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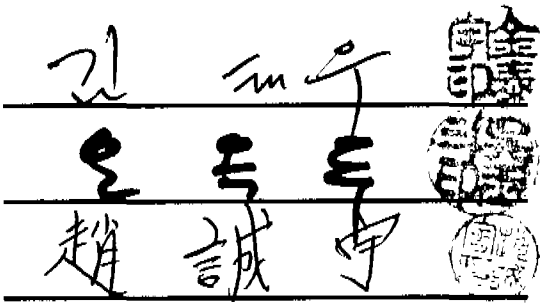
심사위원 

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감사의 글

부족한 제가 이렇게 논문을 완성하고 석사과정을 졸업하기까지 많은 도움과 격려를 아끼지 않으셨던 모든 분들께 이 글을 빌어 진심으로 감사를 드립니다.

항상 아낌없는 가르침과 세심한 배려와 격려로 학문의 길로 이끌어 주신 김태우 교수님과 조성우 교수님께 깊은 감사를 드립니다. 바쁘신 와중에도 저의 논문 심사를 맡아주신 오옥두 교수님과 끊임없는 사랑과 학문적 조언을 아끼지 않으신 박용석 교수님, 양용석 교수님, 김종배 교수님께 고개 숙여 감사 드립니다. 우리 과를 위하여 고생하시는 임병혁 선생님께도 감사의 마음을 전합니다. 아무것도 모르고 처음 우리 과와 인연을 맺은 학부 시절, 학문의 꿈을 키워갈 수 있도록 도와주신 정동주, 김윤석, 양세환, 이원용, 김소영, 엄용빈, 남상민, 최태현, 금준섭, 이양호, 박상욱, 전향, 김성수, 조장온, 김병수, 손병수, 이지숙, 정연우, 박성언, 김영미, 김세중, 이종학 선배님들께 감사 드리며 가장 가까이서 많은 도움과 어려울 때 힘이 되어준 언니, 윤혜영 선배님과 울산의대 생화학 교실 가족들에게 깊은 감사를 드립니다. 대학원의 큰언니, 큰오빠로서 따뜻한 격려로 용기를 주셨던 최현일, 심문정, 임지애, 조윤경, 장재호, 김홍성, 김인식, 이태섭, 김근식 선배님들께 감사드립니다. 대학원 생활을 함께 시작할 수 있어 기뻤던 진연언니와 김재균 선생님, 그리고 같이 졸업하는 용이오빠, 주연언니, 수인 그리고 힘들 때 의지하며 대학원 생활을 보낼 수 있어서 고마웠던 동기, 현정에게 졸업의 기쁨과 감사의 마음을 전하고 싶습니다. 바쁘다고 자주 만나지는 못했지만 어려움이 있을 때 멀리서라도 같이 걱정해주었던 친구들, 정주, 주희, 혜영,

혜경, 그리고 정혁을 비롯한 우리 95학번 동기들과 쌍뚜스 사람들에게 고마운 마음을 전합니다. 대학원 생활에 잘 적응할 수 있도록 도와준 나경언니, 근희언니, 수용오빠에게 감사의 마음을 전하며, 항상 인간적인 따뜻함으로 대학원 생활을 즐겁게 해 준 승주오빠, 그리고 열심히 대학원 생활에서 자신의 모습을 만들어가는 재호, 영삼오빠, 준표오빠, 진현석오빠, 김현석오빠, 호중오빠, 대종오빠, 은숙언니 그리고 종철에게 의미 있는 대학원 생활이 되었으면 하는 마음을 전합니다. 끝으로 언제나 사랑과 믿음으로 지켜봐 주시고, 저를 위해 기도해 주시는 엄마, 아빠, 그리고 사랑하는 우리 인주오빠와 동생 만주에게 기대에 어긋나지 않는 사람이 되겠다는 다짐과 함께 이 작은 결실을 드립니다.

2001년 6월

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ABBREVIATION

$8N_3GTP$: 8-azidoguanosine triphosphate

BME : β-Mercaptoethanol

EDTA : ethylenediaminetetraacetic acid

FPLC: fast protein liquid chromatography

GDH : glutamate dehydrogenase

HPLC : high performance liquid chromatography

IPTG : isopropylthio-β-D-galactoside

NADH : nicotinamide adenine dinucleotide

PMSF : phenymethysulfonylfluoride

SDS/PAGE : sodium dodecyl sulfate/ polyacrylamide gel electrophoresis

ABSTRACT

Identification of GTP Binding Site of Human Glutamate Dehydrogenase Using Cassette Mutagenesis

It has been reported that the hyperinsulinism-hyperammonemia syndrome is caused by mutations in glutamate dehydrogenase (GDH) gene that affects enzyme sensitivity to GTP-induced inhibition. To identify GTP binding site(s) within human GDH, mutant GDHs at Lys450 position were constructed by cassette mutagenesis. While wild type GDH was completely inhibited by 20 μM GTP, all of the five Lys450 mutant GDHs were not inhibited by GTP up to 1 mM and remained a full activity regardless of their size, hydrophobicity, and ionization of the side chains. The binding of GTP to the wild type and mutant GDHs were further examined by photoaffinity labeling with [γ-^{32}P]8-azidoguanosine 5'-triphosphate ($8N_3GTP$). Saturation of

photoinsertion with $8N_3GTP$ occurred apparent K_d value near 15 μM for wild type GDH and the photoinsertion of $8N_3GTP$ was significantly decreased in the presence of GTP. Unlike wild type GDH, no photoinsertion was detected in Lys450 mutant protein. The results with cassette mutagenesis and photoaffinity labeling demonstrate selectivity of the photoprobe for the GTP binding site and suggest that Lys450, is required for efficient binding of GTP to GDH. Interestingly, studies of the steady-state velocity showed that the wild type GDH was inhibited by ATP at concentrations between 10 and 100 μM, while none of the Lys450 mutant GDHs were inhibited by ATP up to 300 μM. These results indicate that Lys450, may be also responsible for the ATP inhibition and so ATP was binding to the GTP site.

Key words : Human glutamate dehydrogenase; GTP binding site; Cassette mutagenesis; hyperinsulinism-hyperammonemia syndrome; Synthetic gene; Photoaffinity labeling

INTRODUCTION

Glutamate is a major excitatory neurotransmitter (Founnum, 1984) but it does not readily cross the blood-brain barrier (McGeer and McGeer, 1976). The major of glutamate in the central nervous system is produced from precursors and regulated by glutamate pyruvate transaminase, glutamine synthase, and glutamate dehydrogenase (GDH) (Christopher et al., 2000; Magali et al., 2000). Glutamate excitotoxicity plays a significant role in the pathogenesis of several acute and chronic neurological conditions. 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; Magali et al., 2000; Plaitakis and Shashidharan, 2000).

GDH (EC 1.4.1.2-4) is one of the central enzyme in glutamate metabolism. GDH is a mitochondrial enzyme that catalyzes the reversible deamination of L-glutamate to 2-oxoglutarate using NAD^+ or $NADP^+$ as coenzyme (Smith et al., 1975).

$$\text{Glutamate} + \text{NAD(P)}^+ + \text{H}_2\text{O} \leftrightarrow \text{2-Oxoglutarate} + \text{NH}_4^+ + \text{NAD(P)H}$$

GDH plays a role in regulating the levels of ammonia and glutamate in the central nervous system and intergrates carbon and nitrogen metabolism via the tricarboxylic acid cycle. Substantial amounts of GDH enzymatic activity have found in several mammalian tissues including liver, brain, kidney, heart, pancreas, ovaries, and lymph nodes (Plaitakis et al., 1980).

The importance of the physiological nature of GDH has attracted considerable interest. It has been reported that patients with neurodegenerative disorders characterized by multisystem atrophy and predominant involvement of the cerebellum and its connections showed a marked reduction of one of the GDH isoproteins (McGeer and McGeer, 1976; Mavrothalassitis et al., 1988; Plaitakis et al., 1982, 1993; Hussain et al., 1989). Although the origin of the GDH polymorphism is not known, the presence of differently sized mRNAs and muliple gene copies for GDH has been reported in the human brain (Amuro et al., 1988; Michaelidis et al., 1993; Shashidharan et al., 1994). Actually, two different types of GDH have been isolated from bovine brain (Cho et al., 1995, 1996; Choi et al., 1999) and regulatory properties

of the two GDH isotypes have showed a difference in their sensitivity to heat (Cho et al., 1995). Similar results have indicated that the presence of two GDH activities in rat brain differs in their relative resistance to thermal inhibition and allosteric regulation characteristics (Colon et al., 1986). It has been reported that a novel nerve tissue specific human GDH shows a different thermostability and ADP regulation to that of previously reported from GDH (Shashidharan et al., 1997). Although the studies of detailed structural and functional description of the various types of brain GDH have been reported (Cho et al., 1996, 1998, 1999; Kim et al., 1997; Cho and Yoon, 1999; Plaitakis et al., 2000), it is not known clearly whether the distinct properties of the GDH isoproteins are essential for the regulation of glutamate metabolism.

Recently, it has been reported that 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). The knowledge on the genetic basis of hyperinsulinism-hyperammonemia syndrome has been rapidly growing during recent years. It has been proposed that hyperinsulinism-hyperammonemia syndrome

is caused by mutations in the gene for the β-cell sulfonylurea receptor or its associated potassium ion pore, *SUR1* and $K_{ir}6.2$, as well as functional mutation of glucokinase (Thomas et al., 1995, 1996; Nestorowicz et al., 1996, 1997). The fact that altered regulation of GDH causes hyperinsulinism-hyperammonemia syndrome indicates the importance of this enzyme in controlling the release of insulin by pancreatic β-cells. Glucose-induced insulin secretion is determined by signals generated in the mitochondria. Maechler and Wollheim (1999) have proposed that glutamate, generated by GDH, participates in insulin secretion as a glucose-derived metabolic messenger. In the pancreas of patients with the hyperinsulinism-hyperammonemia syndrome, the increase in GDH activity associated with impaired GTP inhibition leads the inappropriate secretion of insulin (Stanley et al., 1998; Miki et al., 2000).

Mammalian GDH is composed of six identical subunits and the regulation of GDH is very complex (Fisher, 1985). Mammalian GDH is regulated by numerous factors including pH, aggregation state, diverse compounds such as purine nucleotides, amino acid, and steroid hormones (Smith et al., 1975; Rife and Cleland,

1980; Erencinska and Nelson, 1990). GDH falls into two oligomeric classes. 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 55~57 kDa (vertebrate) (Smith et al., 1975; Cho et al., 1995), whereas the NAD^+-linked enzymes have either six identical subunits of around 48 kDa (e. g. *Clostridium symbiosum*) (Rice et al., 1985), or four identical subunits of 115 kDa (e. g. *Neurospora crassa*) (Veronese et al., 1974). 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 contrast to the extensive allosteric homotrophic and heterotrophic regulation observed in mammalian GDH, bacterial forms of GDH are relatively unregulated. Studies have shown that chemical probes can at least partially desensitize bovine liver GDH to GTP inhibition while not affecting catalytic activity. The amino acids modified by these chemical probes were shown to be a residue in the C-terminus (Coffee et al., 1971; Piszkiewicz et al., 1971). It seems likely that the regulatory GTP binding

domain is located in this region. 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 within the antenna region (Peterson and Smith, 1999).

It has been a major goal to identify substrate and the regulatory binding sites of GDH. Even though there are many reports suggesting the regulatory or substrate binding sites of GDH from various sources, the results are quite controversial. Several classical chemical probes have been used to attempt resolution of these binding sites. However, the studies using classical chemical probes to identify the NADH binding site within bovine liver GDH (Pal et al., 1975; Saradambal et al., 1981; Ozturk et al., 1990; Ozturk et al., 1992) gave a wide scatter of modified residues, i.e., Cys319 (Ozturk et al., 1990; Ozturk and Colman, 1991), Met169 and Tyr262 (Ozturk et al., 1992), and Lys420 and Tyr190 (Pal et al., 1975; Saradambal et al., 1981; Schmidt and Colman, 1984), throughout most of the proposed three-dimensional structure of GDH. It seems, therefore, that the base moiety has not been effective at directing the site of modification by classical chemical probes.

In contrast, 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, 1998; Cho and Yoon, 1999). We previously isolated and characterized two soluble forms of GDH isoproteins (designated GDH I and GDH II) from bovine brain (Cho et al., 1995, 1998; Cho and Lee, 1996; Kim et al., 1997) and identified the GTP binding domain of the GDH isoproteins using [γ-^{32}P]8N_3GTP (Cho et al., 1996). It was proposed that the inhibition involved binding of GTP to a lysine residue (Cho et al., 1996). The mutations identified in the patients with hyperinsulinism-hyperammonemia syndrome are all within exons 11 and 12 of GDH (Stanley et al., 1998, 2000) and exactly lie within a sequence of amino acids that has been identified as a GTP binding site (Ile440 through Arg459) of the bovine brain GDH isoproteins

using [γ-^{32}P] $8N_3GTP$ in our laboratory (Cho et al., 1996). However, these results are contrast to previous reports (Pal and Colman, 1979) suggesting that bovine liver GDH contains two GTP binding sites and Tyr262 (Tyr266 in human sequence) is probably major inhibitory site. Although the atomic structure of bovine liver GDH has suggested a possibility of two GTP binding sites (Peterson and Smith, 1999), there are disagreements on whether there are two GTP sites (Pal and Colman, 1979; Shoemaker and Haley, 1993; Cho et al., 1996) and no direct evidence has been reported for precise sites and functions of the second GTP binding site.

In the present work, we have expanded on GTP binding site(s) of human GDH using a cassette mutagenesis at Lys450 site and photoaffinity labeling with $8N_3GTP$. 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). For the present study, the mutant human GDHs, containing Glu, Gly, Met, Ser, or Tyr at position 450, have been expressed in *E. coli* as a soluble protein, purified, and characterized. To our knowledge this is the first report by site-directed mutagenesis showing that Lys450 is required for efficient binding of GTP and that the second GTP site, if any, is not

required for inhibition of human GDH by GTP.

MATERIALS AND METHODS

Materials

ADP, ATP, NADH, 2-oxoglutarate, IPTG, GTP, and L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin were purchased from Sigma. [γ-^{32}P]8N_3GTP was synthesized by the method as previously described (Shoemaker and Haley, 1993; Cho et al., 1996). 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). Q sepharose and Resource-Q were purchased from Pharmacia. POROS-QM was purchased from Perseptive Biosystems. Protein Pak DEAE-5PW column was purchased from Waters. Pre-cast gels for sodium dodecyl sulfate/ polyacrylamide gel electrophoresis (SDS/PAGE) were purchased from NOVEX. 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 Δ(gltB-F)500 rpsL19 malT1 xyl-7 mtl-2 argH1 thi-1*; kindly provided by Dr. Mary K. B. Berlyn, *E. coli* Genetic Stock Center, Yale University) lacked both GDH and glutamate synthase 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 Lys450 Mutants

A series of single amino acid substitutions of Lys450 was constructed separately by cassette mutagenesis of a synthetic human GDH gene, pHGDH (Cho et al., 2001). For mutagenesis at Lys450 site, plasmid DNA was digested with *Nhe* I and *Tth111* I to remove 15-bp fragment that encodes amino acids 447-451. The 15-bp *Nhe* I/ *Tth111* I fragment was replaced with five 15-bp synthetic DNA duplexes containing a substitution on both DNA strand at positions encoding Lys450 to make K450G, K450S, K450E, K450M, and K450Y mutant proteins. 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 Lys450 mutant proteins were examined by western blot and compared with those of wild type GDH.

Purification and Characterization of Mutant Proteins

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 50 mM Tris, pH 8.0/ 2 mM EDTA/ 2 mM BME/ 2 mM PMSF / 10 mM NaCl / 2 % Triton X-100 and lysed with a sonicator. Cellular debris was removed by centrifugation (11,000 g, 10 min, 4 °C) and the crude extract was loaded onto a Q Sepharose column that was equilibrated with 50 mM Tris, pH 8.0/ 1 mM BME/ 0.5 mM EDTA. The column was washed with 50 mM Tris, pH 7.0/ 1 mM BME/ 0.5 mM EDTA/ 0.1 N NaCl until the

breakthrough peak of protein had been eluted. The enzyme was then eluted by a gradient up to 1 N NaCl. The fractions containing GDH were pooled, concentrated, and buffer changed to buffer A (50 mM Tris, pH 8.0/ 1 mM BME/ 0.5 mM EDTA) using Amicon concentrator and then applied to a FPLC Resource-Q anion exchange column equilibrated with buffer A. The enzyme was then eluted using a linear gradient made with buffer in increasing concentration of NaCl (from 0 mM to 500 mM) at 2 ml/min. After the fractions containing GDH were combined, concentrated and buffer changed to buffer applied to a POROS-QM column equilibrated with 20 mM potassium phosphate, pH 8.0/ 1 mM BME/ 0.5 mM EDTA and the enzyme was then eluted using a linear gradient made with buffer (20 mM potassium phosphate, pH 6.0/ 1 mM BME/ 0.5 mM EDTA) in increasing concentration of NaCl (from 0 mM to 300 mM) 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 (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 °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 °C. 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. 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).

Photolabeling of GDHs

Photolabeling of wild type and mutant GDH were performed by the method of Shoemaker and Haley (1993) with a slight modification. For saturation studies, 0.1 mg of human GDHs in 10 mM Tris-acetate, pH 8.0 were separately incubated with various concentrations of [γ-^{32}P]8N_3GTP in Eppendorf tubes for 5 min with a hand-held UV lamp at a distance of 4 cm. For competition studies, 0.1 mg of GDHs were incubated with various concentrations of GTP for 10 min in the same buffer prior to the addition of 60 μM [γ-^{32}P]8N_3GTP and then allowed to incubate with the photoprobe for 5 min as described above. The samples were then irradiated with a handhold 254-nm UV lamp for 90 s twice at 4 °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 then centrifuged at 10,000 g for 15 min at 4 °C. The pellets were washed and resuspended with 10 mM Tris-acetate, pH 8.0. The remaining free photoprobe, if any, was further removed from the protein by exhaustive washing using Centrifree (Amicon) and ^{32}P incorporation into protein was determined by liquid scintillation counting.

Tryptic Digestion and Isolation of The Photolabeled Peptide

One mg of wild type and mutant GDH in 5 mM Tris-acetate, pH 8.0, were separately incubated with 100 μM [γ-^{32}P]8N_3GTP for 2 min at 4 °C. The mixtures were irradiated for 90 s twice. The reaction was quenched by the addition of ice-cold TCA (final 7 %) and kept at 4 °C for 15 min. The protein was precipitated by centrifugation at 10,000 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. GDHs were proteolyzed by the addition of 1.5 mg of trypsin and kept at room temperature for 3 h after which 1.5 mg of trypsin was added again. After 3 more hours at room temperature, 1.5 mg 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 GTP site and so could be protected by GTP from photomodification, GDH was photolyzed in the presence of 300 μM GTP and proteolyzed as described above. The photolabeled GDHs in the presence or absence of 300 μM GTP were buffer-changed with 50 mM potassium phosphate, pH 7.4, using Centricon (Amicon) and applied to a Protein Pak DEAE-5PW (Waters) column on a Waters HPLC system equipped with a diode array spectral detector. The gradient for HPLC was 0-10 min, 0 N NaCl; 10-60 min, 0-0.5 N NaCl; 60-70 min, 0.5 N NaCl at a flow rate of 0.5 mL/min. The absorbance of the fractions was measured at 220 nm and the photoincorporation was determined by liquid scintillation counting.

RESULTS

Construction and Expression of Lys450 Mutant GDHs

The 15-bp *Nhe* I/ *Tth111* I fragment in pHGDH was replaced with five 15-bp synthetic DNA duplexes (Fig. 1) containing a substitution on both DNA strand at position Lys450 (Fig. 2). These substitutions made mutant proteins K450G, K450S, K450E, K450M, and K450Y at position 450, respectively. The five mutants were designed to have different size, hydrophobicity, and ionization of the side chains. Analysis of crude cell extracts by Western blot showed that Lys450 mutant plasmids encoding an amino acid substitution at position 450 directed the synthesis of a 56.5-kDa protein that interacted with monoclonal antibodies raised against GDH at almost identical level for all Lys450 mutants and wild type GDH (Fig. 3). These results indicate that the mutagenesis at Lys450 sites has no effects on expression or stability of the different mutants.

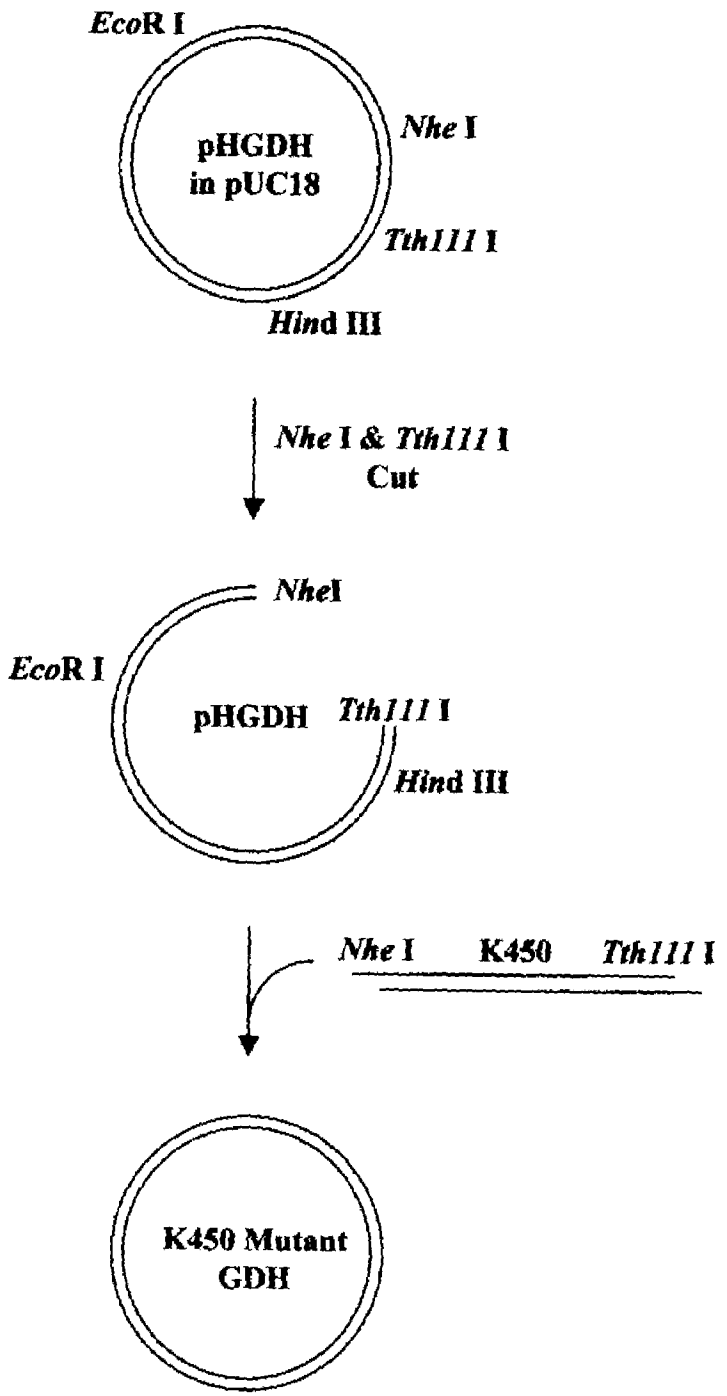

Fig. 1. Cassette mutagenesis of Lys450 mutants.

K450 Mutant GDHs	Oligonucleotide sequences
K450S	5' CT AgC gAA **TCg** gAC C 3' 3' g CTT **AgC** CTg TA 5'
K450G	5' CT AgC gAA **ggC** gAC C 3' 3' g CTT **CCg** CTg TA 5'
K450Y	5' CT AgC gAA **TAT** gAC C 3' 3' g CTT **ATA** CTg TA 5'
K450M	5' CT AgC gAA **ATg** gAC C 3' 3' g CTT **TAC** CTg TA 5'
K450E	5' CT AgC gAA **gAA** gAC C 3' 3' g CTT **CTT** CTg TA 5'

Fig. 2. Oligonucleotides used for Lys450 mutant GDHs

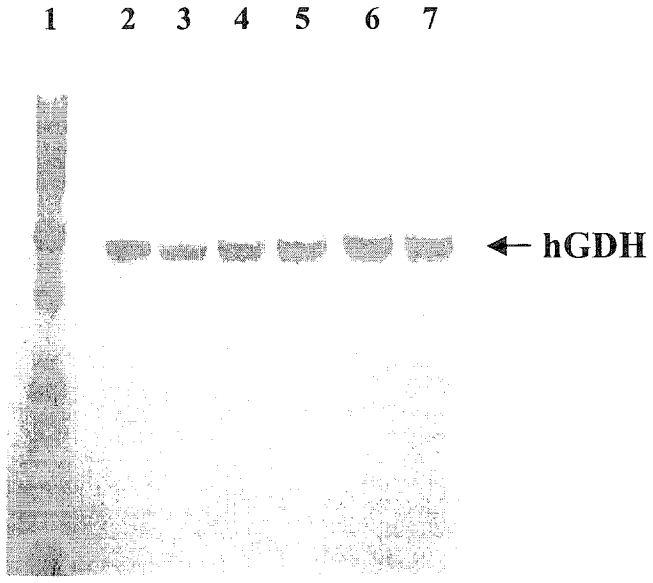

Fig. 3. Western blot analysis of Lys450 mutant GDHs in crude extracts. Land 1, prestained marker proteins (103, 77, 50, 34, 29, 21 kDa); lane 2, wild type GDH; lane 3~7, Lys450 mutants (K450G, K450S, K450E, K450M, and K450Y).

Inactivation of Wild Type and Mutant GDHs by GTP and $8N_3GTP$

The inhibitory effects of GTP on wild type human GDH and Lys450 mutant proteins were examined and summarized in Fig. 4. In enzymatic assays with NADH as a coenzyme, GTP inhibited the wild type GDH with a K_i value of 2.0 μM. There were significant differences between the wild type and Lys450 mutant GDH in their sensitivities to the inhibition by GTP (Fig. 4). While the wild type GDHs were inhibited by GTP with a K_i value of 2.0 μM, none of the Lys450 mutants were inhibited by GTP up to 1 mM and remained a full activity regardless of their size, hydrophobicity, and ionization of the side chains (Fig. 4). The substrate 2-oxoglutarate did not significantly affect the inhibitory effects of GTP on the GDH. To show that $8N_3GTP$ could mimic the inhibitory properties of GTP, the photoanalogue should be able to reversibly inhibit GDH in the absence of activating light. When assayed with NADH as a coenzyme, $8N_3GTP$ was able to inhibit GDH with K_i value of 8.0 μM for the wild type GDH (Fig. 5). Once again, no inhibitions in the reaction of Lys450 mutant GDHs were observed by $8N_3GTP$. These results show that the azidonucleotide, $8N_3GTP$, is able to elicit almost the same biological effects on the

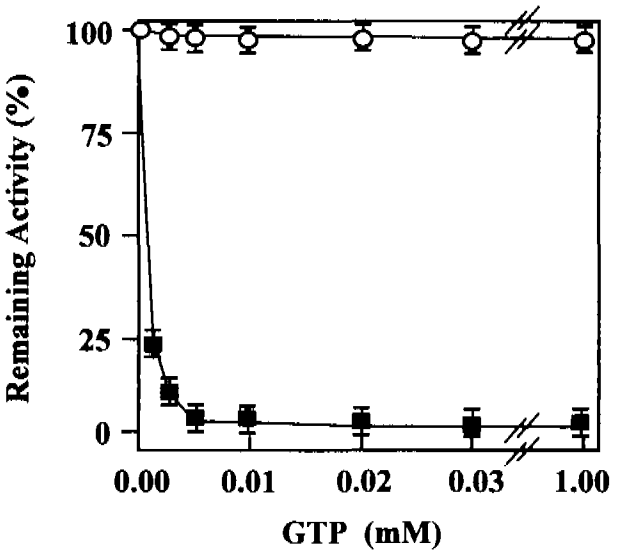

Fig. 4. Inhibition of wild type and mutant GDHs by GTP. The enzymes were preincubated with various concentrations of GTP in the assay buffer and the activities were assayed by the addition of the standard assay mixture with NADH as a coenzyme. Remaining activities are expressed relative to each control. Wild type (closed squares) and K450Y (open circles). Since almost identical results were obtained among the five mutants at position of Lys450, respectively, only the results with K450Y are shown for clarity purpose.

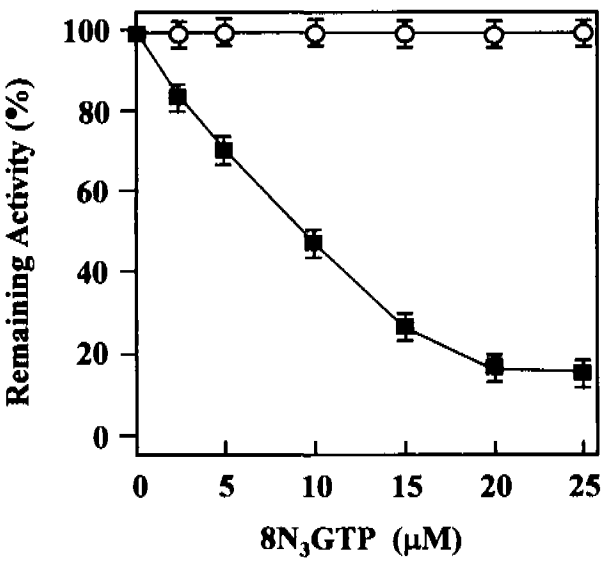

Fig. 5. Inhibition of wild type and mutant GDHs by $8N_3GTP$. The effects of $8N_3GTP$ on wild type and mutant GDHs were examined same as in Fig. 4 except that $8N_3GTP$ was substituted for GTP. Wild type (closed squares) and K450Y (open circles). Since almost identical results were obtained among the five mutants at position of Lys450, respectively, only the results with K450Y are shown for clarity purpose.

wild type and mutant GDHs as the natural nucleotide, GTP. The results of the kinetic parameters are shown (Table 1), and the K_m values and V_{max} values obtained under identical conditions for the five mutants at position of Lys450. Since almost identical results were obtained among the five mutants at position of Lys450, respectively, only one mutant , K450Y, was homogeneously purified for the further studies. The purified K450Y mutant GDH was estimated to be > 98 % pure by SDS/PAGE (Fig. 6).

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 [γ-^{32}P]8N_3GTP occurred at 50 μM photoprobe for the wild type GDH (Fig. 7). The apparent K_d values of this interaction were 15 μM for the wild type GDH. However, no saturation with [γ-^{32}P]8N_3GTP was observed with K450Y mutant GDH (Fig. 7). As shown in the results of the competition experiments (Fig. 8), increasing GTP concentration decreased the photolabeling of 60 μM [γ-^{32}P]8N_3GTP. When 300 μM GTP was present with 60 μM [γ-^{32}P]8N_3GTP,

Table 1. Kinetic parameters of wild type and Lys450 mutant GDHs in crude extracts.

	V_{max}	K_m	
	(μmol.min^{-1}.mg^{-1})	NADH (μM)	2-Oxoglutarate (mM)
Wild type	1.1	81	1.2
K450G	0.9	84	1.3
K450S	1.0	84	1.2
K450E	0.9	86	1.4
K450M	1.0	80	1.4
K450Y	0.8	88	1.7

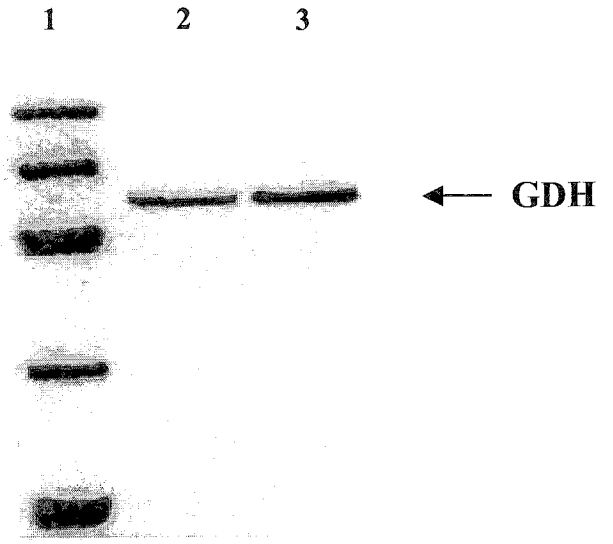

Fig. 6. SDS/PAGE analysis of purified Lys450 mutant GDH. Lane 1, marker proteins (97, 66, 45, 31, 21 kDa); lane 2, purified K450Y mutant GDH; lane 3, purified wild type GDH.

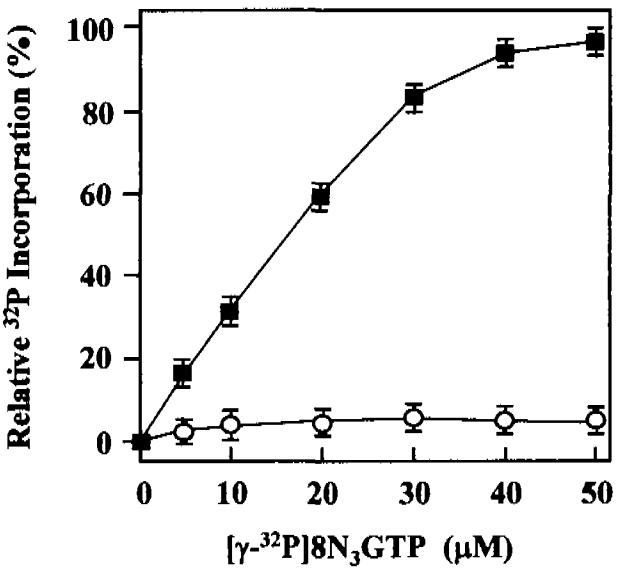

Fig. 7. Saturation of photoinsertion of [γ-^{32}P]8N$_3$GTP into wild type and mutant GDHs. Wild type and mutant GDHs in the reaction buffer were photolyzed with the indicated concentrations of [γ-^{32}P]8N$_3$GTP and ^{32}P incorporation into protein was determined by liquid scintillation counting (see MATERIALS AND METHODS for details). Relative ^{32}P incorporations were expressed relative to each control. Wild type (closed squares) and K450Y (open circles).

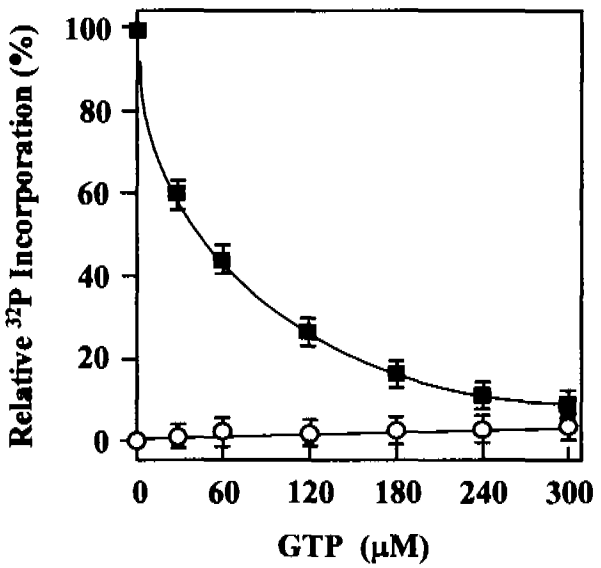

Fig. 8. Effects of GTP on photoinsertion of [γ-^{32}P]8N_3GTP into wild type and mutant GDHs. Wild type and mutant GDHs in the reaction buffer were photolyzed with 60 μM [γ-^{32}P]8N_3GTP in the presence of the indicated concentrations of GTP. Relative ^{32}P incorporation into protein was determined and expressed as described in Fig. 7. Wild type (closed squares) and K450Y (open circles).

90 % of photoinsertion was protected for the wild type GDH. Once again, GTP had no effects on the photoinsertion of [γ-^{32}P]8N_3GTP into the K450Y mutant GDH (Fig. 8). These results show the specificity of 8N_3GTP and the utility of this probe as a good candidate for determining the GTP site.

Tryptic Digestion of Photolabeled Proteins and Isolation of The Photolabeled Peptide

To identify the peptides modified by [γ-^{32}P]8N_3GTP, GDH were photolabeled twice in the absence and presence of 300 μM GTP and digested with trypsin. A higher protein to nucleotide ratio was used to reduce any possible nonspecific labeling. The photolabeled GDHs were separated from most of the noncovalently bound nucleotide by acid precipitation and proteolyzed by trypsin. After overnight trypsin digestion of GDH modified with [γ-^{32}P]8N_3GTP, the digested samples were added to an anion-exchange column (Protein Pak DEAE-5PW, Waters) equilibrated with 50 mM potassium phosphate, pH 7.4. The column was washed by the same buffer and then the peptides were eluted using a linear gradient made with the same

buffer and increasing concentrations of NaCl (from 0.0 to 0.5 N). The results in Fig. 9A show the radioactivity profile of wild type and K450Y mutant modified by 60 μM [γ-^{32}P]8N_3GTP in the absence of GTP. For the wild type GDH, most of the radioactivity was retained on the column and one major radioactive peak around 0.25 N NaCl was recovered from the column. GTP was able to reduce [γ-^{32}P]8N_3GTP photoinsertion into this peak. When 300 μM GTP was originally present in the incubation mixture, more than 90 % of the radioactivity of the peak was eliminated, as shown in Fig. 9B. Unlike the wild type GDH, however, there was no radioactive peak for K450Y mutant GDH in the absence (Fig. 9A) and presence (Fig. 9B) of 300 μM GTP. These results indicate that the radioactive peak represent a guanine binding domain peptide of the GTP binding site of human GDH, and so Lys450 is required for efficient binding of GTP to GDH.

ATP and ADP Effects on Human GDH Activity

Very recently, atomic structure of bovine GDH has suggested that ATP bind to the same site as GTP. If ATP inhibition is due to binding to the GTP site, the mutants

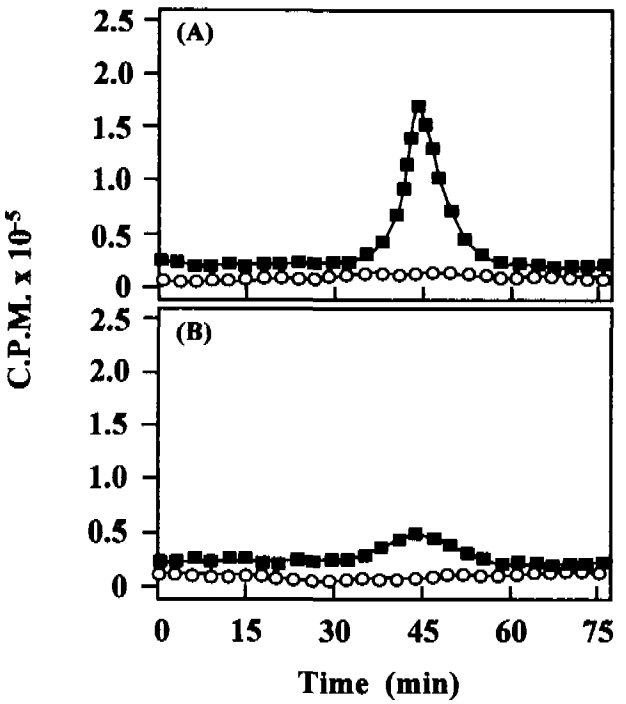

Fig. 9. Anion-exchange HPLC-elution profiles of tryptic peptides photolabeled with [γ-^{32}P]8N_3GTP. The proteins were photolabeled with [γ-^{32}P]8N_3GTP as described in the MATERIALS AND METHODS section in the absence and presence of 300 μM GTP. The tryptic peptides were loaded onto a anion-exchange column (Protein Pak DEAE-5PW, Waters) equilibrated with 50 mM potassium phosphate, pH 7.4 and eluted with an NaCl gradient at a flow rate of 0.5 mL/min (see text for details). One minute fractions were collected. The plot represents the radioactivity profiles from HPLC of the samples photolabeled in the absence (*A*) and presence (*B*) of 300 μM GTP. Wild type (closed squares) and K450Y (open circles).

at the position of GTP binding site should not be inhibited by ATP but still activated by ADP. Studies of the steady-state velocity showed that the wild type GDH was inhibited by ATP at concentrations between 0.01 and 0.1 mM, while none of the Lys450 mutant GDHs were inhibited by ATP up to 0.3 mM (Fig. 10). Unlike the effects of ATP, ADP at concentrations between 0.01 and 1.0 mM activated the reaction of the wild type and Lys450 up to 2.0-fold (Fig. 11). There were no significant differences in their sensitivities to ADP activation between the wild type GDH and the mutant GDHs. These results further support a possibility that Lys450 is also responsible for the ATP inhibition and ATP can inhibit the GDH reaction by binding to the GTP site.

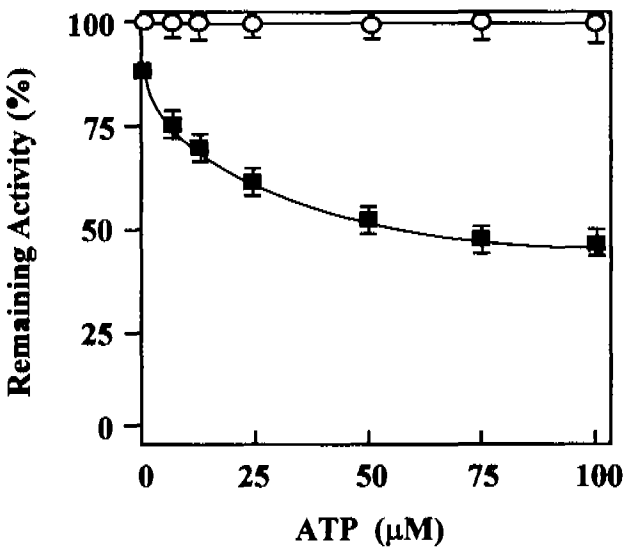

Fig. 10. Effects of ATP on wild type and mutant GDHs. The enzymes were preincubated with various concentrations of ATP or ADP in the assay buffer and the activities were assayed by the addition of the standard assay mixture with NADH as a coenzyme. Remaining activities are expressed relative to each control. Wild type (closed squares), and K450Y (open circles).

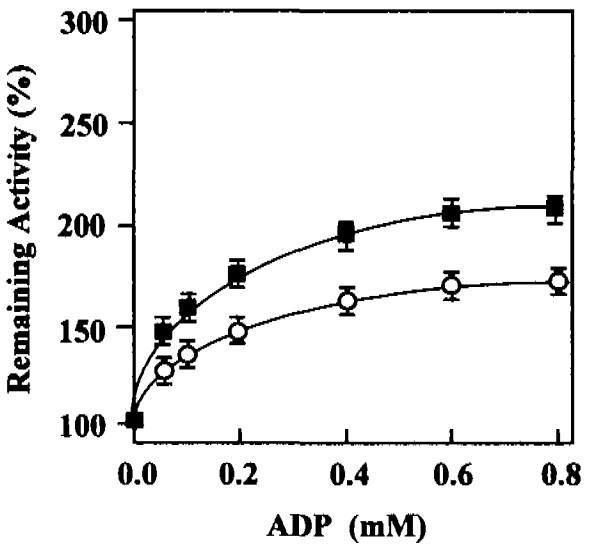

Fig. 11. Effects of ADP on wild type and mutant GDHs. The enzymes were preincubated with various concentrations of ATP or ADP in the assay buffer and the activities were assayed by the addition of the standard assay mixture with NADH as a coenzyme. Remaining activities are expressed relative to each control. Wild type (closed squares), and K450Y (open circles).

DISCUSSION

It has been reported that hyperinsulinism-hyperammonemia syndrome is caused by mutations in GDH gene that affects enzyme sensitivity to GTP-induced inhibition (Stanley et al., 1998). The atomic structure of bovine liver GDH (Peterson and Smith, 1999) has further support that these mutations either have indirect effects on GTP binding or on the allosteric effects caused by GTP. In either case, these results demonstrate the importance of GTP and the regulation of GDH activity in mammalian system. In the present work, we evaluated a role of the Lys450 residue in the binding of GTP to the human GDH using cassette mutagenesis and photoaffinity labeling with [γ-^{32}P]8N_3GTP.

The sequence at the Lys450 position shows a complete identity with that of the other mammalian GDH species known (Cho et al., 1996). These results strongly suggest that the lysine residue plays an important role in the reaction of GDH. In the present work, we evaluated a role of the Lys450 residue in the binding of GTP to the human GDH using cassette mutagenesis, the photoaffinity labeling with [γ-

^{32}P]8N_3GTP, and inhibition studies.

The specificity of 8N_3GTP and the utility of this probe as a good candidate for determining the GTP site were demonstrated by the following. First, in the absence of activating light, wild type GDH is inhibited by 8N_3GTP as well as by GTP and can be photolabeled with the nucleotide analog [γ-^{32}P]8N_3GTP. The K_i value of 8N_3GTP for wild type GDH was around 8 μM (Fig. 5) while this for GTP was 2 μM (Fig. 4). A similar K_i of GTP for bovine liver enzyme has been reported (Shoemaker and Haley, 1993), although these values have varied somewhat (Pal and Colman, 1979; McCarthy and Tipton, 1984). The ability to mimic a native compound before photolysis has an advantage over determination of the enzyme function after modification. Second, the photoinsertion into the wild type GDH was saturated with 50 μM [γ-^{32}P]8N_3GTP. The apparent K_d value of this interaction was 15 μM (Fig. 7). Third, the prevention of photoinsertion of [γ-^{32}P]8N_3GTP by GTP demonstrates that the photoprobe is inserting into a specific GTP site within human GDH (Fig. 8).

Our results are clearly consistent with previous reports that identified the GTP binding site of bovine liver GDH (Shoemaker and Haley, 1993) and bovine brain

GDH isoproteins (Cho et al., 1996) with probable point photomodification at Lys450 using [γ-^{32}P]8N_3GTP. In contrast, the guanine binding site of bovine liver GDH was proposed to be modified by using the classical chemical probe 5'-p-(fluorosulfonyl)benzoyl-1,N6-ethenoadenosine (5'FSBeA) at Tyr262 (Jacobson and Colman, 1982). As indicated from the atomic structure of bovine liver GDH (Peterson and Smith, 1999; Smith et al., 2001), the binding environment for this residue is highly basic. It is not clear why a hydrophobic adenosine-containing probe 5'FSBeA would preferentially bind and react at a hydrophilic GTP binding site and not react at the other adenosine binding sites.

Similar results with quite a discrepancy using classical chemical probes were reported by the same research group to identify other regulatory sites within bovine liver GDH. For instance, the NADH binding site was also proposed to be modified by an ATP analogue at Cys319 (Ozturk et al., 1990; Ozturk and Colman, 1991), by a GMP probe at Met169 and Tyr262 (Ozturk et al., 1992), and by the adenosine analogue at Lys420 and Tyr190 (Pal et al., 1975; Saradambal et al., 1981; Schmidt and Colman, 1984). None of the above sites are consist with the NADH binding site

as presented by atomic structural studies of bovine GDH (Smith et al., 2001). It seems, therefore, that the base moiety has not been effective at directing the site of modification by classical chemical probes. Classical chemical probes may label residues near or outside the binding domain due to their long-lived chemical reactivity. They usually have a much lower affinity and many times require solvents for solubility. Their lack of specificity may be the reason for the wide three-dimensional distribution of the residues identified using classical chemical probes as being in the NADH inhibitory site of GDH (Ozturk et al., 1990, 1992; Ozturk and Colman, 1991).

In contrast, azidopurine photoprobes generate short-lived, very reactive nitrenes which will modify any residue placed near the generated nitrene by binding. Previously, a NAD^+ binding site of bovine liver and brain GDH was identified using [^{32}P]azido-NAD^+ as the peptide containing residues Cys270-Lys289 with photomodification at Glu275 (Kim and Haley, 1991; Cho et al., 1998). This coenzyme binding region is quite consistent with the most recent structure of bovine liver GDH (Kim and Haley, 1991). 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. Saturation of photoinsertion at concentrations corresponding to that expected from the reversible binding affinities also strongly supports the site being labeled within the binding domain. Finally, their selectivity and specificity have been successfully utilized to locate specific binding domains on a variety of proteins (Campbell et al., 1990; Gunnersen and Haley, 1992; Salvucci et al., 1992; Olcott et al., 1994).

It has been reported that bovine liver GDH contains both an NADH-dependent and NADH-independent GTP binding site (Pal and Colman, 1979). However, there are some disagreements on whether there are two GTP sites (Pal and Colman, 1979; Shoemaker and Haley, 1993; Cho et al., 1996; Peterson and Smith, 1999; Smith et al., 2001). We have detected only one GTP binding site which is NADH-independent. NADH and NADPH reduced photolabeling of brain GDH with [γ-^{32}P]8N_3GTP even at oversaturating concentrations. To attempt detection of two different GTP binding sites, saturation studies with [γ-^{32}P]8N_3GTP in the presence of 300 μM NAD(P)H were performed and no results revealed the second GTP binding site on wild type GDH even up to concentrations of 200 μM [γ-^{32}P]8N_3GTP. These results do not show

that there are two GTP binding sites within each subunit of brain GDH (data not shown). The results in Fig. 7 also indicate that under the experimental conditions described only a single K_d is observed. Further more, a perfect protection against to the inhibition by GTP up to 1 mM was observed with Lys450 mutants, whereas the wild type GDH showed a complete lose of enzyme activity by GTP at the concentration of 20 μM GTP (Fig. 4). These results indicate that there is only one GTP binding site and that the second GTP site, if any, is not required for inhibition of human GDH by GTP. The most recent atomic structure of bovine liver GDH (Peterson and Smith, 1999) has also presented that there is no evidence for the second GTP site. The results obtained with our human GDH are consistent with those obtained with bovine liver GDH (Shoemaker and Haley, 1993, Smith et al., 2001) and bovine brain GDH isoproteins (Cho et al., 1996) but contrast to those obtained with the same bovine liver GDH by Pal and Colman (1979). To our knowledge, the guanine binding domain peptide of the second GTP binding site of GDH from any sources has not been identified yet.

ADP and ATP have been shown to have opposite effects on GDH activity. ADP

increases the reductive amination reaction velocity, whereas ATP inhibits the reaction at pH 8.0 and high NADH concentrations (Koberstein and Sund, 1973; Fisher, 1975; Bailey et al., 1982). Since the atomic structure of bovine GDH has suggested that γ-phosphate of GTP dominates the GTP/GDH interactions (Smith et al., 2001), it is possible that ATP can bind to the GTP site. To test this possibility, effects of ATP and ADP on the activities of wild type and Lys450 mutant forms of human GDH. If ATP inhibition is due to binding to the GTP site, the mutants at the position of GTP binding site should not be inhibited by ATP but still activated by ADP. Studies of the steady-state velocity showed that the wild type GDH was inhibited by ATP, whereas none of the Lys450 mutant GDHs were inhibited by ATP (Fig. 10). Unlike the effects of ATP, there were no significant differences in their sensitivities to ADP activation between the wild type GDH and Lys450 mutant GDHs (Fig. 11). These results support a possibility that ATP can inhibit the GDH reaction by binding to the GTP site and Lys450 is responsible for the ATP inhibition.

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.

CONCLUSIONS

1. Construction of Lys450 Mutant GDH

(1) Five mutant GDHs (K450G, K450S, K450E, K450M, and K450Y) at position 450 were designed to have different size, hydrophobicity, and ionization of the side chains and constructed by cassette mutagenesis.

(2) The mutagenesis at Lys450 site had no effects on expression or stability of the different mutants.

2. Photolabeling of GDH with $8N_3GTP$

(1) The specificity of $8N_3GTP$ and the utility of this probe as a good candidate for determining the GTP site were demonstrated by the ability to mimic a native compound before photolysis and the prevention of photoinsertion of [γ-^{32}P]$8N_3GTP$ by GTP.

(2) While wild type GDH was completely inhibited by GTP or $8N_3GTP$, all of the five Lys450 mutant GDHs were not inhibited by GTP or $8N_3GTP$ and remained a full activity regardless of their size, hydrophobicity, and

ionization of the side chains.

(3) Saturation of photoinsertion with [γ-^{32}P]8N_3GTP occurred for wild type GDH and the photoinsertion of [γ-^{32}P]8N_3GTP was significantly decreased in the presence of GTP. Unlike wild type GDH, no photoinsertion was detected in Lys450 mutant protein.

(4) The results with cassette mutagenesis and photoaffinity labeling demonstrate a selectivity of the photoprobe for the GTP binding site and suggest that Lys450, is required for efficient binding of GTP to GDH.

3. Effects of ATP and ADP on Lys450 Mutant GDH

(1) The wild type GDH was inhibited by ATP at concentrations, while none of the Lys450 mutant GDHs were inhibited by ATP.

(2) Unlike the effects of ATP, there were no significant differences in their sensitivities to ADP activation between the wild type GDH and Lys450 mutant GDHs.

(3) These results indicate that Lys450, may be also responsible for the ATP

inhibition and so ATP was binding to the GTP site.

4. Usage of The Synthetic Gene

(1) The construction of a synthetic gene encoding human GDH enabled us to generate a large number of site-directed mutations at any positions in the coding region.

(2) The high level of GDH expression from plasmids that carry the gene facilitated the purification of large quantities of mutant proteins for biochemical and structural studies.

(3) This combination of genetic and biochemical techniques is useful to address a broad range of questions relating to the structure and function of human GDH.

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국 문 요 약

유전자 돌연변이를 이용한 인간 glutamate dehydrogenase의 GTP 결합 부위 규명

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최근 GTP에 의한 억제 효과를 나타내는 glutamate dehydrogenase (GDH) gene에 돌연변이가 발생하는 경우 hyperinsulinism-hyperammonemia 증후군이 유발된다는 보고가 있다. 본 연구에서는 Lys450 부위의 mutant 형태의 GDH를 cassette mutagenesis에 의해 구성하여 인간 GDH 내의 GTP 결합 부위를 규명하였다. Wild 형태 GDH는 20 μM의 GTP 농도에서 완전히 억제되었으나, 5가지 Lys450 부위의 mutant 형태의 GDH는 1 mM 이상의 GTP 농도에서도 억제되지 않고 side chain의 size, hydrophobicity, ionization와 상관없이 충분한 활성도가

남아 있었다. Wild 형태와 mutant 형태에 대한 GTP의 결합은 [γ-^{32}P]8-azidoguanosine 5'-triphosphate ($8N_3GTP$)로 photoaffinity labeling함에 의해 조사하였다. Wild 형태의 경우 $8N_3GTP$의 photoinsertion의 saturation이 대략 15 μM의 K_d 값에서 발생하였으나 GTP 존재 시에는 $8N_3GTP$의 photoinsertion 정도가 상당히 감소하였다. Wild 형태와는 달리 Lys450 mutant 형태는 photoinsertion되지 않았다. cassette mutagenesis와 photoaffinity labeling에 의한 이러한 결과들은 GDH에 있어 GTP의 효과적인 결합에 요구되는 GTP 결합 부위인 Lys450 부위에 대한 photoprobe의 선택성을 나타낸다. 또한, wild 형태의 GDH가 ATP 10과 100 μM 사이의 농도에서 억제되나 Lys450 mutant 형태들은 300 μM 이상의 농도에서도 억제되지 않는 결과를 보이는 steady-state velocity에 대한 연구들은 흥미롭다. 이러한 결과들은 ATP에 의해 억제효과가 Lys450 부위와 관련이 있으며 ATP 가 GTP 결합 부위에 결합한다는 것을 알 수 있었다.

핵심 되는 말 : 인간 glutamate dehydrogenase; GTP 결합 부위; Cassette mutagenesis; hyperinsulinism-hyperammonemia 증후군; Synthetic gene; Photoaffinity labeling