Selective Loss of either the Epimerase or Kinase Activity of UDP-N-acetylglucosamine 2-Epimerase/N-Acetylmannosamine Kinase due to Site-directed Mutagenesis Based on Sequence Alignments*

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Karin Effertz‡, Stephan Hinderlich, and Werner Reutter

From the Institut für Molekularbiologie und Biochemie, Freie Universität Berlin, Arnimallee 22, D-14195 Berlin-Dahlem, Germany

N-Acetyleneuraminic acid is the most common naturally occurring sialic acid, as well as being the biosynthetic precursor of this group of compounds. UDP-GlcNAc 2-epimerase/N-acetylmannosamine kinase has been shown to be the key enzyme of N-acetyleneuraminic acid biosynthesis in rat liver, and it is a regulator of cell surface sialylation. The N-terminal region of this bifunctional enzyme displays sequence similarities with prokaryotic UDP-GlcNAc 2-epimerases, whereas the sequence of its C-terminal region is similar to sequences of members of the sugar kinase superfamily. High level overexpression of active enzyme was established by using the baculovirus/Sf9 system. For functional characterization, site-directed mutagenesis was performed on different conserved amino acid residues. The histidine mutants H45A, H110A, H132A, H155A, and H157A showed a drastic loss of epimerase activity with almost unchanged kinase activity. Conversely, the mutants D413N, D413K, and R420M in the putative kinase active site lost their kinase activity but retained their epimerase activity. To estimate the structural perturbation effect due to site-directed mutagenesis, the oligomeric state of all mutants was determined by gel filtration analysis. The mutants D413N, D413K, and R420M as well as H45A were shown to form a hexamer like the wild-type enzyme, indicating large influence of mutation on protein folding. Histidine mutants H155A and H157A formed mainly trimeric enzyme with small amounts of hexamer. Oligomerization of mutants H110A and H132A was also significantly different from that of the wild-type enzyme. Therefore the loss of epimerase activity in mutants H110A, H132A, H155A, and H157A can largely be attributed to incorrect protein folding. In contrast, the mutation site of mutant H45A seems to be involved directly in the epimerization process, and the amino acids Asp-413 and Arg-420 of UDP-GlcNAc 2-epimerase/N-acetylmannosamine kinase are essential for the phosphorylation process. The fact that either epimerase or kinase activity are lost selectively provides evidence for the existence of two active sites working quite independently.

N-Acetyleneuraminic acid (Neu5Ac) is the precursor of sialic acids, a group of important molecules in biological communication. Sialic acids have been shown to be involved in cellular adhesion (1, 2), and they are important as recognition determinants (3). Glycoproteins can be protected against degradation by sialylation (4, 5), and the metastatic and invasive potential of tumor cells is often correlated with the amount of overexpressed membrane-bound sialic acids (6, 7).

The biosynthesis of Neu5Ac in rat liver is initiated and regulated by its key enzyme, UDP-N-acetylglucosamine 2-epimerase (EC 5.1.3.14)/N-acetylmannosamine kinase (EC 2.7.1.60) (8). Furthermore, it was shown recently that UDP-GlcNAc 2-epimerase is a regulator of cell surface sialylation (9). The bifunctional enzyme catalyzes the conversion of UDP-GlcNAc to ManNAc and the consecutive phosphorylation to form ManNAc 6-phosphate. The homogeneous enzyme from rat liver has an apparent molecular mass of 75 kDa. It assembles as a hexamer possessing both enzyme activities. In vitro it partly decays to dimers, which possess only the kinase activity. CMP-Neu5Ac, the end product of sialic acid biosynthesis, has been shown to be a competitive feed-back inhibitor of UDP-GlcNAc 2-epimerase activity (10). The UDP-GlcNAc 2-epimerases/ManNAc kinases of rat (11), mouse (12), and human (13, 34) have been cloned and sequenced. In all three enzymes, an open reading frame of 2166 base pairs encodes 722 amino acids. The overall amino acid identity between the enzymes from rat and mouse is 99.4%, and between rat and human the identity is 98.6% (13), showing that UDP-GlcNAc 2-epimerase/ManNAc kinase is highly conserved in mammalian organisms.

Bifunctional enzymes are quite rare in mammalian metabolism. Further examples of enzymes catalyzing consecutive steps of a metabolic pathway are heparan sulfate/heparin N-deacetylase/N-sulfotransferase, and 3’-phosphoadenosine 5’-phosphosulfate synthase. Sequence analysis and functional studies show that both of these enzymes might have evolved by gene fusion from two independent enzymes, which in part are still present in lower organisms. In 3’-phosphoadenosine 5’-phosphosulfate synthase the functional domains were expressed separately (14), whereas in heparan sulfate/heparin N-deacetylase/N-sulfotransferase only the sulfotransferase activity can be separately correlated to a distinct region, i.e. the carboxyl half of the enzyme (15).

In the present paper we report the establishment of high level overexpression of UDP-GlcNAc 2-epimerase/ManNAc kinase, results of sequence analysis, and alignment-guided site-directed mutagenesis of the bifunctional enzyme.

ManNAc, N-acetylmannosamine; PAGE, polyacrylamide gel electrophoresis.

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‡ To whom correspondence should be addressed: Tel.: 493084451-545; Fax: 493084451541; E-mail: effertzk@zedat.fu-berlin.de.

The abbreviations used are: Neu5Ac, N-acetyleneuraminic acid;
Functional Domains of UDP-GlcNAc 2-Epimerase/ManNAc Kinase

EXPERIMENTAL PROCEDURES

Materials

UDP-[U-\textsuperscript{14}C]GlcNAc and [\textsuperscript{1-14}C]ManNAc were from ICN (Eschwege, Germany). All other chemicals were from Sigma and Roche Molecular Biochemicals.

Overexpression of UDP-GlcNAc 2-Epimerase/ManNAc Kinase and Mutants in Sf9 Cells

Expression Vector Construction—The UDP-GlcNAc 2-epimerase/ManNAc kinase coding cDNA (11) was amplified by polymerase chain reaction from pBluescript II (Amersham Pharmacia Biotech) using primers designed to contain a forward XhoI site and a reverse 3'-KpnI site. About 0.5 μg of product was excised, eluted from agarose gels, and digested first with XhoI. The restricted DNA was precipitated and afterward restricted with KpnI. To inactive the restriction enzyme, the DNA was extracted with phenol-chloroform. An aliquot of the resulting 5'-XhoI-DNA-3'-KpnI site was ligated to the double-restricted (XhoI, KpnI) vector pFastBac1 (Life Technologies, Inc.). The transformed ligation product was mini-prepped and verified by sequencing (16).

Production of Virus—The recombinant baculovirus containing the coding sequence of the UDP-GlcNAc 2-epimerase/ManNAc kinase was produced by using the Bac to Bac system according to the procedures supplied by the manufacturer (Life Technologies, Inc.). The system is based on transposon-mediated insertion of the foreign gene into the baculovirus genome under transcriptional regulation of the polyhedrin gene (17). Propagation of the recombinant virus as well as wild-type Autographa californica nuclear polyhedrosis virus (strain) was performed according to procedures described by O'Reilly et al. (18).

Cell Culture—Lepidopteran Spodoptera frugiperda cells (Sf9, Life Technologies, Inc.) were maintained as monolayer cultures in plastic flasks (Greiner GmbH, Frickhausen, Germany) or in suspension by using Erlenmeyer flasks (100 ml) on orbital shakers (100–120 rpm) at 27 °C. Cells were grown in TC-100 medium (Biochrom, Berlin, Germany) supplemented with 2 mM l-glutamine, 10% fetal calf serum (Biochrom), or in cell-free SF900 II medium (Life Technologies, Inc.). Antibiotics (100 units/ml penicillin, 40 units/ml streptomycin) were added to both culture media.

Conditions for Overexpression and Cytosol Preparation—Sf9 cells were grown to a density of 2 × 10\textsuperscript{6} cells/ml, then infected at a multiplicity of infection of 0.1. During infection, cells were grown in suspension culture in an orbital shaker at 120 rpm and at 27 °C. After an optimal infection period of 60 h, the cells were pelleted and disrupted in lysis buffer (10 mM Na\textsubscript{2}HPO\textsubscript{4}, pH 7.5, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) by tipping them up and down several times in a 1-ml canule. The ratio of cells/lysis buffer volume was adjusted to 2 × 10\textsuperscript{6} cells/ml. The crude cell lysate was clarified by ultracentrifugation (100,000 × g, 40 min). After harvesting the cells, all procedures were carried out at 4 °C.

Enzyme Assays—UDP-GlcNAc 2-epimerase and ManNAc kinase activity were measured by a modified method of Zeitzer et al. (19). 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 the addition of 350 μl of ethanol. UDP-GlcNAc 2-epimerase assay: 45 mM Na\textsubscript{2}HPO\textsubscript{4}, pH 7.5, 10 mM MgCl\textsubscript{2}, 1 mM UDP-GlcNAc, 25 nCi of UDP-[\textsuperscript{1-14}C]GlcNAc. ManNAc kinase assay: 60 mM Tris/HCl, pH 8.1, 20 mM MgCl\textsubscript{2}, 5 mM ManNAc, 50 nCi of [\textsuperscript{1-14}C]ManNAc, 20 mM ATP (disodium salt). Radiolabeled compounds were separated by paper chromatography as described earlier (19). 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 (20) using bovine serum albumin as a standard. One unit of enzyme activity was defined as the formation of 1 μmol of product/min at 37 °C. Specific activity was expressed as milliunits/mg of protein.

Construction of Mutants

Mutagenic Oligonucleotides and Site-directed Mutagenesis—The mutagenic oligonucleotides used to generate the mutant constructs are shown in Table I. Site-directed mutagenesis was performed using the QuickChange™ site-directed mutagenesis kit (Stratagene, Heidelberg, Germany). In brief, a nonidentical duplicate of the original template is produced by a polymerase chain reaction-like amplification using Pfu polymerase and primers containing the desired mutation. The parental template is then digested specifically by the restriction enzyme DpnI, which cuts only dam-methylated DNA (target sequence 5'-GGAAGATC-3').

| Name | Sequence |
|------|----------|
| H45A | GGTGCTGGCT TCCgCCGTTC TCGACGACTA CGG |
| H110A | CCGACATACT GAGTGTGgCG CCGGACGCTA TTTGAC |
| H132A | CCGCATCCTT gcATGTTAAG GAGG |
| H155A | CCATAACAAA ACGTGG gcTG TACCATCGT GC |
| D413L | GCTGCTCACT AcGcgTGTTG CTGACC |
| D413N | GCTTGGGCTG TTAACTCCGG GGGGAG |
| D413N | GCTTGGGCTG TTAACTCCGG GGGGAG |
| H110A | CCGACATACT GAGTGTGgCG CCGGACGCTA TTTGAC |
| H132A | CCGCATCCTT gcATGTTAAG GAGG |
| H155A | CCATAACAAA ACGTGG gcTG TACCATCGT GC |

The nicked vector DNA incorporating the desired mutations is transformed into Escherichia coli.

Reaction parameters were chosen according to the manufacturer's recommendations. All mutant constructs were controlled by sequencing with the Sanger dyeoxy chain termination reaction for double-stranded DNA.

Multiple Sequence Alignments—Overall sequence similarities were checked using the PSI-BLAST software and the nr data base. Protein sequences of rat UDP-GlcNAc 2-epimerase/ManNAc kinase and four (putative) prokaryotic UDP-GlcNAc 2-epimerases (ESP_CBURSO, WE_BC_ECOLI, RFBC_SALBO, YVVH_BACSU, Swiss-Prot data base) were aligned using the Multalin software (multiple sequence alignment with hierarchical clustering (21), which is based on the blast algorithm). Protein sequences of rat UDP-GlcNAc 2-epimerase/ManNAc kinase, hexokinase of yeast (HXXK YEAST), glucokinase of rat (HXXH RAT), fucokinase of E. coli (FUCK_ECOLI), glycerokinase of E. coli (Bacillus subtilis) (GLPK_ECOLI (GLPK_BACSU), GLPK_ECOLI (GLPK_BACSU), glucokinase of B. subtilis, xylulokinase of E. coli, GNTK_BACSU, XYLK_ECOLI, ribokinase of Salmonella typhimurium (KIRI_SALTY) (all nr data base) were aligned using the same software.

 Determination of Oligomeric Structure—The oligomeric structures of wild-type and mutated UDP-GlcNAc 2-epimerase/ManNAc kinase were determined with freshly prepared cytosol by gel filtration on a Superdex 200 column (Amersham Pharmacia Biotech). For elution, a buffer containing 100 mM NaCl,10 mM NaH\textsubscript{2}PO\textsubscript{4}, pH 7.5, 1 mM dithiothreitol, and 1 mM EDTA was used. Standard proteins were ferritin (440 kDa), γ-globulin (156 kDa), ovalbumin (44 kDa), and myoglobin (17 kDa). Fractions obtained at a flow rate of 0.2 ml/min were analyzed by SDS-PAGE/Western blot analysis as described earlier (8) and assayed for enzyme activity as described above. Gel filtration was also performed with older cytosol fractions to investigate their rate of decay.

RESULTS

Sequence Alignment-guided Site-directed Mutagenesis—Sequence analysis was performed by comparing the sequence of UDP-GlcNAc 2-epimerase/ManNAc kinase of rat with the non-redundant GenBank® CDS using the PSI-Blat software. As reported earlier (22), we found sequence similarities with kinases and epimerases in different halves of the protein, indicating that different regions are involved in the formation of the active sites for epimerization and phosphorylation (Fig. 3 and Table II).

The Multalin software program for multiple protein alignment of related sequences gave the consensus sequences shown in Fig. 1 and Fig. 2.

The N-terminal half of UDP-GlcNAc 2-epimerase/ManNAc kinase shows significant homologies to prokaryotic UDP-GlcNAc 2-epimerases and to synX, a protein involved in prokaryotic ManNAc biosynthesis. The synX protein of E. coli catalyzes either the interconversion of GlcNAc-6-phosphate to ManNAc 6-phosphate or the dephosphorylation of the latter to produce ManNAc (23). The sequence similarities suggest that synX is an epimerase rather than a kinase. In contrast to the eukaryotic epimerase, the prokaryotic epimerase inverts the stereochemistry at C-2 without release of UDP.
etamidoglucal is an intermediate (24–26). This assumption implies the existence of similar protein structures in both cases.

In the prokaryotic enzyme, deprotonation at C-2 is the rate-limiting step of the process (24). Because histidines are often connected with deprotonation reactions, we mutated four conserved and one semiconserved histidine (Fig. 1, 3) to determine their role in catalysis.

In the C-terminal half of the UDP-GlcNAc 2-epimerase/ManNAc kinase, all of the 5 characteristic motifs described for the ATP binding domain common to functionally divergent proteins (27) were identified. Similarities stretching over amino acids 410–684 are highest for four prokaryotic epimerases (Table II), whereas the several eukaryotic hexokinases match best in-between the phosphate 1 motif of ATP binding domain.

Using site-directed mutagenesis, several amino acids in the conserved motif phosphate 1 of mammalian hexokinase have been shown to be essential for catalysis (28–30). Molecular modeling of ATP in the crystal structure of yeast hexokinase predicted interactions of these residues with ATP. The conserved aspartate is predicted to interact with ATP-complexed Mg$^{2+}$; the conserved arginine is predicted to interact with α- and β-phosphate oxygens (30). The analogous positions in UDP-GlcNAc 2-epimerase/ManNAc kinase were mutated to investigate their involvement in the catalytic process (Figs. 2 and 3).

Expression of Wild-type and Mutated UDP-GlcNAc 2-Epimerase/ManNAc Kinase in Sf9 Cells and Characterization of the Expression System—For functional characterization, high level overexpression of UDP-GlcNAc 2-epimerase/ManNAc kinase was established using the baculovirus expression system. Insect cells were infected with a recombinant baculovirus containing the cDNA of the enzyme or its respective mutants.

### TABLE II

| Amino acid identities and similarities of rat UDP-GlcNAc 2-epimerase/ManNAc kinase NH$_2$ and COOH regions with prokaryotic UDP-GlcNAc 2-epimerases and members of the sugar kinase superfamily |
|---|
| **GLK_STRCO**, gluokinase Streptomyces coelicolor; **GLK_RENSAL**, gluokinase Remuntellera salmonea; **GLK_BACME**, glucokinase Bacillus megaterium; **GLK_STAXY**, gluokinase Staphylococcus xylosus; **FRCK_BACSU**, fructokinase B. subtilis; **HXXA_YEAST**, hexokinase a; **HXXA_KLULA**, hexokinase Kluyveromyces lactis. |

| Identities | Positives | Gaps |
|---|---|---|
| **N-terminal region**<sup>a</sup> | | |
| synX_MENSU | 32 | 55 | 4 |
| RFCE_ECOLI | 22 | 39 | 14 |
| YVVH_BACSU | 21 | 39 | 9 |
| EPSC_BURSO | 23 | 46 | 8 |
| RFBC_SALBO | 21 | 39 | 15 |
| **C-terminal region**<sup>b</sup> | | |
| GLK_STRCO | 31 | 45 | 5 |
| GLK_RENSAL | 28 | 45 | 6 |
| GLK_BACME | 26 | 46 | 3 |
| GLK_STAXY | 24 | 43 | 2 |
| FRCK_BACSU | 23 | 40 | 7 |
| HXXA_YEAST | 37 | 52 | |
| HXXA_KLULA | 50 | 65 | |
| HXXA_KLULA | 62 | 74 | |

<sup>a</sup> Aligned amino acid residues 1–376.

<sup>b</sup> Aligned amino acid residues 378–436.

<sup>c</sup> Aligned amino acid residues 405–436.

<sup>d</sup> Aligned amino acid residues 405–428.

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**FIG. 1.** Alignment of UDP-GlcNAc 2-epimerase/ManNAc kinase with 5 (putative) prokaryotic UDP-GlcNAc 2-epimerases, ESPC_BURSO, WEBC_ECOLI, RFCE_ECOLI, RFBC_SALBO, YVVH_BACSU (SWISS-PROT protein sequence data base), and synX (PIR data base), and synX (PIR data base). Sequences were compared using the MultAlign software (21); upper-case letters indicate high consensus levels (>90%), and lowercase letters indicate low consensus levels (50%–90%). In UDP-GlcNAc 2-epimerase/ManNAc kinase, histidines labeled with a star were transformed to alanine by site-directed mutagenesis.

**FIG. 2.** Alignment of UDP-GlcNAc 2-epimerase/ManNAc kinase in the phosphate 1 motif of sugar kinases (27). HXXA_RAT, hexokinase of yeast; HXXA_RAT, glucokinase of rat; FUCK_ECOLI, fucokinase of *E. coli*; GLPK_ECOLI (GLPK_BACSU), glucokinase of *E. coli* (B. subtilis)*; GNTK_BACSU, glucokinase of *B. subtilis*; XYLK_ECOLI, xylulokinase of *E. coli*; KIRI_SALTY, ribulokinase of *S. typhimurium*. Uppercase letters indicate a high consensus level (>90%), and lowercase letters indicate a low consensus level (50%–90%).
recombinant expressed proteins migrated at the same position (75 kDa) as the rat liver enzyme in SDS-PAGE (Fig. 4). As estimated by comparing the signal intensities after staining with Coomassie Brilliant Blue, 20–30 percent of the total cytosolic protein fraction consists of recombinant enzyme. Furthermore, cytosolic extracts of all mutants and the wild-type enzyme are immunoreactive, with a polyclonal antibody specific against UDP-GlcNAc 2-epimerase/ManNAc kinase (8) (data not shown). The native molecular mass of recombinant UDP-GlcNAc 2-epimerase/ManNAc kinase was estimated to be 450 kDa by size exclusion column chromatography, indicating that the expressed protein forms a hexamer. The recombinant enzyme dissociated partly into dimers, which retained only the kinase activity. This phenomenon was reported for the rat liver enzyme as well (Fig. 5) (11).

To quantify the cytosolic background activities, the UDP-GlcNAc 2-epimerase and ManNAc kinase activities of uninfected insect cells were investigated. We found that cytosolic fractions of uninfected Sf9 cells show an epimerase activity of 0.06 milliunits/mg. The fact that this activity can be inhibited by 0.1 mM CMP-Neu5Ac, the feedback inhibitor of UDP-GlcNAc 2-epimerase activity in rat liver, suggests the presence of this enzyme in insect cells (Fig. 6, panel B). The specific activity is about 30 times less than that in rat liver cytosol.

In contrast, the apparent averaged ManNAc kinase activity of insect cell cytosol is 5 milliunits/mg, about 50 times higher than the UDP-GlcNAc 2-epimerase activity. A possible explanation is that other cytosolic kinases are able to phosphorylate ManNAc, as demonstrated recently for the rat liver N-acetylglucosamine kinase (31).

Catalytic Activities of Wild-type and Mutated UDP-GlcNAc 2-Epimerase/ManNAc Kinase—Infection of Sf9 cells with the wild-type enzyme virus resulted in an average specific cytosolic epimerase activity of 890 ± 310 milliunits/mg and in an average specific cytosolic kinase activity of 840 ± 276 milliunits/mg (Fig. 7, panels A and B). Compared with cytosolic extracts of rat liver, the specific activities in insect cell cytosols after infection were increased about 400-fold for both activities. Thus, the specific epimerase activity in insect cytosol after overexpression corresponds to that of the homogeneous enzyme from rat liver cytosol (8). All histidine mutants displayed kinase activities in the same order of magnitude as the wild-type enzyme. On the other hand they all lost their epimerase activity almost completely (Fig. 7, panel A). Differences in the specific kinase activities should correlate with the differences in the expression level of the enzyme. The residual epimerase activities were not significantly higher than those assayed with uninfected cells.

The mutants in the putative kinase active site showed the inverse behavior; they displayed epimerase activities comparable to the wild-type-enzyme, whereas the kinase activities were reduced drastically. Compared with the mutation to lysine, the mutation of aspartate 413 to asparagine seems to result in a slightly higher epimerase and residual kinase activities. The residual kinase activities of all mutants in the ATP binding motif are significantly higher than the background activities measured in the two negative controls (Fig. 7, panel B).
bition of the recombinant enzyme by the native feed-back inhibitor CMP-Neu5Ac was investigated. We could show that 0.1 mM CMP-Neu5Ac inhibits the wild type epimerase activity completely, whereas 0.1 mM \textit{N}-acetylneuraminic acid does not influence the activity at all (Fig. 6, panel A).

To determine whether the allosteric binding site is functionally intact in the epimerase-active mutants D413N, D413K, and R420M, these were assayed in the presence of CMP-Neu5Ac. The epimerase activity of all three mutants was inhibited almost completely by 0.1 mM CMP-Neu5Ac (Fig. 6, panel B).

Size Exclusion Chromatography of Wild-type and Mutated UDP-GlcNAc 2-Epimerase/ManNAc Kinase—To determine whether the loss of activity due to site-directed mutagenesis can be attributed to a disturbed oligomerization process, we performed size exclusion chromatography with all mutant proteins. The obtained fractions were analyzed by Western blotting for the protein and were assayed for epimerase and kinase activity.
Kinase Mutants D413N, D413K, and R420M—Western blot analysis of the fractions obtained after size exclusion chromatography revealed that all three mutants are able to build a hexamer (Fig. 8A); partial dissociation of the hexamer results in a dimer as observed for the wild-type enzyme. Only the hexamer shows epimerase activity. The distribution of residual kinase activity over the molecular weight spectrum shows a major maximum at the molecular mass of the hexamer and a minor peak at the molecular mass of the dimer, which is evidence for specific residual activity (Fig. 8B).

Both mutation of aspartate to lysine (i.e. an inversion of polarity) or the neutralization of the charge of aspartate 413 by mutation to asparagine had no observable effect on oligomerization. In contrast, although the epimerase activities remain equal, the residual kinase activities after gel filtration of D413N is slightly increased over that of D413K. Taken together these results show that site-directed mutagenesis of the positions Asp-413 and Arg-420 does not interfere with enzyme oligomerization, indicating that there is no influence on protein folding.

Histidine Mutants H45A, H110A, H132A, H155A, and H157A—These mutants can be grouped according to three types of oligomerization behavior (Fig. 9). The first one, H45A, forms a kinase-active hexamer (Fig. 9, Type I). In this case, site-directed mutagenesis does not influence the oligomerization process. In cross-linking experiments both the hexameric enzyme and the dimeric enzyme were detected (data not shown).

The neighboring mutants, H155A and H157A, constitute the third group (Fig. 9, Type III); they form only small amounts of hexamer, whereas the maximum protein content and maximum kinase activity are found at molecular weights corresponding to a trimeric state. Because no dissociation to dimers was observed, these mutations seem to inhibit the dimerization process, and consequently, the formation of larger stable amounts of hexameric enzyme.

As shown by Western blot analysis after size exclusion chromatography, the molecular weight distribution for mutant enzymes His-110 and His-132 is wider (Fig. 9, Type II). The Western blot signals are in good agreement with overlapping trimeric and hexameric states of the enzyme. In this case, the second group would be a hybrid of group one and three, where the dimerization process is partially inhibited.

**DISCUSSION**

In this study we have identified amino acids needed for the catalytic activity of the key enzyme of Neu5Ac biosynthesis, UDP-GlcNAc 2-epimerase/ManNAc kinase. The catalytically essential residues were shown to be in different regions of the enzyme for epimerase activity and kinase activity. The data were obtained by overexpression of the enzyme in insect cells, sequence alignment-guided site-directed mutagenesis, and catalytic and structural characterization of the different mutants.

Sequence analysis revealed homologies between prokaryotic epimerases and the N-terminal region of UDP-GlcNAc 2-epimerase/ManNAc kinase, whereas various kinases showed homologies to the C-terminal region of this eukaryotic bifunctional enzyme.

Two strategies of enzyme evolution have been described (32). First, there are structurally related enzymes that catalyze identical reactions with possible differences in substrate specificity. For example, all members of the serine protease superfamily are known to catalyze the same chemistry, although their substrate specificity varied. Also, the sugar kinase superfamly seems to fit with this evolution type. In contrast, there are enzyme superfamilies whose members share a common structural scaffold but catalyze different overall reactions. These enzyme superfamilies probably evolved by incorporation of new catalytic groups within an active site, whereas groups necessary to catalyze the partial reactions common to all of them evolved independently.
them were retained. One might speculate that the prokaryotic and the eukaryotic UDP-GlcNAc 2-epimerases represent an example of the second evolution type. Prokaryotic and eukaryotic UDP-GlcNAc 2-epimerases do not catalyze identical reactions, but they show the same substrate specificity and share a common intermediate, so they probably employ similar mechanistic strategies.

Based on the above-mentioned sequence similarities, site-directed mutagenesis was performed on five conserved histidines in the N-terminal half of the enzyme. Surprisingly, all five mutants lost their epimerase activity. By contrast, the kinase activity was retained, giving a first hint for the existence of two active sites for each reaction working quite independently. To distinguish between general influences on the structural scaffold and specific involvement in the catalytic reaction we investigated whether the histidine mutants were still able to associate as a hexamer, as observed for the wild-type enzyme. The data obtained are consistent with the model shown in Fig. 10.

The histidines His-155 and His-157 might be localized within the enzyme dimerization recognition site (33). Mutation of histidine to alanine leads to a strongly reduced dimer association constant, which is why mainly trimeric protein is found in place of hexameric protein (Fig. 9, Type III). This result is in contrast to the former view, that the kinase active site assembles as a dimer, based on the detection of active kinase dimer, trimer, and hexamer. Therefore, dimerization is not necessary for the formation of the active site, but oligomerization in general may have a positive cooperativity effect. The previously determined Hill coefficients for ManNAc and ATP are consistent with this proposal, as they are greater for the hexameric enzyme than for the dimeric one (8). Furthermore, not only trimerization of the dimer seems to be essential for epimerase activity, but dimerization itself is also essential, because the trimers show no epimerase activity, although even the small amount of detected hexamer showed no epimerase activity. Thus these mutations seem to influence both enzyme oligomerization and the epimerase activity of the hexameric protein.

The mutation of His-110 and His-132 leads qualitatively to the same effect on enzyme oligomerization (Fig. 9, Type II).

**Fig. 9.** Determination of the oligomeric structure of overexpressed mutants H45A, H110A, H132A, H155A, and H157A; fractions were analyzed for UDP-GlcNAc 2-epimerase and ManNAc kinase activity and were also subjected to SDS-PAGE and Western blot analysis. Determination of theoretical elution fractions retention times of hexameric and dimeric enzyme was performed as described under “Experimental Procedures.” Three types of oligomerization are shown depending on the position of the mutated amino acid. No residual epimerase activity was detected. mU, milliunits.

**Fig. 10.** Different pattern of oligomerization of wild-type UDP-GlcNAc-2 epimerase/ManNAc kinase and different mutants. **A**, the wild-type enzyme and the mutants D413K, D413N, R420M, and H45A associate as trimer of dimers. No dissociation resulting in trimers could be observed. Under denaturing conditions, monomer is detected. **B**, association of trimers because of structural disturbance within the dimerization recognition site. This pattern is observed with the mutants H155A and H157A. Under denaturing conditions, monomer is detected.
the effect is clearly less drastic, one can conclude that these residues are at a position less sensitive for structural transformation, for example at the edge of the dimerization recognition site.

Determination of the oligomeric state of the mutants H155A, H157A, H110A, and H132A revealed that they are significantly structurally disturbed. Therefore the loss of epimerase activity can be attributed to this structural perturbation, whereas there is no evidence for a direct involvement in catalysis. On the other hand, the mutant H45A assembles as a hexamer with apparent structural integrity. Since the fully hexameric mutant H45A has no observable epimerase activity, one might speculate that this residue is located in the active site and might be involved in the chemical reaction.

In the C-terminal half of the enzyme two amino acid residues within the phosphate 1 motif common to different families of ATP-binding proteins were transformed. The mutated residues Asp-413 and Arg-420 of UDP-GlcNAc 2-epimerase/ManNAc kinase are well conserved within sugar kinases and phosphotransferases (27). Results of molecular modeling and site-directed mutagenesis suggest that the bifunctional enzyme UDP-GlcNAc 2-epimerase/ManNAc kinase provides a structural scaffold for the transition state. We could show that site-directed mutagenesis of these conserved residues in the UDP-GlcNAc 2-epimerase/ManNAc kinase results in a drastic loss of kinase activity. Furthermore it is quite probable that UDP-GlcNAc 2-epimerase/ManNAc kinase these conserved residues are at a position less sensitive for structural transformation, as all mutants form hexamers. In brief, all parameters investigated correspond to those found for the recombinant wild-type enzyme, apart from the loss of phosphorylation capacity. We conclude that also in the bifunctional enzyme UDP-GlcNAc 2-epimerase/ManNAc kinase these conserved residues play a crucial role in the phosphorylation process. Furthermore it is quite probable that UDP-GlcNAc 2-epimerase/ManNAc kinase provides a structural scaffold for phosphorylation similar to that of the related sugar kinases.

Taken together the results of sequence analysis as well as those of site-directed mutagenesis suggest that the bifunctional enzyme is composed of two catalytic domains. The epimerase active site seems to be localized in the N terminus, whereas the kinase active site seems to be in the C terminus of the protein. Construction of deletion mutants would make it possible to determine whether these postulated domains can be expressed separately while retaining their respective activity.

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Functional Domains of UDP-GlcNAc 2-Epimerase/ManNAc Kinase