Essential Active-Site Lysine of Brain Glutamate Dehydrogenase Isoproteins

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Abstract: Two soluble forms of bovine brain glutamate dehydrogenase (GDH) isoproteins were inactivated by pyridoxal 5'-phosphate. Spectral evidence is presented to indicate that the inactivation proceeds through Schiff's base formation with amino groups of the enzyme. Sodium borohydride reduction of the pyridoxal 5'-phosphate-inactivated GDH isoproteins produced a stable pyridoxyl enzyme derivative that could not be reactivated by dialysis. The pyridoxyl enzyme was studied through fluorescence spectroscopy. No substrates or coenzymes separately gave complete protection against pyridoxal 5'-phosphate. A combination of 10 mM 2-oxoglutarate with 2 mM NADH, however, gave complete protection against the inactivation. Tryptic peptides of the isoproteins, modified with and without protection, resulted in a selective modification of one lysine. In both GDH isoproteins, the sequences of the peptide containing the phosphopyridoxyllysine were clearly identical to sequences of other GDH species. Key Words: Glutamate dehydrogenase isoproteins—Pyridoxal 5'-phosphate—Lysine residue—Schiff's base. J. Neurochem. 69, 418-422 (1997).

Glutamate is a major excitatory neurotransmitter (Fonnum, 1984) and is also known to be the immediate precursor in the biosynthesis of γ-aminobutyric acid, a widely distributed inhibitory neurotransmitter. Due to its neurotoxic potentials, glutamate may be involved in the pathogenesis of human degenerative disorders (McGeer and McGeer, 1976; Plaitakis et al., 1982). One enzyme central to the metabolism of glutamate is glutamate dehydrogenase (GDH) (EC 1.4.1.3). GDHs are a family of enzymes that catalyze the reversible deamination of L-glutamate to 2-oxoglutarate by using NAD⁺, NADP⁺, or both as coenzymes (Stillman et al., 1993).

Because 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. The importance of the pathophysiological nature of the GDH-deficient neurological disorders has attracted considerable interest. 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 isofoms of the enzyme (Plaitakis et al., 1993). The enzyme isolated from one of the patients with a variant form of multisystem atrophy displayed pronounced reduction of one of the GDH isoproteins (Hussain et al., 1989). The origin of the GDH polymorphism is not known. The presence of four differently sized mRNAs and multiple gene copies for GDH in the human brain has been reported (Mavrothalassitis et al., 1988). A novel cDNA encoded by an X chromosome-linked intronless gene has also been isolated from human retina (Shashidharan et al., 1994).

Although the three-dimensional structure of GDH from microorganisms is available (Baker et al., 1992), no crystal structure has been reported for mammalian GDH, and thus, remarkably little is known about the structure of mammalian GDH, especially brain enzyme. Previous knowledge of the active site of GDH from other species has recognized the essential Lys residue (Lilley and Engel, 1992; Valinger et al., 1993). To our knowledge, comparison of the detailed structure of active sites and regulatory sites of any GDH isoproteins has rarely been reported. It is, therefore, essential to have a detailed structural and functional description of the various types of brain GDH to clarify the pathophysiological nature of the GDH-deficient neurological disorders.

Recently, we isolated two soluble forms of GDH isoproteins (designated GDH I and GDH II) from bovine brain (Cho et al., 1995), and the GTP binding site within the GDH isoproteins was identified by using...
photoaffinity labeling (Cho et al., 1996). The results from our recent studies in brain (Cho et al., 1995, 1996) demonstrate that the bovine brain GDH isoproteins are different gene products rather than the results of posttranslational modifications. In the present study, we identified an essential lysine residue by using pyridoxal 5'-phosphate (PLP) and peptide analysis.

MATERIALS AND METHODS

Materials

NADH, 2-oxoglutarate, ADP, and l-1-tosylamido-2-phenylethyl chloromethyl ketone–treated trypsin were purchased from Sigma Chemical Co. PLP was purchased from Fluka. All other chemicals and solvents were reagent grade or better.

Enzyme purification and assay

The GDH isoproteins were purified from bovine brain by the method developed in our laboratory (Cho et al., 1995) and were homogeneous as judged by Coomassie-stained gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis. GDH activity was measured spectrophotometrically in the direction of glutamate oxidation as described previously (Cho et al., 1995).

Inactivation of GDH isoproteins by PLP

The enzymes were incubated with PLP at various concentrations in 0.1 M potassium phosphate, pH 8.0, at 25°C. All solutions containing PLP were protected from photolytic destruction with metal foil. At intervals after the initiation of the inactivation, aliquots were withdrawn for the assay of activity. Whenever possible, a small sample volume was used to minimize artifactual blanks due to the transfer of PLP. In some experiments, the capacity of substrates 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.

Spectroscopic studies

Absorption spectra were recorded at 25°C on a Beckman DU-70 UV/Vis spectrophotometer. Fluorescence spectra were recorded at 25°C on an SLM Aminco DMX-1000 fluorescence spectrophotometer. Excitation was at 280 nm.

Isolation of PLP-labeled peptide and protein sequencing

To identify the PLP-binding site, the enzymes were treated with PLP in the presence or absence of 2 mM NADH and 10 mM 2-oxoglutarate, as described above. The samples were reduced with NaBH4, diacylated against 0.1 M potassium phosphate, pH 8.0. After dialysis at 4°C overnight, samples were treated with urea (final concentration, 8 M) in 0.1 M potassium phosphate, pH 8.0, containing 0.5 mM EDTA and 1 mM dithiothreitol, to give final enzyme concentrations of 2.5–3.0 mg/ml. After incubation at 25°C for 2 h, the samples were dialyzed against four changes of 50 mM NH4HCO3 and 0.1 mM CaCl2, pH 8.0, at 4°C overnight. The dialyzed samples were proteolyzed by the addition of 20 μg trypsin and kept at 25°C for 3 h after which 20 μg trypsin was added again. After 3 more h at 25°C, 50 μg trypsin was added, and the digestion mixtures were kept at 25°C overnight. The digestion mixtures were freeze-dried, resuspended in 0.1% trifluoroacetic acid, and subjected to reversed-phase HPLC by using a Millipore C18 column on a Waters HPLC system equipped with a diode array spectral detector. The mobile system consisted of a 0.1% trifluoroacetic acid and 0.1% trifluoroacetic acid/70% acetonitrile solvent system at a flow rate 0.5 ml/min. The amino acid sequence analysis was performed by the Edman degradation method as described previously (Cho et al., 1995).

RESULTS AND DISCUSSION

Recently, we isolated two soluble forms of GDH isoproteins, 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 (Plaitakis et al., 1993; Rajas and Rouset, 1993), 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 a lysine residue in the overall sequence by a combination of peptide analysis and chemical modification using PLP, a well-characterized labeling reagent for various enzymes (Lo Bello et al., 1992; Paine et al., 1993; Valinger et al., 1993).

The time course of inactivation of bovine brain GDH I and GDH II by PLP was studied in the presence of varying amounts of 2-oxoglutarate. The effects of 2-oxoglutarate in incubations of GDH I or GDH II with PLP, yielding a value of K app for each combination of substrate and PLP concentrations, were examined. The substrate decreases both the rate of inactivation and its final extent. From the sets of pseudo-first-order plots, values of 1/K app were plotted against 1/[PLP] to determine the pattern of protection by 2-oxoglutarate (Fig. 1A and B, for GDH I and GDH II, respectively). There were no significant differences between GDH I and GDH II in sensitivities to inactivation by PLP and protection by 2-oxoglutarate. Although the ordinate intercepts are small, the protection appears to be competitive. The plateau in the replots (Fig. 1, insets) indicates that protection by 2-oxoglutarate for both isoproteins is only partial. This result indicates that the GDH isoproteins saturated with the 2-oxoglutarate are still open to attack by PLP, and therefore PLP and 2-oxoglutarate may be simultaneously bound to the isoproteins. Within experimental error, protection by NADH also appears to be competitive. There were no significant differences between GDH I and GDH II in sensitivity to the action of PLP and NADH (data not shown). The replots of the slopes against the NADH concentration showed that this competition is again only partial. Thus, it appears that NADH also may be bound to the enzyme in the presence of PLP.

A combination of 2 mM NADH and 10 mM 2-oxoglutarate together, however, gave complete protection against PLP for both GDH I and II (Fig. 2). It appears likely that the lysine residue of the bovine brain GDH isoproteins cannot be solely responsible

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FIG. 1. Kinetic analysis of protection by 2-oxoglutarate. The values of $k_{\text{app}}$ for the inactivation of GDH I (A) and GDH II (B) by PLP corresponding to each concentration of 2-oxoglutarate were obtained from the slopes of the appropriate lines from the pseudo-first-order plots and similar plots of results obtained with different concentrations of PLP. Insets: Secondary plots of the slopes obtained for each concentration of 2-oxoglutarate. These slopes are replotted against the concentration of 2-oxoglutarate.

for binding either the substrate or the coenzyme, but it could be essential for the catalytic step.

Studies on the NaBH$_4$ reduction of the PLP-inactivated enzyme afford supporting evidence for a primary Schiff’s base formation in the inactivation process. After NaBH$_4$ reduction of the PLP-inactivated enzyme, dialysis of the enzyme derivative formed did not result in restoration of the catalytic activity. This is in contrast to the complete restoration of activity observed by dialysis of the PLP-inactivated enzyme, which was performed before NaBH$_4$ reduction. The spectrum of

FIG. 2. Protection of 2-oxoglutarate and NADH against PLP-induced inactivation. GDH I (A) and GDH II (B) were treated with PLP in the presence and absence of 2-oxoglutarate or NADH as described in Materials and Methods. At indicated times, the remaining activities were assayed by the addition of the standard assay mixture. This diluted the concentrations of PLP, 2-oxoglutarate, and NADH to 1, 10, and 2 mM, respectively, during the assay. GDH + PLP; ○, GDH + 2-oxoglutarate + PLP; △, GDH + NADH + PLP; □, GDH + 2-oxoglutarate + NADH + PLP.

the reduced and dialyzed PLP-inactivated GDH I shows a characteristic peak of pyridoxamine derivatives (Churchich, 1965a) at 325 nm, which is absent in the spectrum of the native enzyme (Fig. 3A). The

FIG. 3. A: Absorption spectra of pyridoxyl enzyme. Pyridoxyl enzyme was prepared by incubating GDH I with PLP at 25°C for 20 min in 0.1 M potassium phosphate, pH 8.0, at a [PLP]/[GDH I] ratio of 50. The concentration of pyridoxyl enzyme was 1 μM for the spectral studies. The dashed line represents the spectrum of 1 μM native GDH I. B: Fluorescence emission spectra of GDH I derivatives. Spectra were obtained in 0.1 M potassium phosphate, pH 8.0, with excitation at 280 nm. Pyridoxyl enzyme for this study was prepared as described in (A).

FIG. 4. HPLC elution profiles of tryptic peptides from GDH I inactivated with PLP. GDH I was treated with PLP as described in Materials and Methods in the absence (A) or presence (B) of 10 mM 2-oxoglutarate and 2 mM NADH. Peptides were loaded onto a C$_8$ reversed-phase column, eluted with an acetonitrile gradient as shown at a flow rate of 0.5 ml/min, and monitored at 325 nm.
fluorescence spectrum of the pyridoxyl enzyme differs completely from that of the native enzyme when excited at 280 nm. The pyridoxyl enzyme is characterized by two fluorescence peaks at 330 and 390 nm, respectively, and the native enzyme exhibits a maximum emission peak at ~330 nm (Fig. 3B). The peak at 390 nm arises presumably through energy transfer from the aromatic residue to the bound pyridoxyl groups (Churchich, 1965b), and this interpretation is consistent with the finding that the presence of pyridoxyl groups causes a dramatic quenching of the native enzyme fluorescence. GDH II gave absorption and emission spectral profiles almost identical to those of GDH I (data not shown), demonstrating that the microenvironmental structures of the GDH isoproteins are very similar to each other.

To identify the site of inactivation, tryptic peptides were prepared in the presence and absence of protecting ligands. The tryptic peptides were applied to a C_{4} reversed-phase column and eluted with an acetonitrile gradient (Fig. 4). The traces obtained for peptides from both the inactivated isoproteins and the protected isoproteins contained several peaks at 325 nm, but one peak was unique to the inactivated form, and therefore, the peptide corresponding to this peak was purified. GDH II gave almost identical HPLC profiles to GDH I. The results of sequence analysis are summarized in Table 1 and show that the modified residue corresponds to the lysine residue already identified in GDH I from other sources (Julliard and Smith, 1979; Mavrothalassitis et al., 1988; Tzimagiorgis and Moschonas, 1991; Shashidharan et al., 1994).

The labeled lysine residue corresponds to Lys^{128} in the E. coli sequence where the importance of this residue has been demonstrated by site-directed mutagenesis (McPherson et al., 1988). The lysine residue has also been identified in overlapping CNBr fragments of unmodified clostridial GDH (Lilley et al., 1991) and the precise position within the intact sequence is confirmed by comparison with the entire sequence obtained from the cloned gene (Teller et al., 1992). It has been shown through crystallographic studies (Lo Bello et al., 1992) to be involved directly in catalysis rather than in substrate binding. The study presented here clearly establishes that it is involved in the activities of both bovine brain isoproteins.

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REFERENCES


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<td>GDH I</td>
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<td>Bovine liver</td>
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The amino acids are denoted by single-letter code. The amino acid numbering is that of bovine liver GDH and is given at the bottom of the table. X = no identifiable phenylthiohydantoin amino acid was observed at this sequencing cycle.


