Acetyltransfer Precedes Uridylyltransfer in the Formation of UDP-*N*-acetylglucosamine in Separable Active Sites of the Bifunctional GlmU Protein of *Escherichia coli*[†]

Amy M. Gehring, Watson J. Lees, Daniel J. Mindiola, Christopher T. Walsh, and Eric D. Brown*,[‡]

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Avenue, Boston, Massachusetts 02115

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ABSTRACT: The GlmU protein is a bifunctional enzyme with both acetyltransferase and uridylyltransferase (pyrophosphorylase) activities which catalyzes the transformation of glucosamine-1-P, UTP, and acetyl-CoA to UDP-*N*-acetylglucosamine [Mengin-Lecreulx, D., & van Heijenoort, J. (1994) *J. Bacteriol. 176*, 5788−5795], a fundamental precursor in bacterial peptidoglycan biosynthesis and the source of activated *N*-acetylglucosamine in lipopolysaccharide biosynthesis in Gram-negative bacteria. In the work described here, the GlmU protein and truncation variants of GlmU (N- and C-terminal) were purified and kinetically characterized for substrate specificity and reaction order. It was determined that the GlmU protein first catalyzed acetyltransfer followed by uridylyltransfer. The N-terminal portion of the enzyme was capable of only uridylyltransfer, and the C-terminus catalyzed only acetyltransfer. GlmU demonstrated a 12-fold kinetic preference (k_{cat}/K_m, 3.1×10^5 versus 2.5×10^4 L·mol^{-1}·s^{-1}) for acetyltransfer from acetyl-CoA to glucosamine-1-P as compared to UDP-glucosamine. No detectable uridylyltransfer from UTP to glucosamine-1-P was observed in the presence of GlmU; however, the enzyme was competent in catalyzing the formation of UDP-*N*-acetylglucosamine from UTP and *N*-acetylglucosamine-1-P (k_{cat}/K_m 1.2×10^6 L·mol^{-1}·s^{-1}). A two active site model for the GlmU protein was indicated both by domain dissection experiments and by assay of the bifunctional reaction. Kinetic studies demonstrated that a pre-steady-state lag in the production of UDP-*N*-acetylglucosamine from acetyl-CoA, UTP, and glucosamine-1-P was due to the release and accumulation of steady-state levels of the intermediate *N*-acetylglucosamine-1-P.

The GlmU protein is a 456 amino acid bifunctional enzyme with both acetyltransferase and uridylyltransferase (pyrophosphorylase) activities (Mengin-Lecreulx & van Heijenoort, 1993, 1994). Encoded in the *Escherichia coli* gene *glmU*, formerly *Eco* URF1 (Walker *et al*., 1984), the enzyme catalyzes the following overall reaction:

glucosamine 1-phosphate + acetyl-CoA + UTP → UDP-*N*-acetylglucosamine + CoA + PP_i

UDP-GlcNAc is situated at the branchpoint of two important biosynthetic pathways in *E. coli*, namely, peptidoglycan and lipid A biosynthesis. Peptidoglycan is the primary structural polymer of the bacterial cell wall and is composed of alternating units of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) with an attached peptide; UDP-GlcNAc, the nucleotide-activated form of GlcNAc, is also a direct precursor of UDP-MurNAc (Park, 1987). Lipid A serves as the hydrophobic anchor of lipopolysaccharide in the outer membrane and has at its core a disaccharide moiety which is synthesized from UDP-GlcNAc (Raetz, 1987, 1993). As the bulk of the *N*-acetylglucosamine in *E. coli* is found incorporated into peptidoglycan or lipopolysaccharide, the synthesis of UDP-GlcNAc represents a critical step in the biosynthesis of essential components of the bacterial cell envelope.

The bifunctionality of the GlmU protein was recently demonstrated by Mengin-Lecreulx and van Heijenoort (1994) and is consistent with sequence similarities evident in the primary structure of the protein. The protein shows N-terminal sequence similarity with a variety of nucleotidyltransferases (pyrophosphorylases), including UDP-glucose pyrophosphorylases, and demonstrates C-terminal similarity to a number of acetyltransferases, including chloramphenicol acetyltransferase and, in particular, the FirA protein, an *E. coli* UDP-3-*O*-(*R*-3-hydroxymyristoyl)glucosamine *N*-acyltransferase involved in lipid A biosynthesis (Kelly *et al*., 1993). GlmU is most similar over its entire sequence (43% identity) to the *Bacillus subtilis* GcaD protein (Hove-Jensen, 1992), also known as the Tms protein (Nilsson *et al*., 1989), and while GcaD has been shown to catalyze uridylyltransfer to *N*-acetylglucosamine-1-P (Hove-Jensen, 1992), the capacity of GcaD to catalyze acetyltransfer has not been reported.

In this work, we present steady-state kinetic studies describing the substrate specificity of GlmU, aimed primarily at elucidating the order of the bifunctional reaction. The results of this analysis indicate that GlmU catalyzes acetyl-

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* Author to whom correspondence should be addressed.

[‡] Present address: Astra Research Center Boston, Inc., 128 Sidney St., Cambridge, MA 02139.

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transfer, to yield *N*-acetylglucosamine-1-P, prior to uridylyltransfer. We have also localized the respective activities of the GlmU protein to discrete N-terminal and C-terminal protein fragments and present evidence that bifunctional catalysis by the full-length enzyme occurs at distinct acetyltransferase and pyrophosphorylase active sites with the *N*-acetylglucosamine-1-P intermediate equilibrating freely with the bulk solution.

MATERIALS AND METHODS

Chemicals. UDP-glucosamine was synthesized enzymatically and involved anion exchange HPLC purification (Brown *et al*., 1994) of a 1 h incubation (1 mL) of 1 mM glucosamine 1-phosphate, 1 mM UTP, 100 mM Tris·HCl buffer, pH 7.6, 10 mM $MgCl_2$, 20 units of UDP-glucose pyrophosphorylase (EC 2.7.7.9), and 2.5 units of inorganic pyrophosphatase (EC 3.6.1.1). The authenticity of the purified compound was confirmed by fast atom bombardment mass spectrometry (molecular weight 564). The compound α-D-[^{14}C]-*N*-acetylglucosamine-1-P (309 mCi/mmol) was synthesized enzymatically and purified by anion exchange HPLC (Brown *et al*., 1994) from a 4 h incubation (0.5 mL) of 0.38 μCi of uridine diphospho-D-[^{14}C]-*N*-acetylglucosamine (309 mCi/mmol; Dupont NEN), 2 mM sodium pyrophosphate, 5 mM $MgCl_2$, 100 mM Tris·HCl, pH 7.6, and 16 μg of pure full-length GlmU (see below). UDP-*N*-acetylmuramic acid was synthesized as described previously (Benson *et al*., 1993).

Overexpression of the GlmU Protein. The gene *glmU* was cloned from the chromosomal DNA of wild-type *E. coli* using the polymerase chain reaction. Amplification was performed using Vent DNA polymerase (New England Biolabs) and the following primers: 5′-GCGCAATTCCATATGTTGAATAATGCT-3′ and 5′-GCGCCCAAGCTTTCACTTTTTCTTTAC-3′ (*Nde*I and *Hin*dIII sites underlined). The resulting fragment was then blunt-ligated into *Eco*RV-digested cloning vector pBluescript SK (Stratagene). An *Nde*I and *Hin*dIII fragment of the resulting plasmid was then purified and ligated into *Nde*I- and *Hin*dIII-digested expression vector pET22b (Novagen), producing the plasmid pET22b-*glm*U. For protein overproduction, the plasmid was transformed into BL21(DE3) (Novagen) cells [strain BL21(DE3)/pET22b-*glm*U]. The gene sequence of cloned *glmU* was confirmed by dideoxy sequencing using a Sequenase Version 2.0 DNA sequencing kit (USB/Amersham).

The C-terminal glutathione *S*-transferase (GST) fusion protein of GlmU was constructed by cloning a 3′ fragment of the *glmU* gene, from nucleotide 538 in the gene sequence (M180 in the protein sequence) to the stop codon, into a modified pGEX vector, pGEX3b (Payne *et al*., 1993). The 3′ fragment of *glmU* was amplified by PCR using Vent DNA polymerase, plasmid pET22b-*glm*U, and the following primers: 5′-GGTCTAGACATATG-AAACGCTGGCTGGCGAAC-3′ and 5′-GCGCCCAAGCTT-TCACTTTTTCTTTAC-3′ (*Nde*I and *Hin*dIII sites underlined). The resulting PCR fragment was digested with *Nde*I and *Hin*dIII and ligated into *Nde*I- and *Hin*dIII-digested pGEX3b to produce plasmid pGEX3b-3′-*glm*U. For overproduction, this plasmid was transformed into BL21(DE3) cells.

Enzyme Purification. Full-length GlmU was purified from the overproducing strain [BL21(DE3)/pET22b-*glm*U] according to the following procedure. Freshly transformed cells (2 L) were grown to an optical density (600 nm) of 0.5 in Luria−Bertani media. These cells were then induced with 1 mM IPTG, allowed to grow for another 3 h, and harvested by centrifugation. Cells were resuspended in 30 mL of 100 mM Tris·HCl, pH 8, 1 mM DTT, 1 μg each of DNase I and ribonuclease, and the following additives to discourage proteolysis: 2 mM EDTA, 10% glycerol, 1 mM phenylmethanesulfonyl fluoride, 1 mM benzamidine, 1 μM leupeptin, and 20 μg/mL trypsin inhibitor. Cells were lysed by passage through a French press, their volume brought to 60 mL with lysis buffer, and the cell debris pelleted by centrifuging at 200000*g* for 30 min. To the resulting supernatant was added a saturated solution of ammonium sulfate to a final concentration of 1.5 M. This solution was then chromatographed on Pharmacia butyl-Sepharose (1.6 × 25 cm) in a 100 mL gradient from 1.5 to 0 M ammonium sulfate in the resuspension buffer. Pooled fractions (total volume 48 mL) were then dialyzed against several changes of 50 mM Tris·HCl, pH 8, 10% glycerol, 3 mM DTT, and 2 mM EDTA and chromatographed on Pharmacia Resource Q (1 × 20 cm) in a 300 mL gradient from 0 to 1 M KCl in the same buffer. Fractions judged as pure by SDS−polyacrylamide gel electrophoresis were then pooled (total volume 24 mL) and dialyzed against 50 mM Tris·HCl, pH 8, 5% glycerol. Typically the purification yielded 80 mg of GlmU (extinction coefficient of 0.69 cm^2 mg^{-1} calculated from the amino acid composition).

The N-terminal proteolytic fragment of GlmU (M1-R331) was generated serendipitously during purification from the overproducing strain [BL21(DE3)/pET22b-*glm*U] using a procedure similar to that described for the isolation of the full-length enzyme except that additives to discourage proteolysis were omitted from the resuspension buffer. In this procedure, approximately 10 mg of the N-terminal fragment of GlmU was isolated (extinction coefficient of 0.65 cm^2 mg^{-1} calculated from the amino acid composition).

The C-terminal GST-fusion of GlmU (GST-M180-K456) was purified from the overproducing strain [BL21(DE3)/pGEX3b-3′-*glm*U] according to the following procedure. Cells (2 L) were grown, induced, harvested, passed through a French press, and centrifuged as described for the full-length enzyme. While the vast majority of overexpressed fusion protein formed insoluble inclusion bodies, a small proportion remained soluble and was insolated by affinity chromatography. The cell supernatant was loaded onto a 5 mL glutathione−Sepharose 4B (Pharmacia) affinity column and soluble GST-fusion protein eluted with 10 mM glutathione in 50 mM Tris·HCl, pH 8, 5% glycerol. The eluent (about 30 mL) was then membrane-concentrated to a volume of 1 mL and a protein concentration of 0.2 mg/mL (Bradford, 1976).

Enzyme Assays. For the full-length enzyme, acetyltransferase activity was monitored in a discontinuous assay by quantitation of CoA produced. CoA reacts with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to release 4-nitrothiophenolate which can be quantitated at 412 nm (Riddles *et al*., 1983). The standard 400 μL assay mix included 100 mM Tris·HCl, pH 7.6, 5 mM $MgCl_2$, and the substrates glucosamine-1-P and acetyl-CoA. When GlcN-1-P or acetyl-CoA was to be present at saturating concentration, it was added to 2 mM. Assays were initiated by adding GlmU and were stopped, generally at 0, 30, 60, and 90 s, by addition

of 500 μL of a solution of 0.1 M Tris·HCl and 6.40 M guanidine hydrochloride, pH 7.27. To the stopped assay was added 100 μL of a DTNB solution (0.1 M Tris·HCl, pH 7.6, 1 mM EDTA, and 0.01 mM DTNB) and the absorbance at 412 nm determined (extinction coefficient of 13.7 mM^{-1} cm^{-1}). At least three time points were used for each rate determination, and most assays were performed in duplicate.

Uridylyltransferase activity was monitored in a continuous coupled assay for the detection of inorganic pyrophosphate. The assay was a modification of that developed by Webb (1992) for the continuous monitoring of inorganic phosphate and will be referred to here as the MESG-PRPase/pyrophosphatase assay. Pyrophosphate production was monitored with excess inorganic pyrophosphatase, which hydrolyzes 1 equiv of pyrophosphate to 2 equiv of inorganic phosphate, and purine ribonucleoside phosphorylase (PRPase), which catalyzes the phosphorolysis of 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG) with a resulting change in the extinction coefficient at 360 nm of 11.0 mM^{-1} cm^{-1}. Synthesis of MESG and purification of PRPase were as described previously (Etzkorn *et al*., 1994). The standard 400 μL assay mix contained the following: 50 mM Tris·HCl, pH 7.6, 5 mM $MgCl_2$, 1 mM DTT, 0.2 mM MESG, 2.5 units of inorganic pyrophosphatase (EC 3.6.1.1), and 3 μg of purine ribonucleoside phosphorylase (EC 2.4.2.1). When *N*-acetylglucosamine-1-P or UTP was to be present at saturating concentration, it was added to 1 mM. The reaction was started by the addition of GlmU and the A_{360} monitored continuously.

Acetyltransferase activity of the C-terminal GlmU GST-fusion (GST-M180-K456) was assayed continuously by coupling *N*-acetylglucosamine-1-P formation by the fusion protein with uridylyltransfer and pyrophosphate formation by excess N-terminal proteolytic fragment and the MESG-PRPase/pyrophosphatase assay (see above). The assay mixture was that of the standard assay described above with the addition of 1 μM N-terminal proteolytic fragment.

In evaluating k_{cat} and K_m in steady-state kinetic studies, every effort was made to examine substrate concentrations having values ranging from approximately 0.5 to 5 times the K_m. In all cases, apparent steady-state parameters for a given substrate were determined in the presence of saturating amounts of cosubstrate, usually at concentrations between 5 and 10 times the K_m.

RESULTS

Figure 1 depicts an SDS−polyacrylamide gel of the overexpressed GlmU proteins examined in this study: full-length GlmU protein, an N-terminal proteolytic fragment, and a C-terminal GST fusion protein. Overexpression of the GlmU protein allowed the preparation of about 40 mg of highly purified protein from a 1 L fermentation. Purification of the N-terminal proteolytic fragment was, in fact, accidental and was obtained on a first attempt to purify the GlmU protein in the absence of proteinase inhibitors. N-Terminal sequencing demonstrated that the proteolyzed protein (apparent molecular weight by SDS−PAGE of 38 000) had the same N-terminal sequence as full-length GlmU (MLNNAMSVVI). A more accurate determination of the molecular weight of the N-terminal proteolytic fragment by laser desorption mass spectrometry indicated a mass of 36 040 Da, most consistent with cleavage C-terminal to Arg331 (mass by amino acid composition 36 107 Da). Despite good overexpression of the C-terminal GST fusion protein GST-M180-K456, this construct produced only a very small proportion of soluble protein (about 1%), allowing the purification of only 1 mg of protein in which some lower molecular weight species, including free GST (molecular weight 30 000), are evident on the gel. An alternative GST fusion construct, expressing GST-M253-K456, proved too unstable to purify, producing only GST, presumably as a result of degradation.

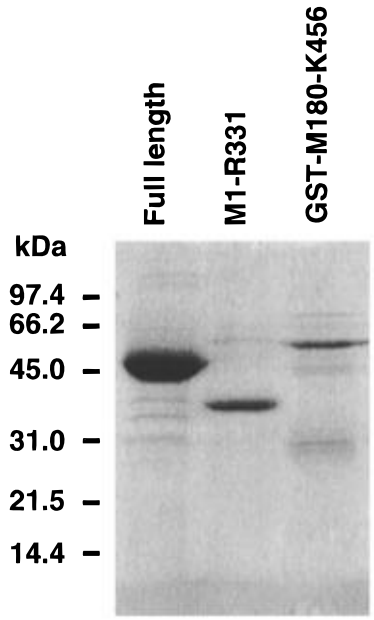


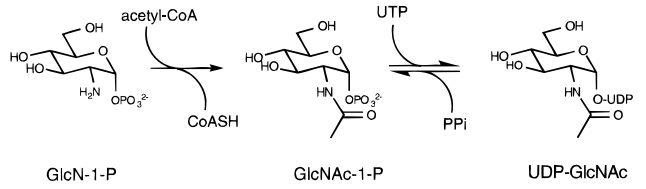
FIGURE 1: SDS−polyacrylamide gel electrophoresis of purified proteins: full-length GlmU; N-terminal proteolytic fragment M1-R331; C-terminal GST-fusion GST-M180-K456. Samples of GlmU (25 μg), N-terminal proteolytic fragment (5 μg), and C-terminal GST-fusion (2 μg) were denatured by boiling in Laemmli (1970) SDS sample buffer containing 8% mercaptoethanol prior to electrophoresis. Apparent molecular weights were 52 000 for full-length GlmU, 38 000 for the N-terminal fragment, and 58 000 for the C-terminal GST-fusion (some contaminating GST of molecular weight 30 000).

Table 1: Substrate Specificity of Acetyltransferase and Pyrophosphorylase (Uridylyltransferase) Activities of GlmU

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\times 10^{-5}$) ($L \cdot mol^{-1} \cdot s^{-1}$)
(1) acetyltransferase			
glucosamine-1-P	0.25	77	3.1
UDP-glucosamine	1.30	33	0.25
glucosamine	>720	0.0005	
galactosamine-1-P	15	11	0.007
acetyl-CoA	0.32	55	1.7
n-propionyl-CoA	0.84	9.5	0.11
n-butyryl-CoA (2 mM)		0.008	
(2) pyrophosphorylase:			
glucosamine-1-P (1 mM)		<0.0001	
N-acetylglucosamine-1-P	0.018	21	12
glucose-1-P	2.0	0.2	0.001
N-acetylgalactosamine-1-P	3.7	0.1	0.0003
UTP	0.017	24	14

Substrate Specificity and Reaction Order. Table 1 depicts the results of steady-state kinetic analysis of both the acetyltransferase and uridylyltransferase activities of full-length GlmU with a variety of substrates. The acetyltransferase reaction proceeds up to 4 times faster than the

Scheme 1



uridylyltransferase reaction at nearly 80 s^{-1} versus 20 s^{-1}, respectively. In establishing reaction order, product identities were confirmed by cochromatography with authentic compounds (i.e., *N*-acetylglucosamine-1-P, UDP-glucosamine, UDP-*N*-acetylglucosamine) on anion exchange (MonoQ) HPLC using a triethylammonium bicarbonate gradient (Brown *et al*., 1994).

Glucosamine-1-P and acetyl-CoA were the preferred substrates for the acetyltransferase reaction, displaying apparent K_m values of 0.25 and 0.32 mM, respectively, with a turnover number approaching 80 s^{-1}. UDP-glucosamine is also a reasonable substrate for acetyltransfer with a k_{cat} of 33 s^{-1}, nearly half of that of glucosamine-1-P; however, its K_m is 5-fold higher. Thus, GlmU shows a 12-fold kinetic preference for the acetylation of glucosamine-1-P versus UDP-glucosamine (k_{cat}/K_m values of 3.1×10^5 and 0.25×10^5 $L \cdot mol^{-1} \cdot s^{-1}$, respectively). On the other hand, glucosamine is a very poor substrate for acetyltransfer with a rate just detectable above the assay background, highlighting the importance of a phosphate group at the C-1 position of the sugar. GlmU is also capable of propionyl transfer from propionyl-CoA to glucosamine-1-P, but butyryl-CoA proved to be a very poor substrate for the enzyme; k_{cat} values were 9.5 s^{-1} and 0.5 min^{-1}, respectively (Table 1). GlmU shows a 15-fold kinetic preference for acetyl-CoA over propionyl-CoA, as indicated by k_{cat}/K_m values of 1.7×10^5 and 0.11×10^5 $L \cdot mol^{-1} \cdot s^{-1}$. The acetyltransferase parameters described above were determined in the absence of UTP. However, no effect on either k_{cat} or K_m for acetyltransfer using the substrates acetyl CoA and GlcN-1-P was observed in the presence of 0.2 mM UTP (data not shown).

For the uridylyltransfer reaction, apparent K_m values for the preferred substrates, *N*-acetylglucosamine-1-P and UTP, were 0.018 and 0.017 mM, respectively (Table 1). In contrast, GlmU-catalyzed uridylyltransfer to glucosamine-1-P was undetectable (<0.0001 s^{-1}). Thus, the acetylation of glucosamine-1-P would appear to occur prior to the uridylyltransfer reaction, indicating the reaction order for catalysis by GlmU which is depicted in Scheme 1. It was noted that GlmU will catalyze the transfer of UMP to glucose-1-P at the slow rate of 0.2 s^{-1} and with a k_{cat}/K_m (100 $L \cdot mol^{-1} \cdot s^{-1}$) which is 10^4-fold lower than that for transfer to GlcNAc-1-P (1.2×10^6 $L \cdot mol^{-1} \cdot s^{-1}$). Thus, the absence of a positive charge at the C-2 position of the sugar may be a necessary condition for catalysis, as is the case for GlcNAc-1-P and glucose-1-P but not for GlcN-1-P.

The ability of galactose sugars (C-4 epimers of glucose) to serve as substrates for GlmU was also investigated. Both galactosamine-1-P and *N*-acetylgalactosamine-1-P could serve as substrates in the acetyltransferase and uridylyltransferase reactions, respectively, with k_{cat} values of 11 and 0.1 s^{-1}, respectively. Glucosamine-1-P and *N*-acetylglucosamine-1-P were, however, strongly preferred substrates with k_{cat}/K_m values on the order of 10^2- and 10^4-fold those for their respective C-4 epimers.

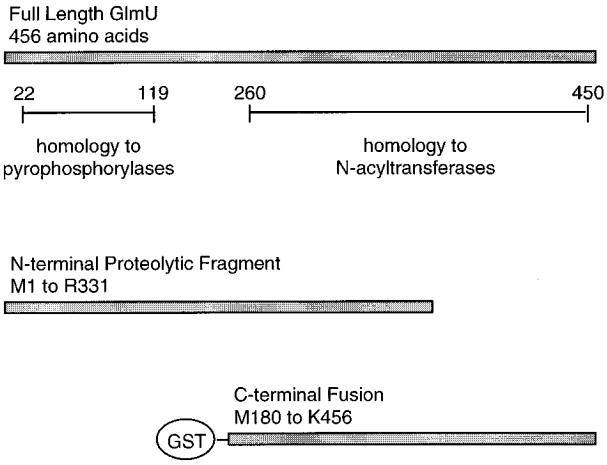


FIGURE 2: Schematic of full-length and truncated GlmU proteins. Full-length GlmU (molecular weight 49 167) shows N-terminal homology with UDP-glucose pyrophosphorylases and C-terminal homology to *N*-acyltransferases [e.g., UDP-3-*O*-(3-hydroxymyristoyl)glucosamine *N*-acyltransferase]. The N-terminal proteolytic fragment (molecular weight 36 108) and the C-terminal glutathione *S*-transferase fusion protein (molecular weight 21 543 fused to GST) were examined for each of the two activities (see Table 2).

UDP-*N*-acetylmuramic acid and *N*-acetylmuramic acid were previously reported to be inhibitory to the acetyltransferase reaction catalyzed by GlmU (Mengin-Lecreulx & van Heijenoort, 1994). In our hands, no inhibition of acetyltransferase activity was observed in the presence of either 0.1 mM UDP-*N*-acetylmuramic acid or 10 mM *N*-acetylmuramic acid (data not shown).

Domain Dissection Experiments. Having addressed the question of reaction order, we were interested in determining whether the acetyltransferase and uridylyltransferase activities reside at a shared or overlapping active site or at two distinct active sites. To explore the two active site possibility, we sought to isolate fragments of GlmU bearing only one of the enzyme's activities. BLAST searching of the protein sequence databases using GlmU as the query sequence revealed that the N-terminal portion of GlmU bears homology to other pyrophosphorylases (e.g., UDP-glucose pyrophosphorylases) while the C-terminal portion shows homology with *N*-acyltransferases [e.g., UDP-3-*O*-(*R*-3-hydroxymyristoyl)glucosamine *N*-acyltransferase]. To determine if uridylyltransferase (pyrophosphorylase) and acetyltransferase domains could be active in isolation, we examined the activity of purified, truncated versions of GlmU: the serendipitously isolated N-terminal proteolytic fragment and an engineered C-terminal GST fusion protein (Figures 1 and 2).

The N-terminal proteolytic fragment had no detectable acetyltransferase activity (<0.0001 s^{-1} at 0.5 mM substrates) but demonstrated appreciable uridylyltransferase activity. K_m values for *N*-acetylglucosamine-1-P (0.01 mM) and UTP (0.012 mM) were similar to those of the full-length enzyme (Table 2). However, enzyme turnover was 20-fold slower with the N-terminal fragment demonstrating a k_{cat} of 1.2 s^{-1}. Conversely, the C-terminal GST-fusion protein had no detectable (<0.0001 s^{-1} at 0.1 mM substrates) uridylyltransferase capacity but was capable of acetyltransfer. The acetyltransferase turnover of the C-terminal GST fusion was approximately 150-fold reduced from full-length GlmU with a k_{cat} of 0.5 s^{-1}. The K_m value for GlcN-1-P was comparable to full-length enzyme at 0.37 mM while that for acetyl-CoA

Table 2: Steady-State Kinetic Parameters Describing the Reactions Catalyzed by the N-Terminal Fragment and C-Terminal GST Fusion

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\times 10^{-5}$) ($L \cdot mol^{-1} \cdot s^{-1}$)
C-Terminal GST Fusion			
acetyltransferase			
glucosamine-1-P	0.37	0.5	0.01
acetyl-CoA	1.1	0.4	0.004
N-Terminal Fragment			
pyrophosphorylase			
N-acetylglucosamine-1-P	0.010	1.2	1.2
glucosamine-1-P		undetectable	
galactosamine-1-P		undetectable	
UTP	0.012	1.2	1.0

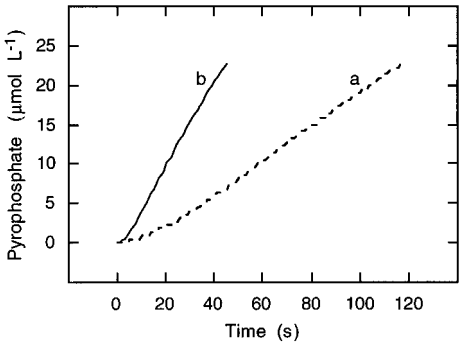
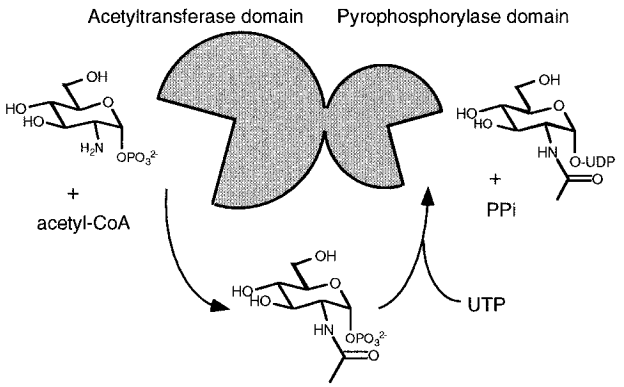


FIGURE 3: Time course of pyrophosphate formation by GlmU in the presence of UTP, acetyl-CoA, and glucosamine-1-P. The MESG-PRPase/pyrophosphatase continuous assay was used to monitor the bifunctional reaction catalyzed by GlmU (0.013 μM) in the absence (a) or presence (b) of 1 μM N-terminal proteolytic fragment. Substrate concentrations were 0.2 mM UTP, 0.5 mM acetyl-CoA, and 0.5 mM glucosamine-1-P. Steady-state turnover numbers were (a) 16 s^{-1} and (b) 44 s^{-1}.

was 3-fold higher at 1.1 mM (Table 2). It has been reported that chromatography of full-length GlmU on gel filtration was consistent with homodimer or homotrimer oligomerization states (Mengin-Lecreulx & van Heijenoort, 1994). We did not investigate the oligomerization state of the truncated GlmU fragments described here.

Release of the Intermediate N-Acetylglucosamine-1-P. Figure 3 (curve a) depicts a typical time course for pyrophosphate production as a result of bifunctional catalysis (substrates acetyl-CoA, glucosamine-1-P, and UTP) by GlmU. Of note is the existence of an approximately 30 s lag in the approach to steady-state catalysis (16 s^{-1}). Further, the addition of an excess (100-fold) of the N-terminal uridylyltransferase fragment in the reaction mixture was found to substantially increase the steady-state reaction rate (44 s^{-1}) and to largely eliminate the pre-steady-state lag in product formation (Figure 3, curve b). The simplest interpretation of these results is that the lag describes the time required for K_m levels of *N*-acetylglucosamine-1-P to accumulate such that the uridylyltransferase reaction can proceed at a substantial rate. Moreover, the 3-fold increase (from 16 to 44 s^{-1}) in reaction rate achieved by adding excess uridylyltransferase activity mirrors the finding that the k_{cat} values for acetyltransfer were 3−4-fold those for uridylyltransfer (Table 1). Indeed, given a rate for acetyltransfer of 0.6 $\mu mol \cdot L^{-1}$ s^{-1} (turnover of 44 s^{-1}, enzyme concentration 0.013 μM), about 30 s would be required to produce K_m quantities (0.018 mM) of *N*-acetylglucosamine-1-P. In sum, these findings are consistent with a two active site model

Scheme 2



where the intermediate *N*-acetylglucosamine-1-P is released from the acetyltransferase domain prior to transformation by the domain for uridylyltransfer (Scheme 2).

To determine independently the amount of *N*-acetylglucosamine-1-P which accumulates in the steady state, GlmU was allowed to react with acetyl-CoA, glucosamine-1-P, and UTP for 90 s, at which point the reaction mixture was spiked with [^{14}C]-*N*-acetylglucosamine-1-P (Figure 4). The steady-state production of pyrophosphate detected with the MESG-PRPase/pyrophosphatase continuous assay after the radioactive spike was 0.11 $\mu M \cdot s^{-1}$. The rate of production of labeled product, [^{14}C]UDP-*N*-acetylglucosamine, however, over the 105 s time period was 0.32 $nM \cdot s^{-1}$ as determined by quantitation of the ratio of [^{14}C]-*N*-acetylglucosamine-1-P to [^{14}C]UDP-*N*-acetylglucosamine by anion exchange HPLC. Given that 229 nM [^{14}C]-*N*-acetylglucosamine-1-P was added to the reaction, the rate of its accumulation into product implies a steady-state accumulation of bulk *N*-acetylglucosamine-1-P to an average concentration of 78 μM over the time period examined.

DISCUSSION

The GlmU protein was recently shown to be bifunctional, catalyzing both acetyltransfer and uridylyltransfer in the transformation of glucosamine-1-P to UDP-*N*-acetylglucosamine (Mengin-Lecreulx & van Heijenoort, 1994). In this work, we present steady-state kinetic evidence which establishes that the chemical reaction order imposed by the bifunctional enzyme is not random, but that acetyltransfer precedes uridylyltransfer. The kinetic data indicate that GlmU first catalyzes acetyltransfer from acetyl-CoA to glucosamine-1-P with release of *N*-acetylglucosamine-1-P and subsequently uridylyltransfer from UTP to *N*-acetylglucosamine-1-P yielding UDP-*N*-acetylglucosamine. A 12-fold kinetic preference (k_{cat}/K_m) was found for acetyltransfer to glucosamine-1-P over UDP-glucosamine. Uridylyltransfer to glucosamine-1-P was undetectable.

We have also shown that acetyltransfer and uridylyltransfer occur in separable active sites of the GlmU protein. We have isolated an N-terminal proteolytic fragment of GlmU with only pyrophosphorylase activity and have constructed a C-terminal GST-fusion protein which displays only acetyltransferase activity. These truncated proteins demonstrated K_m values which were comparable to full-length GlmU; however, in each case enzyme turnover was substantially reduced. The N-terminal proteolytic fragment, consisting of the first 331 amino acids of GlmU, shows homology to other pyrophosphorylases over amino acids 20−120, and the

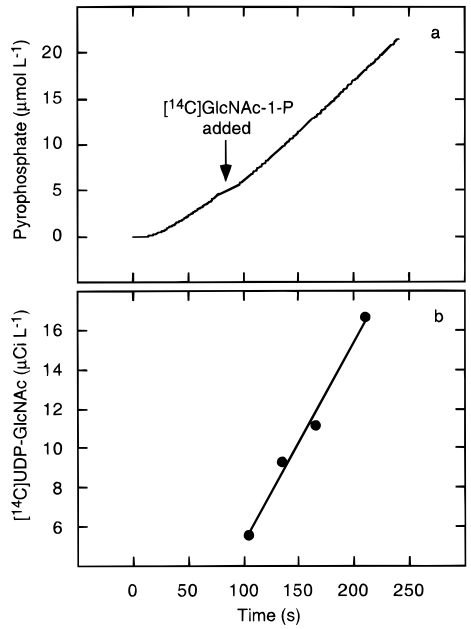


FIGURE 4: Steady-state incorporation of radioactivity from trace quantities of [^{14}C]-GlcNAc-1-P into product during the course of the bifunctional reaction catalyzed by GlmU. The reaction (2.5 mL) of GlmU (0.007 μM) incubated with acetyl-CoA (2 mM), glucosamine-1-P (2 mM), and UTP (0.2 mM) was allowed to proceed for a time of 90 s before 0.20 μCi of [^{14}C]-GlcNAc-1-P (309 Ci mol^{-1}) was added. Aliquots (400 μL) of the reaction were then removed at times 105, 135, 165, and 210 s during the time course and analyzed by anion exchange HPLC with in-line radioactivity detection to determine the relative amounts of radioactivity associated with the GlcNAc-1-P and UDP-GlcNAc peaks. Panel a shows the course of the reaction as determined using the MESG-PRPase/pyrophosphatase continuous assay (steady-state slope after the addition of [^{14}C]-GlcNAc-1-P of 0.11 μM s^{-1}). Panel b shows the production of [^{14}C]-UDP-GlcNAc and demonstrates a slope of 0.10 μCi·L^{-1}·s^{-1}. Given that GlcNAc-1-P is released from the enzyme in the bifunctional reaction (see Figure 2), the rate of production of [^{14}C]-UDP-GlcNAc (0.32 nM s^{-1} based on a specific activity of 309 Ci mol^{-1}) represents a 344-fold drop in the specific activity of the [^{14}C]-GlcNAc-1-P added. Such a drop corresponds to a steady-state concentration of 78 μM GlcNAc-1-P during bifunctional catalysis over the time period.

C-terminal GST-fusion protein, including amino acids 180−456 of GlmU, shows homology to N-acyltransferases over amino acids 260−450 (Figure 2). While the N-terminal proteolytic fragment may include a portion of the acetyltransferase domain of GlmU, it shows no activity in the acetyltransferase assay. Thus, it appears that the acetyltransferase and pyrophosphorylase domains of GlmU are separable, each containing an active site which is functional in isolation.

Kinetic analysis also demonstrated that *N*-acetylglucosamine-1-P is released by GlmU and accumulates to K_m levels for the pyrophosphorylase reaction, a result consistent with evidence provided in this work that acetyltransferase and uridylyltransferase activities reside at separate active sites (Scheme 2). In assaying the GlmU-catalyzed bifunctional transformation of UTP, acetyl-CoA, and glucosamine-1-P to UDP-*N*-acetylglucosamine and pyrophosphate, a substantial pre-steady-state lag (30 s) could be largely eliminated by the addition of excess truncated N-terminal fragment with only uridylyltransferase activity. This addition increased the observed reaction rate by 3-fold, suggesting that the uridylyltransfer reaction was limiting to the overall rate and that the intermediate, *N*-acetylglucosamine-1-P, was released from full-length GlmU during catalysis. Indeed, an investigation of the rate of incorporation of ^{14}C-labeled *N*-acetylglucosamine-1-P into UDP-*N*-acetylglucosamine during the steady-state period of the bifunctional reaction suggested that *N*-acetylglucosamine-1-P accumulates to levels of 78 μM under the conditions investigated, a value 4 times the K_m for this substrate (18 μM).

The substrate specificity for both acetyltransfer and uridylyltransfer by GlmU has been probed in this work with a variety of compounds. For the acetyltransferase reaction, there is some flexibility in the length of the acyl group to be transferred from coenzyme A to the amine of the sugar; a 15-fold kinetic preference was demonstrated for acetyltransfer over propionyltransfer. Obligate to the acetyltransferase reaction was the presence of a phosphate group at the C-1 position of the sugar. Glucosamine-1-P and UDP-glucosamine were both reasonably good substrates while glucosamine, lacking a phosphate group, was an extremely poor substrate. For the pyrophosphorylase reaction, the absence of a positive charge at position 2 of the sugar appeared to be critical for catalysis. Both *N*-acetylglucosamine-1-P and glucose-1-P were substrates for the uridylyltransferase reaction while glucosamine-1-P showed no detectable turnover as a substrate. It is noteworthy that, unlike GlmU, the homolog UDP-glucose pyrophosphorylase is capable of slow but significant uridylyltransfer to glucosamine-1-P, and was used in this work to prepare UDP-glucosamine in order to examine the specificity of the uridylyltransfer reaction. Finally, stereochemistry at C-4 was also important although not critical to the acetyltransferase and uridylyltransferase activity. The substrates galactosamine-1-P and *N*-acetylgalactosamine-1-P showed lower k_{cat}/K_m values on the order of 10^2- and 10^4-fold, compared to their epimers glucosamine-1-P and *N*-acetylglucosamine-1-P, respectively.

The functional similarities of the acetyltransferase and uridylyltransferase activities of GlmU with those of the well-studied enzymes chloramphenicol acetyltransferase and UDP-glucose pyrophosphorylase, respectively, may shed light on the mechanisms of these transformations by GlmU. Stereochemical analysis of the transformation by UDP-glucose pyrophosphorylase has demonstrated inversion of configuration at the α-phosphate of UTP, suggesting a single-step direct attack mechanism for that enzyme (Sheu & Frey, 1978). Furthermore, steady-state kinetic analysis of chloramphenicol acetyltransferase indicated ordered binding and an enzyme ternary complex (Kleanthous & Shaw, 1984). Experiments aimed at a mechanistic understanding of the reactions catalyzed by the GlmU protein will be important to a complete understanding of the enzyme's role at the branchpoint of peptidoglycan and lipopolysaccharide biosynthesis in bacteria. With the understanding that distinct domains of GlmU are responsible for acetyltransfer and uridylyltransfer, future mechanistic work can focus on understanding the respective reactions in isolation.

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