Identification of a Peptide of the Guanosine Triphosphate Binding Site within Brain Glutamate Dehydrogenase Isoproteins Using 8-Azidoguanosine Triphosphate†

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ABSTRACT: Photoaffinity labeling with [γ-^{32}P]8N_3GTP (8-azidoguanosine triphosphate) was used to identify the guanine binding peptides of the GTP binding site within two types of glutamate dehydrogenase isoproteins (GDH I and GDH II) isolated from bovine brain. 8N_3GTP, without photolysis, mimicked the inhibitory properties of GTP on GDH I and GDH II activities. Saturation of photoinsertion of GDH isoproteins revealed an apparent K_d of 8 μM (GDH I) and 24 μM (GDH II) for [γ-^{32}P]8N_3GTP. Ion-exchange and reversed-phase high-performance liquid chromatography (HPLC) were used to isolate photolabel-containing peptides generated with trypsin. This identified a portion of the guanine binding domain within the GTP binding site as the region containing the sequence I-S-G-A-S-E-**X**-D-I-V-H-S-***A***-L-A-Y-T-M-E-R (GDH I) and I-S-G-A-S-E-**X**-D-I-V-H-S-***G***-L-A-Y-T-M-E-R (GDH II). The symbol **X** indicates a position for which no phenylthiohydantoin-amino acid could be assigned. The missing residue, however, can be designated as a photolabeled lysine since the sequences including the lysine residue in question have a complete identity with those of the other GDH species known. Also, trypsin was unable to cleave the photolabeled peptide at this site. Photolabeling of these peptides was prevented by the presence of GTP during photolysis, while other nucleotides could not reduce the amount of photoinsertion as effectively as GTP. These results demonstrate selectivity of the photoprobe for the GTP binding site and suggest that the peptide identified using the photoprobe is located in the GTP binding domain of the brain GDH isoproteins.

Glutamate is a major excitatory neurotransmitter (Fonnum, 1984) and is known to be involved in the pathogenesis of human degenerative disorders due to its neurotoxic potentials (McGeer & McGeer, 1976; Plaitakis et al., 1982). One enzyme central to the metabolism of glutamate is glutamate dehydrogenase (GDH;[1] EC 1.4.1.3), which catalyzes the reversible deamination of L-glutamate to 2-oxoglutarate using NAD^+ or $NADP^+$. Since the pathology of the disorders associated with GDH defects is restricted to the brain, the enzyme may be of particular importance in the biology of the nervous system. Hussain et al. (1989) detected four different forms of GDH isoproteins from human cerebellum of normal subjects and patients with neurodegenerative disorders. The isoproteins are differentially distributed in the two catalytically active isoforms of the enzyme (Plaitakis et al., 1993). The enzyme isolated from one of patient with a variant form of multisystem atrophy displayed marked reduction of one of the GDH isoproteins (Hussain et al., 1989). The origin of the GDH polymorphism is not known. Current studies showed the presence of four different-sized mRNAs and multiple gene copies for GDH in the human (Plaitakis et al. 1993; Amuro et al., 1988; Mavrothalassitis et al., 1994). Recently, a novel cDNA encoded by an X chromosome-linked intronless gene was isolated from human retina (Shashidharan et al., 1994). However, the studies of the human GDH isoproteins were far less encompassing in protein function and structure. Further characterization of the structure and function of these various types of GDH, especially brain enzymes, is needed to elucidate the patho-physiological nature of the GDH-deficient neurological disorders.

Mammalian GDH is composed of six identical subunits and the regulation of GDH is very complex (Fisher, 1985). It has been a major goal to identify the substrate and regulatory binding sites of GDH. Even though there are several reports suggesting the regulatory or substrate binding site, the results are quite controversial. Several classical chemical probes have been used to attempt resolution of these binding sites. The studies using classical chemical probes to identify the NADH and GTP binding sites within bovine liver GDH, however, gave a wide scatter of modified residues throughout most of the proposed three-dimensional structure of GDH. For instance, the NADH binding site was proposed to be modified by an ATP analogue at Cys^{319} (Ozturk et al., 1990; Ozturk & Colman, 1991), by a GMP probe at Met^{169} and Tyr^{262} (Ozturk et al., 1992), and by the adenosine analogue at Lys^{420} and Tyr^{190} (Pal et al., 1975; Saradambal et al., 1981; Schmidt & Colman, 1984). Similarly, the GTP binding site was proposed to be modified by a fluorescent adenosine analogue at Tyr^{262} (Jacobson & Colman, 1982) and the ADP binding site was also proposed to be modified

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[1] Abbreviations: GDH, glutamate dehydrogenase; 8N_3GTP, 8-azidoguanosine 5′-triphosphate.

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by two different AMP analogues at His^{82} (Batra & Colman, 1986; Batra et al., 1986) and Arg^{459} (Wrzeszczynski & Colman, 1994). It seems, therefore, that the base moiety has not been effective at directing the site of modification by classical chemical probes. Recently, identifying nucleotide binding sites of a variety of proteins has been advanced by the use of nucleotide photoaffinity analogs which selectively insert into a site upon photoactivation with ultraviolet light. For instance, $[^{32}P]2N_3NAD^+$ was shown to be a valid active-site probe for several proteins (Kim & Haley, 1990, 1991). The ATP binding site of adenylate kinase and creatine kinase and the protein unique to cerebrospinal fluids of Alzheimer's patients successfully have been identified using $2N_3ATP$ and $8N_3ATP$ (Gunnersen & Haley, 1992; Olcott et al., 1994; Salvucci et al., 1992). The GTP binding site and the ADP regulatory site of bovine liver GDH also have been identified using $[^{32}P]2N_3ADP^+$ and $[^{32}P]8N_3GTP$, respectively (Shoemaker & Haley, 1993, 1996).

Recently, we have isolated two soluble forms of glutamate dehydrogenase isoproteins (designated GDH I and GDH II) from bovine brain (Cho et al., 1995). Unlike most previous reports, which present a soluble and a particulate form of GDH (Fisher, 1985, Smith et al., 1975), both GDH I and GDH II were readily solubilized and no detergents were required for the initial extraction step (Cho et al., 1995). Here we report the identification of an essential lysine residue in the overall sequence by a combination of peptide analysis and photolabeling with $[\gamma\text{-}^{32}P]8N_3GTP$. To our knowledge, this is the first report providing a detailed information about the regulatory-site sequences of any brain GDH isoforms.

MATERIALS AND METHODS

Materials. NADH, NADPH, 2-oxoglutarate, ADP, and L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin were purchased from Sigma Chemical Co. $[\gamma\text{-}^{32}P]8N_3$-GTP was purchased from ICN or RPI Corp. Precast gels for gradient SDS−polyacrylamide gel electrophoresis were purchased from Novex. All other chemicals and solvents were reagent grade or better.

Enzyme Purification and Assay. The GDH isoproteins were purified by the method developed in our laboratory (Cho et al., 1995) and were homogeneous as judged by Coomassie-stained gradient SDS−polyacrylamide gel electrophoresis. Only homogeneously purified GDH isoproteins were used unless otherwise indicated. GDH activity was measured spectrophotometrically in the direction of reductive amination of 2-oxoglutarate by following the decrease in absorbance at 340 as described before (Cho et al., 1995) except that no ADP and EDTA were added.

Inactivation of GDH I and GDH II by GTP. The enzymes were incubated with GTP at various concentrations in 50 mM triethanolamine, pH 8.0, at 25 °C. At intervals after the initiation of the inactivation, aliquots were withdrawn for the assay of activity. In some experiments, the capacity of substrate and coenzyme to protect the enzymes against inactivation was tested by including them in the incubation. If their concentration was sufficient for the small amounts transferred to the assay mixture to affect activity, separate control experiments were included to determine the appropriate value of the 100% "zero time" activity.

Photolabeling of GDH I and GDH II. Photolabeling of GDH isoproteins were performed by the method of Shoemaker and Haley (1993) with a slight modification. For saturation studies, GDH I and GDH II (100 μg each) in 5 mM Tris-acetate, pH 8.0, were separately incubated with various concentrations of $[\gamma\text{-}^{32}P]8N_3GTP$ in Eppendorf tubes for 1 min. For competition studies, 100-μg aliquots of GDH isoproteins were incubated with various concentrations of GTP for 1 min in the same buffer at 4 °C prior to the addition of 60 μM $[\gamma\text{-}^{32}P]8N_3GTP$ and then allowed to incubate with the photoprobe for 1 min as described above. The samples were then irradiated with hand-held 254-nm UV lamp for 90 s twice at 4 °C. The reaction was quenched by the addition of ice-cold trichloroacetic acid (final 7%). The reaction mixtures were kept in an ice bath for 30 min and then centrifuged at 10000*g* for 15 min at 4 °C. The pellets were washed and resuspended with 5 mM Tris-acetate, pH 8.0, containing 2 M urea. The remaining free photoprobe, if any, was further removed from the protein by exhaustive washing using Centrifree (Amicon), and ^{32}P incorporation into protein was determined by liquid scintillation counting.

Tryptic Digestion of Photolabeled GDH Isoproteins. To determine the site modified by $[\gamma\text{-}^{32}P]8N_3GTP$, 1.5-mg samples of each GDH isoprotein in 5 mM Tris-acetate, pH 8.0, were separately incubated with 100 μM $[\gamma\text{-}^{32}P]8N_3GTP$ for 2 min at 4 °C. The mixtures were irradiated for 90 s twice. The reaction was quenched by the addition of ice-cold TCA (final 7%) and kept at 4 °C for 15 min. The protein was precipitated by centrifugation at 10000*g* for 15 min at 4 °C, and the pellet was resuspended in 75 mM NH_4HCO_3, pH 8.5, containing 2 M urea. GDH isoproteins were proteolyzed by the addition of 7.5 μg of trypsin and kept at room temperature for 3 h after which 7.5 μg of trypsin was added again. After 3 more hours at room temperature, 10 μg of trypsin was added, and the digestion mixture was kept at 25 °C overnight. To validate that the isolated peptide(s) was specific for the GTP site and could be protected from photomodification, GDH I and GDH II were photolyzed in the presence of 300 μM GTP and proteolyzed as described above.

Isolation of the Photolabeled Peptide and Protein Sequencing. The photolabeled GDH I and GDH II in the presence or absence of 300 μM GTP were buffer-changed with 50 mM potassium phosphate, pH 7.4, using Centricon (Amicon) and applied to a Protein Pak DEAE-5PW (Waters) column on a Waters HPLC system equipped with a diode-array spectral detector. The gradient for HPLC was 0−10 min, 0% NaCl; 10−60 min, 0−0.5 N NaCl; 60−70 min, 0.5 N NaCl at a flow rate of 0.5 mL/min. The absorbance of the fractions was measured at 200 nm and the photoincorporation was determined by liquid scintillation counting. The fractions containing photolabeled peptides were desalted, freeze-dried, resuspended in 0.1% trifluoroacetic acid, and subjected to reversed-phase HPLC using an Waters C_{18} column on the same HPLC system. The mobile system consisted of 0.1% trifluoroacetic acid solution and 0.1% trifluoroacetic acid/80% acetonitrile solvent system. The gradient for HPLC was 0−10 min, 0% acetonitrile; 10−60 min, 0−80% acetonitrile; 60−70 min, 80% acetonitrile at a flow rate of 0.5 mL/min. HPLC fractions containing photolabeled peptides were sequenced by the Edman degradation method as described elsewhere (Cho et al., 1995).

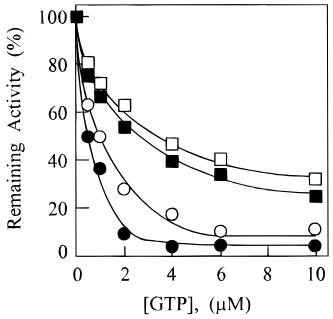

FIGURE 1: Inhibition of GDH isoproteins by GTP. The enzymes were preincubated with various concentrations of GTP in the assay buffer and the activities were assayed by the addition of the standard assay mixture with NADH or NADPH as a coenzyme. Remaining activities are expressed relative to each control. GDH I with (●) NADH and (○) NADPH; GDH II with (■) NADH and (□) NADPH.

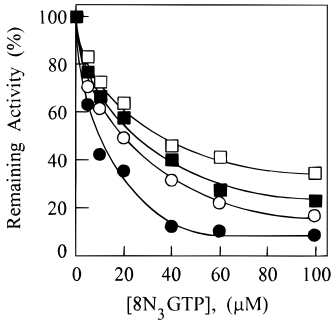

FIGURE 2: Effects of $8N_3GTP$ on GDH isoproteins. The effects of $8N_3GTP$ on GDH isoproteins were examined as in Figure 1 except that $8N_3GTP$ was substituted for GTP. GDH I with (●) NADH and (○) NADPH; GDH II with (■) NADH and (□) NADPH.

RESULTS

Inactivation of GDH Isoproteins by GTP and $8N_3GTP$. The vertebrate GDHs have been known to be regulated by GTP. In the present study, the inhibitory effects of GTP on brain GDH isoproteins were examined and summarized in Figure 1. GTP was able to inhibit GDH isoproteins and there were significant differences between GDH I and GDH II in their sensitivities to the inactivation by GTP. In enzymatic assays with NADH as a coenzyme, GTP inhibited GDH I and GDH II with K_i values of 0.5 μM and 1.2 μM, respectively. When NADPH was used, however, the inhibitory effects of GTP were less effective, with K_i values of 1.2 μM and 3.5 μM for GDH I and GDH II, respectively (Figure 1). The substrate 2-oxoglutarate did not significantly affect the inhibitory effects of GTP on the GDH isoproteins (data not shown). To show that $8N_3GTP$ could mimic the inhibitory properties of GTP, the photoanalogue should be able to reversibly inhibit GDH in the absence of activating light. When assayed with NADH as a coenzyme, $8N_3GTP$ was able to inhibit GDH with K_i values of 10 μM and 25 μM for GDH I and GDH II, respectively (Figure 2). Similar to the inhibitory effects of GTP, $8N_3GTP$ inhibited GDH isoproteins less effectively with K_i values of 20 μM and 35 μM for GDH I and GDH II, respectively, when NADPH was substituted for NADH (Figure 2). These results show that the azidonucleotide, $8N_3GTP$, is able to elicit almost the same biological effects on GDH isoproteins as the natural nucleotide, GTP.

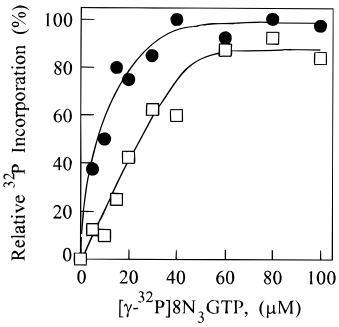

FIGURE 3: Saturation of photoinsertion of $[\gamma\text{-}^{32}P]8N_3GTP$ into GDH isoproteins. GDH I and GDH II in the reaction buffer were photolyzed with the indicated concentrations of $[\gamma\text{-}^{32}P]8N_3GTP$ and ^{32}P incorporation into protein was determined by liquid scintillation counting (see Materials and Methods for details). Relative ^{32}P incorporations were expressed relative to each control. (●) GDH I; (□) GDH II.

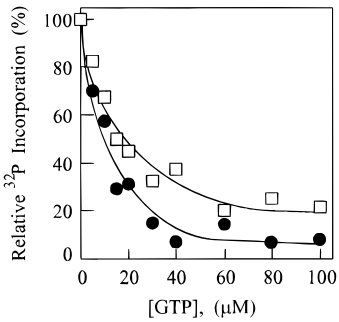

FIGURE 4: Effect of GTP on photoinsertion of $[\gamma\text{-}^{32}P]8N_3GTP$ into GDH isoproteins. GDH I and GDH II in the reaction buffer were photolyzed with 60 μM $[\gamma\text{-}^{32}P]8N_3GTP$ in the presence of the indicated concentrations of GTP. Relative ^{32}P incorporation into protein was determined and expressed as described in Figure 3. (●) GDH I; (□) GDH II.

Saturation and Competition of Photoinsertion. To show specificity of the photoprobe−protein interaction, saturation of photoinsertion should be observed. Under the experimental conditions described, saturation of photoinsertion with $[\gamma\text{-}^{32}P]8N_3GTP$ occurred at 45 μM and 60 μM photoprobe for GDH I and GDH II, respectively (Figure 3). The apparent K_d values of this interaction were 8 μM and 24 μM for GDH I and GDH II, respectively. To demonstrate specific labeling of GDH isoproteins, the enzymes were photolabeled with $[\gamma\text{-}^{32}P]8N_3GTP$ in the presence of increasing GTP concentrations. As shown in the results of the competition experiments (Figure 4), increasing GTP concentration decreased the photolabeling of 60 μM $[\gamma\text{-}^{32}P]8N_3GTP$. The apparent K_d values of this interaction were 10 μM and 19 μM for GDH I and GDH II, respectively. When 300 μM GTP was present with 100 μM $[\gamma\text{-}^{32}P]8N_3GTP$, 96% and 85% of photoinsertion were protected for GDH I and GDH II, respectively, as shown in Table 1. ATP and ADP were able to inhibit photoinsertion, but not as effectively as GTP (Table 1). These results show the specificity of $8N_3GTP$ and the utility of this probe as a good candidate for determining the GTP site.

Previously, it was suggested that bovine liver GDH contains two GTP binding site, one NADH-independent and the other NADH-dependent (Pal & Colman, 1979). There is some disagreement on whether there are two GTP sites. Oversaturating concentrations (100 μM) of $[\gamma\text{-}^{32}P]8N_3GTP$ were incubated with brain GDH isoproteins and increasing amounts of NAD(P)H or $NAD(P)^+$ were added to determine

Table 1: Effect of Nucleotides on Photolabeling of Brain GDH Isoproteins with [γ-^{32}P]8N_3GTP[a]

nucleotide	% of control	
	GDH I	GDH II
control (none)	100	100
GTP (300 μM)	4	15
ATP (300 μM)	22	34
ADP (300 μM)	35	44
NAD^+ (100 μM)	91	97
NAD^+ (300 μM)	65	78
$NADP^+$ (100 μM)	88	95
$NADP^+$ (300 μM)	61	72
NADH (100 μM)	19	30
NADH (300 μM)	10	18
NADPH (100 μM)	17	26
NADPH (300 μM)	9	19

[a] GDH isoproteins were incubated with the indicated concentrations of various nucleotides for 1 min in 5 mM Tris-acetate, pH 8.0, at 4 °C prior to the addition of 100 μM [γ-^{32}P]8N_3GTP. Relative ^{32}P incorporation into protein was determined as described in Figure 4.

the effect on [γ-^{32}P]8N_3GTP photolabeling of brain GDH isoproteins. As shown in Table 1, NAD^+ or $NADP^+$ had little effect on the photoinsertion, whereas NADH or NADPH significantly reduced photolabeling. To attempt detection of two different GTP binding sites, saturation studies with [γ-^{32}P]8N_3GTP in the presence of 300 μM NAD(P)H were performed and no results revealed the second GTP binding site on GDH isoproteins even up to concentrations of 200 μM [γ-^{32}P]8N_3GTP. The results in Figure 4 also indicate that under the experimental conditions described only a single K_d is observed. These results indicate that there is only one GTP binding site which is NADH-independent.

Tryptic Digestion of Photolabeled Proteins and Isolation of the Photolabeled Peptide. To identify the peptides modified by [γ-^{32}P]8N_3GTP, GDH isoproteins were photolabeled twice in the absence and presence of 300 μM GTP and digested with trypsin. A higher protein to nucleotide ratio was used to reduce any possible nonspecific labeling. The photolabeled GDH isoproteins were separated from most of the noncovalently bound nucleotide by acid precipitation and proteolyzed by trypsin. After overnight trypsin digestion of GDH isoproteins modified with [γ-^{32}P]8N_3GTP, the digested samples were added to an anion-exchange column (Protein Pak DEAE-5PW, Waters) equilibrated with 50 mM potassium phosphate, pH 7.4. The column was washed by the same buffer and then the peptides were eluted using a linear gradient made with the same buffer and increasing concentrations of NaCl (from 0.0 to 0.5 N). Figure 5 shows the absorption and radioactivity profile of GDH I modified by 60 μM [γ-^{32}P]8N_3GTP. Most of the radioactivity was retained on the column and one major radioactive peak (indicated by arrow in Figure 5) around 0.25 N NaCl was recovered from the column. GTP was able to reduce [γ-^{32}P]8N_3GTP photoinsertion into this peak. When 100 μM GTP was originally present in the incubation mixture, more than 90% of the radioactivity of the peak was eliminated, as shown in Figure 5. This indicates that the radioactive peak represent a guanine binding domain peptide of the GTP binding site of GDH.

The radioactive eluate (fractions 41−46) from the DEAE column was combined and subjected to reversed-phase HPLC. One major radioactive peak (fractions 30−33) was clearly recovered from the HPLC column (Figure 6).

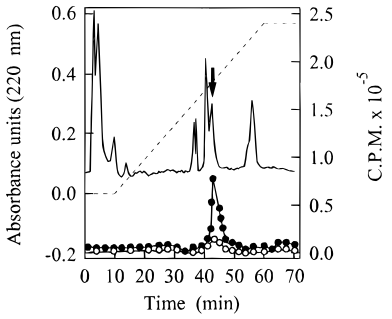

FIGURE 5: Anion-exchange HPLC elution profiles of tryptic peptides from GDH I photolabeled with [γ-^{32}P]8N_3GTP. GDH I was photolabeled with [γ-^{32}P]8N_3GTP as described in the Materials and Methods section in the absence and presence of 300 μM GTP. The tryptic peptides were loaded onto a anion-exchange column (Protein Pak DEAE-5PW, Waters) equilibrated with 50 mM Potassium phosphate, pH 7.4, and eluted with an NaCl gradient (dashed line) at a flow rate of 0.5 mL/min (see text for details). One-minute fractions were collected. The plain solid line represents the OD at 220 nm from HPLC of the sample photolabeled in the absence of GTP. An almost identical UV absorption profile at 220 nm was obtained in the presence of GTP and was omitted for clarity purposes. The plot also represents the radioactivity profiles from HPLC of the samples photolabeled in the presence (○) and absence (●) of 300 μM GTP.

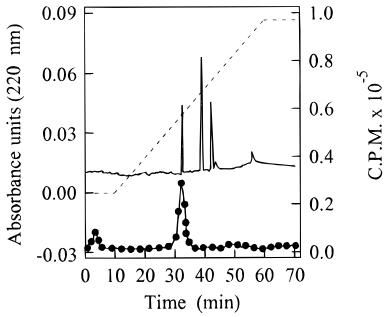

FIGURE 6: Reversed-phase HPLC elution profiles of tryptic peptides eluting from an anion-exchange column. The radioactive eluates (fractions 41−46 in Figure 5) from a DEAE anion-exchange column were loaded onto a C_{18} reversed-phase column, eluted with an acetonitrile gradient (dashed line) at a flow rate of 0.5 mL/min, and monitored at 220 nm (plain solid line). One-minute fractions were collected. The solid line with closed circles represents radioactivity.

Although some radioactivity was found in the flowthrough and wash fractions, over 85% of the total radioactivity coeluted with the first peak (fractions 30−33). The radioactivity associated with the HPLC flowthrough fractions represents unbound probe including any decomposition products of photoadduct produced as peptide binds to the HPLC column matrix. These flowthrough fractions were subjected to analysis and no significant amounts of amino acids were detected. The radioactive peptides (fractions 30−33) were collected and identified by sequence analysis. GDH II gave almost identical chromatographic profiles to GDH I on both DEAE and reversed-phase HPLC columns, even though the intensity of the radioactivity was slightly lower than that of GDH I (data not shown). These results demonstrate that the microenvironmental structures of the two GDH isoproteins are very similar to each other. The photolabeled peptides of GDH II were, therefore, treated and sequenced by the same method as described above.

Sequence Analysis of the Photolabeled Peptide of GDH I and GDH II. The results of sequencing are summarized in Table 2. The sequences obtained were also compared with

Table 2: Alignment of [γ-^{32}P]8N_3GTP-Labeled Peptides with Homologous Sequence from Various GDHs[a]

Source	Reference	Isotypes	Amino acid sequence
Bovine brain	This work	GDH I	I S G A S E **X**[b] D I V H S ***A*** L A Y T M E R
Bovine brain	This work	GDH II	I S G A S E **X** D I V H S ***G*** L A Y T M E R
Human brain	(Banner et al., 1987)	GDH1	I S G A S E K D I V H S G L A Y T M E R
Human retina	(Shashidharan et al., 1994)	GDH2	I S G A S E K D I V H S A L A Y T M E R
Rat brain	(Tzimagiorgis et al., 1991)		I S G A S E K D I V H S G L A Y T M E R
Bovine liver	(Jullard & Smith., 1979)		I S G A S E K D I V H S G L A Y T M E R
Human liver	(Mavrothalassitis et al., 1988)		I S G A S E K D I V H S G L A Y T M E R
			440[c] 445 450 455

[a] The amino acids are denoted by the single-letter code. [b] *X* = no identifiable phenylthiohydantoin-amino acid was observed at this sequencing cycle. [c] The amino acid numbering is that of human brain GDH1 and given at the bottom of the table.

those of various GDHs. The amino acid sequence analysis revealed that the peak fractions contained the amino acid sequence I-S-G-A-S-E-**X**-D-I-V-H-S-***A***-L-A-Y-T-M-E-R (GDH I) and I-S-G-A-S-E-**X**-D-I-V-H-S-***G***-L-A-Y-T-M-E-R (GDH II). The symbol **X** indicates a position for which no phenylthiohydantoin-amino acid could be assigned. The missing residue, **X**, can be designated as a photolabeled lysine since the sequences including the lysine residue in question have a complete identity with those of the other GDH species known. Also, trypsin was unable to cleave the photolabeled peptide at this site. Photolabeling of these peptides was prevented by the presence of 300 μM GTP during photolysis. These results strongly suggest that the lysine residue is located in the guanine binding domain peptide of the GTP binding site of the brain GDH isoproteins. The major difference in the sequence between GDH I and GDH II is that one Ala in GDH I is replaced with Gly in GDH II. This result suggests that the GDH isoproteins are different gene products rather than the results of posttranslational modifications.

DISCUSSION

The existence of brain GDH isoproteins has been only recently recognized (Hussain et al., 1989; Shashidharan et al., 1994). The studies of the brain GDH isoproteins are far less encompassing in protein function and structure of these isotypes. It is only recently that detailed information about the three-dimensional structure of any GDH has become available (Teller et al., 1995; Baker et al., 1991). No crystal structure is available for mammalian GDH and thus remarkably little is known about the chemistry of the active site of GDH, especially brain enzyme. It is, therefore, essential to have a detailed structural description of different types of GDH. Recently, we have isolated two types of soluble glutamate dehydrogenase isoproteins (designated GDH I and GDH II) from bovine brain (Cho et al., 1995). In the present work, we identified a guanine binding domain peptide of the GTP binding site of the brain GDH isoproteins using the photoaffinity probe [γ-^{32}P]8N_3GTP and peptide analysis.

The specificity of 8N_3GTP and the utility of this probe as a good candidate for determining the GTP site were demonstrated by the following. First, in the absence of activating light, GDH is inhibited by 8N_3GTP as well as by GTP and can be photolabeled with the nucleotide analog [γ-^{32}P]8N_3GTP. The K_i values of 8N_3GTP for GDH isoproteins were around 10−35 μM (Figure 2) while those for GTP were 0.5−3.5 μM (Figure 1). A similar K_i of GTP for bovine liver enzyme have been reported (Shoemaker & Haley, 1993), although these values have varied somewhat (Pal & Colman, 1979; McCarthy & Tipton, 1984. The ability to mimic a native compound before photolysis has an advantage over determination of the enzyme function after modification. Second, the photoinsertion into GDH I and GDH II was saturated with 45−60 μM [γ-^{32}P]8N_3GTP. The apparent K_d values of this interaction were 8 μM and 24 μM for GDH I and GDH II, respectively (Figure 3). Third, the prevention of photoinsertion of [γ-^{32}P]8N_3GTP by GTP demonstrates that the photoprobe is inserting into a specific GTP site within GDH isoproteins (Figure 4). Due to the possibility that GDH contains several distinct but overlapping nucleotide binding sites (Gordin & Frieden, 1971; Jacobson & Colman, 1983), other purine nucleotides would be expected to reduce the binding of GTP into GDH isoproteins and seem to do so, but not as effectively as GTP (Table 1).

There are high levels of sequence identity between GDHs compared (Table 2) and the sequence identified in the present study corresponds to residues 439−458 of the amino acid sequence of the well-known bovine liver GDH (Julliard & Smith, 1979). Our results are clearly consistent with those reported by Shoemaker and Haley (1993), who identified the GTP binding site of bovine liver GDH as the peptide containing residues Ile^{439}−Tyr^{454} with probable point photomodification at Lys^{445} using [α-^{32}P]8N_3GTP and [γ-^{32}P]8N_3-GTP. In contrast to our result, the guanine binding site of bovine liver GDH was proposed to be modified by using the classical chemical probe 5′-*p*-(fluorosulfonyl)benzoyl-1,N^6-ethenoadenosine (5′FSBϵA) at Tyr^{262} (Jacobson & Colman, 1982). As indicated elsewhere (Shoemaker & Haley, 1993), it is not clear why a hydrophobic adenosine-containing probe 5′FSBϵA would preferentially bind and react at a hydrophobic GTP binding site and not react at the other adenosine binding sites. Similar results with quite a discrepancy using classical chemical probes were reported by the same research group to identify other regulatory sites within bovine liver GDH. For instance, the ADP binding

site was proposed to be modified by two different AMP analogues at His^{82} (Batra & Colman, 1986; Batra et al., 1986) and Arg^{459} (Wrzeszczynski & Colman, 1994). These two residues are outside the catalytic cleft. The NADH binding site was also proposed to be modified by an ATP analogue at Cys^{319} (Ozturk et al., 1990; Ozturk & Colman, 1991), by a GMP probe at Met^{169} and Tyr^{262} (Ozturk et al., 1992), and by the adenosine analogue at Lys^{420} and Tyr^{190} (Pal et al., 1975; Saradambal et al., 1981; Schmidt & Colman, 1984). It seems, therefore, that the base moiety has not been effective at directing the site of modification by classical chemical probes. Classical chemical probes may label residues near or outside the binding domain due to their long-lived chemical reactivity. They usually have a much lower affinity and many times require solvents for solubility. Their lack of specificity may be the reason for the wide three-dimensional distribution of the residues identified using classical chemical probes as being in the NADH inhibitory site of GDH (Ozturk et al., 1990; Ozturk & Colman, 1991; Ozturk et al., 1992).

In contrast, azidopurine photoprobes generate short-lived, very reactive nitrenes which will modify any residue placed near the generated nitrene by binding. Previously, $2N_3NAD^+$ was shown to be an effective biomimic of NAD^+. Using $[^{32}P]2N_3NAD^+$, a NAD^+ binding site of bovine liver GDH was identified as the peptide containing residues Cys^{270}–Lys^{289} with photomodification at Glu^{275} (Kim & Haley, 1991). The structure of GDH from *Clostridium symbiosum* were characterized and the subunits were found to consist of two globular domains separated by a deep cleft (Stillman et al., 1992). The structures of *C. symbiosum* and mammalian GDH were suggested to be similar due to considerable identity and the conservation of 13 glycine residues, which probably conserves the structure among species, and to consist of two domains. This coenzyme binding region is speculated to be deep within this cleft, which is consistent with the peptide within bovine liver GDH identified with the photoaffinity probe $[\alpha\text{-}^{32}P]2N_3NAD^+$ (Kim & Haley, 1991). The ability of the photoaffinity probes, in the absence of activating light, to mimic the native nucleotides as substrate and regulatory constituents further supports their specificity. Saturation of photoinsertion at concentrations corresponding to that expected from the reversible binding affinities also strongly supports the site being labeled within the binding domain. Finally, their selectivity and specificity have been successfully utilized to locate specific binding domains on a variety of proteins (Campbell et al., 1990; Olcott et al., 1994; Gunnersen & Haley, 1992; Salvucci et al., 1992).

GDH from *Neurospora crassa* is not regulated by GTP or ADP. This enzyme contains 48 less residues than mammalian GDH, and there is little identity between the 100 residues in the C-terminus (Wootton et al., 1974). Studies have shown that chemical probes can at least partially desensitize bovine liver GDH to GTP inhibition while not affecting catalytic activity. The amino acids modified by these chemical probes were shown to residue in the C-terminus (Coffee et al., 1971; Piszkiewicz et al., 1971). It seems likely that the regulatory ADP and GTP binding domains are located in this region. Although a crystal structure of any mammalian GDH is not available yet, the regulatory purine nucleotide binding sites are thought to reside in the C-terminal half of GDH (Smith et al., 1975). An analysis of the three-dimensional structure of the mammalian enzyme should supplement the understanding of the nature and location of these regulatory sites(s).

It has been reported that bovine liver GDH contains both an NADH-dependent and NADH-independent GTP binding site (Pal & Colman, 1979). There is some disagreement on whether there are two GTP sites (Shoemaker & Haley, 1993). We have detected only one GTP binding site which is NADH-independent. NADH and NADPH reduced photolabeling of brain GDH isoproteins with $[\gamma\text{-}^{32}P]8N_3GTP$ even at oversaturating concentrations. The saturation studies with $[\gamma\text{-}^{32}P]8N_3GTP$ in the presence of 300 μM NADH did not reveal the second GTP site up to a range of 200 μM photoprobe (data not shown). These results do not show that there are two GTP binding sites within each subunit of brain GDH isoproteins. The results obtained with our brain GDH isoproteins are consistent with those obtained with bovine liver GDH by Shoemaker and Haley (1993) but contrast to those obtained with the same bovine liver GDH by Pal and Colman (1979). To our knowledge, the guanine binding domain peptide of the second GTP binding site of GDH from any sources has not been identified yet.

Even though the presence of four different-sized mRNAs and multiple gene copies for GDH have been reported in human (Hussain et al., 1989; Plaitakis et al., 1993; Amuro et al., 1988), only one cDNA encoding human brain GDH is known (Banner et al., 1987). Very recently, a novel cDNA encoded by an X chromosome-linked intronless gene was isolated (Shashidharan et al., 1994). It was reported that the novel cDNA is expressed in human retina, testis, and, at a lower level, brain (Shashidharan et al., 1994). To our knowledge, comparison of the detailed structure of active sites and regulatory sites of any GDH isoproteins has not been reported yet. The work presented here clearly establishes that Lys is involved in the GTP binding site of bovine brain isoproteins and identifies it in the overall sequence. Qualitatively, both GDH I and GDH II were similarly inhibited by GTP; however, quantitatively, there were differences (Figure 1). Also, the differences in their amino acid sequences shown in Table 1, together with N-terminal amino acid sequences of GDH I and GDH II (Cho et al., 1995), demonstrate the possibility that the two GDH isoproteins are different gene products rather than the results of posttranslational modifications. An issue not addressed in this work is whether our GDH isoproteins are the bovine counterparts of the human GDH pair. Further sequence characterization is in progress.

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