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Enzymatic analysis of UDP-*N*-acetylglucosamine

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Abstract

The *Methanococcus maripaludis* MMP0352 protein belongs to an oxidoreductase family that has been proposed to catalyze the NAD⁺-dependent oxidation of the 3"-position of uridine diphosphate *N*-acetyl-D-glucosamine (UDP-GlcNAc), forming a 3-hexulose sugar nucleotide. The heterologously expressed MMP0352 protein was purified and shown to efficiently catalyze UDP-GlcNAc oxidation forming one NADH equivalent. This enzyme was used to develop a fixed endpoint fluorometric method to analyze UDP-GlcNAc. The enzyme is highly specific for this acetamido sugar nucleotide, and the procedure had a detection limit of 0.2 μM UDP-GlcNAc in a 1-ml sample. Using the method of standard addition, UDP-GlcNAc concentrations were measured in deproteinized extracts of *Escherichia coli*, *Saccharomyces cerevisiae* and HeLa carcinoma cells. Equivalent concentrations were determined by both enzymatic and chromatographic analyses, validating this method. This procedure can be adapted for the high-throughput analysis of changes in cellular UDP-GlcNAc concentrations during time series experiments or inhibitor screens.

Introduction

Cells from all three domains of life use *N*-acetyl-D-glucosamine (GlcNAc)¹ in their cell walls, extracellular matrices, protein post-translational modifications or glycolipids [1]. The uridine diphosphate activated form of this sugar (UDP-GlcNAc) is the universal GlcNAc donor for the biosynthesis of all these structures, as well as the precursor for many modified acetamido sugars. In mammalian cells, external glucose or glucosamine levels affect the UDP-GlcNAc concentration, which can modulate the levels of protein *O*-GlcNAc modification [2]. Inhibitors of bacterial protein synthesis cause an increase in the concentrations of peptidoglycan precursors, including UDP-GlcNAc [3]. These physiological changes in UDP-GlcNAc pools have created a need for rapid assays to analyze the large number of samples produced during time course experiments [4].

All cells contain a complex mixture of ribonucleotides and deoxyribonucleotides along with a variety of sugar nucleotides that complicate analyses. Many of these molecules have similar charges and spectral properties. In an extreme case, cells can contain three different epimers: UDP-GlcNAc, UDP-*N*-acetyl-D-galactosamine (UDP-GalNAc) and UDP-*N*-acetyl-D-mannosamine. Some microorganisms also produce hexuronate sugar nucleotides or 4"-

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¹The abbreviations used are: GlcNAc, *N*-acetyl-D-glucosamine; UDP-GlcNAc, uridine diphosphate *N*-acetyl-D-glucosamine; UDP-GalNAc, UDP-*N*-acetyl-D-galactosamine; CHES, 2-(cyclohexylamino)ethanesulfonate; TCA, trichloroacetic acid; and DTT, dithiothreitol.

deoxysugar nucleotides with similar properties. Because glycosyltransferase enzymes discriminate among these analogs, it is important to develop highly specific analytical methods.

Previous analyses of UDP-GlcNAc used HPLC or capillary electrophoresis to determine intracellular concentrations of nucleotides and sugar nucleotides, quantitatively detecting their base absorbance at 254 nm. Ion-pair reversed-phase chromatography resolved 8 sugar nucleotides using a 42 min HPLC method, but could not separate UDP-GlcNAc from UDP-GalNAc [5]. An improved ion-pair reversed-phase chromatographic method offered some separation of these two epimers in a 35 min analysis [6]. High performance anion exchange chromatography using a CarboPac PA1 column separated 20 nucleotides and sugar nucleotides, although UDP-GlcNAc and UDP-GalNAc peaks overlapped in that 55 min method [7]. Ion chromatography afforded improved separation of UDP-GlcNAc and UDP-GalNAc in a 65 min method [8]. Lectin affinity chromatography showed significant improvements in discrimination between epimers, but has not been widely adopted [9]. Liquid chromatography-tandem mass spectrometry operated in multiple reaction monitoring mode was used to determine sugar nucleotides in three trypanosomatids during 45 min analyses [10]. Capillary electrophoresis showed excellent separation of UDP-GlcNAc and UDP-GalNAc in a method that required at least 18 min [11]. A similar capillary zone electrophoresis method was used to resolve the sugar nucleotides from *Giardia intestinalis* [12]. While each of these methods simultaneously determines many sugar nucleotide concentrations, they are limited by low-throughput, difficulty resolving sugar nucleotide epimers or high fixed costs.

While investigating the biosynthesis of an unusual 2,3-diacetamidoglucose sugar in the methanogen *Methanococcus maripaludis*, we identified a novel UDP-GlcNAc oxidoreductase enzyme encoded by the gene at locus MMP0352. The protein is homologous to the GnnA protein from *Acidithiobacillus ferrooxidans* that acts in concert with the GnnB aminotransferase to convert UDP-GlcNAc to 3-amino UDP-GlcNAc [13]. In that study, no UDP-GlcNAc oxidoreductase activity was detected in reactions containing only UDP-GlcNAc, NAD⁺ and recombinant GnnA protein. Both the MMP0352 and *A. ferrooxidans* GnnA proteins are homologous to the uncharacterized *Pseudomonas aeruginosa* WbpB and *Bordetella pertussis* WlbA proteins [14;15]. All of these proteins are believed to catalyze similar reactions involving the oxidation of the 3''-position of a UDP-acetamido sugar to produce a 3-hexulose nucleotide (Figure 1). Alternatively, the *Streptomyces fradiae* pathway for mycaminose production involves the biosynthesis of an analogous TDP-6-deoxy-3-keto sugar using 6''-dehydratase and 3'',4''-isomerase enzymes [16].

We show here that heterologously expressed, purified MMP0352 protein catalyzes the NAD⁺-dependent oxidation of UDP-GlcNAc in an alkaline buffer with a methoxyamine trapping agent. This enzyme was used to develop a sensitive and specific fixed endpoint assay for UDP-GlcNAc based on the fluorescence of the NADH product. As little as 0.2 nmol UDP-GlcNAc could be detected in a 1-ml reaction after 1 h incubation. A standard addition method was used to determine concentrations of this sugar nucleotide in extracts from *Escherichia coli*, *Saccharomyces cerevisiae* and HeLa carcinoma cells. These values were equivalent to chromatographically determined UDP-GlcNAc concentrations, and were consistent with values from the literature. This method can provide a high-throughput alternative to chromatographic analysis of UDP-GlcNAc in a complex matrix of deproteinized cell extract.

Materials and Methods

Cloning and molecular biology

The gene at *M. maripaludis* locus MMP0352 was amplified by PCR using oligonucleotide primers 5MMP0352BN (5'-CGAGGATCCCATATGTGTTAAAAGTGGCAGTTG-3') and 3MMP0352B (5'-GCAGGATCCTTAATTACCGTTAGAGCTTTTC-3') (Invitrogen) and *M.*

maripaludis S2 chromosomal DNA. The product was ligated in the NdeI and BamHI sites of vector pET-19b (Novagen) to produce vector pDG441. Plasmids were propagated in *E. coli* DH5 α cells (Invitrogen), and protein was expressed using *E. coli* BL21(DE3) cells (Novagen). Recombinant DNA was sequenced at the Institute for Cellular and Molecular Biology Core Labs DNA Sequencing facility (UT-Austin), using T7 and T7-terminator primers. The MMP0352 protein sequence has the RefSeq accession number NP_987472.1.

Protein expression and purification

The amino-terminal polyhistidine-tagged MMP0352 protein (His₁₀-MMP0352) was heterologously expressed in *E. coli* BL21 (DE3) (pDG441) cells and purified by Ni²⁺-affinity chromatography using standard methods [17]. The molecular mass and purity of the protein was estimated by SDS-PAGE using the Laemmli buffer system and 12% total acrylamide. The apparent mass and Stokes radius of the native protein were determined by analytical size exclusion chromatography [18]. For the determination of UDP-GlcNAc, the His₁₀-MMP0352 protein was desalted using a HiTrap Sephadex G-25 column (5 ml, GE Healthcare) in 20 mM Tris-HCl (pH 8.0). The total protein concentration was determined using the Bradford protein assay with bovine serum albumin as a standard. The purified protein was stored at -80°C in a solution containing 15 mM Tris-HCl (pH 8) and 20% (v/v) glycerol.

UDP-GlcNAc dehydrogenase assay

Continuous assays were performed using a DU-800 spectrophotometer attached to a Peltier temperature-controlled stage (Beckman Coulter). Reactions (300 μ l) containing 1 mM tris-(2-carboxyethyl)phosphine (TCEP), 200 mM KCl, 2 mM NAD⁺, 50 mM Tris-HCl (pH 8.5), and 0.3 μ g His₁₀-MMP0352 were pre-incubated at 37°C for 4 min in a quartz cell (Starna). The reactions were then initiated with 30 to 400 μ M UDP-GlcNAc substrate. The reduction of NAD⁺ to NADH was monitored by following the increase in absorbance at 340 nm at 37°C. The linear portion of the reaction progress curve provided the initial rates, using a molar absorptivity of 6.2 mM⁻¹ cm⁻¹ for NADH. One unit of dehydrogenase activity catalyzed the conversion of 1 μ mole substrate to product per min. Initial rate data were fitted to the Michaelis-Menten-Henri equation using nonlinear regression (KaleidaGraph program, Synergy Software) to estimate the apparent steady-state rate constants. To test the inhibitory properties of substrate analogs, standard reactions were initiated with mixtures containing 0.2 mM UDP-GlcNAc and various concentrations of analogs.

Development of a fluorescent assay for UDP-GlcNAc dehydrogenase activity

To optimize the assay, reactions (1 ml) contained various concentrations of UDP-GlcNAc (1 μ M to 4 μ M), 60 mM potassium chloride, 90 to 300 μ M NAD⁺, 5 or 10 μ g His₁₀-MMP0352, and buffer at pH 9.5. The buffer salts tested were 0.15 M glycine-HCl, 30 mM ammonium bicarbonate, 30 mM 2-(cyclohexylamino)ethanesulfonate [CHES]-KOH or 30 mM sodium borate. Some reactions contained 30 mM methoxyamine hydrochloride to trap carbonyl products. These mixtures were incubated at 37°C for 1 h. NADH fluorescence was measured in an acrylate cuvette using a FP-6300 spectrofluorometer (Jasco) with an excitation wavelength of 340 nm and emission measured at 460 nm, each with band widths of 10 nm.

Determination of UDP-GlcNAc in cell extracts

The continuous standard variation method of standard addition was used to determine the concentration of UDP-GlcNAc in deproteinized cell extracts. A typical reaction mixture (1 ml) contained 150 μ M NAD⁺, 60 mM KCl, 30 mM methoxyamine, 15 μ l of neutralized cell extract, 5 μ g His₁₀-MMP0352 and 30 mM CHES-KOH (pH 9.5). Similar reactions supplemented with 1 to 3 μ M UDP-GlcNAc were prepared and incubated simultaneously. Two control reactions were prepared to measure background fluorescence. The first control solution omitted cell

extract and UDP-GlcNAc, measuring the rate of enzyme-catalyzed NAD⁺ adduct formation. The second control reaction contained 15 μ l of extract in 30 mM methoxyamine and 30 mM CHES-KOH (pH 9.5), accounting for background fluorescence in the extract. All solutions were incubated in microcentrifuge tubes at 37°C for 1 h. NADH fluorescence was measured as described above. The fluorescence due to the two control reactions was subtracted from each reaction to obtain the corrected fluorescence. A least-squares analysis of the response curve was used to estimate slope and intercept parameters and their standard errors. These values were used to calculate UDP-GlcNAc concentrations in the cell extracts, and standard errors were propagated to estimate the associated error [19]. All of the assays were performed in triplicate.

HPLC analysis of sugar nucleotide

The UDP-GlcNAc estimates from fluorometric analysis were confirmed by chromatographic analysis. Deproteinized extracts were applied to a CarboPac PA1 column (250 by 4 mm, Dionex) with a guard column (4 by 50 mm) of the same material. The analytes were separated using an ammonium acetate gradient (0.6 ml min⁻¹) and detected by a photodiode array [20]. The standard addition method was used to estimate UDP-GlcNAc concentrations from integrated peak areas. Standard compounds eluted with the following retention times: UDP-GalNAc (15.0 min), UDP-GlcNAc (15.5 min), UDP-Man (17.7 min), UDP-Glc (19.4 min) and UDP (43 min).

Cell culture and preparation of extracts

A wild-type *E. coli* B culture was obtained from the Coli Genetic Stock Center (CGSC 5365). Luria-Bertani medium (50 ml) was inoculated with 1% (v/v) of an overnight culture of *E. coli* B, followed by continuous shaking at 37°C. After the optical density at 600 nm reached 0.9, the cells were divided into equal parts of 3 mg (dry mass) each and harvested by centrifugation at 14,000 $\times$ g for 10 min. The pellets were frozen at -20°C until extraction. Each pellet was resuspended in 100 μ l of 5% trichloroacetic acid (TCA) at room temperature for 20 min. Cell debris was removed by centrifugation at 10,000 $\times$ g for 5 min. After 15 min at room temperature, the supernatant was neutralized by the addition of 15 μ l of 2.5 M potassium hydroxide in 1.5 M K₂HPO₄ and stored at -20°C. The cells' dry weight was determined after drying at 100°C for 2 days. Measurements were performed on three separate samples.

A *Saccharomyces cerevisiae* DAY4 (*MATa ser1 leu2 his4 trp1 ura3-52*) culture was a gift from Drs. Gisela Kramer and Dean Appling (UT-Austin) [21]. Rich medium containing 1% yeast extract, 2% peptone and 2% dextrose was inoculated with an overnight culture (1% v/v) of DAY4 cells and grown for 23 h with continuous shaking at 30°C to an optical density at 600 nm of 1.9. The cells were harvested and divided into aliquots of 28 mg dry mass. Glass beads (0.5 mm, 1.5 gm) and 600 μ l of 10% TCA were added to each cell pellet followed by bead-beating, using a Mini-BeadBeater homogenizer (BioSpec), for 4 min with intermittent cooling on ice. Complete lysis of the yeast cells was confirmed by microscopy. The lysed cells were centrifuged at 1,000 $\times$ g for 2 min to separate the glass beads from the lysate. This lysate was further centrifuged at 14,000 $\times$ g for 10 min at 4°C. The supernatant (170 μ l) was neutralized by adding 40 μ l of 2.5 M potassium hydroxide in 1.5 M K₂HPO₄. After centrifugation at 14,000 $\times$ g for 10 min, the supernatant was stored at -20°C.

Frozen pellets of HeLa carcinoma cells were a gift from Susan Anderson and Dr. Lara Mahal (UT-Austin). Cells were cultured in minimal essential medium/Earle's balanced salt solution supplemented with 10% fetal bovine serum, 1% sodium pyruvate, and 1% essential amino acids at 37°C in presence of 5% CO₂. After confluent growth was obtained, the cells were washed with phosphate-buffered saline, trypsinized, collected by centrifugation and stored at -80°C. The cells were suspended in 60 μ l 5% TCA and lysed using a sonifier water bath

(Branson Ultrasonics) for 15 min. Following centrifugation at $14,000 \times g$ for 10 min, the supernatant was neutralized and stored at -20°C .

Results and Discussion

Expression and purification of MMP0352

N-terminal decahistidine-tagged MMP0352 (His₁₀-MMP0352) was expressed in soluble form from *E. coli* BL21(DE3) (pDG441) cells, and it was purified by Ni²⁺-affinity chromatography. SDS-PAGE analysis showed that the protein was substantially pure, comprising 98% of the total protein (Figure 2A). The apparent molecular mass of His₁₀-MMP0352 protein was 39 kDa, close to its expected mass of 37.1 kDa. Analytical size exclusion chromatography of this protein identified a single peak corresponding to a protein with an apparent mass of 305 kDa and a Stokes radius of 55 Å, which suggests the protein forms an octamer. A total of 13.5 mg of pure His₁₀-MMP0352 protein was obtained from 4.2 g (wet mass) of cells. The protein was stored stably for at least several weeks in 20% glycerol at -80°C .

Oxidoreductase activity of MMP0352

NAD⁺-dependent oxidoreductase activity was measured using a continuous, spectrophotometric assay that monitors the increase in absorbance at 340 nm due to the production of NADH. The enzyme specifically catalyzed the oxidation of UDP-GlcNAc using NAD⁺, consistent with the reaction scheme proposed in Figure 1. No activity was detected when NAD⁺ was replaced with NADP⁺. The MMP0352 protein did not catalyze the oxidation of the substrate analogs UDP-Glc, UDP-GalNAc, *N*-acetylglucosamine or glucosamine: reaction rates for mixtures containing these compounds were below the limit of detection for enzymatic activity ($< 2 \times 10^{-5} \text{ U mg}^{-1}$). Dehydrogenase activity was unaffected by metal ions and did not require dithiothreitol (DTT) or other reductants for activity. Activity was 85% lower in the absence of KCl as compared to reactions containing 200 mM KCl.

Steady-state kinetic parameters were obtained by fitting the initial rates of oxidation at various UDP-GlcNAc and NAD⁺ concentrations to the Michaelis-Menten-Henri equation (Figure 2B). This analysis showed the MMP0352 enzyme efficiently catalyzed UDP-GlcNAc oxidation with apparent K_m and turnover values comparable to parameters reported for the UDP-GlcNAc 6"-dehydrogenases from *Pseudomonas aeruginosa* [22] and *Salmonella typhi* [20] (Table 1).

The products of the MMP0352-catalyzed reaction were analyzed by HPLC using a CarboPac PA1 column. However, no new peak corresponding to the expected 3-oxo-UDP-GlcNAc product was detected. Instead, a peak corresponding to UDP was identified, suggesting that a spontaneous elimination reaction degraded the sugar nucleotide product. The analogous TDP-6-deoxy-3-oxoglucose is also hydrolytically unstable [23]. This degradation pathway could be analogous to the proposed reaction mechanism for the family 4 NAD⁺ and Mn²⁺-dependent glucosidases [24]. Liquid chromatography-mass spectrometry analysis of the filtered reaction mixture in negative ion mode identified peaks corresponding to NADH ([M - H]⁻ at 664 *m/z*) and UDP ([M - H]⁻ at 403 *m/z*) products. Collision induced dissociation of the ion with 403 *m/z* produced a characteristic peak at 306 *m/z* corresponding to UMP. In positive ion mode, only NAD⁺ ([MH]⁺ at 664 *m/z*) and NADH ([MH]⁺ at 666 *m/z*) ions were identified. Further analysis will be required to identify the decomposed form of the enzymatic reaction product.

Potential inhibitors of MMP0352 oxidoreductase activity were screened in reactions containing 0.2 mM UDP-GlcNAc and various concentrations of substrate analogs. The following concentrations of analogs reduced UDP-GlcNAc oxidoreductase activity by 50%: 2 mM UDP,

2 mM UDP-Glc and 3 mM UDP-GalNAc. The sugars *N*-acetyl-D-glucosamine, D-glucose and D-glucosamine did not inhibit the reaction at 3 mM concentrations.

Fluorometric endpoint assay for UDP-GlcNAc determination

To develop a highly sensitive assay for UDP-GlcNAc, we used fluorescence spectroscopy to detect NADH in reactions with low, biologically relevant concentrations of UDP-GlcNAc [25]. Endpoint assays containing UDP-GlcNAc, His₁₀-MMP0352 protein and excess NAD⁺ produced a linear response in a 1-ml reaction (Figure 3). A standard curve from mock reactions containing 1 to 4 μM NADH showed a stoichiometry of one NADH molecule produced per UDP-GlcNAc molecule oxidized. Nevertheless, the slope of this standard curve was significantly higher than the slope for the endpoint assay curve ($p < 0.05$). Therefore a standard curve of UDP-GlcNAc is required to calibrate this enzymatic assay. The limit of detection ($S/N = 3$) was 0.2 μM UDP-GlcNAc and the limit of quantification ($S/N = 10$) was 0.7 μM UDP-GlcNAc for this method.

In the absence of UDP-GlcNAc the MMP0352 enzyme catalyzed nucleophilic addition reactions to NAD⁺, an activity previously observed in alcohol dehydrogenase [26]. These adducts have UV absorbance and fluorescence properties similar to NADH. This activity is negligible in initial rate assays, but can be a significant interference during prolonged incubations using high enzyme and low substrate concentrations –the usual conditions for endpoint assays. In reactions with MMP0352 enzyme, the background fluorescence increased almost twofold over a 2 h incubation period when compared to 1 h of incubation at 37°C. The net fluorescence (due to UDP-GlcNAc oxidation alone) did not appreciably increase after 1 h. The background fluorescence was 43% higher for samples in 0.15 M glycine buffer (pH 9.5) as compared to 30 mM CHES (pH 9.5) and 30 mM ammonium bicarbonate (pH 9.5). No UDP-GlcNAc dependent NADH formation was observed in 30 mM sodium borate buffer. The fluorescent signal was 21% higher in the presence of 30 mM methoxyamine when tested with 30 mM CHES (pH 9.5). The background fluorescence was more than two-fold lower with 5 μg of MMP0352 when compared to 10 μg; 5 μg of enzyme was sufficient to oxidize UDP-GlcNAc in these assays. Based on these results, standard assays for UDP-GlcNAc included 30 mM CHES-KOH (pH 9.5) and 30 mM methoxyamine buffer salts.

Measurement of UDP-GlcNAc in *E. coli*

E. coli cells use large amounts of GlcNAc in their peptidoglycan and lipopolysaccharides, so the concentration of the UDP-GlcNAc precursor is expected to be high in actively growing cells. Extraction with TCA released UDP-GlcNAc from cells and destroyed intrinsic NADH and NADPH that would interfere with this endpoint assay. The residual background fluorescence, probably due to flavin and pterin compounds, was subtracted from values measured by enzymatic analysis with MMP0352. From a standard addition curve, we determined that *E. coli* B cells contain 1.5 ± 0.24 μmoles UDP-GlcNAc per g dry cells (Figure 4). HPLC analysis of the same extracts determined a similar UDP-GlcNAc composition of 1.2 μmoles per g. Therefore enzymatic analysis provided a precise and accurate measurement of UDP-GlcNAc, with a minimal amount of sample processing.

Previous reports of the UDP-GlcNAc composition in *E. coli* B and K-12 strains ranged from 0.93 to 1.4 μmoles per g dried cells, analyzed by HPLC with reversed-phase and amine columns [3]. The UDP-GlcNAc content of cells in that study varied, depending on growth conditions. Treatment with chloramphenicol significantly increased UDP-GlcNAc concentrations (5.7 to 6.0 μmoles per g), as did tetracycline treatment (2.6 to 4.0 μmoles per g). Assuming that the dry weight of an *E. coli* cell is 2.8×10^{-13} g and the cell volume is 1 fl, the intracellular UDP-GlcNAc concentration measured here is approximately 430 μM [27].

Measurement of UDP-GlcNAc in *S. cerevisiae*

Yeast cells use GlcNAc in *N*-linked protein glycosylation, in glycosylphosphatidylinositol protein-anchors to the membrane, and in the chitin layer of their cell walls [28]. While *S. cerevisiae* cell walls have a relatively low chitin content (1-2%), the walls of other fungi contain a high proportion of chitin [29]. Metabolites were extracted from *S. cerevisiae* cells by bead-beating in the presence of TCA. The enzymatic assay measured 0.17 ± 0.03 μ moles UDP-GlcNAc per g of dry yeast cells (Figure 5). This value was confirmed by HPLC analysis, which estimated the UDP-GlcNAc concentration to be 0.14 μ moles per g. However, this sugar nucleotide pool also contained a compound that co-eluted with UDP-GalNAc, close to the UDP-GlcNAc peak. HPLC analysis of UDP-GlcNAc in eukaryotic extracts is limited by the resolving power of the method and the accuracy of peak integration.

A previous chromatographic analysis of UDP-GlcNAc in *S. cerevisiae* reported 0.4 μ moles UDP-GlcNAc per g wet cells (approximately 1.6 μ moles per g dry weight) [30]. In that report, UDP-GlcNAc concentrations increased almost 10-fold in cells grown on medium supplemented with glucosamine; however, it is not clear whether the analytical method used for those measurements (a normal phase amino HPLC column) completely separated UDP-GlcNAc from UDP-Glc and other sugar nucleotides. Assuming that the dry weight of a *S. cerevisiae* cell is 15×10^{-12} g and the cell volume is 70 fl, the intracellular UDP-GlcNAc concentration measured here is approximately 34 μ M [31].

Measurement of UDP-GlcNAc in HeLa cells

In mammalian cells, GlcNAc is a key component of *N*- and *O*- linked protein glycosylation as well as the proteoglycans that form the extracellular matrix. Enzymatic analysis determined the UDP-GlcNAc concentration of HeLa carcinoma cells to be 12×10^8 molecules per cell or 0.50 ± 0.07 μ moles per g of dry cells (assuming 19% dry weight [32]) (Figure 6). HPLC analysis confirmed that value, determining 0.44 μ moles UDP-GlcNAc per g of dry HeLa cells.

For comparison, a previous chromatographic analysis of UDP-GlcNAc concentrations in HT29 human colon cancer cells measured approximately 8×10^7 UDP-GlcNAc molecules per cell [33]. An ion-pair chromatographic analysis of mammalian CHO cells measured 5×10^7 UDP-GlcNAc molecules per cell [6]. An optimized extraction procedure for Madin–Darby canine kidney cells measured 3×10^8 UDP-GlcNAc molecules per cell [34]. Adipocytes grown without glucose contained approximately 0.04 μ moles per g of dry cells, but this value almost doubled when the cells were grown with glucose [35]. The broad range of UDP-GlcNAc concentrations found in mammalian cells illustrates the need for analytical methods to monitor sugar nucleotide pools.

Potential applications and enhancements

Recent advances in liquid chromatography and capillary electrophoresis have fostered increasingly sensitive analytical methods that identify and quantify a large number of analytes simultaneously. Each of these methods has significant fixed and variable costs. Analysis times range from 20 min to more than an hour per sample, excluding sample preparation that is required to remove interferences, concentrate analytes and protect columns or capillaries. Therefore there has been little improvement in the throughput of these methods. In contrast, the enzymatic method described here only measures UDP-GlcNAc, but requires minimal processing time and could be readily adapted to high-throughput methods using quartz microwell plates. By coupling NADH formation to tetrazolium dye reduction, it may be possible to perform this assay using a standard spectrophotometer without sacrificing sensitivity [36].

In principle, the UDP-GlcNAc 6-dehydrogenase enzyme could be used to develop a UDP-GlcNAc assay with enhanced sensitivity, because each UDP-GlcNAc molecule reduces two NAD⁺ molecules to NADH. The *S. typhi* TviB enzyme specifically catalyzed this reaction with kinetic parameters similar to those of the MMP0352 described here [20]. However, the TviB enzyme requires 10 mM DTT to prevent enzyme inactivation, probably due to oxidation of a catalytic cysteine thiol. Therefore the TviB enzyme cannot be purified by a single affinity chromatographic procedure [20], and it may be susceptible to oxidation by components of the sample matrix.

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Figure 1.

The MMP0352 enzyme is proposed to catalyze the NAD⁺-dependent oxidation of UDP-GlcNAc at the 3''-position to produce the keto-sugar nucleotide UDP-2-acetamido-3-oxo-2,3-dideoxy-α-D-glucopyranose.

Figure 2.

Purification and kinetic characterization of the MMP0352 UDP-GlcNAc oxidoreductase. Part A shows 10 μ g of His₁₀-MMP0352 protein separated by SDS-PAGE (lane 1) adjacent to protein standards with the indicated molecular masses in kDa (lane M). Proteins were stained with Coomassie blue dye. Part B shows the UDP-GlcNAc oxidoreductase activity catalyzed by 0.3 μ g MMP0352 enzyme in continuous assays at various substrate concentrations. The initial rates were fit to the hyperbolic Michaelis-Menten-Henri equation with an apparent K_M of 0.18 ± 0.03 mM and a k_{cat} of 0.9 s⁻¹. Reaction conditions are described in the Materials and Methods section.

Figure 3.

A response curve for the fluorescent UDP-GlcNAc assay shows a linear relationship between UDP-GlcNAc substrate concentration and measured fluorescence (filled circles with solid line). Reactions contained UDP-GlcNAc, 5 μg His₁₀-MMP0352, 150 μM NAD⁺, 60 mM KCl, 30 mM methoxyamine, and 30 mM CHES-KOH (pH 9.5). The NADH fluorescence produced in reactions with MMP0352 protein and UDP-GlcNAc standards was fit by least-squares linear regression ($r^2 = 0.998$, $p < 0.001$). For comparison, a standard curve was prepared using NADH standards (open squares with dashed line), which was fit to a different line ($r^2 = 0.98$, $p < 0.001$).

Figure 4.

UDP-GlcNAc determination in *E. coli* B extract. Part A shows the standard addition plot, which was obtained by incubating three samples with MMP0352 protein, NAD^+ and the indicated concentrations of UDP-GlcNAc standard. Least-squares linear regression produced a calibration curve, whose negative intercept at the x-axis estimates the UDP-GlcNAc concentration in the extract ($1.5 \pm 0.24 \mu\text{mol g}^{-1}$ dry mass). Part B shows a chromatogram of nucleotides and sugar nucleotides from *E. coli* extract separated on a CarboPac PA1 column, as described in the Materials and Methods section. Nucleotides were detected by their absorbance at 262 nm in milli-absorbance units (mAU). The peak corresponding to UDP-

GlcNAc is identified by an asterisk. Part C shows a chromatogram of the same extract after the standard addition of UDP-GlcNAc, indicating a concentration of $1.2 \mu\text{mol g}^{-1}$ dry mass.

Figure 5.

UDP-GlcNAc determination in *S. cerevisiae* DAY4 extract. Part A shows the standard addition plot for enzymatic reactions containing extract, MMP0352, NAD^+ and the indicated concentrations of UDP-GlcNAc. These cells contained $0.17 \pm 0.03 \mu\text{mol UDP-GlcNAc g}^{-1}$ dry mass. Part B shows a chromatogram of compounds in the *S. cerevisiae* extract, with the peak corresponding to UDP-GlcNAc indicated by an asterisk. Part C shows a chromatogram of the extract after the standard addition of UDP-GlcNAc, which indicates a concentration of $0.14 \mu\text{mol g}^{-1}$ dry mass.

Figure 6.

UDP-GlcNAc determination in HeLa cell extract. Part A shows the standard addition plot for enzymatic reactions supplemented with the indicated concentrations of UDP-GlcNAc. These cells contained $0.50 \pm 0.07 \mu\text{mol g}^{-1}$ dry mass. Part B shows a chromatogram of nucleotides from the extract, where the UDP-GlcNAc peak is indicated by an asterisk. Part C shows a chromatogram after the standard addition of UDP-GlcNAc, indicating a concentration of $0.44 \mu\text{mol g}^{-1}$ dry mass.

Table 1
Apparent kinetic parameters for the UDP-GlcNAc oxidoreductases^a

Enzyme	Substrate	K_m (μ M) ^b	V_{max} (U mg ⁻¹)	k_{cat} (s ⁻¹)	k_{cat}/K_m (M ⁻¹ s ⁻¹)
MMP0352	UDP-GlcNAc	180 $\pm$ 30	1.5 $\pm$ 0.11	0.9	5.0 $\times$ 10 ³
MMP0352	NAD ⁺	180 $\pm$ 20	1.6 $\pm$ 0.09	0.99	5.5 $\times$ 10 ⁴
<i>S. typhi</i> TviB ^c	UDP-GlcNAc	77 $\pm$ 9	0.33	0.26	3.3 $\times$ 10 ³
<i>S. typhi</i> TviB ^c	NAD ⁺	276 $\pm$ 52	0.33	0.26	9.3 $\times$ 10 ²
<i>P. aeruginosa</i> WbpA ^d	UDP-GlcNAc	94 $\pm$ 3	N.D. ^e	1.4	1.5 $\times$ 10 ⁴
<i>P. aeruginosa</i> WbpA ^d	NAD ⁺	220 $\pm$ 4	N.D.	1.4	6.4 $\times$ 10 ³

^aContinuous assays for UDP-GlcNAc oxidoreductase activity at various substrate concentrations were performed as described in Materials and Methods.

^bApparent K_m values were estimated using an excess of NAD⁺ (2 mM for MMP0352 and 1.6 mM for TviB) or UDP-GlcNAc (1 mM).

^cThe parameters for *S. typhi* TviB UDP-GlcNAc 6"-dehydrogenase were reported by Zhang et al. [20].

^dThe $K_{0.5}$ values and turnover number for *P. aeruginosa* WbpA UDP-GlcNAc 6"-dehydrogenase were reported by Miller et al. [22].

^eN.D., not determined.