Characterization of Ligand Binding to the Bifunctional Key Enzyme in the Sialic Acid Biosynthesis by NMR

I. INVESTIGATION OF THE UDP-GlcNAc 2-EPIMERASE FUNCTIONALITY*

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The bifunctional enzyme UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosaminosamine kinase is the key enzyme for the biosynthesis of sialic acids. As terminal components of glycolipids and glycoproteins, sialic acids are associated with a variety of pathological processes such as inflammation and cancer. For the first time, this study reveals characteristics of the interaction of the epimerase site of the enzyme with its natural substrate, UDP-N-acetylglucosamine (UDP-GlcNAc) and derivatives thereof at atomic resolution. Saturation transfer difference NMR experiments were crucial in obtaining ligand binding epitopes and to rank ligands according to their binding affinities. Employing a fragment based approach, it was possible to assign the major component of substrate recognition to the UDP moiety. In particular, the binding epitopes of the uridine moieties of UMP, UDP, UDP-GalNAc, and UDP-GlcNAc are rather similar, suggesting that the binding mode of the UDP moiety is the same in all cases. In contrast, the hexopyranose units of UDP-GlcNAc and UDP-GalNAc display small differences reflecting the inability of the enzyme to process UDP-GalNAc. Surprisingly, saturation transfer difference NMR titrations show that UDP has the largest binding affinity to the epimerase site and that at least one phosphate group is required for binding. Consequently, this study provides important new data for rational drug design.

Sialylation of glycoproteins and glycolipids on eukaryotic cell surfaces plays an important role during development and regeneration and in the pathogenesis of diseases (1–3, 43). Terminal sialic acids are involved in a variety of cellular interactions, such as cell-cell adhesion or cell migration (4, 5), including metastasis formation and progression of a variety of tumors (6). They are also known to be involved in the formation and masking of recognition determinants (7) and the biological stability of glycoproteins (8).

N-Acetylmuramic acid (Neu5Ac)1 is the biosynthetic precursor of virtually all of the naturally occurring sialic acids (9). In mammals, Neu5Ac is synthesized in the cytosol from UDP-N-acetylglucosamine by four consecutive reactions. The first two steps in this biosynthesis are catalyzed by the bifunctional enzyme UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosaminesaminosamine kinase (UDP-GlcNAc 2-epimerase/ManNAc kinase, EC 5.1.3.14 and EC 2.7.1.60, respectively) (10, 11), which catalyzes the conversion of UDP-GlcNAc to ManNAc and the consecutive phosphorylation at the sixth position. Neu5Ac-9-phosphate is then formed by the addition of phosphoenolpyruvate to ManNAc-6-phosphate catalyzed by the Neu5Ac-9-phosphate synthase (12, 13). After release of the phosphate group, Neu5Ac is activated by the CMP-Neu5Ac synthase (14) in the cell nucleus.

The bifunctional UDP-GlcNAc 2-epimerase/ManNAc kinase was first purified and cloned from rat liver (10, 11). Later, the murine and human enzymes were also cloned and characterized (15, 16). The enzyme consists of 722 amino acids and has a molecular mass of 79 kDa. Natively, it assembles as a homohexamer, possessing both functionalities. Recently, it has been demonstrated that individuals suffering from hereditary inclusion body myopathy and sialuria carry point mutations in the UDP-GlcNAc 2-epimerase part of the gene (17, 18). The biological importance of the enzyme is further reflected by a drastic reduction of cellular sialylation upon loss of enzyme activity, for example (19), and by the fact that a knockout of the gene in mice is lethal to the embryo at day 8.5 (20). Therefore, specific inhibition of UDP-GlcNAc 2-epimerase activity is an important means of regulating cell surface sialylation and therefore in dealing with disease states. Consequently, the characterization of the ligand binding properties of the enzyme at an atomic resolution is key for the design of such inhibitors and also yields insights into the mechanistic aspects of sialic acid biosynthesis. With this aim, the interactions of the enzyme with a number of ligands have been characterized, using saturation transfer difference (STD) NMR spectroscopy (21).

STD NMR allows one to detect and characterize the binding

1 The abbreviations used are: Neu5Ac, N-acetylmuramic acid; GlcNAc, N-acetylglucosamine; STD, saturation transfer difference; ManNAc, N-acetylmannosamine; GalNAc, N-acetylgalactosamine.
of ligands to large receptor proteins. The technique discriminates binding from non-binding ligands and furnishes binding epitopes of ligands at an atomic resolution (22, 23). Recently, it has been shown that STD NMR data may even be used to derive bound conformations of ligands provided that the three-dimensional structure of the receptor protein is known (24, 25). STD NMR has been applied to study rather diverse biological binding reactions (26). Most noteworthy, there is no size limit for the protein receptor and an assignment of protein resonances is not required. For example, this has rendered possible the investigation of ligands binding to liposome-integrated membrane proteins (27) or to native viruses (28). Until today, only the monofunctional UDP-GlcNAc 2-epimerase from Escherichia coli, which shows only 22% homology of the amino acid sequence to the mammalian bifunctional enzyme, has been characterized and crystallized (29). To date, little is known with regard to the mammalian counterpart of this enzyme. STD NMR has a proven potential to study ligand binding, and here, it is applied to the large and rather fragile bifunctional UDP-GlcNAc 2-epimerase/ManNAc kinase.

**EXPERIMENTAL PROCEDURES**

**Materials**—All of the chemicals with the exception of 2-acetamido-2-deoxyglucose, which was synthesized according to the method of Pravdic et al. (30), were obtained from Sigma.

**Expression and Purification of UDP-GlcNAc 2-Epimerase/ManNAc Kinase**—Rat UDP-GlcNAc 2-epimerase/ManNAc kinase was recombinantly expressed in SF90 insect cells with a His8 tag as previously described (31). Cells from a 50-ml culture were harvested, resuspended in 5 ml of 10 mM sodium phosphate, 1 mM EDTA, 1 mM dithiothreitol, pH 7.5, and lysed by passage through a narrow bore needle 20 times. After centrifugation at 20,000 × g at 4 °C for 30 min, UDP-GlcNAc 2-epimerase/ManNAc kinase was purified as described earlier (31). The resulting supernatant was applied to a 1 ml nickel-nitrilotriacetic acid column (Qiagen), which had been equilibrated with 10 ml of 502-epimerase/ManNAc kinase was purified as described earlier (31). The follow-up by 4 ml of the same buffer with imidazole-GlcNAc 2-epimerase activity, the enzyme was incubated with 0.1 mM UDP-GlcNAc is the natural substrate of the UDP-GlcNAc 2-epimerase. The enzyme converts UDP-GlcNAc to ManNAc and UDP. In the absence of ATP, the reaction stopped at this point because the kinase activity that normally converts ManNAc into ManNAc-6-phosphate in a second step remained turned off. In the presence of UDP-GlcNAc 2-epimerase/ManNAc kinase, significant STD effects were obtained for UDP-GlcNAc showing that UDP-GlcNAc is bound to the enzyme under these conditions (Fig. 1A). The STD spectrum also showed signals of ManNAc and UDP, which were generated by the epimerization reaction. At the same time, this observation showed that the enzyme is active under the experimental conditions chosen. The epimerase reaction was followed by 1H NMR spectra that showed the β-anomer of ManNAc to be the product of the epimerization reaction. The β-anomer was formed later by mutarotation. This observation is in accordance with results that have been published recently (33).

**Epitope Mapping of Ligands with STD NMR**—From the STD spectra shown in Fig. 1, relative STD values were obtained. These data reflected the relative amount of saturation transferred from the protein onto the ligand, and thus, the protons with a high STD value were assumed to be in more intimate contact with protons on the protein surface than those with lower STD values. From Fig. 2, it is immediately obvious that the nucleotide part of UDP-GlcNAc is receiving the largest relative saturation transfer and therefore is most intimately bound to the protein.

To confirm the role of the nucleotide and the sugar moiety in binding, additional STD NMR experiments were conducted using a fragment-based approach. The experiments showed that UDP, UMP, GlcNAc, and GlcNAc-1-phosphate all bind to UDP-GlcNAc 2-epimerase/ManNAc kinase as exemplified for UDP in Fig. 1B. The binding epitopes of the nucleotide moieties in UMP, UDP, UDP-GlcNAc, and UDP-GalNAc showed a remarkable similarity (Fig. 2), suggesting that the nucleotide occupies a common binding pocket in all cases.

The binding epitopes of GlcNAc and GlcNAc-1-phosphate (Fig. 2, D and E) differed from each other with the epitope of GlcNAc-1-phosphate being similar to the sugar moiety of UDP-GlcNAc. This is most evident from Fig. 3 that compares the binding epitopes of GlcNAc and GlcNAc-1-phosphate to the one of UDP-GlcNAc. The protons in most intimate contact with the enzyme were H2 and H3 of GlcNAc, whereas the protons of the N-acetyl group and H1 received the largest saturation transfer.
in the cases of GlcNAc-1-phosphate and UDP-GlcNAc. Although the small differences between GlcNAc-1-phosphate and UDP-GlcNAc may be attributed to slightly different binding modes due to the absence or presence of the nucleotide, GlcNAc displayed a clearly distinct binding epitope proposing either a more significant deviation from the binding mode of UDP-GlcNAc and GlcNAc-1-phosphate or binding to a different binding site. It has been shown that GlcNAc can be metabolized by the other functionality of the bifunctional enzyme, the ManNAc kinase, to furnish GlcNAc-6-phosphate (34). Therefore, it is
very likely that GlcNAc binds to the ManNAc kinase instead of the UDP-GlcNAc 2-epimerase. To test this hypothesis, the enzyme was incubated with the selective irreversible inhibitor α-UDP-GlcNAc (32). Subsequent STD NMR experiments with the ligands showed no appreciable STD signals with the exception of the case of GlcNAc where an identical STD spectrum was obtained. Therefore, GlcNAc binds to the ManNAc kinase site of the bifunctional enzyme. An identical result was also obtained for ManNAc (34).

**STD NMR Titrations**—To further substantiate that it is the nucleotide part that dominates the binding of ligands to the epimerase site of UDP-GlcNAc 2-epimerase/ManNAc kinase, competitive STD titrations were performed as has been described previously (23, 28). The titration experiments yielded a qualitative ranking of the binding affinities of the ligands discussed above as shown in Fig. 4. UDP had the highest affinity of the ligands examined. Interestingly, the substrate analogue UDP-GalNAc that was not metabolized by the enzyme had the same affinity as UDP-GlcNAc. Since it has been postulated that the epimerization reaction

![Figure 2. Binding epitopes as determined by group epitope mapping with $^1$H STD NMR: UDP-GlcNAc (A), UMP (B), UDP (C), GlcNAc-1-phosphate (D), GlcNAc (E), and UDP-GalNAc (F).](image-url)
sugar moiety is not a dominant factor in binding or recognizing the substrate. The observations that the binding epitopes of the hexopyranosne moieties of UDP-GlcNAc and UDP-GalNAc are similar and that binding is also observed for UDP and UMP alone lends further credit to the hypothesis that UDP guides the ligand into the epimerase site. Also in accordance with this notion is the observation that all of the ligands investigated here compete with each other for binding, indicating that they bind to the same site, i.e., the epimerase site. Competitive titrations conducted with UDP-GlcNAc, UDP, and UMP re-

FIG. 3. Binding epitopes as determined by group epitope mapping with $^1$H STD NMR: UDP-GlcNAc (left), GlcNAc-1-phosphate (middle), and GlcNAc (right). To simplify the direct comparison of the epitopes, the group epitope mapping was in this case performed not by normalizing with respect to the largest STD effect but rather against the largest STD effect observed for the GlcNAc moiety.

Relative binding affinity

UDP > UDP-GlcNAc > UDP-GalNAc > UMP > GlcNAc-1-phosphate

FIG. 4. Schematic representation of the relative binding affinities of various ligands to the epimerase functionality of the UDP-GlcNAc 2-epimerase/ManNAc kinase.

of UDP-GlcNAc proceeds via 2-acetamidoglucal as an intermediate (35), we also subjected this ligand to STD experiments. The STD signals were very weak, indicating that 2-acetamidoglucal had either a very large or a very small binding affinity. In both cases, the detection limits of STD NMR were reached and weak signals were expected (26). To discriminate between the two possibilities, competitive titrations with UDP-GalNAc were performed, revealing 2-acetamidoglucal as a ligand with a rather poor binding affinity.

DISCUSSION

Several studies have utilized STD NMR experiments to detect and characterize the binding of ligands to receptor proteins (26). This report describes the application of this approach to the key enzyme for the biosynthesis of sialic acids in mammals. Because this enzyme, UDP-GlcNAc 2-epimerase/ManNAc kinase, is rather large and fragile, structural data are unavailable and little is known regarding the atomic details of ligand binding to the active sites of the enzyme, i.e., the epimerase and the kinase site. Employing a fragment-based approach, we were able to describe the binding of ligands to the epimerase site at atomic resolution.

An inspection of the binding epitope (Fig. 2A) of the natural substrate, UDP-GlcNAc, when bound to the enzyme revealed that the nucleotide moiety is dictating the binding mode. In particular, the protons H5 of the uridine ring and H1' of the ribose moiety received the largest amount of saturation transfer. It is informative to compare the binding epitope of UDP-GlcNAc bound to UDP-GlcNAc 2-epimerase/ManNAc kinase to the binding epitope of UDP-galactosidase bound to $\beta$-1,4-galactosyltransferase T1 (36) where the UDP moiety was also found to receive the largest saturation transfer and therefore presumably positioned the ligand in the binding pocket. In the latter case, it was possible to perform a quantitative analysis of STD effects (24) and obtain the conformation of UDP-galactosidase bound to $\beta$-1,4-galactosyltransferase T1 at atomic resolution in aqueous solution (25). This analysis will also be applicable to ligands binding to UDP-GlcNAc 2-epimerase/ManNAc kinase once structural data on the protein are available. The overall binding modes of UDP-GlcNAc to UDP-GlcNAc 2-epimerase and of UDP-galactosidase to $\beta$-1,4-galactosyltransferase T1 are rather similar.

For a bacterial counterpart of UDP-GlcNAc 2-epimerase/ManNAc kinase, a monofunctional UDP-GlcNAc 2-epimerase, the crystallographic structure at a 2.5-Å resolution, has been published (29). In this structure, a stacking interaction was observed between the uracil and Phe-276. A similar stacking interaction of uracil with Phe-226 was observed in the x-ray structure of UDP-galactosidase bound to $\beta$-1,4-galactosyltransferase T1 (37). It appears likely that such a stacking interaction with an aromatic amino acid is also responsible for positioning the ligand into the epimerase site of UDP-GlcNAc 2-epimerase/ManNAc kinase. A key role of the nucleotide moiety during the binding process has already been suggested by inhibition experiments with UDP-GlcNAc 2-epimerase/ManNAc kinase where nearly no difference in enzyme inhibition was detected between the irreversible inhibitors $\omega$-UDP-GlcNAc and $\omega$-UDP (32). This indicates that the sugar moiety is not a dominant factor in binding or recognizing the substrate.

The observations that the binding epitopes of the hexopyranosne moieties of UDP-GlcNAc and UDP-GalNAc are similar and that binding is also observed for UDP and UMP alone lends further credit to the hypothesis that UDP guides the ligand into the epimerase site. Also in accordance with this notion is the observation that all of the ligands investigated here compete with each other for binding, indicating that they bind to the same site, i.e., the epimerase site. Competitive titrations conducted with UDP-GlcNAc, UDP, and UMP re-
revealed UDP to have the highest affinity. This implies that the sugar moiety in UDP-GlcNAc even impedes binding either by imposing unfavorable steric strains on the binding pocket of the protein or by the lack of a negative charge as compared with UDP. Inside the cells, the concentration of UDP-GlcNAc is ~4 times higher than the concentration of UDP (38). Given this concentration difference, it is likely that, under physiological conditions, the enzyme is constantly inhibited to a substantial extent by UDP. The titration experiments further show that the affinity of a ligand to the enzyme critically depends on the presence of phosphate residues, because the binding affinity of UMP is significantly reduced compared with UDP. Interestingly, one phosphate residue is sufficient to direct GlcNAc-1-phosphate into the epimerase site, whereas GlcNAc itself binds to the kinase site (34) and has virtually no binding affinity for the epimerase site, as was concluded from STD experiments performed with covalently inhibited UDP-GlcNAc 2-epimerase.

Inhibition experiments with o-uridine have shown that this compound inhibits UDP-GlcNAc 2-epimerase much less efficiently than UDP (32). Therefore, in conjunction with the results presented here, it is reasonable to assume that two or even three negative charges as provided by the diphosphate moiety are essential for efficient binding to the epimerase site.

Although most intermediates in enzyme reactions bind tightly, here the intermediate 2-acetamidoglucal is observed to bind very weakly. However, to date, the intermediate 2-acetamidoglucal has never been detected free in solution except in the case of the sialuria disorder (39). The bacterial UDP-GlcNAc 2-epimerase produces UDP-ManNAc instead of ManNAc via 2-acetamidoglucal, and crystallographic studies suggest that the enzyme reaction proceeds via a closed form of the enzyme (29). This closed form is required to maintain the intermediate within the active site of the enzyme to allow the second stage of the epimerization to occur, the syn addition of UDP, and a proton to yield UDP-ManNAc. It is highly likely that the mammalian UDP-GlcNAc 2-epimerase follows a similar mechanism with the exception of the addition of H₂O instead of UDP in the final step. Consequently, the affinity of the enzyme toward the intermediate 2-acetamidoglucal is irrelevant, because it is formed in situ inside a closed form of the enzyme. In accordance with this mechanism is the observation that only 6% of the intermediate 2-acetamidoglucal is converted to ManNAc after 27 h at 37 °C (33), whereas the natural substrate UDP-GlcNAc is 100% converted at ~2 h. In our experiments, no conversion of 2-acetamidoglucal could be detected after 48 h at 5 °C. This mechanism predicts weak STD signals for 2-acetamidoglucal as observed. In this regard, it is interesting to mention that a protein from E. coli K1, termed NeuC, has recently been identified as an UDP-GlcNAc 2-epimerase (40). As the mammalian counterpart, this enzyme also hydrolyzes UDP-GlcNAc via the 2-acetamidoglucal.

This mechanistic model where the reaction occurs inside a closed form of the enzyme also explains why UDP binds with a higher affinity than the natural substrate UDP-GlcNAc. In order for the reaction to complete, the enzyme must remain closed for a specific time. The binding of the UDP moiety is thought to induce and subsequently stabilize the closed form of the enzyme, thereby allowing the reaction to proceed. It has been observed that the enzyme releases UDP prior to ManNAc (35), further substantiating the hypothesis that the binding of UDP stabilizes the closed conformation.

The observation that UDP-GlcNAc and UDP-GalNAc are bound with similar affinities and display similar binding epitopes suggests that both ligands are bound to the same conformational state of the enzyme, presumably the closed form. The inability of the enzyme to convert UDP-GalNAc may be due to the fact that UDP-GalNAc has an axial hydroxyl group attached to C4 of the GalNAc residue and is thus located in close proximity to GalNAc-H2 that, according to the recently proposed mechanism (33), is attacked by a basic residue of the enzyme to yield the 2-acetamidoglucal. In the case of UDP-GalNAc, the more acidic hydroxyl proton of OH-4 would deliver this proton and therefore would prohibit the formation of a corresponding 2-acetamidoglactal. It is also possible that a 1,4-anhydro derivative is formed that would prohibit further processing.

According to our results, it is clear that any approach for the design of selective and potent inhibitors must take into account the importance of the UDP moiety. In the light of our new results, it is not surprising that synthetic derivatives of UDP (41, 42) have been found to effectively inhibit the enzyme. According to our studies, another apparent requirement for a potent inhibitor is the presence of two or three negative charges. Summarizing these results, a general model for potential UDP-GlcNAc-2-epimerase inhibitors can be predicted as indicated in Fig. 5. Our results also highlight that glycosyltransferases and epimerases have similar principles to bind and process activated sugars and therefore support the view that these enzymes may have a common ancestor, as has been previously proposed (29).

This study provides new insights into the epimerase activity of UDP-GlcNAc-2-epimerase/ManNAc kinase, the key enzyme of mammalian sialic acid biosynthesis. For the first time, the binding of ligands including the natural substrate UDP-GlcNAc has been described at atomic resolution, paving the road for the rational design of novel inhibitors of the epimerase activity of UDP-GlcNAc 2-epimerase/ManNAc kinase that have a potential in the treatment of cancer or inflammation, for example.

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