Identification of an NAD$^+$ Binding Site of Brain Glutamate Dehydrogenase Isoproteins by Photoaffinity Labeling*

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Photoaffinity labeling with $[^{32}P]2N_3NAD^+$ was used to identify the NAD$^+$ binding site within two types of glutamate dehydrogenase isoproteins (GDH I and GDH II) isolated from bovine brain. In the absence of photolysis, 2N$_3$NAD$^+$ is a substrate for the GDH isoproteins. When the enzymes were covalently modified by photolysis in the presence of saturating amounts of photoprobe, about 50% inhibition of the GDH activities was observed. Photoinsertion of probe was increased by GTP or glutarate and decreased by NAD$^+$ or ADP. With the combination of immobilized boronate affinity chromatography and reversed-phase HPLC, photolabeled containing peptides generated with trypsin were isolated. This identified a portion of the adenine ring binding domain of GDH isoproteins as the region containing the sequence, CIAGVXSDGWSWPQGDIDPK for both GDH isoproteins, corresponding to Cys$^{270}$ through Lys$^{289}$ of the amino acid sequence of well known bovine liver GDH. The X indicates a position for which no phenylthiohydantoin-derivative could be assigned. The missing residue, however, can be designated as a photolabeled glutamate since the sequences including the glutamate residue in question have a complete identity with those of the other GDH species known. Photolabeling of these peptides was prevented by the presence of NAD$^+$ during photolysis. These results demonstrate selectivity of the photoprobe for the NAD$^+$ binding site and suggest that the peptide identified using the photoprobe is located in the NAD$^+$ binding domain of the brain GDH isoproteins. Both amino acid sequencing and compositional analysis identified Glu$^{275}$ as the site of photoinertion.

Glutamate is a major excitatory neurotransmitter (1) and is known to be involved in the pathogenesis of human degenerative disorders because of its neurotoxic potentials (2, 3). One enzyme central to the metabolism of glutamate is glutamate dehydrogenase (GDH); EC 1.4.1.3), which catalyzes the reversible deamination of l-glutamate to 2-oxoglutarate using NAD$^+$ or NADP$^+$. Mammalian GDH is composed of six identical subunits, and the regulation of GDH is very complex (4). It has been a major goal to identify the substrate and regulatory binding sites of GDH. It is only in recent years that the three-dimensional structure of GDH from microorganisms is available (5, 6). Very recently, crystallization of bovine liver GDH was reported for the first time from the mammalian sources (7). However, remarkably little is known about the detailed structure of mammalian GDH, especially the brain enzymes.

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$^{319}$ (8), by a GMP probe at Met$^{169}$ and Tyr$^{262}$ (9), and by the adenosine analogue at Lys$^{429}$ and Tyr$^{100}$ (10). It seems, therefore, that the base moiety has not been effective at directing the site of modification by classical chemical probes.

Alternatively, identifying nucleotide binding sites of a variety of proteins has been advanced by the use of nucleotide photoaffinity analogues that 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 (11–14). The ATP binding site of adenylyl kinase and creatine kinase and the protein unique to cerebrospinal fluids of Alzheimer’s patients have been identified successfully using 2N$_3$ATP and 8N$_3$ATP (15, 16). The ADP regulatory site and the GTP binding site of bovine liver GDH also have been identified using $[^{32}P]2N_3NAD^+$ and $[^{32}P]8N_3GTP$, respectively (17, 18).

Because the pathology of the disorders associated with GDH defects is restricted to the brain, this enzyme may be of particular importance in the biology of the nervous system. Hussain et al. (19) detected four different forms of GDH isoproteins from human cerebral spinal fluids of normal subjects and patients with neurodegenerative disorders. The enzyme isolated from one patient with a variant form of multisystem atrophy displayed marked reduction of one of the GDH isoproteins (19). The isoproteins are differentially distributed in the two catalytically active isoforms of the enzyme (20). The origin of the GDH polymorphism is not known. Current studies showed the presence of four different sized mRNA and multiple gene copies for GDH in the human (20, 21). A novel cDNA encoded by an X chromosome-linked intronless gene was also isolated from human retina (22). Although the existence of brain GDH isoproteins has been recognized, the comparative studies of the GDH isoproteins from any sources are far less encompassing in protein function and structure. Further characterization of the structure and function of these various types of brain GDH is

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¶ The abbreviations used are: GDH, glutamate dehydrogenase; 2N$_3$NAD$^+$, nicotinamide 2-azidoadenosine dinucleotide; FSBA, 5'-p-fluorosulfonylbenzoyl adenine; HPLC, high performance liquid chromatography.
NAD$^+$ Binding Site of Brain GDH Isoproteins

Experimental Procedures

Materials—NADH, NAD$^+$, 2-oxoglutarate, ADP, and 1:1-tosylamido-2-phenethyl chloromethyl ketone-treated trypsin were purchased from Sigma. 2N$_3$NAD$^+$ was purchased from RPI Corp. [32P]2N$_3$NAD$^+$ was synthesized by the method described previously (12). [1,4-Dihydroxy-boryl)phenyl]sucinylimid derivatives of aminoethyl polyacrylamide (Affi-Gel 601) were obtained from Bio-Rad. All other chemicals and solvents were reagent grade or better.

Enzyme Purification and Assay—The GDH isoproteins were purified from bovine brains by the method developed in our laboratory (23) 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 220 nm, and the absorbance of the fractions was measured at 220 nm, and the photoincorporation was determined by liquid scintillation counting. For saturation studies, GDH I and GDH II (25–27) were photolyzed in the presence of 150 μM GTP and ADP on photoinsertion (Table II). It is well known that GTP increases the binding of NAD$^+$ while ADP weakens the binding with or without glutarate present (30, 31). Similar regulatory effects of these nucleotides were observed using [32P]2N$_3$NAD$^+$ when the enzymes were photolabeled with the photoprobe in the presence of GTP or ADP (Table II). Compared with a control, the addition of 1 mM GTP caused an approximate 2.3-fold increase of photoinsertion whereas the presence of 1 mM ADP caused an approximate 55% reduction. This reduction by ADP was lower than an 80% decrease in photoinsertion caused by the addition of NAD$^+$. When concentrations of the nucleotides were reduced, these effects were less extensive.

Saturation and Competition of Photoinsertion—To show specificity of the photoprobe-protein interaction, saturation of photoinsertion should be observed. The binding of NAD$^+$ to GDH I has been studied by equilibrium dialysis and initial rates (32). In the presence of glutarate, NAD$^+$ was bound more tightly (31). To demonstrate saturation effects with the photoprobe, the enzymes were photolabeled with increasing concentrations of [32P]2N$_3$NAD$^+$ in the presence of glutarate. Under the experimental conditions described, saturation of photoinsertion with [32P]2N$_3$NAD$^+$ occurred at around 40 μM photoprobe with apparent $K_d$ values near 10–15 μM for GDH isoproteins (Fig. 1). In all photolabeling experiments, the ion 

| Additions | Remaining activity$^a$ | % |
|-----------|------------------------|---|
| GDH I     | GDH II                 |
| No addition | 100 ± 3 100 ± 2       |   |
| 2 μM 2N$_3$NAD$^+$ | 99 ± 4 100 ± 3        |   |
| 3.5 μM NAD$^+$    | 98 ± 3 101 ± 4        |   |
| 4.5 μM 2N$_3$NAD$^+$ | 51 ± 4 52 ± 2        |   |
| 5.0 μM 2N$_3$NAD$^+$ + 500 μM NAD$^+$ | 88 ± 3 90 ± 3 |   |

$^a$ Mean of duplicates.

Both 2N$_3$NAD$^+$ and [32P]2N$_3$NAD$^+$ were present during the photolysis.

Results

Effects of 2N$_3$NAD$^+$ on GDH Activity—In the absence of photolysis, 2N$_3$NAD$^+$ is a substrate for the GDH isoproteins (Table I). The ability of 2N$_3$NAD$^+$ to inhibit the activities of GDH isoproteins by photoinsertion on irradiation under different conditions was investigated. GDH activities were not affected by UV light under these conditions. When the enzymes were covalently modified by photolysis in the presence of saturating amounts of 2N$_3$NAD$^+$, about 50% inhibition of the GDH activities was observed (Table I). When the enzymes were photolyzed in the presence of 500 μM NAD$^+$ in addition to 50 μM 2N$_3$NAD$^+$, about 88–90% of the initial activities of GDH isoproteins remained. These results suggest that 2N$_3$NAD$^+$ was photoinserting into a NAD$^+$ binding site on GDH isoproteins in a specific manner.

Active site involvement was also supported by the effects of GTP and ADP on photoinsertion (Table II). It is well known that GTP increases the binding of NAD$^+$ while ADP weakens the binding with or without glutarate present (30, 31). Similar regulatory effects of these nucleotides were observed using [32P]2N$_3$NAD$^+$ when the enzymes were photolabeled with the photoprobe in the presence of GTP or ADP (Table II). Compared with a control, the addition of 1 mM GTP caused an approximate 2.3-fold increase of photoinsertion whereas the presence of 1 mM ADP caused an approximate 55% reduction. This reduction by ADP was lower than an 80% decrease in photoinsertion caused by the addition of NAD$^+$. When concentrations of the nucleotides were reduced, these effects were less extensive.

Isolation of the Photolabeled Peptide and Protein Sequencing—The tryptic digested peptides were applied to a boronate column equilibrated with 0.1 M ammonium acetate. The absorbance of the fractions was measured at 220 nm, and the photoincorporation was determined by liquid scintillation counting. The fractions containing photolabeled peptides were desalted, freeze-dried, reconstituted in 0.1% trifluoroacetic acid, and subjected to reversed-phase HPLC using a Waters C$_{18}$ column on the same HPLC system. The mobile phase consisted of 0.1% trifluoroacetic acid solution and 0.1% trifluoroacetic acid, 80% acetonitrile solvent system. The gradient was 0–10 min, 6% acetonitrile; 10–60 min, 0–80% acetonitrile; 60–70 min, 80% acetonitrile at a flow rate 0.5 ml/min. HPLC fractions containing photolabeled peptides were pyridylated by the method described elsewhere (29) and sequenced by the Edman degradation method as described previously (23).

The pellet was resuspended in 75 mM NH$_4$HCO$_3$, pH 8.5, containing 2 M urea. GDH isoproteins were proteolyzed by the addition of 15 μg of trypsin, and the reaction mixtures were kept at 4 °C for 30 min. The reaction was quenched by the addition of ice-cold trichloroacetic acid (final 7%). The reaction mixtures were kept at 4 °C for 30 min, twice. The reaction was quenched by the addition of 75 mM NH$_4$HCO$_3$, pH 8.5. This procedure was repeated with a control, the addition of 1 mM GTP caused an approximate 2.3-fold increase of photoinsertion whereas the presence of 1 mM ADP caused an approximate 55% reduction. This reduction by ADP was lower than an 80% decrease in photoinsertion caused by the addition of NAD$^+$. When concentrations of the nucleotides were reduced, these effects were less extensive.
TABLE II

| Effector    | Photoincorporation* | Photoincorporation (GDH I) | Photoincorporation (GDH II) |
|-------------|---------------------|-----------------------------|-----------------------------|
| Control     | 100 ± 3             | 100 ± 2                     |                             |
| NAD⁺ (0.15 mM) | 21 ± 2             | 20 ± 3                      |                             |
| NAD⁺ (0.10 mM) | 21 ± 3             | 23 ± 2                      |                             |
| NAD⁺ (0.05 mM) | 27 ± 5             | 28 ± 2                      |                             |
| GTP (1.0 mM)  | 230 ± 3            | 225 ± 4                     |                             |
| GTP (0.5 mM)  | 161 ± 3            | 156 ± 2                     |                             |
| ADP (1.0 mM)  | 45 ± 4             | 44 ± 2                      |                             |
| ADP (0.5 mM)  | 49 ± 3             | 48 ± 3                      |                             |

* Mean of duplicates.

FIG. 2. The effect of NAD⁺ on [³²P]2N₃NAD⁺ phosphoincorporation into GDH isoproteins. GDH I and GDH II in the reaction buffer were photolabeled with the indicated concentrations of [³²P]2N₃NAD⁺, and ³²P incorporation into protein was determined by liquid scintillation counting (see "Experimental Procedures" for details). Relative ³²P incorporation into protein was determined and expressed as described in Fig. 1. ●, GDH I; □, GDH II.

strength was kept low to enhance binding affinity, as we have observed in general that the lower the ionic strength the tighter the binding of nucleotide photoaffinity probes and the more efficient the photoinsertion. Therefore, when interpreting the reported apparent Kᵥ values obtained from photoaffinity labeling one should consider that photolabeling is done under conditions that enhance binding site occupancy. The results in Fig. 1 indicate the saturability of the NAD⁺-specific site of GDH isoproteins with this photoprobe and therefore decrease the possibility of nonspecific photoinsertion.

To further demonstrate specific labeling of GDH isoproteins, the enzymes were photolabeled with [³²P]2N₃NAD⁺ in the presence of increasing NAD⁺ concentrations. As shown in the results of the competition experiments (Fig. 2), NAD⁺ was able to protect photolabeling from 10 μM [³²P]2N₃NAD⁺ at concentrations in the range of known Kᵥ values (32). Approximately, 80% protection was observed with 150 μM NAD⁺ for both GDH isoproteins (Fig. 2). These results show the specificity of [³²P]2N₃NAD⁺ and the utility of this probe as a good candidate for determining the NAD⁺ binding site.

Tryptic Digestion of Photolabeled Proteins and Isolation of the Photolabeled Peptide—To identify the peptides modified by [³²P]2N₃NAD⁺, GDH isoproteins were photolabeled twice in the absence and presence of 150 μM NAD⁺ and digested with trypsin. To reduce any possible nonspecific labeling and at the same time to optimize the specific labeling of the enzymes, 50 μM [³²P]2N₃NAD⁺ was used, which is the concentration at which photoinsertion approaches saturation. In addition, 1 mM GTP and 12 mM glutarate were included in the reaction mixture since they were shown to increase the binding affinity of NAD⁺ (30, 31) and [³²P]2N₃NAD⁺ (Table II) to GDH isoproteins. The photolabeled GDH isoproteins were separated from most of the noncovalently bound nucleotide by acid precipitation and proteolyzed by trypsin. The digested samples were applied to a boronate column equilibrated with 0.1 M ammonium acetate, pH 9.0. All of the unlabelled peptides were eluted with ammonium acetate buffer, pH 9.0, in the void volume, whereas photolabeled peptides were selectively retained on the column. The radioactive peptides were eluted with a pH gradient of 9.0–5.0 (Fig. 3). One major radioactive peak (indicated by the arrow in Fig. 3) around pH 6.5 was recovered from the column. NAD⁺ was able to reduce [³²P]2N₃NAD⁺ photoinser- tion into this peak. When 150 μM NAD⁺ was originally present in the incubation mixture, more than 90% of the radioactivity of the peak was eliminated as shown in Fig. 3. This result indicates that the radioactive peak represents a peptide in the NAD⁺ binding domain of the GDH isoproteins.

When the radioactive eluates from the boronate column were subjected to reversed-phase HPLC, one major radioactive peak (fractions 25–27) was clearly observed (Fig. 4). Although some radioactivity was found in the HPLC flow-through and wash fractions, over 90% of the total radioactivity co-eluted with the major peak. The radioactivity associated with the HPLC flow-through fractions represents unbound probe including any decomposition products of photoaduct produced as peptide binds to the HPLC column matrix. These flow-through fractions were subjected to analysis, and no significant amounts of amino acids were detected. The radioactive peptides (fractions 25–27) were collected and identified by sequence analysis.

GDH II gave almost identical chromatographic profiles to GDH I on both boronate and reversed-phase HPLC column, even though the intensities of the radioactivity were slightly higher than those 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 amino acid sequence analysis revealed that the peak fractions contained the amino acid sequence CIAVGIGDGSIWNPDGIDPK for both GDH isoproteins. The sequences obtained were also compared with those of various GDHs (Table III). As judged by comparison with the well known amino acid sequence of bovine liver GDH, this site was identified as residues 270–289 of bovine liver GDH. The X indicates a position for which no phenylthiohydantoin-derivative could be assigned. The missing residue, however, can be designated as a photolabeled glutamate since the sequences including the glutamate residue in question have a complete identity with those.
was photolabeled with $[^{32}P]2N_3NAD^+$ as described under "Experimental Procedures" in the absence and presence of 150 μM NAD$^+$. The tryptic peptides were loaded onto a 3-ml boronate affinity column equilibrated with 0.1 M ammonium acetate, pH 8.5. Unmodified peptides were eluted with the same buffer, and photolabeled peptide was eluted with a 200-ml gradient of pH 8.5–5.5 in 0.1 M ammonium acetate. Fractions (3 ml) were monitored for absorbance at 220 nm for radioactivity. The solid and dashed line represent the absorbance at 220 nm of the sample photolabeled in the absence and presence of 150 μM NAD$^+$, respectively. The plot also represents the radioactivity profiles from a boronate affinity column of the samples photolabeled in the absence (●) and the presence (○) of 150 μM NAD$^+$.

Fig. 4. Reversed-phase HPLC purification of tryptic peptides eluting from a boronate affinity column. The radioactive eluates (fractions 25–27) from a boronate affinity 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 (solid line). One-minute fractions were collected. The solid line with closed circles represents radioactivity. Levels of $[^{32}P]$ were determined by liquid scintillation counting in aqueous phase.

of the other GDH species known. The amino acid composition of the photolabeled peptide revealed that the peptide had a composition that was compatible with that of the tryptic peptide spanning residues 270–289 with the exception that there was a significant reduction in Glu$^275$ (data not shown). Photolabeling of the peptide was prevented by the presence of NAD$^+$ during photolysis. These results demonstrate selectivity of the photoprobe for the NAD$^+$ binding site and suggest that the peptide identified using the photoprobe is located in the NAD$^+$ binding domain of the brain GDH isoproteins. Both sequencing and compositional analysis identified Glu$^{275}$ as the site of photoinsertion.

**DISCUSSION**

In the present work, we identified an adenine binding domain peptide of the NAD$^+$ binding site of two GDH isoproteins from bovine brain using photoaffinity probe $[^{32}P]2N_3NAD^+$ and peptide analysis. $[^{32}P]2N_3NAD^+$ is a probe that, on photolysis, generates a very reactive nitrene that has the capacity of photoinserting into any residue. The data showing decreased photoinsertion by addition of NAD$^+$ demonstrates that photolysis occurs only by the bound form of $[^{32}P]2N_3NAD^+$. This indicates that proximity controls photoinsertion and that the residues modified are within the adenine binding domain. In addition, pre-photolysis followed by immediate addition of GDH isoproteins did not lead to covalent labeling (data not shown), eliminating the existence of any long lived chemically reactive intermediate that could be involved in covalently modifying enzymes. Saturation of photoinsertion at concentrations corresponding to that expected from the reversible binding affinities also strongly suggests that the site being labeled is within the binding domain. Their selectivity and specificity have been utilized successfully to locate the specific base binding domains of nucleotide binding site peptides of many proteins (11, 14–18).

The specificity of $[^{32}P]2N_3NAD^+$ and the utility of this probe as a good candidate for determining the NAD$^+$ binding site were further supported by the following. First, in the absence of activating light, 2N$_3$NAD$^+$ is a substrate for the GDH isoproteins (Table I). 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 $[^{32}P]2N_3NAD^+$. Saturation of photoinsertion with $[^{32}P]2N_3NAD^+$ occurred at around the 40 μM photoprobe with apparent K$_d$ values near 10 μM for both GDH isoproteins (Fig. 1). Third, active site involvement was also supported by the effects of GTP and ADP on photoinsertion (Table II). These results, together with the enhancement of photoinsertion by glutarate, present evidence of active site labeling of GDH isoproteins with 2N$_3$NAD$^+$.

To identify the site of photoinsertion, the photolabeled GDH isoproteins were digested with trypsin to produce peptides. Taking advantage of the existence of the two cis-hydroxyl groups on the ribose groups of the photoincorporated probe, the photolabeled peptides were isolated by using immobilized boronate column chromatography. It has been shown that immobilized boronates can be successfully used to fractionate adenosine and pyridine nucleotides (33) and nucleosidyl peptides (10, 34). By using a pH gradient elution, a highly purified radiolabeled peptide was obtained from each isoprotein, and the peptide exhibited an unusual UV spectrum with a maximum absorption of 262 nm and a shoulder near 278 nm, which verifies that an adduct of the $[^{32}P]2N_3NAD^+$ photoprobe is still covalently attached to the peptides. The boronate chromatography greatly reduces the possibility of any non-photolabeled peptide co-eluting on HPLC with the photolabeled peptide, which could give misleading results. The sequences identified in the present study correspond to residues 270–289 of the amino acid sequence of well known bovine liver GDH (Table III). On the basis of information obtained on the amino acid sequence determination and composition analysis of the isolated peptide, we suggest that the attachment site of $[^{32}P]2N_3NAD^+$ is Glu$^{275}$.

In contrast to our approach, several classical chemical probes have been used to attempt resolution of the binding sites and have shown quite discrepant results. The NADH binding site was proposed to be modified by an ATP analogue at Cys$^{319}$ (8), by a GMP probe at Met$^{169}$ and Tyr$^{262}$ (9), and by the adenosine analogue at Lys$^{420}$ and Tyr$^{190}$ (10). Similar results with discrepancies using classical chemical probes were also 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$^{262}$ (35) and Arg$^{459}$ (36). These two residues are outside the catalytic cleft. The GTP binding site was also proposed to be modified by a fluorescent FSBA adenosine analogue at Tyr$^{262}$ (37). As indicated elsewhere (17), it is not clear why a hydrophobic adenosine-containing probe 5’FSBeA preferentially
binds and react at a hydrophilic GTP binding site and does not react at the other adenosine binding sites. It seems, therefore, that the base moiety has not been effective at directing the site of modification by classical chemical probes. Chemically reactive probes, because of their long-lived reactive state, have an increased opportunity to react with the most nucleophilic residue within an enzyme and may not necessarily react with a less reactive or nonreactive residue that may be located within the binding domain. This is especially likely if they display low affinity for the binding site being studied. 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 (8, 9).

Very recently, Stanley et al. (38) have reported that the hyperinsulinism-hyperammonemia syndrome is caused by mutations in GDH gene that affect enzyme sensitivity to GTP-induced inhibition. The mutations identified in the patients with hyperinsulinism and hyperammonemia (38) lie exactly within a sequence of 15 amino acids that we previously suggested contains the GTP binding site of the brain GDH isoproteins (24). On the other hand, the location of the mutations on GDH in those mutations are quite distinct from the GTP binding site identified by using the classical chemical probe (37). These results prove selectivity and specificity of the photoaffinity probe as a valid active site probe.

The crystal structure of Clostridium symbiosum GDH has been aligned with the primary sequence of the bovine liver GDH (28, 39). The structures of C. symbiosum and mammalian GDHs were suggested to be similar due to considerable identity of 13 glycine residues, which probably accounts for the inability of NADP$^+$ to be a substrate for these dehydrogenases whereas it is a good substrate for mammalian GDH. This concept is supported by the observation that the equivalent position of Glu$^{275}$ in NADP$^+$-specific dihydrofolate reductase is replaced by a positively charged Arg residue. It is likely that the reason for the Glu replacement for Asp in GDH is to produce a different active site conformation that allows both NAD$^+$ and NADP$^+$ binding.

To our knowledge, comparison of the detailed structure of active sites and regulatory sites of any GDH isoproteins rarely has been reported. The work presented here clearly identifies the NAD$^+$ binding site of the brain GDH isoproteins in the overall sequence.

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**TABLE III**

Alignment of [13P]2NAD$^+$-modified peptide with homologous sequence from various GDHs

| Source             | Reference          | Isotypes | Amino acid sequence$^a$ |
|--------------------|--------------------|----------|------------------------|
| Bovine brain       | This work          | GDH I    | C I A V G$^a$b S D G S I W N P D G I D P K |
| Bovine brain       | This work          | GDH II   | C I A V G$^a$b S D G S I W N P D G I D P K |
| Human brain        | (45)               | GDH 1    | C I A V G$^a$b S D G S I W N P D G I D P K |
| Human retina       | (22)               | GDH 2    | C I A V G$^a$b S D G S I W N P D G I D P K |
| Mouse brain        | (46)               |          | C I A V G$^a$b S D G S I W N P D G I D P K |
| Rat brain          | (46)               |          | C I A V G$^a$b S D G S I W N P D G I D P K |
| Bovine liver       | (47)               |          | C I A V G$^a$b S D G S I W N P D G I D P K |

$^a$ The amino acids are denoted by the single-letter code.

$^b$ X indicates that no identifiable phenylthiohydantoin-derivative was observed at this sequencing cycle.

$^c$ The amino acid numbering is that of bovine liver GDH (46).
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