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Overexpression, One-Step Purification and Characterization of UDP-Glucose Dehydrogenase and UDP-*N*-Acetylglucosamine Pyrophosphorylase

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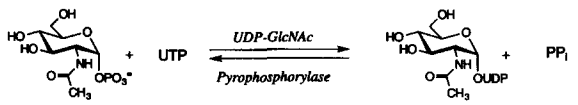
Abstract—Two enzymes of the Leloir pathway, UDP-GlcNAc pyrophosphorylase and UDP-Glc dehydrogenase, which are involved in the synthesis of activated sugar nucleotides have been cloned, overexpressed in *Escherichia coli*, and purified to homogeneity in only one step by chelation-affinity chromatography. The gene *KfaC* of *E. coli* K5 was thus demonstrated to encode UDP-Glc DH. Some properties of the cloned enzymes, such as stability, pH dependence, and substrate kinetics, were studied in order to facilitate the use of these enzymes in carbohydrate synthesis, especially in the synthesis of hyaluronic acid.

Introduction

As part of a program to develop enzyme-catalyzed syntheses of complex oligosaccharides, polysaccharides, glycoproteins, and glycolipids,[1] we report here the overexpression in *E. coli*, one-step purification, and characterization of two enzymes of the Leloir pathway: uridyldiphosphoglucose dehydrogenase (UDP-Glc DH) and uridyldiphospho-*N*-acetylglucosamine pyrophosphorylase (UDP-GlcNAc PP).

The glycosyltransferases of the Leloir pathway,[2] responsible for the synthesis of most glycoconjugates in mammalian systems, utilize sugar nucleotides as an activated monosaccharide donor (GDP-Man, GDP-Fuc, CMP-NeuAc, UDP-Glc, UDP-GlcA, UDP-Xyl, UDP-Gal, UDP-GalNAc, and UDP-GlcNAc). The in vivo synthesis of these sugar nucleotides generally proceeds through a scheme that starts with a kinase-mediated phosphorylation of the corresponding monosaccharide to produce a glycosyl phosphate, which is followed, in some instances, by a phosphomutase-catalyzed transfer of the phosphate group from position 6 to position 1. The sugar phosphate then reacts with a nucleoside triphosphate catalyzed by a pyrophosphorylase to yield the activated nucleoside diphosphate. Further oxidation, epimerization, or decarboxylation of the sugar nucleotides such as UDP-Glc, GDP-Man, and UDP-GlcNAc leads to the formation of UDP-GlcA, UDP-Xyl, UDP-GalNAc, and GDP-Fuc. For the synthesis of nucleoside monophosphate sugars such as CMP-NeuAc and CMP-KDO, the corresponding unactivated monosaccharide reacts with CTP catalyzed by a synthetase. As is typical for complex metabolic pathways, these general schemes have many exceptions and differences within living organisms where different pools of activated sugars and different allosteric regulations are needed.

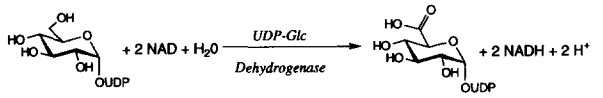
The enzyme UDP-GlcNAc pyrophosphorylase (EC 2.7.7.23) reversibly catalyzes the synthesis of UDP-GlcNAc from GlcNAc-1-P and UTP (eqn 1).



It was first purified from calf liver and from *Staphylococcus aureus* more than three decades ago,[3] but the sequence of the *E. coli* gene *glmU*[4a] encoding UDP-GlcNAc pyrophosphorylase has only recently been elucidated.[4b]

The activated sugar UDP-GlcNAc produced by this enzymatic reaction plays a very important role in the biochemistry of all living organisms. In fact, it is one of the main cytoplasmatic precursors of bacterial cell wall peptidoglycans, as well as a precursor of the disaccharide moiety of the lipid A[5] and of the nod-factor oligosaccharides, which induce nodulation in plants.[6] UDP-GlcNAc is also necessary for the biosynthesis of N-glycoproteins and polymers like chitin.

Uridinediphosphoglucose dehydrogenase (UDPG-DH, EC 1.1.1.22) is a pyridine nucleotide-linked dehydrogenase[7] which catalyzes the reaction leading to the formation of UDP-glucuronate in all organisms except plants. The NAD-dependent oxidation of UDP-glucose reaction is irreversible and 2 mol of NADH are formed per mole of UDP-sugar (equation 2).



UDP-Glc DH has been found in many prokaryotes and eukaryotes tissues and is the most thoroughly studied of the four-electron transport enzyme with respect to structure and mechanism (the more detailed studies have been performed with the bovine liver enzyme[8]). From a genetic point of view, the recently cloned *hasB* gene from *Streptococcus pyogenes* is, to our knowledge, the only gene that has been demonstrated to encode a UDP-glucose dehydrogenase.[9] The gene, together with two other genes, resides on a contiguous stretch of 3.2 kilobase pair streptococcal DNA that seems able to direct hyaluronate biosynthesis in heterologous bacteria.[10]

The product of the dehydrogenase reaction, UDP-GlcA, is the precursor of UDP-xylose in mammals and also the precursor of many tissue extracellular matrix glycosaminoglycans. In mammalian liver, UDP-GlcA is transferred to a nonpolar acceptor molecule such as bilirubin or some compound foreign to the body (e.g., a drug); the formation of water soluble glucuronides occurs so that these compounds can be eliminated from the body in the aqueous media of urine or bile. The activated sugar nucleotides, UDP-GlcA and UDP-GlcNAc together, are precursors of heparin and hyaluronic acid.[11] In a preliminary study, we have used these two enzymes in the synthesis of hyaluronic acid[12] (Fig. 1) with regeneration of sugar nucleotides. We report here the overexpression, purification, and characterization of these two enzymes.

Results and Discussion

Cloning of UDP-GlcNAc PP and UDP-Glc DH

The strategy for the cloning of the two enzymes, as well as the main characteristics of the plasmids constructed, are shown in Figure 2.

UDP-GlcNAc PP. Based on the recently published[4b] *glmU* gene sequence[4a] encoding the enzyme, two oligonucleotides were designed for PCR amplification using *E. coli* K12 DNA as a template. The PCR insert (1.4 kb), corresponding to UDP-GlcNAc PP, was digested with BamH-I and EcoR-I restriction endonucleases and was ligated into the vector *p*Trc-His-A to yield the plasmid *p*Trc-Pyr. The plasmid was transformed into epicurean supercompetent *E. coli* XL1-Blue MRF′ strain[13] and plated on the LB-ampicillin plates. Screening for the positive clones was carried out by using the PCR method (see Experimental), and five clones showing the most intense amplification were selected.

UDP-Glc DH. Several attempts to clone and overexpress the streptococcal gene *hasB*[10] in *E. coli* have been carried out in our laboratories, but no active UDP-Glc DH has been obtained. During a search for

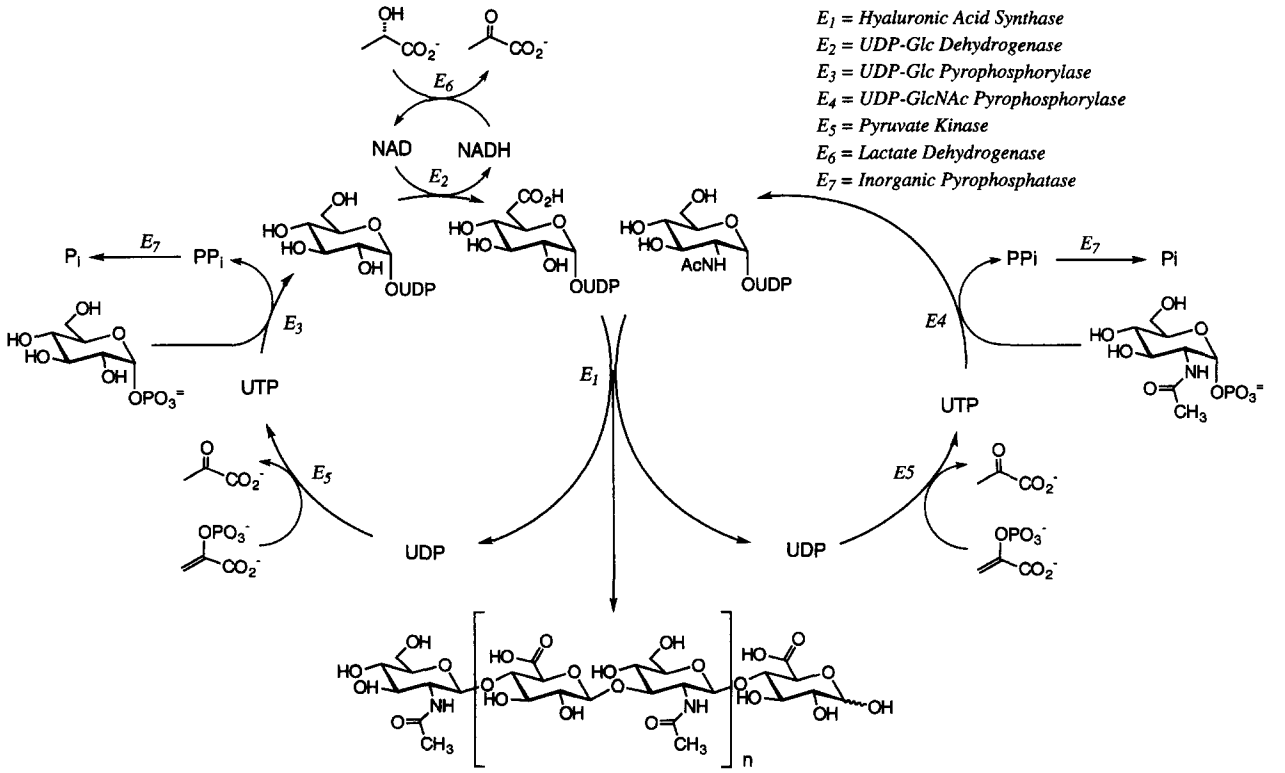


Figure 1. Enzymatic synthesis of hyaluronic acid with regeneration of sugar nucleotides.

sequence similarity within the GenBank using the FASTA program,[14] we found two genes revealing a significant degree of homology with the UDPG-DH from streptococcus (Fig. 3). Both genes are from *E. coli*: one is from the strain 0111 (M92), coding for a not defined 'hypothetical protein' of 43.3 kDa;[15a,15b] the other, named *kfaC*, is from *E. coli* strain K5 and resides in a contiguous stretch of 8 kb pair DNA, named 'region 2 of the K5 antigen gene cluster',[16] a region that seems involved in the synthesis of the K5 polysaccharide (a polysaccharide very similar to the hyaluronic acid). The percent of identity between the streptococcal UDPG-DH and the protein deduced from *E. coli* 0111 gene is 53.5% over 402 residues (init = 481), and the comparison of the streptococcal protein with the protein deduced from the gene *kfaC* of *E. coli* K5 revealed 53.8% identity over 400 residues (init = 450). The identity of the two genes from *E. coli* is 75.3% over 388 residues (init = 1518). To our knowledge, there is no published correlation of the expression of these two genes with dehydrogenasic activity. In order to verify this hypothesis of correlation, we undertook the cloning of *kfaC*. Two oligonucleotides were designed to amplify the gene *kfaC* in *E. coli* K5 by PCR reaction. A band of the expected molecular weight (1.18 kb) was obtained. After purification, the DNA was digested with Sac I and Hind III restriction endonucleases and ligated into the vector *p*TrcHis-A. The plasmid yielded plasmid, *p*Trc-DH, was then transformed into the competent *E. coli* XL1-Blue MRF′ strain and five positive clones were selected.

Expression of cloned UDP-GlcNAc PP and UDP-Glc DH

The selected positive clones (five for UDP-GlcNAc PP and five for UDP-Glc DH) were grown in LB medium containing 250 μg mL^{-1} ampicillin. In the expression system used in this work (*p*TrcHis, Invitrogen Corp.), the transcriptions of the two cloned genes are controlled by *Trc* promoter and can be induced by IPTG (isopropyl β-D-thiogalactopyranoside). The 10 cultures were induced with the same concentration of IPTG (250 μM) when mid-logarithmic phase was reached ($OD_{600} = 0.5$) and the expression of the recombinant proteins was detected using SDS-PAGE and by measuring enzyme activity. The two colonies performing the best expression of the enzyme, as judged by the number of units per liter of culture, were selected. Finally, the conditions allowing the best productivity of the recombinant protein were investigated by using different concentrations of the inducer and different temperatures after induction (Figs 4A and 4B).

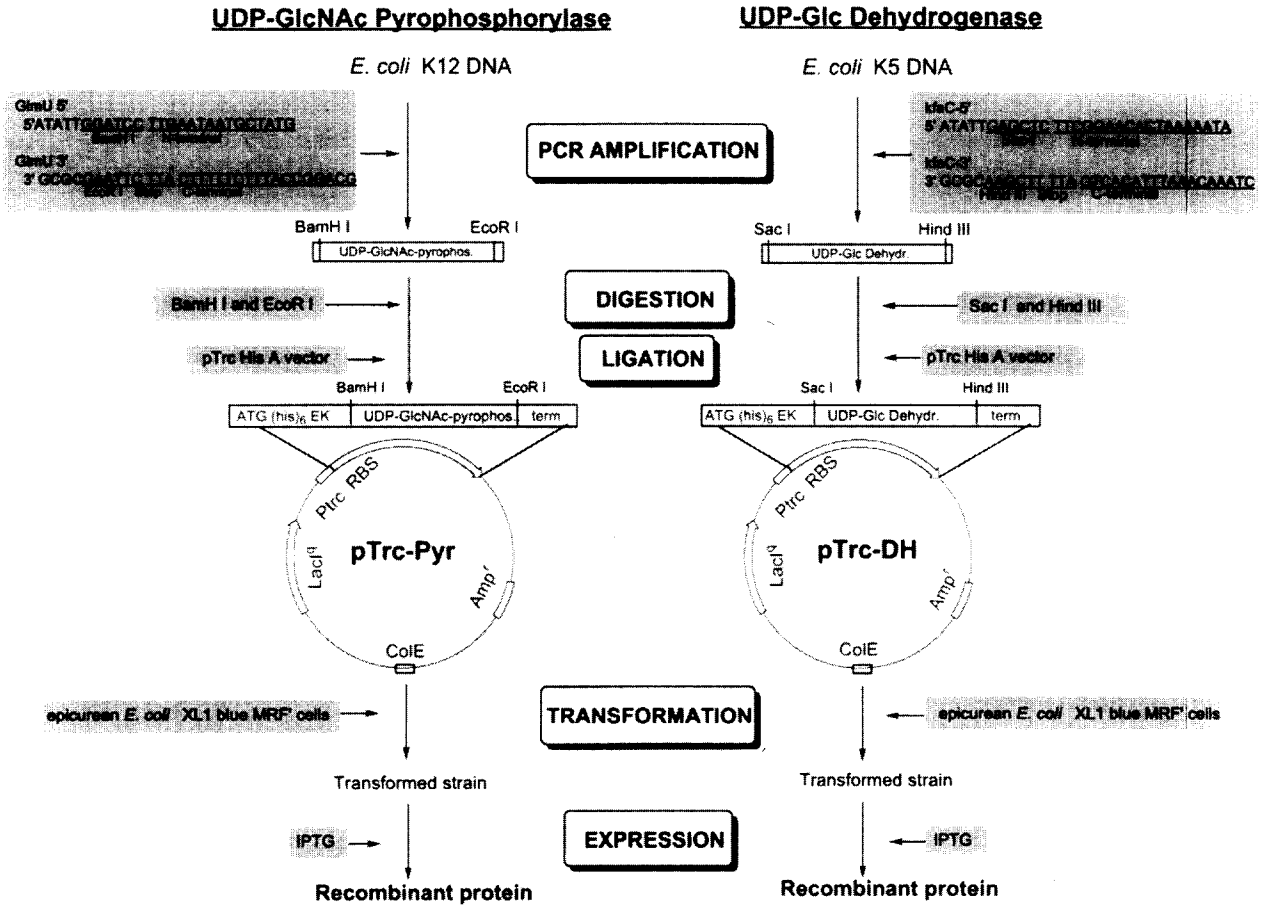


Figure 2. Strategy for the cloning of UDP-GlcNAc PP and UDP-Glc DH.

The optimal concentration of the inducer was found to be 250 μM for the expression of UDP-GlcNAc PP and 50 μM for UDP-Glc DH. Using this concentration and shifting the temperature from 37 to 30 °C, 1 L of culture typically yields, respectively, 300 U and 40 U of enzyme in the crude cell lysate.

Purification of the enzymes

Using a French press, crude cell lysate was obtained from a 1-L culture of *E. coli* harboring *p*Trc-Pyr plasmid and 4 L of *E. coli* harboring *p*Trc-DH. The crude extract was subsequently clarified by centrifugation and ultracentrifugation (see Experimental for details) to yield 40 mL of cell-free extract for each enzyme (276 U and 168 U, respectively). In the *p*TrcHis expression system used in this work, each recombinant protein is expressed with a polyhistidine tag on the N-terminus. This metal binding domain displays a high affinity for Ni^{2+} resins and allows one step purification using chelation affinity chromatography. We therefore added 6 mL of Ni^{2+}-NTA resin (Quiagen) to each cell-free extract. The resin was then washed 10 times with low stringency buffer and loaded onto a column. The column was washed again with low stringency buffer and subsequently with high stringency buffer. UDP-GlcNAc PP was recovered from the resin using a low pH buffer (pH 4.0). The fractions with UDP-GlcNAc PP activity were pooled and concentrated by ultrafiltration. 112 Units were recovered with an overall yield of 40% and a purification factor of 25 (Table 1). Although the specific activity of the concentrated pool was 20.4 U mg^{-1}, the most active fraction eluted from the column demonstrated an activity of 50 U mg^{-1}. In contrast, UDP-Glc DH was recovered using a stepwise gradient of imidazole. Also in this case the active fractions were pooled and concentrated by ultrafiltration. The enzyme was purified almost 50-fold (Table 1) with an overall yield of 42% (70 units). The specific activity of the concentrated pool was 6.8 U mg^{-1}, while the most active fraction was 14 U mg^{-1}. The purity of both recombinant proteins was assessed by SDS-PAGE and coomassie blue staining (Figs 5A and 5B) and estimated to be greater than 90%.

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DH-EcK5      MFGTLKITVSGAGYVGLSN-GILMAQNHE--VVAFDTHQKKVDLLNDKLSPI-EDKEIENYLST--KIL   63
DH-Bovine    MFEIKKICCIGAGYVGGPTCSVIAHMCPEIRVTVVDINESRINAWNSPTLPIYEPGLKEVVESCRGKNL`  69

DH-Strept    SIKATLDSKAAYKEAELVIIA--TPTN-YNSRINYFDTQHVETVIKEVLSVNSHATLII--KSTIPIGF  123
DH-Ec0111    NFRATTDKYDAYRDGTYVIIA--TPTD-YDPKTNYFNTSSVESVIRDVVDINPNAVMVI--KSTIPVGF  123
DH-EcK5      NFRATTNKYEAYKNANYVIIA--TPTN-YDPGSNYFDTSSVEAVIRDVTEINPNAIMVV--KSTVPVGF  127
DH-Bovine    FFS-TNIDD-AIKEADLVFISVNTPTKTYGMGKGPAADLKYIEACARRIVQNSHGYKIVTEKSTVPVRA  136

DH-Strept    ITEMRQKFQTD-----RIIFS-PEFLRESKALYDNLYPSRIIVSCEENDSPKVKADAEKFALLLKSAAK  186
DH-Ec0111    TNLLKERLGID-----NIFFS-PEFLREGRALYDNLHPSRIVIGER-SER------AGRFAALLQEGAV  179
DH-EcK5      TKTIKEHLGIN-----NIIFS-PEFLREGRALYDNLHPSRIIIGEC-SER------AERLAVLFQEGAI  183
DH-Bovine    AESIRRIFDANTKPNLNLQVSNPEFLAEGTAIKLDKNPDRVLIGGDETPEGQRAVQA-LCAVYEHWVPR  204

DH-Strept    KNNVPVLIMGASEAEAVKLFANTYLALRVAYFNELDTYAESRKLNSHMIIQGISYDDRIGMEYNNPSFG  255
DH-Ec0111    KKDIPTLFTDSTEAEAIKLFANTYLALRVAYFNELDSYAESLGLNSRQIIEGVCLDPRIGNEYNNPSFG  248
DH-EcK5      KQNIPVLFTDSTEAEAIKLFSNTYLAMRVAFFNELDSYAESFGLNTRQIIDGVCLDPRIGNYYNNPSFG  251
DH-Bovine    ---EKILTTNTWSSELSKLTANAFLAQRISSINSISALCEATGADVEEVATAIGMDQRIGNKFLKASVG  270

DH-Strept    YGGYCLPKDTKQLL--ANYNNIPQ--TLIEAIVSSNNV-RKSYIAKQIINVLKEQESPVKVVGVYRLIM  319
DH-Ec0111    YGGYCLPKDTKQLL--ANYASVPN--NIIGAIVDANRT-RKDFIADSILARKP------KVVGVYRLIM  306
DH-EcK5      YGGYCLPKDTKQLL--ANYQSVPN--KLISAIVDANRT-RKDFITNVILKHRP------QVVGVYRLIM  309
DH-Bovine    FGGSCFQKDVLNLVYLCEALNLPEVARYWQQVIDMNDYQRRRFASR-IIDSL-FNTVTDKKIAILGFAF  337

DH-Strept    KSNSDNFRESAIKDVIDILKSKDIKIIIYEPMLNKLESEDQSVLVNDLENFKKQANIIVTNRYDNELQD  388
DH-Ec0111    KSGSDNFRASSIQGIMKRIKAKGVPVIIYEPVMVEDEFFE-SRVVRDLTAFKNEADIIISNRMTSELAD  374
DH-EcK5      KSGSDNFRDSSILGIIKRIKKKGVKVIIYEPLISGDTFFN-SPLERELAIFKGKADIIITNRMSEELND  405

DH-Strept    VKNKVYSRDIFGRD                                                          402
DH-Ec0111    VADKVYTRGLFGSD                                                          388
DH-EcK5      VVDKVYSRDLFKCD                                                          392
DH-Bovine    GAHAVVICTEWDMFKELDYERIHKKMLKPAFIFDGRRVLDGLHNELQTIGFQIETIGKKVSSK        468
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Figure 3. Multiple alignment of the deduced amino acid sequences from the gene *hasB* (DH-Strept) of *S. pyogenes*,[11] the gene of *E. coli* 0111[14] (DH-Ec0111), the gene *kfaC* of *E. coli* K5[15] (DH-EcK5), and bovine liver dehydrogenase[17] (DH-Bovine). Strictly conserved residues among the four proteins are given in red. The residues conserved only within the three bacterial proteins are given in blue.

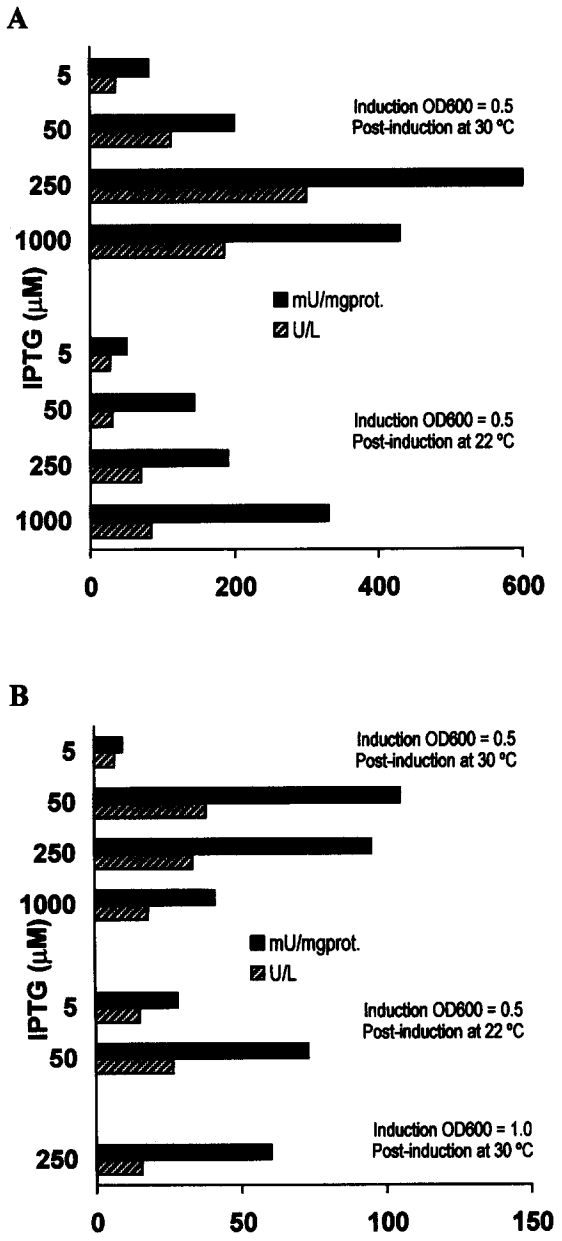

Figure 4. Influence of IPTG concentration and temperature on the productivity of UDP-GlcNAc PP (A) and of UDP-Glc DH (B).

Stability of the enzymes

UDP-Glc DH. During the initial experiments, stability problems were encountered. Although the cell free extract was stable for at least a month when stored at −70 °C, loss of greater than 70% of the UDP-Glc DH activity was experienced after storing the protein eluted from the Ni^{2+}-NTA column (in imidazol buffer) overnight at either 4 °C, −20 °C, or −70 °C. Almost a complete loss of activity was encountered when the elute was concentrated by ultrafiltration. By simply adding 1 mM of the substrate (UDP-Glc) to the imidazol buffer, nearly complete activity could be maintained during the enzyme concentration step and for at least a few days at −20 °C. Moreover, by adding the substrate and either 1 mM β-mercaptoethanol or 1 mM dithiothreitol (DTT), we noticed that enzymatic activity was completely recovered after five days at 4 °C, even in fractions which had lost all activity (Fig. 6). When stored at 4 °C with 1 mM UDP-Glc, 2 mM DTT in 3 M ammonium sulfate, pH 6, the purified UDP-Glc DH was stable for at least one month at 4 °C.

The stability at 25 °C was also investigated (Fig. 7A). The enzyme is quite stable for more than 20 h in the presence of either substrate, indicating that the enzymes are suitable for use in synthesis.

UDP-GlcNAc PP. Both cell-free extract and purified enzymes are stable for months at −20 °C (90% of the original activity after a month) with lyophilize enzyme being the most stable form (data not shown). UDP-GlcNAc pyrophosphorylase lost about 30% of the initial activity after 20 h at 25 °C in the presence of DTT (1 mM) and GlcNAc-1P (1 mM). In the absence of reducing agent this loss is much higher, even if the substrates are dissolved in the reaction solution (Fig. 7B).

It is worth noting that the two enzymes are active when they are still 'immobilized' in the Ni^{2+}-NTA resin (even though the activity is at least 2-fold lower than the subsequently eluted enzyme). The immobilized enzyme is stable for days (Fig. 8).

Table 1. Summary of purification data for the two enzymes

	Protein (mg)	Total activity (units)	Specific activity (units mg^{-1})	Yield (%)	Purification factor
UDP-GlcNAc PP					
1 Liter of culture					
Step:					
Cell Free Extract	345	276	0.8	100	1
NiNTA column + conc.	5.5	112	20.4	40.6	25.5
UDP-Glc DH					
4 Liters of culture					
Step:					
Cell Free Extract	1200	168	0.14	100	1
NiNTA column + conc.	10.3	70	6.8	41.6	48.6

Effect of pH

The effect of pH on the enzymatic activity is shown in Figures 9A and 9B. Phosphate buffer, HEPES and Tris–HCl were used in the desired pH range. The optimum pH range for UDP-GlcNAc PP is quite broad (from 7 to 9); UDP-Glc DH shows a maximum of activity between pH 8 and 9, depending upon the buffer used.

Kinetic constants

The effect of substrate concentration was investigated for the two enzymes.

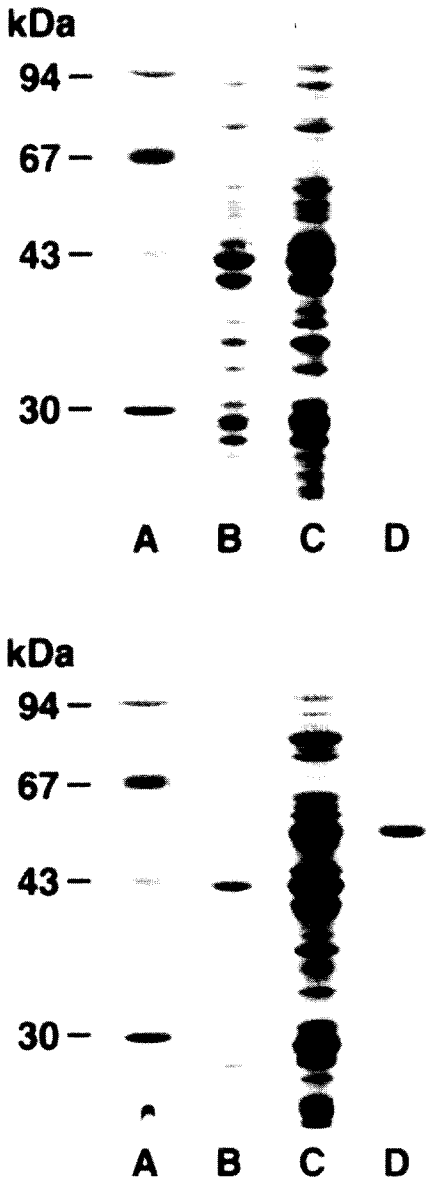

Figure 5. SDS-PAGE analysis of UDP-Glc DH (top) and UDP-GlcNAc PP (bottom). (A) molecular weight markers; (B) crude extract before IPTG induction; (C) crude extract after 4 h (DH) or 6 h (PP) from the induction; (D) enzyme purified by chelation-affinity chromatography. (Photo was electronically enhanced.)

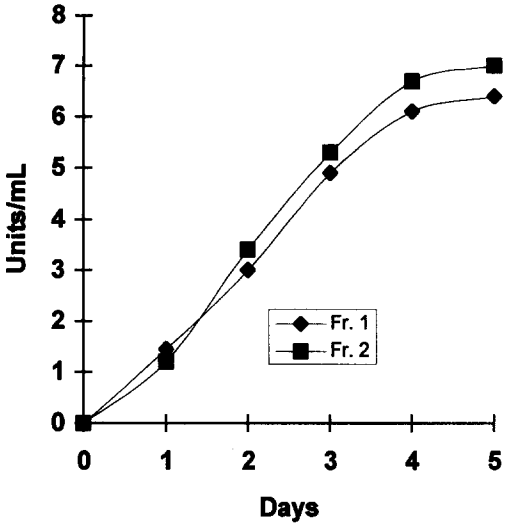

Figure 6. Restoration of activity in two UDP-Glc DH fractions eluted from the Ni^{2+}-NTA column by addition of 1 mM UDP-Glc and 1 mM β-mercaptoethanol.

The results are shown as Lineweaver–Burk plots in Figures 10 and 11. However, the apparent Michaelis constants listed in Table 2 were calculated by a nonlinear least-square fit to the rate equation[17] to give more precise values. From the specific activity of 50 U mg^{-1} of protein determined for the most pure fraction of UDP-GlcNAc PP and assuming a molecular weight of 53 kDa for the recombinant enzyme (49.1 kDa for the *glmU* gene product plus 3.9 kDa for the peptide containing the hystidine domain), a turnover number (kcat) of 2660 min^{-1} was calculated. The turnover number of UDP-Glc DH is 676 min^{-1} when a specific activity of 14 U mg^{-1} of protein and a molecular weight of 48.3 kDa (44.1 plus 4.2 kDa) are assumed.

Conclusions

We have developed an efficient system for the overproduction and facile purification of UDP-GlcNAc PP and UDP-GlcA DH. Optimization of the culture condition yields the best productivity for the cells and, thus, a better yield of the recombinant enzymes. It is worth noting that by using this one-step purification, the specific activity of the most active fractions is in the same range (UDP-Glc DH[18]), or higher (UDP-GlcNAc PP[3,4b,19]), than that previously reported for both these enzymes.

The product of the gene *kfaC* in *E. coli* K5 has now been clearly identified as UDP-Glc DH. This enzyme was purified to homogeneity from *E. coli* strain MC 153 almost two decades ago[18] and has been shown to consist of two identical subunits of 47 kDa each, in contrast to the six subunits of 52 kDa demonstrated for the bovine liver enzyme. The primary structure of the bovine UDP-Glc DH has been recently determined.[20] It was shown to have a relatively high number of identical residues when aligned with the available prokaryotic sequences (Fig. 2). However, among the

prokaryotic UDP-Glc DH sequences, the degree of homology is far higher. The higher molecular weight and the possibly greater structural complexity of the mammalian enzyme reflects the more complex control requirements in eukaryotes, where the UDP-glucuronate is used not only in the synthesis of glucoronides or polysaccharides but also is the precursor of UDP-D-xylose.

Some properties of UDP-Glc DH and UDP-GlcNAc PP, such as the stability at room temperature, the pH dependence of the activity, and the substrate kinetics were also investigated, providing useful information for the use in the synthesis of complex glycoconjugates.

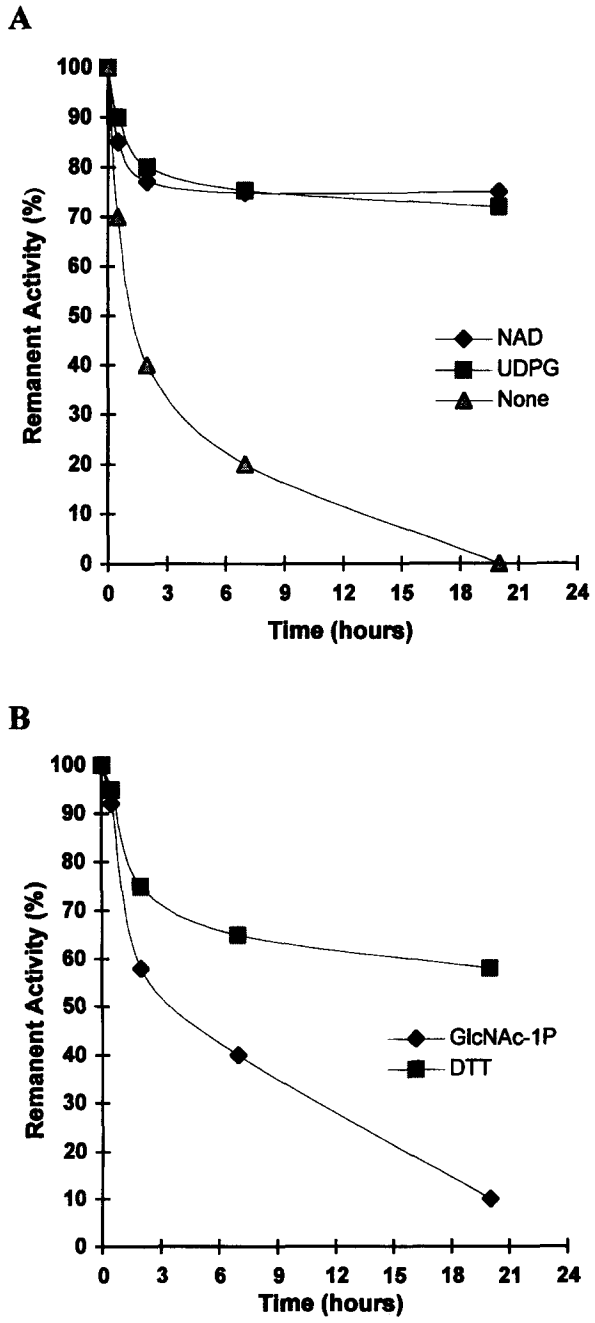

Figure 7. Stability at 25 °C of UDP-Glc DH (A) and UDP-GlcNAc PP (B).

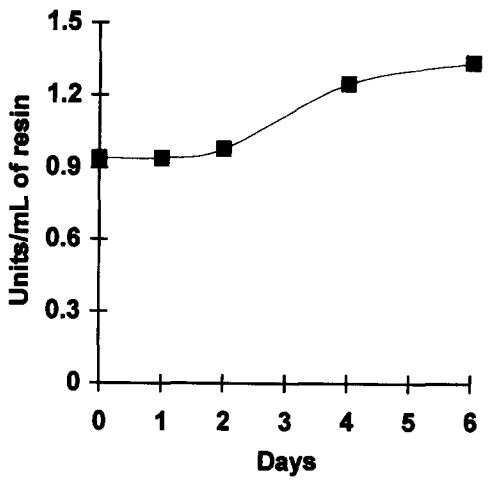

Figure 8. Analysis of the stability of UDP-Glc DH immobilized on NI^{2+}-NTA resin.

Experimental

Materials

All chemicals were purchased from commercial sources as reagent grade.

5–6 ^{3}H-UTP (38 Ci mmol^{-1}) was purchased from ICN. *E. coli* K12 (ATCC 10798) and *E. coli* K5 (ATCC 23508) were obtained from American Type Culture Collection. The vector *p*TrcHis was obtained from Invitrogen Co. (San Diego, CA). The host strain XL1-Blue MRF' was purchased from Stratagene Co. (San Diego, CA). The microorganisms were maintained on LB (Luria-Bertani) medium. When host strains harbored with plasmids, LB medium containing 250 μg mL^{-1} of ampicillin was used.

Methods

PCR amplification. The *E. coli* K12 and *E. coli* K5 DNAs were isolated according to the method described by Maniatis, et al.[13] PCR amplification was performed in a 100 μL reaction mixture containing 1 μL (approximately 1.5 μg) of DNA template, 300 nmol of primers, 200 mM of different dNTPs, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 2 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, and 2 units of *Thermus aquaticus* DNA polymerase. The reaction was overlaid with mineral oil and subjected to 30 cycles of amplifications. The cycle conditions were set as follows: denaturation, 94 °C for 2 min, 94 °C for 1 min, 55 °C for 2 min; and elongation, 72 °C for 1.5 min, 35 cycles.

Construction of a UDP-GlcNAc pyrophosphorylase expression vector. The DNA obtained from PCR amplification was extracted with phenol/chloroform and precipitated with ethanol at −70 °C for 30 min (70% of final ethanol concentration containing 10% of 3 N Na-acetate, pH 5.2). The extracted DNA was double digested with the corresponding restriction enzymes (Boehringer Mannheim Biochemical Co, Indianapolis, IN). The digested DNA was then recovered by phenol/chloroform extraction and ethanol

precipitation, and purified by agarose (0.8%) gel electrophoresis. The DNA bands corresponding to 1370 bp (UDP-GlcNAc PP) and 1180 pb (UDP-Glc DH) were isolated from the agarose gel, extracted with QIAEX gel extraction kit (Qiagen Co., Chatworth, CA) and eluted with TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 7.5). This DNA was used as insert. The vector *p*TrcHis-A was also digested with 5 U mg^{-1} DNA with the corresponding restriction enzymes and recovered with ethanol precipitation after the extraction of phenol/chloroform. The restriction enzyme-digested vector was further purified in agarose gel as described above. The insert was then ligated with the vector by T4 DNA ligase.[13] The ligated DNA was then transformed into supercompetent epicurean *E. coli* XL1-Blue MRF′ strain and plated on LB agar plates which contained 250 mg/mL ampicillin

Screening for positive clones. The PCR method was used to screen for the positive clones. 20 Colonies were randomly selected from plates and grown in 100 mL of LB medium with 250 μg mL^{-1} of ampicillin: 100 μL of this culture was then centrifuged and the pellet resuspended with 50 μL of cell lysing buffer (20 mM Tris–HCl containing 1% Triton X-100 and 2 mM EDTA, pH 8.5). After heating with boiling water for 5 min, the solution was used directly as the DNA template for PCR amplification. The procedure for the PCR amplification was the same as that described in the amplification of this gene except 3 μL of the cell lysing solution was used to replace the *E. coli* DNA. Since the host *E. coli* XL1-Blue also contains a gene encoding UDP-GlcNAc PP, there may be, in this case, some background amplification for non-recombinants. However, the positive clones showed very intensive amplification which form a dense band on agarose gel (0.8%) due to the higher copy number of the target gene presented in the cells. Three clones which gave

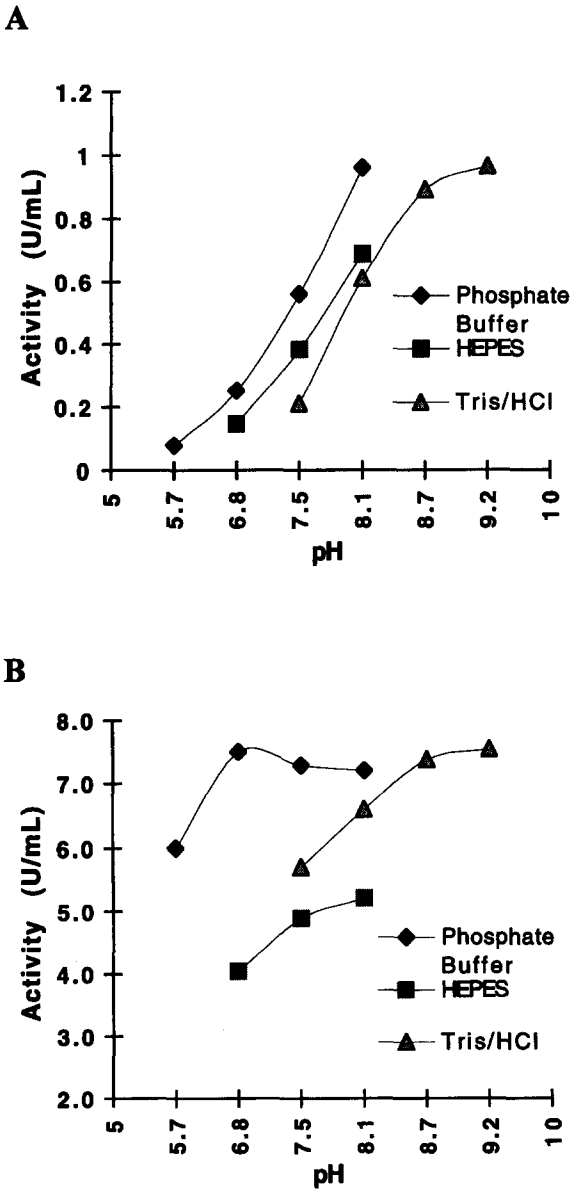

Figure 9. Effect of pH on the enzymatic activity of UDP-Glc DH (A) and UDP-GlcNAc PP (B).

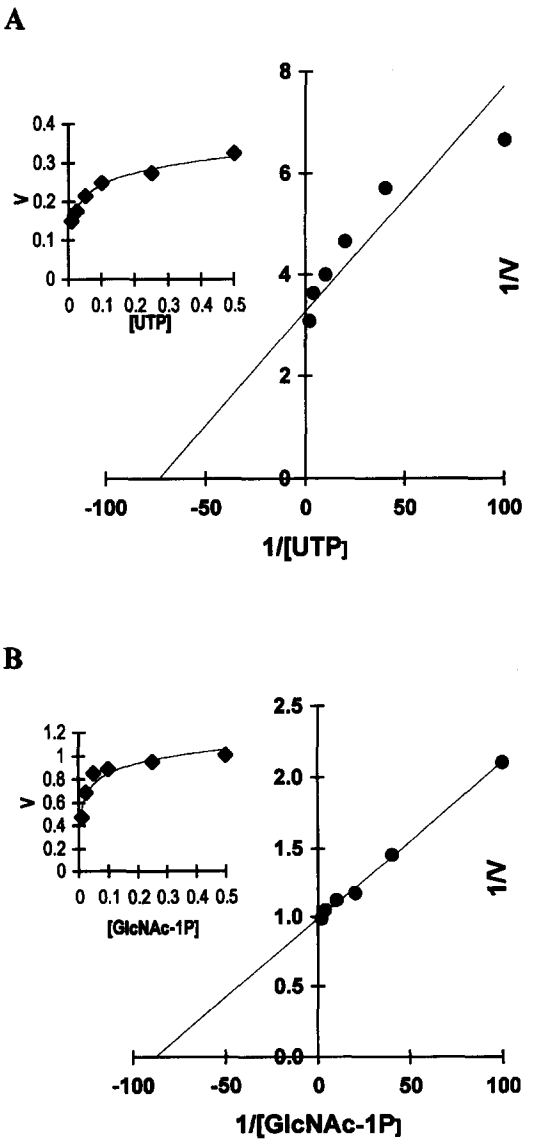

Figure 10. Effect of UTP (A) and GlcNAc-1P (B) concentrations on UDP-GlcNAc PP activity.

the best amplification were selected and investigated for the level of protein expression. At this stage, the plasmids were also extracted from an aliquot of culture; the isolated plasmids were then used as the template for another PCR reaction and the product was analyzed on agarose gel to confirm the gene insert.

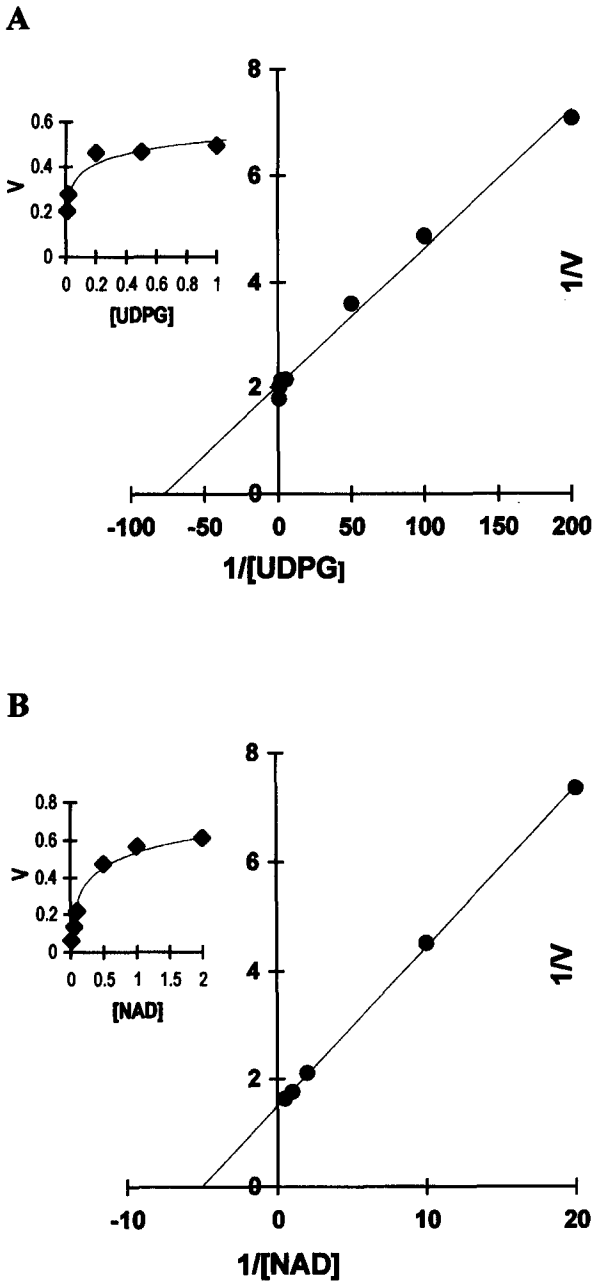

Figure 11. Effect of UDP-Glc (A) and NAD (B) concentrations on UDP-Glc DH activity.

Table 2. Summary of purification data for the two enzymes

UDP-GlcNAc PP		UDP-Glc DH	
K_{mUTP}	12.5±4 μM	$K_{mUDP\text{-}Glc}$	15±2.5 μM
$K_{mGlcNAc1P}$	11.3±1 μM	K_{mNAD}	199±20 μM
kcat	2660 min^{-1}	kcat	676 min^{-1}

Expression of the recombinant proteins. The transformed *E. coli* strains were grown on LB medium containing 250 μg mL^{-1} of ampicillin to mid-logarithmic phase (OD_{600} 0.4–0.5) at 37 °C and then induced with 250 μM of IPTG. After induction the temperature was reduced to 30 °C and the bacterial grown for another 6 (UDP-GlcNAc PP) or 4 h (UDP-Glc DH). The expression level of the recombinant enzyme was followed with time and examined by SDS-PAGE in a Phastsystem (Pharmacia Co.) using precasted gels with a 10–15% gradient of polyacrilamide. The most productive clone for each enzyme was selected and analysis of the influence of IPTG and of the temperature post-induction was carried out.

Preparation of a cell free extract. A cell free extract of the enzyme was obtained from 1 L (UDP-GlcNAc PP) and 4 liters (UDP-Glc DH) of culture broth. Briefly, the cells were harvested and washed with 20 mM NaH_2PO_4, pH 7.8. The cells were then suspended in 30 mL of the same solution and disrupted by a French Press (1600 psig). The lysed cells were then centrifuged at 18,000×*g* for 20 min, and the supernatant was ultracentrifuged for 1 h at 100,000×*g*.

One step chelation affinity purification. The cell free extracts described above were made up to 40 mL containing 500 mM NaCl by using a native binding buffer solution of 20 mM NaH_2PO_4 and 2 M NaCl, pH 7.8. To this solution, 6 mL of Ni^{2+}-NTA agarose resin (Quiagen) was added. The resin was then washed 10 times with native binding buffer at low-force centrifugation (800×*g*) and loaded onto a column (1×15 mL). The column was subsequently washed on with another 500 mL of native binding buffer (0.8 mL min^{-1}) and 500 mL of high stringency buffer (20 mM NaH_2PO_4, 500 mM NaCl, pH 6.0). At this point the OD_{280} of the elute was <0.01. UDP-GlcNAc PP was recovered from the resin by using a lower pH buffer (20 mM NaH_2PO_4, 0.5 M NaCl, pH 4.0), while UDP-Glc DH was recovered from the corresponding column by using an imidazol buffer (20 mM NaH_2PO_4, 0.5 M NaCl, 0.3 M imidazol, pH 6.0). Fractions of 1 mL were collected, analyzed by SDS-PAGE[21] and tested for enzymatic activity. The active fractions were pooled together, concentrated by ultrafiltration (Amicon), and membrane with a 30 kDa cut-off.

Enzyme activity assay

UDP-GlcNAc PP. The assay mixture contained 1 mM GlcNAc-1-P, 5 mM UTP, 5 mM $MgCl_2$ and the enzyme in 100 mM HEPES, pH 7.5. The mixture was incubated at 25 °C for 10, 20, and 30 min. The reaction was terminated by the addition of acetic acid (10% of the mixture's volume). The mixture was filtered by an Ultrafree-MC (Millipore, MA) membrane (cut-off, 10,000 kDa). The reaction products were then separated by HPLC on a Parsital SAX column (Whatman) eluted with a sodium phosphate buffer

(100 mM, pH 3.25, flow rate: 0.5 mL min^{-1}). Quantification of the UDP-GlcNAc was determined by the elution Pick's area. In another assay method, H^3-UTP was used. 4 μL of reaction mixture was then mixed with 1 μL of a solution 10 mM UDP-GlcNAc and 10 μM UTP, loaded on a TLC silica gel plate (aluminum flexible plate, Whatman), and developed within isopropanol:H_2O:NH4-OAc(1N) with a ratio of 7:2:1. The spots corresponding to UTP and UDP-GlcNAc were located by UV absorption and cut out of the plate. The radioactivity was then counted by a Beckman liquid scintillation system LS-3801. One unit of enzyme activity is defined as the amount of enzyme required to produce 1 μmole of UDP-GlcNAc min^{-1}.

UDP-Glc DH. UDP-Glc dehydrogenase was assayed spectrophotometrically following the reduction of NAD at 340 nm at 25 °C in a 1 cm light path cuvette. The reaction mixture contained 1 mM UDP-Glc, 2 mM NAD, and 100 mM Tris–HCl buffer, pH 8.7. The reaction was initiated by the addition of the enzyme solution. The initial velocity was estimated during the first minute. When the activity of the enzyme immobilized on the Ni^{2+}-NTA resin was assayed, 5 or 10 μL of resin were incubated with the reaction mixture, and, after 1 min, the solution was centrifuged and the OD_{340} was measured. The background was calculated by measuring the OD_{340} of the reaction mixture before adding the resin. One unit of enzyme activity is defined as the amount of enzyme required to produce 2 μmol of NADH min^{-1}.

Enzymes stability study. The enzymes were incubated at 25 °C in 100 mM HEPES, pH 8.0, in the presence or absence of one substrate or reducing agent. At different time intervals, aliquots were taken and assayed for the activity. These studies were carried out using pure enzymes.

pH dependence. The pH influence on the activity was studied using the pure enzyme. In the case of UDP-GlcNAc PP, to 30 μL of a stock solution containing the enzyme, $MgCl_2$ and ^{3}H-UTP was added 30 μL of a solution containing GlcNAc-1P and the buffer. The solution was then loaded on a TLC plate as described above. For UDP-Glc DH, to 300 μL of a stock solution containing the enzyme and UDP-Glc was added 300 μL of a solution containing NAD and buffer.

Enzymes kinetics. The influence of the substrate concentration on the initial velocity was measured using pure enzymes and with all other conditions maintaining constant. Also in this case, the radioactivity assay was used for UDP-GlcNAc PP. The K_m values were calculated by a nonlinear least-square fit to the rate equation curve

$$v = \frac{v_{max} \cdot S}{K_m + S}.$$

A computer program (Hyperl) was used according to Cleland.[17]

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