Importance of Gly13 for the Coenzyme Binding of Human UDP-Glucose Dehydrogenase*

Jae-Wan Huh, Hye-Young Yoon¶, Hyun-Ju Lee, Won-Beom Choi, Seung-Ju Yang, and Sung-Woo Cho§

From the Department of Biochemistry and Molecular Biology, University of Ulsan College of Medicine, Seoul 138-736, Korea

Running title: NAD⁺-Binding Site of Human UDP-Glucose Dehydrogenase

* This work was supported by a grant of the Korea Health 21 R&D Project from the Ministry of Health and Welfare (03-PJ1-PG3-20900-0047) to S.-W. Cho.

§ To whom correspondence should be addressed: Department of Biochemistry and Molecular Biology, University of Ulsan College of Medicine, 388-1 Poongnap-2Dong, Songpa-Ku, Seoul 138-736, Korea. (FAX) 82-2-3010-4278; (E-mail) swcho@amc.seoul.kr

¶ Present address: Laboratory of Cellular Oncology, Center for Cancer Research, National Cancer Institute, Building 37 Room 4118, Bethesda, MD20892, USA

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession number: AY212254).

The abbreviations used are: UGDH, UDP-glucose dehydrogenase; 2N₃NAD⁺, nicotinamide 2-azidoadenosine dinucleotide; HPLC, high performance liquid chromatography; GAG, glycosaminoglycan
SUMMARY

UDP-glucose dehydrogenase (UGDH) is the unique pathway enzyme furnishing in vertebrates UDP-glucuronate for numerous transferases. In this report, we have identified an NAD$^+$ binding site within human UGDH by photoaffinity labeling with a specific probe, $[^{32}\text{P}]$nicotinamide 2-azidoadenosine dinucleotide (2$\text{N}_3\text{NAD}^+$) and cassette mutagenesis. For this work, we have chemically synthesized a 1509-base-pair gene encoding human UGDH and expressed it in *Escherichia coli* as a soluble protein. Photolabel-containing peptides were generated by photolysis followed by tryptic digestion and isolated using the phosphopeptide isolation kit. Photolabeling of these peptides was effectively prevented by the presence of NAD$^+$ during photolysis, demonstrating a selectivity of the photoprobe for the NAD$^+$ binding site. Amino acid sequencing and compositional analysis identified the NAD$^+$ binding site of UGDH as the region containing the sequence, ICCIGAYVGGPT, corresponding to I7 through T19 of the amino acid sequence of human UGDH. The unidentified residue, X, can be designated as a photolabeled G13 since the sequences including the glycine residue in question have a complete identity with those of other UGDH species known. The importance of G13 residue in the binding of NAD$^+$ was further examined with a G13E mutant by cassette mutagenesis. The mutagenesis at G13 has no effects on expression or stability of the mutant. Enzyme activity of the G13E point mutant was not measurable under normal assay conditions, suggesting an important role for G13 residue. No incorporation of $[^{32}\text{P}]2\text{N}_3\text{NAD}^+$ was observed for G13E mutant. These results indicate that G13 plays an important role for efficient binding of NAD$^+$ to human UGDH.

**Key words:** UDP-glucose dehydrogenase; NAD$^+$ binding site; Photoaffinity labeling; Reactive Gly; Synthetic gene; Cassette mutagenesis
INTRODUCTION

UDP-glucose dehydrogenase (UGDH, EC 1.1.1.22) is a member of a small group of NAD⁺-linked, 4-electron-transferring oxidoreductases (1), and converts UDP-glucose to UDP-glucuronate. Kinetic studies of UGDH from bovine (2, 3), Streptococcal (4, 5), and plant (6, 7) show that the reaction of UGDH involves two successive oxidations to convert the 6’ hydroxyl of UDP-glucose to a carboxylate, together with reduction of 2 moles of NAD⁺ to NADH. In animals, it constitutes the unique pathway for glucuronate formation (8). Because glucuronate is a component of glycosaminoglycans (GAGs), the mutation inactivation of UGDH (sugarless) abolishes GAG assembly and, consequently, abolishes GAG-dependent growth factor signaling (9-11). The primary structure of the mammalian enzyme was obtained from the protein sequence of bovine UGDH (12). The human gene was also recently cloned and assigned to chromosome 4p15.1 (13). It contains 12 exons, extends over 26 kb, and has one major transcription start site (14).

GAG chains of proteoglycans and hyaluronan are ubiquitous components of extracellular matrix and pericellular spaces. There is a growing body of information on the implication of GAGs in cell behavior, including signal transduction, cell proliferation, spreading, migration, and cancer growth and metastasis (15-17). GAG synthesis is influenced by cytokines and growth factors. Transforming growth factor-β is the most potent stimulator of proteoglycan and GAG synthesis, including that of hyaluronan. Its action, however, depends on the cell type (18). The synthesis of GAGs is also modulated by oxygen cell status. Hypoxic endothelial cells and lung fibroblasts enhanced heparan sulfate/chondroitin sulfate ratio, which led to an increase of basic fibroblast growth factor reactivity on the cell surface (19, 20). It was also shown that the level of intracellular UDP-glucuronate could influence GAG synthesis (8). Interestingly, the human UGDH was shown to be an early response gene after interleukin-1 treatment of ocular fibroblasts (21), as well as an early androgen response gene in breast cancer (22).

Although a clone of human UGDH has been reported (13, 15), specific catalytic residues are not yet available. Therefore, further characterization of the active sites of human UGDH is needed to elucidate the physiological nature of the UGDH. In the
present study, we have identified an NAD$^+$ binding site using photoaffinity labeling and cassette mutagenesis to gain a deeper insight into the structural basis of human UGDH. For this study, a 1509-base-pair gene that encodes human UGDH has been chemically synthesized and expressed in *E. coli* as a soluble protein. Identification of the nucleotide binding sites of a variety of proteins has been advanced by the use of nucleotide photoaffinity analogues that selectively insert into a site upon photoactivation with ultraviolet light. For instance, $[^{32}\text{P}]2\text{N}_3\text{NAD}^+$ was shown to be a valid active-site probe for several proteins (23-26). GTP and ADP binding sites of the bovine glutamate dehydrogenase have been reported using $[^{32}\text{P}]8\text{N}_3\text{GTP}$ and $[^{32}\text{P}]8\text{N}_3\text{ADP}$, respectively (27-30). The ATP binding site of adenylylate kinase and creatine kinase and the protein unique to cerebrospinal fluids of Alzheimer's patients successfully also has been identified using $2\text{N}_3\text{ATP}$ and $8\text{N}_3\text{ATP}$ (31, 32).

Our results indicate that G13 plays an important role for efficient binding of NAD$^+$ to human UGDH. To our knowledge, this is the first report identifying reactive residue critically involved in the coenzyme binding of the human UGDH.

**EXPERIMENTAL PROCEDURES**

*Materials*—UDP-glucose, NAD$^+$, ampicillin, isopropyl thio-β-D-galactoside, phenylmethanesulfonyl fluoride, and L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin were purchased from Sigma Chemical Co. $2\text{N}_3\text{NAD}^+$ and $[^{32}\text{P}]2\text{N}_3\text{NAD}^+$ were synthesized using NMN adenylyltransferase according to the same method as previously described (26). Monoclonal antibody against human UGDH was kindly provided by Dr. S. Choi (Hallym University, Chunchon, Korea). Pre-cast gels for SDS-polyacrylamide gel electrophoresis were purchased from NOVEX. All other chemicals and solvents were of reagent grade or better.

*Bacterial Strains*—*E. coli* DH5α (33) was used as the host strain for plasmid-mediated transformations during the assembly of the synthetic human UGDH gene (pHUGDH) and for cassette mutagenesis. *E. coli* BL21 (DE3) (34) was used for high level expression of the recombinant hUGDH.
Design and Assembly of the Synthetic hUGDH Gene—The design of the synthetic hUGDH gene was based on the amino-acid sequence of hUGDH (15) and used the following strategy. First, a DNA sequence containing 37 restriction sites located approximately every 42 bp throughout the entire length of the coding region was selected from the large number of possibilities. Only those sites 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 hUGDH gene is flanked by unique EcoRI and HindIII restriction site that render the gene portable to various E. coli expression vectors. Second, the codon usage of the resulting hUGDH gene was modified to include those triplets that are utilized in highly expressed E. coli genes (35, 36) while retaining the largest possible number of unique restriction sites. In some cases, suboptimal codons were used either to allow the inclusion of unique restriction sites or to preclude redundant sites. Third, a ribosome binding site AGGAGG (37) 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 (37). Addition of a ribosome binding site made the synthetic hUGDH gene portable to any of a number of commonly available plasmid vectors that carry inducible E. coli promoters. The hUGDH gene was assembled from seven gene segments that were initially cloned into pUC18. Each of the gene segments was constructed with two to four oligonucleotides. Six to seven isolates of each segment were examined by restriction analysis and DNA sequencing. The final synthetic hUGDH gene, designated as pHUGDH, was used for gene expression and mutagenesis studies.

Protein Purification and Characterization—Fresh overnight cultures of DE3/pHUGDH were used to inoculate 1 L of Luria-Bertani (LB) containing 100 µg of ampicillin per mL. DE3/pHUGDH was grown at 37°C until the $A_{600}$ reached 1.0, and then isopropyl thio-β-D-galactoside was added to a final concentration of 1 mM. After isopropyl thio-β-D-galactoside induction, DE3/pHUGDH was grown for an additional 3 h at 37°C and then harvested by centrifugation. Cell pellets were suspended in 100 mL
of 100 mM Tris-HCl, pH 7.4/1 mM EDTA/5 mM dithiothreitol and lysed with a sonicator. Cellular debris was removed by centrifugation and the crude extract was loaded onto a blue Sepharose CL-6B column that was equilibrated with buffer A (20 mM Tris/HCl, pH 6.5/5 mM MgCl₂/10 mM β-mercaptoethanol). The column was washed with buffer A until the breakthrough peak of protein had been eluted. The enzyme was then eluted by a gradient up to 500 mM NaCl. The fractions containing hUGDH were pooled, concentrated, and the buffer changed to buffer B (50 mM Tris/HCl, pH 8.0/0.5 mM EDTA/10 mM β-mercaptoethanol) using Amicon concentrator and then applied to a FPLC Resource-Q 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 300 mM) at 3 ml per min. The fractions containing hUGDH were pooled and concentrated. The purified enzymes were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and recognized by western blot using monoclonal antibodies against the hUGDH. Protein concentration was determined by the method of Bradford (38) using bovine serum albumin as a standard.

Enzyme Assay and Kinetic Studies—hUGDH activity was measured with NAD⁺ in 100 mM sodium glycine, pH 8.7 and 1 mM UDP-glucose at 25°C. The reaction started by the addition of NAD⁺ to 1 mM final concentration. One unit of enzyme was defined as the amount of enzyme required to reduce 2 µmol of NAD⁺ per min at 25°C. For determination of \(K_m\) and \(V_{max}\) values, the assays were carried out 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 fitting the data to the Michaelis-Menten equation and assuming a single binding site each for substrate and cofactor. Catalytic efficiency was estimated by use of the equation \(v/[E_0] = (k_{cat}/K_m)[S]\) (39).

Photolabeling of hUGDH with \(^{32}\text{P}\)2N₃NAD⁺—Photolabeling of the wild-type hUGDH with \(^{32}\text{P}\)2N₃NAD⁺ was performed by the method of Yoon et al. (26) with a slight modification. For saturation studies, the wild-type hUGDH (100 µg) in 10 mM Tris-acetate, pH 8.0 was incubated with various concentrations of \(^{32}\text{P}\)2N₃NAD⁺ in Eppendorf tubes for 5 min. For competition studies, 100 µg of enzyme was incubated with various concentrations of NAD⁺ for 10 min in the same buffer prior to the addition
of 50 μM $[^{32}\text{P}]2\text{N}_{3}\text{NAD}^+$ and then allowed to incubate with the photoprobe for 5 min as described above. The samples were irradiated with a handheld 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 the ice bath for 30 min and centrifuged at 10000g 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 $^{32}\text{P}$ incorporation into protein was determined by liquid scintillation counting.

*Tryptic Digestion and Isolation of Photolabeled Peptide*—To determine the site modified by $[^{32}\text{P}]2\text{N}_{3}\text{NAD}^+$, 1.0 mg of the wild-type hUGDH in 10 mM Tris-acetate, pH 8.0 was incubated with 200 μM $[^{32}\text{P}]2\text{N}_{3}\text{NAD}^+$ for 5 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 30 min. The protein was precipitated by centrifugation at 10000g for 15 min at 4°C, and the pellet was resuspended in 75 mM NH$_4$HCO$_3$, pH 8.5, containing 2 M urea. The enzymes were proteolyzed by the addition of 15 μg of trypsin and kept at room temperature for 3 h, after which time 15 μg of trypsin was added again. After an additional 3 h at room temperature, 20 μg of trypsin was added, and the digestion mixture was kept at 25°C overnight. To validate that the isolated peptide was specific for the NAD$^+$ binding site and could be protected from photomodification, the hUGDH proteins were also photolyzed in the presence of 300 μM NAD$^+$ and proteolyzed as described above. The tryptic-digested peptides were separated by the 'Phosphopeptide Isolation Kit' from Pierce Biotechnology (Rockford, IL, USA) following the manufacturer's protocol and sequenced by the Edman degradation method as described before (26).

*Construction and Purification of G13E Mutant*— Single amino-acid substitution of G13 was constructed by cassette mutagenesis of plasmid pHUGDH. Plasmid DNA (5 μg) was digested with BstBI and SalI to remove 98-bp fragment that encodes amino acids 3-35 and vector DNA was purified by electrophoresis using 1% low melting point agarose. The 98-bp BstBI/SalI fragment was replaced with 98-bp synthetic DNA duplex containing a substitution on both DNA strand at positions encoding G13 to make G13E mutant protein. The mutagenic oligonucleotide was annealed, ligated, and transformed
into DH5α as described above and G13E mutant was identified by DNA sequencing using plasmid DNA as a template. G13E mutant was expressed in *E. coli* strain DE3 and purified to homogeneity as described above.

**RESULTS**

*Construction of Synthetic hUGDH Gene and Expression*—The gene was initially constructed as three segments using the plasmid pUC18 as a cloning vector (Fig. 1). A total of 20 synthetic oligonucleotides that varied from 56 to 92 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 486-bp *Eco*RI/*Pst*I fragment composed of six oligonucleotides, a 580-bp *Pst*I/*Age*I fragment composed of seven oligonucleotides, and a 443-bp *Age*I/*Hind*III fragment composed of seven oligonucleotides (Fig. 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 37 restriction sites in the hUGDH coding region of pHUGDH are shown in Fig. 2. High level expression of the synthetic hUGDH gene was achieved by transformation of pHUGDH into *E. coli* strain DE3. Upon induction with 1 mM of IPTG at 37°C for 3 h, expression of hUGDH in soluble extracts was about 0.163 units/mg. SDS/PAGE analysis of crude cell extracts and measurement of specific activities indicated that pHUGDH directed hUGDH expression to a level of ~ 16% of total cellular protein upon induction with IPTG (Fig. 3).

*Purification and Characterization of Synthetic hUGDH*—hUGDH encoded by pHUGDH in DE3 was purified by several chromatographic methods. Since the recombinant hUGDH was readily solubilized, no detergents were required throughout the entire purification steps. The purified hUGDH was estimated to be >98% pure by SDS/PAGE (Fig. 3). No additional amino acids such as N-terminal histidine tags or
protease recognition sites were introduced in the synthetic hUGDH gene product (Fig. 2). N-terminal sequence analysis of the first 7 amino acids (MFEIKKI) is in agreement with published sequence of human UGDH (15). The subunit size (57 kDa) and the native size (340 kDa) of the hexameric recombinant hUGDH were determined by SDS/PAGE (Fig. 3) and HPLC gel filtration chromatography (data not shown), respectively. The 57-kDa protein was absent in extracts from DE3 transformed with pUC18 (Fig. 3). The highly purified enzyme was a mixture of hexamer, tetramer, and dimer as determined by HPLC gel filtration. The hUGDH was predominantly observed as a hexamer together with significant tetrameric and dimeric species, indicating some subunit dissociation. Although relative species ratios were conserved over several different preparations of the enzyme, the ratio of the oligomeric state was changed by storage conditions and periods.

UGDH catalyzes conversion of a UDP-glucose substrate to a UDP-glucuronic acid product, concomitantly reducing two molecules of NAD$^+$ to NADH. The enzymatic activity of hUGDH was characterized by steady state kinetic analysis. Measurements to determine dependence of the reaction on cofactor concentration were done using purified hUGDH incubated with increasing concentrations of NAD$^+$ in the presence of saturating UDP-glucose substrate. Similarly, dependence of reaction kinetics on substrate was measured by increasing UDP-glucose concentration in the presence of saturating NAD$^+$. Saturation kinetic data were observed for both conditions. Data were fitted to the Michaelis-Menten equation to obtain $K_m$ and $V_{\text{max}}$ for the reaction catalyzed by the wild-type enzyme. Both sets of conditions yielded a similar $V_{\text{max}}$ of 157 ± 5 nmol of NAD$^+$/min/mg of enzyme (Fig. 4A & 4B). The $K_m$ for UDP-glucose was 17 ± 3 µM (Fig. 4A) and the $K_m$ for NAD$^+$ was 133 ± 4 µM (Fig. 4B). For more detailed catalytic properties of hUGDH, the enzyme efficiency ($k_{\text{cat}}/K_m$) for the individual substrates were determined (Table I). The $k_{\text{cat}}$ value of hUGDH was 105 ± 4 s$^{-1}$ and the $k_{\text{cat}}/K_m$ values for NAD$^+$ and UDP-glucose (UDP-Glc) were 7.9 ± 1.0 s$^{-1}$·µM$^{-1}$ and 6.2 ± 1.3 s$^{-1}$·µM$^{-1}$, respectively (Table I).

Specific Binding of 2N3NAD$^+$ to hUGDH—The reactive amino acid in the direct binding of NAD$^+$ to hUGDH was identified by photoaffinity labeling with [$^{32}$P]nicotinamide 2-azidoadenosine dinucleotide (2N3NAD$^+$). To show specificity of
the photoprobe-protein interaction, saturation of photoinsertion should be observed. For demonstrating saturation effects with the photoprobe, the purified enzymes were photolabeled with increasing concentrations of \([^{32}P]2N_3NAD^+\). Under the experimental conditions described, saturation of photoinsertion with \([^{32}P]2N_3NAD^+\) occurred at around 120 µM photoprobe (Fig. 5). 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 (26). The data in Fig. 5 demonstrate the apparent \(K_d\) value of approximately 48 µM, which is in reasonably good agreement with recently reported \(K_d\) value (118 µM) of NAD\(^+\) with hUGDH (40). The stoichiometry of \([^{32}P]2N_3NAD^+\) incorporation was estimated to be 3 moles of the label per hexameric enzyme. The results in Fig. 5 indicate the saturability of NAD\(^+\) specific site of hUGDH with this photoprobe and therefore decrease the possibility of nonspecific photoinsertion. To further demonstrate specific labeling of hUGDH, the wild-type hUGDH was photolabeled with \([^{32}P]2N_3NAD^+\) in the presence of increasing NAD\(^+\) concentrations. As shown in the results of the competition experiments (Fig. 6), NAD\(^+\) at concentrations in the range of \(K_m\) values was able to protect photolabeling from \([^{32}P]2N_3NAD^+\). Approximately, 95% protection was observed with 160 µM NAD\(^+\) for the wild-type hUGDH (Fig. 6). These results show the specificity and utility of \([^{32}P]2N_3NAD^+\) as a good probe for determining the NAD\(^+\) binding site.

Tryptic Digestion of Photolabeled Proteins—To identify the peptides modified by \([^{32}P]2N_3NAD^+\), the wild-type hUGDH was photolabeled twice in the absence and presence of 300 µM NAD\(^+\) and digested with trypsin. To reduce any possible nonspecific labeling and at the same time to optimize the specific labeling of the enzymes, 100 µM \([^{32}P]2N_3NAD^+\) was used, which is the concentration at which photoinsertion approaches saturation. The photolabeled proteins were separated from most of the noncovalently bound nucleotide by acid precipitation and proteolyzed by trypsin. The tryptic-digested photolabeled peptides were isolated by the 'Phosphopeptide Isolation Kit' from Pierce Biotechnology (Rockford, IL, USA). The radioactive peptides were eluted with 100 mM ammonium bicarbonate, pH 9 containing 0.3 M ammonium hydroxide and 20% acetonitrile. Photolabeling of the peptide was
prevented by the presence of NAD$^+$ during photolysis. When 300 µM NAD$^+$ was originally present in the incubation mixture, ~ 95% of the radioactivity of the eluent was eliminated. These results demonstrate a selectivity of the photoprobe and suggest that the radioactive fractions represent a peptide in the NAD$^+$ binding domain of the hUGDH. The radioactive fractions were collected and identified by amino acid sequence analysis.

Sequence Analysis of Photolabeled Peptide—The amino acid sequence analysis revealed that the radioactive eluent contained the amino acid sequence ICCIGAXYVGGPT. As judged by comparison with the amino acid sequence of mammalian UGDHs (Table II), this site was identified as residues 7-19 of hUGDH. 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 glycine because the sequences including the glycine residue in question have a complete identity with those of the other UGDH species known. Because the photolabeled peptides are tryptic digests, it was expected to produce a sequence ending with Arg or Lys. The amino acid composition of the photolabeled peptide revealed that the peptide had a composition that was compatible with that of the tryptic peptide spanning residues 7-31 with an exception that there was a significant reduction in glycine (Table III). On the basis of information obtained on the amino acid sequence determination and composition analysis of the isolated peptide, we suggest that the attachment site of $[^{32}P]2N_3NAD^+$ is G13. The importance of G13 residue in the binding of NAD$^+$ was further examined by substituting Gly into Glu, Leu, Arg, and Tyr at position 13. Since all four mutants (G13E, G13L, G13R, and G13Y) at G13 site were studied to the same extent as G13E and showed almost identical results in protein expression, purification, kinetic parameters, and photoaffinity labeling study, only the results from G13E are presented for clarity purpose.

Construction, Expression, and Properties of G13E Mutant—The 98-bp BstBI/SalI fragment in pHUGDH was replaced with 98-bp synthetic DNA duplexes containing a substitution on both DNA strands at positions encoding G13. This substitution made mutant protein G13E. Analysis of crude cell extracts by western blot showed that G13E mutant plasmid directed the synthesis of a protein that interacted with monoclonal
antibodies raised against hUGDH at almost identical levels for wild-type hUGDH (Fig. 7). These results indicate that the mutagenesis at G13 has no effects on expression or stability of the mutant. In addition, the mutant enzymes were purified homogeneously by the same method as was the wild-type enzyme, indicating that no gross conformational change of the enzyme had occurred.

Catalytic activity of hUGDH point mutant G13E was not measurable under standard assay conditions. To evaluate the role of G13 residue in catalysis and determine the extent of impaired catalytic function, we performed a time course over 3 h, measuring enzyme activity every 10 minutes by absorbance wavelength scans from 290-400 nm. Less than 0.05% activity relative to wild-type enzyme was observed for G13E mutant was found up to 3 h, demonstrating that loss of enzymatic activity was the result of the mutagenesis at G13 site. In addition, no photoaffinity labeling of \([^{32}P]2N_3\text{NAD}^+\) was measured for G13E mutant in the presence and absence of NAD\(^+\) under the same experimental conditions for the wild-type enzyme (Fig. 5 & Fig. 6). These results are consistent with the postulated role of G13 in NAD\(^+\)-binding and support its position in the reaction mechanism for the human enzyme.

**DISCUSSION**

Major functional significance of UGDH in animals is developmental, and its regulation may be related to several pathologies such as cancer progression (22). It would be necessary having a detailed understanding of its structure and mechanism to design specific targeted inhibitors for a therapeutic avenue. The majority of previous studies on UGDH have focused on the bovine liver enzyme, and until recently very little was known about the human enzyme. The structure of the enzyme is well conserved between the species and phyla. The cloned mammalian proteins from different species showed overall 97% identity (21, 41). The human enzyme cDNA codes for 494 amino acids and has 27% homology to the *E. coli* ortholog. The conserved amino acids are uniformly distributed along the molecule. Interestingly, the upstream NAD\(^+\)-binding region (GXGXXG) is 100% identical in mammals and *E. coli*
enzymes (42, 43). The term fold was introduced by Rossmann (44-47) to describe a nucleotide-binding domain found in families of oxidoreductases such as lactate dehydrogenase and flavodoxin. This fold begins with a β-strand connected by a short loop to an α-helix (45) and contain a conserved sequence motif, GX_{1-2}GXXG (48), where the glycine residues are located on the ligand-binding loop in between the β-strand and α-helix, although not all Rossmann folds bind to the nucleotides FAD or NAD(P).

The importance of the glycine residues has been previously explained (49): the first glycine allows a tight turn of the main-chain from the β-strand into the loop, and the second glycine permits close contact of the main-chain to the pyrophosphate of the nucleotide. The third glycine allows close packing of the helix with the β-strand. Mutations in the conserved glycine residues of the loop have been correlated with attenuation or elimination of enzyme activity (50-52) and also with disease (53). The conserved structure suggests that UGDH may take part of a minimal genome for multicellular species and the rigid basal structure is necessary for its activity (14, 42). To our knowledge, the crystallographic structure of hUGDH has not been determined and no studies by site-directed mutagenesis for the coenzyme binding site(s) of the mammalian UGDHs have been reported yet. In the present study, hUGDH was chemically synthesized, expressed, and purified to homogeneity. Specific point mutant was generated based on homology modeling of the enzyme and used to identify the coenzyme binding site of hUGDH.

We identified an NAD\(^+\) binding site of hUGDH using photoaffinity probe, \([^{32}\text{P}]2N_3\text{NAD}^+\). \([^{32}\text{P}]2N_3\text{NAD}^+\) 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\(^+\) (Fig. 6) demonstrates that photoinsertion occurs only by the bound form of \([^{32}\text{P}]2N_3\text{NAD}^+\). This indicates that proximity controls photoinsertion and that the residues modified are within the NAD\(^+\) binding domain. In addition, pre-photolysis followed by immediate addition of hUGDH did not lead to covalent labeling (data not shown), eliminating the existence of any long lived chemically reactive intermediate which could be involved in covalently modifying enzymes. The selectivity and specificity has been successfully utilized to locate the
specific base binding domains of nucleotide binding site peptides of many proteins (23-32).

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 with similar properties as NAD$^+$ for hUGDH (data not shown). The ability to mimic a native compound before photolysis has an advantage over determination of the enzyme function after modification. Second, the photoinsertion into hUGDH shows saturation with \([^{32}P]2N_3NAD^+\). Saturation of photoinsertion with \([^{32}P]2N_3NAD^+\) occurred at around 120 µM photoprobe (Fig. 5). In addition, the sites of attachment of the photoaffinity label were more precisely defined by generating small peptide fragments of the labeled protein, and separating the labeled peptides efficiently by one-step affinity chromatography. Third, the photolabeled peptide of the hUGDH identified is located within the proposed NAD$^+$ binding domain of many proteins (42-48), confirming that this sequence is expected to interact with NAD$^+$.

It has been reported that the two substrates bind in equimolar amounts rather than in a two to one ratio in favor of NAD$^+$ over UDP-glucose in view of the sequential mechanism of the two stage oxidation (40, 54, 55). Interestingly, our data show that only three moles of \([^{32}P]2N_3NAD^+\) are incorporated per hexameric hUGDH. From what is known for the subunit nature of hUGDH, it was suspected that six NAD$^+$-binding sites would exist in the native hexameric enzyme. Therefore, these results suggest that the binding sites are located in the interfaces between pairs of subunits and support that the hUGDH quarternary structure adopts a trimer of dimers arrangement (40, 55).

There were differences in the biochemical properties between wild-type hUGDH and G13E mutant. Catalytic activity of hUGDH point mutant G13E was not measurable under normal assay conditions up to 3 h, measuring enzyme activity every 10 minutes. This result suggests a possibility that loss of enzymatic activity is due to a change in binding of NAD$^+$ by the G13E mutant. In addition, no detectable amounts of photoaffinity labeling were observed for G13E mutant when treated with \([^{32}P]2N_3NAD^+\) both in the presence and absence of NAD$^+$ under the same experimental conditions for the wild-type enzyme (Fig. 5 & Fig. 6) and therefore no photolabeled
peptides were detected for G13E mutant (Table II). The results from the western blot analysis show that the mutagenesis at G13 site has no effects on expression or stability of the mutant (Fig. 4). The results of HPLC gel filtration analysis also showed no differences in their elution profiles in terms of the native molecular size (data not shown), suggesting that the mutagenesis at the G13 did not cause a gross structural changes of hUGDH. Therefore, the results with cassette mutagenesis and photoaffinity labeling techniques suggest that the loss of enzyme activity of G13E mutant is mainly caused by its inability to bind NAD$^+$ and G13 is required for efficient base-binding of NAD$^+$ to hUGDH.

The construction of a synthetic gene encoding hUGDH will enable us to generate a large number of site-directed mutations at several positions in the coding region. The high level of hUGDH expression as a soluble protein in *E. coli* will facilitate the purification of large quantities of mutant proteins and address a broad range of questions relating to the structure and function of hUGDH.

*Acknowledgments*—We wish to thank Dr. Mathias Ziegler (Institut für Biochemie, Freie Universität Berlin) for providing NMN adenylyltransferase.
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23590-23596.


TABLE I

**Kinetic properties of hUGDH and G13E mutant**

$K_m$ and $k_{cat}/K_m$ values of wild-type hUGDH and G13E mutant were obtained from the initial velocity data and linear regression analysis of double-reciprocal plots as described in Materials and Methods. The data represent the mean of two independent experiments with the difference expressed as a ± deviation. Duplicate of the kinetic values differed less than 5%.

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<th></th>
<th>$k_{cat}$</th>
<th>$K_m$-NAD$^+$</th>
<th>$K_m$-UDP-Glc</th>
<th>$k_{cat}/K_m$-NAD$^+$</th>
<th>$k_{cat}/K_m$-UDP-Glc</th>
</tr>
</thead>
<tbody>
<tr>
<td>hUGDH</td>
<td>105 ± 4</td>
<td>133 ± 4</td>
<td>17 ± 3</td>
<td>7.9 ± 1.0</td>
<td>6.2 ± 1.3</td>
</tr>
<tr>
<td>G13E mutant</td>
<td>negligible</td>
<td>negligible</td>
<td>negligible</td>
<td>negligible</td>
<td>negligible</td>
</tr>
</tbody>
</table>

*a* not detectable under standard reaction conditions and found less than 0.05% activity relative to wild-type enzyme after measuring activity for 3h.
<table>
<thead>
<tr>
<th>Source</th>
<th>Reference</th>
<th>Amino acid sequence&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human UGDH (wild-type)</td>
<td>This work</td>
<td>I C C I G A&lt;sup&gt;X&lt;/sup&gt;Y V G G P T&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Human UGDH (G13E)</td>
<td>This work</td>
<td>not detected</td>
</tr>
<tr>
<td>Bovine</td>
<td>(12)</td>
<td>I C C I G A G Y V G G P T C S V I</td>
</tr>
<tr>
<td>Mouse</td>
<td>(15)</td>
<td>I C C I G A G Y V G G P T C S V I</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>(56)</td>
<td>I A V A G S G Y V G L S L G V L L</td>
</tr>
<tr>
<td>Streptococcus pneumoniae</td>
<td>(57)</td>
<td>I A I A G S G Y V G L S L A V L L</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>(58)</td>
<td>I T V S G A G Y V G L S N G I L M</td>
</tr>
<tr>
<td>Soybean</td>
<td>(59)</td>
<td>I C C I G A G Y V G G P T M A V I</td>
</tr>
<tr>
<td>Fruitfly</td>
<td>(60)</td>
<td>V C C I G A G Y V G G P T C A V M</td>
</tr>
<tr>
<td>Poplar</td>
<td>(61)</td>
<td>I C C I G A G Y V G G P T M A V I</td>
</tr>
</tbody>
</table>

<sup>a</sup> The amino acids are denoted by the single-letter code.

<sup>b</sup> Only first 13 cycles were sequenced.

<sup>c</sup> The amino acid numbering is that of the mature human UGDH (15).
### TABLE III

**Amino acid composition of \([^{32}P]2N_3NAD^+\)-labeled peptide**

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Predicted</th>
<th>Determined $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>2</td>
<td>1.8 (2)</td>
</tr>
<tr>
<td>Arg</td>
<td>1</td>
<td>1.3 (1)</td>
</tr>
<tr>
<td>Cys $^b$</td>
<td>4</td>
<td>4.2 (4)</td>
</tr>
<tr>
<td>Glu</td>
<td>1</td>
<td>0.9 (1)</td>
</tr>
<tr>
<td>Gly</td>
<td>4</td>
<td><strong>2.9 (3)</strong></td>
</tr>
<tr>
<td>His</td>
<td>1</td>
<td>0.9 (1)</td>
</tr>
<tr>
<td>Ile</td>
<td>4</td>
<td>4.0 (4)</td>
</tr>
<tr>
<td>Met</td>
<td>1</td>
<td>1.2 (1)</td>
</tr>
<tr>
<td>Pro</td>
<td>2</td>
<td>1.9 (2)</td>
</tr>
<tr>
<td>Ser</td>
<td>1</td>
<td>1.2 (1)</td>
</tr>
<tr>
<td>Thr</td>
<td>1</td>
<td>1.1 (1)</td>
</tr>
<tr>
<td>Tyr</td>
<td>1</td>
<td>0.8 (1)</td>
</tr>
<tr>
<td>Val</td>
<td>2</td>
<td>2.2 (2)</td>
</tr>
</tbody>
</table>

$^a$ Normalized values. Values of molar ratio less than 0.1 are not indicated. The numbers in parentheses are the nearest integer values.

$^b$ Cys was pyridylethylated before it was quantified (62). It represents a sum of cysteine and cystine.
FIGURE LEGENDS

Fig. 1. Assembly of the synthetic hUGDH gene. A total of 40 oligonucleotides were used to assemble three gene fragments that varied from 443 to 580 bp in length. DNA fragments corresponding to the gene segments were isolated and used to create the functional hUGDH gene (pHUGDH) via stepwise ligation into pUC18 as shown. R, *Eco*RI; P, *Pst*I; A, *Age*I; H, *Hind*III.

Fig. 2. DNA sequence of the synthetic hUGDH gene carried in pHUGDH. Numbers on the left refer to amino acids (upper) and DNA (lower). Only the unique restriction sites are shown. Position 1 of the amino acid sequence corresponds to the first amino acid (Met) of the human UGDH (15).

Fig. 3. SDS/PAGE analysis of hUGDH. Lane 1, marker proteins (Bio-Rad, 97, 66, 45, 31, 21, 14 kDa); lane 2, crude extract from DE3 transformed with pUC18 only; lane 3, crude extract from DE3 transformed with pHUGDH; lane 4, FPLC-purified recombinant hUGDH; lane 5, HPLC-purified recombinant hUGDH.

Fig. 4. $V_{\text{max}}$ and $K_m$ values of hUGDH for NAD$^+$ (A) and UDP-glucose (B). Steady state enzyme activity was measured as conversion of NAD$^+$ to NADH detected by absorbance at 340nm. Purified hUGDH was incubated in 100 mM sodium glycine, pH 8.7 at 25°C with increasing concentrations of NAD$^+$ in the presence of 1 mM UDP-glucose (A), or with increasing concentrations of UDP-glucose in the presence of 1 mM NAD$^+$ (B). The $K_m$ and $V_{\text{max}}$ values were calculated by linear regression analysis of double-reciprocal plots.

Fig. 5. Saturation of photoinsertion of [$^{32}$P]2N$_3$NAD$^+$ into hUGDH. The wild-type hUGDH and G13E mutant in the reaction buffer was photolyzed with the indicated concentrations of [$^{32}$P]2N$_3$NAD$^+$. $^{32}$P incorporations into proteins were determined by liquid scintillation counting and expressed relative to each control. ■, wild-type; ○, G13E mutant.
Fig. 6. Effect of NAD$^+$ on photoinsertion of $[^{32}\text{P}]2\text{N}_{3}\text{NAD}^+$ into hUGDH. The wild-type hUGDH and G13E mutant in the reaction buffer was photolyzed with 50 μM $[^{32}\text{P}]2\text{N}_{3}\text{NAD}^+$ in the presence of the indicated concentrations of NAD$^+$. $^{32}\text{P}$ incorporations into proteins were determined by liquid scintillation counting and expressed relative to each control. ■, wild-type; ○, G13E mutant.

Fig. 7. Western blot analysis of the G13E mutant in crude extracts. Lane 1, prestained marker proteins (Novex); lane 2, wild-type hUGDH; lane 3, G13E mutant; lane 4, vector only.
Fig. 1

pHUGDH (1509 bp)
<table>
<thead>
<tr>
<th>Restriction Enzyme</th>
<th>Sequences</th>
<th>Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRI</td>
<td>AA TTC CAA AGG AGG TAA TAA</td>
<td>N-terminal and C-terminal sites for cloning</td>
</tr>
<tr>
<td>BstBI</td>
<td>Phc TTC Glu GAA Ala ATP</td>
<td>Multiple cloning sites</td>
</tr>
<tr>
<td>NcoM IV</td>
<td>Glu GAA Ala ATP</td>
<td>Multiple cloning sites</td>
</tr>
<tr>
<td>SalI</td>
<td>Val Val Gly Gly Pro Thr Cys Ser Val Ile Ala His</td>
<td></td>
</tr>
<tr>
<td>NaiI</td>
<td>Ala Ala Trp GAG</td>
<td></td>
</tr>
<tr>
<td>EcoR I</td>
<td>Gly Gly Ser TGT</td>
<td></td>
</tr>
<tr>
<td>Met Phe Glu Ile Lys Lys Ile Cys Cys Ile Gly Ala Gly</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA TTC CAA AGG AGG TAA TAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyr Val Gly Gly Pro Thr Cys Ser Val Ile Ala His</td>
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<td></td>
</tr>
<tr>
<td>Met Cys Pro Glu Ile Arg Val Thr</td>
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<tr>
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<td>Nsi I</td>
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</tr>
<tr>
<td>54 Tyr Val Gly Gly Pro Thr Cys Ser Val Ile Ala His</td>
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<tr>
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<td></td>
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</tr>
<tr>
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<tr>
<td>Stu I</td>
<td>Ser Ser Ser Ser Ser Ser</td>
<td></td>
</tr>
<tr>
<td>Sac II</td>
<td>Ser Ser Ser Ser Ser Ser</td>
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</tr>
<tr>
<td>54 Tyr Val Gly Gly Pro Thr Cys Ser Val Ile Ala His</td>
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<tr>
<td>Met Cys Pro Glu Ile Arg Val Thr</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>Hind III</td>
<td>Ser Ser Ser Ser Ser Ser</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 2**
Fig. 4
Fig. 6

The graph shows the relative incorporation of $^32$P as a function of NAD$^+$ concentration. The x-axis represents NAD$^+$ concentration (µM) ranging from 0 to 200, while the y-axis shows the relative incorporation (%) ranging from 0 to 100. The data points indicate a decrease in relative incorporation with increasing NAD$^+$ concentration.
Importance of Gly13 for the coenzyme binding of human UDP-glucose dehydrogenase

J. Biol. Chem. published online July 7, 2004

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