Kinetic and Physical Characterization of the Inducible UDP-\(N\)-acetylglucosamine Pyrophosphorylase from *Giardia intestinalis*

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The UDP-\(N\)-acetylglucosamine pyrophosphorylase in *Giardia intestinalis* (GiUAP) is one of the five inducible enzymes to synthesize UDP-GalNAc, which is an important precursor for cyst wall synthesis. The recombinant UDP-\(N\)-acetylglucosamine pyrophosphorylase (rGiUAP) and its mutants G108A and G210A were expressed and identified by SDS-PAGE, size-exclusion chromatography, Western hybridization, and MALDI mass spectrometry. Sequence comparison with other eukaryotic UAPs has identified three specific motifs. Within these motifs alanine substitution for Gly\(^{108}\) or Gly\(^{210}\) dramatically reduced the pyrophosphate synthesis, suggesting these amino acids are catalytic residues. Besides, the rGiUAP was found to have relaxed binding to other uridine-based nucleotides, suggesting the substrate binding pocket is specific to uridine rather than phosphate group(s). Moreover, thermal denaturation analysis showed a significant increase in \(T_m\) for the rGiUAP and G108A upon binding of the substrate Mg-UTP. In contrast, G210A showed a decreased \(T_m\) upon binding of Mg-UTP. These results showed that binding of Mg-UTP increases protein stability of the rGiUAP, and the catalytic residue Gly\(^{210}\) plays a significant role in stabilizing the protein structure. Such stabilization effect induced by substrate binding might be physiologically important as it favors the production of UDP-GlcNAc and hence the downstream GalNAc, which is crucial to survival of *Giardia*. These results help to define the essential amino acids for catalysis in the GiUAP and reveal the role of Mg-UTP binding in regulation of protein stability.

*Giardia intestinalis* has long been recognized as one of the most early branching eukaryotes (1). It is also one of the most common causes of gastrointestinal infection in human and other mammals (2). When this parasite travels down the intestine in the host, the transformation from a vegetative trophozoite to an infectious cyst requires the synthesis of a rigid cyst wall to survive the adverse environmental conditions outside the host. As this transformation represents a basic adaptive response of a eukaryote to the environment for propagation, *G. intestinalis* provides a simple eukaryotic model for differentiation and, the biochemical mechanisms ofencystation and the cyst wall synthesis have been the subject of intensive investigations in the past decade.

Previous studies have shown that cyst wall contains both carbohydrate and protein components (3–9). One of the major components of the outer cyst wall has been identified as a \(\alpha\)-GalNAc(\(\beta1\rightarrow3\))-GalNAc\(_n\) homopolymer by chemical methods, mass spectrometry, and hydrogen nuclear magnetic resonance spectroscopy (10). This GalNAc in *G. intestinalis* is synthesized from endogenous Glc rather than salvaged from external source (5). The precursor of GalNAc, UDP-GalNAc, is synthesized by five inducible enzymes: glucosamine-6-phosphate isomerase (EC 3.5.99.6); glucosamine-6-phosphate \(N\)-acetylace (EC 2.3.1.4); phosphoacetylglucosamine mutase (EC 5.4.2.3, AGM); UDP-\(N\)-acetylglucosamine pyrophosphorylase (EC 2.7.7.23, UAP); and UDP-\(N\)-acetylglucosamine 4-epimerase (EC 5.1.3.7). UDP-\(N\)-acetylglucosamine pyrophosphorylase is the fourth enzyme catalyzing the reaction: UTP plus GlcNAc-1-P\(\rightarrow\)UDP-GlcNAc plus PP\(_\text{}\). The activities of these enzymes have been shown to increase from 8- to 4000-fold during encystation of this parasite (5). To date UAP has been purified and characterized from bacteria (11), yeast (12), *Neurospora crassa* (13), calf liver (11), pig liver (14), and human (12, 15, 16).

However, only the UAP from *G. intestinalis* has been reported to be developmentally controlled, with 20-fold increase in activity during encystment (5), which makes it distinct from its counterparts in other systems.

The gene of UDP-\(N\)-acetylglucosamine pyrophosphorylase from *G. intestinalis* Portland I strain (GiUAP) has previously been cloned and characterized (17). Analysis of the GiUAP amino acid sequence showed <30% of sequence identity to the other eukaryotic UAP sequences (17). It has been identified that GiUAP has two developmentally regulated transcripts of \(~1.8 \text{ and } ~4.3\) kb, which contain the same coding sequence but different unusually long 3’-untranslated regions, and these two transcripts were detectable in both vegetative and encysting trophozoites (17). These results were significantly different from the smaller transcripts of \(~1.2\), \(~1.4\), and \(~1.6\) kb reported for the *GiUAP* isolated from MR4 strain (18), implying the *GiUAP* is under different transcription control in different strains of *G. intestinalis*. In the previous studies the native GiUAP purified from MR4 strain has been characterized kinetically (19) and reported to be allosterically activated by physiological levels of glucosamine 6-phosphate (20). However, the identity of the native GiUAP has never been verified except the enzyme was shown to process pyrophosphorylase activity. This concern is intensified as the latest molecular studies suggest that the purified native GiUAP does not correspond to the protein encoded by the *GiUAP* gene (17, 18). Although the reported kinetic data of the GiUAP becomes questionable, there is a lack of structural insight about this enzyme and no catalytic or binding residue(s) have ever been identified. To further our understandings of this enzyme, the recombinant GiUAP (rGiUAP) and its mutants have been expressed and characterized in this study, with extensive kinetic and physical analysis performed to search for the essential catalytic residues and help to define the role of GiUAP in the inducible biosynthesis pathway of GalNAc.

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2. The abbreviations used are: AGM, phosphoacetylglucosamine mutase; UAP, UDP-\(N\)-acetylglucosamine pyrophosphorylase (EC 2.7.7.23); P, phosphate; Glc, glucose; GlcN-6-P, glucosamine 6-phosphate; Mg-nucleotide, \([\text{Mg}^{2+}]\) nucleotide; CD, circular dichroism.
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EXPERIMENTAL PROCEDURES

Parasite Culture—*G. intestinalis* trophozoites, Portland 1 strain ATCC30888, were cultured in TYI-S-33 medium as described previously (21).

Amino Acid Sequence Analysis and Protein Structure Analysis—Protein similarity alignments were performed using the ClustalW, WWW Service at the European Bioinformatics Institute. Protein sequence motif search and identification were performed using EMOTIF (22, 23). Three-dimensional protein structure was generated and manipulated using PyMol (pymol.sourceforge.net).

Total RNA Extraction—Total RNA was prepared as described before (24) from trophozoites.

Reverse Transcription—The cDNA was prepared by reverse transcription of 2 μg of total RNA using Moloney murine leukemia virus reverse transcriptase (Promega) according to the manufacturer’s instructions. The oligo(dT)-anchored primer dT-A 5′-GGC CAC GCG TCG ACT AGT AC(T)17-3′ (antisense) was used to prime the poly(A⁺) tail of the messenger RNA for the first strand synthesis.

Site-directed Mutagenesis—The coding sequences of the two mutants G108A and G210A harboring an alanine substitution for Gly108 and Gly210 were generated by overlap extension (25). PCR was performed using TripleMaster PCR system (Eppendorf) according to the manufacturer’s instructions. The two fragments with overlapping ends for each mutant were amplified from cDNA, using primers EP5 5′-ctt aag gat cca ctc CAG GCC TGG AGG AGT TTC-3′ (sense), 108R 5′-GTG CTG CTG ATG AGA A TA CAA CTG TG TCC-3′ (antisense), 108F 5′-GTG CTG ATG AGA A TA CAA CTG TG TCC-3′ (sense) and EP3 5′-aca tat ggg tac TCG ACT AGT AC(T)17-3′ (antisense), 210F 5′-ACA TAT GGG TAC TCG ACT AGT AC(T)17-3′ (sense), 210R 5′-ACA TAT GGG TAC TCG ACT AGT AC(T)17-3′ (antisense) for G108A; EP5 (sense), 210F 5′-ACA TAT GGG TAC TCG ACT AGT AC(T)17-3′ (antisense), 210F 5′-ACA TAT GGG TAC TCG ACT AGT AC(T)17-3′ (antisense) and EP3 (antisense) for G210A. The resulting PCR products were polished by Klenow (Promega) and gel-purified. The final fusion products were amplified using EP5 (sense) and EP3 (antisense) and cloned into pGEM®-T Easy vector (Promega) to produce pT108 and pT210 for sequencing.

Cloning and Expression of the Recombinant Protein—The coding sequences of GiUAP, G108A, and G210A with restriction sites BamHI and KpnI attached were amplified by PCR using primers EP5 (sense) and EP3 (antisense) from the cDNA, pT108, and pT210, respectively, using Pfx polymerase (Invitrogen) according to manufacturer’s instructions. The resulting PCR products were cloned into the pQE-30 expression vector (Qiagen) to generate expression constructs pQUAP, pQ108, and pQ210, which were transformed into *Escherichia coli* M15 cells (Qiagen). Preparation of cell cultures was modified as described previously (26).

Purification and Quantitation of the Recombinant Protein—The recombinant proteins with an N-terminal His₆ tag were purified using a His-trap chelating column (Amersham Biosciences). The purification was performed following the manufacturer’s instructions, except that 10 mM Hepes, pH 7.4, 200 mM NaCl was used for column equilibration. Unbound proteins were removed by washing with 20 column volume of 40 mM imidazole in Hepes buffer, prior to elution of the recombinant proteins using 10 column volume of 100 mM imidazole in Hepes buffer. The purified recombinant proteins were immediately desalted using a PD-10 desalting column (Amersham Biosciences) and concentrated by Centriprep YM-10 (Millipore) according to manufacturer’s instructions. The recombinant proteins were quantified using the Bradford assay (27) and spectrophotometry at 280 nm (28).

SDS-PAGE and Western Hybridization—The protein fractions collected along the purification procedures were analyzed on 12% SDS-PAGE, and protein bands were visualized using Coomassie staining (29). The presence of the His₆ tag of the recombinant proteins was verified by Western hybridization using nickel-nitrilotriacetic acid conjugates (Qiagen). The procedures were performed as described by the manufacturer except that a polyvinylidene difluoride membrane (Novex) was used instead of nitrocellulose membrane; 3% bovine serum albumin was replaced by 5% skim milk powder and an ECL detection kit (Amersham Biosciences) was used for signal determination.

MALDI Mass Spectrometry Analysis and Size-exclusion Chromatography—MALDI mass spectrometry was performed after tryptic digestion of the purified recombinant protein using sequencing-grade trypsin (Roche Applied Science) in 100 mM Tris-HCl, pH 7.5 (trypsin:protein mass ratio, 1:100). The reaction mixture was incubated at 37 °C for 20 h, with subsequent desalting by ZipTip (Millipore). The resulting peptide fragments were analyzed on a Voyager-DE STR MALDI-TOF mass spectrometer (PerSeptive Biosystems). The masses of the peptide fragments were analyzed using MOWSE peptide mass fingerprint search (30). Size-exclusion chromatography was performed by applying proteins to a Superose 12 HR 10/30 column (Amersham Biosciences) equilibrated with 10 mM Tris-HCl or phosphate, pH 7.5, 200 mM NaCl, 1 mM dithiothreitol, and 1 mM Na₃VO₄. The protein size standards used were IgG (bovine γ globulin, 158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and cyanocobalamin (vitamin B₁₂, 1350 Da). The column was calibrated with the protein size standards each time before a protein sample was applied. The corresponding absorbance was measured by the AKTA purifier fast-protein liquid chromatography system (Amersham Biosciences).

Enzyme Assay—The high flux assay has been modified and described in a previous report (31). In the forward direction to quantify the pyrophosphate, the incubation mixtures contained 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 10% (v/v) glycerol, 1 mM dithiothreitol, GlcNAc-1-P, and UTP at 2 to 10 times greater than its *Kₘ* values, 0.04 unit of pyrophosphatase, and various amounts of recombinant protein in a final volume of 100 μl. Mg²⁺ was used in excess in the assay to ensure all UTP was saturated to form the Mg-UTP complex. The assay was performed in a 96-well plate at 37 °C for 10 min, stopped by adding 100 μl of color reagent (0.03% (w/v) malachite green, 0.2% (w/v) ammonium molybdate, and 0.05% (v/v) Triton X-100 in 0.7 N HCl) followed by a 5-min incubation at 37 °C. The absorbance was measured at 655 nm. Each enzyme assay was performed at least twice in at least two different experiments. The standard curve was constructed by including different concentrations of inorganic phosphate in the incubation mixture, except that GlcNAc-1-P, pyrophosphatase, and recombinant proteins were omitted. Other necessary controls were included as described (31).

Intrinsic Fluorescence Quenching Analysis—Fluorescence spectra were recorded on a PerkinElmer Life Sciences LS-50-B luminescence spectrometer with the use of a 1-cm X 1-cm quartz cell. Titrations with ligands were performed at room temperature by sequentially adding aliquots (up to 100 μl) of the concentrated quencher stock solution to 2 ml of 2 μM recombinant protein in 10 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM dithiothreitol, 1 mM Na₃VO₄. The excitation wavelength was set at 300 nm, and the fluorescence emission spectra were scanned between 350 and 500 nm. All spectra were corrected for background fluorescence. The diminution effect and the inner filter effect of recombinant protein solution due to addition of ligand were corrected by using Equation 1 (32).
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\[
F_{\text{cor}} = (F_{\text{exp}} - B) \times \left( \frac{V_o + V_i}{V_o} \right) \times 10^\lambda \left( A_{\text{ex}} + A_{\text{em}} \right)
\]

(Eq. 1)

where \( F_{\text{cor}} \) is corrected and \( F_{\text{exp}} \) the experimentally measured values of the fluorescence intensities, \( B \) is the background fluorescence of the buffer, \( V_o \) is the initial sample volume, \( V_i \) is the total volume of ligands added, and \( A_{\text{ex}} \) and \( A_{\text{em}} \) are the additional absorbance values of the sample at the excitation and emission wavelengths, respectively, owing to the added ligand.

The equilibrium constants, \( K_d \), for the association of recombinant protein with various ligands were analyzed as described (33), by Equation 2,

\[
\frac{[L]_{\text{tot}}}{\Delta F} = \frac{K_d}{\Delta F_{\text{max}} - \Delta F} + \frac{[P]_{\text{tot}}}{\Delta F_{\text{max}}}
\]

(Eq. 2)

where \([L]_{\text{tot}}\) and \([P]_{\text{tot}}\) are the total concentration of the ligand and recombinant protein, respectively. The \( \Delta F \) is defined by,

\[
\Delta F = F_{\text{cor}} - F
\]

(Eq. 3)

where \( F_{\text{cor}} \) and \( F \) refer to the corrected fluorescence intensities of the recombinant protein in the absence of the ligand and at a given total concentration of the ligand. The \( \Delta F_{\text{max}} \) is estimated using the Equation 4 (34),

\[
\frac{1}{\Delta F} = \frac{1}{\Delta F_{\text{max}}} + \frac{1}{([L] - [P]_{\text{int}}) \times K' \times \Delta F_{\text{max}}}
\]

(Eq. 4)

where \( \Delta F \) is described as above, \([L] \) is the concentration of ligand, \([P]_{\text{int}} \) is the initial concentration of the recombinant protein, and \( K' \) is the apparent binding constant. The linear double reciprocal plot of \( 1/\Delta F \) against \( 1/([L] - [P]_{\text{int}}) \) is extrapolated to the ordinate to obtain the value of \( \Delta F_{\text{max}} \) from the intercept. The equilibrium constant, \( K_d \), of association is defined by Equation 5,

\[
K_d = \frac{[LP]}{[L] \times [P]}
\]

(Eq. 5)

where \([L], [P], \) and \([LP] \) stand for the concentration of the free ligand, free recombinant protein, and the associate of the ligand and recombinant protein, respectively.

The stoichiometry of binding was established from a linear version of the Hill equation (35),

\[
\log \left( \frac{\Delta F}{\Delta F_{\text{max}} - \Delta F} \right) = n \times \log [L] - \log K
\]

(Eq. 6)

where \( \Delta F, \Delta F_{\text{max}}, \) and \([L] \) are described as above, \( n \) is the hill coefficient representing the order of binding reaction with respect to ligand concentration, and \( K \) is the concentration of ligand that yields 50% of \( \Delta F_{\text{max}} \).

Circular Dichroism Analysis—CD measurements of recombinant proteins were made using a lasco J-810 spectropolarimeter. The recombinant proteins were freshly desalted into 10 mM phosphate buffer, pH 7.5, and filtered through a 0.22-μm sterile filter before the measurement. For the ligand binding studies, the desalted recombinant proteins were either preincubated with GlcNAc-1-F or Mg-UTP up to 1 mM. For the far-UV CD measurement, 0.4 ml of 0.1 mg/ml recombinant protein and a 0.1-cm path length quartz cell were used for scans between 190 and 260 nm at 20 °C; for the near-UV CD measurement, 3 ml of 1 mg/ml recombinant protein and a 1-cm path length quartz cell were used for scans between 240 and 350 nm at 20 °C, for the temperature melt CD measurement, the conditions were similar to far-UV CD measurement except that temperature was varied manually from 20 to 90 °C. The CD of the samples was monitored in two ways: the spectrum scan between 190 and 260 nm and the time course measurement at 208 nm for a time interval of 2 min. All spectra were corrected for background CD. Mean residue ellipticity (\( [\Phi]_{\text{mrw,A}} \)) at a given wavelength \( \lambda \) in units of deg.cm².dmool⁻¹ was obtained by Equation 7,

\[
[\Phi]_{\text{mrw,A}} = \frac{\Phi \times \text{MRW}}{10 \times d \times c}
\]

(Eq. 7)

where MRW is obtained by dividing the molecular weight by \( N - 1 \) (\( N \) is the number of amino acids of the protein sample), \( \Phi \) is the observed ellipticity (deg), \( d \) is the path length (cm), and \( c \) is the protein concentration (mg/ml) (36).

RESULTS

Identification of UAP Motifs—An amino acid sequence motif of L(X)₆GXXGM(X)₉PK, where \( X \) represents any amino acid was proposed for UAP by comparing the amino acid sequence of the bifunctional N-acetylglucosamine-1-phosphate uridylyltransferase from \( E. \) coli and UDP-glucose pyrophosphorylase from Saccharomyces cerevisiae and human, respectively (12). Although this motif has been successfully applied to search and identify the UDP-N-acetylglucosamine pyrophosphorylase (UAP) from the yeast data base (12), it cannot be the representative of UAP family. Moreover, the amino acid sequences of prokaryotic UAPs were found to differ significantly from those of eukaryotic UAPs (data not shown). By comparing the identified eukaryotic UAP sequences to date, including GiUAP using the multiple sequences alignment, three highly conserved regions were identified (Fig. 1). These highly conserved amino acid fragments were analyzed by EMOTIF, and this resulted in the identification of three putative motifs from the amino acid sequence of GiUAP: 107-GGQXTRGLG(X)₉PKG-209, 208-D/(N)/GN(G/A)G(X)₉-217 and 382KXEEXFDXT₃₉₁. These motifs were found to surround the substrate binding site in the human UAPs: AGX1 and AGX2 (37) (Fig. 2). Searches in the Swiss-Prot data base using any of these three putative motifs resulted in exclusive identification of eukaryotic UAPs, indicating that the identified putative motifs are highly specific for UAP conserved sequences.

Expression of the Recombinant Enzymes—The rGiUAP and its mutants G108A and G210A were expressed and purified under the similar conditions. The denatured molecular masses of rGiUAP, G108A, and G210A were estimated to be ~50 kDa from SDS-PAGE analysis (Fig. 3A), which were consistent with the predicted 49,624 Da for rGiUAP and 49,638 Da for the mutants from the gene sequences. The presence of a His₈ tag in rGiUAP, G108A, and G210A was verified by Western hybridization (Fig. 3B). The identity of the expressed rGiUAP, G108A, and G210A was confirmed by peptide mass fingerprinting. The observed masses of the digested peptide fragments were measured by MALDI mass spectrometry and subsequently compared with the predicted masses of the digested peptide fragments obtained from the NCBI protein data base. The rGiUAP was shown as the only significant match with 20 peptide fragments identified and 64% of the amino acid sequence covered (data not shown). The G108A and G210A were also distinguished from the wild type rGiUAP by identifying the mass of the specific peptide containing the mutated amino acid (data not shown). All the recombinant proteins were eluted as single peaks in
size-exclusion chromatography (data not shown), and their molecular masses estimated from the standard curve were ~47 kDa, which is in good agreement with the 49 kDa predicted from the amino acid sequence.

**Enzyme Activity of the Recombinant Enzymes**—For the rGiUAP, the crude, soluble and His-trap®-purified fractions were measured to monitor the enzyme activities during purification, as well as the desalted fraction (TABLE ONE). The final desalted fraction was used for the relative kinetics for different substrates (TABLE TWO). The related data reported for the native GiUAP (19) was also included in both tables for comparisons. The single step purification using His-trap® chelating column resulted in a purification of ~5-fold with ~57% of enzyme activity being recovered. The abnormal increase of total activity from the His-trap®-purified fraction to the desalted fraction suggested that some enzyme inhibitors might be present in the former fractions before the desalting procedures. It was subsequently confirmed that Hepes has unknown inhibitory effects on the rGiUAP, because the total activity of the desalted fraction decreased significantly when Hepes was added to the buffer (data not shown). It has also been noticed that the protein concentration of rGiUAP quantified by the commonly used Bradford assay was usually overestimated by 1.5- to 2-fold compared with that determined from absorbance measurement at 280 nm, which led to an...
underestimation of the specific activity of the enzyme. Besides, the specific activity of the rGiUAP was ~10-fold higher than that previously described for the native GiUAP (19). Moreover, the $K_m$ values obtained for GlcNAc-1-P and UTP were, respectively, ~7.5- and ~32-fold lower, and the $V_{max}$ values obtained for GlcNAc-1-P and UTP were, respectively, ~34- and ~6-fold higher than that of the native GiUAP, in accord with the higher specific activity observed at fixed substrate concentrations. The enzyme activities of the purified mutants G108A and G210A were also measured (TABLE THREE). Although detectable levels of phosphate were observed in assays of both mutants, the activities were so low that it was impossible to obtain reliable estimates for the $K_m$ values of the substrates.

Bulik et al. (19) who have previously characterized the native GiUAP showed that GlcNac-1-P can be replaced by GalNac-1-P or Glc-1-P in the anabolic reaction. These substrates gave 58 and 53%, respectively, of the activity with GlcNAc-1-P. In another paper Bulik et al. (20) reported that GlcN-6-P is an allosteric activator for the native GiUAP, the activity of which in anabolic direction was enhanced ~3- to 6-fold in the presence of 3 mM GlcN-6-P. To investigate whether the rGiUAP has similar properties to those reported for the native GiUAP, the desalted fraction of rGiUAP was tested for the use of the alternative substrates. Because GalNac-1-P was no longer commercially available, only Glc-1-P was used in this study. No enzyme activity was observed for Glc-1-P concentration of up to 10 mM. In addition, GlcN-6-P was included in the

| Enzyme   | Sequence                                                                 | $K_m$ (mM) | $V_{max}$ (μM/min) |
|----------|---------------------------------------------------------------------------|------------|--------------------|
| DmtUAP   | AHSVDNILIKVAVDFPVGYCVQKEKACAKVVE--KAPNEAVGVVAVD--GYQV--                      | 338        | 301                |
| DmtUAP   | AHSVDNILIKVAVDFPVGYCVQKEKACAKVVE--KAPNEAVGVVAVD--GYQV--                      | 301        | 301                |
| HsUAP    | VYCDNILKVDPRIFGCIKQGACADGKVEK--KTNPREVGVCVCRV--GVYQV--                       | 301        | 301                |
| HsUAP    | VYCDNILKVDPRIFGCIKQGACADGKVEK--KTNPREVGVCVCRV--GVYQV--                       | 301        | 301                |
| AtUAP    | CYGYNMVLVRVFDDFLNYDIAAASAKKCVR--KYFQEVGTFVRKRGGMPL--                       | 348        | 301                |
| ScUAP    | MLCYVRLKIAEFIPVGIKHIKPLKAKVR--KRDANSGVIGLAEQLNGKQFV--                       | 300        | 300                |
| CaUAP    | MLCYNMLKVDKDFIPVFAPKAFKAPKLKAKVR--KRDANSGVIGLAEQLNGKQFV--                       | 307        | 307                |
| CeUAP    | VYCDNILKVDPRIFGCIKQGACADGKVEK--KTNPREVGVCVCRV--GVYQV--                       | 301        | 301                |
| GiUAP    | IVGDNLPLPLCCGLVATLTVFQAKKSLDDLN1RRNVPQC6K9GKLVGVRSTETEWQAPLVRD                | 356        | 356                |

FIGURE 1—continued
anabolic reaction of the desalted fraction of rGiUAP to test the potential allosteric activation. Nevertheless, no activation effect was observed, and the activity of rGiUAP remained relatively constant in the presence of GlcN-6-P up to 0.1 mM (data not shown).

The Ligand Binding Studies—Sequence analysis showed that each recombinant protein contains 1 tryptophan, 17 tyrosines, and 20 phenylalanines, which can contribute to protein intrinsic fluorescence. Therefore intrinsic fluorescence quenching analysis was performed, and the rGiUAP and its mutants were shown to fluoresce with two absorption maxima (at ~229 and ~277 nm) and an emission maximum at ~389 nm (data not shown). The emission maximum was found to red-shift relative to that of free L-tryptophan, which might be caused by the change of microenvironments around the fluorophores (38). The absorption spectra of different nucleotides are well known to overlap considerably with the absorption spectra of the three fluorescent amino acids (33, 39, 40) leading to significant inner filter effect. Therefore the absorption spectra of different potential nucleotides to be used in the binding experiments were measured at relevant concentrations, in search of an alternative excitation wavelength for the recombinant proteins. It was found that at concentration of 500 μM, Mg-UTP, Mg-UDP, Mg-UMP, and Mg-uridine all absorbed significantly up to ~300 nm (data not shown). Hence 300 nm was chosen as the excitation wavelength as a reasonable compromise between interference by nucleotides and maintaining the strength of fluorescence emissions.

The fluorescence quenching of the recombinant proteins by different ligands is summarized in TABLE FOUR. For the rGiUAP, fluorescence quenching was only observed when the enzyme was saturated with the ligand Mg-UTP but not GlcNAc-1-P or PPi. Binding of Mg-UTP to the enzyme showed a maximal ~33% decrease in fluorescence and a Hill coefficient of ~1. To investigate the role of the triphosphate in binding, Mg-uridine, Mg-UMP, and Mg-UDP were also employed. It was found that they all bound to the enzyme. No significant difference in binding constant was observed, although the $K_d$ value for Mg-UMP was slightly higher than that of Mg-UTP. Ribose 5’-phosphate and inorganic phosphate were also used in binding experiments, but no change to protein fluorescence could be observed.

Secondary and Tertiary Structures of the Recombinant Enzymes—

The far-UV spectra of the recombinant proteins were measured from 260 to 190 nm in transparent phosphate buffer to investigate protein secondary structure (Fig. 4A). The spectra of rGiUAP, G108A, and G210A were found to be largely superimposable, indicating there is no
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| TABLE ONE |
| --- |
| Enzyme activities of the rGiUAP and the native GiUAP (Ref. 19) |
| Purification step | Total protein* | Total activityb | Specific activity | Purification factor | Yield |
| --- | --- | --- | --- | --- | --- |
| --- | mg | units | units/mg | --- | % |
| rGiUAP | --- | --- | --- | --- | --- |
| Crude fraction | 159 | 400 | 2.5 | 1.0 | 100 |
| Soluble fraction | 89 | 274 | 3.1 | 1.2 | 68 |
| His-trappurified | 18 | 227 | 12.9 | 5.1 | 57 |
| Desalted fraction* | 11 | 432 | 39.3 | NA | NA |
| Desalted fraction | 6 | 432 | 72 | NA | NA |
| GiUAP | --- | --- | --- | --- | --- |
| GF-HPLC | 0.4 | 1.4 | 3.4 | 170 | 15 |

* The amount of total protein was from 100 ml of E. coli culture.

b The protein fraction was prepared in Tris buffer and quantified by Bradford assay.

rGiUAP, G108A, and G210A were similar to each other, supporting the inference from far-UV CD spectra that there is no major difference in their secondary structures and hence no significant conformational modification was induced by either mutation. To determine if the binding of reaction substrate results in a change of secondary structure, the far-UV CD spectrum of the rGiUAP was also recorded in the presence of substrates GlcNAc-1-P or Mg-UTP. It was found that no major difference in their secondary structures and hence no significant loss of structure was estimated to be 27% for G108A and ~48% for G210A. The Tm was estimated to be ~56 °C for G108A and ~55 °C for G210A. These results suggested that the mutants unfolded in a similar way to the rGiUAP in the absence of the substrate, although the final loss of structure may have been greater for G210A.

For the heat-induced unfolding in the presence of GlcNAc-1-P, the CD spectra of the rGiUAP were found to shift in a similar way to those observed in the absence of this substrate (data not shown). The unfolding was shown to be irreversible after heating to 90 °C, because the ellipticity could not be fully recovered when the starting temperature was restored (data not shown). As the maximum change of ellipticity was at 208 nm, the CD for the rGiUAP was monitored at this wavelength for estimation of protein structure loss and determination of the Tm of the unfolding process. The overall loss of structure was estimated to be ~34%, and the Tm was estimated to be ~54 °C. For the unfolding induced for the mutant G108A and G210A, the shifts of spectra between 200 and 260 nm were similar to those observed for the rGiUAP (data not shown). The unfolding of the mutants was irreversible, as for the rGiUAP. The overall loss of structure was estimated ~27% for G108A and ~48% for G210A. The Tm was estimated to be ~56 °C for G108A and ~55 °C for G210A. These results suggested that the mutants unfolded in a similar way to the rGiUAP in the absence of the substrate, although the final loss of structure may have been greater for G210A.

**TABLE TWO**

Characteristics of the rGiUAP and the native GiUAP (19)

| Enzyme | Ligand | Maximum quenching |
| -- | -- | -- |
| --- | --- | --- |
| GlcNAc-1-P | 3 | NAa |
| PPI | 1 | NA |
| Mg-UTP | 33 | 0.64 | 1.01 |
| Mg-UDP | 23 | 0.45 | 1.03 |
| Mg-UMP | 27 | 1.05 | 0.98 |
| Mg-uridine | 26 | 0.68 | 1.01 |
| R5P | 1 | NA |
| Pi | 1 | NA |
| G108A | Mg-UTP | 28 | 0.84 | 1.02 |
| G210A | Mg-UTP | 33 | 0.60 | 1.04 |

a NA, not applicable.

**TABLE THREE**

Enzyme activities of G108A and G210A (anabolic direction)

| Desalted fraction | Total protein* | Total activityb | Specific activity |
| --- | --- | --- | --- |
| --- | mg | units | units/mg |
| G108A | 13.75 | 0.028 | 0.002 |
| G210A | 1.53 | 0.032 | 0.022 |

* The amount of total protein was from 100 ml of E. coli culture.

b 1 unit of enzyme activity is 1 μmol of pyrophosphate formed per min.

c The protein fraction was prepared in Tris buffer and quantified by Bradford assay.

d The protein fraction was prepared in Hepes buffer and quantified by absorbance at 280 nm.

e The amount of total protein was from 100 ml of E. coli culture.

f 1 unit of enzyme activity is 1 μmol of pyrophosphate formed per min.

g The protein fraction was prepared in Tris buffer and quantified by Bradford assay (19).
loss of structure was estimated to be ~39%, and the $T_m$ was estimated to be ~56 °C. As no significant changes in the CD spectra were observed for the rGiUAP upon incubation with GlcNAc-1-P, the unfolding of the mutants was not studied in the presence of this substrate.

For the heat-induced unfolding in the presence of Mg-UTP, there were significant differences in the CD spectra of the rGiUAP (Fig. 5B) compared with those observed in the absence of this substrate. The ellipticity between 200 and 240 nm was remarkably increased and that between 240 and 260 nm was slightly decreased, indicating a nearly complete loss of structure. Furthermore, the $T_m$ was estimated to be ~60 °C, which is significantly higher than the ~54 °C estimated in the absence of substrate. As with the rGiUAP, there were dramatic changes in the CD spectra of the G108A during unfolding in the presence of Mg-UTP (data not shown). The $T_m$ was estimated to be ~60 °C, compared with the ~56 °C estimated in the absence of substrate. Although dramatic changes similar to that of the rGiUAP and G108A were also observed in the CD spectra of the G210A during unfolding in the presence of Mg-UTP (data not shown), the $T_m$ estimated was ~48 °C, which is significantly lower than the ~55 °C estimated in the absence of substrate.

**DISCUSSION**

The UDP-N-acetylglucosamine pyrophosphorylase (UAP) plays an important role in synthesis of UDP-GlcNAc, which is an essential metabolite for different biological functions. In prokaryotes the most well characterized UAP is the GlmU from *E. coli* (EcGlmU) (41–43). The EcGlmU is a bifunctional enzyme that processes both pyrophosphorylase and acetyltransferase activities (44). The recently resolved crystal structure of a truncated EcGlmU has revealed two domains for its bifunctional nature: a pyrophosphorylase N-terminal domain with a motif GXGT(R/S)(X)_4PK, resembling the dinucleotide-binding Rossmann fold, and an acetyltransferase C-terminal domain with the hexapeptide repeat (L/I/V)(G/A/E/D)X_2(S/T/A/V)X (45). However, the bifunctionality of the prokaryotic GlmU is not observed in eukaryotes, which have evolved with two distinct enzymes: a pyrophosphorylase and an acetyltransferase. In contrast to the trimeric form found in the prokaryotic GlmUs (43, 46), the eukaryotic UAPs usually exist as a monomer (13, 47) or dimer (14, 37). Yet the native GiUAP was reported to have a dimeric structure consisting of two ~33 kDa subunits (19), the rGiUAP was found to exist as a monomer of ~50 kDa, which corresponds to the GiUAP gene (17, 18) and is more comparable with the subunit size of other eukaryotic UAPs. Besides, the kinetic data showed that the rGiUAP has a significantly higher activity with tighter binding to both substrates when compared with the native GiUAP (19). In addition, the partial activity with Glc-1-P (51%) and the allosteric activation by GlcN-6-P (3 μM) reported for the native GiUAP (19, 20) were not observed on the rGiUAP. These results agree and reinforce the previous conclusion that the smaller native GiUAP purified in the previous stud-
ies is likely to be another bi-functional enzyme with partial pyrophosphorylase activity or a degraded or post-translational modified GiUAP, rather than the one directly translated from the GiUAP gene (17).

Although it was found that the overall amino acid sequence identity between GiUAP and other eukaryotic UAPs is low, there is significant segmental similarity in several distinct regions. Analysis of these regions has identified three highly specific motifs for the eukaryotic UAP family. The first motif GGQ TRLG(3) PKG has the most conserved pyrophosphorylase sequence, which is similar to the motif GXG R(S)(4) PK identified in the pyrophosphorylase domain of EcGlmU (45). Mutagenesis data on the ScUAP has shown that Ala substitution for Gly112, Arg116, or Lys123 within the first motif dramatically impaired the pyrophosphorylase activity (12), whereas similar results were observed for the EcGlmU when Gly14, Arg18, or Lys25 within the motif was substituted by Ala (43). Because mutated Gly112 but not other residues of the ScUAP showed significant decrease of binding to the substrate GlcNAc-1-P (12), the corresponding residue Gly108 in the rGiUAP was substituted by Ala to create the mutant G108A, and dramatic decrease in pyrophosphorylase activity similar to the G108A was observed. It suggested the Gly108 in the rGiUAP is a possible catalytic residue.

Although the activities of the mutants G108A and G210A were too low for estimation of $K_m$ values for both substrates, the fluorescence quenching data obtained from the rGiUAP, G108A, and G210A suggested that the substrate Mg-UTP binds to these recombinant proteins in a similar way, which is different from the AGX, which lost binding to the substrate when the Gly222 (corresponding to Gly210 in the rGiUAP) was mutated to Ala (16). The similar binding of Mg-UTP to the rGiUAP and its mutants implied the loss of activities in the mutants could be due to: 1) impaired binding to another substrate GlcNAc-1-P; 2) modified orientation of one or more bound substrates that inhibit the catalysis. Analysis of the crystal structure of the AGX (37) has revealed that Gly111 and Gly222 (corresponding to Gly108 and Gly210 in the rGiUAP) are in close proximity to the uridine ring (3–4 Å) but far away from the GlcNAc (7–10 Å) (Fig. 2). Provided the rGiUAP and its mutants share significant segmental similarity and assume they have a similar binding pocket for the substrates, it is unlikely for the mutants G108A and G210A to lose binding to GlcNAc-1-P while the binding to Mg-UTP is unaffected. Therefore the impaired activities of the mutants G108A and G210A were possibly due to the inhibited catalysis induced by the modified orientation of bound Mg-UTP. Furthermore, the Hill coefficient of $\sim$1 obtained from the rGiUAP and its mutants suggested there is no cooperativity in binding to Mg-UTP, which is similar to the AGX (37).
Kinetic and Physical Characterization of the Inducible GiUAP

In addition, the rGiUAP was found to have relaxed binding to the other uridine-nucleotides, which has never been reported for other UAPs, suggesting the binding pocket is specific to the uridine rather than the phosphate(s) group.

The far/near-UV CD analysis showed that the rGiUAP and its mutants have similar secondary structure (with or without substrate) and tertiary structure (without substrate). Because no data has ever been reported on UAPs related to the structural stability and the effect of substrate binding, heat-induced unfolding was performed on the rGiUAP and its mutants in the presence or absence of the substrate. Although there was no difference observed in the unfolding of rGiUAP in the presence of GlcNac-1-P, unexpected and remarkable changes were observed in the presence of Mg-UTP that the $T_m$ was found to increase significantly (−5°C), and the unfolding was catastrophic once initiated (−95% overall loss of structure). These results provided direct evidence for binding of Mg-UTP to the rGiUAP, which is consistent with the fluorescence quenching data. It suggested that binding of Mg-UTP induced conformational changes and stabilized the protein structure of the rGiUAP. Such induced protein stability upon binding of Mg-UTP strongly favors the anabolic reaction to produce UDP-GlcNAc, leading to synthesis of a downstream product GalNAc, which is a crucial component during encystation of Giardia. Taken together with the inducible nature of the transcription of GiUAP in the biosynthetic pathway of GalNAc, such stabilization effect at protein level by ligand binding might have a physiological role to induce and secure the synthesis of GalNAc.

Besides, the G108A was found to exhibit similar unfolding properties during the unfolding process, implying the mutation of Gly108 has no effect on the protein stability upon binding of Mg-UTP. On the contrary, the G210A was found to start unfolding at much lower temperatures in the presence of Mg-UTP, and the stability of protein structure was compromised, compared with the rGiUAP and G108A where binding of Mg-UTP increased the stability. This is consistent with the previous hypothesis that in the mutants G108A and G210A the conformation of uridine binding pocket is changed, which leads to inhibition of catalysis. Moreover, in this case of G210A, such conformational changes not only alter the orientation of bound Mg-UTP, but also impair the overall protein stability, suggesting the Gly210 has a significant structural role in addition of being an essential catalytic residue.

Giardia has long been regarded as one of the most deeply branching eukaryotes. Studies of the GiUAP not only further our insight into the significant developmentally regulated encysting pathways but also provide a clue of how this enzyme has evolved from the prokaryotic bifunctional GlmUs into the eukaryotic UAPs. Further experiments on the GiUAP might reveal some novel structures that would help to explain the low overall homology between the GiUAP and other eukaryotic UAPs and possibly lead to the design of new therapeutic drugs to act against this parasite in human.

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