

A Bifunctional Enzyme Catalyzes the First Two Steps in *N*-Acetylneuraminic Acid Biosynthesis of Rat Liver

PURIFICATION AND CHARACTERIZATION OF UDP-*N*-ACETYLGLUCOSAMINE 2-EPIMERASE/*N*-ACETYLmannosamine KINASE*

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Biosynthesis of *N*-acetylneuraminic acid (Neu5Ac), a prominent component of glycoconjugates, is initiated by the action of UDP-*N*-acetylglucosamine 2-epimerase (UDP-GlcNAc 2-epimerase, EC 5.1.3.14) and *N*-acetylmannosamine kinase (ManNAc kinase, EC 2.7.1.60). We demonstrate for the first time that the two activities are parts of one bifunctional enzyme in rat liver. The enzyme was purified to homogeneity from rat liver cytosol using salmine sulfate precipitation and chromatography on phenyl-Sepharose, ATP-agarose, and Mono Q. The purification resulted in one polypeptide with an apparent molecular mass of 75 kDa. Immunoprecipitation with a polyclonal antibody against the polypeptide reduced both enzyme activities in equal amounts.

Gel filtration analysis of purified UDP-GlcNAc 2-epimerase/ManNAc kinase showed that the polypeptide self-associates as a dimer and as a hexamer with apparent molecular masses of 150 and 450 kDa, respectively. The hexamer was fully active for both enzyme activities, whereas the dimer catalyzed only the phosphorylation of *N*-acetylmannosamine (ManNAc). Incubation of the dimer with UDP-*N*-acetylglucosamine led to reassembly of the fully active hexamer; maximal quantities of the hexamer were produced after incubation for 3 h.

Kinetic analysis of purified hexameric and dimeric enzyme revealed significantly lower Michaelis constants (93 ± 3 to $121 \pm 15 \mu\text{M}$ for ManNAc and 1.18 ± 0.13 to $1.67 \pm 0.20 \text{ mM}$ for ATP) and higher cooperativity (Hill coefficients of 1.42 ± 0.16 to 1.17 ± 0.06 for ManNAc and 1.30 ± 0.09 to 1.05 ± 0.14 for ATP) for the hexamer for both substrates of ManNAc kinase. The Michaelis constant of UDP-GlcNAc 2-epimerase for its substrate was $11 \pm 2 \mu\text{M}$. The Hill coefficient of 0.45 ± 0.07 represents strongly negative cooperativity in substrate binding. UDP-GlcNAc 2-epimerase was feedback inhibited by CMP-Neu5Ac. Complete inhibition was achieved with $60 \mu\text{M}$ CMP-Neu5Ac, and highly positive cooperativity (Hill coefficient of 4.1) was found for inhibitor binding.

N-Acetylneuraminic acid (Neu5Ac)¹ is an important component within the oligosaccharide residues of glycoconjugates. Neu5Ac and its activated form, CMP-Neu5Ac, are biosynthesized in rat liver by five consecutive reactions (1): UDP-*N*-acetylglucosamine (UDP-GlcNAc) \rightarrow *N*-acetylmannosamine (ManNAc) \rightarrow ManNAc 6-phosphate \rightarrow Neu5Ac 9-phosphate \rightarrow Neu5Ac \rightarrow CMP-Neu5Ac. UDP-*N*-acetylglucosamine 2-epimerase (UDP-GlcNAc 2-epimerase, EC 5.1.3.14) catalyzes the formation of ManNAc from UDP-GlcNAc (2), and ManNAc is phosphorylated by *N*-acetylmannosamine kinase (ManNAc kinase, EC 2.7.1.60) (3). Activities of both enzymes are found in the cytosol of liver, salivary glands, and intestinal mucosa, and the ratios of their respective specific activities are apparently the same in each tissue cytosol (4). UDP-GlcNAc 2-epimerase (5, 6) and ManNAc kinase (7) activities have previously been enriched from rat liver cytosol with different purification methods, but until now they have not been purified to homogeneity.

It has been suggested that UDP-GlcNAc 2-epimerase is the key enzyme of Neu5Ac biosynthesis because its activity is feedback inhibited by CMP-Neu5Ac (6, 8). Furthermore, it has been shown that the inherited disease of sialuria is due to a defect in the binding of CMP-Neu5Ac to UDP-GlcNAc 2-epimerase (9). The activity of UDP-GlcNAc 2-epimerase is dramatically reduced in hepatoma cell lines in accordance with the lower secretion of plasma proteins in hepatomas (10).

In this study we demonstrate that UDP-GlcNAc 2-epimerase and ManNAc kinase are parts of one bifunctional enzyme. By purification of the enzyme to homogeneity and analysis of its oligomeric structure it is shown that a single 75-kDa polypeptide assembles as a hexamer with both enzyme activities, or as a dimer which is able only to phosphorylate ManNAc.

EXPERIMENTAL PROCEDURES

Materials

Salmine sulfate was from Roth (Karlsruhe, Germany). UDP-[U-¹⁴C]GlcNAc and [1-¹⁴C]ManNAc were from ICN (Eschwege, Germany). All other chemicals were from Sigma (Deisenhofen, Germany) and Boehringer Mannheim (Mannheim, Germany).

Enzyme Assays

UDP-GlcNAc 2-epimerase and ManNAc kinase activities were assayed by a modified method of Zeitler *et al.* (11). In brief, the final volume of incubation mixtures was 225 μl , incubations were carried out at 37 °C for 30 min, and reactions were stopped by addition of 350 μl of ethanol. UDP-GlcNAc 2-epimerase assay: 45 mM Na₂HPO₄, pH 7.5, 10 mM MgCl₂, 1 mM UDP-GlcNAc, 25 nCi of UDP-[¹⁴C]GlcNAc. ManNAc kinase assay: 60 mM Tris/HCl, pH 8.1, 20 mM MgCl₂, 5 mM ManNAc, 50 nCi [¹⁴C]ManNAc, 20 mM ATP (disodium salt). Radiolabeled compounds

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¹ The abbreviations used are: Neu5Ac, *N*-acetylneuraminic acid; ManNAc, *N*-acetylmannosamine; PAGE, polyacrylamide gel electrophoresis; UDP-GlcNAc, UDP-*N*-acetylglucosamine.

were separated by paper chromatography as described earlier (11). Radioactivity was determined in the presence of Ultima Gold XR (Packard; Groningen, Netherlands) in a Tri-Carb 1900 CA liquid scintillation analyzer (Packard).

Protein concentration was measured by the method of Bradford (12), using bovine serum albumin as standard. One unit of enzyme activity was defined as the formation of 1 μ mol of product per min at 37 °C. Specific activity was expressed as milliunits per mg of protein.

Purification of UDP-GlcNAc 2-Epimerase/ManNAc Kinase

Step 1: Preparation of Rat Liver Cytosol—Female Wistar rats were perfused with 20 ml of 150 mM NaCl while the animals were under light ether anesthesia. Livers were removed and transferred to 2 volumes of homogenizing buffer containing 10 mM NaH_2PO_4 , pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM ManNAc (buffer A), and 1 mM phenylmethylsulfonyl fluoride. The tissue was homogenized with an ultraturax for 1 min at 10,000 rpm. The homogenate was ultracentrifuged at $100,000 \times g$ for 60 min.

Step 2: Saline Sulfate Precipitation—The supernatant obtained from step 1 was fractionately precipitated with 0.085% and 0.145% salmine sulfate (w/v), pH 7.0. The precipitates were collected by centrifugation at $35,000 \times g$ for 15 min. The pellet after the second centrifugation was eluted with 100 mM NaH_2PO_4 , pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM ManNAc, 1 mM phenylmethylsulfonyl fluoride and centrifuged at $35,000 \times g$ for 15 min.

Step 3: Hydrophobic Chromatography on Phenyl-Sepharose FF—The supernatant from step 2 was applied to a column (26-mm inner diameter \times 50 mm) of phenyl-Sepharose FF (Pharmacia, Freiburg, Germany). The column was washed with 60 ml of buffer A containing 20% ethylene glycol (v/v). The enzyme was eluted with 100 ml of a linear gradient of 20 to 70% ethylene glycol (v/v) in buffer A. Fractions were collected at a flow rate of 1 ml/min.

Step 4: Affinity Chromatography on ATP-agarose—Fractions containing enzyme activity from step 3 were applied to a column (16-mm inner diameter \times 20 mm) of ATP-agarose (Sigma). The column was washed with 30 ml of buffer A and nonspecifically bound proteins were eluted with 30 ml of buffer A containing 100 mM NaCl. The enzyme was eluted with 50 ml of a linear gradient of 0 to 10 mM ATP in buffer A at a flow rate of 1 ml/min.

Step 5: Anion Exchange on Mono Q—Fractions with the highest specific enzyme activities from step 4 were applied to a Mono Q HR 5/5 column (Pharmacia). The column was washed with 5 ml of buffer A and then eluted with 10 ml of a 0 to 0.6 M NaCl gradient. Fractions were collected at 0.2 ml/min.

All procedures were carried out at 0–4 °C. Fractions obtained during purification were assayed for UDP-GlcNAc 2-epimerase and ManNAc kinase activities as described above, and analyzed for purity by SDS-PAGE according to the method of Laemmli (13). Gels were silver stained for protein as described elsewhere (14).

Immunoprecipitation

Polyclonal antibody against UDP-GlcNAc 2-epimerase/ManNAc kinase was obtained as described elsewhere (15). For immunoprecipitation, 10 mg of Protein-A-Sepharose were preincubated in 10 mM NaH_2PO_4 , pH 7.8, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100 (w/v), and 1% bovine serum albumin (w/v) for 30 min at 25 °C. Then 10 or 20 μ l of polyclonal antiserum, preimmunoserum, or (for control) H_2O were added and incubated for 2 h. Sepharose was washed once with 10 mM NaH_2PO_4 , pH 7.8, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100 (w/v), 1% bovine serum albumin (w/v) and three times with 10 mM NaH_2PO_4 , pH 7.8, 500 mM NaCl. A partially purified UDP-GlcNAc 2-epimerase/ManNAc kinase sample from purification step 3 was made dithiothreitol-free by gel filtration as described below, with 10 mM NaH_2PO_4 , pH 7.8, 500 mM NaCl, 1 mM EDTA as eluent. 300 μ l of the sample (25 μ g of protein) was added to the Protein-A-Sepharose and incubated for 6 h at 4 °C. Then the Sepharose was precipitated by centrifugation at $5,000 \times g$ for 5 min and the supernatant was assayed for both enzyme activities.

Determination of Oligomeric Structure

The oligomeric structure of UDP-GlcNAc 2-epimerase/ManNAc kinase was determined with purified UDP-GlcNAc 2-epimerase/ManNAc kinase from step 5 (10 μ g) by gel filtration on a Superdex 200 column (Pharmacia). For elution, buffer A containing 100 mM NaCl was used. Gel filtration was performed with samples preincubated in the absence or presence of 1 mM UDP-GlcNAc for 3 h at 0 °C. Fractions obtained at a flow rate of 0.2 ml/min were analyzed by SDS-PAGE and assayed for enzyme activities as described above, with the exception that the assay

time was reduced to 15 min in order to avoid reassociation of the hexamer (see below).

Reassociation of Hexameric UDP-GlcNAc 2-Epimerase/ManNAc Kinase

Dimeric enzyme was prepared by gel filtration as described above. Reassociation was performed in a mixture containing 45 mM NaH_2PO_4 , pH 7.5, 10 mM MgCl_2 , 1 mM UDP-GlcNAc, 0.2 mM NADH, 2 mM phosphoenolpyruvate, 2 units of pyruvate kinase, 2 units of lactate dehydrogenase, and 1.25 μ g/ml UDP-GlcNAc 2-epimerase/ManNAc kinase. UDP-GlcNAc 2-epimerase activity was monitored at 340 nm by the oxidation of NADH. The ratio of dimer to hexamer was determined by gel filtration of aliquots withdrawn from an equivalent reassociation assay, but without pyruvate kinase and lactate dehydrogenase, followed by peak integration.

Kinetic Analysis

Kinetic data were determined separately for dimeric and hexameric UDP-GlcNAc 2-epimerase/ManNAc kinase prepared by gel filtration as described above. Hexameric enzyme was stabilized with 0.1 mM UDP. The hexameric state was completely stable under these conditions for 6 h as tested by re-chromatography. The UDP-GlcNAc 2-epimerase assay contained 45 mM NaH_2PO_4 , pH 7.5, 10 mM MgCl_2 , 0.2 mM NADH, 2 mM phosphoenolpyruvate, 2 units of pyruvate kinase, 2 units of lactate dehydrogenase, 0.25 to 0.5 μ g/ml UDP-GlcNAc 2-epimerase/ManNAc kinase, and variable amounts of UDP-GlcNAc. The ManNAc kinase assay contained 60 mM Tris/HCl, pH 8.1, 20 mM MgCl_2 , 0.2 mM NADH, 2 mM phosphoenolpyruvate, 2 units of pyruvate kinase, 2 units of lactate dehydrogenase, 0.05 to 0.2 μ g/ml UDP-GlcNAc 2-epimerase/ManNAc kinase, and variable concentrations of ManNAc in the presence of 20 mM ATP or variable concentrations of ATP in the presence of 5 mM ManNAc. Initial velocities were determined by measuring the decrease in extinction at 340 nm. Michaelis constants (K_m) were determined by Lineweaver-Burk plots, and Hill coefficients (n_H) were determined by Hill plots.

Inhibition of UDP-GlcNAc 2-epimerase activity by CMP-Neu5Ac was determined in an assay containing 45 mM NaH_2PO_4 , pH 7.5, 10 mM MgCl_2 , 0.2 mM NADH, 2 mM phosphoenolpyruvate, 2 units of pyruvate kinase, 2 units of lactate dehydrogenase, 0.5 μ g/ml UDP-GlcNAc 2-epimerase/ManNAc kinase, 1 mM UDP-GlcNAc, and variable concentrations of CMP-Neu5Ac. The Hill coefficient for inhibitor binding was determined as described by Levitzki and Koshland (16).

RESULTS

Purification of UDP-GlcNAc 2-Epimerase/ManNAc Kinase as a Bifunctional Enzyme—The purification of UDP-GlcNAc 2-epimerase/ManNAc kinase from rat liver cytosol involved salmine sulfate precipitation, followed by hydrophobic interaction chromatography on phenyl-Sepharose FF (Fig. 1A), affinity chromatography on ATP-agarose (Fig. 1B), and anion exchange on Mono Q HR 5/5 (Fig. 1C). The enzyme activities of UDP-GlcNAc 2-epimerase and ManNAc kinase were co-purified during all purification steps (Table I). The chromatograms in Fig. 1 displayed the same elution behavior for both enzyme activities. After the last purification step, SDS-PAGE showed only one polypeptide, and this had a molecular mass of 75 kDa (Fig. 2). A polyclonal antibody against the 75-kDa polypeptide precipitated both enzyme activities from a partially purified fraction in apparently equal amounts (Fig. 3). All these results led to the conclusion that the 75-kDa polypeptide is a bifunctional enzyme possessing both enzyme activities. Surprisingly, the ratio of UDP-GlcNAc 2-epimerase activity to ManNAc kinase activity was not constant during purification (Table I), as expected for a bifunctional enzyme. The explanation for this observation is given below.

The enzyme fraction from step 5 was stored at –70 °C. No loss of activity was observed under these conditions for several months.

Determination of Oligomeric Structure—Purified UDP-GlcNAc 2-epimerase/ManNAc kinase from the ultimate purification step was used to determine the oligomeric structure of the enzyme. Gel filtration resulted in two peaks corresponding to proteins with native molecular masses of 450 and 150 kDa,

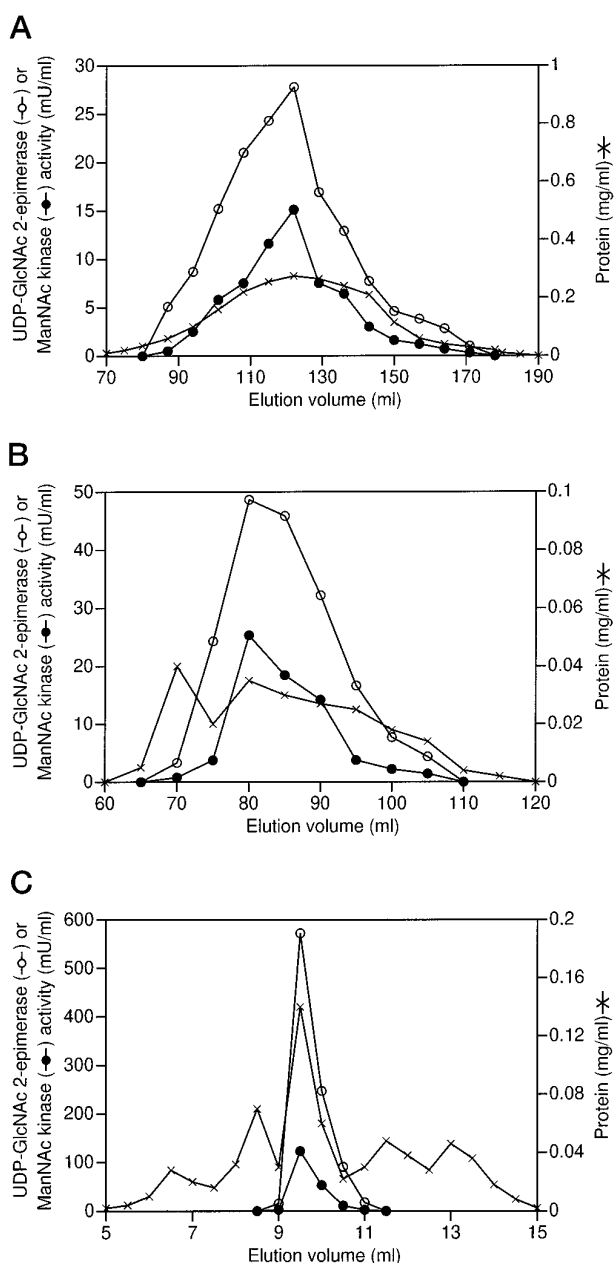


FIG. 1. Purification of UDP-GlcNAc 2-epimerase/ManNAc kinase. A, phenyl-Sepharose chromatography of salmine sulfate precipitate. B, affinity chromatography of active peak from A on ATP-agarose. C, ion exchange chromatography of active peak from B on Mono Q HR 5/5. Purification methods are described in detail under "Experimental Procedures." Fractions were assayed for UDP-GlcNAc 2-epimerase activity (●), ManNAc kinase activity (○), and protein (×).

respectively (Fig. 4A). SDS-PAGE of fractions obtained during chromatography demonstrated that both protein peaks contained the same 75-kDa polypeptide, associated either as a hexamer or as a dimer. The hexamer showed both enzyme activities, whereas the dimer only catalyzed the phosphorylation of ManNAc (Fig. 4).

The ratio of hexamer to dimer in fractions of purification step 5 depended on the duration of the purification procedure. Fig. 4A shows the ratio after a 3-day purification with overnight storage on ice. After 5 days of storage the hexamer was completely dissociated to the dimer, whereas no further dissociation was observed if the enzyme was frozen. It was possible to stabilize the hexameric state in buffers containing 0.1 mM UDP or 0.1 mM UDP-GlcNAc, but this prevents binding of the en-

zyme to ATP-agarose. Dissociation of the hexamer to the UDP-GlcNAc 2-epimerase-inactive but ManNAc kinase-active dimer over a period of some days explains the non-constant ratio of the two enzyme activities during purification. After gel filtration of freshly prepared cytosol, enzyme activities were found only in fractions corresponding to the hexamer (data not shown).

The dimer reassembled to the fully active hexamer by induction with the UDP-GlcNAc 2-epimerase-substrate, UDP-GlcNAc. Fig. 4B demonstrates that the dimer reassociated to the hexamer in a dimer-hexamer mixture obtained after step 5 of the purification. The purified dimer also reassociated to the hexamer (Fig. 5). The time course of hexamer assembly showed a lag phase of about 20 min. The maximal amount of hexamer was present after 3 h of incubation with UDP-GlcNAc. The presence of ManNAc and/or ATP did not influence the dimer-hexamer ratio (data not shown).

In both reassociation experiments the hexamer was only partially restored. This may depend on the *in vitro* conditions used in the experiments or on the enzyme concentration, which may be critical for optimal reassociation (17). At a UDP-GlcNAc 2-epimerase/ManNAc kinase concentration of 100 $\mu\text{g/ml}$, a reassociation rate of 70% was achieved (Fig. 4), whereas a concentration of 1.25 $\mu\text{g/ml}$ resulted in only 37% reassociation (Fig. 5).

Kinetic Analysis—Michaelis constants and Hill coefficients for the hexameric and the dimeric enzyme were determined for the substrates used by UDP-GlcNAc 2-epimerase/ManNAc kinase (Table II). The data for UDP-GlcNAc could be determined only for the hexamer. For UDP-GlcNAc, the K_m was 11 ± 2 mM and the Hill coefficient was 0.45 ± 0.07 , corresponding to a strongly negative cooperativity for the binding of this substrate. For ManNAc and ATP it was possible to obtain data for both oligomeric states. For both substrates we found significantly lower K_m values and higher Hill coefficients for the hexamer than for the dimer. This may explain the slightly higher specific ManNAc kinase activity of the hexamer (4.88 units/mg; calculated from the data of Fig. 4) compared with that of the dimer (3.85 units/mg).

Inhibition of UDP-GlcNAc 2-epimerase activity by CMP-Neu5Ac has already been reported (8). In the present study we also showed that the UDP-GlcNAc 2-epimerase activity of purified hexameric UDP-GlcNAc 2-epimerase/ManNAc kinase is inhibited in a concentration-dependent manner by CMP-Neu5Ac (Fig. 6). Complete inhibition was achieved with 60 μM CMP-Neu5Ac. A Hill coefficient of 4.1 was found for the binding of the inhibitor. The results were in good agreement with those for the partially purified enzyme from rat liver (6) and for the enzyme in human fibroblasts (18). The ManNAc kinase activity was not influenced by CMP-Neu5Ac, either in the dimeric or the hexameric enzyme (data not shown).

DISCUSSION

Biosynthesis of Neu5Ac is initiated by the action of UDP-GlcNAc 2-epimerase and ManNAc kinase. In this study we have purified a protein which occurs as a bifunctional enzyme combining both UDP-GlcNAc 2-epimerase and ManNAc kinase activities. This finding is supported by the following results. First, during all steps of the purification procedure presented above, both enzyme activities were co-purified. Second, UDP-GlcNAc 2-epimerase and ManNAc kinase activities show the same elution profile from phenyl-Sepharose, ATP-agarose, and Mono Q. Third, the purification resulted in a single 75-kDa polypeptide; fractions containing only this polypeptide displayed both UDP-GlcNAc 2-epimerase and ManNAc kinase activity. Fourth, a polyclonal antibody against the 75-kDa polypeptide precipitated both enzyme activities in equal

TABLE I
Purification of UDP-GlcNAc 2-epimerase/ManNAc kinase

Purification step	Protein	Specific activities		Purification factors		Yields	
		UDP-GlcNAc 2-epimerase	ManNAc kinase	UDP-GlcNAc 2-epimerase	ManNAc kinase	UDP-GlcNAc 2-epimerase	ManNAc kinase
	mg	milliunits/mg		-fold		%	
Cytosol	530	1.9	4.3	1	1	100	100
Salmine sulfate	50	12.5	36.6	7	8	62	80
Phenyl-Sepharose	6	71	165	37	38	42	43
ATP-agarose	0.5	283	1210	149	281	14	27
Mono Q	0.1	878	4114	462	957	9	18

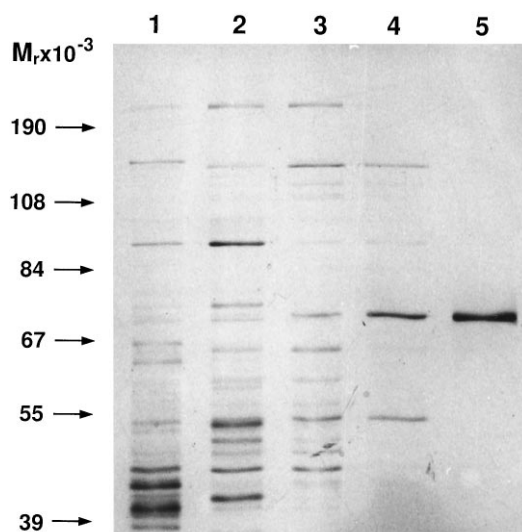


FIG. 2. SDS-PAGE of samples obtained during purification of UDP-GlcNAc 2-epimerase/ManNAc kinase. 150 ng of protein per lane were applied to a 10% polyacrylamide gel and silver stained. Lane 1, cytosol; lane 2, salmine sulfate precipitation; lane 3, phenyl-Sepharose chromatography; lane 4, ATP-agarose chromatography; lane 5, Mono Q chromatography.

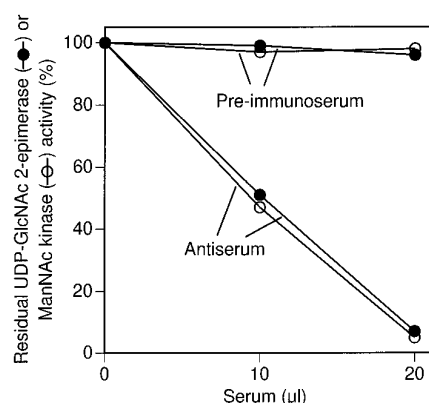


FIG. 3. Immunoprecipitation of a partially purified UDP-GlcNAc 2-epimerase/ManNAc kinase sample. UDP-GlcNAc 2-epimerase/ManNAc kinase was precipitated with a polyclonal antibody against the 75-kDa polypeptide. Immunoprecipitation and the determination of residual UDP-GlcNAc 2-epimerase (●) and ManNAc kinase activity (○) were performed as described under "Experimental Procedures." Values are means of duplicate experiments.

amounts. Fifth, as shown in the accompanying paper (19), the corresponding cDNA of the 75-kDa polypeptide was overexpressed in COS7 cells whereby both enzyme activities were increased proportionately.

Bifunctional enzymes are rare in mammalian metabolism. Three other known bifunctional enzymes, which catalyze two subsequent steps of a metabolic pathway, are: peroxisomal 2-enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (20),

formiminotransferase/cyclo-deaminase (21), and ATP sulfurylase/adenosine 5-phosphosulfate kinase (22). Possible advantages conferred by the evolution of a bifunctional enzyme are: (i) the enzyme is more stable than isolated catalytic domains (23); (ii) allosteric control is possible between the two domains (17); (iii) the two catalytic centers may interact to "channel" an intermediate (21). Further investigations are necessary to determine whether any of these advantages apply to UDP-GlcNAc 2-epimerase/ManNAc kinase. However, it may be concluded that "channeling" of intermediates is advantageous, in that ManAc is not released into the cytosol, where it would be available to aminosugar metabolism. Thus, in the cytosol ManNAc can directly be epimerized to GlcNAc by *N*-acetylglucosamine 2-epimerase, an activity present in rat liver (24). ManNAc, which is essential for the biosynthesis of Neu5Ac, could be converted to GlcNAc whereas ManNAc 6-phosphate is not a substrate of *N*-acetylglucosamine 2-epimerase.

Gel filtration analysis of purified UDP-GlcNAc 2-epimerase/ManNAc kinase resulted in two oligomeric structures of the enzyme, a fully active hexamer with a molecular mass of 450 kDa and a partially active dimer with a molecular mass of 150 kDa. These results are consistent with the works of Sommar and Ellis (5) in which the native molecular mass of the UDP-GlcNAc 2-epimerase was estimated to be in the range of 400 to 600 kDa. The two oligomeric structures can dissociate and reassociate. Association is promoted by the presence of the UDP-GlcNAc 2-epimerase substrate, UDP-GlcNAc, whereas dissociation occurs in the absence of this substrate. Several enzymes are known in which a change in subunit assembly is accompanied by a change in enzymatic activity, thereby providing a possible mechanism for regulation (17). In the case of UDP-GlcNAc 2-epimerase/ManNAc kinase, the UDP-GlcNAc 2-epimerase activity is influenced by a change in quaternary structure whereas the hexamer and the dimer display only slightly different specific activities of ManNAc kinase. This suggests that UDP-GlcNAc 2-epimerase activity may be regulated by the dissociation-association mechanism in accordance with the proposed key role of UDP-GlcNAc 2-epimerase in the metabolism of Neu5Ac. However, this suggestion appears to be inconsistent with the finding that freshly prepared cytosol does not contain a dimer. As mentioned by Traut (17), most results of the dissociation-reassociation of enzymes come from *in vitro* experiments wherein it is impossible to show the existence of an *in vivo* regulatory mechanism. Thus, it cannot be concluded from our results for UDP-GlcNAc 2-epimerase/ManNAc kinase that UDP-GlcNAc 2-epimerase activity is really regulated by a dissociation-reassociation mechanism *in vivo*. Other bifunctional enzymes are known with different oligomeric structures (17, 23), but to our knowledge, UDP-GlcNAc 2-epimerase/ManNAc kinase is the first reported enzyme whereby one of the oligomeric structures catalyzes both reactions, whereas an alternative oligomeric structure catalyzes only a single enzyme activity.

As demonstrated by gel filtration, UDP-GlcNAc 2-epimerase/

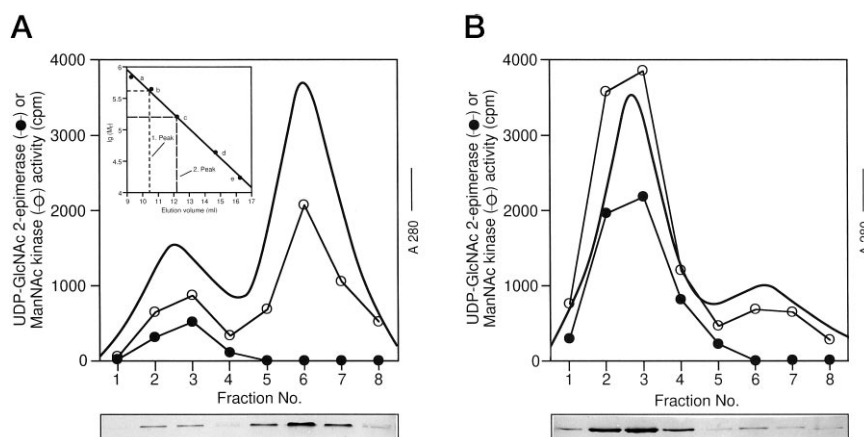


FIG. 4. **Determination of oligomeric structure of purified UDP-GlcNAc 2-epimerase/ManNAc kinase.** Panel A, gel filtration analysis of a UDP-GlcNAc 2-epimerase/ManNAc kinase sample obtained from purification step 5 as described under "Experimental Procedures." Fractions were analyzed for UDP-GlcNAc 2-epimerase (●) and ManNAc kinase activity (○) and were also subjected to SDS-PAGE. Protein was determined by its absorbance at 280 nm (—). Inset, determination of native molecular masses of the proteins in the two observed peaks; standard proteins were: a, thyroglobulin (670 kDa); b, ferritin (440 kDa); c, γ -globulin (156 kDa); d, ovalbumin (44 kDa); and e, myoglobin (17 kDa). Panel B, gel filtration analysis of an equivalent UDP-GlcNAc 2-epimerase/ManNAc kinase sample after incubation with UDP-GlcNAc.

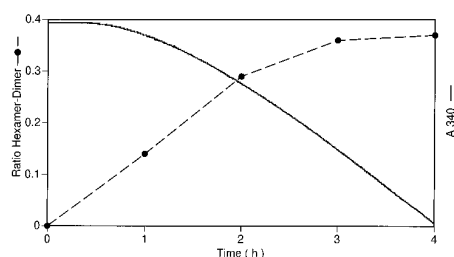


FIG. 5. **Time course of the reassociation of dimeric UDP-GlcNAc 2-epimerase/ManNAc kinase to the hexamer.** Purified dimer reassociated in the presence of UDP-GlcNAc. UDP-GlcNAc 2-epimerase activity was followed by the decrease of NADH (A_{340} , —) coupled to the release of UDP in the assay described under "Experimental Procedures." Ratio of hexamer to dimer (●) was determined by gel filtration in fractions withdrawn from the reassociation assay.

TABLE II
Kinetic data of UDP-GlcNAc 2-epimerase/ManNAc kinase
All values are means \pm S.D. of three independent determinations.

Substrate	K_m		n_H	
	Dimer	Hexamer	Dimer	Hexamer
	μM			
UDP-GlcNAc	ND ^a	11 ± 2	ND	0.45 ± 0.07
ManNAc	121 ± 15	93 ± 3	1.17 ± 0.06	1.42 ± 0.16
ATP	1670 ± 200	1180 ± 130	1.05 ± 0.14	1.30 ± 0.09

^a ND, not determined.

ManNAc kinase assembles as a hexamer and as a dimer of 75-kDa subunits. Kinetic data for the enzyme confirm this observation. The binding of CMP-Neu5Ac to the enzyme occurs with positive cooperativity and a Hill coefficient of 4.1. This suggests that the oligomeric state responsible for inhibitor binding must be greater than a tetramer and is likely to be a hexamer. Hill coefficients between one and two for the binding of ManNAc and ATP, respectively, reveal that the functionally important structure for ManNAc kinase activity is dimeric, both in the hexameric and in the dimeric enzyme. On the basis of these results we assume that hexameric UDP-GlcNAc 2-epimerase/ManNAc kinase assembles as a trimer of dimers. Only two other enzymes are known which form hexamers by the assembly of three dimers: chick embryo dCMP deaminase (17) and DNA replication helicase of bacteriophage T4 (25).

Kinetic analysis of UDP-GlcNAc 2-epimerase/ManNAc kinase suggests further regulatory mechanisms for UDP-GlcNAc 2-epimerase activity. As observed in this and previous studies

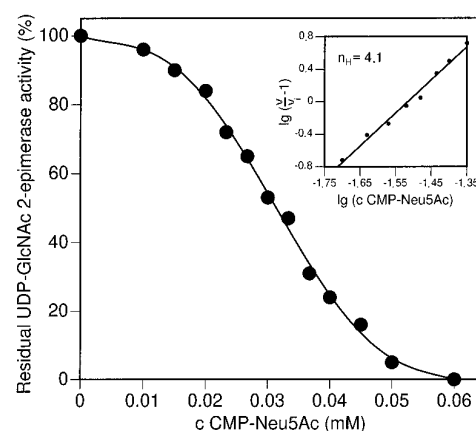


FIG. 6. **Inhibition of UDP-GlcNAc 2-epimerase activity by CMP-Neu5Ac.** Residual UDP-GlcNAc 2-epimerase activity of purified hexameric UDP-GlcNAc 2-epimerase/ManNAc kinase was determined as described under "Experimental Procedures." Inset, Hill plot.

(6, 18), CMP-Neu5Ac inhibits UDP-GlcNAc 2-epimerase activity in a concentration dependent manner, the effective concentrations lying in the normal concentration range of CMP-Neu5Ac in the cytosol (10). This provides for a very sensitive regulation of UDP-GlcNAc 2-epimerase activity depending on the consumption of CMP-Neu5Ac for oligosaccharide biosynthesis. The binding of UDP-GlcNAc to UDP-GlcNAc 2-epimerase/ManNAc kinase is regulated by a strongly negative cooperativity ($n_H = 0.45$). It should be considered that UDP-GlcNAc is used not only for Neu5Ac biosynthesis, but also as a substrate for *N*-acetylglucosaminyl transferases in the biosynthesis of oligosaccharides (26). The negative cooperativity of UDP-GlcNAc binding, together with the limited synthetic rate of UDP-GlcNAc 2-epimerase, which is largely independent of the higher substrate concentration, guarantees that the amounts of UDP-GlcNAc required for oligosaccharide biosynthesis are not diverted to sialic acid biosynthesis.

Kinetic data for ManNAc kinase (K_m and n_H values) indicate that the higher oligomeric state results in better substrate binding. For this reason, the specific ManNAc kinase activity of the hexamer is higher than that of the dimer although the difference is too small to be physiologically significant. There is no need for the regulation of ManNAc kinase activity, because its efficiency is directly linked to UDP-GlcNAc 2-epimerase by the bifunctionality of the enzyme. Furthermore, it has been

shown that Madin-Darby canine kidney cells fed with high amounts of ManNAc have a 6.5-fold increased CMP-Neu5Ac pool (27). This indicates that Neu5Ac biosynthesis is controlled only by the UDP-GlcNAc 2-epimerase activity.

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