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Copurification of Glucosamine-1-Phosphate Acetyltransferase and *N*-Acetylglucosamine-1-Phosphate Uridyltransferase Activities of *Escherichia coli*: Characterization of the *glmU* Gene Product as a Bifunctional Enzyme Catalyzing Two Subsequent Steps in the Pathway for UDP-*N*-Acetylglucosamine Synthesis

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The *glmU* gene product of *Escherichia coli* was recently identified as the *N*-acetylglucosamine-1-phosphate uridyltransferase activity which catalyzes the formation of UDP-*N*-acetylglucosamine, an essential precursor for cell wall peptidoglycan and lipopolysaccharide biosyntheses (D. Mengin-Lecreulx and J. van Heijenoort, J. Bacteriol. 175:6150–6157, 1993). Evidence that the purified GlmU protein is in fact a bifunctional enzyme which also catalyzes acetylation of glucosamine-1-phosphate, the preceding step in the same pathway, is now provided. Kinetic parameters of both reactions were investigated, indicating in particular that the acetyltransferase activity of the enzyme is fivefold higher than its uridyltransferase activity. In contrast to the uridyltransferase activity, which is quite stable and insensitive to thiol reagents, the acetyltransferase activity was rapidly lost when the enzyme was stored in the absence of reducing thiols or acetyl coenzyme A or was treated with thiol-alkylating agents, suggesting the presence of at least one essential cysteine residue in or near the active site. The acetyltransferase activity is greatly inhibited by its reaction product *N*-acetylglucosamine-1-phosphate and, interestingly, also by UDP-*N*-acetylmuramic acid, which is one of the first precursors specific for the peptidoglycan pathway. The detection in crude cell extracts of a phosphoglucosamine mutase activity finally confirms that the route from glucosamine-6-phosphate to UDP-*N*-acetylglucosamine occurs via glucosamine-1-phosphate in bacteria.

UDP-*N*-acetylglucosamine (UDP-GlcNAc) is one of the main cytoplasmic precursors of bacterial cell wall peptidoglycan (13, 28). In *Escherichia coli* and most related gram-negative bacteria, it is also the precursor for the disaccharide moiety of lipid A, which is an essential component of outer membrane lipopolysaccharide, as well as for the synthesis of the enterobacterial common antigen (16, 30). While the genes and enzymes involved in the steps which occur downstream from this branchpoint (shown schematically in Fig. 1) in the different pathways have in most cases been identified and studied in detail (1, 15, 16, 19–21, 28, 30), the metabolic route leading to the formation of UDP-GlcNAc has been only poorly characterized.

It is generally assumed that UDP-GlcNAc is synthesized from fructose 6-phosphate by four enzyme-catalyzed reactions (7, 11, 38) (Fig. 1). The first of these reactions is catalyzed by the glucosamine-6-phosphate (GlcN-6-P) synthase (L-glutamine:D-fructose-6-phosphate amidotransferase) (9). Mutants altered in this activity which required glucosamine or GlcNAc for growth have been characterized (33, 39), and the corresponding *glmS* gene has been located at 84 min on the *E. coli* map (3, 9, 37).

The fourth step concerns the formation of UDP-GlcNAc from *N*-acetylglucosamine-1-phosphate (GlcNAc-1-P) and UTP. It is catalyzed by the GlcNAc-1-P uridyltransferase (UDP-GlcNAc pyrophosphorylase) (34). We and others recently noted that the *glmS* gene was preceded by an open reading frame of unknown function named *Eco*URF-1, which theoretically encoded a polypeptide of 456 amino acids with a molecular weight of 49,130 (24, 37). The short intergenic distance between *Eco*URF-1 and *glmS* and the absence of an obvious promoter sequence upstream of *glmS* suggested that these genes were cotranscribed (29) and that the function of *Eco*URF-1 might also be related to cell wall synthesis. A conditional mutant in which the synthesis of the *Eco*URF-1 gene product was impaired at 42°C was constructed (24). We observed that the inactivation of this gene in exponentially growing cells was associated with various alterations of cell shape and cell lysis which resulted from an arrest of peptidoglycan synthesis. The high level of accumulation of GlcNAc-1-P in the mutant cells and the concomitant depletion of all precursors located downstream in the pathway indicated that the mutational block was in the step leading from GlcNAc-1-P and UTP to the formation of UDP-GlcNAc. Enzymatic assays finally confirmed that the *Eco*URF-1 gene coded for the GlcNAc-1-P uridyltransferase (24), and this gene was named *glmU* for glucosamine uridyltransferase, according to the nomenclature previously adopted for the *glmS* gene encoding GlcN-6-P synthase (37, 39).

The enzymes catalyzing the two intermediate steps from GlcN-6-P to GlcNAc-1-P, as well as their genes, remained to be characterized. These activities were tentatively named phosphoglucosamine mutase and glucosamine-1-phosphate

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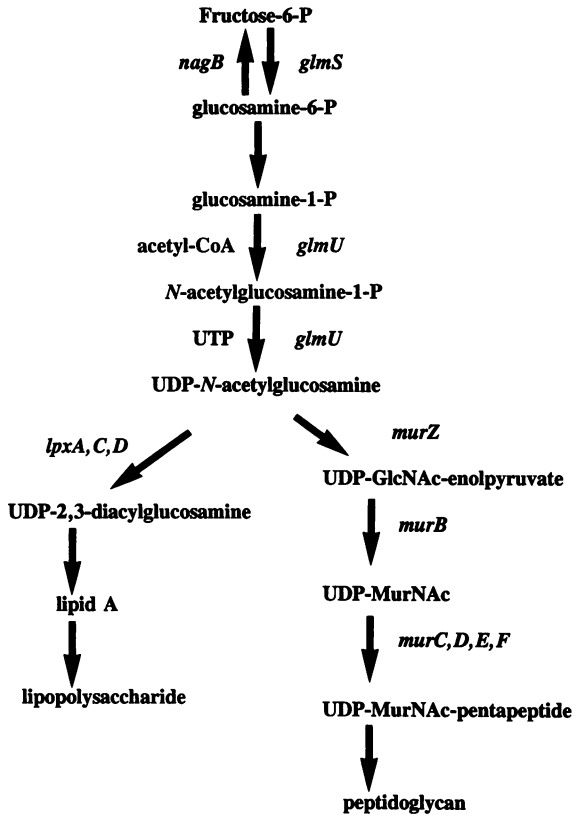

FIG. 1. Biosynthesis and cellular utilization of UDP-GlcNAc in *E. coli*.

(GlcN-1-P) acetyltransferase, assuming that the route for UDP-GlcNAc was via GlcN-1-P, as shown in Fig. 1. Here we demonstrate the presence of both activities in crude extracts of *E. coli* and prove that the GlcN-1-P acetyltransferase activity is in fact also carried by the *glmU* gene product, which is thus a bifunctional enzyme catalyzing two subsequent steps in the pathway for UDP-GlcNAc synthesis.

MATERIALS AND METHODS

Bacterial strain, plasmids, and growth conditions. *E. coli* JM83 [*ara* Δ(*lac-proAB*) *rpsL thi* φ80 d*lacZ* ΔM15] (40) was used as the host strain for plasmids and for the large-scale purification of the overproduced *glmU* gene product (24). Cloning vectors pUC18 and pUC19 were purchased from Pharmacia. Plasmids pMLD71 and pMLD78, carrying the *glmU* gene expressed under the control of the *lac* or lambda p_R promoter, respectively, and plasmid pMLD76, carrying the *glmU* gene inactivated by a kanamycin resistance cartridge, were previously described (24). Small- and large-scale plasmid isolations and *E. coli* cell transformation were performed according to standard procedures (6, 32). Unless otherwise stated, 2YT was used as a rich medium for growing cells (26) and growth was monitored at 600 nm with a spectrophotometer (model 240; Gilford Instrument Laboratories, Inc., Oberlin, Ohio). For strains carrying drug resistance genes, antibiotics were used at 100 $\mu g \cdot ml^{-1}$ for ampicillin and 30 $\mu g \cdot ml^{-1}$ for kanamycin.

Preparation of crude enzyme. Cells of JM83 harboring the different plasmids listed in Table 1 (except pMLD78) were grown exponentially at 37°C in rich medium supplemented with ampicillin (1-liter culture). Cells were harvested in the cold when the optical density of the cultures reached 0.7 (4×10^8 cells $\cdot ml^{-1}$). A different protocol was used with cells carrying the pMLD78 plasmid, in which the *glmU* gene was under the control of the lambda p_R promoter, as follows. Cells were grown first at 30°C, and at an absorbance of 0.2 the cultures were shifted to 42°C for 3 h (to inactivate the thermosensitive repressor encoded by the *c*I857 gene present on the plasmid), during which time the absorbance reached a plateau of 0.7. In all cases, cells were harvested and washed with 40 ml of cold 20 mM potassium phosphate buffer (pH 7.4) containing 10 mM β-mercaptoethanol, 5 mM $MgCl_2$, and 10% (vol/vol) glycerol (buffer A). The wet cell pellet was suspended in 10 ml of the same buffer and disrupted by sonication (Sonicator 150; T.S. Ultrasons, Annemasse, France) for 10 min with cooling. The resulting suspension was centrifuged at 4°C for 20 min at 200,000 × *g* with a Beckman TL100 centrifuge. The supernatant was dialyzed overnight at 4°C against 100 volumes of the same phosphate buffer, and the resulting solution (about 80 mg of protein in 9 ml), designated crude enzyme, was stored at −20°C. Protein concentrations were determined by the method of Lowry et al. (18), using bovine serum albumin as a standard.

Analysis of a crude protein extract of *E. coli* on DEAE–Trisacryl-M. The total extract (80 mg of protein) secured from wild-type strain JM83 as described above was loaded onto a column (12.5 by 2.5 cm) of DEAE–Trisacryl-M (IBF, Villeneuve-la-Garenne, France) that had been preequilibrated with buffer A. The elution was run at a flow rate of 1 $ml \cdot min^{-1}$, first with 70 ml of buffer A and then with a linear gradient (400 ml) of NaCl (0 to 500 mM) in buffer A. Elution of proteins was monitored at 280 nm, and fractions (10 ml) were assayed as described below for phosphoglucosamine mutase, GlcN-1-P acetyltransferase, and GlcNAc-1-P uridyltransferase activities.

Enzymatic assays. (i) Phosphoglucosamine mutase activity. A coupled assay in which the GlcN-1-P produced from GlcN-6-P was quantitatively converted to UDP-GlcNAc by the purified GlmU activity was used. The standard assay mixture contained 50 mM Tris-hydrochloride buffer (pH 8.2), 1 mM GlcN-6-P, 1 mM [^{14}C]acetyl coenzyme A (acetyl-CoA) (500 Bq), 5 mM UTP, 3 mM $MgCl_2$, pure GlmU activity (1 μg), and crude extract (1 to 10 μg of protein) in a final volume of 100 μl.

(ii) GlcN-1-P acetyltransferase activity. The standard assay mixture contained 50 mM Tris-hydrochloride buffer (pH 8.2), 2 mM GlcN-1-P, 1 mM [^{14}C]acetyl-CoA (500 Bq), 3 mM $MgCl_2$, and enzyme (0.01 to 1 μg of protein, depending on overexpression and purification factors) in a final volume of 100 μl.

(iii) GlcNAc-1-P uridyltransferase activity. The standard assay mixture contained 50 mM Tris-hydrochloride buffer (pH 8.2), 1 mM UTP, 0.2 mM [^{14}C]GlcNac-1-P (500 Bq), 3 mM $MgCl_2$, and enzyme (0.01 to 1 μg of protein) in a final volume of 100 μl.

In all cases, reaction mixtures were incubated at 37°C for 30 min, and the reaction was terminated by the addition of 20 μl of acetic acid. Reaction products were separated by high-voltage electrophoresis on Whatman 3MM filter paper in either 2% formic acid (pH 1.9) or pyridine-acetic acid-water (6:23:971) (pH 4.0) for 2 h at 40 V/cm with an LT36 apparatus (Savant Instruments, Hicksville, N.Y.). The radioactive spots were located by overnight autoradiography using type R2 films (3M, St. Paul, Minn.) or with a radioactivity scanner (Multi-Tracermaster LB285; Berthold France, Elancourt, France). The spots were cut out, and radioactivity was measured in an Intertechnique SL 30 liquid scintillation spectrophotometer

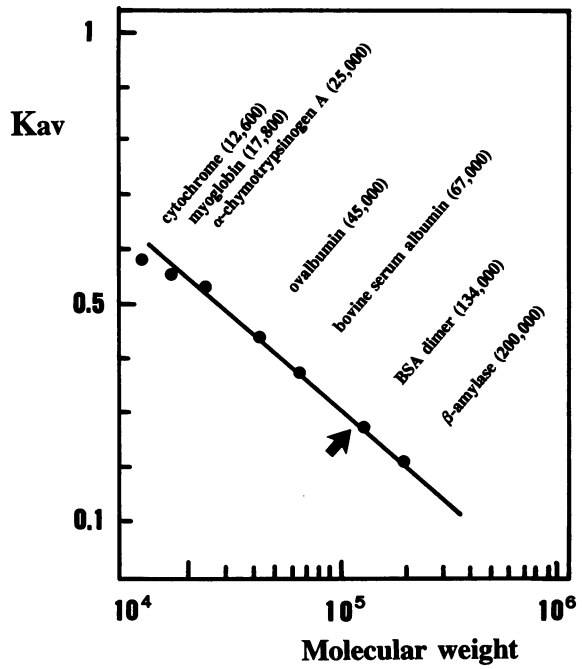

FIG. 2. Molecular weight of the native GlmU enzyme. A sample of pure enzyme (40 μg) was loaded onto a Pharmacia Superose 12 column (10 by 300 mm) equilibrated at 0.5 ml · min^{-1} with 50 mM potassium phosphate (pH 7.4), 150 mM NaCl, and 10 mM β-mercaptoethanol. Standards were detected by A_{280}, and GlmU was detected by both A_{280} and uridyltransferase activity in the fractions (200 μl each). The K_{av} for GlmU is indicated (arrow). BSA, bovine serum albumin.

with a solvent system consisting of 2 ml of water and 13 ml of Aqualyte mixture (J. T. Baker Chemicals, Deventer, The Netherlands). One unit of enzyme activity was defined as the amount which catalyzed the synthesis of 1 μmol of product in 1 min.

Estimation of molecular weight. The GlmU enzyme was overproduced and purified to homogeneity by a previously described procedure (24). A sample of pure GlmU (50 μl) containing 40 μg of protein in buffer A was applied onto a column of Superose 12 HR 10/30 connected to a Pharmacia fast protein liquid chromatography device. The column was equilibrated with 50 mM potassium phosphate buffer (pH 7.4) containing 150 mM NaCl and 10 mM β-mercaptoethanol, and the sample was eluted at 0.5 ml · min^{-1}. Calibration was carried out with cytochrome *c*, myoglobin, α-chymotrypsinogen A, ovalbumin, bovine serum albumin, and β-amylase. Standards were detected by A_{280}, and GlmU was detected by both A_{280} and enzyme activity in the fractions (200 μl each). The voided volume (V_0) and V_t were determined with blue dextran 2000 and tyrosine, respectively. A dimer of bovine serum albumin (134 kDa) eluted with a slightly higher K_{av} value than GlmU (Fig. 2), suggesting that the native GlmU enzyme was likely to be a dimer or trimer of identical 49-kDa units.

Chemicals. UDP-[^{14}C]GlcNAc (7.4 GBq · mmol^{-1}) was purchased from Amersham and [^{14}C]acetyl-CoA (1.9 GBq · mmol^{-1}) was from ICN (Irvine, Calif.). [^{14}C]GlcNAc-1-P was obtained from UDP-[^{14}C]GlcNAc by using the pyrophosphorylase activity of the purified GlmU enzyme. GlcN-1-P and GlcN-6-P, their *N*-acetyl derivatives, UTP, UDP-GlcNAc, and the thiol reagents DTNB [5,5′-dithiobis(2-nitrobenzoic acid)] and NTCB (2-nitro-5-thiocyanobenzoic acid) were from Sigma Chemical Co. (St. Louis, Mo.). *N*-Acetylmuramic acid-1-phosphate (MurNAc-1-P), UDP-*N*-acetylmuramic acid (UDP-MurNAc), and UDP-MurNAc peptides were prepared as previously described (10, 19, 20, 25).

TABLE 1. Specific activities of enzymes involved in UDP-GlcNAc synthesis in *E. coli* cells

Enzyme source	Sp act (U · mg of protein^{-1})[a]		
	Phosphoglucosamine mutase	GlcN-1-P acetyltransferase	GlcNAc-1-P uridyltransferase
Crude extract			
JM83(pUC19)	0.01	0.1	0.02
JM83(pMLD71)	0.01	2.0 (×20)	0.5 (×25)
JM83(pMLD76)	0.01	0.1	0.02
JM83(pMLD78)	0.01	48.0 (×480)	10.0 (×500)
Pure GlmU	0	2.2	15.1

[a] Overproduction factors are indicated in parentheses. Plasmids pMLD71 and pMLD78 contain the *glmU* gene expressed under the control of the *lac* and lambda p_R promoters, respectively. In the pMLD76 plasmid, the *glmU* gene sequence is interrupted by a kanamycin resistance cartridge.

RESULTS

Identification of the route for UDP-GlcNAc synthesis in *E. coli*. To detect phosphoglucosamine mutase and GlcN-1-P acetyltransferase activities in crude extracts of *E. coli*, we first used a convenient coupled assay in which the formation of UDP-GlcNAc from either GlcN-6-P or GlcN-1-P was followed by a previously described high-pressure liquid chromatography procedure (20). In all cases, acetyl-CoA and UTP were present at saturating concentrations, and pure *glmU*-encoded GlcNAc-1-P uridyltransferase was added to ensure that the final step was not limiting. In this way, we were able to detect both activities in crude extracts (Table 1), but surprisingly, appropriate controls showed that the pure GlmU enzyme alone catalyzed the two-step formation of UDP-GlcNAc from GlcN-1-P. Confirmation that this purified preparation of uridyltransferase also contained a GlcN-1-P acetyltransferase activity was obtained in a direct assay in which the synthesis of [^{14}C]GlcNAc-1-P from GlcN-1-P and [^{14}C]acetyl-CoA was monitored. No apparent acetylation of GlcN-6-P was observed under similar assay conditions, with either purified GlmU enzyme or crude cell extract. These results confirmed the initial assumption that UDP-GlcNAc was synthesized via GlcN-1-P and not via GlcNAc-6-P in *E. coli* (7, 38). The specific activities of the three enzymes involved in the stepwise formation of UDP-GlcNAc from GlcN-6-P in a freshly prepared crude extract of a wild-type *E. coli* strain were estimated (Table 1). Under the in vitro conditions used, the level of the acetyltransferase activity appeared fivefold higher than those of the mutase and uridyltransferase activities, which were more or less equivalent.

Characterization of GlmU as a bifunctional enzyme. The fact that samples of pure *glmU*-encoded uridyltransferase also catalyzed acetylation of GlcN-1-P could be interpreted in two different ways. (i) The acetyltransferase and uridyltransferase activities are encoded by two distinct genes, but these proteins either are tightly bound together or behave similarly during the two-step procedure used for the purification of the uridyltransferase activity. (ii) A more likely hypothesis was that both activities were carried out by the sole *glmU* gene product, which was thus a bifunctional enzyme in this metabolic pathway. To discriminate between the two hypotheses, the specific

TABLE 2. Inhibition of GlcN-1-P acetyltransferase activity[a]

Compound	Concn (mM)	Relative enzyme activity
None		100
GlcNAc-1-P	0.1	55
	1	15
MurNAc-1-P	0.1	20
UDP-MurNAc	0.01	40
	0.1	11
	1	2
UDP-GlcNAc	1	100
UDP-GlcNAc-enolpyruvate	0.1	100
UDP-MurNAc-L-Ala	0.1	97
UDP-MurNAc-dipeptide	0.1	80
UDP-MurNAc-tripeptide	0.1	72
UDP-MurNAc-pentapeptide	0.1	73
	1	27
Dihydro-UDP-MurNAc	0.1	30
GlcNAc	1	80
MurNAc	1	50

[a] Assays for GlcN-1-P acetyltransferase activity were performed under standard conditions with pure GlmU enzyme and nonsaturating concentrations of both substrates (0.25 mM acetyl-CoA and 0.15 mM GlcN-1-P).

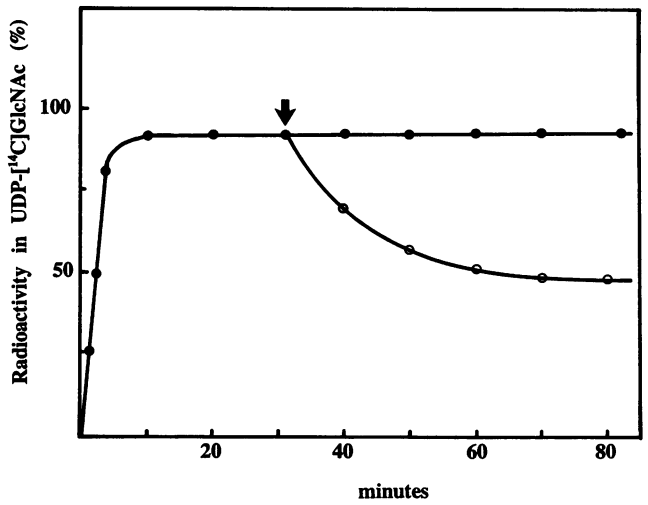

FIG. 3. Demonstration that an unfavorable equilibrium constant limits the uridyltransferase reaction. The reaction was initiated with 10 μM [^{14}C]GlcNAc-1-P and 1 mM UTP in a standard assay mixture (500 μl) containing 2 μg of pure GlmU. After the formation of UDP-[^{14}C]GlcNAc has reached a plateau, the addition of 1 mM unlabeled GlcNAc-1-P (arrow) causes the disappearance of the radiolabel from the product, which is indicative of a progressive reequilibration of substrate and product concentrations. Symbols: ○ and ●, addition or no addition, respectively, of unlabeled GlcNAc-1-P at t = 30 min. The corresponding final percentages of radiolabels in UDP-GlcNAc were 35 and 93%, respectively.

activities of both enzymes in crude extracts from cells overexpressing the *glmU* gene were determined. As previously described (24), a 25- to 500-fold overproduction of uridyltransferase was detected in cells carrying the pMLD71 or pMLD78 plasmid, in which the gene is under the control of the *lac* or lambda p_R promoter, respectively (Table 1). It was in all cases accompanied by an equivalent overproduction of acetyltransferase activity (Table 1), a result thus corroborating the hypothesis of a bifunctional enzyme. This finding was consistent with the previous observation that the purified GlmU formed a single band with an electrophoretic mobility corresponding to the molecular mass of 49 kDa expected from the gene sequence when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (24).

Kinetic properties of the bifunctional GlmU enzyme. The optimal pH for both acetyltransferase and uridyltransferase reactions catalyzed by the pure enzyme was 8.2. Both activities absolutely required the presence of Mg^{2+} in the sharp range of concentrations from 1 to 10 mM, with an optimal value of 3 mM. The ability of other divalent cations to replace Mg^{2+} was not investigated.

The double-reciprocal plots for the acetyltransferase activity showed that the K_m values for GlcN-1-P and acetyl-CoA were 0.15 and 0.6 mM, respectively. The acetyltransferase is competitively inhibited by its reaction product GlcNAc-1-P (50% inhibition at 0.1 mM) (Table 2). A search for other compounds related to peptidoglycan metabolism which could act as effectors of this enzyme revealed UDP-MurNAc as a potent inhibitor (more than 50% enzyme inhibition at 10 μM). This effect was quite specific, since the other peptidoglycan nucleotide precursors, from UDP-GlcNAc to UDP-MurNAc-pentapeptide, only weakly inhibited the acetylase when assayed at much higher concentrations, from 0.1 to 1 mM (Table 2). The effects of some modifications of the UDP-MurNAc structure on the inhibitory effect were also investigated. Table 2 shows that MurNAc-1-P, dihydro-UDP-MurNAc, and MurNAc also inhibit the enzyme but to a much lesser extent, indicating that all parts of the UDP-MurNAc molecule might be implicated in its binding to the enzyme.

The double-reciprocal plots for uridyltransferase activity showed that the K_m values for GlcNAc-1-P and UTP were 0.07 and 0.1 mM, respectively. These values were similar to those previously determined with a crude enzyme preparation (24). The uridyltransferase activity was not at all inhibited by a 1 mM concentration of MurNAc-1-P or UDP-MurNAc, which otherwise completely inhibits the acetyltransferase activity. It was only weakly inhibited by its reaction product UDP-GlcNAc (25% enzyme inhibition at 1 mM). MurNAc-1-P could not replace GlcNAc-1-P as a substrate in the reaction.

As expected from its other name (UDP-GlcNAc pyrophosphorylase), the GlmU uridyltransferase also catalyzed the reverse reaction (34). When a mixture of 1 mM UTP and 0.1 mM [^{14}C]GlcNAc-1-P was incubated under standard assay conditions with purified enzyme, the forward reaction ceased after only 70% of the labeled substrate had been consumed. This extent of conversion could be increased to 90% by decreasing the initial concentration of GlcNAc-1-P to 0.01 mM (Fig. 3). If fresh enzyme was added to such reaction mixtures, no additional product was formed, indicating that enzyme instability could not account for the failure of the reaction to go to completion. Furthermore, addition of a large excess of unlabeled GlcNAc-1-P after product formation had ceased caused a rapid disappearance of the radiolabel from the product UDP-GlcNAc (Fig. 3). This indicated that the reaction remained readily reversible at the plateau, implying that net product formation had ceased because of an unfavorable equilibrium in the forward direction. The equilibrium constant, defined as K_{eq} = (UDP-GlcNAc) (PP_i)/(GlcNAc-1-P)(UTP), was estimated between 0.15 and 0.3. When a crude extract of *E. coli* was used instead of pure enzyme, the extent of conversion in the forward reaction was not limited (data not shown), probably because a pyrophosphorylase activity from the crude extract continuously cleaved the PP_i produced by the reaction, thus shifting the equilibrium towards UDP-GlcNAc.

Inactivation of GlcN-1-P acetyltransferase by thiol-alkylat-

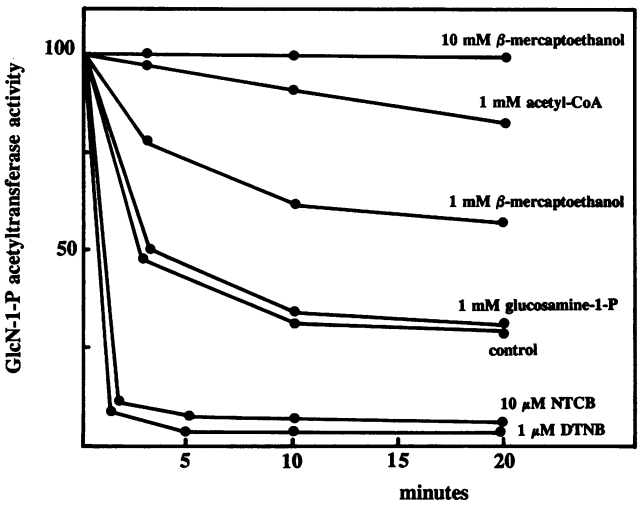

FIG. 4. Inactivation of GlcN-1-P acetyltransferase by thiol-alkylating agents. The pure GlmU enzyme, appropriately diluted in 20 mM potassium phosphate buffer (pH 7.4), was preincubated for various periods in the presence of the indicated compounds. The remaining acetyltransferase activity was then determined under standard assay conditions for 30 min at 37°C. Activity without preincubation is indicated as 100%.

ing agents. We mentioned above that the level of acetyltransferase activity was fivefold higher than that of the uridyltransferase in crude cell extracts of *E. coli*. It was therefore quite surprising to observe that the acetyltransferase activity of the purified GlmU enzyme was about sevenfold lower than its uridyltransferase activity (Table 1). Two different hypotheses could explain this finding: (i) the presence of another GlcN-1-P acetyltransferase activity in crude extracts which should be much more active than the GlmU enzyme and (ii) the fact that an important part of the acetyltransferase activity of the GlmU enzyme had been lost during the two-step chromatographic procedure used for its purification. The latter hypothesis was confirmed in assays demonstrating the great instability of the acetyltransferase activity of GlmU. In particular, preincubation of the pure enzyme at 37°C in the absence of its substrates led to a rapid loss of its acetylase activity (Fig. 4). The presence of either acetyl-CoA or β-mercaptoethanol (at a high concentration, 10 mM) clearly protected the acetyltransferase from inactivation, whereas its other substrate, GlcN-1-P, did not (Fig. 4). Interestingly, the enzyme was only partially protected by 1 mM β-mercaptoethanol, which was the concentration previously used in all buffers during the purification of the uridyltransferase activity. We also showed that the thiol-alkylating agents DTNB and NTCB readily inactivated the acetyltransferase within a few minutes (Fig. 4). All these results were consistent with the presence of a cysteine residue(s) in or near the active site of the protein, which may play a catalytic role in the acetylation reaction.

It was noteworthy that the uridyltransferase activity of GlmU was comparatively very stable (only 5% of the activity was lost during a 30-min preincubation period in the absence of substrates and β-mercaptoethanol) and completely insensitive to millimolar concentrations of the thiol reagents (data not shown). This result indicated that the cysteine mentioned above was not involved in the second reaction catalyzed by the bifunctional GlmU enzyme.

In order to control that only one GlcN-1-P acetyltransferase was present in *E. coli* cells, proteins from a crude extract were separated on a column of DEAE–Trisacryl-M, and all fractions were assayed for acetyltransferase activity. Only one peak of activity could be detected (Fig. 5), coeluting with that corresponding to the uridyltransferase activity, as expected. The phosphoglucosamine mutase activity was also detected and

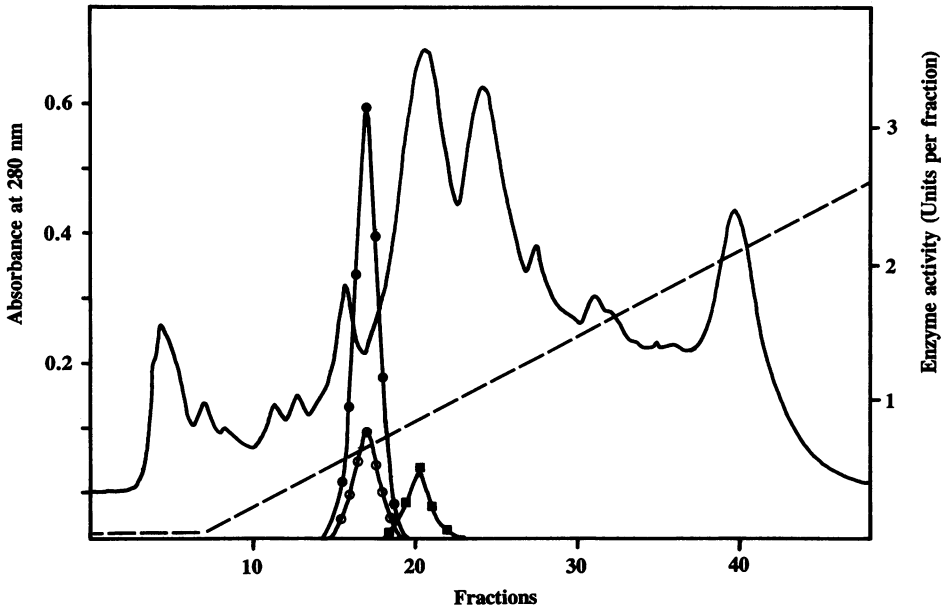

FIG. 5. Detection of enzymes involved in UDP-GlcNAc biosynthesis following chromatography of a crude extract of *E. coli* on DEAE–Trisacryl-M. The total crude extract (80 mg of protein) secured from wild-type strain JM83 was analyzed on a column of DEAE–Trisacryl-M as described in the text. Elution of proteins was monitored at 280 nm, and all fractions were assayed for the following enzymatic activities: phosphoglucosamine mutase (■), GlcN-1-P acetyltransferase (●), and GlcNAc-1-P uridyltransferase (○).

appeared to elute with a slightly higher NaCl concentration than the GlmU enzyme (Fig. 5).

DISCUSSION

GlcN-1-P acetyltransferase and GlcNAc-1-P uridyltransferase of *E. coli*, which catalyze the last two steps of the UDP-GlcNAc synthetic pathway, were copurified from extracts of a strain overproducing to high levels the *glmU* gene product. Evidence that the two enzyme activities are carried by a bifunctional protein rather than a complex of associated monofunctional enzymes is provided here. Although a number of multifunctional enzymes which catalyze two or more reactions of the same biosynthetic pathway in *E. coli* are known, this is the first report of the occurrence of a bifunctional enzyme in the cytoplasmic steps of the pathways for peptidoglycan and lipopolysaccharide synthesis.

The *firA* (*lpxD*) gene product of *E. coli* was recently identified as the UDP-3-*O*-(*R*-3-hydroxymyristoyl)-glucosamine *N*-acyltransferase which catalyzes the third step of lipid A biosynthesis (15). Interestingly, Vaara and coworkers (35, 36) reported earlier that in a protein homology search the FirA protein matched with LpxA (UDP-GlcNAc 3-hydroxymyristoyl transferase) (1), three other bacterial acetyltransferases (LacA, CysE, and NodL), and also a hypothetical protein of unknown function, Yglm, corresponding to the actual *glmU* gene product. It was shown that all these proteins possess a 41-amino-acid-long highly conserved region which had a peculiar repeated hexapeptide pattern (35). This region contained in addition an I-G-A-G-S-[L, I, V, M]-V motif, which was earlier proposed to be an acetyltransferase consensus sequence (8). In the case of GlmU, this particular sequence was located between residues 268 and 309 of the 456-amino-acid polypeptide and the acetyltransferase motif appeared as I-G-T-G-C-V-I (36). The significant homology of GlmU with other bacterial acyl- or acetyltransferases was thus consistent with its present identification as a bifunctional enzyme also exhibiting GlcN-1-P acetyltransferase activity.

We previously constructed a mutant strain, UGS83, with the chromosomal *glmU* gene inactivated, that was viable only in the presence of a plasmid carrying the wild-type gene (24). Since the plasmid bore a thermosensitive replicon, the effects of this inactivation were visualized by shifting growing cells to the restrictive temperature. Cells were shown to accumulate GlcNAc-1-P to high levels while pools of the nucleotide peptidoglycan precursors located downstream in the pathway (from UDP-GlcNAc to UDP-MurNAc-pentapeptide) were rapidly depleted. As a result, peptidoglycan synthesis (and probably also lipopolysaccharide synthesis) was inhibited and cells progressively lost their rod shape and finally lysed. All these results and in particular the opposite variations of the pools of GlcNAc-1-P and UDP-GlcNAc clearly indicated that the *glmU* gene coded for the GlcNAc-1-P uridyltransferase. It was thus quite surprising, considering that GlmU also catalyzed the preceding acetylation reaction of the same pathway, that UGS83 accumulated GlcNAc-1-P and not GlcN-1-P when grown at the restrictive temperature. This unexpected finding was not due to the presence in *E. coli* cells of a GlcN-1-P acetyltransferase other than GlmU, as demonstrated here by the failure to detect such an additional enzyme in crude cell extracts. However, it should be remembered that the acetyltransferase activity of GlmU was about fivefold higher than its uridyltransferase activity. This relative excess of acetyltransferase probably explains why the first observable effects of the progressive disappearance of the bifunctional enzyme in UGS83 cells grown at 43°C were those expected for a defect in the sole uridyltransferase activity.

It will be interesting to construct a *glmU* mutant strain expressing a GlmU enzyme modified in such a way that only its GlcN-1-P acetyltransferase is altered. For this purpose, it is essential to identify the amino acid residues of the bifunctional enzyme that are specifically involved in the first acetylation reaction. In this regard, the observation that GlmU completely lost its acetyltransferase activity but retained the uridyltransferase activity when treated with thiol-alkylating agents is of particular interest. It suggests that the active sites for the two enzyme activities are separated from each other on the same polypeptide and that one (or more) essential cysteine might be implicated in substrate binding or catalysis in the acetylation reaction. This residue that remains to be identified is therefore a good candidate for future mutagenesis experiments allowing the construction of a strain defective only in GlcN-1-P acetyltransferase.

The purified GlmU protein exhibits a number of characteristics which suggest that the acetyltransferase and uridyltransferase activities may reside in separate catalytic domains. First, the acetyltransferase activity (but not the uridyltransferase activity) is inhibited by sulfhydryl reagents. Second, the substrates, products, and effectors of the acetyltransferase reaction do not inhibit the uridyltransferase activity and vice versa. Third, there are large structural differences between the substrates for the two enzymes as well as mechanistic differences between an acetylation reaction and the charging of an acetylamino sugar phosphate with a nucleotide. Fourth, portions of the GlmU amino acid sequence exhibiting homologies with that of other XDP-sugar pyrophosphorylase and acetylase activities are located in the N-terminal portion and the second third of the protein, respectively (data not shown). Further work using site-directed mutagenesis or limited proteolysis is now required to identify regions of the bifunctional enzyme necessary for the acetyltransferase and uridyltransferase activities. Considering the apparent oligomeric nature of the native enzyme, eventual interactions between specific domains of its monomers should be also taken into consideration.

A search of databases for sequences homologous to *glmU* previously revealed the *tms* gene of *Bacillus subtilis* (27). The *tms* gene was identified on the basis of the complementation of a *tms-26* mutant strain that was thermosensitive for growth (5). The effects of inactivating the chromosomal *glmU* gene on cell growth and peptidoglycan metabolism in *E. coli* were clearly reproduced with the *Bacillus tms-26* strain grown under restrictive conditions (5, 24). Hove-Jensen recently demonstrated the accumulation of GlcNAc-1-P in the *tms-26* strain and presented evidence that the *tms* gene coded for the GlcNAc-1-P uridyltransferase (14). Extensive conservation of amino acids is observed throughout the GlmU and Tms polypeptides (27). Both sequences contain 456 amino acids. Of these, 196 are identical, suggesting 44% similarity. A careful examination of these sequences revealed that the four cysteines from GlmU are not conserved in Tms (27). Since one (or more) of these cysteines seems to be essential for the acetyltransferase activity of the *E. coli* enzyme, it would be interesting to know whether the uridyltransferase from *Bacillus* spp. also exhibits an acetyltransferase activity.

A turnover number of 742 min^{-1} was previously calculated for the pure GlmU uridyltransferase, and the copy number of this enzyme in a plasmid-free *E. coli* strain was estimated at about 2,500 per cell (24). Under the in vitro conditions used, the uridyltransferase is able to catalyze the formation of 50×10^6 molecules of UDP-GlcNAc in each cell during a 30-min generation time. Even though a 50% turnover of peptidoglycan

should be taken into account (12), this value is much higher than that required for the formation of the average peptidoglycan and lipopolysaccharide contents of normally growing cells (22, 31). This is a fortiori true for the GlcN-1-P acetyltransferase, whose specific activity is fivefold higher, and it could be concluded that both enzymes are in relative excess in cells compared with the amounts needed for the synthesis of these different cell envelope components. However, a number of in vivo conditions (e.g., substrate concentration or pH) can differ substantially from the optimal in vitro conditions we used. A comparison of K_m values of substrates with their pool levels previously showed that the uridyltransferase seems to work in vivo with saturating concentrations of UTP and with concentrations of GlcNAc-1-P close to its K_m value (24). In the case of the acetyltransferase, the pool level of GlcN-1-P is unknown but that of acetyl-CoA was estimated at 0.2 mM (4) and is thus lower than the corresponding K_m value.

The question of how the flow of metabolites is regulated in this reaction sequence remains. From the data reported here, a few comments regarding possible regulatory mechanisms can be made. The inhibition of GlcN-1-P acetyltransferase by its reaction product GlcNAc-1-P, as well as by UDP-MurNAc, which is one of the first precursors specific for the peptidoglycan pathway, is of particular interest. Concentrations of GlcNAc-1-P that significantly inhibit the enzyme in vitro are higher than 0.1 mM (Table 2). Considering that the pool level of GlcNAc-1-P in normally growing cells was estimated at about 0.05 mM (24), this inhibitory effect could have some physiological significance, particularly under specific conditions in which this compound is accumulated. The pool level of UDP-MurNAc was previously estimated in the range from 0.01 to 0.05 mM (19, 22). We report here that such concentrations inhibit the acetyltransferase activity in vitro by more than 50%. This is a very sensitive and specific effect, since the other nucleotide peptidoglycan precursors only weakly inhibit the enzyme at concentrations lower than 0.1 mM. However, the 25% inhibition observed with UDP-MurNAc-pentapeptide at 0.1 mM should be taken into consideration, since this compound always appears in cells at a relatively high concentration that exceeds 0.1 mM in most cases (19, 22). Further work will be necessary to determine whether these various inhibitory effects correspond to specific control mechanisms. Furthermore, it should be kept in mind that GlmU is an enzyme located at a branchpoint which produces UDP-GlcNAc molecules for both peptidoglycan and lipopolysaccharide synthetic pathways. It is thus tempting to speculate that the activity of this enzyme could also be regulated by some specific lipopolysaccharide precursor. Interestingly, Anderson et al. suggested earlier that UDP-GlcNAc pyrophosphorylase was a possible control point in the synthesis of cell wall polymers in *Bacillus licheniformis* (2).

Compounds which are potent inhibitors of the GlcN-1-P acetyltransferase of GlmU are without effect on its uridyltransferase activity. The latter activity is only weakly inhibited by its reaction product UDP-GlcNAc (25% inhibition at 1 mM), an effect of little physiological significance when the fact that the pool of this compound does not exceed 0.1 mM under normal growth conditions is considered (20, 22). The reversibility of the reaction catalyzed by the uridyltransferase probably explains why UDP-GlcNAc did not accumulate extensively in *E. coli* cells treated with fosfomycin (a MurZ inhibitor) (Fig. 1) (23). This finding was presumably due to the equilibration of the pool of UDP-GlcNAc with that of GlcNAc-1-P, as well as the continued use of UDP-GlcNAc by the unaffected lipopolysaccharide pathway. We have shown that the unfavorable equilibrium of the uridyltransferase reaction was not observed when a crude protein extract was used instead of pure GlmU, probably because a pyrophosphatase activity continuously cleaved the PP_i product and thus shifted the reaction towards UDP-GlcNAc formation. The activity of this pyrophosphatase and the pool level of PP_i are factors which could influence the rate of UDP-GlcNAc synthesis in vivo. Kukko-Kalske et al. (17) have reported that the intracellular concentration of PP_i was constant and relatively high, about 0.5 mM in *E. coli*.

We mentioned previously that the lack of an obvious promoter consensus on the DNA sequence upstream of the *glmS* gene encoding GlcN-6-P synthase suggested that the *glmU* and *glmS* genes were cotranscribed. Interestingly, Plumbridge et al. recently showed that the *glmS* gene of *E. coli* was subject to a control mechanism which causes its expression to be reduced when the *nag* regulon of genes coding for amino sugar-degrading enzymes is derepressed (29). The transcription in this particular chromosomal region and the possible coregulation of *glmU* and *glmS* gene expression are being investigated.

Finally, the detection of a phosphoglucosamine mutase activity in crude extracts of *E. coli* confirms that the steps leading from GlcN-6-P to UDP-GlcNAc occur via GlcN-1-P. This is consistent with the previous demonstration that GlcNAc had to be deacetylated before it could be incorporated into cell walls (7), a finding suggesting that any isomerase converting GlcNAc-6-P to GlcNAc-1-P has insignificant activity and that the major (or only) flux goes via GlcN-1-P, which has to be reacetylated before joining the nucleoside. The gene encoding the phosphoglucosamine mutase is the last gene of this pathway that remains to be identified. No other open reading frame of unknown function in the *unc-phoS* region has been described, indicating that the gene encoding the phosphoglucosamine mutase is not linked to *glmU* and *glmS* and thus may belong to a separate chromosomal region.

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