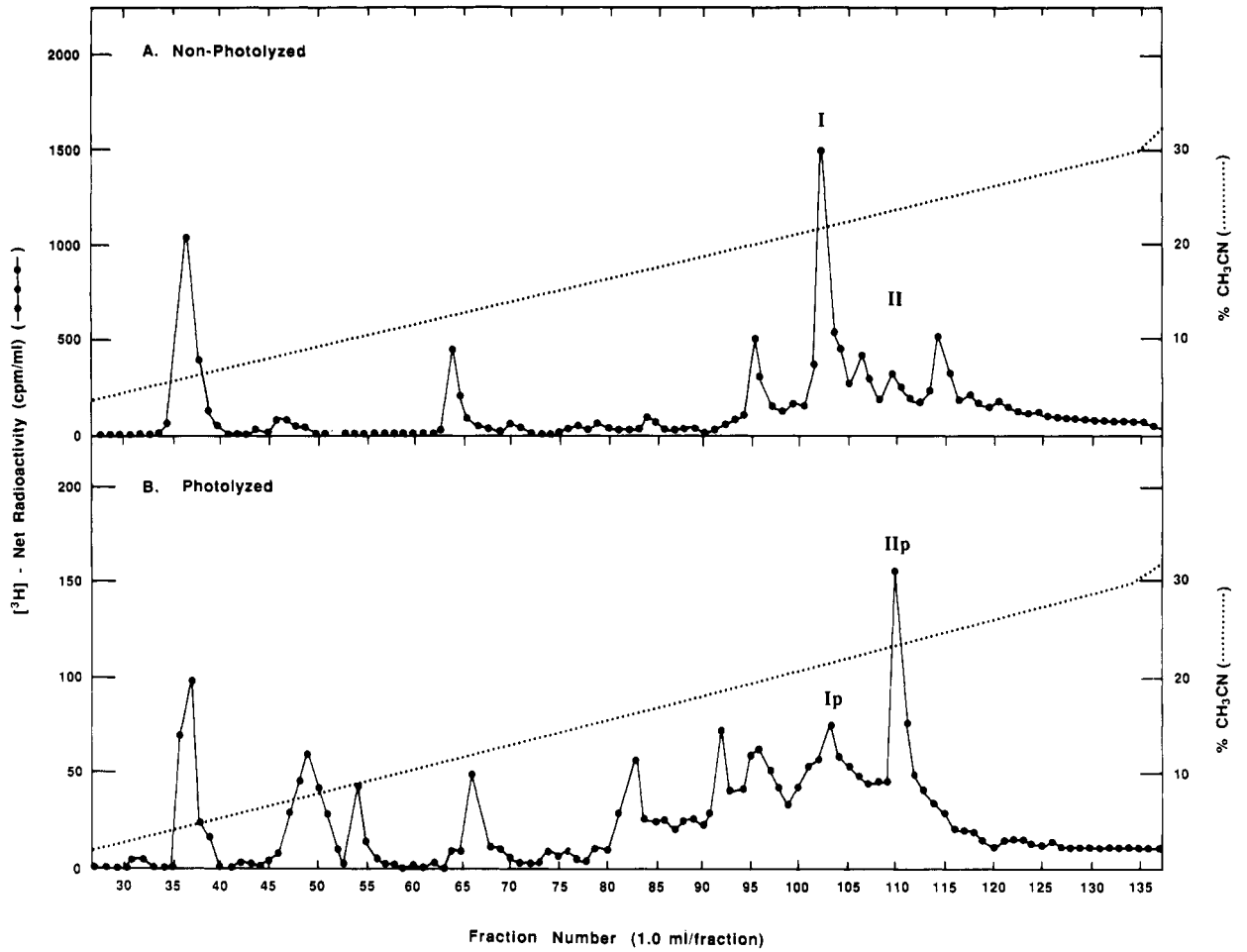

FIGURE 4: Fractionation by HPLC of nucleotidyl peptides from chymotrysin digests. A lyophilized peak II pool of nucleotidyl peptides from a chymotryptic digest of (A) nonphotolyzed and (B) photolyzed 5′-FSBAzA-modified enzyme was dissolved in 1 mL of solvent A and applied to a Vydac C_{18} reverse-phase column. A linear gradient in acetonitrile was used as described in Materials and Methods, and fractions of 1 mL were collected. Aliquots of 0.4 mL were counted for radioactivity.

(Julliard & Smith, 1979). Thus, it was concluded that Tyr^{190} in the GTP-dependent NADH inhibitory site was the major amino acid target modified in the dark reaction by 5′-FSBAzA.

Support for the modification of Tyr^{190} as the predominant modified amino acid came from analysis of a chymotryptic digest of nonphotolyzed, modified enzyme. The major radioactive peptide peak of this digest on HPLC is peak I (Figure 4A), which yields a dodecapeptide upon sequencing (Table II, column 4). This dodecapeptide corresponds to the sequence Ala^{184}–His^{195}. No recognizable amino acid derivative appeared in cycle 7, which again corresponds to Tyr^{190}.

When glutamate dehydrogenase was modified in the presence of the protectants 200 μM GTP plus 2 mM NADH, an incorporation of 0.19 mol of -SBAzA/mol of subunit was obtained. The partially modified enzyme was found to be inhibited by GTP to the same extent as that of native enzyme; the K_I for this "protected" enzyme was indistinguishable from that of native enzyme. [In contrast, enzyme modified with 5′-FSBAzA in the absence of GTP plus NADH contained 0.5 mol of reagent/peptide chain and exhibited a K_I for GTP that was 3.6-fold higher than that of native enzyme (Dombrowski & Colman, 1989).] Digestion with thermolysin of this "protected" enzyme, followed by fractionation of the nucleotidyl peptides by PBA-30 chromatography and HPLC, again yielded two peaks of radioactive peptides (data not shown) which exhibited the same retention times as those shown in Figure 3. These peaks were decreased in magnitude but were present in the same relative proportion as in the unprotected sample. Peaks II and III contained, respectively, labeled Lys^{143} and Tyr^{190}. These results indicate that both modified amino acids are within the GTP-dependent NADH inhibitory site and that more than one subunit of the enzyme hexamer must be modified before the enzyme exhibits a decreased response to regulatory nucleotides.

Amino Acids Modified through the Azido Moiety during the Photolytic Reaction. Glutamate dehydrogenase which had been modified to an extent of 0.5 mol of -SBAzA/mol of subunit was photolyzed with light above 300 nm, carboxymethylated, and digested with proteolytic enzymes as described in Materials and Methods. The nucleotidyl peptides were purified initially by chromatography on PBA-30 followed by fractionation by HPLC, as described for the enzyme samples modified only through the dark reaction.

Figure 4 shows the distribution of radioactivity from chymotryptic digests of nonphotolyzed and photolyzed -SBAzA-modified glutamate dehydrogenase. Peak I (Figure 4A) contains the peptide which corresponds to the modification of Tyr^{190} through the fluorosulfonyl group as discussed previously. However, following photolysis, several changes were noted in the HPLC profile (Figure 4B): peak I (Figure 4A) decreased significantly with a concomitant increase of a peak eluting later in the gradient (peak IIp, Figure 4B). Fractions eluting before 70 yielded no amino acid sequences. Peaks at these positions have been observed in HPLC runs of 5′-FSBAzA incubated under various conditions before and after photolysis and in the absence or presence of DTT; presumably these peaks represent reagent decomposition products.

Table III: Amino Acid Sequences of -SBAzA Peptides from Photolyzed Modified Enzyme

cycle no.	chymotrypsin peptide[a]				thermolysin peptides[b]							
					sample 1				sample 2			
1	Ala^{184}	(37);	Asp^{476}	(70)	Ile^{187}	(81);	Leu^{475}	(45)	Ile^{187}	(65);	Leu^{477}	(22)
2	Ser	(30);	X_2		Gly	(41);	Asp^{476}	(108)	Gly	(39);	X_3	
3	Thr	(10);	Arg^{478}	(5)	His	(5);	X_2		His	(4);	Thr^{479}	(12)
4	Ile	(14)			X_1				X_1			
5	Gly	(11)			Asp^{191}	(6)			Asp^{191}	(5)		
6	His	(3)										
7	X_1											
8	Asp	(7)										
9	Ile	(10)										
10	Asn	(10)										
11	Ala^{194}	(9)										

[a] Peak IIp from the chymotryptic digest shown in Figure 4B. Values in parentheses are the amount (in picomoles) of the corresponding amino acid in the cycle. Fractions 92 and 96 contained the same sequence, but in smaller amounts. More than one peak with the same amino acid sequence has previously been observed for peptides modified by (fluorosulfonyl)benzoyl nucleotides (Jacobson & Colman, 1984). [b] From thermolysin digest of photolyzed, -SBAzA-modified enyme.

Amino acid sequence analysis of peak IIp is shown in Table III (column I). The first cycle contains two amino acids, suggesting the presence of a cross-linked peptide. Further analysis reveals a peptide of 11 amino acids corresponding to the sequence Ala^{184}–Ala^{194} of glutamate dehydrogenase (Julliard & Smith, 1979); however, no recognizable amino acid derivative was found in the seventh cycle for this peptide, a position corresponding again to Tyr^{190}. The observation that Asp and Arg are also present at cycles 1 and 3, respectively, for this sample is consistent with the comigration of the tripeptide Asp^{476}-Leu^{477}-Arg^{478} of glutamate dehydrogenase (Julliard & Smith, 1979), but with the second position yielding no amino acid derivative. [It has been pointed out that the PTH derivatives of amino acids modified by photoreaction are generally not directly observed during gas-phase sequencing (Knight & McEntee, 1985; Kim & Haley, 1991).] This result suggest that Tyr^{190} is cross-linked to Leu^{477} during the photolytic reaction.

Further evidence for this cross-link was obtained from a thermolysin digest of photolyzed 5′-FSBAzA-modified glutamate dehydrogenase. Sequence analysis of two HPLC samples from this digest are shown in Table III, columns 2 and 3. Both samples exhibit evidence for the pentapeptide Ile^{187}–Asp^{191}, with no PTH derivative in the fourth cycle, corresponding to the modified Tyr^{190}. In sample 1, the sequence Leu^{475}-Asp^{476} was also observed. This is a unique sequence in glutamate dehydrogenase (Julliard & Smith, 1979). Leu^{477} is the next amino acid in the sequence (Julliard & Smith, 1979), suggesting that modification of Leu^{477} may occur in the photolytic reaction. Sample 2 contains, in addition to the pentapeptide Ile^{187}–Asp^{191}, Leu at cycle 1 and Thr at cycle 3 but no additional PTH amino acid at cycle 2. These results are consistent with the presence of the tripeptide Leu^{477}-Arg^{478}-Thr^{479} (Julliard & Smith, 1979). These samples from the thermolysin digest suggest the existence after photolysis of alternative cross-links between Tyr^{190} and residues in the enzyme region Leu^{475}-Asp^{476}-Leu^{477}-Arg^{478}-Thr^{479}.

Discussion

The bifunctional nucleotide affinity label 5′-(*p*-(fluorosulfonyl)benzoyl)-8-azidoadenosine has been used in this study to extend the identification of amino acids in the vicinity of nucleotide binding sites of bovine liver glutamate dehydrogenase. The functional site modified by 5′-FSBAzA was determined on the basis of the properties of the modified enzyme, as well as on the basis of the ligands which decrease the observed rate constant for reaction. The modified enzyme, with 0.5 mol of -SBAzA/mol of average enzyme subunit or 3 mol of reagent/mol of hexameric enzyme (both before and after photolysis), exhibited a small decrease in the maximum extent of activation by ADP, with a marked decrease in the inhibition by high concentrations of NADH and weakened affinity for GTP (Dombrowski & Colman, 1989). Since ADP does not affect the rate of functional change, while 2 mM NADH reduces the reaction rate constant 7-fold and 2 mM NADH plus 200 μM GTP decreases the rate almost to zero, we conclude that the functional site attacked by 5′-FSBAzA is the GTP-dependent NADH site. Indeed, "protected" enzyme, prepared by reaction with 5′-FSBAzA in the presence of NADH + GTP, is kinetically indistinguishable from native enzyme. The "protected" enzyme had 0.19 mol of reagent/mol of average enzyme subunit or about 1 mol of reagent/mol of hexameric enzyme. This result suggests that the modification of one of the six subunits is not sufficient to elicit a change in the affinity of the enzyme for regulatory nucleotides; rather, intersubunit communication, which requires modification of at least three of the six subunits, seems to be involved. A variety of biophysical techniques as well as cross-linking experiments have indicated that the subunits in hexameric glutamate dehydrogenase are arranged in two layers of trimers (Goldin & Frieden, 1972; Hucho et al., 1975; Sund et al., 1975), which may provide a structural foundation for this putative subunit interaction.

The monofunctional nucleotide analogue 5′-(*p*-(fluorosulfonyl)benzoyl)adenosine effected functional changes in glutamate dehydrogenase that are strikingly similar to those caused by the bifunctional 5′-FSBAzA, and the ligands which protect the enzyme against modification by 5′-FSBA (Pal et al., 1975; Saradambal et al., 1981) are the same as those which reduce the reaction rate with 5′-FSBAzA. In addition, the stoichiometry for the incorporation of 5′-FSBA into glutamate dehydrogenase exhibiting maximum functional change (Saradambal et al., 1981) is in agreement with that observed for 5′-FSBAzA (Dombrowski & Colman, 1989). In all probability, the structurally related monofunctional and bifunctional adenosine analogues 5′-FSBA and 5′-FSBAzA modify the same nucleotide site on glutamate dehydrogenase: the GTP-dependent NADH inhibitory site.

The predominant amino acids modified by 5′-FSBAzA both in the dark reaction through the fluorosulfonyl group and in the photolytic reaction through the azido group are reported in this paper. In the dark reaction, Tyr^{190} was the major amino group modified by the fluorosulfonyl group; Lys^{143} was also modified, but to a lesser extent. This distribution of labeled amino acids differs in part from that produced by 5′-FSBA, the structural predecessor of 5′-FSBAzA. In the case of 5′-

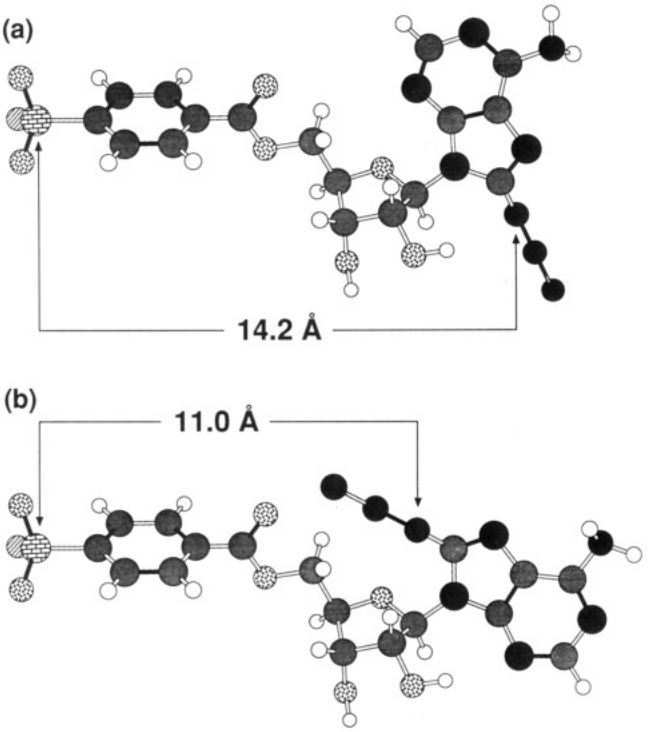

FIGURE 5: Distance relationships between the azido and fluorosulfonyl groups of 5′-FSBAzA. Distance measurements were made using the Chem 3D computer program (Cambridge Scientific Corp.) with the purine ring of 5′-FSBAzA in the (A) syn and (B) anti conformations about the glycosidic bond. The distances labeled are those between the nitrogen of the nitrene and the sulfur of the sulfonyl fluoride.

FSBA, equal amounts of Tyr^{190} and Lys^{420} of glutamate dehydrogenase were modified (Schmidt & Colman, 1984). It is likely that this difference in reaction products is attributable to a distinction in the predominant conformation of the reactive nucleotide analogues. When adenine nucleotides are free in solution, the orientation of the purine ring about the glycosidic bond is equally distributed between the syn and the anti conformation (Ikehara et al., 1972; Gueron et al., 1973; Sundaralingam et al., 1975; Evans & Kaplan, 1976). While the conformation of the glutamate dehydrogenase-bound 5′-FSBA and 5′-FSBAzA have not been established, it seems reasonable that the equal labeling of Tyr^{190} and Lys^{420} is due to reaction of each of these residues with, respectively, the syn or anti conformation of 5′-FSBA. In contrast, substitution of the 8-position of the adenine ring with a bulky group, such as the azido group of 5′-FSBAzA, causes a shift in the conformational equilibrium such that the syn form predominates (Gueron et al., 1983; Evans & Kaplan, 1976; Tavale & Sobel, 1970; Uesugi & Ikehara, 1977). The major reaction of 5′-FSBAzA with Tyr^{190} may thus correspond to reaction with the fluorosulfonyl group of 5′-FSBAzA bound in the syn conformation. The smaller amount of reaction of 5′-FSBAzA with Lys^{143} may indicate that residues 143 and 190 are close in the native structure of glutamate dehydrogenase and that both are accessible to 5′-FSBAzA bound in the syn conformation.

Amino acids modified upon photolysis of the tethered 5′-FSBAzA have also been identified. Although 30% of the reaction of the fluorosulfonyl group of 5′-FSBAzA with the enzyme occurred at Lys^{143}, no crosslinks involving Lys^{143} were detected. Either -SBAzA tethered to the lysine residue encounters solvent rather than another part of the enzyme or the yield of such cross-links is below the detection level. While several peptides were isolated and sequenced, they all were products of the cross-linkage of Tyr^{190} and either Leu^{477} or Arg^{478}. The activation of the azido group results in the formation of a highly reactive nitrene radical which can react with amino acid side chains or the polypeptide backbone (Bayley & Staros, 1974). Since nitrenes are known to react by insertion into carbon–hydrogen or nitrogen–hydrogen bonds (Bayley & Knowles, 1977), leucine and arginine are reasonable target amino acids. It has been pointed out that variation in the point of insertion of a photoprobe within a particular site is a common occurrence (King et al., 1991).

The only other photoaffinity reagent used to label glutamate dehydrogenase in sufficiently high yield to permit peptide isolation is 2-azido-NAD^+ (Kim & Haley, 1990, 1991). This compound, in the presence of glutarate, appears to photolabel the catalytic coenzyme site (Kim & Haley, 1990) by reaction with a peptide spanning residues 270–289 (Kim & Haley, 1991). The target site of 2-azido-NAD is obviously distinct from that of 5′-(*p*-fluorosulfonyl)benzoyl)-8-azidoadenosine.

The modification and cross-linkage of the GTP-dependent NADH regulatory site of glutamate dehydrogenase by the bifunctional reagent 5′-FSBAzA provides the basis for postulating a spatial arrangement of amino acids and an approximation of the size of this regulatory coenzyme site. Figure 5 shows the bifunctional nucleotide analogue 5′-FSBAzA in the syn (Figure 5a) or in the anti (Figure 5b) conformation. The maximum distance between the nitrogen of the nitrene and the sulfur of the fluorosulfonyl group is 14.2 Å with 5′-FSBAzA in the syn conformation and 11.0 Å in the anti conformation. Although a range of distances between the two functional groups is possible, a value of approximately 14 Å is most likely if the syn conformation, as expected, predominates in this 8-substituted nucleotide.

This study demonstrates the usefulness of the bifunctional nucleotide analogue 5′-(*p*-(fluorosulfonyl)benzoyl)-8-azidoadenosine in identifying amino acid residues which are distinct in the linear sequence of bovine liver glutamate dehydrogenase but are near within the GTP-dependent NADH regulatory site of the native enzyme. With its electrophilic functional group and photoactivatable moiety, 5′-FSBAzA should have applications in identifying pairs of amino acids and estimating the interresidue distances for the numerous enzymes for which 5′-(*p*-(fluorosulfonyl)benzoyl)adenosine acts as an affinity label of a nucleotide binding site (Colman, 1990).

ACKNOWLEDGMENTS

We thank Dr. Sara H. Vollmer for her help in obtaining the distance estimates reported in this paper.

REFERENCES

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

Batra, S. P., & Colman, R. F. (1986b) *Biochemistry 25*, 3508–3515.

Bayley, H., & Knowles, J. R. (1977) *Methods Enzymol. 46*, 69–114.

Bayley, H., & Staros, J. V. (1984) in *Azides and Nitrenes: Reactivity and Utility*, pp 433–490, Academic Press, New York.

Cartwright, I. L., Hutchinson, D. W., & Armstrong, V. W. (1976) *Nucleic Acids Res. 3*, 2331–2339.

Colman, R. F. (1990) in *Enzymes (3rd Ed.) 19*, 283–321.

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

Dombrowski, K. E., & Colman, R. F. (1989) *Arch. Biochem. Biophys. 275*, 302–308.

Dombrowski, K. E., Huang, Y.-C., & Colman, R. F. (1991) *FASEB J. 5*, A1523.
Evans, F. E., & Kaplan, N. O. (1976) *J. Biol. Chem. 251*, 6791-6797.
Frieden, C. (1963) *J. Biol. Chem. 238*, 3286-3299.
Goldin, B. R., & Frieden, C. (1972) *Curr. Top. Cell. Regul. 4*, 77-117.
Gueron, M., Chachaty C., & Son, T. D. (1973) *Ann. N.Y. Acad. Sci. 222*, 307-322.
Holmes, R. E., & Robins, R. K. (1963) *J. Am. Chem. Soc. 87*, 1772-1776.
Hucho, F., Rasched, I., & Sund, H. (1975) *Eur. J. Biochem. 52*, 221-230.
Ikehara, M., & Kaneko, M. (1970) *Tetrahedron 26*, 4251-4259.
Ikehara, M., Usegui, S., & Yoshida, K. (1972) *Biochemistry 11*, 830-836.
Jacobson, M. A., & Colman, R. F. (1984) *Biochemistry 23*, 6377-6382.
Julliard, J. H., & Smith, E. L. (1979) *J. Biol. Chem. 254*, 3427-3438.
Kim, H., & Haley, B. E. (1990) *J. Biol. Chem. 265*, 3636-3641.
Kim, H., & Haley, B. E. (1991) *Bioconjugate Chem. 2*, 142-147.
King, S. M., Kim, H., & Haley, B. E. (1991) *Methods Enzymol. 196*, 449-466.
Knight, K. L., & McEntee, K. (1985) *J. Biol. Chem. 260*, 10185-10191.
Koberstein, R., & Sund, H. (1973) *Eur. J. Biochem. 36*, 545-552.
Krause, H., Buhner, M., & Sund, H. (1974) *Eur. J. Biochem. 41*, 593-602.
Olson, J. A., & Anfinsen, C. B. (1951) *J. Biol. Chem. 197*, 67-79.
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.
Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891-2899.
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.
Sund, H., Markau, K., & Koberstein, R. (1975) *Subunits in Biological Systems*, Vol. 7C, pp 225-287, Dekker, New York.
Sundaralingam, M. (1975) *Ann. N.Y. Acad. Sci. 225*, 3-42.
Tavale, S. S., & Sobel, H. M. (1970) *J. Mol. Biol. 48*, 109-123.
Uesugi, S., & Ikehara, M. (1977) *J. Am. Chem. Soc. 99*, 3250-3253.
Wower, J., Aymie, M., Hixson, S. S., & Zimmerman, R. A. (1989) *Biochemistry 28*, 1563-1567.

Primary Structure of Rat Liver D-β-Hydroxybutyrate Dehydrogenase from cDNA and Protein Analyses: A Short-Chain Alcohol Dehydrogenase[†,‡]

Perry Churchill,*[,§] John Hempel,[‖] Hana Romovacek,[‖] Wei-Wei Zhang,[§] Mark Brennan,[⊥] and Sharon Churchill[§]

Department of Biological Sciences, University of Alabama, Tuscaloosa, Alabama 35487, Department of Molecular Genetics and Biochemistry, University of Pittsburgh Medical School, Pittsburgh, Pennsylvania 15261, and Department of Biochemistry, University of Louisville Medical School, Louisville, Kentucky 40292

Received October 15, 1991; Revised Manuscript Received January 22, 1992

ABSTRACT: The amino acid sequence of D-β-hydroxybutyrate dehydrogenase (BDH), a phosphatidylcholine-dependent enzyme, has been determined for the enzyme from rat liver by a combination of nucleotide sequencing of cDNA clones and amino acid sequencing of the purified protein. This represents the first report of the primary structure of this enzyme. The largest clone contained 1435 base pairs and encoded the entire amino acid sequence of mature BDH and the leader peptide of precursor BDH. Hybridization of poly(A+) rat liver mRNA revealed two bands with estimated sizes of 3.2 and 1.7 kb. A computer-based comparison of the amino acid sequence of BDH with other reported sequences reveals a homology with the superfamily of short-chain alcohol dehydrogenases, which are distinct from the classical zinc-dependent alcohol dehydrogenases. This protein family, initially discerned from *Drosophila* alcohol dehydrogenase and bacterial ribitol dehydrogenase, is now known to include at least 20 enzymes catalyzing oxidations of distinct substrates.

D-β-Hydroxybutyrate dehydrogenase (BDH)[1] is a lipid-requiring enzyme with a specific requirement of phosphatidylcholine for enzymatic activity. The activated enzyme catalyzes the reversible oxidation of D-β-hydroxybutyrate to acetoacetate utilizing NAD as coenzyme and is localized on the matrix side of the inner mitochondrial membrane (McIntyre et al., 1978). BDH is synthesized on cytoplasmic free ribosomes as a larger size precursor protein (Mihara et al., 1982). Precursor BDH must be transported into and processed by mitochondria in

[†] This research was supported in part by a grant from Pittsburgh Supercomputing Center through the NIH Division of Research Resources Cooperative Agreement 1P41 RR06009.

[‡] The genetic sequence of D-β-hydroxybutyrate dehydrogenase in this paper has been submitted to GenBank under Accession Number M89902.

[§] University of Alabama.
[‖] University of Pittsburgh Medical School.
[⊥] University of Louisville Medical School.

[1] Abbreviations: BDH, D-β-hydroxybutyrate dehydrogenase (EC 1.1.1.30); NEM, *N*-ethylmaleimide; DCCD, *N,N'*-dicyclohexylcarbodiimide; NBRF, National Biomedical Research Foundation; PIR, Protein Identificiation Resource; GCG, Genetics Computer Group; 17HSD, 17β-hydroxysteroid dehydrogenase (EC 1.1.1.62).

0006-2960/92/0431-3793$03.00/0