Importance of Glutamate 279 for the Coenzyme Binding of Human Glutamate Dehydrogenase*

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Although the structure of glutamate dehydrogenase (GDH) has been reported from various sources including mammalian GDH, there are conflicting views regarding the location and mechanism of actions of the coenzyme binding. We have expanded these speculations by photoaffinity labeling and cassette mutagenesis. Photoaffinity labeling with a specific probe, [32P]nicotinamide 2-azidoadenosine dinucleotide, was used to identify the NAD⁺ binding site within human GDH encoded by the synthetic human GDH gene and expressed in Escherichia coli as a soluble protein. Photolabel-containing peptides generated with trypsin were isolated by immobilized boronate affinity chromatography. Photolabeling of these peptides was most effectively prevented by immobilized boronate affinity chromatography. Photolabeling of these peptides was most effectively prevented by the presence of NAD⁺ during photolysis, demonstrating a selectivity of the photoprobe for the NAD⁺ binding site. Amino acid sequencing and compositional analysis identified Glu279 as the site of photoinertion into human GDH, suggesting that Glu279 is located at or near the NAD⁺ binding site. The importance of the Glu279 residue in the binding of NAD⁺ was further examined by cassette mutagenesis with mutant enzymes containing Arg, Gly, Leu, Met, or Tyr at position 279. The mutagenesis at Glu279 has no effects on the expression or stability of the differently involved GDHs. The Kₘ values for NAD⁺ were 10–14-fold greater for the mutant GDHs than for wild-type GDH, whereas the Vₘₐₓ values were similar for wild-type and mutant GDHs. The efficiency (Kₘ/Vₘₐₓ) of the mutant GDH was reduced up to 18-fold. The decreased efficiency of the mutants results from the increase in Kᵥ values for NAD⁺. In contrast to the Kₘ values for NAD⁺, wild-type and mutant GDHs show similar Kᵥ values for glutamate, indicating that substitution at position 279 had no appreciable effect on the affinity of enzyme for glutamate. There were no differences in sensitivities to ADP activation and GTP inhibition between wild-type and mutant GDH, suggesting that Glu279 is not directly involved in allosteric regulation. The results with photoaffinity labeling and cassette mutagenesis studies suggest that Glu279 plays an important role for efficient binding of NAD⁺ to human GDH.

Glutamate dehydrogenase (GDH; EC 1.4.1.3) catalyzes the reversible reaction of 2-oxoglutarate to L-glutamate using NADH or NADPH (1). There are three types of GDH that vary according to the coenzyme they use: NAD(H)-specific GDH, NADP(H)-specific GDH, and GDH with mixed specificity. The bacterial and fungal NADP⁺-linked and vertebrate dual specificity GDHs have six identical subunits, with a subunit size between 48 kDa (Escherichia coli) (2) and 55–57 kDa (vertebrate) (1, 3), whereas the NAD⁺-linked enzymes have either four identical subunits with a size of ~115 kDa (Neurospora crassa) (4) or six identical subunits with a subunit size of ~48 kDa (Clostridium symbiosum) (5). NAD(H)-specific enzymes are believed to participate mainly in the catabolism of glutamate, but NADP(H)-specific enzymes have a mainly anabolic role. Unlike GDH from primitive organisms, mammalian GDH uses both forms of coenzyme with comparable efficacy, and the anabolic/catabolic balance is therefore tightly controlled by a complex network of allosteric regulators.

The structures of GDHs from microbial and mammalian sources have been reported previously (6–11). The largest difference between mammalian GDH and bacterial GDH is a long antenna domain in mammalian GDH formed by the 48-amino acid insertion starting at residue 385 (10–12). Mammalian GDH is strictly regulated by allosteric activators and inhibitors (1–3). GTP inhibits enzyme turnover over a wide range of conditions by increasing the affinity of the enzyme for the product, making product release rate-limiting under all conditions in the presence of ADP (10–14). ADP is a potent activator decreasing product affinity (11, 15, 16). In contrast to vertebrate GDH, bacterial GDH is not regulated by the allosteric regulators (4, 5). Therefore, it has been suggested that the antenna domain that is unique to mammalian GDH has important roles in allosteric regulation (10, 11). However, it is well documented that the regulatory pattern of GDH is very complicated (1, 3, 15–18), and therefore it cannot be completely explained only by the existence of the antenna region. For results, ADP is generally considered as an activator of mammalian GDH (11, 18, 19), but it can also inhibit GDH activity under some conditions, such as low pH (3, 15). The most recent study shows that GTP binds to GDH from E. coli at an allosteric site and reverses the destabilizing effects of the coenzyme (20). This result strongly suggests that the unique 48-amino acid antenna region in mammalian GDH may not be wholly responsible for the observed regulation of ADP and GTP.

It has been a major goal to identify the substrate and regulatory binding sites of GDH. Identification of the nucleotide

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** The abbreviations used are: GDH, glutamate dehydrogenase; 2N,NAD⁺, nicotinamide 2-azidoadenosine dinucleotide; HPLC, high performance liquid chromatography.
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, $[^{32}P]2N\text{NAD}^+$ was shown to be a valid active-site probe for several proteins (21–24). GTP and ADP binding sites of bovine GDH have been reported using $[^{32}P]5\text{NTP}$ and $[^{32}P]5\text{NAD}$, respectively (25–28). The ATP binding site of adenylate kinase, creatine kinase, and the protein unique to cerebrospinal fluids of Alzheimer patients has also been sequenced by means of $5\text{NTP}$ and $5\text{NAD}$ (29, 30). Recent studies of the x-ray structure of bovine liver GDH indicate that Glu275 (Glu279 in human enzyme) forms a hydrogen bond with the coenzyme (10, 11). However, no information about the importance of this residue in the direct binding with coenzyme has been reported. Studies of the effects of site-directed mutagenesis at the Glu279 site on the affinity and kinetics of coenzyme binding are necessary to obtain such information.

Recently, a 1557-bp gene that encodes human GDH has been synthesized and expressed in E. coli in our laboratory (31). Using this synthetic human GDH gene, reactive amino acid residues for catalysis (31), GTP base binding (32), and ADP base binding (33) have been identified by cassette mutagenesis. In the present work, we report identification of an NAD$^+$ binding site of human GDH by a combination of cassette mutagenesis and photolabeling. For the present study, the mutant human GDHs containing Gly, Leu, Met, Arg, or Tyr at the Glu279 site have been expressed in E. coli as a soluble protein and characterized. Our data place the NAD$^+$ binding domain within a proposed catalytic cleft defined in the crystal structure of GDH (10, 11, 34, 35). To our knowledge, this is the first report by site-directed mutagenesis showing an involvement of Glu279 of mammalian GDH in NAD$^+$ binding.

**EXPERIMENTAL PROCEDURES**

**Materials**—NADH, NAD$^+$, 2-oxoglutarate, ADP, and 1-tosylamido-2-phenylthyl chloromethyl ketone-treated trypsin were purchased from Sigma. Human GDH gene (pHGDH) has been chemically synthesized and expressed in Escherichia coli in our laboratory as described elsewhere (31). 2N$\text{NAD}^+$ and 2N$\text{NAD}$ were synthesized using NNN adenylyltransferase according to the same method described previously (21). NNN adenylyltransferase was kindly provided by Dr. Mathias Ziegler (Institut für Biochemie, Freie Universität Berlin). Precast gels for SDS-PAGE were purchased from Novex. All other chemicals and solvents were of reagent grade or better.

**Bacterial Strains**—E. coli DH5α (36) was used as the host strain for plasmid-mediated transformations for cassette mutagenesis. E. coli PA340 (thr–1 flbA2 leuB6 lacYI supE44 gal–1 hisG1 rfbD1 gdiP83 sgtF8/F900 rpaL1 malI1 tyl-7 mtl-2 argH1 thi–1; kindly provided by Dr. Mary K. B. Berlyn, Biochemie, Freie Universität, Berlin) was used as a host strain for cassette mutagenesis. Precast gels for SDS-PAGE were purchased from Novex. All other chemicals and solvents were of reagent grade or better.

**Photolabeling of GDH**—Photolabeling of wild-type human GDH was performed by the method of Kim and Haley (21), with a slight modification. For saturation studies, wild-type GDH (100 μg) in 10 mM Tris acetate, pH 8.0, containing 12 mM glutarate was incubated with various concentrations of $[^{32}P]2N\text{NAD}^+$ in Eppendorf tubes for 5 min. Glutamate was included in the reaction mixture because it has been reported by equilibrium dialysis and initial rates that glutamate makes NAD$^+$ bind to GDH more tightly (39–41). For competition studies, 100 μg of enzyme was incubated with various concentrations of NAD$^+$ for 10 min in the same buffer before the addition of 100 μM $[^{32}P]2N\text{NAD}^+$ and then incubated with the photoprobe for 5 min as described above. The samples were irradiated twice with a hand-held 254 nm UV lamp for 90 s at 4 °C. The reaction was quenched by the addition of ice-cold trichloroacetic acid (final concentration, 7%). The reaction mixtures were kept on ice for 30 min and centrifuged at 10,000 g for 15 min at 4 °C. The pellets were washed and resuspended with 10 mM Tris acetate, pH 8.0. 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.

**Construction and Characterization of Glu279 Mutants**—Cassette mutagenesis at the Glu279 site was performed using a synthetic human GDH gene, pHGDH (31). The plasmid DNA (5 μg) was digested with NsiI and EspDI to remove the 46-bp fragment that encodes amino acids 275–290, which was replaced with five 46-bp synthetic DNA duplexes containing a substitution on both DNA strands at positions encoding Glu279 to make E279G, E279L, E279M, E279R, and E279Y mutants. Recombinant proteins were identified using 2N3ATP and 8N3ATP (29, 30). Recent advances of the x-ray structure of bovine liver GDH indicate that Glu275 (Glu279 in human enzyme) forms a hydrogen bond with the coenzyme (10, 11). However, no information about the importance of this residue in the direct binding with coenzyme has been reported. Studies of the effects of site-directed mutagenesis at the Glu279 site on the affinity and kinetics of coenzyme binding are necessary to obtain such information.

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RESULTS

Specific Binding of \(2\text{N}_{\text{AD}}^{+}\) to Human GDH—The role of Glu-279 in the direct binding of \(\text{NAD}^{+}\) to human GDH was examined by photoaffinity labeling with \([^{32}\text{P}]2\text{N}_{\text{AD}}^{+}\). To show specificity of the photoprobe-protein interaction, saturation of photoinsertion should be observed. To demonstrate saturation effects with the photoprobe, the purified enzymes were photolabeled with increasing concentrations of \([^{32}\text{P}]2\text{N}_{\text{AD}}^{+}\) in the presence of glutarate. Under the experimental conditions described, saturation of photoinsertion with \([^{32}\text{P}]2\text{N}_{\text{AD}}^{+}\) occurred at around 95 \(\mu\text{M}\) photoprobe (Fig. 1). In all photolabeling experiments, the ionic strength was kept low to enhance binding affinity because we have observed in general that the lower the ionic strength, the tighter the binding of nucleotide photofluorescence probes and the more efficient the photoinsertion. The results in Fig. 1 indicate the saturability of the \(\text{NAD}^{+}\)-specific site of GDH with this photoprobe and therefore decrease the possibility of nonspecific photoinsertion. To further demonstrate specific labeling of GDH, wild-type GDH was photolabeled with \([^{32}\text{P}]2\text{N}_{\text{AD}}^{+}\) in the presence of increasing \(\text{NAD}^{+}\) concentrations. As shown in the results of the competition experiments (Fig. 2), \(\text{NAD}^{+}\) was able to protect photolabeling from 10 \(\mu\text{M}\) \([^{32}\text{P}]2\text{N}_{\text{AD}}^{+}\) at concentrations in the range of known \(K_{d}\) values (41). Approximately 85\% protection was observed with 150 \(\mu\text{M}\) \(\text{NAD}^{+}\) for wild-type GDH (Fig. 2). These results show the specificity and utility of \([^{32}\text{P}]2\text{N}_{\text{AD}}^{+}\) as a good probe for determining the \(\text{NAD}^{+}\) binding site.

Tryptic Digestion of Photolabeled Proteins and Boronate Affinity Column—To identify the peptides modified by \([^{32}\text{P}]2\text{N}_{\text{AD}}^{+}\), wild-type GDH was photolabeled twice in the absence and presence of 300 \(\mu\text{M}\) \(\text{NAD}^{+}\) and digested with trypsin. To reduce any possible nonspecific labeling and, at the same time, optimize the specific labeling of the enzymes, 10 \(\mu\text{M}\) \([^{32}\text{P}]2\text{N}_{\text{AD}}^{+}\) was used, which is the concentration at which photoinsertion approaches saturation. The photolabeled proteins were separated from most of the noncovalently bound nucleotide by acid precipitation and proteolyzed by trypsin. 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 boronate can be successfully used to fractionate adenine and pyridine nucleotides (21, 45) and nucleosidyl peptides (46, 47). The trypic digested GDHs were applied to the boronate column equilibrated with 0.1 m ammonium acetate, pH 9.0. After washing the column with ammonium acetate buffer, pH 9.0, the radioactive peptides were eluted with a pH gradient of 9.0 to 5.0 (Fig. 3). One major radioactive peak around pH 6.5 was observed for wild-type GDH. The peptides 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 \([^{32}\text{P}]2\text{N}_{\text{AD}}^{+}\) photoprobe is still covalently attached to the peptides. Photolabeling of the peptide was prevented by the presence of \(\text{NAD}^{+}\) during photolysis. When 300 \(\mu\text{M}\) \(\text{NAD}^{+}\) was originally present in the incubation mixture, 90\% of the radioactivity of the peak was eliminated, as shown in Fig. 3. These results demonstrate the selectivity of the photoprobe and suggest that the radioactive peak represents a peptide in the \(\text{NAD}^{+}\) binding domain of human GDH.

When the radioactive eluates from the boronate column were subjected to reversed-phase HPLC, one major radioactive peak was clearly observed (data not shown). The radioactive peptide was collected and identified by amino acid sequence analysis.

Sequence Analysis of Photolabeled Peptide—The amino acid sequence analysis revealed that the peak fractions contained the amino acid sequence CIAVGXS6G6SIWNPDG1. The sequences obtained were also compared with those of various GDHs (Table I). As judged by comparison with the amino acid sequence of mammalian GDHs, this site was identified as residues 274–290 of human GDH. 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 Glu because the sequences including the Glu residue in question have a complete identity with those 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 274–293, with the exception that there was a significant reduction in Glu (Table II). 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 \([^{32}\text{P}]2\text{N}_{\text{AD}}^{+}\) is Glu-279. The impor-
The 46-bp NsiI/EspDI fragment in pHGDH was replaced with five different 46-bp synthetic DNA duplexes containing a substitution on both DNA strands at positions encoding Glu279. These substitutions made mutant proteins E279G, E279L, E279M, E279R, and E279Y at position 279. The five mutants were designed to have different size, hydrophobicity, and ionization of the side chains at each position. Analysis of crude cell extracts by Western blot showed that Glu279 mutant plasmids encoding an amino acid substitution at position 279 directed the synthesis of a protein that interacted with monoclonal antibodies raised against GDH at almost identical levels for all Glu279 mutants and wild-type GDH (Fig. 4). These results indicate that the mutagenesis at Glu279 has no effects on expression or stability of the different mutants. The mutants also appeared to be as stable as wild-type human GDH, on the basis of their stability toward proteolysis and retention of activity upon prolonged storage at 4°C. In addition, the mutant enzymes were purified homogeneously by the same method as the wild-type enzyme (data not shown), indicating that no gross conformational change in the enzyme had occurred.

**Kinetic Properties for Mutant GDH**—To evaluate the kinetic properties for the mutants, the $K_m$ values for the individual substrates were determined. Detailed investigation of the catalytic activities of the mutant enzymes revealed an ~10–14-fold decrease in the respective apparent $K_m$ values and an 11–18-fold decrease in the $k_{cat}/K_m$ values for NAD$^+$ compared with those of wild-type GDH (Table III). Although the mutations at position 279 produce an increase in the apparent $K_m$ values for NAD$^+$, all the mutants and the wild-type enzyme show similar $k_{cat}$ values of the same order of magnitude (Table III). Therefore, the dramatic reductions in the catalytic efficiencies ($k_{cat}/K_m$) of the Glu279 mutants primarily reflect changes in the $K_m$ values for NAD$^+$ and suggest that the mutations at the Glu279 site reduced the affinity of the enzyme for NAD$^+$ binding. These results suggest that Glu279 plays an important role for efficient binding of the coenzyme to human GDH.

The $K_m$ values for glutamate for wild-type and Glu279 mutants were also determined in the standard assay mixture at various concentrations of glutamate. In contrast to the $K_m$ values for NAD$^+$, the apparent $K_m$ values for glutamate obtained from Lineweaver-Burk plots increased only slightly (3.05 and 3.44–4.12 mM for wild-type and mutant GDHs, respectively) (Table IV). These slight changes may be due to a local conformational change in substrate binding by the mutant enzymes. However, the similarities in $k_{cat}/K_m$ values (14–19 s$^{-1}$ mM$^{-1}$) for glutamate between wild-type and mutant enzymes suggest that substitution at position 279 might have no appreciable effect on the affinity of the enzyme for glutamate.

**TABLE I**

| Source GDH | Reference | Amino acid sequence |
|-----------|-----------|---------------------|
| Human GDH | This work | CIAGXXDCUWPDGI$^*$  |
| Human liver | 55 | CIAGXXDCUWPDGI |
| Human retina | 17 | CIAGXXDCUWPDGI |
| Bovine brain | 3 | CIAGXXDCUWPDGI |
| Bovine liver | 56 | CIAGXXDCUWPDGI |
| Rat brain | 57 | CIAGXXCULWPDGI |

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

$^b$ Only the first 17 cycles were sequenced.

$^c$ The amino acid numbering is that of the mature human liver GDH (55).

**TABLE II**

| Amino acid analysis of the photolabeled peptide |
|-----------------------------------------------|
| Amino acid | Predicted | Determined$^a$ |
|-----------|-----------|---------------|
| Ile       | 3         | 3.2 (3)$^a$   |
| Ala       | 1         | 1.2 (1)       |
| Val       | 1         | 0.9 (1)       |
| Gly       | 3         | 2.8 (3)       |
| Glu       | 1         | 0.1 (0)       |
| Ser       | 2         | 2.3 (2)       |
| Asp       | 3         | 2.9 (3)       |
| Trp       | 1         | 0.9 (1)       |
| Asn       | 1         | 1.2 (1)       |
| Pro       | 2         | 2.1 (2)       |
| Lys$^c$   | 1         | 1.1 (1)       |
| Cys$^c$   | 1         | 0.9 (1)       |

$^a$ Normalized values. Values of molar ratio less than 0.1 are not indicated.

$^b$ Nearest integer.

$^c$ Cys was pyridylethylated before it was quantified (58). It represents a sum of cysteine and cystine.

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ADP and GTP Effects on Human GDH Activity—Finally, the effects of ADP and GTP, well-known allosteric regulators, on the activities of wild-type and mutant GDHs were compared. There were no differences between wild-type and mutant GDHs in sensitivity to ADP activation at concentrations between 0.1 and 1.0 mM and GTP inhibition at concentrations between 1 and 50 μM (Table V). These results indicate that Glu279 is not responsible for the allosteric regulation of human GDH by ADP or GTP.

DISCUSSION

The construction of a synthetic gene encoding human GDH will enable us to generate a large number of site-directed mutations at several positions in the coding region. The high level of GDH expression as a soluble protein in E. coli will facilitate the purification of large quantities of mutant proteins for biochemical and structural studies. This combination of genetic and biochemical techniques could be used to address a broad range of questions related to the structure and function of human GDH. In the present work, we identified an adenine binding domain peptide of the NAD⁺ binding site of human GDH using cassette mutagenesis of the synthetic human GDH gene and photoaffinity probe [32P]2N3NAD⁺. [32P]2N3NAD⁺ is a probe that, on photolysis, generates a very reactive nitrone that has the capacity to photoinsert into any residue. The data showing decreased photoinsertion by addition of NAD⁺ demonstrate that photoinsertion occurs only by the bound form of [32P]2N3NAD⁺. 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 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. The selectivity and specificity have been successfully utilized to locate the specific base binding domains of the nucleotide binding site peptides of many proteins (23–30).

The specificity of [32P]2N3NAD⁺ and the utility of this probe as a good candidate for determining the NAD⁺ binding site were further supported by the following observations. First, in the absence of activating light, 2N3NAD⁺ is a substrate for GDH (data not shown). 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 was saturated with [32P]2N3NAD⁺. Saturation of photoinsertion with [32P]2N3NAD⁺ occurred at around 95 μM photoprobe (Fig. 1). Third, the prevention of photoinsertion of [32P]2N3NAD⁺ by NAD⁺ demonstrates that the photoprobe is inserted into a specific NAD⁺ site within GDH (Fig. 2). In addition, the sites of attachment of the photoaffinity label were more precisely defined by generating small peptide fragments of the labeled protein and separating the labeled peptides. For separation of the [32P]-labeled peptide fragments generated in the proteolytic digest, immobilized boronate column chromatography was carried out before HPLC separation. The boronate chromatography greatly reduces the possibility of any nonphotolabeled peptide co-eluting on HPLC with the photolabeled peptide, which could give misleading results.

There were differences in the biochemical properties between wild-type GDH and Glu279 mutants. The Kₘ values for NAD⁺ increased 10–14-fold in the Glu279 mutants compared with those of wild-type GDH (Table III). Although the mutations at position 279 produce changes in the apparent Kₘ values for NAD⁺, all the mutants and wild-type enzyme show similar kₗₜ values of the same order of magnitude (Table III). The results from the Western blot analysis (Fig. 4) show that the mutagenesis at the Glu279 site has no effects on expression or stability of the different mutant GDHs. Therefore, the dramatic reductions in the catalytic efficiencies (kₗₜ/Kₘ) of the Glu279 mutants primarily reflect changes in the Kₘ values for NAD⁺ and suggest that Glu279 plays a role in NAD⁺ binding. The importance of Glu279 in NAD⁺ binding is supported by the interesting information that the invariant, functional residue Asp is found in the β-sheet region of four known dehydrogenases and is conservatively changed to Glu 279 in mammalian GDHs (34). With these dehydrogenases, Asp is proposed to be involved in hydrogen bond formation with the O-2' atom of the ribose group, which could not occur, due to charge repulsion, with NAD⁺. This probably accounts for the inability of NAD⁺ 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 Glu279 in NADP⁺-specific dihydrofolate reductase is replaced by a positively charged Arg residue. It is likely that the reason for Glu replacement of Asp in GDH is to produce a different active site conformation that allows both NAD⁺ and NADP⁺ binding.

The crystal structure of C. symbiosum GDH has been
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