Cassette Mutagenesis and Photoaffinity Labeling of Adenine Binding Domain of ADP Regulatory Site within Human Glutamate Dehydrogenase†

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ABSTRACT: The adenine binding domain of the ADP site within human glutamate dehydrogenase (GDH) was identified by cassette mutagenesis at the Tyr187 position. The wild type GDH was activated 3-fold by ADP at a concentration of 1 mM at pH 8.0, whereas no significant activation by ADP was observed with the Tyr187 mutant GDH regardless of the size, hydrophobicity, and ionization of the side chains. Studies of the steady-state velocity of the mutant enzymes revealed essentially unchanged apparent K_m values for 2-oxoglutarate and NADH, but an ∼4-fold decrease in the respective apparent V_{max} values. The binding of ADP to the wild type or mutant GDH was further examined by photoaffinity labeling with [α-^{32}P]8-azidoadenosine 5′-diphosphate (8N_3ADP). 8N_3ADP, without photolysis, mimicked the stimulatory properties of ADP on GDH activity. Saturation of photoinsertion with 8N_3ADP occurred with apparent K_d values near 25 μM for the wild type GDH, and the photoinsertion of [α-^{32}P]8N_3ADP was decreased best by ADP in comparison to other nucleotides. Unlike the wild type GDH, essentially no photoinsertion was detected for the Tyr187 mutant GDH in the presence or absence of 1 mM ADP. For the wild type GDH, photolabel-containing peptide generated by tryptic digestion was identified in the region containing the sequence EMSWIADTYASTIG, and the photolabeling of this peptide was prevented >95% by the presence of 1 mM ADP during photolysis, whereas no such a peptide was detected for the Tyr187 mutant GDH in the presence or absence of ADP. These results with cassette mutagenesis and photoaffinity labeling demonstrate selectivity of the photoprobe for the ADP binding site and suggest that the photolabeled peptide is within the ADP binding domain of the human GDH and that Tyr187 is responsible for the efficient base binding of ADP to human GDH.

Mammalian glutamate dehydrogenase (GDH)[1] reversibly catalyzes the reductive amination of 2-oxoglutarate L-glutamate using NADH or NADPH as coenzyme (*1*). It plays a role in regulating the levels of ammonia and glutamate in the central nervous system and integrates carbon and nitrogen metabolism via the citric acid cycle. The importance of the physiological nature of GDH has attracted considerable interest (*2*, *3*−*6*). The existence of the hyperinsulinism−hyperammonemia syndrome caused by mutations in a human GDH gene that affects enzyme sensitivity to GTP-induced inhibition highlights further the importance of GDH (*7*−*9*). The mutated residues responsible for this pathology were found to lie mainly around the GTP site (*9*). These observations demonstrate that allosteric regulation of GDH plays a crucial role in the regulation of insulin secretion and hepatic ureagenesis. GTP is a potent inhibitor that acts by slowing the product release (*10*−*12*). In addition, NADH, a substrate for the reverse reaction, has also been reported to inhibit the forward reaction (*13*). In contrast, ADP acts as an activator by facilitating product release (*3*, *14*). However, it has been well documented that ADP regulation is quite complex (*14*). A further understanding of the mechanism and details of this regulation will help us to elucidate the metabolic role that GDH has in cellular homeostasis.

The recently elucidated atomic structure of bovine liver GDH has suggested that the allosteric regulation and negative cooperativity observed in mammalian GDH may be facilitated by the subunit interactions and performed by changing the energy required to open and close the catalytic cleft during enzymatic turnover (*10*, *11*). Previous studies using classical chemical probes to identify the ADP binding sites within bovine liver GDH also have given a wide scatter of modified residues throughout most of the proposed three-dimensional structure of GDH. For instance, the ADP binding site has been proposed to be modified by different ADP analogues at His82 (*15*, *16*) and at Arg459 (*17*). Similarly, benzophenone-based nucleotide analogues also have been used to identify the peptides within the terminal phosphate binding domains of ADP binding sites but showed different results when modified at Arg491 (*18*) and at a peptide corresponding to Met411−Arg419 (*19*). Alternatively, various azidonucleotide photoprobes have been used to identify

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[1] Abbreviations: GDH, glutamate dehydrogenase; 8N_3ADP, 8-azidoadenosine 5′-diphosphate; HPLC, high-performance liquid chromatography; IPTG, isopropyl β-D-thiogalactopyranoside.

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the base binding domains of the allosteric sites and to localize the domains within the proposed catalytic cleft (*20*−*24*). Azidonucleotide photoprobes generate short-lived, very reactive nitrenes that will modify any residue placed near the generated nitrene by binding. 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. However, the similarities between the structures of NAD^+ and ADP could contribute to the difficulties in identifying the specific ADP binding site. It was also reported that the affinity of ADP and azido-ADP to GDH is not as tight as that of NAD^+, GTP, and their corresponding photoaffinity probes (*21*). This is most likely due to fewer points of protein−nucleotide contact, which would account for loose binding. Therefore, the exact residues photolabeled within the adenine ring domain within the ADP binding site have not been identified. It is clear that direct evidence such as site-directed mutagenesis would be necessary to identify the ADP binding site within GDH.

Very recently, a 1557 base-pair gene that encodes human GDH has been synthesized and expressed in *Escherichia coli* in our laboratory (*25*). In the present work, we have expanded the identification of the ADP binding site of human GDH using a cassette mutagenesis and photoaffinity labeling with $8N_3$ADP at the Tyr187 residue that has been located within a putative base binding domain of the ADP sites (*19*, *21*, *24*). For the present study, the mutant human GDHs containing Arg, Glu, Gly, Met, or Ser at the Tyr187 site have been expressed in *E. coli* as a soluble protein, purified, and characterized. To our knowledge, this is the first study by site-directed mutagenesis showing that Tyr187 is required for the efficient binding of ADP to human GDH.

MATERIALS AND METHODS

Materials. $NADP^+$, NAD^+, NADH, NADPH, 2-oxoglutarate, ATP, ADP, IPTG, ADP, L-glutamate, and L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin were purchased from Sigma. $8N_3$ADP was purchased from ALT Corp. [α-^{32}P]$8N_3$ADP was synthesized according to the method as previously described (*21*, *24*). Epoxy-activated iminodiacetate Sepharose 6B was obtained from Sigma. Human GDH gene (pHGDH) has been chemically synthesized and expressed in *E. coli* as a soluble protein in our laboratory as described elsewhere (*25*). All other chemicals and solvents were of reagent grade or better.

Bacterial Strains. E. coli DH5α (*26*) was used as the host strain for plasmid-mediated transformations for cassette mutagenesis. *E. coli* PA340 (*thr-1 fhuA2 leuB6 lacY1 supE44 gal-6 gdh-1 hisG1 rfbD1 galP63* Δ*(gltB*−*F)500 rpsL19 malT1 xyl-7 mtl-2 argH1 thi-1*; kindly provided by Dr. Mary K. B. Berlyn, *E. coli* Genetic Stock Center, Yale University) lacked both GDH and glutamate synthtase activities (*27*) and was used to test plasmids for GDH activity. *E. coli* BL21 (DE3) (*28*) was used for high-level expression of the recombinant human GDH.

Construction and Characterization of Tyr187 Mutant. A series of single amino acid substitutions of Tyr187 was constructed separately by cassette mutagenesis of a synthetic human GDH gene, pHGDH (*25*). Plasmid DNA (5 μg) was digested with *Age*I and *Eco*RV to remove the 60 base-pair fragment that encodes amino acids 176−195 and replaced with five 60 base-pair synthetic DNA duplexes containing a substitution on both DNA strands at positions encoding Tyr187 to make Y187E, Y187G, Y187M, Y187R, and Y187S. The mutant enzymes were identified by DNA sequencing using plasmid DNA as a template. Each of these mutants has been expressed in *E. coli* strain DE3 and purified to homogeneity as described above and has had its steady-state kinetic parameters determined. The gene expression levels of the Tyr187 mutant GDHs were examined by western blot and compared with those of wild type GDH.

Purification and Characterization of Mutant GDHs. Fresh overnight cultures of DE3/pHGDH were used to inoculate 1 L of LB containing 100 μg of ampicillin/mL. DE3/pHGDH was grown at 37 °C until the A_{600} reached 1.0, and then IPTG was added to a final concentration of 1 mM. After IPTG induction, DE3/pHGDH was grown for an additional 3 h at 37 °C and then harvested by centrifugation. Cell pellets were suspended in 100 mL of 100 mM Tris-HCl, pH 7.4/1 mM EDTA/5 mM dithiothreitol and lysed with a sonicator. The wild type GDH and the mutant GDHs were purified according to the method developed in our laboratory (*25*, *29*). Because the wild type GDH and the mutant GDHs were readily solubilized, no detergents were required throughout the entire purification steps. The purified GDHs were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and recognized by western blot using monoclonal antibodies previously produced in our laboratory against the bovine brain GDH (*30*). Protein concentration was determined as described previously (*29*).

Enzyme Assay and Kinetic Studies. GDH activity was measured spectrophotometrically in the direction of reductive amination of 2-oxoglutarate by following the decrease in absorbance at 340 nm as described before (*29*). All assays were performed in duplicate, and initial velocity data were correlated with a standard assay mixture containing 50 mM triethanolamine, pH 8.0, 100 mM ammonium acetate, 0.1 mM NADH, 10 mM 2-oxoglutarate, and 2.6 mM EDTA at 25 °C. Because *E. coli* has only an NADP(H)-dependent GDH (*26*, *31*), the enzyme assay was performed with NADH as a coenzyme as described elsewhere (*29*). One unit of enzyme was defined as the amount of enzyme required to oxidize 1 μmol of NADH per minute at 25 °C. For determination of K_m and V_{max} values, the assays were carried out in the presence of 1 mM ADP by varying the substrate under investigation while keeping the other substrate and reagents at the optimal concentration indicated above. The K_m and V_{max} values were calculated by linear regression analysis of double-reciprocal plots and given along with standard errors. Effects of allosteric regulators on the wild type GDH and the mutant GDHs were examined by incubating the enzymes with the allosteric effectors at various concentrations in 50 mM triethanolamine, pH 8.0 at 25 °C. At intervals after the initiation with the effectors, aliquots were withdrawn for the assay of GDH activity.

Photolabeling of GDH. Photolabeling of the wild type GDH and the mutant GDHs was performed as described elsewhere (*21*, *24*) with a slight modification. For saturation studies, 0.1 mg of human GDH in 10 mM Tris−acetate, pH 8.0, was separately incubated with various concentrations of [α-^{32}P]$8N_3$ADP in Eppendorf tubes for 5 min. The samples were then irradiated with a handheld 254-nm UV lamp at a distance of 4 cm for 90 s at 4 °C. For competition

studies, 0.1 mg of GDHs was incubated with various concentrations of ADP for 10 min in the same buffer prior to the addition of 60 μM [α-^{32}P]8N$_3$ADP and then allowed to incubate with the photoprobe for 5 min. The samples were then irradiated as described above. The reaction was quenched by the addition of ice-cold trichloroacetic acid (TCA; final 7%). The reaction mixtures were kept on the ice bath for 30 min and then centrifuged at 10000*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.

Tryptic Digestion and Isolation of Photolabeled Peptide. One milligram of the wild type and mutant GDHs was separately incubated with 60 μM [α-^{32}P]8N$_3$ADP for 5 min at 4 °C in 5 mM Tris−acetate, pH 8.0, containing 5 mM glutamate and 0.1 mM NAD^+. Glutamate and NAD^+ were included in the reaction mixture to saturate their respective binding sites. After irradiation for 90 s at 4 °C, 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. The resuspended sample was proteolyzed by the addition of 15 μg of trypsin and kept at room temperature for 3 h, after which time 15 μg of trypsin was added again. After an additional 3 h at room temperature, 15 μg of trypsin was added, and the digestion mixture was kept at 25 °C overnight. To validate that the isolated peptide(s) was (were) specific for the ADP site and so could be protected by ADP from photomodification, the enzymes were also photolyzed in the presence of 1 mM ADP and proteolyzed as described above. The photolabeled GDHs in the presence or absence of 1 mM ADP were applied to an immobilized aluminum chromatography by using the same methods as described elsewhere (*21*, *24*). The photolabeled peptides were eluted with 5 mM KH_2PO_4 in 50 mM ammonium acetate, pH 8.0. The absorbance of the fractions was measured at 220 nm, and the photoincorporation was determined by liquid scintillation counting. Fractions from immobilized aluminum column were further purified by reversed-phase HPLC and sequenced according to the same method as described elsewhere (*23*, *24*).

RESULTS

Construction and Expression of Tyr187 Mutants. The 60 base pair *Age*I/*Eco*RV fragment was replaced with five 60 base pair synthetic DNA duplexes containing a substitution on both DNA strands at positions encoding Tyr187. These substitutions made the mutant GDHs Y187E, Y187G, Y187M, Y187R, and Y187S at position 187. These mutants were designed to have different sizes, hydrophobicities, and ionizations of the side chains at each position. Analysis of crude cell extracts by western blot showed that Tyr187 mutant plasmids encoding an amino acid substitution at position 187 directed the synthesis of a 56.5 kDa protein that interacted with monoclonal antibodies raised against GDH at almost identical levels for all mutant GDHs and wild type GDH (Figure 1A). These results indicate that the mutagenesis at Tyr187 site has no effects on expression or stability of the different mutant GDHs. When assayed in the presence of 1 mM ADP, specific activities of the Tyr187 mutant GDHs in the crude extracts were 0.34 ± 0.06 unit/mg, compared with a wild type value of 1.12 ± 0.02 units/mg. This represent a 3.5-fold reduction in the activities of the Tyr187 mutant GDHs compared with those of the wild type GDH. However, the specific activities in the absence of ADP were 0.39 ± 0.03 and 0.30 ± 0.05 unit/mg, respectively, for the wild type GDH and the Tyr187 mutant GDHs. A more detailed investigation of the catalytic activities of the mutant enzymes revealed an ~4-fold decrease in the respective apparent V_{max} values (Table 1). However, the apparent K_m values for 2-oxoglutarate and NADH were essentially unchanged. The K_m values for 2-oxoglutarate were 1.25 and 1.29−1.46 mM, respectively, for the wild type and mutant GDHs, whereas the K_m values for NADH were 80 and 82−88 μM, respectively, for the wild type and mutant GDHs (Table 1). The similarity of the K_m values for the wild type and mutant enzymes indicates that the mutagenesis at 187 position does not affect the affinity of the enzyme for these two substrates.

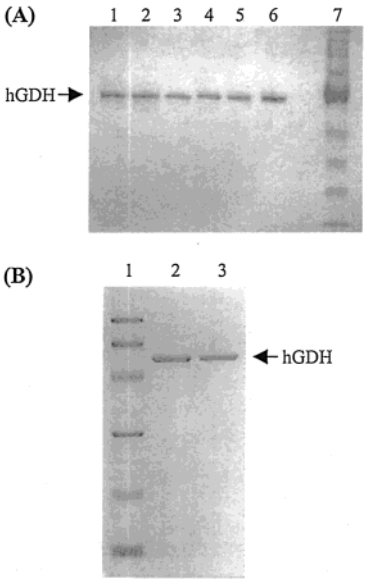

FIGURE 1: (A) Western blot analysis of the Tyr187 mutant GDHs in crude extracts: (lane 1) wild type GDH; (lanes 2−6) Tyr187 mutant GDHs (Y187E, Y187G, Y187M, Y187R, and Y187S); (lane 7) prestained marker proteins (Novex). (B) SDS−PAGE analysis of purified wild type and mutant GDHs: (lane 1) marker proteins (Bio-Rad); (lane 2) Y187M mutant GDH; (lane 3) wild type GDH.

Table 1: Kinetic Parameters of Wild Type GDH and Mutant GDHs in Crude Extracts[a]

	V_{max} (μmol·min^{-1}·mg^{-1})	K_m NADH (μM)	K_m 2-oxoglutarate (mM)
wild type	1.20 ± 0.02	80 ± 1	1.25 ± 0.04
Y187E	0.26 ± 0.02	82 ± 3	1.29 ± 0.05
Y187G	0.32 ± 0.03	85 ± 2	1.45 ± 0.03
Y187M	0.36 ± 0.04	84 ± 4	1.31 ± 0.03
Y187R	0.33 ± 0.03	82 ± 2	1.44 ± 0.04
Y187S	0.31 ± 0.04	88 ± 3	1.46 ± 0.03

[a] Values are expressed as means ± SD with all measurements performed in duplicate.

Activation of Wild Type GDH and Mutant GDHs by ADP and 8N$_3$ADP. The stimulatory effects of ADP on the wild type GDH and the Tyr187 mutant GDHs were examined.

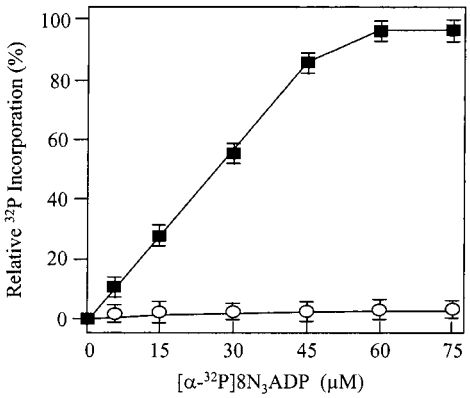

FIGURE 2: Saturation of photoinsertion of [α-^{32}P]8N$_3$ADP into the wild type and mutant GDHs: wild-type GDH (■); Y187M mutant GDH (○). The wild type GDH and the mutant GDHs in the reaction buffer were photolyzed with the indicated concentrations of [α-^{32}P]8N$_3$ADP. ^{32}P incorporations into proteins were determined by liquid scintillation counting and expressed relative to each control.

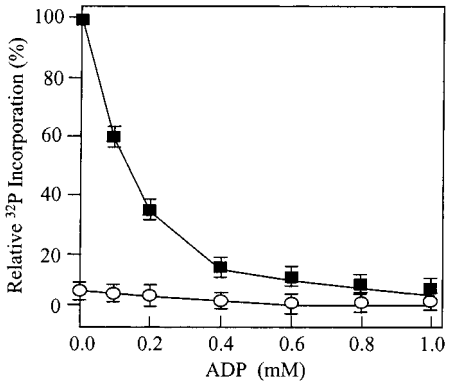

FIGURE 3: Effect of ADP on photoinsertion of [α-^{32}P]8N$_3$ADP into the wild type and mutant GDHs: wild type GDH (■); Y187M mutant GDH (○). The wild-type GDH and the mutant GDHs in the reaction buffer were photolyzed with 60 μM [α-^{32}P]8N$_3$ADP in the presence of the indicated concentrations of ADP. Relative ^{32}P incorporations into proteins were determined and expressed as described for Figure 2.

There were big differences between the wild type GDH and the Tyr187 mutant GDHs in their sensitivities to the activation by ADP. Whereas the wild type GDH was activated 3-fold by ADP at 1 mM at pH 8.0, no detectable activation was observed for the Tyr187 mutant GDHs regardless of their size, hydrophobicity, and ionization of the side chains. These results suggest that the activation of GDH by ADP is mainly due to the binding of ADP to the Tyr187 residue. The binding of ADP to the wild type GDH and the mutant GDHs was further examined by photoaffinity labeling with [α-^{32}P]8-azidoadenosine 5′-triphosphate (8N$_3$-ADP). To show that 8N$_3$ADP could mimic the stimulatory properties of ADP, the photoanalogue should be able to reversibly activate GDH in the absence of activating light. When assayed with NADH as a coenzyme, 8N$_3$ADP was able to activate the wild type GDH, although maximal activity with 8N$_3$ADP was ~80% of maximal ADP-stimulated activity (data not shown). Unlike the wild type GDH, there were no detectable activations by 8N$_3$ADP in the reaction of the Tyr187 mutant GDHs. These results show that the azidonucleotide, 8N$_3$ADP, is able to elicit almost the same biological effects on the wild type GDH and the mutant GDHs as the natural nucleotide, ADP. Because almost identical results were obtained among the five mutant GDHs at the position of Tyr187, only one mutant GDH, Y187M, was homogeneously purified for the further studies. The purified Y187M mutant GDH was estimated to be >98% pure by SDS−PAGE (Figure 1B).

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 [α-^{32}P]8N$_3$ADP occurred at 60 μM for the wild type GDH (Figure 2). The apparent K_d value of this interaction was 25 μM for the wild type GDH. However, the photoinsertion of [α-^{32}P]8N$_3$ADP into the Y187M mutant GDH was <5% of that obtained with the wild type GDH (Figure 2). To demonstrate specific labeling of GDH, the enzymes were photolabeled with [α-^{32}P]8N$_3$ADP in the presence of increasing ADP concentrations. As shown in the results of the competition experiments (Figure 3), increasing ADP concentration decreased the photolabeling of 60 μM [α-^{32}P]8N$_3$-ADP. When 1 mM ADP was present with 60 μM [α-^{32}P]8N$_3$-ADP, 95% of the photoinsertion was reduced for the wild type GDH. For Y187M mutant GDH, there was only a little effect of ADP on the photoinsertion of [α-^{32}P]8N$_3$ADP, and this was a decrease from an already very low starting level (Figure 3). These results show the specificity and utility of 8N$_3$ADP as a good candidate for determining the ADP site and suggest the involvement of Tyr187 for the efficient binding of ADP to the human GDH.

Tryptic Digestion and Isolation of Photolabeled Peptide. To identify the peptides modified by [α-^{32}P]8N$_3$ADP, the enzymes were photolabeled in the absence or presence of 1 mM ADP and digested with trypsin. The enzymes were photolabeled in the presence of 5 mM glutamate and 0.1 mM NAD$^+$ to saturate the binding sites of the two substrates and reduce photoinsertion into NAD$^+$ site. Normally, multiple photolysis is performed in these experiments to increase photoinsertion levels (*32*). However, due to the uncertainty of the number of ADP sites and possible wobble associated with them (*33*), GDHs were incubated with photoprobe and irradiated only once. This was done to enhance site selective modification. A higher protein-to-nucleotide ratio was also used to reduce any possible nonspecific labeling. The photolabeled GDH was separated from most of the noncovalently bound nucleotide by acid precipitation and proteolyzed by trypsin. After overnight trypsin digestion of GDH modified with [α-^{32}P]8N$_3$ADP, the digested samples were purified by an immobilized aluminum chromatography. The results in Figure 4A show the radioactivity profile of wild type GDH and Y187M mutant GDH modified by 60 μM [α-^{32}P]8N$_3$ADP in the absence of ADP. For the wild type GDH, most of the radioactivity was retained on the column and one major radioactive was recovered from the column. ADP was able to reduce [α-^{32}P]8N$_3$ADP photoinsertion into this peak. When 1 mM ADP was originally present in the incubation mixture, ~95% of the radioactivity of the peak was eliminated as shown in Figure 4B. Unlike the wild type GDH, there was no detectable radioactive peak for the Y187M mutant GDH in the absence (Figure 4A) or presence (Figure 4B) of 1 mM ADP. These results indicate that the radioactive peak represents an adenine binding domain peptide of the ADP binding site within human GDH, and so

Table 2: Alignment of [α-^{32}P]8N_3ADP-Labeled Peptides with Homologous Sequences from Various GDHs

source	reference	amino acid sequence[a]																
human GDH (wild type)	this work	E	M	S	W	I	A	D	T	Y	A	S	T	I	G[b]			
human GDH (Y187M)	this work	not detected																
human liver	*41*	E	M	S	W	I	A	D	T	Y	A	S	T	I	G	H	D	I
human retina	*3*	E	M	S	W	I	A	D	T	Y	A	S	T	I	G	H	D	I
bovine brain	*24*	E	M	S	W	I	A	D	T	Y	A	S	T	I	G	H	D	I
bovine liver	*42*	E	M	S	W	I	A	D	T	Y	A	S	T	I	G	H	D	I
rat brain	*43*	E	M	S	W	I	A	D	T	Y	A	S	T	I	G	H	D	I
		179[c]																195

[a] Amino acids are denoted by the single-letter code. [b] Only the first 14 cycles were sequenced. [c] Amino acid numbering is that of the mature human GDH (*3*).

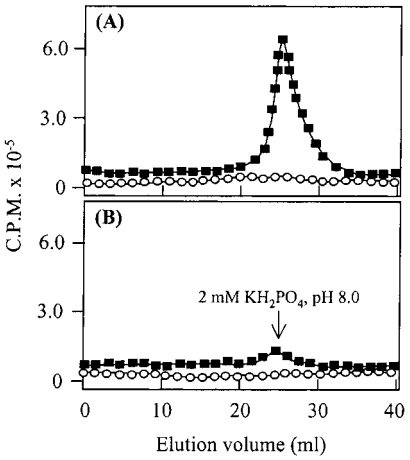

FIGURE 4: Chromatogram of immobilized aluminum column of tryptic peptides from the wild type and mutant GDHs photolabeled with [α-^{32}P]8N_3ADP: wild-type GDH (■); Y187M mutant GDH (○). The proteins were photolabeled with [α-^{32}P]8N_3ADP as described under Experimental Procedures. The tryptic peptides were loaded onto an immobilized aluminum column. Each 1 mL fraction was collected and monitored for absorbance at 220 nm by UV spectrophotometer and for radioactivity by liquid scintillation. The plot represents the radioactivity profiles from the immobilized aluminum column of the samples photolabeled in the absence (A) and presence (B) of 1 mM ADP.

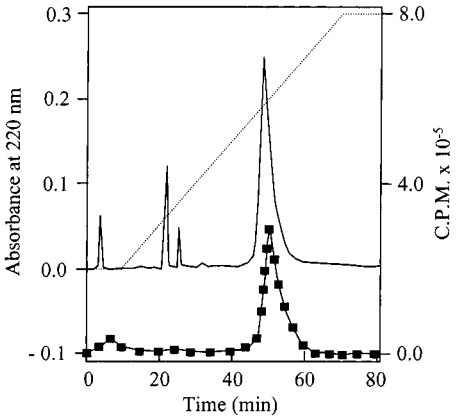

FIGURE 5: Reversed-phase HPLC purification of tryptic peptides eluting from an aluminum chelate column: radioactivity determined by liquid scintillation counting in aqueous phase (■); UV absorption profile at 220 nm (−). The radioactive eluates from the aluminum chelate column were loaded onto a C_{18} reversed-phase column, eluted with an acetonitrile gradient (dashed line) at a flow rate of 0.5 mL/min.

Tyr187 is required for efficient binding of [α-^{32}P]8N_3ADP to GDH.

When the flow-through fractions from the aluminum column were subjected to separation on a reversed-phase HPLC column, multiple peaks were observed as determined with a UV absorption spectrophotometer at 220 nm, indicating that many of the peptides were not retained on the resin (data not shown). The radioactive eluates of the wild type GDH from the immobilized aluminum column were combined and subjected to reversed-phase HPLC. As shown in Figure 5, one major radioactive peak and minor nonradioactive peaks were clearly observed, and the radioactive eluates were collected and identified by sequence analysis. Although some radioactivity was found in the flow-through and wash fractions, >95% of the total radioactivity coeluted with the radioactive peak. The radioactivity associated with the HPLC flow-through fractions represents unbound probe including any decomposition products of photoadduct 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.

Sequence Analysis of Photolabeled Peptides. The results of the amino acid sequencing are summarized in Table 2. The sequences obtained were also compared with those of various GDHs. The amino acid sequence analysis revealed that the peak fractions contained the amino acid sequence EMSWIADTYASTIG for the wild type GDH. Because these are tryptic digests, it was expected to produce a sequence ending with Arg or Lys. 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 Glu179−Lys205 of the amino acid sequence of the mature human GDHs (Table 3). Photolabeling of these peptides was prevented >95% by the presence of 1 mM ADP during photolysis. Unlike the wild type GDH, no peptides containing amino acids around 187 position were

Table 3: Amino Acid Composition of [α-^{32}P]8N_3ADP-Modified Peptide

amino acid	molar ratio[a]	amino acid	molar ratio[a]
Ala	3.8 (4)	Met	1.2 (1)
Asp	2.2 (2)	Ser	1.9 (2)
Asn	1.2 (1)	Thr	3.1 (3)
Cys[b]	1.1 (1)	Tyr	2.1 (2)
Glu	0.9 (1)	Trp[c]	1.1 (1)
Gly	1.9 (2)	Val	1.0 (1)
His	2.1 (2)		
Ile	3.0 (3)	total residues	27
Lys	1.2 (1)		

[a] Molar ratio of amino acids recovered following hydrolysis under vacuum with 6 N HCl for 24 h at 100 °C. Values of molar ratio <0.1 are not indicated. Values in parentheses are the nearest integer. [b] Cys was pyridylethylated before it was quantified (*44*). It represents a sum of cysteine and cystine. [c] Trp was determined by monitoring the decrease in absorbance at 280 nm following oxidation with *N*-bromosuccinimide.

detected with the Y187M mutant GDH. Once again, these results demonstrate selectivity of the photoprobe for the ADP binding site and suggest that Tyr187 is mainly involved in the binding of [α-^{32}P]8N_3ADP to human GDH.

DISCUSSION

The allosteric mechanisms of GDH by ADP and the importance of the regulation of GDH activities by ADP in mammalian system have been well documented (*3*−*6*). However, no direct experimental evidences have been reported to identify the specific amino acid residues involved in ADP binding, although the crystal structure of the bovine liver GDH has been reported (*10*, *11*). Previous studies using chemical probes to identify the ADP binding sites within bovine liver GDH have given a wide scatter of modified residues as proposed to be His82 (*15*, *16*), Arg459 (*17*), Arg491 (*18*), and peptides corresponding to Met411−Arg419 (*19*) and Glu179−Asn197 (*21*, *24*). These diversities may due to the specificities of the probes, making identification of peptides as involved in direct contact with ADP quite problematic. Alternatively, a number of regions are actually involved in ADP binding. As expected from the structure of ADP, it is quite possible that base binding domains are different from those of the phosphate binding domains. These issues will be addressed by site-directed mutagenesis on the human GDH gene and these newly identified contact residues. In the present work, we identified an amino acid required for the efficient binding of ADP to the human GDH using cassette mutagenesis, the photoaffinity labeling with [α-^{32}P]8N_3ADP, and protection studies.

The specificity of 8N_3ADP and the utility of this probe as a good candidate for determining the ADP base binding site were demonstrated by the following. First, in the absence of activating light, wild type GDH is activated by 8N_3ADP as well as by ADP and can be photolabeled with the nucleotide analogue [α-^{32}P]8N_3ADP, whereas no such activation or photolabeling is observed with Tyr187 mutant GDHs. 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 at low micromolar concentrations of [α-^{32}P]8N_3ADP. The apparent K_d values of this interaction were 25 μM (Figure 2). Third, the prevention of photoinsertion of [α-^{32}P]8N_3ADP by ADP demonstrates that the photoprobe is inserting into a specific ADP site within the human GDH (Figure 3). The ability of a nucleotide analogue to mimic the natural nucleotide in its binding properties to the enzyme is essential to secure the specificity of photoinsertion into the active site. ADP afforded better protection against the photolabeling of GDH by 8N_3ADP than by other nucleotides (data not shown). Thus, 8N_3ADP has been shown to photomodify GDH by specifically binding to the ADP base binding site.

There were differences in the biochemical properties between the wild type GDH and the Tyr187 mutant GDHs. There were 4-fold reductions in V_{max} values of the Tyr187 mutant GDHs compared to those of the wild type GDH (Table 1). The results from the western blot analysis (Figure 1A) show that mutagenesis at the Tyr187 site has no effects on expression or stability of the different mutant GDHs. The similarity of K_m values for 2-oxoglutarate and NADH between the two types of enzymes indicates that the mutagenesis at position 187 does not affect the affinity of the enzyme for these two substrates. This site is very close to Tyr194, which is modified with the ADP analogue, 5′-*p*-fluorosulfonylbenzoyladenosine (*34*). This modification diminishes NADH inhibition. However, on the basis of the most recent crystal structure of bovine liver GDH, it is not likely that NADH binds at this location, although 5′-*p*-fluorosulfonylbenzoyladenosine may penetrate to the core of the hexamer (*11*). It has been documented that there are two NADH binding sites, one at the catalytic site and the other at the inhibitory site, and that the second site NADH binding and that of the activator ADP are mutually antagonistic (*10*−*13*). The possibility that the 4-fold reduction in V_{max} values in Table 1 may at least in part be due to the antagonistic interactions between the ADP binding and the second NADH binding remains to be studied.

Another difference between the wild type GDH and the Tyr187 mutant GDHs is their sensitivities to activation by ADP. The mutagenesis at position 187 resulted in a dramatic decrease in the sensitivities to the activation by ADP regardless of their size, hydrophobicity, and ionization of the side chains. The decrease of the sensitivities to the activation by ADP in the Tyr187 mutant GDHs is consistent with the reduction of the activities of the mutant GDHs (Table 1). These results strongly suggest that the activation of GDH by ADP is mainly due to the binding of ADP to the Tyr187 residue.

The kinetic properties of the modified human GDH provide more information on the site of the enzyme with 8N_3ADP. The 8N_3ADP-modified human GDH is no longer activated by ADP, as expected if the allosteric ADP site is occupied by 8N_3ADP. In contrast, the modified enzyme is still inhibited by GTP, although at higher concentrations than for native enzyme (data not shown). These results suggest that the modification of the ADP binding site has an indirect effect in weakening binding at other allosteric sites. There have been several studies indicating the competition between ADP and GTP (*35*, *36*), as well as between ADP and NADH (*37*, *38*). Interestingly, the Tyr187 site identified as within or near the ADP base binding domain in this work is located near one of the GTP phosphate binding domains (*19*) in the three-dimensional structure. Similarly, it also has been reported that the ADP phosphate binding domain (*19*) is near the GTP base binding domain (*22*, *32*, *39*). It seems likely that ADP and GTP can be imagined to be oriented with their terminal phosphates in almost opposite directions. Therefore, ADP and GTP probably bind in an antagonistic manner and cause opposite effects on GDH activity as proposed by crystal structures of GDH (*10*, *11*).

It is clear that the ADP base binding domain established using [α-^{32}P]8N_3ADP in this work is distinct from the ADP phosphate binding domains performed with benzophenone-based nucleotide probes to be modified at Arg491 (*18*) and at a peptide corresponding to Met411−Arg-419 (*19*). Previously, irreversible activation of bovine liver GDH by adenosine 5′-*O*-[*S*-(4-bromo-2,3-dioxobutyl)thiophosphate] identified Arg459 (Arg 463 in human GDH) as part of the ADP regulatory site by facilitating the binding of ADP by electrostatic interaction with the β-phosphate of ADP (*17*). However, the most recent crystal structures show that ADP can bind to either the open or closed conformation and that

the Arg459 residue is too far away to interact with the phosphate of ADP in the closed conformation, although it may rotate down and interact with the phosphate moieties of the coenzyme in the open mouth conformation (*11*). This suggests that Arg459 may not be wholly responsible for the observed ADP binding. From the crystal structures of bovine GDH (*10*, *11*), it also has been suggested that the ADP site, with its deep purine binding pocket, is very selective at the adenine-ribose end but makes few contacts with the ligand beyond the phosphate moieties. In contrast, it appears that the GTP site favors triphosphate binding with only marginal purine selectivity. The most recent study shows that GTP binds to GDH from *E. coli* at an allosteric site and reverses the destabilizing effects of coenzyme (*40*). This result strongly suggests that the 48 antenna region existing only in the mammalian GDHs may not be wholly responsible for the observed binding of ADP and GTP. Therefore, it is reasonable to speculate that the base binding domains, in addition to the phosphate binding domains, may be required for the efficient binding of ADP to GDH. However, at this point, we cannot exclude the possibility that the mutations within the core might disrupt interactions between the subunits that form the regulatory site(s) in mammalian GDH because six Tyr187 residues are located at the core of the hexamer. Therefore, although our data show that Tyr187 is required for activation of human GDH by ADP, further studies are required to prove the direct interaction between ADP and Tyr187.

The construction of a synthetic gene encoding human GDH and the high level of GDH expression as a soluble protein in *E. coli* will enable us to generate a large number of site-directed mutations at several positions in the coding region and facilitate the purification of large quantities of mutant proteins for biochemical and structural studies. Data obtained using photoaffinity probes (*20*−*24*, *39*) place all of the base binding domains of NAD^+, GTP, and ADP at different locations within a proposed catalytic cleft defined in the crystal structure (*10*, *11*, *27*). Therefore, photoaffinity labeling appears to be a useful procedure for identifying active or regulatory sites. The combination of genetic and photolabeling techniques, together with the recent crystal structures of GDH from various sources, could be used to address a broad range of questions relating to the structure and function of human GDH.

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