Glutamine-fructose-6-phosphate amidotransferase (GFAT) catalyzes the first committed step in the pathway for biosynthesis of hexosamines in mammals. A member of the N-terminal nucleophile class of amidotransferrases, GFAT transfers the amino group from the L-glutamine amide to D-fructose 6-phosphate, producing glutamic acid and glucosamine 6-phosphate. The kinetic constants reported previously for mammalian GFAT implicate a relatively low affinity for the acceptor substrate, fructose 6-phosphate (Fru-6-P, $K_m = 0.2–1$ mM). Utilizing a new sensitive assay that measures the production of glucosamine 6-phosphate (GlcN-6-P), purified recombinant human GFAT1 (hGFAT1) exhibited a $K_m$ for Fru-6-P of 7 $\mu$M, and was highly sensitive to product inhibition by GlcN-6-P. In a second assay method that measures the stimulation of glutaminase activity, a $K_i$ of 2 $\mu$M was measured for Fru-6-P binding to hGFAT1. Further, we report that the product, GlcN-6-P, is a potent competitive inhibitor for the Fru-6-P site, with a $K_i$ measured of 6 $\mu$M. Unlike other members of the amidotransferase family, where glutamate production is loosely coupled to amide transfer, we have demonstrated that hGFAT1 production of glutamate and GlcN-6-P are strictly coupled in the absence of inhibitors. Similar to other amidotransferases, competitive inhibitors that bind at the synthase site may inhibit the synthase activity without inhibiting the glutaminase activity at the hydrolase domain. GlcN-6-P, for example, inhibited the transfer reaction while fully activating the glutaminase activity at the hydrolase domain. Inhibition of hGFAT1 by the end product of the pathway, UDP-GlcNAc, was competitive with a $K_i$ of 4 $\mu$M. These data suggest that hGFAT1 is fully active at physiological levels of Fru-6-P and may be regulated by its product GlcN-6-P in addition to the pathway end product, UDP-GlcNAc.

The first step in the de novo biosynthesis of hexosamines, formation of glucosamine 6-phosphate from fructose 6-phosphate and glutamine, is catalyzed by the rate-limiting enzyme glutamine-fructose 6-phosphate amidotransferase, EC 2.6.1.16, (GFAT1). The bacterial form, glucosamine 6-phosphate synthetase, has been purified to homogeneity and studied extensively. The properties and enzyme mechanism of the amide transfer were reviewed for glucosamine 6-phosphate synthetase (1) and other members of the N-terminal nucleophile (NTN) class (2, 3). Like other NTN amidotransferases, GFAT is a modular enzyme with two distinct domains (1). The crystal structures of the separate hydrolase and synthase domains of glucosamine 6-phosphate synthetase have been solved and are highly homologous to the other NTN amidotransferases (4–6).

Early studies by Ghosh et al. (7) illustrated that liver GFAT catalyzes an irreversible reaction in which the transfer of the amino group from L-Gln and isomerization of Fru-6-P yields the products L-Glu and D-GlcN-6-P. Unlike other amidotransferases in this class, neither bacterial nor mammalian forms of GFAT are capable of utilizing $NH_3$ as a nitrogen donor (7–9). Huynh et al. (10) reported the characterization of rat liver GFAT, and Milewski et al. (8) reported the biochemical properties of the Candida enzyme, which exhibits similarities to the mammalian forms. Recent descriptions of the GFAT1 isoform, GFAT1A1t, demonstrated that GFAT1 and GFAT1A1t differ in their sensitivity to inhibition by UDP-GlcNAc (11, 12). These studies agree that feedback regulation by the pathway end product, UDP-GlcNAc, is a common feature of all mammalian GFATs studied to date, unlike the bacterial forms (13, 14). The products GlcN-6-P and L-Glu are weak inhibitors of the Escherichia coli GFAT (15); feedback inhibition of eukaryotic GFAT by GlcN-6-P has not been reported. Although biochemical studies of several mammalian forms of GFAT have been reported previously, the instability of the enzyme preparations and its relatively low abundance have prevented detailed studies of the pure mammalian enzymes.

The increased production and tissue accumulation of UDP-GlcNAc, the end product of the hexosamine pathway, has recently been implicated in the development of insulin resistance (16–22). Increasing the cellular level of UDP-GlcNAc by modest overexpression of GFAT1, or the provision of exogenous GlcN, can induce insulin resistance both in vivo and in cultured cells.

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adipocytes (20–23). The mechanism by which GlcN exerts these physiological effects is unclear. The hypothesis has been proposed that elevated cytosolic UDP-GlcNAc promotes the hyperglycosylation of Ser or Thr phosphorylation sites, thereby disrupting insulin signaling pathways (24, 25). The possible role for hexosamine biosynthesis during the pathological development of insulin resistance highlights the need for thorough biochemical characterization of hGFAT1.

Previous studies have measured the kinetic properties of mammalian GFAT1 using one of three assay methods: the Morgan Elson colorimetric reaction with GlcN 6-phosphate, derivitization of GlcN 6-phosphate by orthothaldehyde, or coupling l-Glu formation to glutamate dehydrogenase and production of NADPH. Using these methods, previously reported $K_m$ values range between 0.2 and 1.0 mM for Fru-6-P and 0.4 and 2 mM for l-Gln (10, 13, 26–28). The presence of phosphoglucoisomerase in some studies was carefully monitored and was recognized as a competing activity that can rapidly convert Fru-6-P to Glu-6-P, confusing any kinetic measurements at the synthase domain. However, because of their relative insensitivity, both the Morgan Elson and Glu dehydrogenase methods require appreciable GlcN-6-P accumulation. In this study we report the kinetic characterization of hGFAT1 using a sensitive method similar to that reported by Callahan et al. (29) which allows the measurement of GFAT activity with less than 0.2 μM product accumulation (30). Our results indicate that hGFAT1 binds Fru-6-P with much greater affinity than previously realized. Additionally our data demonstrate that the GFAT amidotransferase product, GlcN-6-P, is a potent feedback inhibitor of hGFAT1.

EXPERIMENTAL PROCEDURES

Materials—Ion exchange resin AG1 × 8 (400 mesh, formate form) and disposable polypropylene columns were purchased from Bio-Rad. The custom synthesis of [U-14C]Fru-6-P (300 mCi/mmol) was performed by American Radiolabeled Chemicals, Inc., St. Louis, MO, according to the methods described elsewhere (30). l-[14C]Gln (300 mCi/mmol) was purchased from Amersham Biosciences. Other reagents were purchased from Sigma.

Overexpression and Purification of hGFAT1—Purified hGFAT1 was overexpressed by infecting S9F cells with viral vector containing the hGFAT1 transcript as described (31). The cDNA clone of hGFAT1 was a kind gift of Dr. Donald McClain, University of Utah School of Medicine (32). The overexpressed hGFAT1 was purified from the cytosolic fraction by chromatography on Q-Sepharose and hydroxyapatite (10).2 The purified enzyme concentrate (1–4 mg/ml) was stored with 1 mM Fru-6-P in Buffer A (50 mM MOPS, pH 7.0, 1 mM DTT, 1 mM EDTA, 10 mM KCl, Roche Molecular Biochemicals complete protease mixture, 10% glycerol) at −70 °C with no detectable loss of enzymatic activity for at least 6 months. Just prior to assay, the enzyme sample was diluted into assay buffer containing 10 mM KCl, 1 mg/ml bovine serum albumin, 20 mM imidazole buffer, pH 6.8, 1 mM EDTA, 1 mM DTT, and 10% glycerol. Residual Fru-6-P (maximally 0.2 μM in the assay) was included in the calculation of total available substrate.

Determination of GlcN-6-P Production—The direct measure of [U-14C]GlcN-6-P formed from [U-14C]Fru-6-P by hGFAT1 was determined by an ion exchange method, which separates the product and substrate on small disposable columns (30). Assay of hGFAT1 activity was measured at room temperature for initial rate measurements. The reaction with hGFAT1 was initiated by the addition of enzyme to the substrate mixtures. The reaction was terminated by adding 1 ml of 10 mM sodium formate, pH 3.0. Diluted reactants were immediately applied to a 0.75 ml bed volume AG1 × 8 columns equilibrated in the same buffer. [14C]GlcN-6-P eluted from the column without binding in a 7-ml wash (10 mM sodium formate, pH 3.0) and was collected in scintillation vials, mixed with Ultima Gold XR (Packard), and counted in a scintillation counter. Experiments to measure the kinetic constants of inhibitors, or to measure the IC50 of an inhibitor, were conducted as described above with the addition of enzyme to initiate the assay.

2 Q. Khai Huynh, H. Boddupalli, E. Gulve, and T. Dian, manuscript in preparation.

**RESULTS**

Stabilization of hGFAT1 by Fru-6-P—Many previous reports have documented the instability of mammalian GFAT activity (13, 14, 27, 28). The inability to isolate a stable enzyme preparation has been a serious obstacle to biochemical studies and has prevented a full kinetic characterization of mammalian GFAT. Further, some properties of the unstabilized enzyme preparations may affect the results of biochemical studies. To allow the thorough and accurate characterization of hGFAT1, we first identified conditions that preserved its activity and properties during isolation and storage. We evaluated the ability of 10 mM l-Gln, 0.5 mM UDP-GlcNAc, 1 mM Fru-6-P, and DTT to protect the purified hGFAT1 preparations from loss of activity with time. Shown in Fig. 1, inclusion of Fru-6-P and DTT effectively protected hGFAT1 from the rapid loss of activity.
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amidotransferase activity at 4 °C. UDP-GlcNAc and glutamine (data not shown) slowed the rate of enzyme decay, but were far less effective than Fructose-6-P. Reducing agents alone did not stabilize hGFAT1; the half-life of hGFAT1 was 2 h in the absence and 4 h in the presence of 1 mM DTT. Conversely, without a reducing agent, 1 mM Fructose-6-P alone extended the hGFAT1 half-life to 4 h. Following an initial loss of activity, hGFAT1 amidotransferase activity was stable for several days at 4 °C in the presence of 1 mM Fructose-6-P and 1 mM DTT (75% of initial activity at 48 h). Inclusion of either 1 mM Fructose-6-P or GlcN-6-P with 10 mM DTT preserved 96% of hGFAT1 activity for 48 h. However, neither UDP-GlcNAc (half-life is 18 h) nor 1 mM DTT (67% residual activity at 48 h) were as effective for long term stabilization of hGFAT1. In similar studies conducted at room temperature, Fructose-6-P slowed but did not prevent the loss of GFAT1 activity (data not shown.) All enzyme preparations were isolated and stored in buffers containing 1 mM Fructose-6-P and 1 mM DTT unless specified in the studies described below.

Coupling of Amidotransferase and Glutaminase Activities of hGFAT1—The coupling of the amidotransferase activity with the hydrolysis of L-Gln to L-Glu was measured by measuring the production of radiolabeled GlcN-6-P in parallel assays. Three samples of hGFAT1 were evaluated: the insect cell cytosolic extract containing overexpressed hGFAT1 (extract), DEAE column purified fraction without DTT during purification (DEAE), and a replicate preparation column purification contained 1 mM DTT (DEAE + DTT). Data are expressed as the molar ratio of Glu divided by moles of GlcN-6-P produced. In the crude extract and in the DEAE-purified sample protected with 1 mM DTT, the molar ratio of Glu/GlcN-6-P was near 1. Uncoupled glutaminase activity was a 5-fold molar excess when hGFAT1 was purified without DTT. The error bars represent the standard deviation of three separate experiments.

The apparent Km for Fructose-6-P and L-Gln was determined by measuring the rate of GlcN-6-phosphate production at a constant L-Gln concentration (2 mM.) The Km was determined by measuring the rate of GlcN-6-phosphate production at increasing L-Gln concentrations at 50 μM Fructose-6-P. The measured Km for L-Gln was 285 μM.

FIG. 2. Coupling of hGFAT1 glutaminase and amidotransferase activities. The rate of hydrolysis of L-Gln to L-Glu and the rate of production of GlcN-6-P were measured in parallel assays as described under “Experimental Procedures.” Insect cell cytosolic extract containing overexpressed hGFAT1 (extract), DEAE column purified fraction without DTT during purification (DEAE), and a replicate preparation column purification contained 1 mM DTT (DEAE + DTT). Data are expressed as the molar ratio of Glu divided by moles of GlcN-6-P produced. In the crude extract and in the DEAE-purified sample protected with 1 mM DTT, the molar ratio of Glu/GlcN-6-P was near 1. Uncoupled glutaminase activity was a 5-fold molar excess when hGFAT1 was purified without DTT. The error bars represent the standard deviation of three separate experiments.

FIG. 3. Km determination for Fructose-6-P and L-Gln. A, the Km was determined for Fructose-6-P by measuring the rate of GlcN-6-phosphate production at a constant L-Gln concentration (2 mM.) The Km was derived from the data by curve fitting or from linearized double reciprocal plots (inset). The average Km from 5 determinations was 7.1 μM for Fructose-6-P. B, the Km for L-Gln was determined from the initial rate of GlcN-6-P production at increasing GlcN concentrations at 50 μM Fructose-6-P. The measured Km for L-Gln was 285 μM.

The apparent Km values of hGFAT1 for Fructose-6-P and L-Gln were determined by measuring the initial rate of GlcN-6-P formation at varying substrate concentrations while holding the opposing substrate at a constant saturating concentration. Assay parameters of length and enzyme concentration were chosen so that GlcN-6-P accumulation did not exceed either 2 μM or 5% of the available substrate by the end of the incubation period. Plots of the rate of GlcN-6-P formation with increasing concentrations of Fructose-6-phosphate are shown in Fig. 3A. When these data were fitted to the Michaelis-Menten equation and solved for the Km, using curve fitting software (Grafit version 4.0.13), a Km of 7 μM was derived for Fructose-6-P (n = 5). The results of similar experiments to measure the Km for L-Gln are shown in Fig. 3B. Both methods of deriving the Km yielded values of 260 μM for L-Gln. Evaluation of both substrate saturation curves for Fructose-6-phosphate showed cooperative interaction suggested that neither substrate exhibited competitive binding (Hill coefficients were 1.0 and 1.1 for Fructose-6-P and L-Gln, respectively.) The Km for L-Gln was similar to that reported previously (10, 12, 13, 27, 28). However, the Km we measured for Fructose-6-P was at least an order of magnitude more effective than that of the L-Gln.
lower than any previous report.

**Determination of the Kᵢ of GlcN-6-P and UDP-GlcNac for hGFAT1**—Early experiments indicated that the product of hGFAT1, GlcN-6-P, had substantial inhibitory activity. The inhibition constant of GlcN-6-P for hGFAT1 was measured in end point assays detecting GlcN-6-P production, shown in Fig. 4. GlcN-6-P was found to be a potent competitive feedback inhibitor of the amidotransferase activity of hGFAT1. A Kᵢ of 6 μM was measured from plotting the Kᵢ( app) versus inhibitor and from curve fit analysis. The inhibition of hGFAT by the pathway end product, UDP-GlcNac, was also measured in similar experiments (data not shown).

**Inhibition of hGFAT1 by Analogs of Fru-6-P and UDP-GlcNac**—The selective inhibition of hGFAT1 by GlcN-6-P and UDP-GlcNac was explored using several analogs. Shown in Table I are the results of that study where IC₅₀ values were measured in the amidotransferase assay. Similar to the *E. coli* GFAT1, the transition state analog, 2-amino-2-deoxyaminoglycitol 6-phosphate, was a potent inhibitor of hGFAT1 (1). The calculated Kᵢ for that compound, 0.8 μM, suggests that it has greater affinity for the hGFAT1 active site than the product GlcN-6-P. Other Fru-6-P analogs were less potent inhibitors of hGFAT1. Human GFAT1 was highly specific for the nucleotide sugar, UDP-GlcNac, as UDP-GalNac was much less potent as an inhibitor. Nucleoside monophosphates acted as weak inhibitors of hGFAT1, whereas other nucleotides did not inhibit at any concentration tested. A strict dependence upon the presence of the phosphate for any monosaccharide inhibitor was observed; neither Fru nor GlcNac inhibited hGFAT1. These data suggest that the interaction between amino acids in the active site of hGFAT1 and the charged phosphate and amino groups of GlcN-6-P contribute significantly to the free energy of binding to that site.

**Uncoupling of Amidotransferase and Glutaminase Activities by hGFAT1 Inhibitors**—The inhibition of hGFAT1 by UDP-GlcNac has been measured previously using assays that monitor either GlcN-6-P or L-Glu production. We compared the ability of UDP-GlcNac, GlcN-6-P, and UMP to inhibit both the amidotransferase and glutaminase activities of hGFAT1. Shown in Fig. 5, at 1 mM concentrations of each inhibitor, essentially complete inhibition of the amidotransferase reaction was measured with all of the inhibitors. However, despite the blockade of the transfer reaction, the glutaminase activity was completely blocked only by UDP-GlcNac. Modest inhibition of the glutaminase activity by UMP was observed under conditions that inhibited the amidotransferase reaction by 80%. In the case of GlcN-6-P, an inhibitor concentration that completely blocked the transfer reaction fully preserved the rate of L-Glu hydrolysis to L-Glu. In assays where Fru-6-P was not added to levels above that contained within the enzyme buffer solution, stimulation of the glutaminase activity was observed by the addition of exogenous GlcN-6-P (data not shown). Because the fully activated glutaminase rate was measured at a concentration of GlcN-6-P that completely interfered with the transfer reaction, it seems likely that the Fru-6-P site was fully saturated with GlcN-6-P. Yet the glutaminase activity remained fully activated. These data suggest that GlcN-6-P (or GlcNac-6-P) was bound to the Fru-6-P site of hGFAT1 and preserved the conformation of the activated glutaminase domain. The maximal glutaminase rate was observed with three ligands tested in this experiment: Fru-6-P, GlcN-6-P, and UDP-GlcNac.

![Inhibition of hGFAT1 by GlcN-6-P](image1)

**FIG. 4. Inhibition of hGFAT1 by GlcN-6-P.** The Kᵢ for GlcN-6-P was determined for the Fru-6-P site. Fitting the data to a competitive model using curve fit analysis derived the Kᵢ of 6 μM. The linearized plot also indicates that GlcN-6-P acts as a competitive inhibitor of Fru-6-P.

![Uncoupling of the hGFAT1 amidotransferase and glutaminase activities by inhibitors](image2)

**FIG. 5. Uncoupling of the hGFAT1 amidotransferase and glutaminase activities by inhibitors.** Inhibitors of hGFAT1 production of GlcN-6-P were tested for their effects on the glutaminase activity. In parallel assays that separately measured production of GlcN-6-P or L-Glu, 1 mM GlcN-6-P, UDP-GlcNac, UMP, and GlcNac-6-P were each tested for inhibition of L-Glu and GlcN-6-P formation. Open bars, [14C]GlcN-6-P product formation; shaded bars, [14C]Glu product formation. Data presented are the average of two separate experiments consisting of duplicate measurements.

**TABLE I**  
**Inhibitor specificity of hGFAT1**  
The IC₅₀ of several potential inhibitors of hGFAT1 were measured in assays that detected synthesis of GlcN-6-P. The IC₅₀ was calculated using a SAS assisted curve fit program. For these experiments, the substrate concentrations were 20 μM Fru-6-P and 400 μM L-Glu in the assay buffer described under “Experimental Procedures.”
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**DISCUSSION**

Increased hexosamine biosynthesis resulting in the increased production and tissue accumulation of UDP-GlcNAc has recently been implicated as a key step in the development of insulin resistance (16–22, 34). Modest overexpression of hGFAT1 in fat and skeletal muscle of transgenic mice, elevating enzyme activity and UDP-GlcNAc pools only 2-fold, was sufficient to confer insulin resistance (23, 35). Elevated GFAT activity has been demonstrated in biopsies of skeletal muscle from patients with Type II diabetes (36). Serum HbA1c levels, which reflect a patient’s cumulative glycemia, also correlate with GFAT activity (36). These data, given that GFAT catalyzes the first committed and rate-limiting step in the pathway for de novo biosynthesis of GlcN, implicate GFAT1 as a potential target enzyme for modulating or preventing insulin resistance. Efforts to understand the role that GFAT plays in normal physiology and disease have increased focus on the biochemical characterization and regulation of GFAT. A second gene, GFAT2, has recently been reported (37). The relative role of GFAT1 and GFAT2 in normal physiology and in disease states has yet to be elucidated. The discovery that the splice variant, GFAT1Alt, has a lower $K_i$ for UDP-GlcNAc than GFAT1 emphasizes the importance of understanding the expression and regulation of GFAT isoforms (12). Prior to the cloning and overexpression of the mammalian enzymes, biochemical studies of GFAT have been limited by the practical challenges of enzyme instability and assay sensitivity. The biochemical characterization of the hGFAT1 described in this report capitalizes on the ability to stabilize the enzyme during isolation and upon the use of a sensitive assay for the amidotransferase activity.

The high affinity of the product, GlcN-6-P, for the Fru-6-P site of hGFAT1 has several important consequences. First, it seems likely that previous attempts to measure the kinetic parameters of mammalian GFAT were affected by product inhibition. GlcN-6-P accumulation would be predicted to have the greatest impact on kinetic measurements at the Fru-6-phosphate site. In this study, the $K_m$ measured for Fru-6-P was at least an order of magnitude lower than from previous reports, whereas the $K_i$ for L-Gln was similar to previous reports with mammalian GFAT (10, 12, 13, 27, 28). In our studies, the $K_m$ for Fru-6-P and the $K_i$ for GlcN-6-P were roughly equivalent. Consequently, one might predict that results from methods requiring GlcN-6-P accumulation to levels above the $K_m$ may be affected by product inhibition. The glutaminase assay coupled to glutamate dehydrogenase or the Morgan-Elson colorimetric assay requires at least 10 $\mu$M product accumulation (13, 27, 28). The $K_m$ for Fru-6-P measured with overexpressed mouse GFAT1 and GFAT1Alt also differs significantly from that of hGFAT1 in this report (12). Although species or methodological differences might affect these measurements, it is also possible that the presence of phosphoglucoisomerase activity in cytosolic extracts can rapidly deplete Fru-6-P (30). Direct comparison of the biochemical properties of purified GFAT isoforms should be undertaken to better understand the regulation of this important enzyme.

The feedback inhibition of GFAT by the pathway end product, UDP-GlcNAc, was previously demonstrated as a key regulatory mechanism for limiting hexosamine biosynthesis by eukaryotic GFAT (12, 13, 27, 28, 38). Differences in the magnitude of measured $K_i$ for UDP-GlcNAc have not been resolved for mammalian GFAT isoforms. Tourian et al. (38) reported inhibition of human fibroblast GFAT by $>25 \mu$M UDP-GlcNAc. Also, the measured $K_i$ for mGFAT1 (54 $\mu$M) was significantly higher than that reported in this study (12). Although the absolute values differ for the measured $K_i$ perhaps due to differences in assay methodologies, these findings consistently demonstrate that UDP-GlcNAc is a key feedback regulator of mammalian GFAT isoforms.

The next step in the hexosamine biosynthetic pathway after the conversion of Fru-6-P to GlcN-6-P is the acetylation of GlcN-6-P. The biosynthesis of GlcNAc-6-P has previously been thought to rapidly remove GlcN-6-P from the cytosol because very low levels of GlcN-6-P have been measured in bovine thyroid (39). The concentration of GlcN-6-P in relevant tissues such as skeletal muscle or adipose is unknown. Our results demonstrate that GlcN-6-P was a potent (while GlcNAc-6-P was a weak) feedback inhibitor of hGFAT1. Unlike the complete inhibition of both transferase and glutaminase activities by UDP-GlcNAc, inhibition of hGFAT1 by GlcN-6-P fully activated the glutaminase activity of hGFAT1. The metabolic consequence of GlcN-6-P inhibition of GFAT1 to the cell or tissue would be to block synthesis of GlcN-6-P while promoting the hydrolysis of L-Gln to ammonia and L-Glu. Whether the cellular levels of GlcN-6-P achieve a sufficient level to be physiologically relevant before rapid acetylation depletes the pool of GlcN-6-P, thereby relieving product inhibition, is not known.

Native hGFAT1 is tightly coupled between the glutaminase and transferase reactions. Data included in this report illustrate that uncoupling of the glutaminase and transferase ac-
activities can be observed in preparations that have not been fully protected from oxidation. It seems likely that the uncoupling we observed was an artifact of the isolation procedure. However, it is important to note that enzyme preparations with these characteristics might be unusual in other biochemical parameters. Enzyme samples that exhibited uncoupled amidotransferase and glutaminase activities also contained a UDP-GlcNAc-resistant enzyme activity. The loss of GFAT inhibition by UDP-GlcNAc has been reported previously and is thought to implicate loss of normal function (13, 14, 29, 40, 41). Winterburn and Phelps (14) limited their studies of liver GFAT to those preparations that were inhibited “fully” by UDP-GlcNAc. The protection of hGFAT1 by reducing agents such as dithiothreitol suggests that oxidation of a sensitive Cys may affect the conformation of the enzyme, or its ability to undergo conformational changes. Because the glutaminase activity was not affected by the oxidizing environment, it seems likely that the affected residues were distinct from Cys-1, which is required for catalysis (1, 2). The efficiency of hGFAT1 amide transfer and the ability of UDP-GlcNAc to inhibit hGFAT1 might be affected by conformational changes resulting from oxidation of hGFAT1 at a site distal to the glutaminase domain.

As a member of the NTN amidotransferase class, hGFAT1 likely shares the mechanism of hydrolysis and ammonia transfer that has been demonstrated for the other members of this class (1–4). Solution of the crystal structures of bacterial PRPP-amidotransferase and asparagynase synthetase revealed a hydrophobic channel that facilitates diffusion of ammonia from the glutaminase active site to the acceptor substrate site (43–47). Teplyakov et al. (5, 6) speculated that residues adjacent to the glutaminase active site of GFAT might represent the mouth of the ammonia channel. Because the GFAT domains have been crystallized separately, evidence confirming a common mechanism awaits the solution of the GFAT holoenzyme crystal structure (5, 6, 48). In the case of PRPP-amidotransferase, which is also tightly coupled between glutaminase and transferase reactions, the binding of the first substrate triggers a conformational change that rearranges the glutaminase active site (43, 44). This conformational rearrangement of the glutaminase domain increases both the catalytic efficiency and the affinity for substrate (1–3). Kinetic characterization of all three members of this class has illustrated the common feature of glutaminase activation by the acceptor substrate (1–3, 15).

For PRPP-amidotransferase the crystallographic studies demonstrated that the ammonia channel is formed only in the presence of bound substrates (43–46). These dramatic conformational rearrangements, which constitute the mechanism of ammonia transfer for the NTN class of amidotransferases, may conversely make them more susceptible to damage by chemical or mechanical manipulations.

The activation of the hGFAT1 glutaminase domain by the product GlcN-6-P suggests that occupancy of the Fru-6-P site by GlcN-6-P is sufficient to invoke the conformational rearrangements that form the activated glutaminase domain of hGFAT1. Badet et al. (15, 49) demonstrated that E. coli GFAT follows an ordered bi-bi mechanism where Fru-6-P binds first, enabling the binding of L-Gln. Following hydrolysis of glutamine and amide transfer, GFAT releases the products glutamate and GlcN-6-P. E. coli GFAT is weakly inhibited by L-Glu, although the separate glutaminase domain binds L-Glu with high affinity (4, 15). However, hGFAT1 was not inhibited by L-Glu (10 mM L-Glu) (data not shown). The effect of GlcN-6-P on hGFAT1 is consistent with hGFAT1 following an ordered bi-bi mechanism; however, direct demonstration of the mechanism should be conducted. Our finding that the glutaminase of hGFAT1 was fully activated by saturating concentrations of GlcN-6-P suggests that the conformation of hGFAT1 does not relax to the basal state until the release of GlcN-6-P. This property of hGFAT1 is not unprecedented; uncoupling of the glutaminase and transferase activities by product or other inhibitors has been observed with asparagynase synthetase (4). Whether inhibition of the transferase activity with retention of glutaminase activity is physiologically relevant is not known. However, biochemical studies conducted with glutaminase activity as the sole measure of hGFAT1 amidotransferase activity should be validated by measurement of GlcN-6-P production in those cases where product accumulation or possible inhibition at the Fru-6-P site might have disparate effects on the glutaminase and transferase activities of hGFAT1.

In conclusion, we have demonstrated that hGFAT1 exhibits a high affinity binding site for the substrate, Fru-6-P. Because the K_m for Fru-6-P measured in this study was well below the estimated cytosolic concentration of substrate in normal tissues, hGFAT1 activity should not be limited by substrate availability (26). The primary biochemical regulation of hGFAT1 may be accomplished through feedback inhibition by GlcN-6-P and the pathway end product UDP-GlcNAc.

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