2′,3′-Dialdehydo-UDP-*N*-acetylglucosamine inhibits UDP-*N*-acetylglucosamine 2-epimerase, the key enzyme of sialic acid biosynthesis

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Abstract Sialic acids comprise a family of terminal sugars essential for a variety of biological recognition systems. UDP-*N*-acetylglucosamine 2-epimerase catalyzes the first step of their biosynthesis. Periodate-oxidized UDP-*N*-acetylglucosamine, namely 2′,3′-dialdehydo-UDP-α-D-*N*-acetylglucosamine, was found to be an effective inhibitor of this enzyme, compared with the periodate oxidation products of compounds such as UDP, uridine or methyl riboside. It bound covalently to amino acids in the active site causing an irreversible inhibition. This compound may therefore represent a basis for the synthesis of potent inhibitors of UDP-*N*-acetylglucosamine 2-epimerase and, as a consequence, of the biosynthesis of sialic acids. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: 2′3′-Dialdehydo-UDP-α-D-*N*-acetylglucosamine; Inhibition; Sialic acid; UDP-*N*-acetylglucosamine 2-epimerase

1. Introduction

Sialic acids are terminal sugars of the oligosaccharide chains in glycoconjugates. They are well suited to their role as molecular determinants of specific biological processes such as cellular adhesion [1], formation or masking of recognition determinants [2,3], and stabilization of the structure of glycoproteins [4]. Also, cell surface sialylation has been implicated in the tumorigenicity and metastatic behavior of malignant cells [5,6].

N-Acetylneuraminic acid (Neu5Ac), the most common naturally occurring sialic acid, is the biosynthetic precursor of this group of compounds. In mammals biosynthesis of Neu5Ac is initiated by UDP-*N*-acetylglucosamine 2-epimerase (UDP-GlcNAc 2-epimerase), followed by phosphorylation of the released *N*-acetylmannosamine by *N*-acetylmannosamine kinase (ManNAc kinase). In mammalian cells both enzymes are combined in a single bifunctional enzyme, UDP-GlcNAc 2-epimerase/ManNAc kinase [7,8]. Neu5Ac is then formed by Neu5Ac 9-phosphate synthase [9,10] and consecutive release of the phosphate moiety. CMP-Neu5Ac synthase catalyzes the final step of the pathway, the activation of Neu5Ac in the nucleus [11,12]. CMP-Neu5Ac then serves as substrate of the sialyltransferases in glycoconjugate biosynthesis [13].

Neu5Ac biosynthesis is regulated by feedback inhibition of UDP-GlcNAc 2-epimerase by CMP-Neu5Ac [14]. Strongly positive cooperative binding of the inhibitor connects Neu5Ac biosynthesis effectively to the consumption of sialic acids in glycoconjugate synthesis [7]. Loss of UDP-GlcNAc 2-epimerase leads to a drastic decrease of cellular sialylation, influencing several sialic acid-dependent functions [15]. Therefore, inhibition of UDP-GlcNAc 2-epimerase activity is an appropriate means of regulating cell surface sialylation. Zeitler et al. [16] showed that UDP-GlcNAc 2-epimerase activity was sensitive to metal ions, but this inhibitory effect was mainly attributed to an oxidation of cysteine residues in the active site. Here we present the first potent in vitro inhibitor of the enzyme, 2′,3′-dialdehydo-UDP-α-D-*N*-acetylglucosamine (oxidized UDP-GlcNAc, o-UDP-GlcNAc), which may serve as a basis for the chemical synthesis of further inhibitors.

2. Materials and methods

2.1. Materials

All chemicals and enzymes, if not otherwise indicated, were from Sigma (Deisenhofen, Germany).

2.2. Chemical synthesis of o-UDP-GlcNAc, o-uridine, methyl o-riboside and r-UDP-GlcNAc

o-UDP-GlcNAc was synthesized following the procedure of Prehm [17]. 100 mg (154 μmol) UDP-GlcNAc was dissolved in 1 ml 40 mM Na_2HPO_4, pH 6.8. One equivalent of $NaIO_4$ was added and allowed to react for 1 h at 0°C with continuous stirring. The reaction was stopped by addition of 10 μl glycerol. The products were desalted on a Bio-Gel P2 column (Pharmacia, Freiburg, Germany) using water as eluent. The eluate was lyophilized, dissolved in ethyl acetate/methanol/water (3:2:1) and applied to a silica gel column. The compounds were eluted with ethyl acetate/methanol/water (3:2:1) and fractions were collected and checked by thin-layer chromatography. Fractions containing o-UDP-GlcNAc were pooled, the solvent was evaporated and 85.7 mg (132 μmol, 86%) of the white foam was analyzed by nuclear magnetic resonance (NMR; Bruker DRX 600; Bruker, Rheinstetten, Germany), mass spectrometry (Kompact MALDI 2-Spectrometer; Kratos Analytical, Hofheim, Germany) and elemental analysis (CHN-analysator; Heraeus, Hofheim, Germany).

o-Uridine and methyl o-riboside were synthesized in a similar man-

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Abbreviations: ManNAc kinase, *N*-acetylmannosamine kinase; Neu5Ac, *N*-acetylneuraminic acid; o-UDP-GlcNAc, 2′,3′-dialdehydo-UDP-α-D-*N*-acetylglucosamine; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; UDP-Gal 4-epimerase, UDP-galactose 4-epimerase; UDP-GlcNAc 2-epimerase, UDP-*N*-acetylglucosamine 2-epimerase

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ner with the exception that the periodate oxidation was performed at room temperature for 2 h, o-uridine was eluted with ethyl acetate/methanol (10:1), and methyl o-riboside was eluted with ethyl acetate from the silica gel column. 56 mg white foam (231 μmol, 56%) o-uridine and 24 mg (148 μmol, 49%) methyl o-riboside were analyzed by ^{1}H NMR and mass spectrometry. o-UDP, o-ADP and o-GDP were obtained from Sigma.

Reduced UDP-GlcNAc (r-UDP-GlcNAc) was synthesized by reduction of o-UDP-GlcNAc with sodium borohydride. 40 μmol o-UDP-GlcNAc was dissolved in 1.5 ml water. 400 mg sodium borohydride was added and the reaction was performed overnight at room temperature. The progress of the reaction was followed by TLC. The products were desalted on a Bio-Gel P2 column and purified by silica gel chromatography as described above for o-UDP-GlcNAc. 8.5 mg (13 μmol, 33%) of the white foam was analyzed by NMR.

2.2.1. Chemical analysis.

o-UDP-GlcNAc

TLC (Ethyl acetate/methanol/water 3:2:1): $R_f = 0.32$

^{1}H NMR (400 MHz, D_2O): δ (ppm) = 2.09 (s, 3H, Ac), 3.50–4.02 (m, 9H, H-4′, H-5′a, H-5′b, 7 H Glc), 5.18–5.68 (m, 2H, H-2′, H-3′), 5.88–6.01 (m, 2H, H-1′, H-5), 7.83–7.95 (m, 1H, H-6)

^{31}P NMR (162 MHz, D_2O): δ (ppm) = −10.6 (mc, 1P, αP), −12.4 (d, 1P, $J = 17$ Hz, βP)

MALDI-MS: $[M+H^+] = 650.7$

Elemental analysis calculated for $C_{17}H_{23}N_3O_{17}P_2Na_2 \times 4\ H_2O$ (721,31): N, 5.82; C, 28.31; H, 4.33. Found: N, 5.69; C, 28.62; H, 4.64.

o-Uridine

TLC (Ethyl acetate/methanol 10:1): $R_f = 0.27$

^{1}H NMR (250 MHz, D_2O): δ (ppm) = 3.40–4.07 (m, 3H, H-4′, H-5′a, H-5′b), 4.90–5.80 (m, 4H, H-5, H-1′, H-2′, H-3′), 7.58–7.82 (m, 1H, H-6)

MALDI-MS: $[M+H^+] = 243.8$

Methyl o-riboside

TLC (Ethyl acetate): $R_f = 0.25$

^{1}H NMR (250 MHz, D_2O): δ (ppm) = 3.32 (br.s, 3H, Me), 3.38–4.01 (m, 3H, H-4′, H-5a, H-5′b), 4.27–5.05 (m, 3H, H-1′, H-2′, H-3′)

MALDI-MS: $[M+Na^+] = 184.7$

r-UDP-GlcNAc

TLC (Ethyl acetate/methanol/water 3:2:1): $R_f = 0.20$

^{1}H NMR (250 MHz, D_2O): δ (ppm) = 1.9 (s, 3H, Ac), 3.32–3.88 (m, 13H), 5,34 (dd, 1H, $J_{1'',2''} = 3$ Hz, $J_{1'',P} = 7$ Hz, H-1″), 5.76–5.81 (m, 2H, H-1′, H-5), 7,71 (d, 1H, $J_{5,6} = 8$ Hz, H-6)

^{31}P NMR (243 MHz, D_2O): δ (ppm) = −10.2 (d, 1P, $J = 21$ Hz, αP), −12.0 (d, 1P, $J = 17$ Hz, βP)

2.3. Purification of UDP-GlcNAc 2-epimerase/ManNAc kinase from Sf9 insect cells

UDP-GlcNAc 2-epimerase/ManNAc kinase from rat was recombinantly expressed in Sf9 insect cells as described [18]. Cells (2×10^6) were harvested and resuspended in 10 mM Na_2HPO_4, pH 7.5, 1 mM ethylenediamine tetraacetic acid, 1 mM dithiothreitol (buffer A), 1 mM phenylmethylsulfonyl fluoride. Cells were lysed by 20 strokes of a syringe with a narrow needle, and the lysate was centrifuged at $100\,000 \times g$ for 30 min.

After centrifugation the supernatant was applied to a MonoQ HR 5/5 column (Pharmacia). The column was washed with 5 ml of buffer A, and proteins were eluted by a linear gradient from 0 to 600 mM NaCl in buffer A. Fractions of 1 ml were collected and checked by UDP-GlcNAc 2-epimerase assay 1 as described below. The enzyme eluted by about 300 mM NaCl. Fractions containing UDP-GlcNAc 2-epimerase/ManNAc kinase were applied to a Superdex 200 HR 10/30 gel filtration column (Pharmacia). Proteins were eluted with buffer A containing 100 mM NaCl. Fractions of 0.5 ml were collected, monitored for activity by UDP-GlcNAc 2-epimerase assay 1, and for purity by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using a MiniProteanII system (Bio-Rad, Munich, Germany). UDP-GlcNAc 2-epimerase/ManNAc kinase eluted in fractions corresponding to the expected size of 450 kDa [7] and showed a purity of more than 95%.

2.4. Enzyme assays

UDP-GlcNAc 2-epimerase assay 1 contained 45 mM Na_2HPO_4, pH 7.5, 10 mM $MgCl_2$, 1 mM UDP-GlcNAc and variable amounts of protein in a final volume of 200 μl. The reaction was performed at 37°C for 30 min and stopped by boiling for 1 min. The released ManNAc was detected by the Morgan–Elson method [19]. In brief, 150 μl of sample was mixed with 30 μl of 0.8 M H_2BO_3, pH 9.1, and boiled for 3 min. Then 800 μl of DMAB solution (1% (w/v) 4-dimethylamino benzaldehyde in acetic acid/1.25% 10 N HCl) was added and incubated at 37°C for 30 min. The absorbance was read at 578 nm.

UDP-GlcNAc 2-epimerase assay 2 was performed as described earlier [7]. It contained 45 mM Na_2HPO_4, pH 7.5, 10 mM $MgCl_2$, 1 mM UDP-GlcNAc, 0.2 mM NADH, 2 mM phospho*enol*pyruvate, 4 U (40 μg) pyruvate kinase, 4 U (40 μg) lactate dehydrogenase and variable amounts of protein in a final volume of 800 μl. The reaction was performed at 37°C and the decrease of NADH was continuously monitored at 340 nm. Initial rates of enzyme activities were determined by applying tangents to the extinction curves.

The ManNAc kinase assay was performed as described earlier [7]. It contained 60 mM Tris–HCl, pH 8.1, 10 mM $MgCl_2$, 5 mM ManNAc, 10 mM ATP, 0.2 mM NADH, 2 mM phospho*enol*pyruvate, 4 U (40 μg) pyruvate kinase, 4 U (40 μg) lactate dehydrogenase and variable amounts of protein in a final volume of 800 μl. The reaction was performed at 37°C and the decrease of NADH was continuously monitored at 340 nm. Initial rates of enzyme activities were determined by applying tangents to the extinction curves.

The UDP-galactose 4-epimerase (UDP-Gal 4-epimerase) assay was performed as described earlier [20]. It contained 100 mM glycine, pH 8.7, 5 mU UDP-glucose dehydrogenase, 0.125 mM UDP-Gal, 0.125 mM NAD^+ and variable amounts of yeast UDP-Gal 4-epimerase in 800 μl. The reaction was performed at 37°C for 20 min. The reaction was stopped by boiling for 1 min and the resulting NADH was detected at 340 nm.

2.5. Inhibition reactions

Stock solutions of inhibitors were prepared with a concentration of 100 mM in water. Purified UDP-GlcNAc 2-epimerase/ManNAc kinase (2 μg) was incubated with various concentrations of inhibitors for the indicated periods. Enzyme activities were determined as described above. Subunit crosslinking by o-UDP-GlcNAc was monitored by applying the samples to reducing SDS-PAGE and staining the gels with silver.

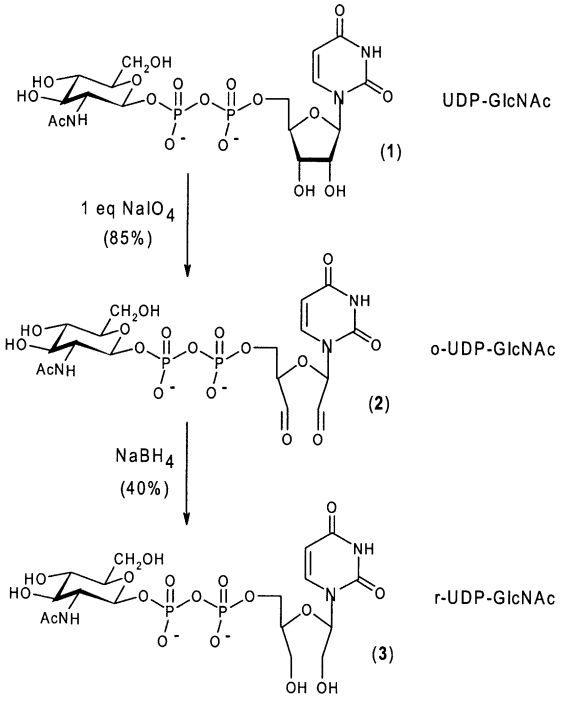

Fig. 1. Structure and synthesis of o-UDP-GlcNAc (2) and r-UDP-GlcNAc (3). The compounds were synthesized from UDP-GlcNAc (1). First, a periodate-oxidation resulted in formation of (2), then borohydride reduction of (2) formed (3). Synthesis was performed as described in detail in Section 2.

UDP-Gal 4-epimerase stock solution (5 μg/ml) was diluted 1:10 in phosphate-buffered saline; 0.05 μg was incubated with the indicated amounts of inhibitors, and enzyme activity was determined as described above.

3. Results and discussion

3.1. Inhibition of UDP-GlcNAc 2-epimerase activity by o-UDP-GlcNAc and related compounds

Prehm [17] reported the inhibition of human hyaluronate synthase by o-UDP-GlcNAc. An inhibitory effect of o-UDP-GlcNAc on the activity of methylcoenzyme M methylreductase from *Methanobacterium thermoautotrophicum*, catalyzing the reduction of methylcoenzyme M to methane and coenzyme M (2-mercaptoethanesulfonic acid) with UDP-GlcNAc as part of an essential cofactor, was also found [21]. It was concluded that the inhibitor bound to UDP-GlcNAc binding sites within both enzymes. Periodate oxidation of UDP-GlcNAc led to a cleavage of the ribose ring between C2 and C3 and the formation of two adjacent aldehyde groups (Fig. 1). We therefore investigated whether o-UDP-GlcNAc was also able to inhibit the activity of UDP-GlcNAc 2-epimerase. o-UDP-GlcNAc was first tested over a wide range of concentrations and incubation times. Inhibitor concentrations in the 100 μM range and incubation times up to 1 h resulted in efficient inhibition of UDP-GlcNAc 2-epimerase (data not shown). The inhibition efficiency was therefore in the same range as that for hyaluronate synthase and methylcoenzyme M methylreductase [17,21].

For more detailed information on the specificity and efficiency of the inhibition of UDP-GlcNAc 2-epimerase activity, we compared o-UDP-GlcNAc with several periodate-oxidized diphosphonucleotides, uridine and methyl riboside (Fig. 2A). o-UDP-GlcNAc appeared to be the most effective inhibitor of the tested compounds. After 30 min incubation, 50 μM o-UDP-GlcNAc inhibited by about 70%, while at 250 μM the inhibitor decreased enzyme activity to less than 10%. An effective enzyme inhibition was also found using o-UDP and o-ADP. A more significant result showing the specificity of o-UDP-GlcNAc was obtained at an inhibitor concentration of 300 μM when the time course of inhibition was monitored continuously by assay 2 (Fig. 3A). o-UDP-GlcNAc was the most effective inhibitor under these conditions. o-UDP and o-ADP again showed a similar effect on UDP-GlcNAc 2-epimerase activity. o-GDP, o-uridine and methyl o-riboside had almost no effect on the enzyme activity.

The overall inhibition efficiency during the continuous assay 2 was lower compared to that observed when using the non-continuous assay 1. The difference between the two assays was the addition of a larger quantity of protein (about 80 μg of pyruvate kinase and lactate dehydrogenase) in the continuous assay, which was 40 times greater than the quantity of UDP-GlcNAc 2-epimerase. Under these conditions most of the inhibitors were probably inactivated by an unspecific reaction of the aldehyde groups of the inhibitors with free amino groups of the proteins (for details see Section 3.3). On the other hand, inhibition of UDP-GlcNAc 2-epimerase activity by o-UDP-GlcNAc, o-UDP and o-ADP showed the high specificity of these inhibitors for the enzyme. o-UDP-GlcNAc seemed to be bound rapidly and tightly by the active site of UDP-GlcNAc 2-epimerase and was therefore able to act as an inhibitor. It is known that UDP can also bind to the active site of the enzyme, because it stabilizes the hexameric state [7], and a reaction mechanism involving an exchange of UDP and UDP-GlcNAc has been proposed. It was therefore also suggested that o-UDP binds to the active site of UDP-GlcNAc 2-epimerase.

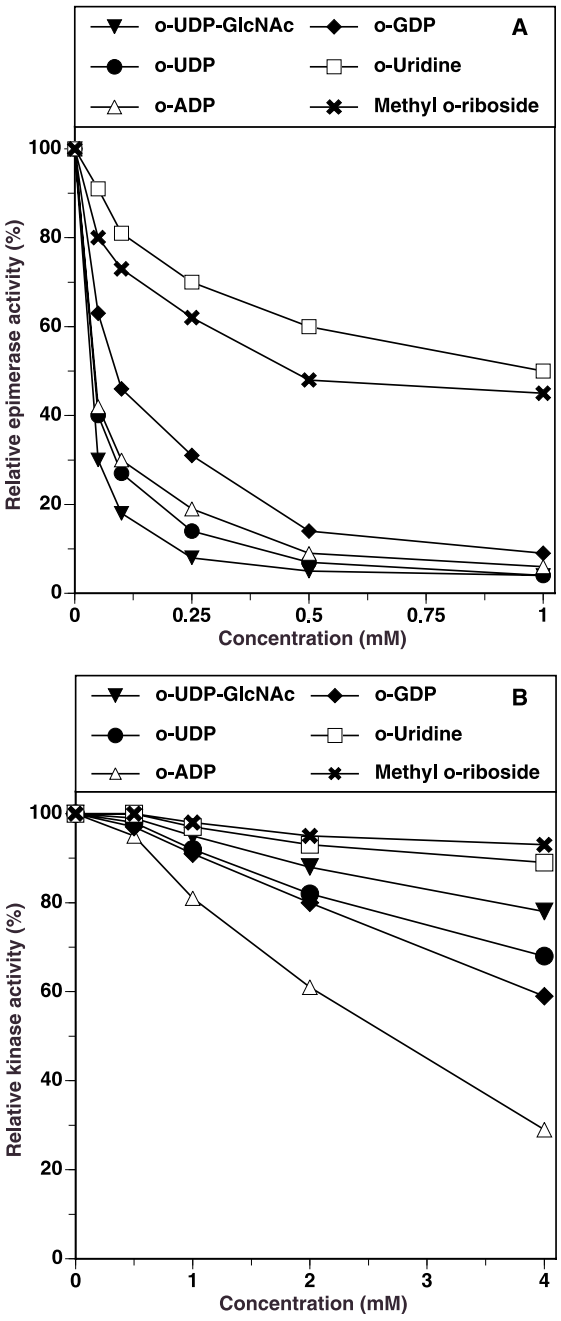

Fig. 2. Inhibition of UDP-GlcNAc 2-epimerase activity (A) and ManNAc kinase activity (B) by different concentrations of o-UDP-GlcNAc and derivatives. Purified UDP-GlcNAc 2-epimerase/ManNAc kinase was incubated with the indicated concentrations of inhibitors for 30 min. Enzyme activities were determined with UDP-GlcNAc 2-epimerase assay 1 and ManNAc kinase assay as described in Section 2. Values are means of two independent experiments.

It should be noted that o-ADP also inhibited UDP-GlcNAc 2-epimerase. o-ADP, like o-UDP, may bind to the active site of UDP-GlcNAc 2-epimerase. Alternatively, it may act on the ATP binding site of ManNAc kinase, which is fused to UDP-

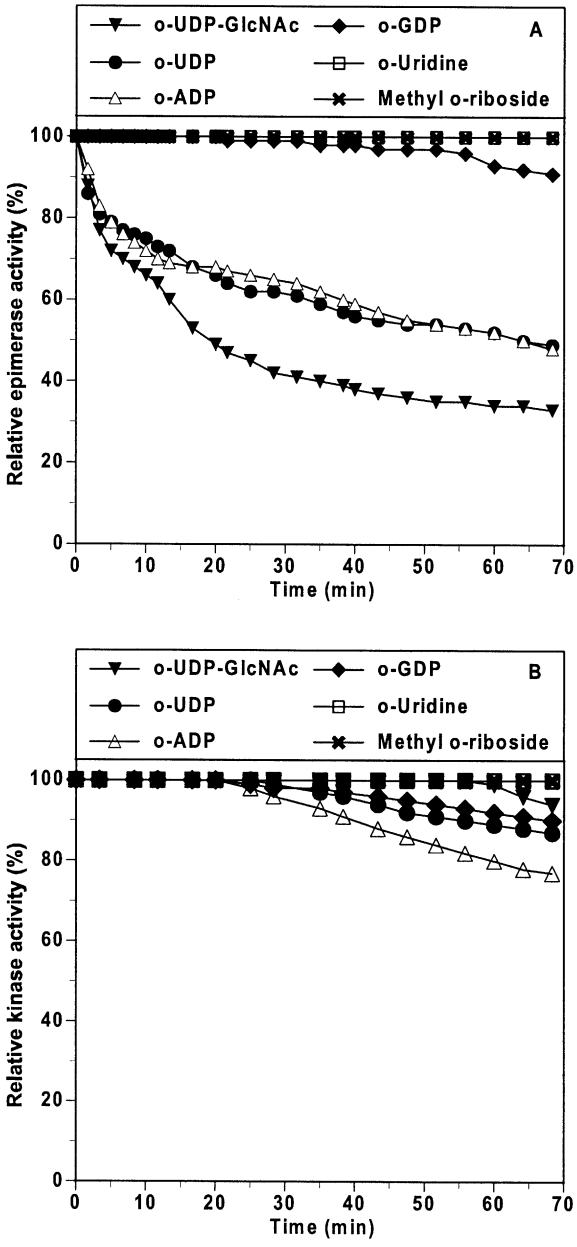

Fig. 3. Time dependence of the inhibition of UDP-GlcNAc 2-epimerase activity (A) and ManNAc kinase activity (B) by o-UDP-GlcNAc and derivatives. Purified UDP-GlcNAc 2-epimerase/ManNAc kinase was incubated with 300 μM inhibitor for the indicated periods of time. Enzyme activities were determined with UDP-GlcNAc 2-epimerase assay 2 and the ManNAc kinase assay as described in Section 2. Values are means of two independent experiments.

GlcNAc 2-epimerase in vivo, but which has been shown to work independently [18]. Nevertheless, an allosteric effect on UDP-GlcNAc 2-epimerase activity by binding of o-ADP to the ManNAc kinase domain remained a possibility. Therefore the panel of inhibitors was also tested for inhibition of ManNAc kinase activity. For an equivalent inhibition of the two enzyme activities, inhibitor concentrations were 40-fold higher for ManNAc kinase than for UDP-GlcNAc 2-epimerase (Fig. 2B). Under conditions used for an efficient inhibition of UDP-GlcNAc 2-epimerase, almost no inhibition of ManNAc kinase was observed (Fig. 3B). o-ADP was still the most effective inhibitor of ManNAc kinase, suggesting a binding to the ATP binding site of the enzyme. This was confirmed by the inhibition efficiency of the other o-modified compounds used, which corresponded to their structural similarity to o-ADP. From these results it is concluded that the inhibition of UDP-GlcNAc 2-epimerase and ManNAc kinase occurred by independent binding of the respective inhibitor to the two active sites of the bifunctional enzyme. Therefore the efficient inhibition of UDP-GlcNAc 2-epimerase by o-ADP was most likely due to the structural similarity to o-UDP and not to an allosteric effect via the ATP binding site.

3.2. Inhibition of UDP-Glc 4-epimerase by o-UDP-GlcNAc and o-UDP

In order to further evaluate the specificity of o-UDP-GlcNAc and o-UDP in the inhibition of UDP-GlcNAc 2-epimerase, the compounds were also tested using commercially available UDP-Gal 4-epimerase. UDP-Gal 4-epimerase catalyzes the interconversion of UDP-Gal and UDP-Glc and it is suggested that at least the mammalian UDP-Gal 4-epimerases [22] also catalyze the interconversion of UDP-GlcNAc and UDP-GalNAc. UDP-Gal 4-epimerase was inhibited by o-UDP-GlcNAc and o-UDP (Fig. 4). o-UDP-GlcNAc was more effective than o-UDP, indicating that the nucleotide sugar had a higher affinity to the enzyme than the corresponding nucleotide. Nevertheless, an about 100-fold higher concentration was necessary to show a comparable effect on UDP-GlcNAc 2-epimerase. Therefore it can be concluded that o-UDP-GlcNAc has a much higher affinity to UDP-GlcNAc 2-epimerase and other enzymes with a specific UDP-GlcNAc binding site [17,21], thus making it possible to avoid numerous side effects when appropriate concentrations are applied to cells.

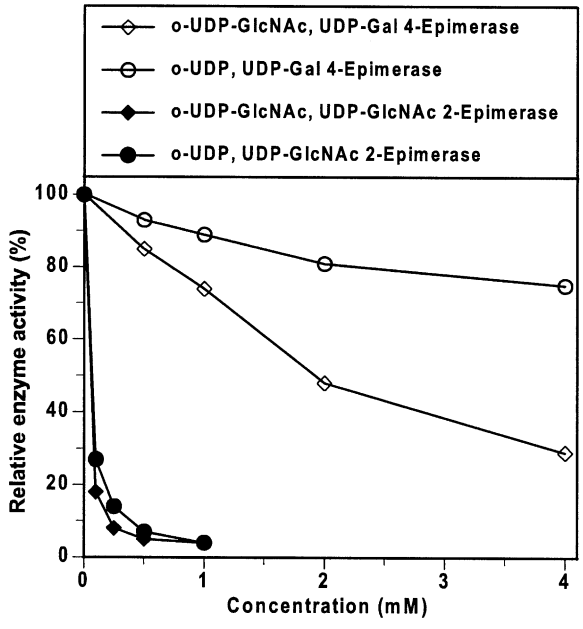

Fig. 4. Inhibition of UDP-Gal 4-epimerase and UDP-GlcNAc 2-epimerase by o-UDP-GlcNAc and o-UDP. UDP-Gal 4-epimerase and purified UDP-GlcNAc 2-epimerase/ManNAc kinase were incubated with the indicated concentrations of o-UDP-GlcNAc and o-UDP for 30 min. Enzyme activities were determined with UDP-GlcNAc 2-epimerase assay 1 and the UDP-Gal 4-epimerase assay as described in Section 2. Values are means of two independent experiments.

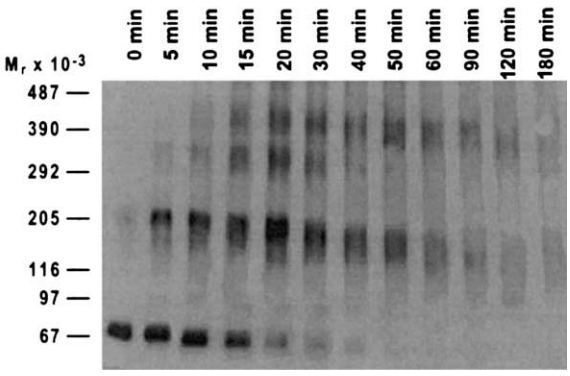

Fig. 5. Crosslinking of UDP-GlcNAc 2-epimerase/ManNAc kinase by o-UDP-GlcNAc. Purified UDP-GlcNAc 2-epimerase/ManNAc kinase was incubated with 2.5 mM o-UDP-GlcNAc for the indicated periods. Crosslinked proteins were separated on a 4.5% SDS–PAGE gel, and the gel was silver stained.

3.3. Characterization of the inhibitory features of o-UDP-GlcNAc

Periodate oxidation of UDP-GlcNAc leads to the formation of two aldehyde groups at the ribose of the sugar nucleotide. These aldehydes may react preferentially with the primary amine groups of lysine residues of proteins. This would lead to a covalent binding of o-UDP-GlcNAc in the substrate binding site of UDP-GlcNAc 2-epimerase, thereby blocking the active site for further reactions. In order to determine if o-UDP-GlcNAc acted as an irreversible or a competitive inhibitor on UDP-GlcNAc 2-epimerase, we tried to influence the inhibition with the natural substrate UDP-GlcNAc. When a mixture of UDP-GlcNAc and o-UDP-GlcNAc was applied to UDP-GlcNAc 2-epimerase the inhibition of enzyme activity was reduced, but when the enzyme was pretreated with o-UDP-GlcNAc, the subsequent addition of UDP-GlcNAc did not restore epimerase activity (data not shown). This indicates that o-UDP-GlcNAc bound covalently to amino acid residues in the active site of UDP-GlcNAc 2-epimerase, causing an irreversible inhibition, which was not competitive.

A further control was performed with r-UDP-GlcNAc, which was synthesized by reduction of o-UDP-GlcNAc with sodium borohydride (Fig. 1). UDP-GlcNAc 2-epimerase was treated with 300 μM o-UDP-GlcNAc and r-UDP-GlcNAc, respectively, under the conditions of assay 2. This resulted in an inhibition of 93% by o-UDP-GlcNAc, but only 16% by r-UDP-GlcNAc. r-UDP-GlcNAc behaved as a competitive inhibitor, since the inhibitory effect could be overcome by an excess of UDP-GlcNAc (data not shown). In contrast, the high inhibitory efficiency of o-UDP-GlcNAc was due to the reactivity of its aldehyde groups. Therefore the irreversible inhibition of UDP-GlcNAc 2-epimerase by o-UDP-GlcNAc or derivatives seems to be a more promising basis for creating a potent inhibitor than using a competitive inhibitor. o-UDP-GlcNAc contains a dialdehyde group which is similar to the structure of glutardialdehyde, a well known reagent for crosslinking of proteins [23]. We therefore tested the ability of o-UDP-GlcNAc to act as a crosslinker for UDP-GlcNAc 2-epimerase/ManNAc kinase. In its fully active state UDP-GlcNAc 2-epimerase/ManNAc kinase is a hexameric enzyme. Storage of the enzyme in the absence of UDP-GlcNAc or UDP results in decay to a dimer with loss of epimerase activity [7], but surprisingly the enzyme still displays ManNAc kinase activity, showing that UDP-GlcNAc 2-epimerase is retained independently of the fused ManNAc kinase domain. Furthermore, point mutations of distinct amino acids in the epimerase domain resulted in formation of epimerase-inactive, but kinase-active trimers [18]. UDP-GlcNAc 2-epimerase/ManNAc kinase was treated with 2.5 mM o-UDP-GlcNAc and the time course of crosslinking was monitored by denaturing and reducing SDS–PAGE (Fig. 5). The control sample showed only monomeric protein (75 kDa), but as early as 5 min after treatment a trimeric form emerged. The reaction then progresses via the formation of an intermediate pentamer, until finally a hexameric, crosslinked protein was observed. The slight shift in molecular masses of the proteins was probably due to the binding of large amounts of negatively charged o-UDP-GlcNAc molecules to the protein, leading to a lower apparent molecular mass in SDS–PAGE. Crosslinking of UDP-GlcNAc 2-epimerase with disuccinimidyl suberate, another often used protein crosslinker [23], confirmed the formation firstly of trimers and finally of hexamers, but without a shift in molecular mass (data not shown). These results indicate that o-UDP-GlcNAc is able to form covalent bonds to amino acid residues of UDP-GlcNAc 2-epimerase.

When treating UDP-GlcNAc 2-epimerase/ManNAc kinase with 2.5 mM o-UDP-GlcNAc, as used above, irreversible inhibition of both enzyme activities was also observed. Therefore the inhibition may be due to a covalent binding of the aldehyde groups of o-UDP-GlcNAc in the active sites of the enzyme. On the other hand, the time course of inhibition of UDP-GlcNAc 2-epimerase activity and of ManNAc kinase activity is not consistent with the time course of the formation of trimers and higher aggregates by crosslinking (Fig. 3 and not shown). Furthermore, crosslinking with o-UDP and o-ADP gave very similar crosslinking results compared to o-UDP-GlcNAc. This indicates that the site of crosslinking is not within the active sites of the enzyme and an independent trimerization site with vicinal reactive amino acids on two separate subunits is postulated. Interestingly, the formation of dimers was not observed, but a formation of dimers of trimers, resulting in hexamers. This suggests the existence of an independent dimerization site, whose amino acids react more slowly with crosslinking agents. Knowledge about the three-dimensional structure of the enzyme is needed to verify this model.

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