UDP-N-acetylglucosamine Pyrophosphorylase, a Key Enzyme in Encysting Giardia, Is AllostERICALLY Regulated*

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Giardia synthesizes UDP-GalNAC during cyst wall formation (encystment) via a pathway of inducible enzymes similar to that used to synthesize chitin or peptidoglycan and that includes the UDP-requiring UDP-N-acetylglucosamine pyrophosphorylase. Although it has never been reported as a regulatory enzyme in any system studied to date, kinetic data including Hill plots demonstrate clearly that UDP-N-acetylglucosamine pyrophosphorylase activity, purified from encysting Giardia, is allosterically activated anabolically by physiological levels of glucosamine 6-phosphate (3 μM). Capillary electrophoresis demonstrates that within 24 h after trophozoites are induced to encyst, the level of glucosamine 6-phosphate increases 3-fold over that of non-encysting cells and that by 48 h into encystment the level of glucosamine 6-phosphate has decreased to non-encysting levels or below. UDP-N-acetylglucosamine pyrophosphorylase protein is present constitutively in encysting as well as non-encysting cells. UDP-N-acetylglucosamine pyrophosphorylase immunoaffinity purified from encysting or non-encysting cells exhibited the same molecular weight, amino acid composition, and circular dichroism spectra. Moreover, regardless of whether the enzyme came from encysting or non-encysting cells, the change in its circular dichroism spectra and up to a 6-fold increase in its specific activity anabolically were due to its activation with glucosamine 6-phosphate. Thus, the data support the idea that UDP-N-acetylglucosamine pyrophosphorylase is a major regulatory point in amino sugar synthesis in encysting Giardia and that its allosteric anabolic activation may shift the equilibrium of this pathway toward UDP-GalNAC synthesis.

Giardia lamblia, a primitive eukaryote (1), parasitizes humans worldwide causing giardiasis. The life cycle of Giardia includes cysts, by which fecal-oral transmission occurs, and vegetative trophozoites, which colonize the upper small intestine and produce cysts in response to bile (2, 3). During cyst formation (encystment), trophozoites become encased within a cyst wall, which has an inner membranous and an outer filamentous portion (4, 5). These filaments are made of a GalNAC polymer complexed with protein (6–8). The precursor for this polysaccharide, UDP-GalNAC, is synthesized from endogenous Glc by inducible enzymes, the activities of which increase dur-
Purification of and Assays for UDP-N-acetylgalactosamine Pyrophosphorylase—UDP-GlcNac pyrophosphorylase (EC 2.7.7.23) was purified from encysting *Giardia* trophozoites and assayed by measuring the formation of [\(^{14}C\)]UDP-GlcNac from [\(^{14}C\)]UTP and GlcNac-1-P (23) and catabolically by measuring the formation of GlcNac using high pressure liquid chromatography. Each experiment was performed on each of the six samples, and the difference between the GlcN-6-P peak areas of the phosphatase inhibitor mixture-treated material and those of the identical material treated with alkaline phosphatase was used to calculate the GlcN-6-P content of the cells under each experimental condition.

**Circular Dichroism (CD)—** CD analyses were performed at room temperature in a Jasco J-715 spectropolarimeter at a λ of 260–280 nm and a speed of 5 mm min⁻¹ using a 0.1 cm path length cuvette. The samples were equilibrated in running buffer (30 mM phosphate buffer, pH 7.5). CD spectra of purified UDP-N-acetylgalactosamine pyrophosphorylase (2.6 μm) from encysting and non-encysting cells were recorded for the enzyme alone and for the enzyme in the presence of 5 μM GlcN-6-P.

**Amino Acid Analysis—** Amino acid analysis was performed by a modified method of Moore and Stein (27). UDP-GlcNac pyrophosphorylase, affinity purified from NB medium and HB medium cell homogenates (50 μl of 3.2 μm), was hydrolyzed in 6 N HCl for 20 h at 120°C. Subsequent amino acid composition analysis was performed on a Beckman 6300 amino acid analyzer.

**Antibody Production, Western Blot, and Immunooaffinity Column—** Monospecific, polyclonal antibody was raised against UDP-N-acetylgalactosamine pyrophosphorylase in a New Zealand White rabbit using purified protein excised from SDS-polyacrylamide gel electrophoresis gels and allowed to diffuse into 10 mM phosphate buffer, pH 7.0, for 48 h at 4°C. Preimmune rabbit serum collected prior to immunization served as a control in immunodetection experiments. TiterMax® (Sigma) adjuvant mixed with ~70 μg (1:1) of the pure protein was injected subcutaneously in the rabbit. IgG fractions of rabbit serum were purified using the Protein A Affinity Avidity columns (Immunopure® IgG purification kit, Pierce); purification was monitored by Western blot analysis (28). Cells grown in NB, 0 h, and HB media were harvested, lysed, and electrophoresed in 10% polyacrylamide gel electrophoresis gels under reducing or nonreducing conditions. Proteins were transferred to a membrane and screened with the antibody (1:500). Peroxidase-conjugated goat anti-rabbit IgG was used as a secondary antibody (1:3000).

**Quantitation of UDP-N-acetylgalactosamine Pyrophosphorylase by Immunooaffinity Column—** An immunooaffinity column was constructed by immobilizing anti-UDP-N-acetylgalactosamine pyrophosphorylase IgG onto an rProtein A column for one-step purification of UDP-N-acetylgalactosamine pyrophosphorylase from cytosolic (S) fractions from either non-encysting or encysting trophozoites (23, 29). Purified anti-UDP-N-acetylgalactosamine pyrophosphorylase-IgG (6 mg) was covalently immobilized on the rProtein A matrix (ImmunoPure® Protein A IgG Plus orientation kit, Pierce). The column binding capacity for UDP-N-acetylgalactosamine pyrophosphorylase was ~160 μg. S fractions from non-encysting cells, diluted 1:1 with binding buffer (50 mM Tris-HCl, pH 7.8), were applied to the column that had been equilibrated with the binding buffer. The column was then washed with at least 6 column volumes of binding buffer. UDP-N-acetylgalactosamine pyrophosphorylase was eluted with 50 mM Tris-HCl buffer (pH 7.8) containing 1 mM NaCl; 2 ml fractions were collected until base-line absorbance at 280 nm was reached. To verify that the protein eluted was the pyrophosphorylase, fractions were assayed for UDP-N-acetylgalactosamine pyrophosphorylase activity and then assayed for purity by SDS-polyacrylamide gel electrophoresis.

**Quantitation of UDP-N-acetylgalactosamine Pyrophosphorylase in Non-encysting and Encysting Trophozoites and Encystment Medium—** S fractions from the growth or encystment medium, and trophozoites were grown for 24 or 48 h in the presence of [\(^{35}S\)Met (0.5 μCi ml⁻¹) and then either harvested or induced to encyst for 24 h without the radioisotope in the encystment medium. Alternatively, cells were grown in the growth medium for 24 or 48 h and transferred to encystment medium containing [\(^{35}S\)Met. Each experiment was performed at least twice.
Trophozoites were harvested, washed three times in 20 mM phosphate buffer, pH 7.2, and homogenized by 10 freeze-thaw cycles. Cell homogenates were applied to the anti-UDP-N-acetylglucosamine pyrophosphorylase immunoaffinity column equilibrated with 50 mM Tris buffer, pH 7.8, and the enzyme was eluted with 50 mM Tris, 1 M NaCl buffer, pH 7.8. Eluates containing UDP-N-acetylglucosamine pyrophosphorylase activity were pooled and concentrated, and radioactivity and protein concentrations were determined. The void volumes were reapplied to the affinity column to ensure that all of the UDP-N-acetylglucosamine pyrophosphorylase was collected.

RESULTS

Enzyme Purity—UDP-N-acetylglucosamine pyrophosphorylase was purified, and it migrated as a single spot with a pI of 7.3 on a two-dimensional gel (Fig. 1A) and appeared as a single peak with the same specific activity along the peak (Fig. 1B) in gel filtration fast protein liquid chromatography.

Activation of UDP-N-acetylglucosamine Pyrophosphorylase—Only GlcN-6-P showed any effect on UDP-N-acetylglucosamine pyrophosphorylase activity. GlcN-6-P enhanced the activity of the pyrophosphorylase by ~3-fold (Fig. 2), but in some experiments activation of up to 6-fold was observed. Notably, this activation was detected only in the anabolic direction; neither GlcN-6-P nor any of the other pathway sugars examined had any effect on UDP-N-acetylglucosamine pyrophosphorylase catabolically at concentrations up to 1 mM.

The binding kinetics of UDP-N-acetylglucosamine pyrophosphorylase for GlcNAc-1-P in the presence or absence of GlcN-6-P gave sigmoidal curves (Fig. 3), indicating binding cooperativeness in both cases. Furthermore, the velocity of enzymatic activity increased 5-fold in the presence of 3 μM GlcN-6-P. Hill plots (Fig. 4) for this enzyme with GlcNAc-1-P as substrate yielded Hill coefficients of ~2 in the absence of and 1.4 in the presence of GlcN-6-P, indicating a greater degree of positive cooperativity in substrate binding of the enzyme alone than in the presence of GlcN-6-P.

GlcN-6-P activation of UDP-N-acetylglucosamine pyrophosphorylase is reversible. Dialysis of the enzyme and the activator against 20 mM Tris-HCl (pH 7.5) returned the activity of the enzyme to that prior to the addition of the activator (data not shown). Preincubation of GlcN-6-P with either of its substrates plus the enzyme or with the enzyme alone had no effect on the activation kinetics (data not shown).

Quantitation of GlcN-6-P—The GlcN-6-P peak was resolved from other related compounds (Fig. 5B). The entire peak produced by the standard GlcN-6-P (Fig. 5C) was eliminated by alkaline phosphatase digestion (Fig. 5D) as were most of the unknown peaks from Giardia (data not shown).

A GlcN-6-P peak (Fig. 5D) is apparent in Giardia extracts whether or not they are grown in the presence of bile. Non-encysting Giardia exhibited a small amount of GlcN-6-P (220 amol cell$^{-1}$). At 24 h after trophozoites were induced to encyst, the amount of GlcN-6-P increased ~3-fold to 710 amol cell$^{-1}$, and by 48 h into the encystment process this concentration had dropped to 140 amol cell$^{-1}$.

Western Blot Analysis and Purification of UDP-N-acetylglu-
cosamine Pyrophosphorylase by Immunoaffinity Column—Fig. 6 shows the results of Western blot analysis using IgG fractions of the monospecific, polyclonal anti-UDP-N-acetylglucosamine pyrophosphorylase. This antibody recognized a single band on the native gel (arrow). Some slight proteolysis of enzyme appears in 48 h and NB media S fractions. Preimmune serum showed no reactivity (data not shown). S fractions from NB, 0 h, or 48 h media encysting cells all contain UDP-N-acetylglucosamine pyrophosphorylase. Whereas the lanes contain equal amounts of S fraction proteins and whereas band intensities suggest that the amount of enzyme for each medium condition is approximately equal, the specific activity of UDP-N-acetylglucosamine pyrophosphorylase was markedly different for each. The results of [35S]methionine labeling confirm that the amount of UDP-N-acetylglucosamine pyrophosphorylase protein remains relatively constant in cells even during encystment.

Amino Acid Analysis—The amino acid composition of UDP-\(N\)-acetylglucosamine pyrophosphorylase purified from Giardia encysting (HB medium) and non-encysting trophozoites (NB medium) have the same amino acid composition (Table I), further confirming that these enzymes are identical.

The Effect of GlcN-6-P on Affinity-purified UDP-N-acetylglucosamine Pyrophosphorylase from Encysting and Non-encysting Giardia Trophozoites—Fig. 7 demonstrates that despite constant levels of UDP-N-acetylglucosamine pyrophosphorylase in Giardia trophozoites during encystment, the specific activity of the enzyme increases with the presence of bile in the growth medium. Furthermore, the affinity-purified enzyme, regardless of the status of the trophozoite from which it is isolated, increases its activity 4–5-fold with the addition of GlcN-6-P.

Circular Dichroism—Fig. 8 shows CD analyses of affinity-
purified UDP-N-acetylglucosamine pyrophosphorylase from non-encysting and encysting cells in the presence or absence of GlcN-6P. The spectra of UDP-N-acetylglucosamine pyrophosphorylase from encysting (48 h encysting medium) and non-encysting (NB medium) cells in the absence of GlcN-6P appear similar, suggesting the presence of the same enzyme in either condition. In both enzymes the addition of GlcN-6-P induces similar spectral changes, suggesting a conformational change in the protein due to activator binding.

**DISCUSSION**

UDP-N-acetylglucosamine pyrophosphorylase has been recognized for years as a key enzyme in the formation of UDP-GlcNAc for the synthesis of peptidoglycan, chitin, and glycoconjugates in a variety of prokaryotic and eukaryotic systems (12, 14–16). In recent years, we have shown that it is a key enzyme in the synthesis of GalNAc for formation of the *Giardia* cyst wall filaments (9). In this report, we demonstrate that a UDP-N-acetylglucosamine pyrophosphorylase is a regulatory point in the pathway leading to the synthesis of UDP-GlcNAc or UDP-GalNAc in *Giardia*. We conclude that purified *Giardia* UDP-N-acetylglucosamine pyrophosphorylase in the presence of physiological levels of GlcN-6-P can be activated allosterically more than 3-fold in anabolic activity with no detectable effect on the catabolic activity of the enzyme. This conclusion is based on the following three considerations. First, exposure to increasing amounts of GlcN-6-P, but not other pathway intermediates or their analogs, increases enzyme specific activity in a sigmoidal fashion. Second, Hill coefficients indicate cooperative binding of GlcNAc-1-P and GlcN-6-P by UDP-N-acetylglucosamine pyrophosphorylase. Third, the $V_{max}$ of UDP-N-acetylglucosamine pyrophosphorylase increased in the presence of GlcN-6-P by $\approx 5$-fold with respect to GlcNAc-1-P.

UDP-N-acetylglucosamine pyrophosphorylase is detectable even in non-encysting *Giardia* cells, indicating that it is a constitutive enzyme. Additionally, Western blot analysis and $[^{35}S]$Met incorporation revealed that equivalent amounts of UDP-N-acetylglucosamine pyrophosphorylase are present in encysting and non-encysting cells. UDP-N-acetylglucosamine pyrophosphorylase immunoaffinity purified from non-encysting and encysting cells appears to be the same enzyme, based on the following data. First, these proteins exhibit the same immunoreactivity with monospecific, polyclonal antibody against UDP-N-acetylglucosamine pyrophosphorylase. Second, these proteins behave in the same fashion with respect to GlcN-6-P, which in both cases enhances the velocity of the enzymatic reaction $3–5$-fold. Third, circular dichroism analysis shows the same spectra for the enzymes alone and similar shifts in spectra in the presence of GlcN-6-P. Fourth, amino acid analysis shows that the enzymes have essentially the same amino acid composition. Notably, the specific activity of

**Fig. 5. Hexosamine analysis by capillary electrophoresis.**

A, sugar phosphate peaks of *Giardia* grown in the absence of bile (non-encysting). B, sugar phosphate peaks of authentic standards: 1, GlcNAc-6-P; 2, α-D-GlcN-1-P; 3, α-D-GalN-1-P; 4, GlcNAc-1-P; 5, α-D-GlcN-6-P; 6, UDP-GalNAc. Other standards were detected after the first 10 min of the electrophoresis shown here. C, authentic GlcN-6-P standard peak. D, authentic GlcN-6-P standard after treatment with alkaline phosphatase.

**Fig. 6. Western blot showing the presence of UDP-N-acetylglucosamine pyrophosphorylase in *Giardia* grown in different media.** A native gel of S fractions of *Giardia* grown in media containing no bile (NB, non-encysting), low bile (0 h, non-encysting), and high bile (48 h, encysting) was transblotted to nitrocellulose and probed with anti-UDP-N-acetylglucosamine pyrophosphorylase. The arrow indicates the detected UDP-N-acetylglucosamine pyrophosphorylase (PPylase). Each lane of the original gel contained 75 µg of S fraction protein lane$^{-1}$. UDP-N-acetylglucosamine pyrophosphorylase specific activity in S fractions is as follows: 0.042 units mg of protein$^{-1}$ in 48 h encysting medium cells, 0.012 units mg of protein$^{-1}$ in 0 h medium cells, and 0.008 units mg of protein$^{-1}$ in NB medium cells.
An Allosterically Regulated UDP-GlcNAc Pyrophosphorylase

A comparison of the amino acid analysis of Giardia from encysting medium (HB medium) and non-encysting medium (NB medium) and other eukaryotic UDP-N-acetylglucosamine pyrophosphorylases

| Amino acid | G. intestinalis | Saccharomyces cerevisiae | Candida albicans | H. sapiens |
|------------|----------------|-------------------------|-----------------|-----------|
|            | HB medium      | NB medium               |                 |           |
| Asx*       | 1.17 ± 0.07    | 1.5 ± 0.06              | 1.85            | 2.87      | 1.48      |
| Thr (T)    | 0.77 ± 0.07    | 0.62 ± 0.05             | 0.68            | 0.87      | 0.42      |
| Ser (S)    | 0.77 ± 0.07    | 0.69 ± 0.04             | 1.23            | 1.61      | 0.67      |
| Glx*       | 1.28 ± 0.05    | 1.34 ± 0.03             | 1.91            | 2.85      | 1.97      |
| Gly (G)    | 0.91 ± 0.09    | 1.2 ± 0.05              | 1.27            | 1.36      | 0.94      |
| Ala (A)    | 1.00           | 1.00                    | 1.00            | 1.00      | 1.00      |
| Val (V)    | 0.75 ± 0.09    | 0.75 ± 0.01             | 0.88            | 1.00      | 1.11      |
| Ile (I)    | 0.55 ± 0.03    | 0.55 ± 0.02             | 1.00            | 1.78      | 0.86      |
| Leu (L)    | 1.03 ± 0.09    | 0.91 ± 0.06             | 1.73            | 1.95      | 1.20      |
| Tyr (Y)    | 0.29 ± 0.03    | 0.22 ± 0.08             | 0.61            | 0.61      | 0.42      |
| Phe (F)    | 0.40 ± 0.02    | 0.44 ± 0.01             | 0.74            | 1.17      | 0.77      |
| His (H)    | 0.21 ± 0.02    | 0.22 ± 0.02             | 0.27            | 0.29      | 0.37      |
| Lys (K)    | 0.84 ± 0.04    | 0.79 ± 0.07             | 1.63            | 1.83      | 1.00      |
| Arg (R)    | 0.61 ± 0.07    | 0.57 ± 0.04             | 0.56            | 0.38      | 0.53      |
| Pro (P)    | 0.61 ± 0.07    | 0.55 ± 0.04             | 0.74            | 0.83      | 0.62      |
| Actual Ala* | 5.65 ± 1.9   | 4.15 ± 2.2              |                 |           |

* Asx = Arg (D) + Asn (N).
* Glx = Glu (E) + Gln (Q).
* In nanomoles per 50 μl injected. Values above are expressed relative to Ala as 1.00.

UDP-N-acetylglucosamine pyrophosphorylase in non-encysting cells is very low and increases during the course of encystment and/or in the presence of GlcN-6-P, reaching peak activity by 48 h into the encystment process (9). The amount of GlcN-6-P increases more than 3-fold in Giardia during encystment (HB medium) and non-encysting trophozoites (0 h and NB medium) by immunoadfinity column as described under “Experimental Procedures.” The effect of GlcN-6-P on UDP-N-acetylglucosamine pyrophosphorylase was measured in the anabolic direction under the standard assay conditions in the presence of 5 μM GlcN-6-P.

In bacterial and yeast systems, GlcN-6-P isomerase is regarded as an enzyme that operates catabolically (30, 31) in the degradation of amino sugars. However, an in vivo anabolic role for GlcN-6-P isomerase has been proposed in Droso phila, Musca domestica, and Homo sapiens and in Escherichia coli mutants lacking glucosamine synthase (l-glutamine:D-fructose-6-phosphate aminotransferase, EC 2.6.1.16) activity (32–35). Despite the fact that the isomerase from Giardia more closely resembles the catabolic isomerase than the anabolic ones and despite the fact that it has a greater affinity for GlcN-6-P than for Fru-6-P and NH4Cl, Steimle et al. (11) proposed an anabolic role for GlcN-6-P isomerase in Giardia because its anabolic activity is greater than its deaminase activity and because Giardia lacks glutamine synthase. These investigators (11) suggested further that the equilibrium of the GlcN-6-P isomerase reaction in vivo might be shifted toward GlcN-6-P production by the increased activity of the subsequent acetylase and mutase. Whereas our preliminary evidence suggests that GlcN-6-P has no activating effect on either

![Fig. 7](http://www.jbc.org/)

**Fig. 7.** The effect of GlcN-6-P on affinity-purified UDP-N-acetylglucosamine pyrophosphorylase from encysting and non-encysting Giardia trophozoites. UDP-N-acetylglucosamine pyrophosphorylase was purified from the S fractions of encysting (48 h medium) and non-encysting trophozoites (0 h and NB media) by immunoadfinity column as described under “Experimental Procedures.” The effect of GlcN-6-P on UDP-N-acetylglucosamine pyrophosphorylase was measured in the anabolic direction under the standard assay conditions in the presence of 5 μM GlcN-6-P.

![Fig. 8](http://www.jbc.org/)

**Fig. 8.** Circular dichroism analyses of UDP-N-acetylglucosamine pyrophosphorylase. Curve 1, spectrum of purified UDP-N-acetylglucosamine pyrophosphorylase from non-encysting cells (NB medium); curve 2, same as curve 1 but in the presence of 5 μM GlcN-6-P; curve 3, spectrum of purified UDP-N-acetylglucosamine pyrophosphorylase from encysting cells (48 h); curve 4, same as curve 3 but in the presence of 5 μM GlcN-6-P.
the acetylase or mutase, GlcN-6-P does exhibit a marked effect on UDP-N-acetylgalactosamine pyrophosphorylase, which might actually be the enzyme that shifts the equilibrium of this pathway in the anabolic direction, thus promoting synthesis of UDP-GalNAc and eventually the cyst wall filaments.

Based on the current evidence, we propose that during Giardia encystment signal transduction events occur that cause the transcription of GlcN-6-P isomerase, which in turn produces GlcN-6-P. This phosphorylated amino sugar then interacts allosterically with constitutive UDP-N-acetylglucosamine pyrophosphorylase to activate this enzyme, shifting the equilibrium of this pathway toward UDP-GalNAc synthesis.

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