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

II. INVESTIGATION OF THE ManNAc KINASE FUNCTIONALITY*

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N-Acetylimmannosamine (ManNAc) is the physiological precursors to all sialic acids that occur in nature. As variations in the sialic acid decoration of cell surfaces can profoundly affect cell-cell, patho-cell, or drug-cell interactions, the enzymes that convert ManNAc into sialic acid are attractive targets for the development of drugs that specifically interrupt sialic acid biosynthesis or lead to modified sialic acids on the surface of cells. The first step in the enzymatic conversion of ManNAc into sialic acid is phosphorylation, yielding N-acetylimmannosamine-6-phosphate. The enzyme that catalyzes this conversion is the N-acetylimmannosamine kinase (ManNAc kinase) as part of the bifunctional enzyme UDP-N-acetylglucosamine 2-epimerase/ManNAcaminosamine kinase. Here, we employed saturation transfer difference (STD) NMR experiments to study the binding of ManNAc and related ligands to the ManNAc kinase. It is shown that the configuration of C1 and C4 of ManNAc is crucial for binding to the enzyme, whereas the C2 position not only accepts variations in the attached N-acetyl side chain but also tolerates inversion of configuration. Our experiments also show that ManNAc kinase maintains its functionality, even in the absence of Mg2+.

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As terminal sugars of membrane-associated glycoconjugates, sialic acids play a fundamental role in biological recognition reactions. Recent studies have revealed the therapeutic potential of biosynthetic modifications of such cellular sialic acids. As a general concept, it has been proposed that pathogens such as viruses or bacteria may not infect their hosts if modified sialic acids are presented on the cell surface of the host (1). As an example, biosynthetic modifications of the N-acetyl group may be achieved through the administration of sialic acid precursor analogues that are finally metabolized into modified sialic acid residues located at the cell surface (2–5). The key enzyme of sialic acid metabolism, the bifunctional enzyme, UDP-N-acetyllactosamine 2-epimerase/ManNAcaminosamine kinase (UDP-GlcNAc2-epimerase/ManNAc kinase) (6, 7), has been identified as an excellent target for this so-called metabolic engineering (8). By providing ManNAc derivatives with chemically modified N-acetyl side chains as substrates, the ManNAc kinase functionality of this enzyme furnishes corresponding 6-phosphates that are finally converted into sialic acid derivatives (8). The concept of metabolic engineering by targeting the ManNAc kinase has been successfully used to inhibit the poly-α,2-sialic acid expression on the surface of tumor cells (9), and there is a potential for a variety of other novel therapeutic applications. Therefore, it is of considerable interest to characterize the binding of ManNAc and related derivatives to the ManNAc kinase at atomic resolution.

The ManNAc kinase part of the bifunctional enzyme, UDP-GlcNAc2-epimerase/ManNAc kinase, belongs to the family of N-acetylatedhexosamine kinases. As such, it is also a member of the functionally rather diverse sugar kinase/hsp 70/actin superfamily that all share a common ATPase domain (10). Therefore, gross conformational properties of the ManNAc kinase may be predicted from this relationship, as has been demonstrated recently for the murine N-acetyllactosamine kinase (11). Such structure predictions can be a valuable tool in analyzing the details and requirements of ligand binding. To experimentally identify and characterize the binding of ligands to receptor proteins, NMR spectroscopy offers a variety of techniques (12). For the analysis of binding epitopes at atomic resolution, STD NMR experiments (13) have been proven to be instrumental, and in an accompanying paper (14), we have successfully applied these experiments to analyze the binding of ligands to the UDP-GlcNAc 2-epimerase. In this second part, we report on the

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¶ The abbreviations used are: GlcNAc, N-acetyllactosamine; ManNAc, N-acetylimmannosamine; STD, saturation transfer difference; GalNAc, N-acetyllactosamine; ManNProp, N-propionylmannosamine; ManNBut, N-butyrylmannosamine.

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binding of ligands to the kinase site of UDP-GlcNAc 2-epimerase/ManNAc kinase.

**EXPERIMENTAL PROCEDURES**

**Materials**—All of the ligands were obtained from Sigma. Propyl and butyl derivatives of ManNAc were synthesized following published procedures (12) and purified via reversed-phase high pressure liquid chromatography using a C8 column and a gradient of 80% water in acetonitrile (v/v) to 100% acetonitrile.

**Synthesis and Purification of ManNAc-6-phosphate**—ManNAc-6-phosphate was enzymatically synthesized using the bifunctional enzyme UDP-GlcNAc 2-epimerase/ManNAc kinase. The synthesis was conducted on 4 mM ATP with 1 mM ManNAc in the presence of the enzyme at 37 °C for 12 h. The enzyme was separated from the educts and products by ultrafiltration. The resulting ManNAc-6-phosphate was separated from ADP as well as the unreacted ManNAc and ATP by ion-exchange chromatography using a 6-ml Resource Q column (Amersham Biosciences). Ion-exchange chromatography was conducted in a 10 mM Tris buffer, pH 8.0, and with increasing NaCl concentrations of 0–1 M. The charged eluants were detected by conductivity measurements. In addition, ADP and ATP were also detected by their UV absorption at 260 nm. The uncharged educt ManNAc was detected solely by UV absorption at 215 nm. NMR spectroscopy was used to identify the single peak for which a conductivity change was observed, and that did not absorb in the UV at 215 or 260 nm as ManNAc-6-phosphate. The ManNAc-6-phosphate was then desalted using a Superdex gel filtration column (Amersham Biosciences). The purity of the material was determined by UV and NMR spectroscopy. The material was found to be at least 95% pure.

**Expression and Purification of UDP-GlcNAc 2-epimerase/ManNAc Kinase**—Expression of UDP-GlcNAc 2-epimerase/ManNAc kinase in Sf-900 insect cells and the consecutive purification was performed as described previously (14). For purification on the PD-10 column, a buffer containing 50 mM Tris-HCl instead of 50 mM sodium phosphate was used when ligands were measured in the presence of MgCl2.

**Enzyme Assay**—The ManNAc kinase activity was detected with a colorimetric assay. It contained 65 mM Tris-HCl, pH 8.1, 20 mM MgCl2, 5 mM ManNAc, 10 mM ATP, 5 mM phosphoenolpyruvate, 0.8 mM NADH, 4 units of pyruvate kinase, 4 units of lactate dehydrogenase, 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 adding 800 µl of 10 mM EDTA to the absorbance was observed at 340 nm. To determine the conversion rates of GlcNAc, ManNProp, and ManNBut, the assay was conducted utilizing these compounds as substrates.

**NMR Experiments**—Saturation transfer difference (STD) NMR spectra were obtained using a 12.1 T Bruker DRX spectrometer equipped with a triple resonance probe head, incorporating gradients in the z-axis. The samples were prepared and investigated similar to the epimerase investigations (14) and contained between 10 and 20 µM protein and a ligand concentration between 200 µM and 4 mM. The investigation of the enzyme kinetics for the kinase functionality used samples dissolved in a 100% D2O containing 50 mM d1-Tris, pH 7.5 buffer (uncorrected reading for the presence of 2H1), which included 100 mM NaCl. Each time point consisted of one of a dimension “pulse and acquire” NMR experiment with 64 scans for acquisition and 8 dummy scans. The resulting data were analyzed using the matNMR toolbox for Matlab. The sweep width and recycle delay were as for the STD experiments (14).

**RESULTS**

Prior to performing any STD NMR experiments, the ManNAc kinase activity was validated by simply acquiring one-dimensional NMR spectra of ManNAc in the presence of ManNAc kinase and ATP. The formation of ManNAc-6-phosphate and ADP was found to be clearly indicated that the ManNAc kinase is active under the NMR conditions employed. This 1H NMR activity assay also revealed that the enzymatic conversion of ManNAc to ManNAc-6-phosphate exclusively yields α-ManNAc-6-phosphate.

**Binding of ATP, ADP, ManNAc, and ManNAc-6-phosphate to ManNAc Kinase**—To study the interactions of the ManNAc kinase with different ligands, we first performed STD NMR spectra as described in the accompanying paper (14) with the educts ManNAc and ATP as well as the products ManNAc-6-phosphate and ADP. The STD spectra obtained for ManNAc in the presence of ManNAc kinase unambiguously showed that from the mixture of α- and β-anomers, present at approximately equimolar amounts in aqueous solution, only the α-anomer binds to the enzyme (Fig. 1). Interestingly, the enzyme is less selective for the recognition of ManNAc-6-phosphate. For ManNAc-6-phosphate, it was observed that the β-anomer also binds to the enzyme with a lower affinity than the α-anomer. The binding epitopes of ATP, ADP, α-ManNAc, and α-ManNAc-6-phosphate are summarized in Fig. 2. It was obvious that the binding epitopes of the corresponding educts and products differed significantly. For α-ManNAc and α-ManNAc-6-phosphate, the relative STD effects deviated for all of the protons with the exception of the protons attached to C1 and C4. The binding epitopes of ADP and ATP displayed particularly large differences for H2', H3', and H2. Qualitative STD NMR titrations with the educt/product pairs ATP/ADP and ManNAc/ManNAc-6-phosphate showed that they compete with each other and therefore bind to the same site. Moreover, these titration experiments suggest that the products ADP and ManNAc-6-phosphate bind more weakly to ManNAc kinase than the educts ATP and ManNAc. Thus, the binding mode changed along with the binding affinity.

To exclude the possibility that the observed STD effects of α-ManNAc were due to binding to the epimerase site of the enzyme, the UDP-GlcNAc 2-epimerase functionality of the bifunctional enzyme UDP-GlcNAc 2-epimerase/ManNAc kinase was irreversibly inhibited by covalently blocking the epimerase active site with α-UDP-GlcNAc (2,3-diolidehydro-UDP-N-acetylglucosamine) (15). The STD NMR spectra obtained for the binding of ManNAc to this covalently modified enzyme showed no difference to the spectrum obtained for the native enzyme (cf. Fig. 1). Therefore, it can be excluded that the effects observed are due to binding of ManNAc to the epimerase site.

**Binding of N-Acylmannosamines and Derivatives to ManNAc Kinase**—From our colorimetric assays, it was shown that ManNAc kinase in addition to ManNAc also phosphorylated ManNProp, ManNBut, and GlcNAc but not GalNAc, ManNH2, or Man (Fig. 3B). From this assay, it was found that, at substrate concentrations of 5 mM each, the conversion into the corresponding 6-phosphates was 13% for GlcNAc, 24% for ManNProp, and 13% for ManNBut.

To explain these different conversion rates, relative binding affinities were assigned to the different ligands by a qualitative STD NMR titration. From this series of titrations, the following ranking of relative binding affinities was obtained: ManNAc < GlcNAc > ManNProp > ManNBut (cf. Fig. 3B). GalNAc, ManNH2, and Man did not display any STD signals and did not compete with the binding of any of the other ligands. Therefore, they do not bind to the ManNAc kinase.

A comparison of the binding epitopes showed that ManNAc and ManNProp have rather similar binding epitopes (Fig. 3A). A further elongation of the N-acetyl chain similar to ManNBut lead to a significantly altered binding epitope with the largest change in relative STD effects observed for the proton attached to C4. This finding suggested that ManNBut binds in a different orientation to the ManNAc kinase than ManNProp, although both ligands have similar binding affinities to the enzyme. As for ManNAc, STD signals were only observed for the α-anomers of ManNProp, ManNBut, as well as for GlcNAc. The binding epitope of α-GlcNAc differed from that of α-ManNAc, although both ligands had a similar affinity to the enzyme. Despite the similar binding affinities of ManNAc and GlcNAc, the rate of conversion to the corresponding 6-phosphate was significantly lower for GlcNAc (cf. Fig. 3B).
Dependence of the ManNAc Kinase Activity on the Mg²⁺ Concentration—For many kinases, it is known that Mg²⁺ is essential for the enzyme reaction to proceed. For the ManNAc kinase, we showed that enzyme activity persists, even in the absence of Mg²⁺. It is unlikely that tightly bound Mg²⁺ plays a role since the enzyme has been purified from the cell culture in the presence of EDTA (14). The influence of Mg²⁺ on the enzyme reaction of the ManNAc kinase was examined by ¹H NMR experiments. The presence of 0.1 mM MgCl₂ in the sample caused a significant increase in enzyme activity as compared with the absence of Mg²⁺ (Fig. 4). For example, for the complete conversion of ManNAc to ManNAc-6-phosphate in the
absence of Mg\(^{2+}\) (12 h) were required, whereas in the presence of Mg\(^{2+}\) the reaction was completed within 80 min. Therefore, Mg\(^{2+}\) cannot be considered as essential for the kinase activity of the enzyme. On the other hand, the reaction rate is profoundly dependent on the Mg\(^{2+}\) concentration. Consequently, it is likely that Mg\(^{2+}\) influences the binding of ATP to ManNAc kinase. Therefore, STD spectra for ATP binding to the kinase were conducted in the presence and absence of Mg\(^{2+}\). The resulting epitopes showed small reproducible changes in the STD effects of the ribose protons closest to the triphosphate moiety. In contrast, the binding of UDP-GlcNac to UDP-GlcNac 2-epimerase, as well as the rate of epimerization, was not found to be dependent on the concentration of Mg\(^{2+}\).

**DISCUSSION**

The STD NMR experiments described above furnished the binding epitopes of ATP, ADP, ManNAc, and ManNAc-6-phosphate at atomic resolution. Likewise, the binding epitopes of the substrate derivatives GlcNac, ManNProp, and ManNBut were obtained and allowed the correlation of binding affinities, enzymatic conversion rates, and binding epitopes with each other. In addition, we could show that the bifunctional enzyme, UDP-GlcNac 2-epimerase/ManNAc kinase, only binds and processes the \(\alpha\)-anomer. Unexpectedly, the \(\beta\)-anomer of ManNAc did not bind to any significant extent. This result reflects the high degree of sophistication that this enzyme has developed during evolution. From the accompanying study (14) on the ligand-binding properties of the epimerase activity of the UDP-GlcNac 2-epimerase/ManNAc kinase, it is clear that only \(\alpha\)-ManNAc is generated by UDP-GlcNac 2-epimerase. Given that the two functionalities of the enzyme are spatially proximate, the major source of ManNAc phosphorylated by the kinase is in all probability directly delivered by the epimerase functionality. Consequently, it is reasonable to postulate that the ManNAc kinase is under an evolutionary pressure to select for the \(\alpha\)-ManNAc. The inability of the ManNAc kinase to bind \(\beta\)-ManNAc indirectly supports this hypothesis that the product of the epimerization reaction is immediately phosphorylated by the kinase without prior release to the bulk solution and that the epimerase and kinase sites are close in space. On the other hand, it was observed that the binding specificity of the enzyme for the product of the phosphorylation reaction is not as pronounced because \(\beta\)-ManNAc-6-phosphate is yielding weak STD effects. This is readily explained by the fact that the 6-phosphate group almost certainly interacts with the protein and that this may, to a degree, offset the unfavorable effect of the \(\beta\)-anomeric configuration. Furthermore, the ManNAc kinase is not required to differentiate between the two products in order to function properly, and consequently, there has never been any evolutionary pressure to discriminate between \(\alpha\)-ManNAc-6-phosphate and \(\beta\)-ManNAc-6-phosphate.

The observed preference for \(\alpha\)-ManNAc-6-phosphate and the fact that ManNAc competes with ManNAc-6-phosphate in com-

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**Fig. 3.** A, binding epitopes as determined by \(^1\)H STD NMR: ManNAc (top left); ManNProp (top right); ManNBut (bottom left); and GlcNac (bottom right). B, enzymatic conversion rates and relative binding affinities of ManNAc, ManNProp, ManNBut, and GlcNac are shown on top of the graph. Although normally the best binding affinity is correlated with the highest enzymatic activity, it can be clearly seen here that, in this case, there is no simple correlation between binding affinity and enzymatic activity because GlcNac has the same affinity as ManNAc but a drastically reduced conversion rate. For an explanation of this observation, one must also consider the binding epitopes.
petitive STD NMR titrations suggests that the educt and product bind to the same site. For UDP-GlcNAc 2-epimerase/ManNAc kinase, our results therefore favor a model in which the binding pocket for the educt ManNAc and the product ManNAc-6-phosphate is identical. This contrasts with the hexokinase I where different binding pockets for the products and educts have been described (16). In the case of the hexokinase I, the binding of the products is associated with an allosteric inhibition of the enzyme. In the case of the UDP-GlcNAc 2-epimerase/ManNAc kinase, this regulatory role is performed by the feedback inhibition of the epimerase functionality by CMP-5-N-acetylneuraminic acid.

Several kinases require Mg\(^{2+}\) as an essential cation for their enzymatic activity (10). Here, it is shown that the ManNAc kinase is also active in the absence of Mg\(^{2+}\). Nevertheless, the addition of small amounts of Mg\(^{2+}\) to the buffer results in a 15-fold increase of enzyme activity. Also, upon the addition of Mg\(^{2+}\), a slight change in the binding epitope of ATP was detected for those protons close to the triphosphate group. X-ray structures from related kinases suggest that this may be due to the complexation of the triphosphate moiety with Mg\(^{2+}\) to facilitate the nucleophilic substitution reaction. Lowe and Potter (17) and Pollard-Knight et al. (18) have suggested that Mg\(^{2+}\) shields the negative charges of the β- and γ-phosphate groups of ATP and thus facilitates the nucleophilic attack of the hexose C6 hydroxyl group on the γ-phosphate of ATP.

It has been shown for different ManNAc derivatives that they were metabolically transformed into corresponding sialic acid derivatives that finally were expressed as modified glycan chains on cell surfaces (3). This strongly influences the biochemical recognition processes such as the adherence of polyomaviruses to their usurped cell surface receptors. Here, we have also shown that ManNAc kinase phosphorylates ManNProp, ManNBut, and GlcNAc, although less efficiently than ManNAc. The binding epitopes of these natural and unnatural substrates differ to varying degrees from the binding epitope of ManNAc reflecting different binding modes. The largest variations of the binding epitopes were observed for the protons attached to C1 and C4. This is most clearly reflected by the inability of the enzyme to bind to GalNAc. Variations at the C2 position are obviously well tolerated by the ManNAc analogue with the NH replaced by a methylene group is not converted.

ManNAc and ManNProp show almost identical binding epitopes apart from the N-acetyl and the N-propionyl groups. Nevertheless, their relative binding affinities toward the ManNAc kinase are significantly different. It may be hypothesized that the larger N-acetyl side chain of ManNProp leads to an unfavorable steric interaction with the protein leading to a
reduced binding affinity. On the other hand, ManNBut displays a distinctly different binding epitope as well as a reduced binding affinity toward ManNAc kinase. This finding suggests that ManNBut favors a different binding mode than either ManNAc or ManNProp. Therefore, the lower conversion rate of ManNProp may be explained by a lower binding affinity. However, ManNBut has the same binding affinity for the ManNAc kinase as ManNProp. Consequently, in this case, the lower conversion rate cannot be a function of the binding affinity but rather the result of a change in the binding mode as reflected by the altered binding epitope. The differences in the conversion rate of GlcNAc and ManNAc can also be explained by the differences in their binding mode, because the binding affinities are similar but their binding epitopes differ. In conclusion, two factors determine the rate of enzymatic conversion: 1) the binding affinity and 2) the mode of binding.

In summary, this study for the first time delivers information on structure-activity and structure-binding relationships of natural and unnatural ligands of the ManNAc kinase. On this basis, it will be possible to design novel ManNAc analogues for engineering membrane-bound glycostructures that may lead to novel therapeutic applications as illustrated in Fig. 5.

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