Structure, Allosteric Regulation, and Reaction Mechanism of Glutamate Dehydrogenase

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Dedicated to my parents, my brothers, my husband and my son, who encouraged me.

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ABBREVIATION

$2N_3NAD^+$: nicotinamide 2-azidoadenosine dinucleotide

$8N_3ADP$: 8-azidoadenosine 5 -diphosphate

CNBr : cyanogen bromide

E. coli : *Escherichia coli*

EDTA : ethylenediaminetetraacetic acid

FPLC : fast protein liquid chromatography

GDH : glutamate dehydrogenase

HPLC : high performance liquid chromatography

IPTG : isopropyl â-D-thiogalactopyranoside

MSG : monosodium glutamate

PLP : pyridoxal 5 -phosphate

SDS-PAGE : sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TAT : transcriptional transactivator

ABSTRACT

Structure, Allosteric Regulation, and Reaction Mechanism of Glutamate Dehydrogenase

Glutamate in the form of its sodium salt (MSG; monosodium glutamate) is a widely used food additive and reported to be a cause of the Chinese restaurant syndrome. To investigate a long-term effect of MSG ingestion on the brain, one-week-old albino rats were kept for 1 year in equal groups with or without MSG in their drinking water. The concentrations of the glutamate in the brain crude extracts of the MSG treated group were 2-fold higher than those of the control group. There were no significant change in body weight, brain weight, and contents of protein, total RNA and DNA in the two groups of rats. The concentration of the enzyme on the western blot analysis was significantly decreased in the MSG treated group, whereas the level of GDH mRNA remained unchanged, suggesting a post-transcriptional control of the expression of GDH or an increased rate of degradation of enzyme protein. These results indicate that the prolonged MSG feeding reduces the activity of GDH and subsequently decreases the catabolism of glutamate in rat brain.

To gain a deeper insight into the structural and regulatory basis of GDH, a 1557-base-pair gene that encodes human GDH has been synthesized and expressed in *Escherichia coli* as a soluble protein. The recombinant enzymes

were indistinguishable in its biochemical properties from those isolated from human and bovine tissues. The results from the site-directed mutagenesis of Lys130 site indicate that Lys130 plays an important role in the catalysis of GDH and that Lys130 is an essential residue required for the catalysis of GDH but not for the substrate or coenzyme binding.

To identify ADP (allosteric activator) and NAD^+ (coenzyme) binding sites within human GDH, a series of cassette mutations at Tyr187 and Glu279 positions were constructed for ADP and NAD^+ binding, respectively. The wild type GDH was activated up to 3-fold by ADP, whereas no significant activation by ADP was observed with the Tyr187 mutant GDH regardless of their size, hydrophobicity, and ionization of the side chains. Studies of the steady-state velocity of Tyr187 mutant enzymes revealed essentially unchanged apparent K_m values for 2-oxoglutarate and NADH, but an approximately 4-fold decrease in the respective apparent V_{max} values. The Glu279 mutant proteins showed a 22 ~ 28-fold decrease in the respective apparent V_{max} values and 20 ~ 25-fold increase in K_m values for NAD^+ with essentially unchanged K_m values for glutamate. The identification of the ADP and NAD^+ binding sites was further performed using photoaffinity labeling with [á-^{32}P]8-azidoadenosine 5 -diphosphate ($8N_3ADP$) and [^{32}P]nicotinamide 2-azidoadenosine dinucleotide ($2N_3NAD^+$). Saturation of photoinsertion with [á-^{32}P]$8N_3ADP$ and [^{32}P]$2N_3NAD^+$ occurred apparent K_d values near 25 ì M and 55 ì M, respectively, for wild type GDH. The photoinsertions of $8N_3ADP$ and $2N_3NAD^+$ were significantly decreased by ADP and NAD^+, respectively. Unlike wild type GDH, none of the mutant enzymes at Tyr187 or Glu279 was able to interact with $8N_3ADP$ and $2N_3NAD^+$, respectively. The results with

cassette mutagenesis and photoaffinity labeling indicate that the Tyr187 and Glu279 are required for efficient base-binding of ADP and NAD^+ to GDH, respectively.

In an effort to replenish the GDH activity in the patients with the GDH-deficient neurodegenerative disorders, human GDH was transduced into PC12 cells by fusing with a gene fragment encoding the protein transduction domain of human immunodeficiency virus TAT protein to produce genetic in-frame TAT-GDH fusion protein. The TAT-GDH protein can enter PC12 cells efficiently when added exogenously in culture media. Once inside the cells, the transduced denatured TAT-GDH protein showed a full activity of GDH indicating that the TAT-GDH fusion protein was correctly refolded after delivery into cells and the activities of GDH in the TAT-GDH fusion protein was not affected by the addition of the TAT sequence. TAT-GDH showed no cytotoxicity as determined by the ability to inhibit protein synthesis. These results may suggest new possibilities for direct delivery of GDH into the patients with the GDH-deficient disorders.

Keywords : Glutamate dehydrogenase, Monosodium glutamate, Post-transcriptional regulation, Cassette mutagenesis, Pyridoxal 5 -phosphate, ADP binding site, NAD^+ binding site, Photoaffinity labeling, TAT fusion protein, Protein therapy, Protein engineering

CHAPTER I

Decreased Expression of Glutamate Dehydrogenase by Prolonged Intake of Monosodium Glutamate in Rat Brains

I. INTRODUCTION

Glutamate, the main substrate of the GDH-catalyzed reaction, is one of the most abundant free amino acids in the central nervous system and is thought to serve as a major excitatory neurotransmitter (Mukhin et al. 1996). Glutamate also involved in most aspects of normal brain function including cognition, memory and learning (Fonnum, 1984; Ottersen and Strom-Mathisen, 1984; Collingridge and Lester, 1989; Headley and Grillner, 1990) and plays major roles in the development of the central nervous system, including synapse induction and elimination, and cell migration, differentiation and death. It is known to an immediate precursor for the inhibitory neurotransmitter ã–aminobutyric acid (GABA), a widely distributed inhibitory neurotransmitter, (Roberts and Frankel, 1950) and present in brain tissue in concentrations that are 2 ~ 5 fold higher than those found in other organs. Furthermore, due to its neurotoxic potentials, glutamate may be involved in the pathogenesis of human degenerative disorders (McGeer and McGeer, 1976; Plaitakis et al., 1982). Substantive evidence supports the involvement of glutamate in neuronal death or injury associated with ischemic brain injury, stroke, status epilepticus, amyotrophic lateral sclerosis, Parkinson's disease and Alzheimer's disease (Ben-Ari and Schwarcz, 1986; Choi, 1988, 1992; Turski et al., 1991; Mattson et al., 1992; Meldrum, 1995).

In patients with recessive, adult-onset olivopontocerebellar atrophy characterized by progressive atrophy of areas of the brainstem, cerebellum, spinal cord, and substatia nigra and by ataxia, corticospinal deficits, dysarthria, dysphagia, and signs of parkinsonism (Greenfield, 1954) associated with a partial deficiency of GDH, the concentration of glutamate in plasma was significantly higher than that in controls (Plaitakis et al., 1982). Since glutamate does not readily cross the blood-brain-barrier (Mc Geer and Mc Geer, 1976), the majority of this amino acid in the central nervous system is produced from precursors and regulated through the action of glutamate pyruvate transaminase, glutamine synthetase, and glutamate dehydrogenase.

Glutamate in the form of its sodium salt (MSG; monosodium glutamate) is a widely used food additive, although it is not a wholly innocuous substance. It was reported that monosodium glutamate is the cause of the Chinese restaurant syndrome (flushing, tightness of the chest, difficulty in breathing, etc.) and can precipitate headache, flushing, and chest pain (Schaumburg et al., 1969). Parentally administered monosodium glutamate produced an acute degenerative lesion in the inner retina of normal neonatal mice and subcutaneous injections of monosodium glutamate induced acute neuronal necrosis in several regions of developing brain in newborn mice (Olney, 1971). The neonatal treatment with MSG resulted in reduction in the number of tyrosine hydroxylase that affects primarily L-DOPA-ergic

neurons (tyrosine hydroxylase positive cells) located in the ventrolateral part of the arcuate nucleus (Bodnar et al., 2001). The neonatal administration of large doses of MSG to rodents causes neuronal necrosis in some brain regions, along with behavioral and metabolic abnormalities (Ali et al., 2000; Bowri et al., 1994, 1995) and induces cell death and dendritic hypotrophy in rat prefrontocortical pyramidal neurons (Gonzalez-Burgos et al., 2001). That monosodium glutamate treatment might have a similar deleterious effect on neurons in other regions of the central nervous system has apparently not been considered. Because of its varied and critical roles in the central nervous system, it is essential that glutamate production and degradation should be finely regulated. One enzyme central to the metabolism of glutamate is glutamate dehydrogenase (GDH). What is the influence of monosodium glutamate on the GDH activity in brain? It was reported that in patients with recessive adult-onset olivopontocerebellar degeneration associated with a partial deficiency of GDH, the concentration of glutamate in plasma was significantly higher than that in controls, suggesting that decreased glutamate catabolism may result in an excess of glutamate in the nervous system and cause neuronal degeneration (Plaitakis et al., 1982; Hussain et al., 1989). Since the metabolic defect is systemic, it was reasonable to speculate that impaired glutamate metabolism probably occurs in the brain, containing the highest concentrations of glutamate (about 5 ~ 15 mmol per kg wet weight

depending on the region) (Schousboe, 1981). Furthermore, when glutamate and its potent analogs are injected into certain brain areas of experimental animals, they produce morphological and biochemical alterations found in patients with degenerative neurological disorders (Olney and Ho, 1970; Coyle and Schwarcz, 1976). Therefore, the accumulation of glutamate in the nervous tissue may well be the cause of the neuronal degeneration. However, the specific mechanism underlying the effect of ingestion of monosodium glutamate on brain has not been definitively understood.

The present work is investigated the long term effect of monosodium glutamate on the activities of enzymes involved in glutamate metabolism in rat brain.

II. MATERIALS AND METHODS

Materials

ADP, ATP, $NADP^+$, NAD^+, NADH, NADPH, L-glutamate, monosodium glutamate and 2-oxoglutarate were purchased from Sigma. One-week old albino female rats were purchased from Asan Institute for Life Sciences. Human GDH gene (Cho et al., 2001) and cDNA for human á-tubulin donated by Dr. O. Hwang (University of Ulsan College of Medicine), were used as probes in northern blot analyses. Precast gels for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from NOVEX. Low molecular weight standards for SDS-PAGE were obtained from Bio-Rad. All other chemicals and solvents were reagent grade or better.

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 (Cho et al., 1995). 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, and 2.6 mM EDTA at 25 ^oC. Since *E. coli* only has an NADP(H)-dependent GDH (Hanahan, 1983; McPherson and Wootton, 1983), the enzyme assay was performed with NADH as a coenzyme

as described elsewhere (Cho et al., 1995). One unit of enzyme was defined as the amount of enzyme required to oxidize 1 ìmol of NADH per min at 25 °C. For determination of kinetic constants (V_{max} and K_m), the assays were carried out by varying one substrate under investigation while keeping the other substrate and reagents at the saturating concentration as described elsewhere (Cho et al., 1995). All analyses were performed using the StatView statistical computer program (Abacus Concepts, Berkeley, CA).

Long term effects of monosodium glutamate

One-week-old albino female rats were kept in equal groups with (experimental group) or without (age-matched control group) monosodium glutamate in their drinking water. The concentration of monosodium glutamate used was 1 mg/g body weight as employed elsewhere (Schaumburg et al., 1969; Olney, 1984). After 1 year, the animals were killed by decapitation and brains were dissected, rinsed with 0.9 % ice-cold saline, weighed, and homogenized in 50 mM Tris-HCl, pH 7.4 containing 0.5 mM ethylenediaminetetraacetate and 1.0 mM β-mercaptoethanol. The homogenates were centrifuged at 8,000 x *g* for 40 min at 4 °C and the supernatants were assayed for protein (Cho et al., 1995), total DNA and RNA (Sambrook et al., 1989), and the activities of GDH (Cho et al., 1995) and glutamine synthetase (Garcia-Dominguez et al., 1996). For determination of kinetic constants, V_{max} and K_m, the assays were carried out by varying one substrate (α-ketoglutarate

or NADH) under investigation while keeping the other substrate (NADH or α-ketoglutarate) and reagents at the optimal concentration. V_{max} and K_m were determined by a least-squares method fitted to the double-reciprocal plots.

The 8,000 x *g* supernatants were applied to 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane for western blot analysis (Choi et al., 1999). The monoclonal antibodies used in this study were produced against bovine brain GDH as described before in our laboratory (Choi et al., 1999). For the isolation of RNA, five volumes of 4 M guanidinium thiocyanate buffer were added to brains and homogenized with a Polytron homogenizer. The isolation of total RNA and northern blot was performed by standard methods (Sambrook et al., 1989). Hybridization was performed by adding saturating amounts of ^{32}P-labeled and denatured cDNA probes along with 100 μg/ml denatured salmon sperm DNA. Autoradiography was performed overnight at -80 ^{o}C. Intensity of the bands was densitometrically determined and normalized to the intensity of the corresponding α-tubulin bands. Ammonia, ornithine, α-ketoglutarate, glutamate and other amino acids concentrations were measured in neutralized brain extracts by enzymatic and fluorometric methods (Heinrikson and Meredith, 1984).

III. RESULTS

Long term effects of monosodium glutamate feeding

There were no significant changes in the body weight, brain weight, and protein contents after 1 year of feeding monosodium glutamate orally (Table I-1). It was reported that subcutaneous injections of monosodium glutamate induced acute neuronal necrosis in several regions of developing brain in newborn mice and as adults, treated animals showed stunted skeletal development and marked obesity (Olney, 1971). None of these conditions, however, was present in our animals. Also, the RNA and DNA content did not vary in the two groups. The monosodium glutamate treated group showed a brain glutamate concentration of 59 ìmol/g wet weight that were 2 times higher than the control group, 29 ìmol/g wet weight (Table I-1). In contrast, the concentrations of NH_4^+ and á-ketoglutarate in the glutamate treated group were 40 % and 45 % less than those in the control group, respectively (Table I-1). There were no differences between the two groups in the concentrations of other amino acids such as Gln, Ser, Leu, Ile, Ala, Val, Met, Tyr, Phe, Arg, and His.

Compared to the control groups, a significant decrement in the GDH activity was observed after one year of feeding the monosodium glutamate. For a detailed comparison, enzyme kinetic studies were performed by varying the substrate under investigation while keeping the other substrate and

reagents at the optimal concentration. As shown in Table I-1, V_{max} values were reduced 45 % in the monosodium glutamate treated group (0.18 ± 0.02 units/mg protein) compared to those in the control group (0.33 ± 0.03 units/mg protein), whereas no significant changes were observed in K_m values for NADH, ammonia, glutamate, and α-ketoglutarate between the two groups. The results in Figure I-1*A* show that the expression level of GDH in the monosodium glutamate treated group on the western blot analysis was 38 % reduced as determined by using densitometer. The level of GDH mRNA, however, remained unchanged as determined by northern blot analysis as shown in Figure I-1*B*, suggesting a post-transcriptional control of GDH expression or an increased rate of degradation of enzyme protein.

Table I-1 Effects of monosodium glutamate feeding on the glutamate content and the activity of GDH in rat brains

Parameters	Control group (n = 5)	Monosodium glutamate treated group (n = 5)
Body weight (g)	480 ± 35	465 ± 41
Brain weight (g)	2.4 ± 0.3	2.3 ± 0.2
Protein (mg/g wet tissue)	248 ± 11	239 ± 16
Glutamate (ì mol/g wet weight)	29.60 ± 0.81	59.22 ± 1.12[a]
α-Ketoglutarate (ì mol/g wet weight)	9.12 ± 0.68	5.13 ± 0.98[a]
NH_4^+ (ì mol/g wet weight)	1.36 ± 0.21	0.71 ± 0.63[a]
Kinetic constants of GDH;		
V_{max} (units/mg protein)	0.33 ± 0.03	0.18 ± 0.02[a]
$K_{m(NADH)}$ (ì M)	40.09 ± 2.91	38.43 ± 3.76
$K_{m(Ammonia)}$ (mM)	6.24 ± 1.99	5.53 ± 2.88
$K_{m(Glutamate)}$ (mM)	3.14 ± 0.03	3.25 ± 0.04
$K_{m(\alpha\text{-Ketoglutarate})}$ (mM)	0.98 ± 0.02	0.97 ± 0.02

Values are expressed as means ± S.D. for five rats with all measurements performed in duplicate. Statistical comparisons between control and treated groups were made by ANOVA using Fisher's protected least significant different test at the 0.5 significance level.

[a]Significant differences between the two groups, $P < 0.01$.

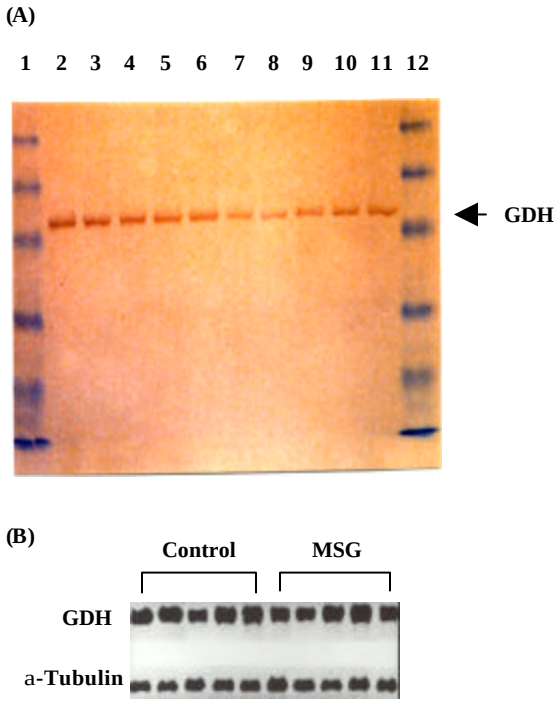


Figure I-1. (A) Effects of monosodium glutamate feeding on the expression of GDH in rat brains. The supernatants of the crude extracts were loaded on a 12 % SDS-PAGE gel, submitted to electrophoresis and immunoblotted with the monoclonal antibody directed against bovine brain GDH. The final amount of total proteins loaded on each lane (lane 2 ~ 11) was 0.5 mg. Lanes 2 ~ 6, control group; lanes 7 ~ 11, monosodium glutamate treated group, and lane 1 and 12, prestained molecular weight marker proteins. **(B) Northern blot analysis.** GDH mRNA levels of rat brains from the control group (lane 1 ~ 5) and the monosodium treated group (lane 6 ~ 10) were compared and normalized against α-tubulin. Densitometric analyses show no significant differences between the two groups.

IV. DISCUSSION

Glutamate taken up by cells may be used for metabolic purposes (protein synthesis, energy metabolism, ammonia fixation) or be reused as transmitter. In nerve terminals, reuse as transmitter is straightforward. Glutamate also involved in most aspects of normal brain function including cognition, memory and learning (Fonnum, 1984; Ottersen and Strom-Mathisen, 1984; Collingridge and Lester, 1989; Headley and Grillner, 1990) and plays major roles in the development of the central nervous system, including synapse induction and elimination, and cell migration, differentiation and death. Although glutamate does not readily cross the blood-brain barrier (Bradbury, 1979), doses of monosodium glutamate in the range of those sometimes fed unwittingly to human young as a food additive destroy central nervous system neurons when administered orally to immature animals (Olney, 1984). Neurons most vulnerable to destruction by orally administered monosodium glutamate are those lying in certain brain regions that lack blood-brain barriers, such as those in the arcuate nucleus of the hypothalamus that regulate neuroendocrine function. As destruction of hypothalamic neurons in immature animals results in a complex neuroendocrine deficiency syndrome, the question arises as to whether ingestion of monosodium glutamate by human young contributes to the occurrence of neuroendocrinopathies in later life

(Olney, 1984).

In contrast to synaptic glutamate transporters such as NMDA-receptors, AMPA/kainite receptors and metabotropc receptors (Hollmann and Heinemann, 1994; Schepfer et al., 1994; Nakanishi et al., 1998; Dingledine et al., 1999), most glutamate-degrading enzymes have much lower affinities for glutamate, but higher capacity for glutamate elimination. The known glutamate-degrading enzymes such as the ATP-dependent, glia-specific enzyme glutamate synthetase (Martinez-Hernandez, et al., 1977; Laake et al., 1995; Ottersen et al., 1996), glutamate dehydrogenase (GDH), and glutamate pyruvate transaminase already have been shown to have neuroprotective value in models of glutamate excitotoxicity (Matthews et al. 2000). In astrocytes, glutamate taken up from the extracellular fluid may be converted to glutamine, which is released to the extracellular fluid, taken up by neurons and reconverted to glutamate inside neurons. This trafficking of glutamate and glutamine between astrocyte and neurons has been proposed to be a major pathway by which transmitter glutamate is recycled. It is commonly referred to as the glutamine-glutamate cycle. GDH has been suggested to be associated with the pool of glutamate that is released as a neurotransmitter at the nerve endings (Akoi et al., 1987). Because there are many reports demonstrating high GDH activity in synaptic terminals, especially enriched in astrocytic rather than neuronal mitochondria (Wenthold and Altschuler, 1986; Akoi et al.,

1987; Rothe et al., 1994). The precise roles of GDH in the central nervous system as well as the predominant direction of the reaction it catalyzes remain unclear. However, given the extensive nature of the glutamatergic pathways in brain (Young and Fagg, 1990), GDH may play a role in a number of human neurodegenerations. These results suggest a possibility that the prolonged exposure to monosodium glutamate may be one of the factors involved in the control of GDH expression in brain. Subsequently decreased catabolism (oxidative deamination) of glutamate at the nerve terminals could result in an increased amount of the neurotransmitter at the synapses, leading to overexcitation and neuronal degeneration. Actually, a partial deficiency of GDH with a high level of glutamate was reported in fibroblasts and leukocytes of humans with a late adult-onset form of olivopontocerebellar atrophy (Plaitakis, 1982), supporting the possibility that the accumulation of glutamate in the nervous system may be the cause of the neuronal degeneration. The studies on specific glutamate antagonists also suggested the possibility that glutamate excitotoxicity might be involved in epilepsy (Plaitakis, 1982). Early mitochondrial damage plays a key role induction of glutamate neurotoxicity (Schinder et al., 1996). Glutamate-induced cell death can follow a rapid path or a slow path. The rapid path is characterized by massive influx of ions (Na^+ and Cl^-), cell swelling and a necrotic type of cell death. A delayed type of death, which can occur several hours after the

initiating stimulus, characterizes the slow path. Studies of cultured cortical neurons suggest that glutamate is more prone to induce necrotic cell death at high concentrations and more prone to induce a delayed type at lower concentrations (Cheung et al., 1998). Therefore, the regulation of GDH that is important in adjusting the levels of the neurotransmitter glutamate might be worth examining in the context of experimental and clinical neurodisorders.

No significant changes were detected in the activity of glutamaine synthetase between the two groups even after feeding monosodium glutamate for 1 year (data not shown). Similarly, it was reported that degeneration of serotonergic neurons produced a large induction in GDH expression that was mediated at the post-transcriptional level, whereas the level of glutamine synthetase remained unchanged (Hardin et al., 1994). This stability of glutamine synthetase in response to lesions of the glutamatergic or serotonergic inputs may be to avoid perturbation of concentrations of the glutamine and NH_4^+ in brain. Indeed, an increase of glutamine level causes cerebral edema (Hawkins et al., 1963). A fall in the level and subsequent acidosis could lead to an impairment of neuronal excitability, to cell swelling and even to cell death. It was also reported that the majority of glutamine is eliminated in the blood stream or oxidized into CO_2 and only a small part of glutamine is converted into glutamate (Hertz, 1992). Therefore, a specific perturbation in GDH gene expression suggests that glutamate metabolism is

preferentially regulated by a control of GDH rather than glutamine synthetase in brain. The mechanism of the regulation of GDH gene expression by long-term exposure to monosodium glutamate in brain remains to be determined.

CHAPTER II

Cassette Mutagenesis of Lysine130 of Human Glutamate Dehydrogenase: An Essential Residue in Catalysis

I. INTRODUCTION

Mammalian glutamate dehydrogenase (GDH) plays a role in regulating the levels of ammonia and glutamate in the central nervous system. The study of GDH is of particular interest since the enzyme activity has been found to be altered in patients with neurodegenerative disorders characterized by multisystem atrophy and predominant involvement of the cerebellum and its connections (Plaitakis et al., 1984; Hussain et al., 1989). Hussain et al. (1989) detected four different forms of GDH isoproteins from human cerebellum of normal subjects and patients with neurodegenerative disorders. The isoproteins are differentially distributed in the two catalytically active isoforms of the enzyme (Plaitakis et al., 1993). The enzyme isolated from one of the patients with a variant form of multisystem atrophy displayed marked reduction of one of the GDH isoproteins (Hussain et al., 1989). The origin of the GDH polymorphism is not known. It was reported that the presence of four differently sized mRNAs and multiple gene copies for GDH in the human brain (Mavrothalassitis et al., 1988). A novel cDNA encoded by an X chromosome-linked intronless gene also has been isolated from human retina (Shashidharan et al., 1994).

Recently, it also has been reported that the hyperinsulinism-hyperammonemia syndrome is caused by mutations in GDH gene that affects

enzyme sensitivity to GTP-induced inhibition (Stanley et al., 1998; Miki et al., 2000), demonstrating that allosteric regulation of GDH plays a crucial role *in vivo*. The mutations identified in the patients with hyperinsulinism and hyperammonemia (Stanley et al., 1998) exactly lie within a sequence of amino acids that we previously suggested to contain GTP binding site of the bovine brain GDH (Cho et al., 1996). Thus, it is essential to have a detailed structural and functional description of the human GDH to elucidate the pathophysiological nature of the GDH-related disorders.

The atomic structures of several forms of GDH from nonmammalian (Rice et al., 1987; Knapp et al., 1997) and bovine liver (Peterson et al., 1999; Smith et al., 2001) have proposed roles of three conserved lysine residues, Lys94, Lys118, and Lys130 in human GDH. Lys118 and Lys94 are proposed to be involved in salt bridge to the á- and ã-carboxyl groups of the substrate, respectively (Peterson and Smith, 1999). Recently, it has been reported that the NAD^+-binding domain is closed over the ligands, bringing Lys130 and the nicotinamide ring in close proximity to the bound glutamate (Peterson and Smith, 1999). There are, however, conflicting views as to whether Lys130 is involved in Schiff's base formation with catalytic intermediates (Hochreiter and Schellenberg, 1969), stabilization of negatively charged groups or the carbonyl group of 2-oxoglutarate during catalysis (Rife and Cleland, 1980), or some other function (Chen and Engel, 1975). In the present work, it has been

expanded on these observations using a cassette mutagenesis at Lys130 site.

Pyridoxal 5 -phosphate (PLP) has been widely used in structural and functional studies to selectively label particularly reactive lysyl residues, which ard often involved directly in the catalytic mechanism of active sites (Lo Bello et al., 1992; Paine et al., 1993; Valinger et al., 1993). Its molecular structure contains an aromatic moiety and a negatively charged group; these features are in common with other lysyl-modifying reagents, as dicussed by Jeffry et al. (1985). Therefore, the present work has been performed spectroscopic studies of the complexes formed between GDH and several mutants with PLP and several PLP analogs.

For these studies, a 1557-base-pair gene that encodes human GDH has been synthesized based on the amino acid sequence of human GDH (Mavrothalassitis et al., 1988) and expressed in *Escherichia coli* as a soluble protein. The mutant enzymes, containing Glu, Gly, Met, Ser, or Tyr at position 130, have been expressed in *E. coli* as a soluble protein, purified, and characterized. This is the first report by site-directed mutagenesis showing a direct involvement of Lys130 of mammalian GDH in catalysis rather than in the binding of coenzyme or substrate. From such studies, ideas have emerged as to how one might approach the design of new inhibitors of this enzyme.

II. MATERIALS AND METHODS

Materials

Pyridoxal 5-phosphate and pyridoxal were purchased from Fluka. 5-Deoxypyridoxal was purchased from Eastman Kodak and purified further by vacuum sublimation. Pyridoxol phosphate was synthesized as reported (Santi et al., 1993) and characterized by mass spectrometry. ADP, ATP, $NADP^+$, NAD^+, NADH, NADPH, L-glutamate and 2-oxoglutarate were purchased from Sigma. Precast gels for sodium dodecil sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from NOVEX. Hydroxylapatite and low molecular weight standards for SDS-PAGE were obtained from Bio-Rad. FPLC Resource Q column was purchased from Pharmacia. HPLC Protein-Pak 300SW column was purchased from Waters. All other chemicals and solvents were reagent grade or better.

Bacterial strains

E. coli DH5á (Hanahan, 1983) was used as the host strain for plasmid-mediated transformations during the assembly of the synthetic GDH gene (pHGDH) and for cassette mutagenesis. *E. coli* PA340 (*thr-1 fhuA2 leuB6 lacY1 supE44 gal-6 gdh-1 hisG1 rfbD1 galP63 D(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 synthase activities (Teller et al., 1992) and was used to test plasmids for GDH activity. *E. coli* BL21 (DE3) (Studier and Moffatt et al., 1986) was used for high level expression of the recombinant GDH.

Oligonucleotide synthesis

Oligonucleotides were purchased from the University of California, San Francisco, Biomolecular Resource Center at a scale of 50 ~ 200 nmol. Oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis using 8 ~ 12 % acrylamide/ 8 M urea gels and desalted by passage over a Waters Sep-Pack cartridge or Pharmacia NAP-25 column as described elsewhere (Sambrook et al., 1989).

Design and assembly of the synthetic gene

The design of the synthetic GDH gene was based on the amino acid sequence of human GDH (Mavrothalassitis et al., 1988) and used the following strategy. First, a DNA sequence that includes 45 sites located approximately every 35 bp throughout the entire length of the coding region was selected from the large number of possibilities. Only those sites that are recognized by commercially available restriction enzymes and that are not located in pUC18 (except in the polylinker region) were included in the final

sequence of the gene. The GDH gene is flanked by unique *Eco*R I and *Hin*d III restriction sites that render the gene portable to any of several *E. coli* expression vectors. Second, the codon usage of the resulting GDH gene was modified to include those triplets that are utilized in highly expressed *E. coli* genes (An et al., 1981; Gouy and Gautier, 1982) while retaining the largest possible number of unique restriction sites. In some cases, suboptional codons were used either to allow the inclusion of unique restriction site or to preclude redundant sites. Third, a ribosome binding site AGGAGG (Stormo, 1986) was added 10 bases upstream of the coding region to direct the initiation of translation in *E. coli*. The sequence adjacent to the ribosome binding site included an A at position -3 relative to the ATG, and the spacer region (-1 to -9) was made A + T rich to reduce potential mRNA secondary structure in the vicinity of the translation start site (Stormo, 1986). Addition of a ribosome binding site made the synthetic GDH gene portable to any of a number of commonly available plasmid vectors that carry inducible *E. coli* promoters. Finally, the synthetic GDH gene contains the sequence coding for the recognition site of the specific protease Factor Xa, located just before the first amino acid (Ser) of the authentic human GDH. Factor Xa cleaves after its four amino acid recognition sequence, I-E-G-R (Nagai and Thogersen, 1987), so that no additional residues are attached to the protein.

The GDH gene was assembled from three gene segments that were

initially cloned into pUC18. Each of the gene segments was constructed with six to seven oligonucleotides. Methods for plasmid purification, subcloning, and bacterial transformation were as described (Sambrook et al., 1989). Six to seven isolates of each segment were examined by restriction analysis and DNA sequencing. The final synthetic human GDH gene, designated as pHGDH, was used for gene expression and mutagenesis studies.

Protein purification and characterization

Fresh overnight cultures of DE3/pHGDH were used to inoculate 1 L of LB containing 100 μg of ampicillin per 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 lysis buffer (100 mM Tris-HCl, pH 7.4/ 1 mM EDTA/ 5 mM dithiothreitol) and lysed with a sonicator. Cellular debris was removed by centrifugation and the crude extract was precipitated by 30 ~ 65 % ammonium sulfate. After centrifugation at 12,000 x g for 30 min at 4 °C, the pellet was dissolved in a minimum amount of buffer A (2 mM potassium phosphate, pH 7.0/ 1 mM EDTA/ 5 mM dithiothreitol) and dialyzed against buffer A. The dialyzed sample was loaded onto a hydroxylapatite column (2.5 x 10 cm) (Bio-Gel HTP, Bio-Rad) that was equilibrated with buffer A. The column was

washed with washing buffer (10 mM potassium phosphate, pH 7.0/ 1 mM EDTA/ 5 mM dithiothreitol) until the breakthrough peak of protein had been eluted. The enzyme was then eluted by a gradient up to 200 mM potassium phosphate. The fractions containing GDH were pooled, concentrated, and buffer changed to buffer B (20 mM Tris-HCl, pH 8.0/ 1 mM EDTA/ 5 mM dithiothreitol) using Amicon concentrator and then applied to a FPLC Resource-Q anion exchange column equilibrated with buffer B. The enzyme was then eluted using a linear gradient made with buffer B in increasing concentration of NaCl (from 0 mM to 100 mM) at 0.5 ml/min. The fractions containing GDH were combined and applied to a HPLC Protein-Pak 300SW gel filtration column (0.15 x 30 cm) equilibrated with buffer B and the proteins were eluted by the same buffer at 1 ml/min.

The purified GDH was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and recognized by western blot using monoclonal antibodies previously produced in our laboratory (Choi et al., 1999) against the bovine brain GDH. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard. Since *E. coli* only has an $NADP^+$-dependent GDH (McPherson and Wootton, 1983; Teller et al., 1992), the enzyme assay was performed with NAD^+ as a coenzyme as described elsewhere (Cho et al., 1995).

To remove five additional N-terminal residues (M-I-E-G-R), the purified

human GDH was treated with Factor Xa (10 ì g per 1 mg GDH) in reaction mixtures of 20 mM Tris-HCl, pH 8.0 containing 100 mM NaCl, and 2 mM $CaCl_2$. After incubation at room temperature for 3 hr, the reaction mixtures were purified by a HPLC Protein-Pak 300SW gel filtration column as described above. HPLC-purified human GDH was subjected to automated Edman degradation.

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 (Cho et al., 1995). 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, and 2.6 mM EDTA at 25 ^{o}C. Since *E. coli* only has an NADP(H)-dependent GDH (Hanahan, 1983; McPherson and Wootton, 1983), the enzyme assay was performed with NADH as a coenzyme as described elsewhere (Cho et al., 1995). One unit of enzyme was defined as the amount of enzyme required to oxidize 1 μmol of NADH per min at 25 ^{o}C. For determination of kinetic constants (V_{max} and K_m), the assays were carried out by varying one substrate under investigation while keeping the other substrate and reagents at the saturating concentration as described elsewhere

(Cho et al., 1995). All analyses were performed using the StatView statistical computer program (Abacus Concepts, Berkeley, CA).

Construction and characterization of Lys130 mutants

A series of single amino acid substitutions of Lys130 was constructed by cassette mutagenesis of plasmid pHGDH. Plasmid DNA (5 μg) was digested with *Sal* I and *Ngo*M I to remove 27-bp fragment that encodes amino acids 123 ~ 131 and vector DNA was purified by electrophoresis using 1 % low melting point agarose. The 27-bp *Sal* I/*Ngo*M I fragment was replaced with five 27-bp synthetic DNA duplexes containing a substitution on both DNA strand at positions encoding Lys130 to make K130G, K130S, K130E, K130M, and K130Y mutant proteins. Mutagenic oligonucleotides were annealed, ligated, and transformed into DH5á as described above and Lys130 mutants were identified by DNA sequencing using plasmid DNA as a template. Each of these mutants has been expressed in *E. coli* strain DE3, purified to homogeneity as described above, and has had its steady state kinetic parameters determined. The gene expression levels of Lys130 mutant proteins were examined by western blot and compared with those of wild type GDH.

Interaction of GDH with PLP and its analogs

The wild type and mutant proteins were separately incubated with PLP or

its analogs at various concentrations in 100 mM potassium phosphate, pH 7.4 at 25 °C. All solutions containing PLP were protected from photolytic destruction with metal foil. At intervals after the initiation of the inactivation, aliquots were withdrawn for the assay of activity. For spectroscopic studies, aliquots of the PLP-modified proteins were treated with freshly prepared ice-cold 40 mM $NaBH_4$. After 3 h at 4 °C, the samples were dialyzed against four changes of 100 mM potassium phosphate, pH 7.4 at 4 °C overnight and clarified by centrifugation. Absorption spectra were recorded at 25 °C on a Beckman DU-70 UV/Vis spectrophotometer. Fluorescence spectra were recorded at 25 °C on a SLM Aminco DMX-1000 fluorescence spectrophotometer. Excitation was at 280 nm.

III. RESULTS

Gene synthesis and expression

The gene was initially constructed as three segments using the plasmid pUC18 as a cloning vector (Figure II-1). A total of 19 synthetic oligonucleotides that varied from 35 to 120 nucleotides in length were used to assemble the three segments. The ends of the segments were chosen from restriction sites present in the polylinker of pUC18 to allow stepwise assembly in that vector. The three segments were, respectively, a 467-bp *Eco*R I/*Sac* I fragment composed of six oligonucleotides, a 526-bp *Sac* I/*Xba* I fragment composed of seven oligonucleotides, and a 564-bp *Xba* I/*Hin*d III fragment composed of six oligonucleotides (Figure II-1). Several isolates of each of the gene segments were characterized by DNA sequencing. Based on the sequencing of several isolates of each gene segment, an overall mutation frequency of ~3 per 1000 bp synthesized was observed and corrected to the designed sequence using a standard cassette mutagenesis procedure. The designed sequence and position of 45 restriction sites in the GDH coding region of pHGDH are shown in Figure II-2.

E. coli strain PA340 lacks both GDH and glutamate synthase activities (Teller et al., 1992). Activity measurements and SDS-PAGE of the crude extracts from *E. coli* strain PA340 transformed with pHGDH showed that the

plasmid was directing the synthesis of catalytically active GDH (0.11 units/mg) to a level of ~1 % of the total soluble protein. However, higher level expression of the synthetic GDH gene was achieved by transformation of pHGDH into *E. coli* strain DE3. Upon induction with 1 mM of IPTG at 37 ^{o}C for 3 h, expression of GDH in soluble extracts increased to 1.24 units/mg. SDS-PAGE analysis of crude cell extracts and measurement of specific activities indicated that pHGDH directed GDH expression to a level of ~ 11 % of total cellular protein upon induction with IPTG (Figure II-3*A*).

Purification and characterization of the gene product

GDH encoded by pHGDH in DE3 was purified by several chromatographic methods (see MATERIALS ANS METHODS). Since the recombinant human GDH was readily solubilized, no detergents were required throughout the entire purification steps. The purified GDH was estimated to be >98 % pure by SDS-PAGE (Figure II- 3*A*). The subunit size (56.5 kDa) and the native size (320 kDa) of the hexameric recombinant GDH as determined by SDS-PAGE (Figure II-3*A*) and HPLC gel filtration chromatography (data not shown), respectively, was similar to those of authentic GDHs (Smith et al, 1975; Hussain et al., 1989; Cho et al., 1995). The 56.5-kDa protein were absent in extracts from DE3 transformed with pUC18 (Figure II-3*A*). The catalytic properties of purified recombinant GDH

were also indistinguishable from those of the authentic GDHs (Smith et al., 1975; Mavrothalassitis et al., 1988; Hussain et al., 1989; Cho et al., 1995); the kinetic parameters were 120 units/mg, K_m for 2-oxoglutarate = 1.4 mM, and K_m for NADH = 80 ì M.

To remove five additional N-terminal residues (M-I-E-G-R) that were introduced to create initiation codon and Factor Xa recognition site, the purified human GDH was treated with Factor Xa, purified by a HPLC Protein-Pak 300SW gel filtration column, and subjected to automated Edman degradation. N-terminal sequence analysis of the first 8 amino acids (S-E-A-V-A-D-R-E) was in good agreement with published sequence of authentic human GDH (Mavrothalassitis et al., 1988). Indeed, the five additional residues (M-I-E-G-R) did not show any significant changes in specific activities, heat stability, and solubility of the recombinant human GDH.

Construction of Lys130 mutants

To test the utility of the synthetic GDH gene, it was constructed a series of mutants at Lys130 by cassette mutagenesis. The 27-bp *Sal* I/*Ngo*M I fragment in pHGDH was replaced with five 27-bp synthetic DNA duplexes containing a substitution on both DNA strand at positions encoding Lys130 to make K130G, K130S, K130E, K130M, and K130Y mutant proteins (Figure II-4, Table II-1). Activities of the mutants were 0.3 ~ 0.5 units/mg, compared

with wild type value of 120 units/mg. This represents an ~ 400-fold reduction in the activities of Lys130 mutants compared with wild type, suggesting that Lys130 is strictly required for catalytic activity. A more detailed investigation of the catalytic activities of the mutant enzymes revealed essentially unchanged apparent K_m values for 2-oxoglutarate and NADH (Table II-2), despite an approximately 400-fold decrease in the respective apparent V_{max} values. Analysis of crude cell extracts by western blot showed that the five Lys130 mutants plasmids encoding an amino acid substitution at position 130 directed the synthesis of a 56.5-kDa protein that interacted with monoclonal antibodies raised against GDH at almost identical level for all five Lys130 mutants and wild type GDH (Figure II-3*B*). These results indicate that the absence of catalytic activity is not due to a lack of production or stability of the different mutants.

Interaction of GDH with PLP and its analogs

It has been reported that GDH is inactivated by PLP and suggested a possibility that Lys130 may be involved in the formation of GDH-PLP complex (Lilley and Engel, 1992; Kim et al., 1997). To unambiguously identify that Lys130 is responsible for PLP binding on GDH, interaction of wild type and Lys130 mutant GDHs with PLP was examined using absorption and fluorescence emission spectroscopy. For spectroscopic studies, attempts

were made to stabilize the inactive form of the enzyme by $NaBH_4$ reduction. After $NaBH_4$ reduction and dialysis, the spectrum of PLP treated wild type GDH shows a characteristic peak of pyridoxamine derivatives (Churchich, 1965a) at 340 nm that is absent in the spectrum of the Lys130 mutant proteins (Figure II-5*A*). The fluorescence spectrum of the pyridoxyl-wild type GDH also differs completely from that of the Lys130 mutant proteins when excited at 280 nm. The pyridoxyl-wild type GDH is characterized by two fluorescence peaks at 330 nm and 380 nm, respectively, while the mutant proteins exhibits a maximum emission peak around 330 nm (Figure II-5*B*). The peak at 380 nm presumably arises through energy transfer from the aromatic residue to the bound pyridoxyl groups (Churchich, 1965b), and this interpretation is consistent with the finding that the presence of pyridoxyl groups causes a dramatic quenching of the wild type enzyme fluorescence, which does not occur in the Lys130 mutant proteins. All five mutant proteins show almost identical absorption and emission spectra and only data obtained with K130M are shown in Figure II-5 for clarity purpose.

To test the importance of phosphate and aldehyde moieties in the interaction of PLP with the Lys residue, the enzyme was treated with PLP analogs having no phosphate or aldehyde moieties. First, it was examined the interaction of 5-deoxypyridoxal (Figure II-7), which lacks the phosphate moiety of PLP. Treatment of GDH with 5-deoxypyridoxal showed an

inactivation of GDH activity as effective as PLP (Figure II-6). The results of spectroscopic studies of GDH complexed with 5 -deoxypyridoxal also showed a similar pattern to those obtained with PLP (data not shown). These results indicate that 5 -deoxypyridoxal is clearly an effective inactivator and the phosphate moiety is not necessary for binding of PLP to GDH. Unlike the phosphate group, the importance of aldehyde moiety of PLP in binding to GDH was demonstrated by showing that reduction of the aldehyde moiety of PLP to alcohol, giving pyridoxol phosphate (Figure II-7), only caused a minor inactivation (Figure II-6).

Table II-1. Oligonucleotides used for Lys130 mutant GDHs

K130 Mutant GDHs		Oligonucleotide Sequences					
		122		130		132	
K130G	5	TC gAC … gCC	**ggC**	g			3
	3	g … Cgg	**CCg**	Cgg	CC		5
K130S	5	TC gAC … gCC	**TCC**	g			3
	3	g … Cgg	**Agg**	Cgg	CC		5
K130E	5	TC gAC … gCC	**gAA**	g			3
	3	g … Cgg	**CTT**	Cgg	CC		5
K130M	5	TC gAC … gCC	**ATg**	g			3
	3	g … Cgg	**TAC**	Cgg	CC		5
K130Y	5	TC gAC … gCC	**TAC**	g			3
	3	g … Cgg	**ATg**	Cgg	CC		5

Table II-2. Kinetic parameters of wild type and Lys130 mutant GDHs

	V_{max}	K_m	
	($\mu mol.min^{-1}.mg^{-1}$)	NADH (μM)	2-Oxoglutarate (mM)
Wild type	115	80	1.4
K130G	0.4	81	1.3
K130S	0.3	86	1.5
K130E	0.5	83	1.5
K130M	0.4	79	1.7
K130Y	0.3	80	1.4

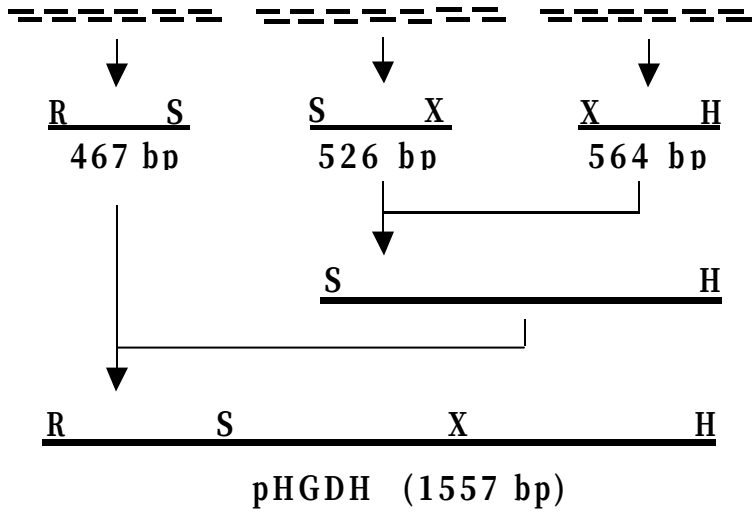


Figure II-1. Assembly of the synthetic GDH gene. A total of 38 oligonucleotides were used to assemble three gene fragments that varied from 467 to 564 bp in length. DNA fragments corresponding to the gene segments were isolated and used to create the functional GDH gene (pHGDH) via stepwise ligation into pUC18 as shown. R, *Eco*R I; S, *Sac* I; X, *Xba* I; H, *Hin*d III.

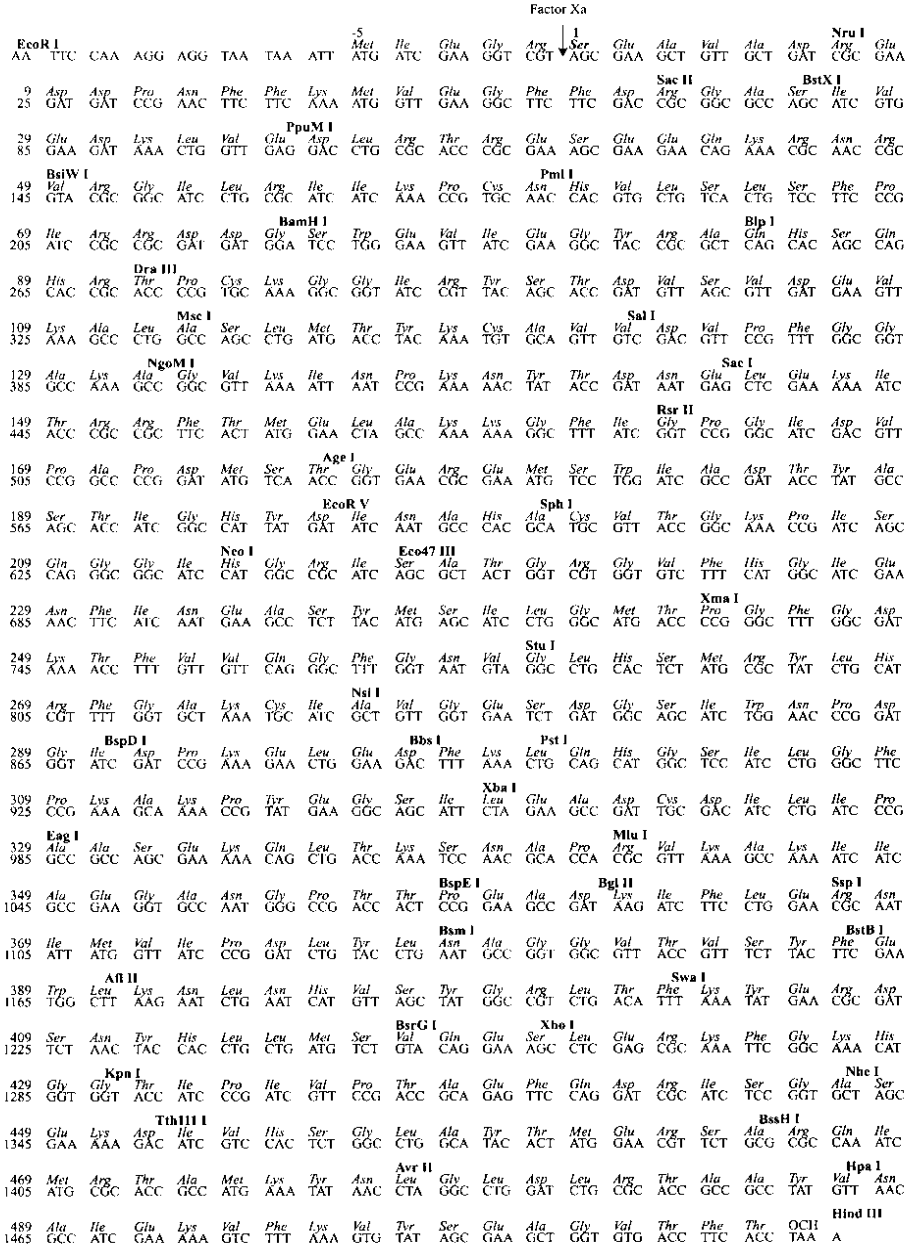


Figure II-2. DNA sequence of the synthetic human GDH gene carried in pHGDH. Numbers on the left refer to amino acids (upper) and DNA (lower). Only the unique restriction sites are shown. Factor Xa site is shown by a vertical arrowhead. Position 1 of the amino acid sequence corresponds to the first amino acid (Ser) of the authentic human GDH (Mavrothalassitis et al., 1988)

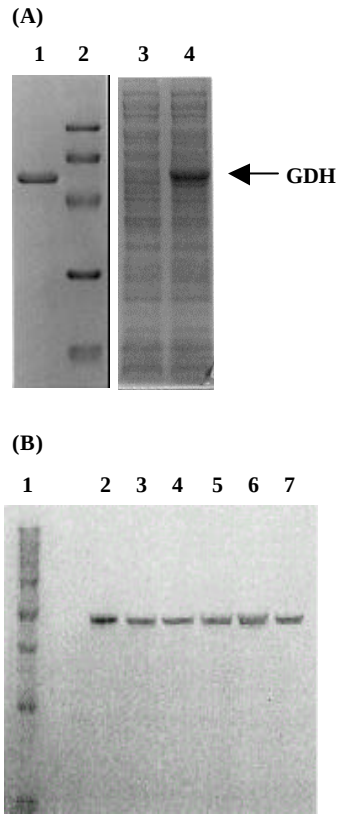


Figure II-3. Electrophoretic analysis of human GDH. **(A)** SDS-PAGE analysis of recombinant human GDH. Lane 1; purified recombinant human GDH, lane 2; marker proteins (97, 66, 45, 31, 21 kDa), lane 3; crude extract from DE3 transformed with pUC18, and lane 4; crude extract from DE3 transformed with pHGDH. **(B)** Western blot analysis of recombinant human GDH and Lys130 mutants in crude extracts. Lane 1; prestained marker proteins (103, 77, 50, 34, 29, 21 kDa), lane 2; wild type GDH, and lane 3 ~ 7; Lys130 mutants (K130G, K130S, K130E, K130M, and K130Y).

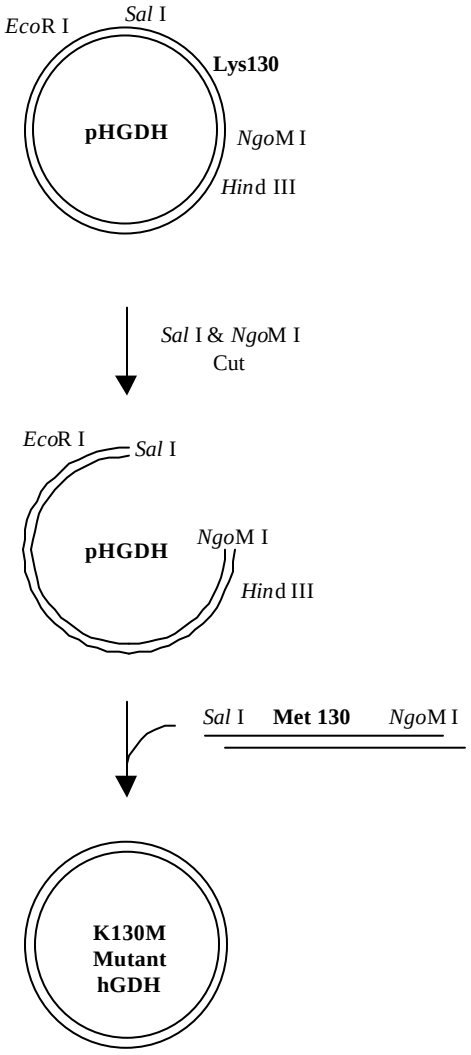


Figure II-4. Cassette mutagenesis of Lys130 site of human GDH.

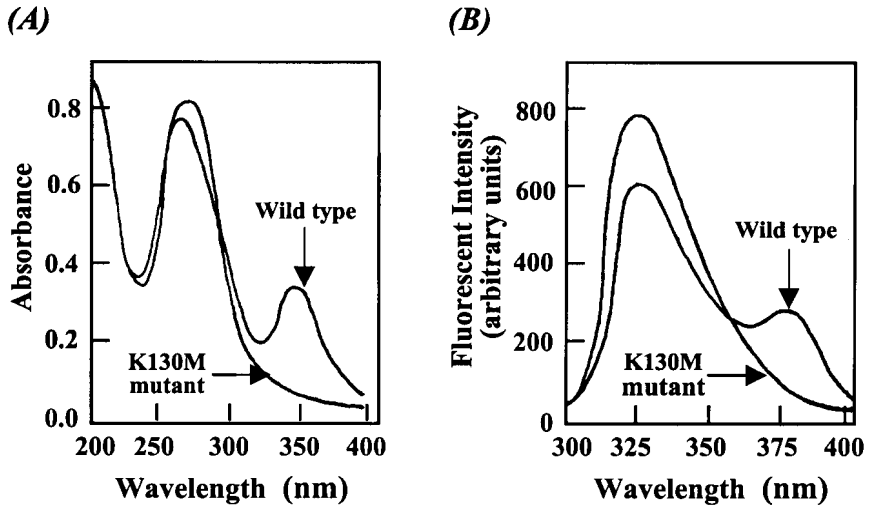


Figure II-5. Spectroscopic studies of the interaction of the wild type GDH and Lys130 mutant enzymes with PLP. All five mutant proteins show identical absorption and emission spectra and only data from the K130M mutant protein is shown in this figure as clarity purpose. Pyridoxyl enzyme (2 ìM) was prepared as described in MATERIALS AND METHODS at a [PLP]/[GDH] ratio of 100. (**A**) Absorption spectra of pyridoxyl enzyme. (**B**) Fluorescence emission spectra of wild type and K130M mutant GDH.

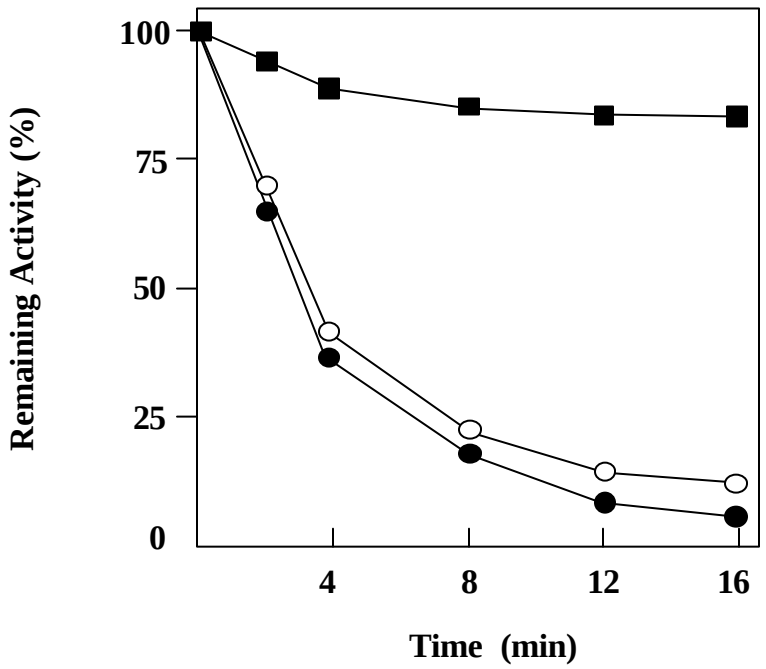
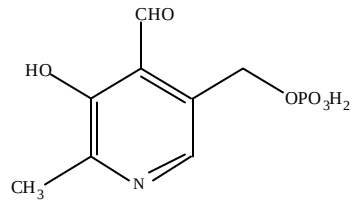
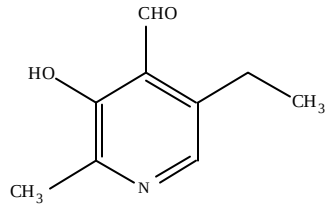


Figure II-6. Time-dependent inactivation of GDH by with PLP analogs. GDH (2 ìM) was incubated with PLP and its analogs 25 ºC in 100 mM potassium phosphate, pH7.4. Aliquots were withdrawn at the indicated time and the remaining activities were measured. The concentration of the reagents was 200 ìM. ●, PLP; ○, 5 ′-deoxypyridoxal; ■, pyridoxol phosphate.

Pyridoxal 5′-phosphate



5′-Deoxypyridoxal



Pyridoxol phosphate

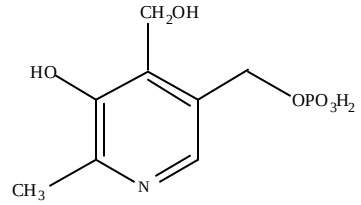


Figure II-7. Structure of PLP and its analogs.

IV. DISCUSSION

The reaction mechanism of GDH has been extensively studied using a variety of methods (Srinivasan and Fisher, 1985; Singh et al., 1993; Plaitakis et al., 2000). Previous knowledge of chemical modification studies also has suggested that the reactive essential Lysine residues may be involved in the GDH reaction (Valinger et al., 1993; Kim et al., 1997). For bovine liver GDH, it has been reported that closing the active-site cleft provides a hydrophobic environment suitable for hydride transfer (Singh et al., 1993). This closure removes or neutralizes four positive charges; an á-amino group of glutamate and three conserved lysine residues. It has been proposed (Singh et al., 1993) that Lys130, having an abnormally low pK_a, spontaneously loses an H^+ to solvent and triggering three events in a concerted manner: a single water molecule becomes hydrogen bonded to the NH_2 group of Lys130; the bulk water is expelled from the cleft; and the α-hydrogen atom of the substrate is brought into the correct orientation and position to permit hydride transfer to the nicotinamide ring of NAD^+. Lys130 is fully conserved in all GDH sequences obtained so far. It has been reported that GDH is inactivated by PLP and suggested that one lysine residue, probably Lys130, may be responsible for the formation of GDH-PLP complex based on the results from chemical modification and tryptic digested peptide analysis (Lilley and Engel,

1992; Kim et al., 1997). The Lys130 residue of human GDH corresponds to Lys128 in the *E. coli* sequence where the importance of this residue has been demonstrated (McPherson et al., 1988). This lysine residue has been identified also in overlapping CNBr fragments of unmodified clostridial GDH (Lilley et al., 1991) and the precise position within the intact sequence is confirmed by comparison with the entire sequence obtained from the cloned clostridial GDH gene (Teller et al., 1992). There are, however, conflicting views as to whether Lys130 is involved in Schiff's base formation with catalytic intermediates (Hochreter and Schellenberg, 1969), stabilization of negatively charged groups or the carbonyl group of 2-oxoglutarate during catalysis (Rife and Cleland, 1980), or some other function (Chen and Engel, 1975). Recently, it has been reported through crystallographic studies that the NAD^+-binding domain is closed over the ligands, bringing Lys130 and the nicotinamide ring in close proximity to the bound glutamate (Peterson and Smith, 1999).

To unambiguously test the role of Lys130, we have performed a cassette mutagenesis at Lys130 position using chemically synthesized human GDH gene. The synthetic GDH gene facilitates the rapid generation of large numbers of site-directed mutations by the simple replacement of small restriction fragments with synthetic DNA duplexes that carry the desired mutations. This approach is made possible by the inclusion of 45 restriction sites within GDH coding sequence (Figure II-2). The synthetic gene can be

used to generate a large variety of mutations ranging from single amino acid substitutions to the replacement of entire structural domains. The use of the synthetic gene is further enhanced by its high level of expression in *E. coli*. It is prepared a mutagenic oligonucleotide cassette in which the codon for Lys130 was replaced by five codons that encode 5 different amino acids (Table II-1, Figure II-4). Enzyme assays of crude extracts showed 500-fold reduction in V_{max} values (Table II-2) in all five mutant proteins regardless of their size, hydrophobicity, and ionization of the side chains. This present work suggests that Lys130 is critically involved in the catalysis of human GDH. The kinetics of Lys130 mutants in more detail, in particular by rapid-reaction kinetics, remains to be studied in order to elucidate the role of this residue more fully.

The phosphate moiety of PLP has been implicated as an important site of interaction with GDH because pyridoxal, which lacks the phosphate moiety of PLP, is much less potent inactivator of bovine liver GDH (Metzler and Snell, 1955). From this study, it is found that treatment of human GDH with pyridoxal, which lacks the phosphate moiety of PLP, gave only a minor inactivation (data not shown). However, since pyridoxal exists as a stable intramolecular hemiacetal in solution (Nakamoto and Martell, 1959), its ability to form an intermolecular interaction with GDH is compromised and the analog is not a valid test of the importance of the phosphate group in the

interaction. Unlike pyridoxal, 5 -deoxypyridoxal lacks the hydroxyl group of pyridoxal and thus retains the aldehyde moiety in aqueous solution (Metzler and Snell, 1955). Therefore, I examined the interaction of 5 -deoxypyridoxal with GDH. As shown in Figure II-6, 5 -deoxypyridoxal is clearly an effective inactivator. From the view of the experiments reported here, I suggest that a negative charge, such as that of the phosphate group, is not required for efficient inactivation. The importance of aldehyde moiety of PLP in binding to GDH was observed with pyridoxol phosphate, in which the aldehyde moiety of PLP is reduced to alcohol. The result from Figure II-6 indicates that pyridoxol phosphate is not an effective inactivator, demonstrating that the aldehyde moiety, unlike phosphate moiety, is required for efficient binding to GDH (Figure II-7). None of the PLP analogs were able to bind to Lys130 mutant proteins (data not shown).

The enzyme activity of GDH is controlled by allosteric effectors, namely, inhibition by GTP and activation by ADP (Smith et al., 1975; Plaitakis et al., 2000). Recently, it has been reported that in patients with hyperinsulinism-hyperammonemia syndrome, activating mutations in GDH gene have been identified and the mutations have been located within the GTP-binding site in conjunction with a loss of GTP inhibition (Stanley et al., 1998; Miki et al., 2000). A knowledge of the interaction of PLP with human GDH may provide insights into approaches for the design of a new class of drugs which do not

resemble the allosteric regulators such as GTP of the enzyme. It is clear that the aldehyde moiety of PLP is important to binding (Figure II-7). The lack of requirement for the phosphate group may be promising in drug design, since phosphate monoesters do not readily penetrate cells and, therefore, must undergo appropriate metabolic activation. We do not know whether the pyridine ring of PLP is important for binding to GDH, although we think it plays some role. An understanding of the interaction of the PLP pyridine moiety with the enzyme may permit design of even more potent inhibitors of GDH, either by optimizing favorable interactions or by using the heterocycles as a scaffold for other groups which may interact favorably with the enzyme.

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 from plasmids that carry the gene 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 relating to the structure and function of human GDH.

CHAPTER III

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

I. INTRODUCTION

Glutamate dehydrogenase (GDH; EC 1.4.1.3) has been found in nearly every organism and has a pivotal role in nitrogen and carbon metabolism via the tricarboxylic acid cycle. GDH catalyzes the reversible deamination of L-glutamate to 2-oxoglutarate using NAD^+, $NADP^+$ or both as coenzymes (Smith et al., 1975).

$$\text{Glutamate} + NAD(P)^+ + H_2O \quad \text{2-Oxoglutarate} + NH_4^+ + NAD(P)H$$

It plays a role in regulating the levels of ammonia and glutamate in the central nervous system. Therefore, the activity of this enzyme should be tightly regulated to respond appropriately to the metabolic needs of the cell. The enzyme has been purified from the liver of several species and sequenced (Smith et al., 1975). Mammalian GDH is known to be a homohexameric mitochondrial enzyme. Mammalian GDH is strictly regulated by several effectors such as pH, aggregation state, a variety of activators and inhibitors (Smith et al., 1975). 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 (Peterson and Smith, 1999; Smith et al., 2001; Pal and Colman, 1979). In addition, high concentrations of NADH, a substrate for the reverse reaction, has been reported to inhibit the forward reaction (Frieden, 1959). In contrast,

ADP is a potent activator by decreasing product affinity (Hussain et al., 1989; Bailey et al., 1982). Paradoxically, ADP and ATP have been shown to have opposite effects on GDH activity. At pH 8.0 and high NADH concentrations, ADP increases the reductive amination reaction velocity by about threefold, wherease ATP inhibits the reaction by about fourfold (Friden, 1959). Recently, a new class of GDH from *Streptomyces clavuligerus* is reported (Minambres et al., 2000). This enzyme, which is composed of 183-kDa subunits and NAD-specific, requires AMP as an essential activator. Aspartate and asparagines were found to be allosteric activators and excess of NADH or ammonia was inhibited the activity of the enzyme. It is noteworthy that GDHs purified from various sources, independently from coenzyme requirements and allosteric modulators, show a complex pattern of allosteric effects of the coenzymes on the rate of reaction and allosteric interaction among the identical subunits (Syed et al., 1994).

The recent 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 (Peterson and Smith, 1999; Smith et al., 2001). However, the ADP binding site has not been identified. 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 (Batra and Colman, 1986; Batra et al., 1989) and at Arg459 (Wrzeszczynski and Colman, 1994). 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 a different results to be modified at Arg491 (Madhusoodanan and Colman, 2001) and at a peptide corresponding to Met411 ~ Arg419 (Rajagopalan et al., 1999). Alternatively, various azidonucleotide photoprobes have been used to identify the base-binding domains of the allosteric sites and to localize the domains within the proposed catalytic cleft (Kim and Haley, 1991; Cho et al., 1996; Shoemaker and Haley, 1996; Cho and Yoon, 1999). 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 (Shoemaker and

Haley, 1996). 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.

The importance of the physiological nature of GDH has attracted considerable interest (Hussain et al., 1989; Shashidharan et al., 1997; Plaitakis et al., 2000; Herrero-Yraola et al, 2001). 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 (Fahien et al., 1988; Bryla et al., 1994; Stanley et al., 1998). These observations demonstrate that allosteric regulation of GDH plays a crucial role in the regulation of insulin secretion and hepatic ureagenesis. The mutated residues responsible for this pathology (Stanley et al., 1998) exactly lie within a sequence that we previously suggested to contain GTP binding site of the brain GDH isoproteins using photoaffinity labeling (Cho et al., 1996). These results prove selectivity and specificity of the photoaffinity probe as a valid active-site probe. A further understanding of the regulation and details of structure will help us to elucidate the metabolic role that GDH has in cellular homeostasis.

Very recently, a 1557-base-pair gene that encodes human GDH has been

synthesized and expressed in *Escherichia coli* in our laboratory (Cho et al., 2001). This present work have expanded on the identification of ADP binding site of human GDH using a cassette mutagenesis and photoaffinity labeling with $8N_3$ADP at Tyr187 residue that has been located within a putative base-binding domain of the ADP sites (Shoemaker and Haley, 1996; Cho and Yoon, 1999; Rajagopalan et al., 1999). For the present study, the mutant human GDHs containing Arg, Glu, Gly, Met, or Ser at Tyr187 site have been expressed in *E. coli* as a soluble protein, purified, and characterized. This is the first report by site-directed mutagenesis showing that Tyr187 is required for the efficient binding of ADP to human GDH.

II. 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. $8N_3ADP$ was purchased from ALT Corp. [á-^{32}P]$8N_3ADP$ was synthesized by the method as previously described (Shoemaker and Haley, 1996; Cho and Yoon, 1999). Q-Sepharose and FPLC Resource-Q column were purchased from Pharmacia. POROS-QM column was purchased from Perceptive Biosystems. 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 (see MATERIALS AND METHODS of CHAPTER II). All other chemicals and solvents were reagent grade or better.

Bacterial strains

E. coli DH5α (Hanahan, 1983) 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 D(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 (Teller et al., 1992) and was used to test plasmids for GDH activity. *E. coli* BL21 (DE3) (Studier and Moffatt, 1986) was used for high level expression of the recombinant human GDH.

Construction and characterization of Tyr187 mutants

A series of single amino acid substitutions of Tyr187 was constructed separately by cassette mutagenesis of a synthetic human GDH gene, pHGDH. Plasmid DNA (5 μg) was digested with *Age* I and *Eco*R V to remove 62-bp fragment that encodes amino acids 175 ~ 195 and replaced with five 62-bp synthetic DNA duplexes containing a substitution on both DNA strand 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, 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 per ml. DE3/pHGDH was grown at 37 ^{o}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 ^{o}C and then harvested by centrifugation. Cell pellets were suspended in 100 ml of 50 mM Tris-HCl, pH 8.0 containing 2 mM EDTA, 10 mM NaCl, 2 mM phenylmethylsulfonylfluoride, 1 mM dithiothreitol and 2 % Triton X-100 and lysed with a sonicator. The homogenate was centrifuged at 11,000 x g, 10 min, 4 $^{\circ}$C and the pellet was discarded. The supernatant was loaded onto to a column (5 x 16 cm) of Q-Sepharose was equilibrated with 50 mM Tris-HCl, pH 8.0 containing 0.5 mM EDTA and 1 mM â-mercaptoethanol. The column was washed with 50 mM Tris-HCl, pH 7.0 containing 0.5 mM EDTA, 0.1 N NaCl and 1 mM â-mercaptoethanol (buffer I) until the breakthrough peak of protein had been eluted. The enzyme was then eluted using a step gradient with buffer I containing 0.5 N and 1N NaCl, respectively. The fractions containing GDH were combined, concentrated and buffer changed to buffer II (50 mM Tris-HCl, pH 8.0/ 0.5 mM EDTA/ 1 mM â-mercaptoethanol) using Amicon concentrator and then applied to a FPLC Resource-Q anion exchange column (6 ml) equilibrated with buffer II. The enzyme was then eluted using a linear gradient made with buffer II in increasing concentration of NaCl (from 0 mM to 500 mM) at 2 ml/min. The fractions containing GDH were combined, concentrated and desalted with buffer III (20 mM potassium phosphate, pH 8.0/ 0.5 mM EDTA/ 1 mM â-mercaptoethanol) and applied to

a POROS-QM anion exchange column equilibrated with buffer III. The enzyme was then eluted using a linear gradient made with 20 mM potassium phosphate, pH 6.0 containing 0.5 mM EDTA, 0.3 M NaCl and 1 mM â-mercaptoethanol at 1 ml/min. Since the wild type and 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 (Choi et al., 1999) against the bovine brain GDH. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

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 (Cho et al., 1995). 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 ^{o}C. Since *E. coli* only has an NADP(H)-dependent GDH (Hanahan, 1983; McPherson and Wootton, 1983), the enzyme assay was

performed with NADH as a coenzyme as described elsewhere (Cho et al., 1995). One unit of enzyme was defined as the amount of enzyme required oxidizing 1 μmol of NADH per min 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 were performed as described elsewhere (Shoemaker and Haley, 1996; Cho and Yoon, 1999) 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 [á-^{32}P]8N_3ADP in Eppendorf tubes for 5 min. The samples were then irradiated with a handhold 254-nm UV lamp at a distance of 4 cm for 90 s at 4 °C. For competition studies, 0.1 mg of GDHs were

incubated with various concentrations of ADP for 10 min in the same buffer prior to the addition of 100 µM [á-^{32}P]8N_3ADP 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 (final 11 %). The reaction mixtures were kept on ice bath for 30 min and then centrifuged at 10,000 x g for 15 min at 4 ^{o}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

1.0 mg of the wild type and mutant GDHs were separately incubated with 100 µM [á-^{32}P]8N_3ADP for 5 min at 4 ^{o}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 ^{o}C, the reaction was quenched by the addition of ice-cold trichloroacetic acid (final 11 %) and kept at 4 ^{o}C for 15 min. The protein was precipitated by centrifugation at 10,000 x g for 15 min at 4 ^{o}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 15 μg of trypsin was added again. After 3 more hours at room temperature, 20 μg of trypsin was added, and the digestion mixture was kept at 25 ^{o}C overnight. To validate that the isolated peptide(s) was 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 the same methods as described elsewhere (Shoemaker and Haley, 1996, Cho and Yoon, 1999). For preparation of the immobilized aluminum column, 1.5 ml of an Epoxy-activated iminodiacetate Sepharose 6B was chelated to Al_3^+ by slowly passage through 20 ml of 50 mM $AlCl_3$. The resin was then washed successively with H_2O and 50 mM ammonium acetate, pH 5.9 (buffer A), 0.5 M NaCl in buffer A (buffer B), buffer A, 2 M urea in buffer A (buffer C), buffer A, 5 mM glutamate in buffer A (buffer D), and buffer A. Buffer A was added to a fraction of the tryptic digestion mixture (1:1), and dithiothreitol was added to a final concentration of 1 mM. The pH was adjusted to 6.0, and the tryptic-digested peptides were applied to the column. The column was washed with approximately 15 ml of buffers A, B, A, C, A, D, and A, respectively (0.5 mM/min). 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. Fractions containing photolabeled peptides were desalted, freeze-dried, resuspended in 0.1 % trifluoroacetic acid, and subjected to reversed-phase HPLC using an Waters C_{18} column on the same HPLC system. The mobile system consisted of 0.1 % trifluoroacetic acid solution and 0.1 % trifluoroacetic acid/ 80 % acetonitrile solvent system. The gradient for HPLC was 0 ~ 10 min, 0 % acetonitrile; 10 ~ 60 min, 0 ~ 80 % acetonitrile; 60 ~ 70 min, 80 % acetonitrile at a flow rate 0.5 ml/min. HPLC fractions containing photolabeled peptides were pyridylethylated by the method described elsewhere (Mak and Jones, 1978) and sequenced by the Edman degradation method as previously (Cho et al., 1998b, Cho and Yoon, 1999).

III. RESULTS

Construction and expression of Tyr187 mutants

The 62-bp *Age* I/*Eco*R V fragment was replaced with five 62-bp synthetic DNA duplexes containing a substitution on both DNA strand at positions encoding Tyr187. These substitutions made the mutant GDHs Y187E, Y187G, Y187M, Y187R, and Y187S at position 187 (Figure III-1). These mutants were designed to have different size, hydrophobicity, and ionization of the side chains at each position (Table III-1). 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 level for all mutant GDHs and wild type GDH (Figure III-2). 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 units/mg, compared with 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 units/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 approximately 4-fold decrease in the respective apparent V_{max} values (Table III-2). 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, while the K_m values for NADH were 80 and 82 ~ 88 μM, respectively, for the wild type and mutant GDHs (Table III-2). 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.

Activation of wild type GDH and mutant GDHs by ADP and $8N_3ADP$

The stimulatory effects of ADP on the wild type GDH and the Tyr187 mutant GDHs were examined and summarized in Figure III-3. There were big differences between the wild type GDH and the Tyr187 mutant GDHs in their sensitivities to the activation by ADP (Figure III-3). While the wild type GDH were activated 3-fold by ADP at 1 mM at pH 8.0, no detectectable activation was observed for the Tyr187 mutant GDHs regardless of their size, hydrophobicity, and ionization of the side chains (Figure III-3). 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 [á-

^{32}P]8-azidoadenosie 5 -diphosphate ($8N_3ADP$) (Figure III-4). To show that $8N_3ADP$ 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, $8N_3ADP$ was able to activate the wild type GDH, although maximal activity with $8N_3ADP$ was about 80 % of maximal ADP-stimulated activity (data not shown). Unlike the wild type GDH, there were no detectable activations by $8N_3ADP$ in the reaction of the Tyr187 mutant GDHs. These results show that the azidonucleotide, $8N_3ADP$, is able to elicit almost the same biological effects on the wild type GDH and the mutant GDHs as the natural nucleotide, ADP. Since almost identical results were obtained among the five mutant GDHs at 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 III-5).

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 [á-^{32}P]$8N_3ADP$ occurred at 60 µM for the wild type GDH (Figure III-6). The apparent K_d values of this interaction were 25 µM for the wild type GDH. However, the photoinsertion

of [á-^{32}P]8N_3ADP into the Y187M mutant GDH was less than 5 % of that obtained with the wild type GDH (Figure III-6). To demonstrate specific labeling of GDH, the enzymes were photolabeled with [á-^{32}P]8N_3ADP in the presence of increasing ADP concentrations. As shown in the results of the competition experiments (Figure III-7), increasing ADP concentration decreased the photolabeling of 100 µM [á-^{32}P]8N_3ADP. When 1 mM ADP was present with 100 µM [á-^{32}P]8N_3ADP, 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 [á-^{32}P]8N_3ADP and this was a decrease from an already very low starting level (Figure III-7). These results show the specificity and utility of 8N_3ADP 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 of photolabeled proteins and isolation of the photolabeled peptide

To identify the peptides modified by [á-^{32}P]8N_3ADP, 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 (Shoemaker and Haley, 1993). However, due to the uncertainly of the number of ADP sites and possible wobble associated with them (Colen et al., 1974), 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 [á-^{32}P]8N_3ADP, the digested samples were purified by an immobilized aluminum chromatography. The results in Figure III-8*A* show the radioactivity profile of wild type GDH and Y187M mutant GDH modified by 60 µM [á-^{32}P]8N_3ADP in the absence of ADP. For the wild type GDH, most of the radioactivity was retained on the column and one major radioactivity was recovered from the column. ADP was able to reduce [á-^{32}P]8N_3ADP photoinsertion into this peak. When 1 mM ADP was originally present in the incubation mixture, approximately 95 % of the radioactivity of the peak was eliminated, as shown in Figure III-8*B*. Unlike the wild type GDH, there was no detectable radioactive peak for the Y187M mutant GDH in the absence (Figure III-8*A*) or presence (Figure III-8*B*) of 1 mM ADP. These results indicate that the radioactive peak represents a adenine binding domain peptide of the ADP binding site within human GDH, and so Tyr187 is required for

efficient binding of [á-^{32}P]8N_3ADP to GDH.

The radioactive eluates from the immobilized aluminum column were combined and subjected to reversed-phase HPLC. One major radioactive peak was recovered from the HPLC column (data not shown) and the radioactive eluates were collected and identified by sequence analysis. Although some radioactivity was found in the flow-through and wash fractions, over 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 the photolabeled peptide of GDH

The results of the amino acid sequencing are summarized in Table III-3. 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. Since 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 residue Glu179 ~ Lys205 of the amino acid sequence of the

mature human GDHs (Table III-4). Photolabeling of these peptides was prevented over 95 % by the presence of 1 mM ADP during photolysis. Unlike the wild type GDH, no peptides containing amino acids around 187 position were detected with the Y187M mutant GDH. Once again, these results strongly suggest Tyr187 is mainly involved in the binding of [á-^{32}P]8N_3ADP to human GDH.

Table III-1. Oligonucleotides used for Tyr187 mutant GDHs

Y187 Mutant GDHs		Oligonucleotide Sequences							
		175			187		195	
Y187E	5	CC	ggT …	ACC	**gAA**	gCC …	gAT	3
	3		A …	Tgg	**CTT**	Cgg …	CTA	5
Y187G	5	CC	ggT …	ACC	**ggC**	gCC …	gAT	3
	3		A …	Tgg	**CCg**	Cgg …	CTA	5
Y187M	5	CC	ggT …	ACC	**ATg**	gCC …	gAT	3
	3		A …	Tgg	**TAC**	Cgg …	CTA	5
Y187R	5	CC	ggT …	ACC	**CgC**	gCC …	gAT	3
	3		A …	Tgg	**gCg**	Cgg …	CTA	5
Y187S	5	CC	ggT …	ACC	**TCC**	gCC …	gAT	3
	3		A …	Tgg	**Agg**	Cgg …	CTA	5

Table III-2. Kinetic parameters of wild type GDH and mutant GDHs in crude extracts

	V_{max}	K_m	
		NADH	2-Oxoglutarate
	(ì mol.min^{-1}.mg^{-1})	(ì M)	(mM)
Wild type	1.20 ± 0.02	80 ± 1	1.25 ± 0.04
Y187E	0.26 ± 0.02 [a]	82 ± 3	1.29 ± 0.05
Y187G	0.32 ± 0.03 [a]	85 ± 2	1.45 ± 0.03
Y187M	0.36 ± 0.04 [a]	84 ± 4	1.31 ± 0.03
Y187R	0.33 ± 0.03 [a]	82 ± 2	1.44 ± 0.04
Y187S	0.31 ± 0.04 [a]	88 ± 3	1.46 ± 0.03

Values are expressed as means ± S.D. with all measurements performed in duplicate. Statistical comparisons between the wild type GDH and the Tyr187 mutant GDHs were made by ANOVA using Fisher's protected least significant different test at the 0.5 significance level.

[a] Significantly different from the wild type ($p < 0.01$).

Table III-3. Alignment of [á-^{32}P]8N_3ADP-labeled peptides with homologous sequence from various GDHs

Source	Reference	Amino acid sequence[a]
Human GDH (Wild type)	This work	E M SWIAD TYAST IG[b]
Human GDH (Y187M)	This work	not detected
Human liver	(Mavrothalassitis et al., 1988)	E M SWIAD TYAST IGHDI
Human retina	(Shashidharan et al., 1997)	E M SWIAD TYAST IGHDI
Bovine brain	(Cho and Yoon, 1999)	E M SWIAD TYAST IGHDI
Bovine liver	(Julliard and Smith, 1979)	E M SWIAD TYAST IGHDI
Rat brain	(Tzimagiorgis and Moschonas, 1991)	E M SWIAD TYAST IGHDI
		179[c] 195

[a] The amino acids are denoted by the single-letter code.

[b] Only first 14 cycles were sequenced.

[c] The amino acid numbering is that of the mature human GDH (Shashidharan et al., 1997).

Table III-4. Amino acid composition of [á-^{32}P]8N$_3$ADP-modified peptide

Amino acids	Molar ratio[a]
Ala	3.8[b]
Asp	2.2
Asn	1.2
Cys	1.1
Glu	0.9
Gly	1.9
His	2.1
Ile	3.0
Lys	1.2
Met	1.2
Ser	1.9
Thr	3.1
Tyr	2.1
Trp	1.1
Val	1.0
Total residues	28

[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 less than 0.1 are not indicated. Values in parentheses are the nearest integer.

[b] Cys was pyridylethylated before it was quantified (Mak and Jones, 1978). 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.

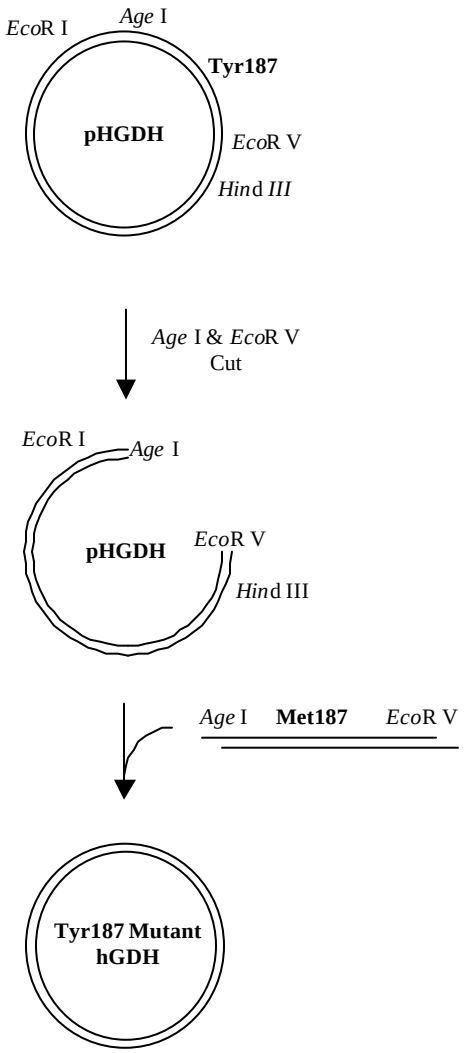


Figure III-1. Construction of Tyr187 mutant.

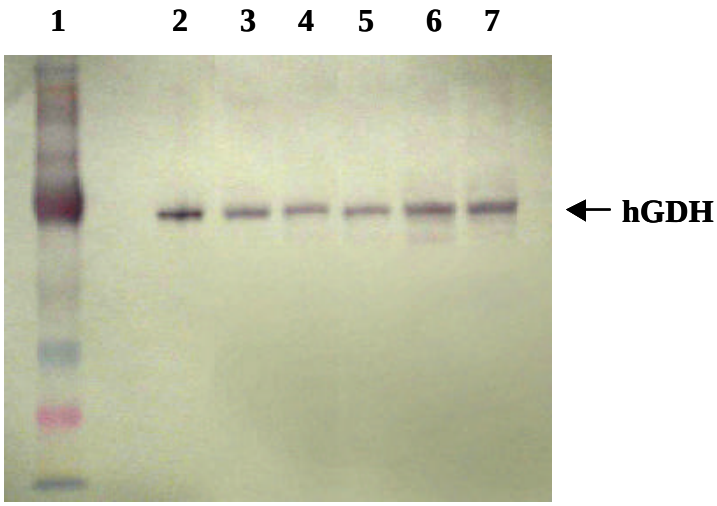


Figure III-2. Western blot analysis of the Tyr187 mutant GDHs in crude extracts. Lane 1, prestained marker proteins (Novex); lane 2, wild type GDH; lane 3 ~ 7, Tyr187 mutant GDHs (Y187E, Y187G, Y187M, Y187R, and Y187S).

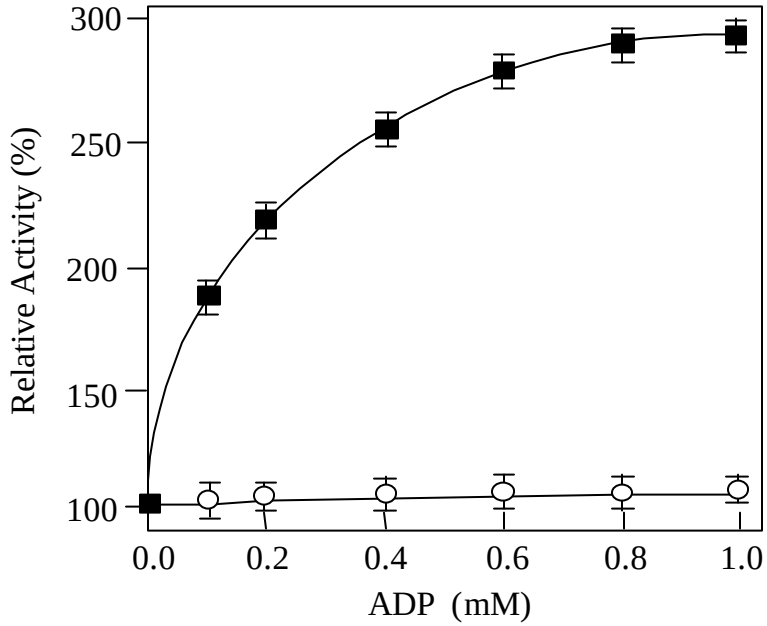


Figure III-3. Activation of the wild type and mutant GDHs by ADP. The enzymes were preincubated with various concentrations of ADP in the assay buffer and the activities were assayed by the addition of the standard assay mixture with NADH as a coenzyme. Remaining activities are expressed relative to each control. Wild type (closed squares), and Y187M (open circles).

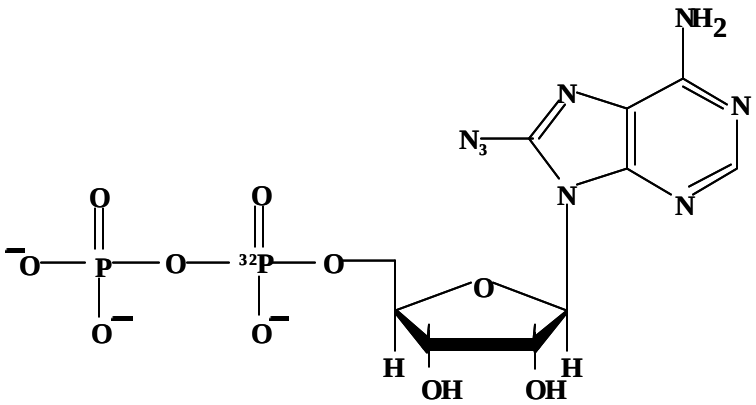


Figure III-4. Structure of [á-^{32}P]8-azidoadenosine 5 - diphosphate (8N_3ADP).

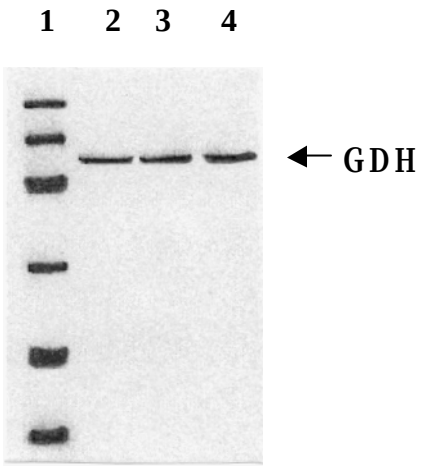


Figure III-5. SDS-PAGE analysis of purified the wild type and mutant GDHs. Lane 1; marker proteins (Bio-Rad), lane 2; Y187M mutant GDH, lane 3; E279G mutant GDH, and lane 4; wild type GDH.

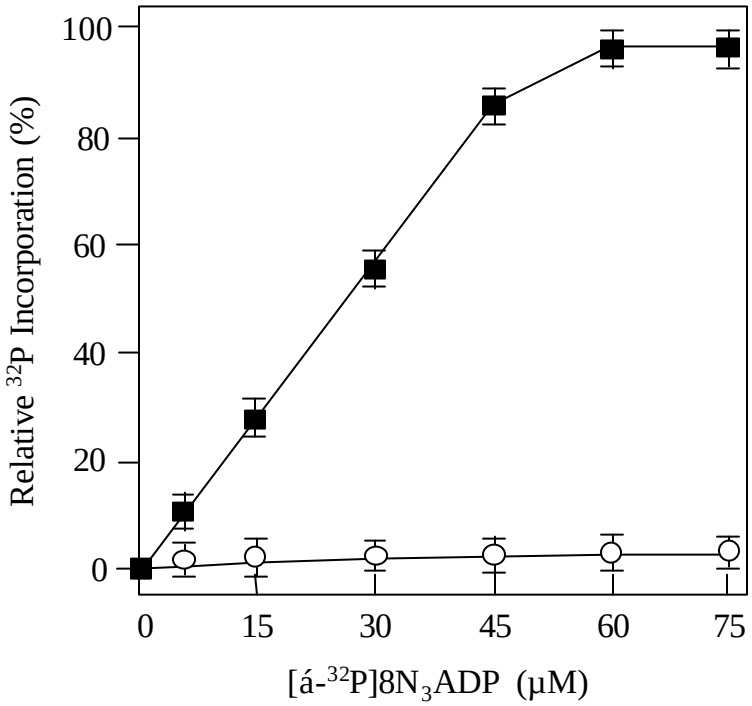


Figure III-6. Saturation of photoinsertion of [á-^{32}P]8N_3ADP into the wild type and mutant GDHs. The wild type and mutant GDHs in the reaction buffer were photolyzed with the indicated concentrations of [á-^{32}P]8N_3ADP and ^{32}P incorporation into protein was determined by liquid scintillation counting. Relative ^{32}P incorporations were expressed relative to each control. Wild type (closed squares) and Y187M (open circles).

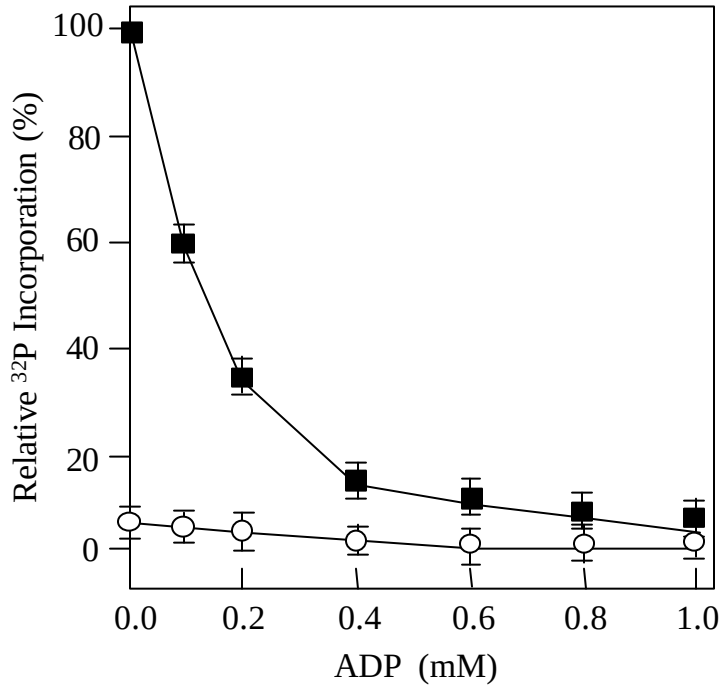


Figure III-7. The effect of ADP on photoinsertion of [á-^{32}P]8N_3ADP into the wild type and mutant GDHs. Wild type and mutant GDH in the reaction buffer were photolyzed with 100 µM [á-^{32}P]8N_3ADP in the presence of the indicated concentrations of ADP. Relative ^{32}P incorporation into protein was determined. Wild type (closed squares) and Y187M (open circles).

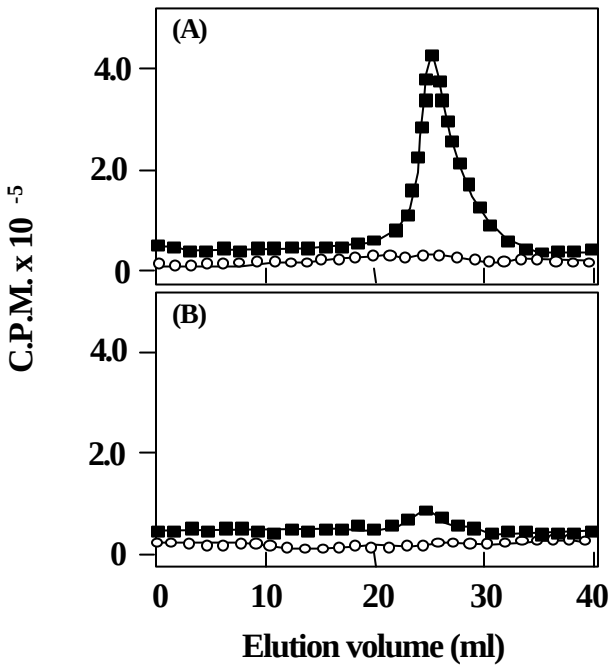


Figure III-8. Chromatogram of immobilized aluminum column of tryptic peptides from the wild type and mutant GDHs photolabeled with [á-^{32}P]8N_3ADP. Wild type GDH and Y187M mutant GDH were separately photolabeled with [á-^{32}P]8N_3ADP in the absence and presence of 1 mM ADP. The tryptic peptides were loaded onto an immobilized aluminum chelate column equilibrated with 50 mM ammonium acetate, pH5.9 and eluted with 5 mM potassium phosphate in 50 mM ammonium acetate, pH 8.0 at a flow rate of 0.5 ml/min. One minute fractions were collected.The plot represents the radioactivity profiles from the aluminum chelate column of the samples photolabeled in the absence **(*A*)** and presence **(*B*)** of 1 mM ADP. Wild type GDH (closed squares) and Y187M mutant GDH (open circles).

IV. 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 (Hussain et al., 1989; Shashidharan et al., 1997; Plaitakis et al., 2000; Herrero-Yraola et al., 2001). 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 (Peterson and Smith, 1999; Smith et al., 2001). 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 (Batara and Colman, 1986; Batra et al., 1989), Arg459 (Wrzeszczynski and Colman, 1994), Arg491 (Madhusoodanan and Colman, 2001), and peptides corresponding to Met411 ~ Arg419 (Rajagopalan et al., 1999) and Glu179 ~ Asn197 (Shoemaker and Haley, 1996; Cho and Yoon, 1999). 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. This present work is identified an amino acid required for the efficient binding of ADP to the human GDH using cassette mutagenesis, the photoaffinity labeling with [á-^{32}P]8N_3ADP, and protection studies.

There were differences in their 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 compare to those of the wild type GDH (Table III-2). The results from the western blot analysis (Figure III-2) show that the mutagenesis at 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 187 position does not affect the affinity of the enzyme for these two substrates. This site is very close to Tyr194 that is modified with the ADP analog, 5 -p-fluorosulfonylbenzoyladenosine (Schmidt and Colman, 1984). This modification diminishes NADH inhibition. However, based on 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 (Smith et al., 2001). Another difference between the wild type GDH and the Tyr187 mutant GDHs are their sensitivities to activation by ADP. The mutagenesis at 187 position resulted in a dramatic decrease in the sensitivities to the activation by ADP regardless of their size,

hydrophobicity, and ionization of the side chains (Figure III-3). 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 III-2). 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 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 (Dieter et al., 1981), as well as between ADP and NADH (Koberstein and Sund, 1973). 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 (Rajagopalan et al., 1999) in the three-dimensional structure. Similarly, it also has been reported that the ADP phosphate-binding domain (Rajagopalan et al., 1999) is near the GTP base-binding domain (Shoemaker and Haley, 1993; Cho et al., 2001; Lee et al., 2001). It seems likely that ADP and GTP can 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 (Peterson and Smith, 1999; Smith et al., 2001).

It is clear that the ADP base-binding domain established using [á-^{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 (Madhusoodanan and Colman, 2001) and at a peptide corresponding to Met411 ~ Arg419 (Rajagopalan et al, 1999). 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 (Wrzeszczynski and Colman, 1994). 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 interacts with the phosphate moieties of the coenzyme in the open mouth conformation (Smith et al., 2001). This suggests that Arg459 may not be wholly responsible for the observed ADP binding. From the crystal structures of bovine GDH (Peterson and Smith, 1999; Smith et al., 2001), 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. Therefore, it is reasonable to speculate that the base-binding domains, in addition to the phosphate-binding domains, are necessary for the efficient binding of ADP to GDH.

The specificity of $8N_3$ADP 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 $8N_3$ADP as well as by ADP and can be photolabeled with the nucleotide analog [á-^{32}P]$8N_3$ADP, whereas no such an 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 µM concentrations of [á-^{32}P]$8N_3$ADP. The apparent K_d values of this interaction were 25 µM (Figure III-6). Third, the prevention of photoinsertion of [á-^{32}P]$8N_3$ADP by ADP demonstrates that the photoprobe is inserting into a specific ADP site within the human GDH (Figure III-7). The ability of a nucleotide analog 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.

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. The use of photoaffinity labeling also appears to be a useful procedure for identifying active or regulatory sites. Therefore, the combination of genetic and biochemical 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.

CHAPTER IV

Site-Directed Mutagenesis and Photoaffinity Labeling of an NAD^+ Binding Site of Human Glutamate Dehydrogenase

I. INTRODUCTION

Glutamate is a major excitatory neurotransmitter (Founnum, 1984) and is also known to be the immediate precursor in the biosysthesis of γ-amino butyric acid (GABA), a widely distributed inhibitory neurotransmitter (Roberts and Frankel, 1950). It is present in brain tissue in concentrations that are 2 ~ 5 fold higher than those found in other organs. Furthermore, due to its neurotoxic potentials, glutamate may be involved in the pathogenesis of human degenerative disorders (McGeer and McGeer, 1976; Plaitakis et al., 1982). Substantive evidence supports the involvement of glutamate in neuronal death or injury associated with ischemic brain injury, stroke, status epilepticus, amyotophic lateral sclerosis, Parkinson's disease and Alzheimer's disease (Ben-Ari and Schwarcz, 1986; Choi, 1988, 1992; Turski et al., 1991; Mattson et al., 1992; Meldrum, 1995; Plaitakis and Shashidharan, 2000). Since glutamate does not readily cross the blood-brain-barrier (McGeer and McGeer, 1976), the majority of this amino acid in the central nervous system is produced from precursors and regulated through the action of several enzymes.

One enzyme central to the metabolism of glutamate is glutamate dehydrogenase (Smith et al., 1975). There are three types of GDH that vary according to the coenzyme they use: NAD(H)-specific, NADP(H)-specific,

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 (bacterial, e.g. *Escherichia coli*) (McPherson and Wootton, 1983) and 55- to 57-kDa (vertebrate) (Smith et al., 1975; Cho et al., 1995), whereas the NAD^+-linked enzymes have either four identical subunits with an size of appromaximately 115-kDa (e.g. *Neurospora crassa*) (Veronese et al., 1974) or six identical subunits with a subunit size of approximately 48-kDa (e.g. *Clostridium symbiosum*) (Rice et al., 1985). 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 largest difference between mammalian and bacterial GDH is a long antenna domain formed by the 48 amino acid insertion starting at residue 395 and there is little identity between the 100 residues in the C-terminus (Wootton et al., 1974). In fact, mammalian GDH is strictly regulated by several effectors such as pH, aggregation state, a variety of activators and inhibitors (Smith et al., 1975).

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 (Pal and Colman,

1979; Peterson and Smith, 1999; Smith et al., 2001). In addition, NADH, a substrate for the reverse reaction, has also been reported to inhibit the forward reaction (Frieden, 1959). In contrast, ADP and AMP are potent activators by decreasing product affinity (Bailey et al., 1982; Hussain et al., 1989; Dai et al., 2000). L-Leucine also enhances the activity of the GDH with a relief of high substrate inhibition (McGivan et al., 1973; Couee and Tipton, 1989; Lee et al., 1997). A breakdown in this regulation is serious in humans (Holt et al., 1983). Hussain et al. (1989) detected four different forms of GDH isoproteins from human cerebellum of normal subjects and patients with neurodegenerative disorders.

It is the only recent years that the three-dimensional structure of GDH from microorganisms is available (Baker et al., 1992; Yip et al., 1995). More recently, crystallization of bovine liver GDH was reported for the first time from the mammalian sources (Peterson et al., 1997; Peterson and Smith, 1999; Smith et al., 2001). The recent 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 (Peterson and Smith, 1999; Smith et al., 2001).

Identifying nucleotide binding sites of variety of proteins has been

advanced by the use of nucleotide photoaffinity analogs that selectively insert into a site upon photoactivation with ultraviolet light. Azidopurine 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. Their selectivity and specificity have been successfully utilized to locate specific binding domains on mammalian GDH and other proteins (Campbell et al., 1990; Gunnersen and Haley, 1992; Salvucci et al., 1992; Olcott et al., 1994; Cho et al., 1996, 1998b; Cho and Yoon, 1999). For instance, [^{32}P]2N_3NAD$^+$ was shown to be a valid active-site probe for several proteins (Campbell et al., 1990; Kim and Haley, 1990, Kim and Haley, 1991; Vaillancourt et al., 1995). Recently, it has been identified GTP, NAD$^+$, and ADP binding sites of the bovine brain GDH using [ã-^{32}P]8N_3GTP, [^{32}P]2N_3NAD$^+$, and [á-^{32}P]8N_3ADP, respectively (Cho et al., 1996; Cho et al., 1998b; Cho and Yoon, 1999). The ATP binding site of adenylate kinase and creatine kinase and the protein unique to cerebrospinal fluids of Alzheimer's patients successfully also have been identified using 2N_3ATP and 8N_3ATP (Gunnersen and Haley, 1992; Olcott et al., 1994). Very recently, Stanley et al. (1998) has reported that the hyperinsulinism-hyperammonemia syndrome is caused by mutations in GDH gene that affects enzyme sensitivity to GTP-

induced inhibition. The mutations identified in the patients with hyperinsulinism and hyperammonemia (Stanley et al., 1998) exactly lie within a sequence of 15 amino acids previously suggested to contain GTP binding site of the brain GDH isoproteins (Cho et al., 1996). 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 (Jacobson and Colman, 1982). These results prove selectivity and specificity of the photoaffinity probe as a valid active-site probe.

This present work reports the identification of an NAD^+ binding site of human GDH by a combination of site-directed mutagenesis and photolabeling with $[^{32}P]2N_3NAD^+$ at Glu279 residue. This data place the NAD^+ base-binding domain within a proposed catalytic cleft defined in the crystal structure of mammalian GDH (Teller et al., 1992; Peterson and Smith, 1999; Smith et al., 2001). For the present study, the mutant human GDHs containing Arg, Gly, Leu, Met, or Tyr at Glu279 site have been expressed in *E. coli* as a soluble protein, purified, and characterized. This study is the first report by site-directed mutagenesis showing a direct involvement of Glu279 of mammalian GDH in the base-binding of NAD^+.

II. MATERIALS AND METHODS

Materials

NADH, NAD^+, 2-oxoglutarate, ADP, and L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin were purchased from Sigma Chemical Co. Human GDH gene (pHGDH) has been chemically synthesized and expressed in *E. coli* as a soluble protein in our laboratory as described elsewhere (Cho et al., 2001). $2N_3NAD^+$ was purchased from ALP Corp. $[^{32}P]2N_3NAD^+$ was synthesized by the method as previously described (Kim and Haley, 1990; Cho and Yoon, 1999). Q-Sepharose and FPLC Resource-Q column were purchased from Pharmacia. POROS-QM column was purchased from Perceptive Biosystems. [(Dihydroxyboryl)-phenyl]succinamyl derivatives of aminoethyl polyacrylamide (Affi-gel 601) were obtained from Bio-Rad. Human GDH gene (pHGDH) has been chemically synthesized and expressed in *E. coli* as a soluble protein (see MATERIALS AND METHODS of CHAPTER II). Pre-cast gels for sodium dodecyl sulfate-polyacrylamide gel electrophoresis were purchased from NOVEX. All other chemicals and solvents were reagent grade or better.

Bacterial strains

E. coli DH5á (Hanahan, 1983) 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 D(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 (Teller et al., 1992) and was used to test plasmids for GDH activity. *E. coli* BL21 (DE3) (Studier and Moffatt, 1986) was used for high level expression of the recombinant human GDH.

Construction and characterization of Glu279 mutants

A series of single amino acid substitutions of Glu279 was constructed separately by cassette mutagenesis of a synthetic human GDH gene, pHGDH (Cho et al., 2001). The plasmid DNA (5 ì g) was digested with *Nsi* I and *Bsp*D I to remove 46-bp fragment that encodes amino acids 275 ~ 290 and vector DNA was purified by electrophoresis using 1 % low melting point agarose. The 46-bp *Nsi* I/*Bsp*D I fragment was replaced with five 46-bp synthetic DNA duplexes containing a substitution on both DNA strand at positions encoding Glu279 to make E279G, E279L, E279M, E279R, and E279Y mutant proteins. Glu279 mutants were identified by DNA sequencing using plasmid DNA as a template. Each of these mutants has been expressed in *E. coli* strain DE3, purified to homogeneity as described above, and has had its steady state kinetic parameters determined. The gene expression levels of Glu279 mutant

proteins 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 ì g of ampicillin per 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 50 mM Tris-HCl, pH 8.0 containing 2 mM EDTA, 10 mM NaCl, 2 mM phenylmethylsulfonylfluoride, 1 mM dithiothreitol and 2 % Triton X-100 and lysed with a sonicator. The homogenate was centrifuged at 11,000 x g, 10 min, 4 °C and the pellet was discarded. The supernatant was loaded onto to a column (5 x 16 cm) of Q-Sepharose was equilibrated with 50 mM Tris-HCl, pH 8.0 containing 0.5 mM EDTA and 1 mM â-mercaptoethanol. The column was washed with 50 mM Tris-HCl, pH 7.0 containing 0.5 mM EDTA, 0.1 N NaCl and 1 mM â-mercaptoethanol (buffer I) until the breakthrough peak of protein had been eluted. The enzyme was then eluted using a step gradient with buffer I containing 0.5 N and 1N NaCl, respectively. The fractions containing GDH were combined, concentrated and buffer changed to buffer II (50 mM Tris-HCl, pH 8.0/ 0.5 mM EDTA/ 1 mM â-mercaptoethanol)

using Amicon concentrator and then applied to a FPLC Resource-Q anion exchange column (6 ml) equilibrated with buffer II. The enzyme was then eluted using a linear gradient made with buffer II in increasing concentration of NaCl (from 0 mM to 500 mM) at 2 ml/min. The fractions containing GDH were combined, concentrated and desalted with buffer III (20 mM potassium phosphate, pH 8.0/ 0.5 mM EDTA/ 1 mM â-mercaptoethanol) and applied to a POROS-QM anion exchange column equilibrated with buffer III. The enzyme was then eluted using a linear gradient made with 20 mM potassium phosphate, pH 6.0 containing 0.5 mM EDTA, 0.3 M NaCl and 1 mM â-mercaptoethanol at 1 ml/min. Since the wild type and 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 (Choi et al., 1999) against the bovine brain GDH. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

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 (Cho et al., 1995). 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, 1 mM ADP, and 2.6 mM EDTA at 25 °C. One unit of enzyme was defined as the amount of enzyme required to oxidize 1 ìmol of NADH per min at 25 °C. GDH activity was also measured in the direction of glutamate oxidation in 50 mM Tris-HCl, pH 9.5 containing 1.4 mM NAD^+, 25 mM glutarate, 2.6 mM EDTA, and 1 mM ADP at 25 °C. V_{max} and K_m values were obtained from the initial velocity data using a least-squares method to the double-reciprocal plots. Effects of wild type and mutant GDHs by allosteric regulators such as GTP, ADP, and ATP 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 Glu279 mutant proteins were performed by the method of Kim and Haley (1990) with a slight modification. For saturation studies, the wild type GDH and Glu279 mutant proteins (0.1 mg each) in 10 mM Tris-acetate, pH 8.0, containing 12 mM glutarate were

separately incubated with various concentrations of [^{32}P]2N$_3$NAD$^+$ in Eppendorf tubes for 5 min. For competition studies, 0.1 mg of the enzymes were incubated with various concentrations of NAD$^+$ for 10 min in the same buffer prior to the addition of 50 ì M [^{32}P]2N$_3$NAD$^+$ and then allowed to incubate with the photoprobe for 5 min as described above. The samples were irradiated with a handhold 254-nm UV lamp for 90 s twice at 4 ^{o}C. The reaction was quenched by the addition of ice-cold trichloroacetic acid (final 7 %). The reaction mixtures were kept on ice bath for 30 min and centrifuged at 10,000 x g for 15 min at 4 ^{o}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 the photolabeled peptide

To determine the site modified by [^{32}P]2N$_3$NAD$^+$, 2.0 mg of the wild type and Glu279 mutant proteins in 10 mM Tris-acetate, pH 8.0 were separately incubated with 50 ì M [^{32}P]2N$_3$NAD$^+$ in the presence of 1 mM GTP, 12 mM glutarate, and 10 ì M EDTA for 5 min at 4 ^{o}C. The mixtures were irradiated for 90 s twice. The reaction was quenched by the addition of ice-cold

trichloroacetic acid (final 7 %), and kept at 4 °C for 30 min. The protein was precipitated by centrifugation at 10,000 x 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 enzymes were proteolyzed by the addition of 15 ìg of trypsin and kept at room temperature for 3 h after which 15 ìg of trypsin was added again. After 3 more hours at room temperature, 20 ìg of trypsin was added, and the digestion mixture was kept at 25 °C overnight. To validate that the isolated peptide(s) was specific for the NAD^+ binding site and could be protected from photomodification, the GDH proteins were photolyzed in the presence of 300 ìM NAD^+ and proteolyzed as described above.

The tryptic-digested peptides were applied to boronate column equilibrated with 0.1 M ammonium acetate, pH 8.8. Unmodified peptides were washed with the same buffer and photolabeled peptides were eluted with a 200-ml gradient of pH 8.8 ~ 5.5 in 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, resuspended in 0.1 % trifluoroacetic acid, isolated by reversed-phase HPLC using an Waters C_{18} column on the same HPLC system, and sequenced by the Edman degradation method as described before (Cho et al., 1998b, Cho and Yoon, 1999).

III. RESULTS

Construction and expression of Glu279 mutant GDHs

The 46-bp *Nsi* I/*Bsp*D I fragment in pHGDH were replaced with five different 46-bp synthetic DNA duplexes containing a substitution on both DNA strand at positions encoding Glu279. These substitutions made mutant proteins E279G, E279L, E279M, E279R, and E279Y at position 279 (Figure IV-1). The five mutants were designed to have different size, hydrophobicity, and ionization of the side chains at each position (Table IV-1). 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 56.5-kDa protein that interacted with monoclonal antibodies raised against GDH at almost identical level for all Glu279 mutants and wild type GDH (Figure IV-2). These results indicate that the mutagenesis at Glu279 has no effects on expression or stability of the different mutants. The mutant proteins showed a 22 ~ 28-fold decrease in the respective apparent V_{max} values and 20 ~ 25-fold increase in K_m values for NAD^+ with essentially unchanged K_m values for glutamate (Table IV-2). Since the all five mutant proteins showed almost identical biochemical properties, only wild type GDH and E297G mutant protein were purified homogeneously (Figure IV-3) and used for the following experiments.

Effects of nucleotides on binding of $2N_3NAD^+$ to GDH

The binding of NAD^+ to the wild type and mutant GDHs were further examined by photoaffinity labeling with [^{32}P]nicotinamide 2-azidoadenosine dinucleotide ($2N_3NAD^+$) (Figure IV-4) and by the effects of GTP and ADP on photoinsertion. It is well known that GTP increases the binding of NAD^+ while ADP weakens the binding with or without glutarate present (Frieden, 1963; Dalziel and Egan, 1972). Similar regulatory effects of these nucleotides were observed using [^{32}P]$2N_3NAD^+$ when the enzymes were photolabeled with the photoprobe in the presence of GTP or ADP (Table IV-3). Compared to a control, the addition of 1 mM GTP caused an approximate 220 % increase of photoinsertion into the wild type GDH, whereas the presence of 1 mM ADP caused an approximate 60 % reduction. This reduction by ADP was lower than a 90 % decrease in photoinsertion caused by addition of NAD^+. When concentrations of the nucleotides were reduced, these effects were less extensive. Unlike the wild type GDH, none of the mutant proteins were affected by ADP or GTP.

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 has been studied by equilibrium dialysis and initial rates (Dalziel, 1975). In the

presence of glutarate, NAD^+ was bound more tightly (Dalziel and Egan, 1972). To demonstrate saturation effects with the photoprobe, the purified enzymes were photolabeled with increasing concentrations of [^{32}P]2N_3NAD^+ in the presence of glutarate. Under the experimental conditions described, saturation of photoinsertion with [^{32}P]2N_3NAD^+ occurred at around 40 ìM photoprobe with apparent K_d values near 35 ìM for wild type GDH (Figure IV-5). However, less than 10 % of saturation with [^{32}P]2N_3NAD^+ was observed with Glu279 mutant proteins (Figure IV-6). In all photolabeling experiments, the ionic 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, the reported apparent K_d values obtained from photoaffinity labeling should be interpreted considering that photolabeling is done under conditions that enhance binding site occupancy. The results in Figure IV-3 indicate the saturability of NAD^+ specific site of GDH with this photoprobe and therefore decrease the possibility of nonspecific photoinsertion.

To further demonstrate specific labeling of GDH, the wild type GDH and mutant proteins were photolabeled with [^{32}P]2N_3NAD^+ in the presence of increasing NAD^+ concentrations. As shown in the results of the competition experiments (Figure IV-6), NAD^+ was able to protect photolabeling from 50 ìM [^{32}P]2N_3NAD^+ at concentrations in the range of known K_d values (Dalziel,

1975). Approximately, 80 % protection was observed with 300 ì M NAD^+ for the wild type GDH (Figure IV-6). These results show the specificity and utility of $[^{32}P]2N_3NAD^+$ as a good probe for determining the NAD^+ binding site. Once again, NAD^+ had no effects on the photoinsertion of $[^{32}P]2N_3NAD^+$ into the Glu279 mutant proteins (Figure IV-6), suggesting that Glu279 site is critically involved in the NAD^+ binding.

Tryptic digestion of photolabeled proteins and boronate affinity column

To identify the peptides modified by $[^{32}P]2N_3NAD^+$, the wild type and Glu279 mutant GDHs were photolabeled twice in the absence and presence of 150 ì M 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 ì M $[^{32}P]2N_3NAD^+$ 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^+ (Frieden, 1963; Dalziel and Egan, 1972) and $[^{32}P]2N_3NAD^+$ (Table IV-3) to GDH. The photolabeled proteins were separated from most of the noncovalently bound nucleotide by acid precipitation and proteolyzed by trypsin. Since the $2N_3NAD^+$-labeled peptide binds to a boronate affinity column (Kim and Haley, 1991), the digested GDH

proteins were applied to the boronate column equilibrated with 0.1 M ammonium acetate, pH 8.8. All of the unlabeled peptides were eluted with ammonium acetate buffer, pH 8.8 in the void volume, whereas photolabeled peptides were selectively retained on the column. The radioactive peptides were eluted with a pH gradient of 8.8 ~ 5.5 (Figure IV-7). One major radioactive peak around pH 6.5 was observed for the wild type GDH. NAD^+ was able to reduce $[^{32}P]2N_3NAD^+$ photoinsertion into this peak. When 300 ì M NAD^+ was originally present in the incubation mixture, more than 90 % of the radioactivity of the peak was eliminated as shown in Figure IV-7. However, no such a radioactive peak was observed for Glu279 mutant proteins. This result indicates that the radioactive peak represent a peptide in the NAD^+ binding domain of the GDH.

When the radioactive eluates from the boronate column were subjected to reversed-phase HPLC, one major radioactive peak (fractions 25 ~ 27) was clearly observed (data not shown). 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 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. The

radioactive peptide was collected and identified by sequence analysis.

Sequence analysis of the photolabeled peptide of GDH

The amino acid sequence analysis revealed that the peak fractions contained the amino acid sequence CIAVGXSDGSIWNPDGIDPK. As judged by comparison with the amino acid sequence of mammalian GDHs, this site was identified as residues 274 ~ 293 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 since 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 residue 274 ~ 293 with an exception that there was a significant reduction in Glu (data not shown). Photolabeling of the peptide was prevented by the presence of NAD^+ during photolysis. Unlike the wild type GDH, no such peptides were detected from the E279G mutant GDH. 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 human GDH. Both sequencing and compositional analysis identified Glu 279 as the site of photoinsertion.

Table IV-1. Oligonucleotides used for Glu279 mutant GDHs

Glu279 Mutant GDHs	Oligonucleotide Sequences											
		274				279			290			
E279G	5		TC	…	ggT	**ggC**	TCT	…	AT			3
	3	ACg	TAG	…	CCA	**CCg**	AgA	…	TAg	C		5
E279L	5		TC	…	ggT	**CTg**	TCT	…	AT			3
	3	ACg	TAG	…	CCA	**gAC**	AgA	…	TAg	C		5
E279M	5		TC	…	ggT	**ATg**	TCT	…	AT			3
	3	ACg	TAG	…	CCA	**TAC**	AgA	…	TAg	C		5
E279R	5		TC	…	ggT	**CgC**	TCT	…	AT			3
	3	ACg	TAG	…	CCA	**gCg**	AgA	…	TAg	C		5
E279Y	5		TC	…	ggT	**TAT**	TCT	…	AT			3
	3	ACg	TAG	…	CCA	**ATA**	AgA	…	TAg	C		5

Table IV-2. Kinetic parameters of Glu279 mutant GDHs

Kinetics[a]		Wild type	E279G	E279L	E279M	E279R	E279Y
V_{max}[b]	(μmol.min^{-1}.mg^{-1})	115.3	4.75	4.12	5.24	4.16	5.03
K_m[b]	(mM)						
	NAD^+	0.94	23.4	18.9	21.3	22.7	23.0
	Glutamate	4.10	4.52	4.49	4.63	4.14	4.33

[a] Mean of triplicates.

[b] K_m and V_{max} values of wild type GDH and E279G mutant protein were obtained from the initial velocity data using a least-squares method to the double-reciprocal plots.

Table IV-3. Photoinsertion of [^{32}P] $2N_3NAD^+$ into the wild type GDH and E279G mutant GDH in the presence of effectors

Effectors	Photoincorporation[a]	
	Wild type	E279G
%		
Control	100 ± 3	4 ± 1
NAD^+ (0.05 mM)	25 ± 2	5 ± 1
NAD^+ (0.15 mM)	10 ± 1	5 ± 3
GTP (0.5 mM)	140 ± 4	5 ± 1
GTP (1.0 mM)	210 ± 1	4 ± 2
ADP (0.5 mM)	49 ± 2	5 ± 1
ADP (1.0 mM)	40 ± 2	4 ± 2

The concentration of [^{32}P] $2N_3NAD^+$ used was 50 μM.

[a] Mean of duplicates.

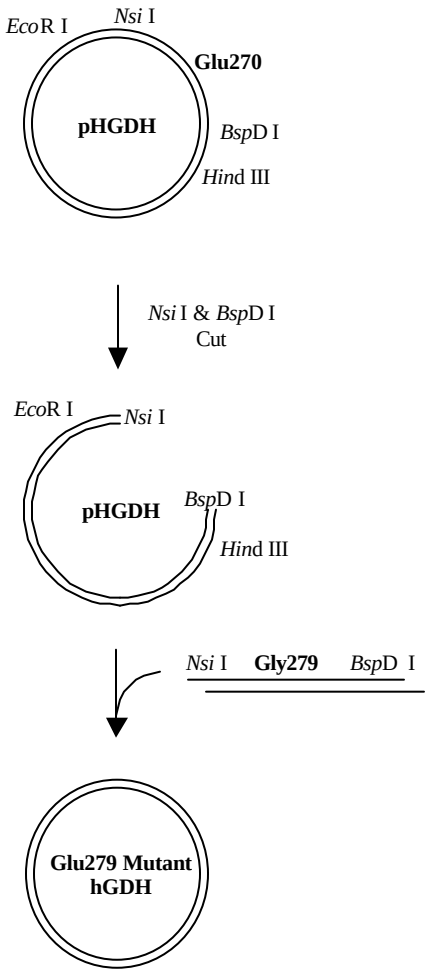


Figure IV-1. Cassette mutagenesis of human GDH at Glu279 site.

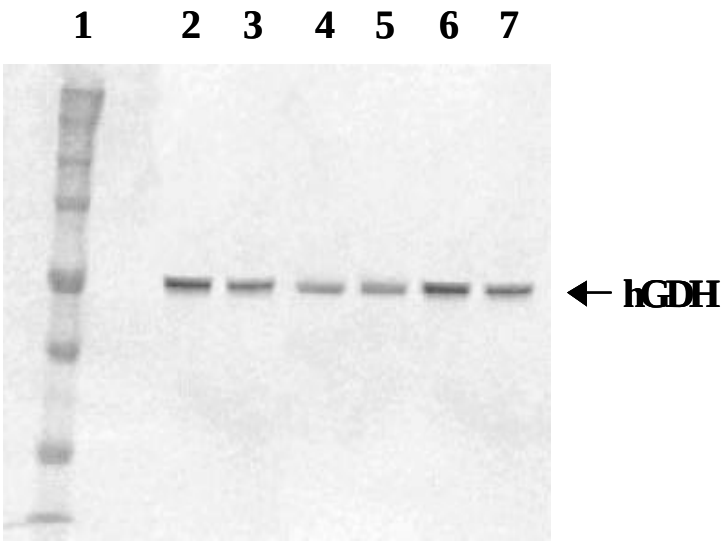


Figure IV-2. Western blot analysis of Glu279 mutant GDHs in crude extracts. Lane 1, prestained marker proteins; lane 2, wild type GDH, and lane 3 ~ 7, Glu279 mutants (E279M, E279R, E279L, E279G and E279Y).

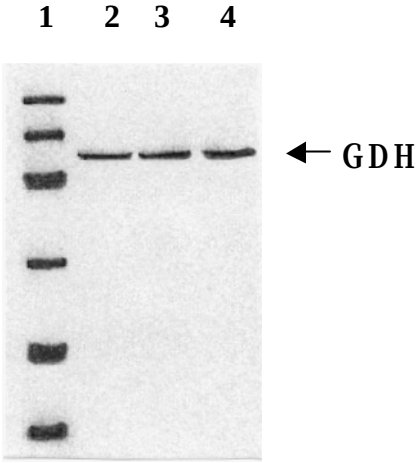


Figure IV-3. SDS-PAGE analysis of purified the wild type and mutant GDHs. Lane 1; marker proteins (Bio-Rad), lane 2; Y187M mutant GDH, lane 3; E279G mutant GDH, and lane 4; wild type GDH.

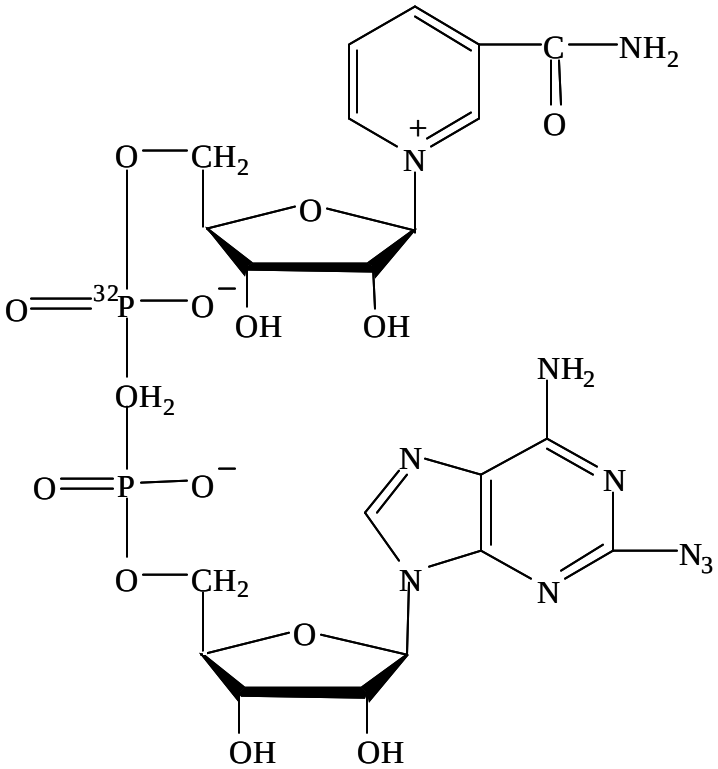


Figure IV-4. Structure of [^{32}P]nicotinamide 2-azidoadenosine dinucleotide ($2N_3NAD^+$).

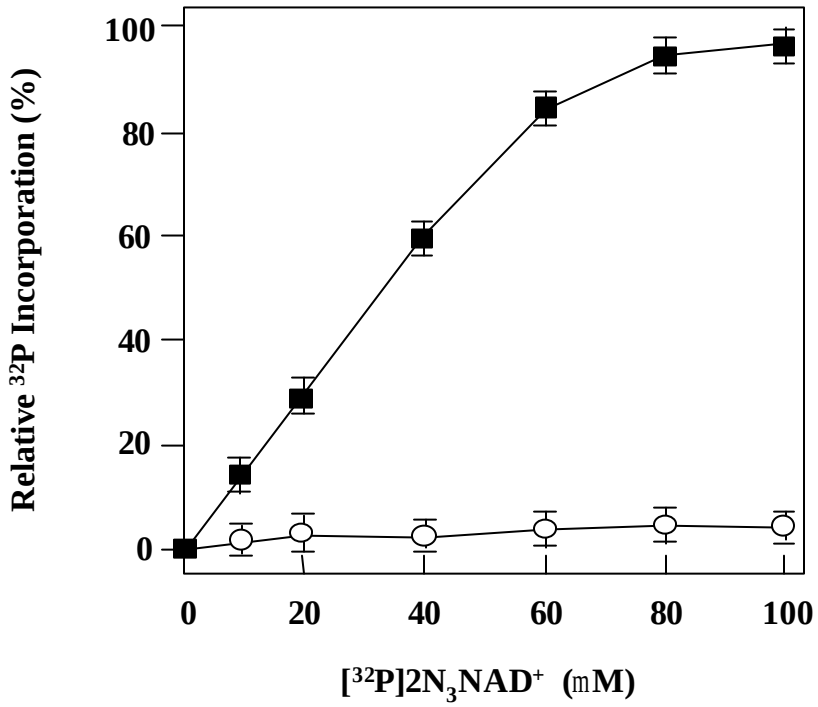


Figure IV-5. Saturation of [^{32}P]2N_3NAD$^+$ photoinsertion into GDH. Wild type and E279G mutant protein in the reaction buffer were photolyzed with the indicated concentrations of [^{32}P]2N_3NAD$^+$ and ^{32}P incorporation into protein was determined by liquid scintillation counting. Relative ^{32}P incorporations were expressed relative to each control. Wild type (closed squares) and E279G (open circles).

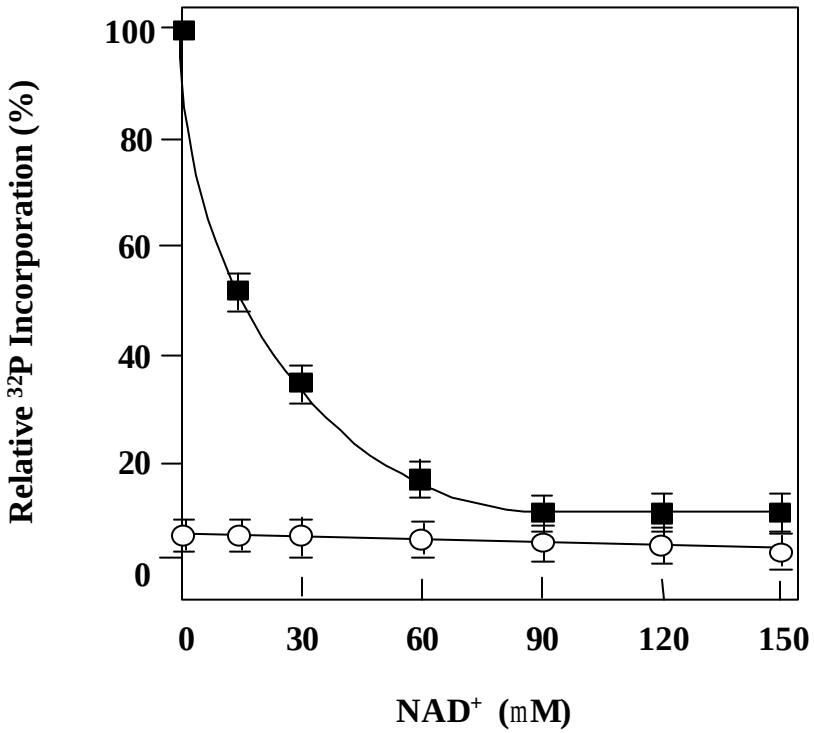


Figure IV-6. The effect of NAD$^+$ on [^{32}P]2N$_3$NAD$^+$ photoincorporation into GDH. Wild type and E279G mutant protein in the reaction buffer were photolyzed with 50 µM [^{32}P]2N$_3$NAD$^+$ in the presence of the indicated concentrations of NAD$^+$. Relative ^{32}P incorporation into protein was determined and expressed as described in Figure IV-5. Wild type (closed squares); E279G mutant (open circles).

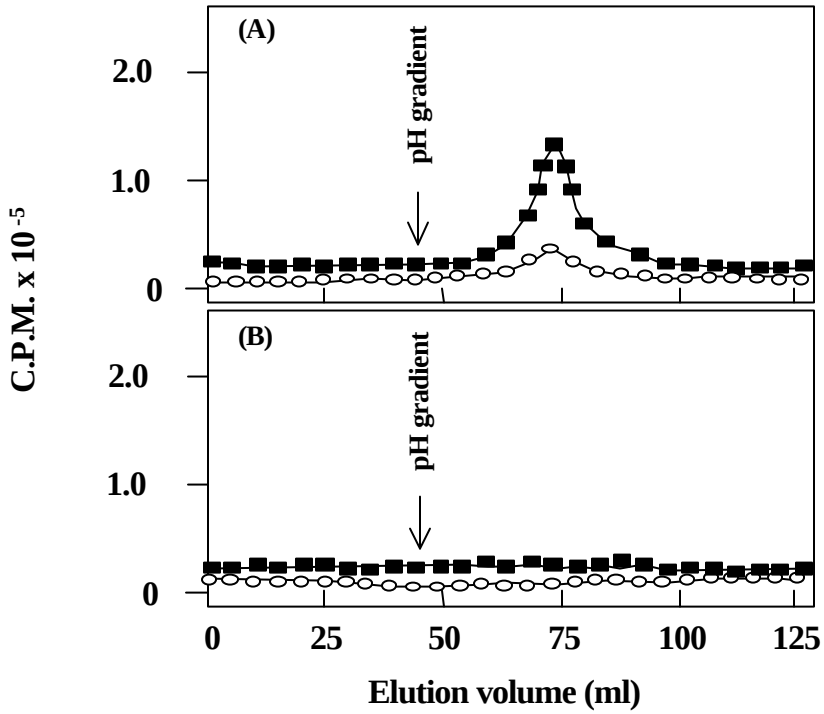


Figure IV-7. Binding of tryptic peptides photolabeled with [^{32}P]2N$_3$NAD$^+$ to a boronate affinity column. Wild type GDH and E279G mutant protein were separately photolabeled with [^{32}P]2N$_3$NAD$^+$ as described in "MATERIALS AND METHODS" in the absence and presence of 300 ìM NAD$^+$. The tryptic peptides were loaded onto 3-ml boronate affinity column equilibrated with 0.1 M ammonium acetate, pH 8.8.Unmodified peptides were eluted with the same buffer and photolabeled peptide was eluted with a 200-ml gradient of pH 8.8 ~ 5.5 in 0.1M ammonium acetate. Fractions (3ml) were monitored for absorbance at 220 nm and for radioactivity. The plan solid and dashed line represents the absorbance at 220 nm of the sample photolabeled in the absence and presence of 300 ìM NAD$^+$, respectively. The plot also represents the radioactivity profiles from a boronate affinity column of the samples photolabeled in the absence (A**)** and presence (B) of 300 ìM NAD$^+$. Wild type (closed squares); E279G mutant (open circles).

IV. DISCUSSION

In the present work, it was identified that 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, $[^{32}P]2N_3NAD^+$. $[^{32}P]2N_3NAD^+$ 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 photoinsertion 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 did not lead to covalent labeling, eliminating the existence of any long lived chemically reactive intermediate which could be involved in covalently modifying enzymes. Saturation of photoinsertion at concentrations corresponding to that expected from the reversible binding affinities also strongly supports the site being labeled is within the NAD^+ binding domain. Their selectivity and specificity have been successfully utilized to locate the specific base binding domains of nucleotide binding site peptides of many proteins (Campbell et al., 1990; Gunnersen and Haley, 1992; Shoemaker and Haley, 1993, 1996; Olcott et al., 1994; Vaillancourt et al., 1995).

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_3NAD$^+$ is a substrate for GDH. 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 [^{32}P]2N_3NAD$^+$. Saturation of photoinsertion with [^{32}P]2N_3NAD$^+$ occurred at around 90 ì M photoprobe with apparent K_d values near 35 ì M (Figure IV-5). Third, active site involvement was also supported by the effects of GTP and ADP on photoinsertion (Table IV-3). These results, together with the enhancement of photoinsertion by glutarate, present evidence of active site labeling of GDH with 2N_3NAD$^+$.

To identify the site of photoinsertion, the photolabeled enzymes 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 adenine and pyridine nucleotides (Alvarez-Gonzalez et al., 1983) and nucleosidyl peptides (Schmidt and Colman, 1984; Bailey and Colman, 1987). By using a pH gradient elution, a highly purified radiolabeled peptide was obtained and 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 $[^{32}P]2N_3NAD^+$ photoprobe is still covalently attaches 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 are corresponds to residues 274 ~ 293 of the amino acid sequence of human GDH with an attachment site of $[^{32}P]2N_3NAD^+$ as Glu279.

The crystal structure of *Clostridium symbiosum* GDH has been aligned with the primary sequence of the bovine liver GDH (Britton et al., 1992; Teller et al., 1992; Peterson and Smith, 1999; Smith et al., 2001). The structures of *Clostridium symbiosum* and mammalian GDHs were suggested to be similar due to considerable identity and the conservation of 13 glycine residues, which probably conserves the structure among species, and to consist of two domains. The first domain has been proposed to contain the catalytically important residues, while the second domain contains a â-á-â motif that is responsible for coenzyme binding (Wierenga et al., 1986; Stillman et al., 1992). The primary sequence of bovine liver GDH also suggests the presence of a â-á-â motif corresponding to the motif within the bacterial subunit. The photolabeled peptide of the human GDH identified in this work is located within the proposed NAD^+ binding domain of bovine

liver GDH (Rossmann et al., 1974; Wooton, 1974), and predicted to form a â-pleated sheet which constitutes one of the six strands of parallel sheet found in the NAD^+ binding domain. This site is also near the active site portion of GDH as previously predicted (Singh et al., 1993). Therefore, this sequence is expected to interact with the adenine ring of the coenzyme.

The importance of Glu279 in coenzyme binding can be further supported from some interesting information that the invariant, functional residue, Asp, is found in the â-pleated sheet region of four known dehydrogenases and is conservatively changed to Glu 279 in mammalian GDH (Rossmann et al., 1974). 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 be occurring, due to charge repulsion, with $NADP^+$. This 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 279 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.

The work presented here clearly identifies the NAD^+ binding site of the human GDH.

CHAPTER V

TAT-Mediated Delivery of Human Glutamate Dehydrogenase into PC12 Cells

I. INTRODUCTION

Mammalian glutamate dehydrogenase (GDH) reversibly catalyzes the reductive amination of 2-oxoglutarate L-glutamate using NADH or NADPH as coenzyme (Smith et al., 1975). 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. Therefore, the activity of this enzyme should be tightly regulated to respond appropriately to the metabolic needs of the cell. Plaitakis et al. (1984) revealed evidence for the cellular presence of two distinct enzyme fractions: one that is bound to membranes (particulate GDH) and another that is readily solubilized (soluble GDH) (Colon et al., 1986). Similar observations have also been made for GDH isolated (Colon et al., 1986; Hussain et al., 1989; Rajas and Rouset, 1993). Although many studies show the subcellular localization of GDH (Schmitt and Kugler, 1999; McKenna et al., 2000), the origin of the GDH polymorphism is not known. It was reported that the presence of four differently sized mRNAs and multiple gene copies for GDH occur in the human brain (Plaitakis et al., 1993). A novel cDNA encoded by an X chromosome-linked intronless gene also has been isolated from human retina (Shashidharan et al., 1994; Shashidharan et al., 1997).

The importance of the GDH-deficient neurological disorders has attracted

considerable interest. Four different forms of GDH isoproteins were detected from the human cerebellum of normal subjects and patients with neurodegenerative disorders (Duvoisin et al., 1983; Plaitakis et al., 1984; Plaitakis et al., 1989; Hussain et al., 1989). The GDH isoproteins are differentially distributed in the two catalytically active isoforms of the enzyme (Colon et al., 1986; Plaitakis et al., 1993). The enzyme isolated from one of the patients with a variant form of multisystem atrophy displayed a marked reduction of one of the GDH isoproteins (Hussain et al., 1989). However, it is not known whether the distinct properties of the GDH isoproteins are essential for the regulation of glutamate metabolism. At present, the functional significance and reaction mechanism of GDH isotypes in nerve tissue remains to be studies.

One way to replenish the GDH activities in the patients with the GDH-deficient neurodegenerative disorders is cellular manipulation by transfection or viral introduction of cDNA expression vectors and microinjection of proteins into cell. Internalization of exogenous macromolecules by live cells provides powerful tools for studying cellular functions and may be applied for developing new therapeutic approaches. Various methods have been proposed for the delivery of proteins and other macromolecules into living cells (Renneisen et al., 1990; Prior et al., 1991; Leamon et al., 1992). These techniques, however, have several difficulties such as massive overproduction,

low percentage of cells targeted, and specific gene and cell type restrictions (Leamon et al., 1992; Matsumoto et al., 1992). Therefore, the approach to manipulate cellular processes by the introduction of full-length proteins in a concentration-dependent manner into 100 % of cells may alleviate these technical problems.

It was reported that the transcriptional transactivator (TAT) protein from human immunodeficiency virus 1 is able to enter cells when added exogenously (Mann et al., 1991). The TAT interaction with the transactivation responsive element (TAR) is mediated by a nine amino acid region RKKRRQRRR (residue 49 ~ 57) of the protein (Calnan et al., 1991a, 1991b). TAT protein can simply be added to medium at concentrations as low as 1 nM, and biological responses can be detected. Small region of the TAT protein called protein transduction domains, 11 ~ 34 amino acids in length, could across the lipid bilayer of cells either alone or as a fused form to some polypeptides or nucleotides (Derossi et al., 1998; Schwarze and Dowdy, 2000). However, the mechanism by which TAT traverses a membrane and the precise intracellular location of this event is not understood and no method to harness this technology potential has been devised. Recently, Nagahara et al. (1998) have described the development of a full-length protein transduction method using urea-denatured, genetic in-frame TAT fusion proteins. If the method can be applied to a broad spectrum of proteins regardless of size or function, it

might be used to overcome inherited diseases and other conditions caused by a malfunctioning or absent intracellular protein.

This study is described transduction of full-length TAT-GDH fusion protein into PC12 cells using synthetic human GDH gene. In this approach, full-length fusion proteins are generated to contain an N-terminal 9-amino acid protein transduction domain from the human immunodeficiency virus TAT protein. These results suggest a possibility that the transduction of TAT-GDH fusion proteins may be one of the ways to replenish the GDH activities in the patients with the GDH-deficient disorders.

II. MATERIALS AND METHODS

Construction of TAT-GDH fusion gene (pTAT-GDH)

The TAT-GDH gene (pTAT-GDH) was constructed by a cassette mutagenesis of synthetic human GDH gene previously constructed in our laboratory (Cho et al., 2001). Briefly mentioned, the synthetic human GDH gene (pHGDH) (Cho et al., 2001) was subcloned into pET28a. The pET/hGDH plasmid was digested with *Eco*R I and *Nru* I and the *Eco*R I/*Nru* I fragment was replaced with 71-bp synthetic DNA duplexes containing Factor Xa cleavage site and 9-amino acid (RKKRRQRRR) TAT protein transduction domain (Nagahara et al., 1998) as shown in Figure V-1. Mutagenic oligonucleotides were annealed, ligated, and transformed into DH5á. The cells were plated on LB agar containing 100 µg of ampicillin per ml and plasmid DNA (pTAT-GDH) was purified and examined by DNA sequencing. General methods for plasmid purification, subcloning, and bacterial transformation were as described (Sambrook et al., 1989).

Isolation of TAT-GDH fusion protein

pTAT-GDH was transformed into *E. coli* BL21 (DE3) and fresh overnight cultures of DE3/pTAT-GDH were used to inoculate 1 L of LB containing 100 µg of ampicillin per ml. DE3/pTAT-GDH was grown at 37°C until the A_{600}

reached 1.0 and then isopropyl â-D-thiogalactopyranoside was added to a final concentration of 1 mM. TAT-GDH was isolated by sonication in 50 mM Tris-HCl, pH 8.0 containing 100 mM NaCl, 2 mM phenylmethylsulfonylfluoride, and 8M urea and purified using Nickel affinity chromatography as described elsewhere (Nagahara et al., 1998). After reducing the concentration of urea into 0.05 M by rapid desalt as described (Nagahara et al., 1998), the isolated TAT-GDH was treated with Factor Xa and further purified by HPLC Protein-Pak 300SW gel filtration. The final TAT-GDH protein was analyzed by SDS-PAGE and recognized by western blot using monoclonal antibodies raised against the bovine brain GDH in our laboratory (Choi et al., 1999). Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard. The purified TAT-GDH was examined further by amino acid sequencing using automated Edman degradation.

Cell culture

PC12 cells were plated onto collagen-coated plastic tissue culture dishes and cultured in RPMI 1640 medium supplemented with 10 % FBS, 5 % horse serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin and kept in a controlled atmosphere (5 % CO_2) incubator at 37°C. After plating, the medium was replaced with RPMI 1640 containing 10 % horse serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin every 4 days. TAT-GDH (25 µg/ml) was

added directly to PC12 cells in culture media and incubated for various time periods (4, 8, 12, and 24 hrs). For control experiments, human GDH having no TAT domain was used under the same condition. The TAT-GDH treated cells were washed twice in PBS and treated with trypsin (20 μg/ml) for 8 h to remove cell surface associated proteins. The cells were then washed three times in PBS and lysed with RIPA buffer (PBS, 1 % Nonidet P-40, 0.5 % sodium deoxycholate, 0.1 % SDS, 10 μg/ml phenylmethanesulfonic acid, 0.3 trypsin inhibitor unit/ml aprotinin, and 1 mM sodium orthovanadate, pH 7.4). After centrifugation at 15,000 x *g* for 30 min at 4 °C, the supernatants were assayed for GDH activity, total protein, and analyzed by western blot using monoclonal antibodies raised against bovine brain GDH as described elsewhere (Cho et al., 1995; Choi et al., 1999).

Cytotoxicity assay

The cytotoxicity of TAT-GDH was assessed by the ability to inhibit protein synthesis, measured by [^{35}S]methionine incorporation into CCl_3COOH-insoluble material as described elsewhere (Fawell et al., 1994). Cells were plated into 24-well dishes at 50 % confluence and allowed to attach as described above. TAT-GDH and controls at the concentrations indicated were added and incubated at 37 °C. After 8 h of incubation, the medium was removed and cells were washed in PBS and then incubated with

1.0 μCi of [^{35}S]methionine per well in PBS. After 2 h, the cells were washed three times with ice cold 5 % CCl_3COOH, washed once with PBS, and then extracted with 0.1 ml of 0.5 M NaOH. The CCl_3COOH-precipitated material was quantified by liquid scintillation counting.

Statistical analysis

The effects of TAT-GDH in the PC12 cells were analyzed by an unpaired two-tailed *t* test. Unless otherwise mentioned, each experimental point represents the mean of triplicate determinations from different preparations and standard deviation is indicated as error bar in each figure. At some points, error bars were omitted in the figures for clarity purpose.

III. RESULTS

Gene synthesis and expression

Human GDH gene was fused with a gene fragment encoding the 9-amino acid TAT protein transduction domain of human immunodeficiency virus 1 in bacterial expression vector to produce genetic in-frame TAT-GDH fusion protein. The design of the TAT-GDH fusion gene was based on the following strategy. First, the 9-amino acid (RKKRRQRRR) TAT protein transduction domain (Nagahara et al., 1998) was added just before the first amino acid (Ser) of the authentic human GDH. Second, the codon usage of the resulting TAT-GDH gene was modified to include those triplets that are utilized in highly expressed *E. coli* genes (An et al., 1981; Gouy and Gautier, 1982). Finally, the TAT-GDH fusion gene contains the sequence coding for the recognition site of the specific Factor Xa protease, located just before TAT protein transduction domain. Factor Xa cleaves after its four amino acid recognition sequence, I-E-G-R (Nagai and Thogersen, 1987), so that no additional residues are attached to the TAT-GDH protein. The designed TAT-GDH fusion protein and position of TAT transduction domain sites together with a Factor Xa site are shown in Figure V-1.

Isolation of TAT-GDH fusion protein

TAT-GDH fusion protein encoded by pTAT-GDH in DE3 was isolated as described in MATERIALS AND METHODS. To remove additional N-terminal residues, the purified human GDH was treated with Factor Xa, purified by a HPLC Protein-Pak 300SW gel filtration column, and subjected to automated Edman degradation. N-terminal sequence analysis of the first 12 amino acids was in good agreement with expected amino acid sequence of TAT-GDH fusion protein (Table V-1).

Delivery of TAT-GDH fusion protein into PC12 cells

The denatured TAT-GDH fusion protein can enter neuronal PC12 cells efficiently at time- and dose-dependent manner when added exogenously in culture media. The 56.5-kDa protein band corresponding to authentic GDH in the crude extracts of the PC12 cells transduced with TAT-GDH protein was detected 9 ~ 11 times stronger than those of cells transduced with denatured GDH that was not fused with TAT as determined by western blot analysis (Figure V-2). The specific activities of GDH (0.74 units/mg) in the crude extracts from PC12 cells transduced with the denatured TAT-GDH protein for 8 h was approximately 6 times higher than that of endogenous GDH (0.13 units/mg) from PC12 cells transduced with plain GDH (Figure V-3). These results indicate that the denatured TAT-GDH protein transduced into PC12

cells and once inside the cells transduced denatured TAT-GDH protein was correctly refolded probably because it can access the cell's normal protein-folding machinery such as chaperones (Schneider et al., 1996; Gottesman et al., 1997). The differences in results between western blot analysis (Figure V-2) and specific activities (Figure V-3) may be due to the incomplete refolding of the tranduced denatured proteins or may reflect a slow posttransduction refolding rate of the protein by intracellular chaperons such as HSP90 (Schneider et al., 1996). This remains to be further studied.

Table V-1. N-Terminal amino acid sequence of the TAT-GDH fusion protein

Cycle[a]	Residue[b]	Picomoles
1	R	37
2	K	34
3	K	33
4	R	30
5	R	29
6	Q	22
7	R	25
8	R	22
9	R	20
10	S	17
11	E	14
12	A	9

[a] Only the first 12 cycles were sequenced.

[b] The amino acids are denoted by the single-letter code.

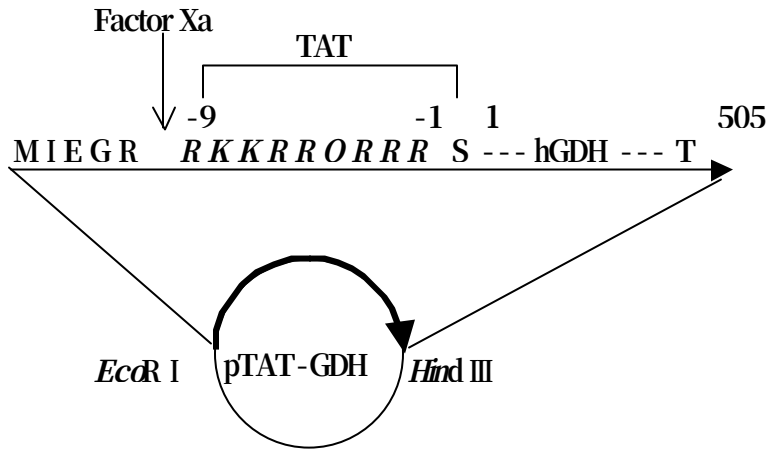


Figure V-1. pTAT-GDH expression vector. The 9 amino acid TAT protein transduction domain is located at position -9 to -1 of the amino acid sequence. Factor Xa site is shown by a vertical arrowhead. Position 1 of the amino acid sequence corresponds to the first amino acid (Ser) of the mature authentic human GDH (Mavrothalassitis et al., 1988).

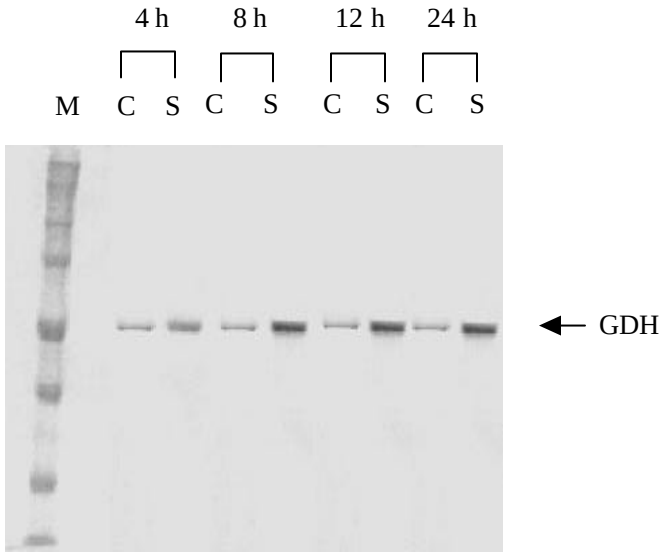


Figure V-2. Transduction of the recombinant TAT-GDH protein into PC12 cells. TAT-GDH was added directly to PC12 cells in culture media and incubated for various time periods (4, 8, 12, and 24 hrs). At different time intervals, the cells were disrupted and the crude extracts were analyzed by SDS-PAGE and western blot using monoclonal antibodies raised against bovine brain GDH (Choi et al., 1999). As a control, the crude extracts of PC12 cells transduced with human GDH without TAT fusion were used. ***M***, marker proteins; ***C***, control (GDH only); ***S***, sample (TAT-GDH).

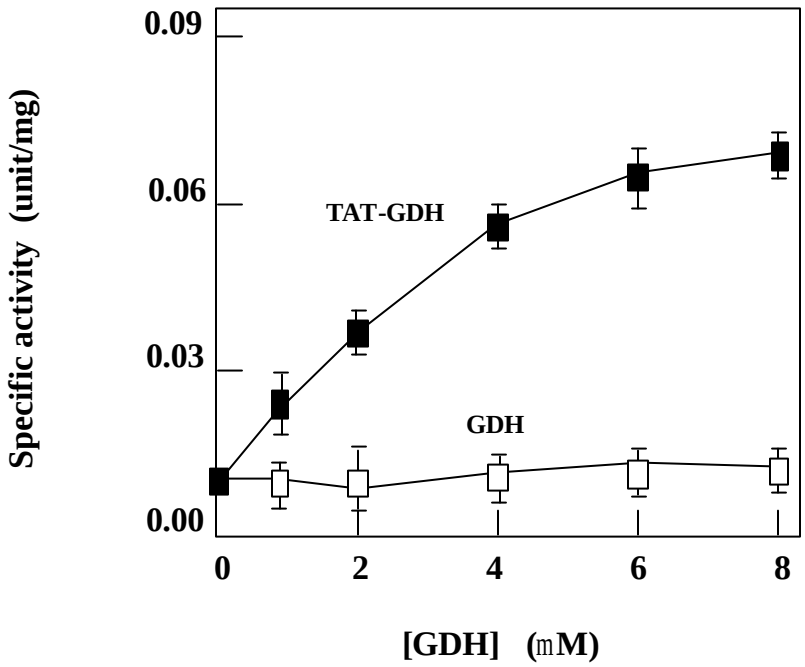


Figure V-3. Dose-dependent transduction of the recombinant TAT-GDH protein into PC12 cells. TAT-GDH was added directly to PC12 cells in culture media in the presence of different concentration of TAT-GDH. After 8 h, the cells were disrupted and the specific activities of GDH in the crude extracts were measured (■). As a control (•), the crude extracts of PC12 cells transduced with human GDH without TAT fusion under the same experimental conditions were used. Experiments were carried out in triplicate, and the results show the average of three independent experiments.

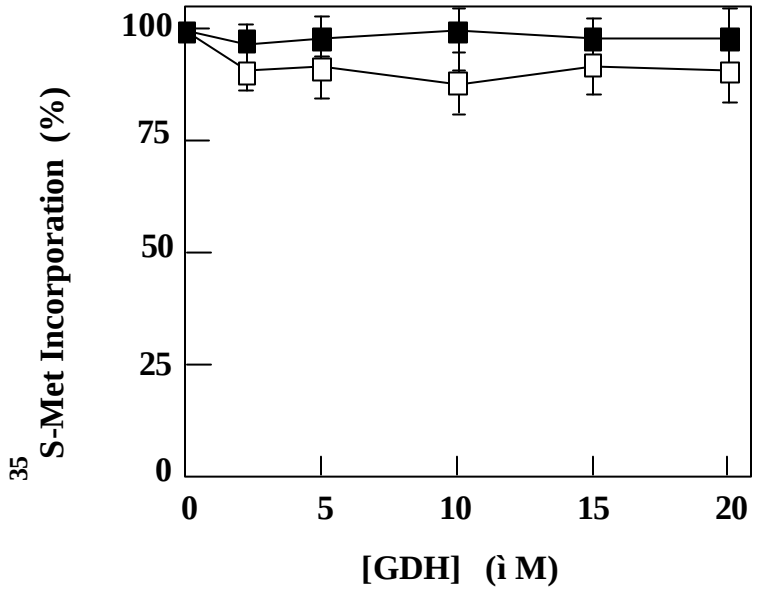


Figure V-4. Cytotoxicity of TAT-GDH. Cytotoxicity was measured by the inhibition of [^{35}S]methionine incorporation into CCl_3COOH-insoluble material. Cells were incubated with GDH control (•) and TAT-GDH (■) at the concentrations indicated and as described in text. Experiments were carried out in triplicate, and the results show the average of three independent experiments.

IV. DISCUSSION

To traverse the cell membrane, TAT fusion proteins may require partial unfolding, presumably because a partially unfolded protein would have more of its oily interior amino acids exposed and might therefore slide more easily through the lipid-rich cell membrane. Similar results have been reported by Nagahara et al. (1998), showing that transduction of denatured TAT-p27 protein into mammalian cells readily induced cell migration at 50 ~ 150 nM concentrations whereas correctly folded TAT-p27 protein failed to induce cell migration at even higher concentrations. They suggested that this is not a failure of correctly folded TAT-p27 protein to transduce into cells but merely represents the inefficiency to achieve the required intracellular threshold level to elicit cell migration (Nagahara et al., 1998). Therefore, the use of energetically unstable denatured fusion proteins seems to enhance the ability to transduce proteins into cells. I do not know how TAT can ferry other proteins into cells. Two laboratories have demonstrated that TAT binds to specific cell surface proteins, raising the possibility that delivery is receptor-mediated (Vogel et al., 1993; Weeks et al., 1993). However, other researchers have reported that the TAT protein transduction is independent of receptors and transporters and instead is thought to target the lipid bilayer component of the cell membrane, suggesting that all mammalian cell types should be

susceptible to protein transduction (Derossi et al., 1996; Schwarze et al., 1999). Whether these specific interactions are involved in uptake of TAT fusion proteins remains to be determined.

There are possibilities that TAT fusion proteins may produce immune responses or other toxic effects, although no signs of problems have appeared yet. To expand these possibilities, the TAT-GDH was applied to PC12 cells and tested for the ability to inhibit protein synthesis. The TAT-GDH showed no detectable cytotoxicity at the concentrations tested (Figure V-4) or at extended incubation up to 24 h (data not shown), suggesting that TAT-GDH was not toxic at least under the experimental conditions used in the present study. Along these lines, a similar result has been reported that injection of a mouse with 1 mg of a TAT fusion protein per kilogram of body weight each day for 14 consecutive days produced no signs of gross neurological problems or systemic distress (Schwarze et al., 1999). However, the toxicity associated with long-term transduction of proteins and potential immune responses together with the exact mechanism of transduction across a membrane still remain to be examined.

The importance of the pathophysiological nature of the GDH-deficient neurological disorders has attracted considerable interest (Plaitakis et al., 1982; Hussain et al, 1989). It is, therefore, essential to have a detailed structural and functional description of GDH to elucidate the

pathophysiological nature of the GDH-deficient neurological disorders. Our lab current understanding of the structure and catalytic activity of GDH has been the result of many years of biochemical study. In an effort to replenish the GDH activity in the patients with the GDH-deficient neurodegenerative disorders (Plaitakis et al., 1984; Hussain et al., 1989), this study was focused on a genetic approach that involves the generation of a genetic in-frame TAT-GDH protein and transduction of the TAT-GDH fusion protein into neuronal cells. Therapy with biologically active full-length proteins will allow access to the built-in evolutionary specificity of these proteins for their targets, thereby potentially avoiding the nonspecific effects sometimes seen with small molecule therapies (Schwarze et al., 1999). Although the exact mechanism of transduction across a membrane and the physiological role of the transduction activity of TAT fusion proteins in human tissues are not clear at this moment, my results of the TAT-GDH transduction into neuronal cells may suggest a possibility of the therapeutic delivery of GDH into the patients caused by a malfunctioning or absent intracellular enzyme.

CONCLUSIONS

I. Decreased Expression of Glutamate Dehydrogenase by Prolonged Intake of Monosodium Glutamate in Rat Brains

1. The concentrations of the glutamate in the brain crude extracts of the monosodium glutamate treated group were 2-fold higher than those of the control group.

2. The activities of glutamate dehydrogenase (GDH) were decreased with 45 % reduction in V_{max} values in the monosodium glutamate treated group compared to those in the control group, whereas no significant changes were observed in K_m values between the two groups.

3. The protein expression of glutamate dehydrogenase was significantly decreased in the monosodium glutamate treated group, whereas the level of GDH mRNA remained unchanged, suggesting a post-transcriptional control of the expression of GDH or an increased rate of degradation of the enzyme.

II. Cassette Mutagenesis of Lysine130 of Human Glutamate Dehydrogenase

1. A 1557-base-pair gene that encodes human glutamate dehydrogenase has been chemically synthesized.

2. The DNA sequence contains 45 unique restriction sites that are located an average of 35 base pairs throughout the entire length of the gene. A ribosome binding site was included 18 base pairs upstream from the translation start site and codon usage was adjusted to ensure efficient translation in *E. coli*.

3. Catalytically active GDH gene encoded by the synthetic gene is expressed and is indistinguishable from that isolated from human and bovine brain tissues.

4. Analysis of the mutants by enzyme kinetics and western blot analysis indicates that Lys130 is strictly required for catalytic activity.

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

1. Five mutant GDHs (Y187M, Y187R, Y187S, Y187G, and Y187E) at position 187 were designed to have different size, hydrophobicity, and ionization of the side chains and constructed by cassette mutagenesis.

2. The mutagenesis at Tyr187 sites had no effects on expression or stability of the different mutants.

3. Saturation and photoinsertion with [α-^{32}P]8N_3ADP occurred for wild type GDH was significantly decreased by ADP, whereas no photoinsertion was detected in Tyr187 mutant protein.

4. Photolabeled peptide of the wild type GDH was identified as in the ADP binding domain of the human GDH.

5. Protection studies demonstrate selectivity of the photoprobe for the ADP binding site and suggest that Tyr187 is responsible for the efficient base-binding of ADP to human GDH.

IV. Site-Directed Mutagenesis and Photoaffinity Labeling of an NAD^+ Binding Site of Human Glutmate Dehydrogenase

1. Five mutant GDHs (E279M, E279R, E279L, E279G, and E279Y) at position 187 were designed to have different size, hydrophobicity, and ionization of the side chains by cassette mutagenesis.

2. The mutagenesis at Glu279 sites had no effects on expression or stability of the different mutants.

3. Saturation and photoinsertion with $[^{32}P]2N_3NAD^+$ was significantly decreased in the presence of NAD^+, whereas no photoinsertion was detected in Glu279 mutant protein.

4. The results with cassette mutagenesis and photoaffinity labeling demonstrate that the Glu279 are required for efficient binding of NAD^+ to human GDH.

V. TAT-Mediated Delivery of Human Glutamate Dehydrogenase into PC12 Cells

1. Human GDH gene was fused with a gene fragment encoding the 9 amino acid TAT protein transduction domain of HIV-1 in bacterial expression vector to produce genetic in-frame TAT-GDH fusion protein.

2. The isolated recombinant TAT-GDH fusion protein can enter neuronal PC12 cells efficiently at dose- and time-dependent manner when added exogenously in culture media.

3. Denatured TAT-GDH protein was transduced much more efficiently into cells than correctly folded proteins. Once inside the cells, however, transduced denatured TAT-GDH protein showed a full activity of GDH.

4. These results suggest a possibility that the transduction of TAT-GDH fusion proteins may be one of the ways to replenish the GDH activities in the patients with the GDH-deficient disorders.

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국문 요약

글루탐산 분해효소(glutamate dehydrogenase)의 구조, 알로스테릭 조절, 및 작용기전

글루탐산 분해효소 (GDH)의 구조 및 분자적 특성을 알아 보기 위하여 다음과 같은 연구들을 수행하였다. 우선 조미료로 많이 이용되고 있으며 Chinese restaurant syndrome을 유발시키는 물질로 잘 알려져 있는 글루탐산나트륨 (MSG)이 뇌조직 내의 GDH에 미치는 영향을 조사하였다. 장기간 MSG 복용에 의한 뇌에 미치는 영향을 알아보기 위해, 1주된 albino 쥐에게 1년간 식수에 MSG를 첨가하여 먹인 그룹과 먹이지 않은 두 그룹으로 나뉘어 실험해 보았다. 쥐의 몸무게와 뇌의 무게, 총 DNA와 RNA양 그리고 단백질 양에는 두 그룹간의 유의한 차이를 보이지 않았으나 뇌의 생 추출물속의 글루탐산 농도를 비교한 결과, MSG를 먹인 그룹에서는 대조군에 비해 2배 정도 높게 나타났다. 또한, 글루탐산 분해효소(GDH)의 mRNA 양은 두 그룹간의 유의한 차이를 보이지 않았으나, western blot 검사를 실시한 결과 효소의 양은 MSG를 먹인 그룹이 현저해 감소한 것으로 보아, GDH 발현에 있어 후전사 조절에 영향을 미치거나 GDH의 분해율을 증가시키는 것으로 보여진다. 이 결과로, 장기간 MSG를 섭취하게 되면 쥐 뇌 속에 존재하는 GDH 활성을 감소시킴으로 글루탐산의 분해가 저하됨을 알 수 있었다.

다음으로, GDH의 구조적, 기능적 조절형태를 심도 있게 연구하

기 위해 1557-base-pair의 인간 GDH 유전자를 합성하여 대장균주를 이용해 활성을 지닌 효소로 발현시켰다. 이렇게 발현된 효소는 사람과 소의 조직에서 추출한 효소와 여러 가지 생화학적 측면에서 유사한 양상을 보였다. 자리지정 돌연변이방법을 이용해 이 효소의 Lys130 위치를 다른 아미노산으로 바꾼 돌연체를 이용해 실험해 본 결과, Lys130 위치는 GDH의 기질이나 다른 조효소가 결합하는 곳이 아닌 촉매작용을 일으키는데 관여하는 중요한 곳임을 알게 되었다.

다음으로, 인간 GDH의 알로스테릭 활성제인 ADP와 조효소인 NAD^+ 결합부위를 규명하기 위해, 각각 이에 해당되는 Tyr187과 Glu279부위에 대해 돌연변이를 일으켜 아미노산의 크기와 소수성, 곁가지의 이온화 정도에 따라 다양한 돌연체들을 만들어 실험해 보았다. GDH의 자연형은 ADP와 반응시키면 3배정도 활성도가 증가된 반면 ADP 결합부위의 각 돌연체 효소들(Tyr187 돌연체)의 활성은 유의한 차를 보이지 않았다. 정지상태의 역학치수를 구한 결과 Tyr187 돌연체 효소들은, 2-oxoglutarate와 NADH에 대한 K_m 값에는 유의한 차이를 보이지 않았으나 V_{max} 값에는 자연형에 비해 4배 정도 감소된 결과를 얻었다. Glu279 돌연체 효소들은 NAD^+ 와 글루탐산에 대해 V_{max} 값은 22 ~ 28배 정도 감소한 반면 K_m 값은 NAD^+에 대해서만 20 ~ 25배 정도 감소하였다. ADP와 NAD^+ 결합부위를 규명하기 위해 [á-^{32}P]8-azidoadenosine 5 -diphosphate ($8N_3ADP$)와 [^{32}P]nicotinamide 2-azidoadenosine dinucleotide ($2N_3NAD^+$)를 이용하여 광흡착표지를 실시한 결과 GDH의 자연형에 대한 [á-^{32}P]$8N_3ADP$와 [^{32}P]$2N_3NAD^+$의 광흡착 포화도에 대한 K_d 값은 25 ìM 과 55 ìM로

나타났다. 이때, 자연형의 경우에는 ADP와 NAD^+를 첨가하면 $8N_3ADP$와 $2N_3NAD^+$의 광흡착이 현저히 감소되었으나, Tyr187와 Glu279 돌연체들은 각각 $8N_3ADP$ 및 $2N_3NAD^+$와 반응하지 않았다. 즉, 인간 GDH 유전자의 돌연변이와 광흡착표지법을 통해 GDH의 Tyr187과 Glu279 위치는 각각 ADP와 NAD^+의 염기가 결합하는 부위임을 증명할 수 있었다.

마지막으로, GDH 결핍으로 신경퇴행성 질환이 생긴 환자에게 부족한 GDH를 단백질 차원에서 보충하기위한 새로운 방법을 시도하였다. 이를 위해서 인간 GDH 유전자에 인간 면역결핍 바이러스에 존재하는 TAT 단백질의 전이 부위를 융합 시켜 TAT-GDH 융합 단백질을 발현시켰다. 이렇게 얻어진 TAT-GDH 융합 단백질을 변성시켜 신경세포 배양액에 분주한 결과 신경세포 내로 잘 침투하였고, 일단 세포 내로 들어가게 되면 변성된 단백질이 다시 제 형태를 갖추게 되어 정상적인 효소 활성을 가지게 되었다. 즉, TAT 잔기를 GDH에 첨부하여도 GDH 활성에는 별 영향을 미치지 않으며, 세포 내 독성도 나타내지 않음을 알게 되었다. 이러한 결과는 GDH 결핍으로 인해 야기된 여러 질환을 앓고있는 환자에게 GDH를 직접 전달할 수 있는 새로운 방법을 제시해 주고 있다고 사료된다.

핵심어: 글루탐산 분해효소, 글루탐산나트륨, 돌연변이, ADP 결합부위, NAD^+ 결합부위, 촉매작용부위, 광흡착표지법, TAT 융합 단백질, 단백질 치료, 퇴행성 신경질환, 유전자 공학, 단백질 공학

감사의 글

처음 학위를 시작한 때가 생각납니다. 학문에 뜻이 있어 다니던 직장도 그만두고 누구보다도 더 많은 설레임과 걱정으로 만학의 길을 시작한 제가 이렇듯 논문을 완성하고 박사학위를 받기까지 너무나 많은 분들의 도움과 배려로 이 순간이 제게 허락된 것 같아 부족하나마 이 글을 빌어 진심으로 감사의 마음을 전해 드리고자 합니다.

변함없는 관심과 격려로 부족한 저에게 많은 학문적 조언과 가르치심을 주시고 지도해주신 김 태우 교수님 그리고 조 성우 교수님께 진심으로 감사를 드립니다. 바쁘신 와중에도 저의 논문 심사를 맡아주시고 끊임없는 사랑과 학문적 조언을 아끼지 않으셨던 오 옥두 교수님, 김 종배 교수님, 그리고 박 용석 교수님께 감사 드립니다. 또한, 학부 때부터 저를 지켜봐 주시고 많은 가르치심을 주신 양 용석 교수님께도 이 자리를 빌어 감사를 드립니다. 학위를 마치기까지 애정어린 관심과 배려를 아끼지 않으셨던 울산의대 생화학교실 나 도선 교수님, 이 재담 교수님, 송 규영 교수님, 황 온유 교수님, 그리고 최 현진 박사님께도 깊이 감사 드립니다. 바쁜 생활 속에서도 언제나 부족한 후배에게 따뜻한 조언을 아끼지 않으시던 김 윤석 선배님과 이 종원 선배님 그리고 우리 과를 위해 항상 애써주시는 임 병혁 선생님께도 감사 드립니다. 인생의 선배로서 방의

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