Journal of Biochemistry and Molecular Biology, Vol. 40, No. 5, September 2007, pp. 690-696

JOURNAL OF Biochemistry and Molecular Biology

Alteration of The Quaternary Structure of Human UDP-Glucose Dehydrogenase by a Double Mutation

Jae-Wan Huh[1,#], Seung-Ju Yang[2,#], Eun Young Hwang[1], Myung-Min Choi[1], Hyun-Ju Lee[1], Eun-A Kim[1], Soo Young Choi[3], Jene Choi[4], Hea-Nam Hong[5] and Sung-Woo Cho[1,*]

[1]Department of Biochemistry and Molecular Biology, University of Ulsan College of Medicine, Seoul 138-736, Korea
[2]Department of Biomedical Laboratory Science, Konyang University, Daejeon 320-718, Korea
[3]Department of Biomedical Sciences, Hallym University, Chunchon 200-702, Korea
[4]Department of Pathology, University of Ulsan College of Medicine, Seoul 138-736, Korea
[5]Department of Anatomy and Cell Biology, University of Ulsan College of Medicine, Seoul 138-736, Korea

Received 27 March 2007, Accepted 18 April 2007

There are conflicting views for the polymerization process of human UDP-glucose dehydrogenase (UGDH) and no clear evidence has been reported yet. Based on crystal coordinates for *Streptococcus pyogenes* UGDH, we made double mutant A222Q/S233G. The double mutagenesis had no effects on expression, stability, and secondary structure. Interestingly, A222Q/S233G was a dimeric form and showed an UGDH activity, although it showed increased K_m values for substrates. These results suggest that Ala222 and Ser233 play an important role in maintaining the hexameric structure and the reduced binding affinities for substrates are attributable to its altered subunit communication although quaternary structure may not be critical for catalysis.

Keywords: Cassette mutagenesis, Gel filtration, Polymerization, UDP-glucose dehydrogenase

Introduction

UDP-glucose dehydrogenase (UGDH; EC 1.1.1.22) catalyzes an NAD^+-dependent two-fold oxidation of UDP-glucose to generate UDP-glucuronate (Franzen *et al*., 1980). UDP-glucuronate is the required substrate for glucuronidation of many substances, including xenobiotics, opioids, androgens, and heme proteins through the action of UDP-glucuronosyl transferases in the liver (Jansen *et al*., 1992). UDP-glucuronate is also an essential precursor for synthesis of glycosaminoglycans such as hyaluronan, which serves a variety of functions within the extracelluar matrix and pericellular spaces, thus having a profound influence on cell behavior and developmental processes (Luca *et al*., 1981; Rizzotti *et al*., 1986). Because the UDP-glucuronate supply is a limiting step in glycosaminoglycan synthesis, it was recently postulated that any modification of UGDH activity may influence the proteoglycan structure and function (Cumberledge and Reichsman, 1997). For example, the mutational inactivation of the *Drosophila* gene suppresses the Wingless activity in developing pups (Hacker *et al*., 1997), confirming the role of glycosaminoglycans in growth-factor-signaling mechanisms.

It has been reported that bovine liver UGDH is a hexamer but due to its 'half-of-the-sites' reactivity, it may be better described as a trimer of dimers (Feingold and Franzen, 1981). Gel filtration studies have indicated that the *Escherichia coli* (*E. coli*) enzyme exists as a dimer in solution (Schiller *et al*., 1973). Similar experiments with the *Streptococcus pyogenes* (*S. pyogenes*) enzyme have indicated that it may exist as a monomer in solution (Campbell *et al*., 1997). Recently, the recombinant human UGDH has been cloned, expressed, and purified (Sommer *et al*., 2004; Huh *et al*., 2004). The human enzyme is active as a hexamer with some evidence for assembly in a trimer of dimers as has been proposed for the bovine enzyme. Steady-state kinetic constants were established and found to be comparable with previously published data in the bovine system (Ridley *et al*., 1975; Ordman and Kirkwood, 1977; Franzen *et al*., 1980). UGDH from *S. pyogenes* consists of two discrete α/β domains, each of which contains a core β-sheet sandwiched between α-helices. These two domains are

Abbreviations: UGDH, UDP-glucose dehydrogenase

[#]Co-first authors

*To whom correspondence should be addressed.
Tel: 82-2-3010-4278; Fax: 82-2-3010-4248
E-mail: swcho@amc.seoul.kr

connected by a long (48Å) central α-helix (α9) that also serves as the core of the dimer interface. The six-stranded parallel β-sheet (β1-β4, β7, β8) that is characteristic of the dinucleotide binding Rossmann fold (Rossmann *et al*., 1975) serves as the core of the N-terminal NAD^+ binding domain (residues 1-196).

Very recently, Easley *et al*. (2007) have reported that the K339A mutant of the human UGDH is exclusively found in a dimeric state and shows with a similar maximum reaction velocity, demonstrating that the overall function of this mutant is not altered. They have postulated that in human UGDH, N-terminal and C-terminal interdomain interactions within individual monomeric subunits are essential for the maintenance of hexameric quaternary structure (Easley *et al*., 2007). It is, therefore, essential to have a detailed structural description of hUGDH. However, no crystal structure of human UGDH has been reported yet, although diffraction data of the human enzyme to a resolution of 2.8 Å have been collected recently in our laboratory (Huh *et al*., 2006). The human enzyme cDNA codes for 494 amino acids and has 23% sequence identity to its ortholog in *Streptococcus pyogenes* (*S. pyogenes*), for which a crystal structure has been published (Campbell *et al*., 2000). The structure of recombinant UGDH from *S. pyogenes* reveals a crystallographic dimer with an interface of greater than 2600 $Å^2$ (Campbell *et al*., 2000). The helical portion of the C-terminal domain (α10-α12) contributes the majority (52%) of the interface solvent-inaccessible surface area, followed by the central α-helix α9 (37%), and the N-terminal domain (12%). There are a total of 24 hydrogen bonds stabilizing the dimer interface, though none of the amino acids involved are strictly conserved. Aromatic residues including Phe206, Tyr210, Tyr217, Tyr224, and Tyr272 dominate the dimer interface. These five residues correspond to Ala222, Phe226, Ser233, Leu240, and Cys288 in the human enzyme.

Recent studies of structure-function relationships, using site-directed mutagenesis of human UGDH at those single sites, showed that the A222Q and the S233G change reproduced some, but not all, of the properties of hUGDH (unpublished data). In the present study, therefore, we have performed double mutagenesis at Ala222 and Ser233 sites in a single polypeptide to examine their roles in the hexameric quaternary structure. Over-expression in *E. coli* of the double mutant led to the production of a soluble protein cross-reacting with an anti-wild-type human UGDH antibody preparation. Here we describe essential roles for Ala222 and Ser233 in preserving quaternary structure of the enzyme.

Materials and Methods

Materials. UDP-glucose, NAD^+, ampicillin, isopropyl-β-D-thiogalactopyranoside (IPTG), and phenylmethanesulfonyl fluoride were purchased from Sigma Chemical Co. DNA ligase, polynucleotide kinase, and restriction enzymes were purchased from New England Biolabs. Human UGDH gene (pHUGDH) has been chemically synthesized and expressed in *E. coli* in our laboratory as described elsewhere (Huh *et al*., 2004). Blue Sepharose CL-6B and Resource-Q were purchased from Amersham Pharmacia Biotech. Protein-Pak 300SW column was purchased from Waters. Restriction enzymes were purchased from New England Biolabs. Monoclonal antibodies against hUGDH were produced as before (Huh *et al*., 2004). Pre-cast gels for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from NOVEX. All other chemicals and solvents were of reagent grade or better. *E. coli* DH5α (Hanahan, 1983) was used as the host strain for cassette mutagenesis. *E. coli* BL21 (DE3) (Studier and Moffatt, 1986) was used for high level expression of the recombinant hUGDH.

Construction of mutants. Amino acid substitution was constructed by cassette mutagenesis using a synthetic human hUGDH gene (pHUGDH) as described before (Huh *et al*., 2004). Plasmid DNA (5 μg) was digested with restriction enzymes (*Bgl*II and *Dra*III) to remove the flanking fragment that encodes target amino acids (Ala222and Ser233) and the flanking fragment (Leu208 through Leu240) was replaced with synthetic DNA duplexes containing a substitution on both DNA strands at the two positions to make double mutant A222Q/S233G. Mutagenic oligonucleotide was annealed, ligated, and transformed into DH5α and the resultant mutant plasmid was identified by DNA sequencing using plasmid DNA as a template. The mutant was expressed in *E. coli* strain DE3 and the gene expression level of the double mutant A222Q/S233G was examined by Western blot using monoclonal antibodies against the human UGDH. For purification of the wild-type and mutant hUGDHs, fresh overnight cultures of DE3/pHUGDH were used to inoculate 1 L of Luria-Bertani (LB) containing 100 μg of ampicillin/mL. DE3/pHUGDH 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/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 at pH 7.4, 1 mM EDTA, and 5 mM dithiothreitol and lysed with a sonicator. Cellular debris was removed by centrifugation, and the proteins were purified by chromatographic methods using a Blue Sepharose CL-6B column and FPLC Resource-Q column as described elsewhere (Huh *et al*., 2004; Huh *et al*., 2005). The purified protein was analyzed by SDS-PAGE and Western blot analysis (Kim *et al*., 2006).

Enzyme assay and kinetic studies. Enzyme assay was performed spectrophotometrically by measuring the reduction of NAD^+ in the presence of UDP-glucose at 340 nm as described previously (Huh *et al*., 2004). The reaction was performed in a total volume of 1 ml with hUGDH adjusted to give a measured rate of less than 0.04 absorbance units/min. One unit of enzyme activity was defined as the amount of enzyme required to reduce 2 μmol of NAD^+/min at pH 8.7 at room temperature. 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 concentrations. The K_m and V_{max} values were calculated by linear regression analysis of double-reciprocal plots and catalytic efficiency was estimated by use of the equation $v/[E_0] = (k_{cat}/K_m)[S]$ (Fersht, 1985).

HPLC gel filtration analysis. The oligomeric states of the wild-type and mutant proteins were determined by a HPLC Protein-Pak 300SW gel filtration column with a Waters HPLC system. The column equilibrated with 100 mM Tris-HCl, pH 7.5 and 50 mM NaCl. The wild-type and mutant hUGDHs were separately loaded onto a Protein-Pak 300SW column (Waters) and eluted with the same buffer at a flow rate of 1 ml/min. The protein was monitored at 280 nm and the hUGDH activity was monitored by the standard assay method as described above.

Circular dichroism. Proteins were incubated 20 mM potassium phosphate, pH 7.0 at 25°C for 30 min and far-UV CD spectra were monitored from 200 to 250 nm in a cuvette of 1 mm path length, 50 mdeg sensitivity, response time of 1 s, and scan speed of 50 nm/min on a Jasco J-715 spectrophotometer. The spectra were recorded as a 5 scan average value and analyzed by the methods of Yang *et al.* (1986) with reading from a smoothed curve through five successive point window average assigned to middle to determine the secondary conformation. After subtracting appropriate blanks, mean residue ellipticities were calculated, using the formula $\theta = \theta_{obs} \times MRW/10cl$, where θ_{obs} is the observed ellipticity in degrees, MRW is the mean residue weight, c is the concentration of protein in g/ml, and l is the path length in centimeters (Balasubramanian and Kumar, 1976).

ANS binding assay. Binding experiment with anilinonaphthalene-8-sulfonic acid (ANS; Molecular Probes) was performed by incubating wild-type or double mutant proteins with various concentrations of ANS in 20 mM phosphate, pH 7.0 for 10 min at 25°C. The protein concentration used was 3 μM. The incubated solution was then excited at 380 nm and the fluorescence intensity at 510 nm was monitored. Fluorescence spectra were obtained using a cuvette with a path length of 10 mm, and subtracted from the spectrum of the ANS solution without hUGDH. The protein samples were also incubated with 10 μM of ANS and fluorescence spectra were obtained at between 400 and 600 nm (380 nm excitation wavelength).

Results and Discussion

UGDH is expressed by most species from bacteria to humans. Human UGDH is closely related to the enzyme characterized from bovine liver but is only 23% identical to the *Streptococcus pyogenes* enzyme, both of which catalyze the same two-fold oxidation of UDP-glucose to UDP-glucuronate, apparently by similar mechanisms. The mechanism used by the bovine liver UGDH is thought to involve an initial oxidation of the C-6 hydroxyl of UDP-glucose to form an aldehyde intermediate and NADH (Ridley *et al.*, 1975; Ordman and Kirkwood, 1977). It has been suggested that the aldehyde is bound via an imine linkage with a lysine residue. The second oxidation is initiated by the addition of an active-site cysteine thiol to the aldehyde to form a thiohemiacetal intermediate. A hydride transfer from the intermediate to NAD^+ then takes place to produce an enzyme-bound thioester intermediate and a second molecule of NADH. In a final irreversible step, the thioester is hydrolyzed to give the product UDP-glucuronate. Both substrate binding assays and chemical modifications of the active site in the bovine enzyme suggest that there is half-site reactivity among the subunits in the trimer of dimers that is induced through subunit communication following the first binding event (Franzen *et al.*, 1973; Franzen *et al.*, 1980). This phenomenon may provide a regulatory mode for human UGDH. Its regulation may be related to several pathologies such as cancer progression (Lapointe and Labrie, 1999). Specific targeted inhibition of this enzyme may offer a therapeutic avenue but requires a detailed understanding of its mechanism. However, until recently, very little was known about the human enzyme. In this study, we have investigated the complex catalytic mechanism of hUGDH with the mutagenesis at Ala222 and Ser233 to make a double mutant A222Q/S233G based on previously published crystal coordinates of the streptococcal homologue and identified putative residues in subunit interaction.

To test the involvement of two putative amino acids in subunit polymerization of human UGDH, we constructed mutant proteins at two different sites by cassette mutagenesis. The flanking fragment in pHUGDH was replaced with synthetic DNA duplexes containing a substitution on both DNA strands at positions encoding Ala222 and Ser233 to make double mutant A222Q/S233G by the general strategy of the cassette mutagenesis as described before (Huh *et al.*, 2004). The entire A222Q/S233G gene has been sequenced in order to check for unwanted secondary mutations. The double mutant protein was efficiently expressed in *E. coli* as a soluble protein and interacted with monoclonal antibodies raised against hUGDH at almost identical level with wild-type UGDH by Western blot analysis (Fig. 1A). The double mutant was homogeneously purified using Blue Sepharose CL-6B column followed by FPLC Resource-Q column. Purified double mutant hUGDH showed a protein band of the right subunit molecular mass of a 57-kDa on SDS-PAGE (Fig. 1B). These results indicate that the mutagenesis at 222 and 233 sites has no effects on expression or stability of the double mutant protein. The secondary structure of the two proteins was also very similar, as monitored by far-UV CD, and comparable with that of the native hexamer (Fig. 2). The intrinsic fluorescence emission spectra of the native enzyme and A222Q/S233G were superimposable, indicating a similar shielding of aromatic residues from the solvent.

To compare the relative hydrophobic surface area of hUGDH between the native and double mutant, binding experiments with anilinonaphthalene-8-sulfonic acid (ANS) were performed. ANS, a fluorescent probe, has been utilized to monitor conformational changes of the protein with the subsequent exposure of hydrophobic binding sites on proteins, as described previously (Stryer, 1965). Figure 3 represents a comparison of the fluorescence titration curves of the double mutant and of the native GDH with ANS. ANS alone did not display any significant fluorescence intensity between 400 and 600 nm. Compared with the free dye in the solution,

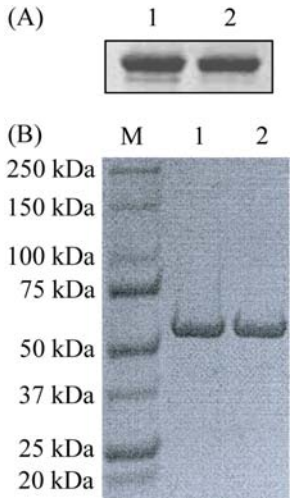

Fig. 1. Electrophoretic analysis of double mutant A222Q/S233G. Recombinant wild-type hUGDH and double mutant A222Q/S233G were over-expressed in *E. coli* and purified to homogeneity from the soluble extract by column chromatography. (A) The gene expression levels of the mutant proteins were examined by Western blot using monoclonal antibodies against hUGDH. (B) Proteins were evaluated by 10% SDS-PAGE followed by staining with Coomassie Blue. Lane *M*, molecular weight marker proteins; lane *1*, wild-type hUGDH; lane *2*, the double mutant A222Q/S233G.

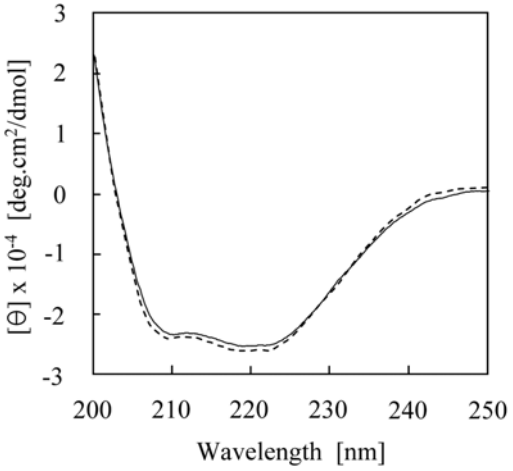

Fig. 2. Circular dichroism spectra. Proteins were incubated 20 mM potassium phosphate, pH 7.0 at 25°C for 30 min and far-UV CD spectra were monitored from 200 to 250 nm. The spectra were recorded as a 5 scan average value. CD spectra are expressed in terms of [θ] (deg · cm^2 · dmol^{-1}). Wild-type (-); double mutant A222Q/S233G (· · ·).

hUGDH resulted in a drastic increase in the fluorescence intensity upon binding ANS (Fig. 3A). When ANS was bound to double mutant A222Q/S233G, the ANS fluorescence was marginally enhanced compared to the hexamer (Fig. 3A). Saturation behavior was observed for all the forms but for double mutant A222Q/S233G the final value of ANS

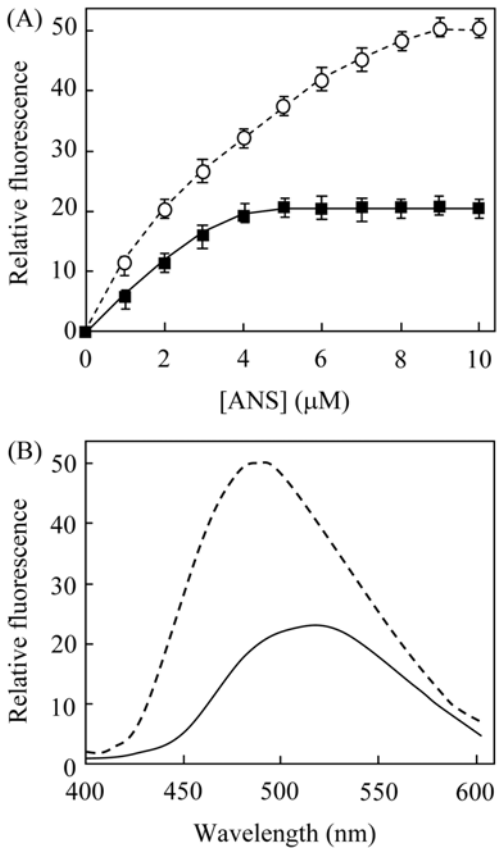

Fig. 3. Interaction of wild-type and double mutant A222Q/S233G of hUGDH with the fluorescent probe anilinonaphthalene-8-sulfonic acid (ANS). (A) The changes in fluorescent intensity at 490 nm (380 nm excitation wavelength) were monitored at 25°C 10 min after each addition of the probe. Protein concentration was 3 μM. Wild-type (■-■); double mutant A222Q/S233G (O · · · O). (B) The protein samples (3 μM) were incubated with 10 μM of ANS fluorescence spectra were obtained at between 400 and 600 nm (380 nm excitation wavelength). Wild-type (-); double mutant A222Q/S233G (· · ·). Results are mean ± S.E.M. of three independent experiments.

emission intensity at 490 nm was higher with respect to the native enzyme with a saturation midpoint at a higher ANS concentration. The quantum yield and the fluorescence emission maximum of ANS are directly related to the polarity of the medium (Stryer, 1965). In our results the fluorescence emission maximum of ANS is centered at 510 nm and 490 nm for the native enzyme and the double mutant protein, respectively (Fig. 3B). This suggests that the observed fluorescence changes of the probe in the presence of double mutant A222Q/S233G are caused by the differences in polarity of the environment of bound ANS molecules and probably by an increased binding stoichiometry. Our results suggest that the overall protein dynamics of double mutant A222Q/S233G differ from the wild-type hUGDH.

The enzymatic activity of the mutant protein was characterized by steady state kinetic analysis. Measurements

Table 1. Kinetic parameters of the purified wild-type and double mutant A222Q/S233G. Results are mean ± SEM of three independent experiments

		Wild-type	A222Q/S233G
V_{max}	(nmol · min^{-1} · mg^{-1})	157 ± 4	145 ± 3
$K_{m\text{-UDPG}}$	(μM)	17 ± 1	980 ± 5
$K_{m\text{-NAD}}$+	(μM)	133 ± 2	401 ± 5
k_{cat}	(s^{-1})	105 ± 3	97 ± 2
$k_{cat}/K_{m\text{-UDPG}}$	($s^{-1} \cdot \mu M^{-1}$)	6.2 ± 0.02	0.10 ± 0.01
$k_{cat}/K_{m\text{-NAD}}$+	($s^{-1} \cdot \mu M^{-1}$)	0.79 ± 0.01	0.24 ± 0.01

to determine dependence of the reaction on cofactor concentration were done using purified mutant enzyme 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_{max} for the reaction catalyzed by the wild-type enzyme. Both sets of conditions yielded a similar V_{max} of 157 nmol and 145 nmol of NAD^+/min/mg of enzyme for wild-type and double mutant A222Q/S233G, respectively (Table 1). A similar maximum reaction velocity for wild-type hUGDH and A222Q/S233G with respect to both substrate and cofactor demonstrates that the overall function of this mutant is not altered. However, double mutant A222Q/S233G showed increased K_m values for both NAD^+ and UDP-glucose compared to those of the wild-type hUGDH. For wild-type, the K_m for UDP-glucose was 17 μM and the K_m for NAD^+ was 133 μM (Table 1). Relative to that of the wild-type, the K_m for UDP-glucose was 980 μM and K_m for NAD^+ was 401 μM for double mutant A222Q/S233G. These results indicate that substitution at position 222 and 233 has appreciable effects on the affinity of the enzyme for both UDP-glucose and NAD^+. Because substrate binding is thought to precede cofactor binding in the mammalian enzyme as reported elsewhere (Ordman and Kirkwood, 1977; Franzen *et al*., 1983), the reduced affinity of double mutant A222Q/S233G for substrate also affects the affinity for the NAD^+ cofactor.

For more detailed catalytic properties of hUGDH, the enzyme efficiency (k_{cat}/K_m) for the individual substrates was determined. The k_{cat} value of the wild-type hUGDH was 105 s^{-1} and the k_{cat}/K_m values for UDP-glucose and NAD^+ were 6.2 $s^{-1} \cdot \mu M^{-1}$ and 0.79 $s^{-1} \cdot \mu M^{-1}$, respectively (Table 1). Unlike to that of the wild-type, the k_{cat} value of double mutant A222Q/S233G was 97 s^{-1} and the k_{cat}/K_m values for UDP-glucose and NAD^+ were 0.1 $s^{-1} \cdot \mu M^{-1}$ and 0.24 $s^{-1} \cdot \mu M^{-1}$, respectively (Table 1). Therefore, the dramatic decrease in the efficiency (k_{cat}/K_m) of double mutant A222Q/S233G was mainly due to an increase in K_m values. The detailed kinetics of double mutant A222Q/S233G, in particular by rapid reaction kinetics and subunit complementation analysis, remains to be studied in order to elucidate the role of these residues more fully.

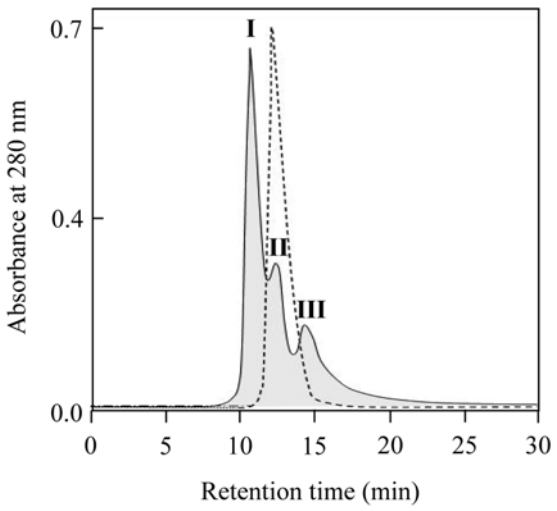

Fig. 4. HPLC gel filtration elution profiles of wild-type and double mutant A222Q/S233G of hUGDH. HPLC gel filtration was performed on a Protein-Pak 300SW column in 50 mM Tris/HCl, pH 7.4 containing 50 mM NaCl with a flow rate of 1.0 mL/min. Calibration was performed with thyroglobulin (669 kDa), ferritin 440 kDa), catalase (232 kDa), aldolase (158 kDa), and glutamate dehydrogenase (56 kDa). Wild-type hUGDH (-) showed a major peak (peak I) observed as a hexamer at a molecular mass corresponding to six times the single subunit mass, together with minor dimeric (peak II) and monomeric (peak III) species. The peak of double mutant A222Q/S233G (· · ·) was superimposed on the wild-type trace for comparison and represented a dimeric form of hUGDH.

Many investigators have reported that the bovine and human UGDHs are considered as homo hexamers (Franzen *et al*., 1980, Huh *et al*., 2004; Sommer *et al*., 2004; Easley *et al*., 2007), while the bacterial enzyme is dimeric in crystal structures (Campbell *et al*., 2000) and may be active as a monomer (Campbell *et al*., 1997). To determine the oligomeric state of double mutant A222Q/S233G, gel filtration analysis by HPLC was performed (Fig. 4). Consistent with our previous data for the human enzyme (Huh *et al*., 2004), wild-type hUGDH predominantly is observed as a hexamer (peak I) eluting from the gel filtration column at a molecular mass corresponding to six times the single subunit mass. Minor dimeric (peak II) and monomeric (peak III) species also are present, indicating some subunit dissociation (Fig. 4). This elution profile was observed repeatedly over several different preparations of the enzyme with relative species ratios conserved so the distribution of subunit structures appears to be an innate property of the enzyme. Similar elution profiles of the wild-type hUGDH also have been reported by other investigators (Sommer *et al*., 2004; Easley *et al*., 2007). Interestingly, double mutant A222Q/S233G that had altered

substrate affinity but an almost identical V_{max} showed a dimeric structure compared by overlay of its elution profile on that of the wild-type enzyme (Fig. 4). This observation is not consistent with the UGDH quaternary structure adopting a "trimer of dimers" arrangement within the hexameric protein (Spicer *et al*., 1998). It is not clear what role subunit associations may play in enzyme function. However, since the double mutant A222Q/S233G showed a similar V_{max} to that of the wild-type enzyme, hexameric quaternary structure is not essential for activity. This is supported by the fact that the bacterial enzyme exists as a dimer. Very recently, Easley *et al*. (2007) have reported that the K339A mutant of the human UGDH shows the reduced affinity for substrate and cofactor with a similar maximum reaction velocity, demonstrating that the overall function of this mutant is not altered. Intriguingly, this K339A mutant is exclusively found in a dimeric state (Easley *et al*., 2007), despite a clear prediction from the homology model that its location is remote from sites likely to be directly involved in maintaining "trimer of dimers" contact as has been proposed (Spicer *et al*., 1998). The overall structure of each enzyme monomer contains two distinctly folded globular domains, between which the active site exists within an extended crevice formed by association of the two domains. Easley *et al*. (2007) have postulated that in human UGDH, N-terminal and C-terminal interdomain interactions within individual monomeric subunits are essential for the maintenance of hexameric quaternary structure. An analysis of the three-dimensional structure of the human enzyme should supplement the understanding of the nature of this subunit interaction. Recently, we have collected diffraction data of the human UGDH to a resolution of 2.8 Å (Huh *et al*., 2006) and we are seeking to optimize the crystals to evaluate the structure and mechanism of this enzyme.

Our results with kinetic studies and HPLC analysis suggest that the double mutagenesis at Ala222 and Ser233 causes a gross structural change of human UGDH and Ala222 and Ser233 appear to function structurally in preserving the quaternary structure of the enzyme. These results also show that the hexameric structure may not be required for the maximal activity of human UGDH. Characterization of residual enzymatic activity in the catalytic double mutant A222Q/S233G may afford new insights into the mechanism of hUGDH.

Acknowledgments. This work was supported by grants from the Korea Research Foundation (KRF-2006-005-J00802).

References

Balasubramanian, D. and Kumar, C. (1976) Recent studies of the circular dichroism and optical rotatory dispersion of biopolymers. *Appl. Spectrosc. Rev.* **11,** 223-286.

Campbell, R. E., Mosimann, S. C., van de Rijn, I., Tanner, M. E. and Strynadka, N. C. (2000) The first structure of UDP-glucose dehydrogenase reveals the catalytic residues necessary for the two-fold oxidation. *Biochemistry* **39,** 7012-7023.

Campbell, R. E., Sala, R. F., van de Rijn, I. and Tanner, M. E. (1997) Properties and kinetic analysis of UDP-glucose dehydrogenase from group A streptococci. Irreversible inhibition by UDP-chloroacetol. *J. Biol. Chem*. **272**, 3416-3422.

Cumberledge, S. and Reichsman, F. (1997) Glycosaminoglycans and WNTs: Just a spoonful of sugar helps the signal go down. *Trends Genet*. **13**, 421-423.

Easley, K. E., Sommer, B. J., Boanca, G., Barycki, J. J. and Simpson, M. A. (2007) Characterization of human UDP-glucose dehydrogenase reveals critical catalytic roles for lysine 220 and aspartate 280. *Biochemistry* **46**, 369-378.

Feingold, D. S. and Franzen, J. S. (1981) Amino acid sequence of the tryptic peptide containing the catalytic-site thiol group of bovine liver uridine diphosphate glucose dehydrogenase. *Trends Biochem. Sci*. **6**, 103-105.

Fersht, A. (1985) Enzyme Structure and Mechanism, pp. 98-120, W.H. Freeman, New York, USA.

Franzen, J. S., Ashcom, J., Marchetti, P., Cardamone, J. J., Jr. and Feingold, D. S. (1980) Induced versus pre-existing asymmetry models for the half-of-the-sites reactivity effect in bovine liver uridine diphosphoglucose dehydrogenase. *Biochim. Biophys. Acta* **614**, 242-255.

Franzen, J. S., Kuo, I., Eichler, A. J. and Feingold, D. S. (1973) UDP-glucose dehydrogenase: Substrate binding stoichiometry and affinity. *Biochem. Biophys. Res. Commun*. **50**, 517-523.

Franzen, J. S., Marchetti, P. S. and Feingold, D. S. (1980) Resonance energy transfer between catalytic sites of bovine liver uridine diphosphoglucose dehydrogenase. *Biochemistry* **19**, 6080-6089.

Franzen, J. S., Marchetti, P. S., Lockhart, A. H. and Feingold, D. S. (1983) Special effects of UDP-sugar binding to bovine liver uridine diphosphoglucose dehydrogenase. *Biochim. Biophys. Acta* **746**, 146-153.

Hacker, U., Lin, X. and Perrimon, N. (1997) The Drosophila sugarless gene modulates Wingless signaling and encodes an enzyme involved in polysaccharide biosynthesis. *Development* **124**, 3565-3573.

Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol*. **166**, 557-580.

Huh, J.-W., Lee, H.-J., Choi, M.-M., Yang, S.-J., Yoon, S. Y., Kim, D. W., Kim, S. Y., Choi, S. Y. and Cho, S.-W. (2005) Identification of a UDP-glucose-binding site of human UDP-glucose dehydrogenase by photoaffinity labeling and cassette mutagenesis. *Bioconjugate Chem*. **16**, 710-716.

Huh, J.-W., Robinson, R. C., Lee, H. S., Lee, J. I., Heo, Y. S., Kim, H. T., Lee, H. J., Cho, S.-W. and Choe, H. (2006) Expression, purification, crystallization, and preliminary X-ray analysis of the human UDP-glucose dehydrogenase. *Protein Peptide Lett*. **13**, 859-862.

Huh, J.-W., Yoon, H.-Y., Lee, H.-J., Choi, W.-B., Yang, S.-J. and Cho, S.-W. (2004) Importance of Gly-13 for the coenzyme binding of human UDP-glucose dehydrogenase. *J. Biol. Chem*. **279**, 37491-37498.

Jansen, P. L., Mulder, G. J., Burchell, B. and Bock, K. W. (1992) New developments in glucuronidation research: Report of a workshop on "glucuronidation, its role in health and disease". *Hepatology* **15**, 532-544.

Kim, S. Y., An, J. J., Kim, D. W., Choi, S. H., Lee, S. H., Hwang, S.-I., Kwon, O.-S., Kang, T.-C., Won, M. H., Cho, S.-

W., Park, J., Eum, W. S., Lee, K. S. and Choi, S. Y. (2006) Tat-mediated protein transduction of human brain pyridoxine-5-P oxidase into PC12 cells. *J. Biochem. Mol. Biol.* **39**, 76-83.

Lapointe, J. and Labrie, C. (1999) Identification and cloning of a novel androgen-responsive gene, uridine diphosphoglucose dehydrogenase, in human breast cancer cells. *Endocrinology* **140**, 4486-4493.

Luca, G., Rindi, S. and Rizzotti, M. (1981) Exogenous UDP-xylose can inhibit UDP-glucose dehydrogenase activity of cultured human skin fibroblasts. *Basic Appl. Histochem.* **25**, 67-69.

Ordman, A. B. and Kirkwood, S. (1977) Mechanism of action of uridine diphoglucose dehydrogenase. Evidence for an essential lysine residue at the active site. *J. Biol. Chem.* **252,** 1320-1326.

Ridley, W. P., Houchins, J. P. and Kirkwood, S. (1975) Mechanism of action of uridine diphosphoglucose dehydrogenase. Evidence for a second reversible dehydrogenation step involving an essential thiol group. *J. Biol. Chem.* **250**, 8761-8767.

Rizzotti, M., Cambiaghi, D., Gandolfi, F., Rindi, S., Salvini, R. and De Luca, G. (1986) The effect of extracellular matrix modifications on UDP-glucose dehydrogenase activity in cultured human skin fibroblasts. *Basic Appl. Histochem.* **30**, 85-92.

Rossmann, M. G., Liljas, A., Branden, C.-I. and Banaszak, L. J. (1975) Evolutionary and structural relationships among dehydrogenases. in: The Enzymes (Boyer, P., Ed.), pp. 61-102, Academic Press, New York.

Schiller, J. G., Bowser, A. M. and Feingold, D. S. (1973) Partial purification and properties of UDPG dehydrogenase from Escherichia coli. *Biochim. Biophys. Acta* **293**, 1-10.

Sommer, B. J., Barycki, J. J. and Simpson, M. A. (2004) Characterization of human UDP-glucose dehydrogenase. CYS-276 is required for the second of two successive oxidations. *J. Biol. Chem.* **279**, 23590-23596.

Spicer, A. P., Kaback, L. A., Smith, T. J. and Seldin, M. F. (1998) Molecular cloning and characterization of the human and mouse UDP-glucose dehydrogenase genes. *J. Biol. Chem.* **273**, 25117-25124.

Stryer, L. (1965) The interaction of naphthalene dye with apomyoglobin and apohemoglobin. A fluorescent probe of non-polar binding sites. *J. Mol. Biol.* **13**, 82-495.

Studier, F. W. and Moffatt, B. A. (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **189**, 113-130.

Yang, J. T., Wu, C.-S. C. and Martinez, H. M. (1986) Calculation of protein conformation from circular dichroism. *Methods Enzymol.* **130**, 208-269.