Inhibition of CMP-Sialic Acid Transport into Golgi Vesicles by Nucleoside Monophosphates†

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ABSTRACT: We examined the interactions of nucleotides with the CMP-sialic acid transporter in order to better understand which features play a role in binding and to investigate the relationship between binding and subsequent transport. With respect to the sugar, the transporter requires a complete ribose ring for tight binding, and the 2′-ara hydrogen makes an important contact. The enzyme exhibits little specificity with respect to the 2′- and 3′-hydroxyls, as it tolerated substitutions ranging from fluorine to an azido group. In the base, the C4 amine and C2 carbonyl groups make important contacts, while the N3 nitrogen does not. However, adding a methyl group to N3 dramatically reduced binding, indicating that mass at this position sterically hinders binding. Adding a group at C5 had either no effect or slightly enhanced binding. To determine if the transporter recognizes these CMP analogues as substrates, we assayed them for their ability to trans stimulate CMP-sialic acid import. These data suggest that the enzyme transports a wide variety of NMPs, and the rate of transport is inversely proportional to the K_I of the analogue. The importance of our findings for understanding the specificities of the different nucleotide−sugar tranlocators and the design of novel glycosylation inhibitors are discussed.

Glycosylation of proteins and lipids has wide-ranging importance for the structure, targeting, and function of biomolecules. For example, glycans are involved in the clearance of proteins from circulation (extracellular sorting), cell−cell adhesion and communication, recognition of bacterial cells by the innate immune system, escape from the immune system by cancer cells, cell movement and communication during growth and development, formation of the glycocalyx that shields the cell surface, and molecular binding at the cell surface (*1*−*4*). N-Linked protein glycosylation begins in the endoplasmic reticulum with the attachment of a core glycan ($GlcNAc_2Man_9Glc_3$)[1] and is followed by glucose removal and transport to the Golgi apparatus. This glycan structure can be modified by mannose trimming and the addition of various other sugars, such as fucose, GlcNAc, and sialic acid. The Golgi complex also carries out the glycosylation of glycolipids and many O-linked glycoproteins (*5*−*11*). For all of these glycosylation reactions, nucleotide−sugars serve as the activated sugar donor (*12*).

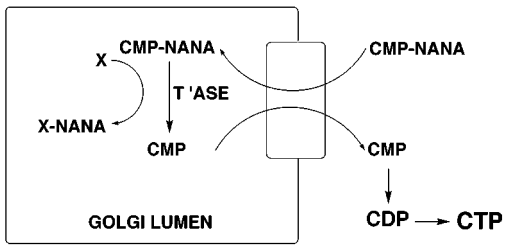

FIGURE 1: Diagram illustrating the key reactions involved in Golgi glycosylation reactions, using sialylation as an example. X represents a lipid or protein acceptor for the sialylation reaction.

Nucleotide−sugars accumulate in the cytosol after synthesis and must be transported into the lumen of the Golgi apparatus. Each nucleotide−sugar is imported into the Golgi apparatus via its own specific nucleotide−sugar transporter, a membrane-bound antiporter protein that will import and export both the nucleotide−sugar and NMPs, the ultimate nucleotide byproduct of glycosylation (*7*, *13*, *14*). Transport does not require an energy source. Rather, previous work indicates that two concentration gradients drive the import of the nucleotide−sugar: one created by the synthesis of nucleotide−sugars in the cytosol and their metabolism in the Golgi lumen,and another arising from the formation of NMPs in the Golgi lumen and their rapid conversion to NTPs in the cytosol [Figure 1 (*15*−*19*)]. Importantly, whereas the transporters slowly import nucleotide−sugars when assays only contain nucleotide−sugars on the cytosolic side of the membrane, the presence of NMPs on the lumenal side of

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[1] Abbreviations: acycloTMP, 1-[(2-phophoethoxy)methyl]thymidine; AZT, 3′-azido-2′-deoxythymidine; CMP-NANA, CMP-sialic acid; FAB, fast atom bombardment; Glc, glucose; GlcNAc, *N*-acetylglucosamine; HIV, human immunodeficiency virus; HRMS, high-resolution mass spectrometry; Man, mannose.

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the membrane dramatically stimulates import (*15*−*18*). This "trans stimulation" observed under these latter conditions results from a catalytic cycle where the transporters import the nucleotide−sugar and export the NMP. Thus, these proteins are typically described as "leaky" antiporters.

As Figure 1 suggests, high NMP concentrations in the cytosol could inhibit nucleotide−sugar transport by competing for the exterior binding face of the transporter. Indeed, we have previously shown that inhibition of nucleotide−sugar transport by AZTMP, the primary metabolite of the HIV chemotherapeutic AZT, dramatically alters the glycosylation of proteins and lipids, and the effects likely account for some of the side effects associated with AZT therapy (*20*, *21*). Changes in N-linked protein glycosylation included reduced synthesis of tri- and tetraantennary glycans, accumulation of biantennary glycans, shortening of polylactosamine chains, and a reduction in sialylation (*20*, *22*). A decrease in the length and/or synthesis of the glycosaminoglycans chondroitin and heparan sulfate was observed (R. Steet and R. Kuchta, unpublished data), as well as inhibition of *N*-acetylgalactosamine and sialic acid incorporation in glycolipids (*20*). The marked decrease in sialylation indicates that AZTMP significantly alters CMP-sialic acid transport into the Golgi complex.

To develop a better understanding of how NMPs interact with the CMP-sialic acid transporter, we examined a series of NMP analogues for their ability to both inhibit the import of CMP-sialic acid and serve as alternate substrates. Whereas the transporter requires an intact ribose ring and makes important contacts with the hydrogen in the 2′-ara position, the C2 carbonyl, and the C4 amine, it will tolerate substantial modifications of both the ribose ring and pyrimidine base. The implications of these results with respect to the development of novel glycosylation inhibitors that would target specific transporters and avoid interference with other biological pathways, such as DNA replication and repair, are discussed.

EXPERIMENTAL PROCEDURES

Materials. All reagents were of the highest quality commercially available. 5-Methyluridine, 2,4-dihydroxypyridine ribofuranoside, 2-hydroxypyridine, 2-hydroxypyrimidine, and 4-hydroxypyrimidine were purchased from Sigma, 4-*N*-acetylcytosine and 1,3,5-tri-*O*-benzoylribofuranside were from Aldrich, Dess−Martin periodinane was from Lancaster, "Ultrapure" sucrose was obtained from USB, CMP-*N*-acetylneuraminic acid synthetase was from Calbiochem, [^{32}P]-CTP (3000Ci/mmol) and CMP-[^{3}H]sialic acid (32.8 Ci/mmol) were from NEN, and nitrocellulose membrane filters (0.45 μm, white HAWP, 25 mm) were from Millipore.

Synthesis of 5-Methyl-5′-UMP and 2,4-Dihydroxypyridine Ribofuranoside MP. The 5′-monophosphate was prepared directly by selective phosphorylation of the nucleoside with phosphorus oxychloride in trimethyl phosphate (*23*, *24*). 5-Methyl-5′-UMP: ^{1}H NMR (500 MHz, D_2O) δ 7.84 (d, J = 1.0 Hz, 1H), 5.99 (dd, J = 2.1, 5.7 Hz, 1H), 4.42 (dt, J = 2.0, 5.5 Hz, 1H), 4.35 (m, 1H), 4.25 (m, 1H), 4.03 (m, 2H), 1.94 (d, J = 1.0 Hz, 3H); ^{31}P NMR (202 MHz, D_2O) δ 2.53 (s); HRMS *m*/*z* (FAB) calcd for $C_{10}H_{16}N_2O_9P^+$ 339.0593, found 339.0589. 2,4-Dihydroxypyridine ribofuranoside MP: ^{1}H NMR (500 MHz, D_2O) δ 7.84 (d, J = 8.0 Hz, 1H), 6.18 (m, 2H), 4.32 (m, 2H), 4.24 (m, 1H), 4.10 (dt, J = 3.1, 11.9 Hz, 1H), 4.03 (dt, J = 4.5, 11.3 Hz, 1H); ^{31}P NMR (202 MHz, D_2O) δ 2.10 (s); HRMS *m*/*z* (FAB) calcd for $C_{10}H_{13}NO_9P$ 322.0328, found 322.0338.

Synthesis of 2-Hydroxypyridine, 2-Hydroxypyrimidine, and 4-Hydroxypyrimidine Ribofuranoside 5′-Monophosphates. These compounds were synthesized in a one-pot procedure from the appropriate free base and the tetra-*O*-acetylribofuranose, followed by deacetylation (*25*). Monophosphorylation was completed as described above. 2-Hydroxypyrimidine ribofuranoside MP: ^{1}H NMR (500 MHz, D_2O) δ 8.71 (dd, J = 2.6, 6.8 Hz, 1H), 8.62 (dd, J = 2.8, 4.4 Hz, 1H), 6.82 (dd, J = 4.4, 6.8 Hz, 1H), 5.96 (d, J = 1.8 Hz, 1H), 4.26−4.36 (m, 4H), 4.09 (ddd, J = 2.2, 5.0, 12.0 Hz, 1H); ^{31}P NMR (202 MHz, D_2O) δ 1.49 (s); HRMS *m*/*z* (FAB) calcd for $C_9H_{14}N_2O_8P$ 307.0331, found 307.0346. 2-Hydroxypyridine ribofuranoside MP: ^{1}H NMR (500 MHz, D_2O) δ 8.12 (dd, J = 1.5, 7.1 Hz, 1H), 7.65 (ddd, J = 2.0, 6.9, 9.0 Hz, 1H), 6.64 (m, 2H), 6.23 (d, J = 2.9 Hz, 1H), 4.27−4.36 (m, 3H), 4.15 (ddd, J = 2.4, 4.2, 11.9 Hz, 1H), 4.05 (ddd, J = 3.1, 5.0, 12.0 Hz, 1H); ^{31}P NMR (202 MHz, D_2O) δ 1.35 (s); HRMS *m*/*z* (FAB) calcd for $C_{10}H_{13}NO_8P$ 306.0379, found 306.0375. 4-Hydroxypyrimidine ribofuranoside MP: ^{1}H NMR (500 MHz, D_2O) δ 8.63 (s, 1H), 4.11 (d, J = 2.4, 7.8 Hz, 1H), 6.53 (d, J = 7.8 Hz, 1H), 5.62 (d, J = 6.6 Hz, 1H), 4.45 (t, J = 5.8 Hz, 1H), 4.38 (m, 2H), 4.07 (m, 2H); ^{31}P NMR (202 MHz, D_2O) δ 1.33 (s); HRMS *m*/*z* (FAB) calcd for $C_9H_{12}N_2O_8P$ 307.0331, found 307.0335.

Synthesis of 2′-Methyl(ara)-5′-CMP. N^4-Acetylcytosine and 1,2,3,5-tetra-*O*-benzoyl-2-*C*-methyl-β-D-ribofuranose (*26*) were coupled in a one-pot procedure as previously described and deprotected using anhydrous ammonia in methanol to yield 2′-ara-methylcytidine which matched previously reported material (*27*). The monophosphate was generated as described above and then purified by RP-HPLC using a Beckman ultrasphere semipreparative column, eluting with 0.1 M triethylammonium bicarbonate. ^{1}H NMR (500 MHz, D_2O): δ 0.97 (3H), 3.87 (1H, d, J = 9.3 Hz), 3.92 (2H, m), 4.07 (1H, m), 5.91 (1H, s), 5.95 (1H, d, J = 6.5 Hz), 7.90 (1H, d, J = 6.5 Hz). ^{31}P NMR (202 MHz, D_2O): δ 2.07 (s). HRMS *m*/*z* (FAB) calcd for $C_{10}H_{17}N_3O_8P^+$ 338.0753, found 338.0759.

Buffers Used. X% SKM = X% sucrose, 0.1 M KH_2PO_4 (pH 7.6), and 5 mM $MgCl_2$; KM = 0.1 M KH_2PO_4 (pH 7.6) and 5 mM $MgCl_2$; STKM = 8.3% sucrose, 150 mM KCl, 10 mM Tris-HCl (pH 7.6), and 1 mM $MgCl_2$.

Preparation of Golgi Vesicles. Golgi-enriched membranes were isolated from rat liver (rats fasted for 24 h prior to sacrifice) using a modification of the procedure of Leelavathi et al. (*28*). Liver was homogenized in four volumes of 16% SKM plus 1 mM PMSF using a Bamix "magic wand" M122 homogenizer. Centrifugation at 8500*g* was followed by filtering the supernatant through two layers of cheesecloth, placing the clarified liquid on top of 10 mL of 38% SKM in ultracentrifuge tubes, and centrifugation for 90 min at 28000 rpm in an SW28 rotor at 4 °C. The crude membranes were removed from the interface using an 18G needle and syringe, pooled, and adjusted slowly to 33% sucrose with either KM or 40% SKM. Crude membranes were transferred to an ultracentrifuge tube, carefully overlayed with 12 mL of 16% SKM, and centrifuged as described above. Highly enriched membranes were collected from the interface as before,

pooled, adjusted slowly (over 10 min) to 8.3% sucrose with KM, and collected by centrifugation as above. The resulting pellet was resuspended in a minimal volume of STKM, divided into aliquots, frozen in N_2(l), and stored at −70 °C.

Analytical Procedures. Protein concentrations were determined using the Bio-Rad DC protein assay kit using bovine serum albumin as a standard. Enrichment of Golgi membranes was measured by determining the enrichment in sialyltransferase activity relative to the homogenate. Assays were performed essentially as previously described (*21*) and contained 0.25 μM CMP-[^{3}H]sialic acid and 2 mg/mL asialofetuin. Proteins were precipitated using trichloroacetic acid, and the amount of [^{3}H]sugar incorporated into proteins was measured by collection on glass microfiber filters followed by scintillation counting. Golgi preparations were typically enriched in sialyltransferase activity 15−20-fold over the homogenate.

Synthesis of [^{32}P]CMP-Sialic Acid. [^{32}P]CMP-sialic acid was prepared from [α-^{32}P]CTP and *N*-acetylneuraminic acid using a modification of the procedure described by Liu et al. (*29*). Briefly, [α-^{32}P]CTP (1 mCi, 0.33 nmol) was incubated with *N*-acetylneuraminic acid (0.85 μmol), 10 mM $MgCl_2$, 100 mM Tris-HCl (pH 9.5), and 0.2 unit of CMP-*N*-acetylneuraminic acid synthetase in a reaction volume of 200 μL at 37 °C for 2 h. The reaction was loaded onto a silica TLC plate and developed in 7:3 EtOH:1 M NH_4OAc (pH7). The product band was identified by phosphorimaging and comparison to a CMP-sialic acid standard. After elution from the silica with H_2O, the [^{32}P]CMP-sialic acid was lyophilized to dryness, resuspended in a minimal volume of 10 mM Tris-HCl (pH 7.6), and stored at −20 °C. Typically, yields were >90% and contained <5% radiochemical impurities.

Transport Assay for Measuring the Import of CMP-Sialic Acid into Golgi Vesicles. Assays were based on that described previously by Waldman and Rudnick (*16*). Golgi membranes (100 μg of protein) were incubated at 37 °C with 0.25 M (8.3%) sucrose, 150 mM KCl, 10 mM Tris-HCl (pH 7.6), 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 5 mM 2,3-dimercaptopropanol, and 1 μM [^{32}P]CMP-sialic acid (ca. 44000 cpm/pmol) in a final volume of 100 μL. Reactions were stopped by addition of 1 mL of ice-cold STKM, membranes were collected on a nitrocellulose filter, and the filters were washed three times with 5 mL of cold STKM prior to scintillation counting.

Trans Stimulation Assay. A modification of the mechanical loading protocol of Waldman and Rudnick (*30*) was used. Golgi vesicles (ca. 0.9 mg of protein) were triturated in the presence of the desired intravesicular buffer (final volume of 650 μL) using a 21G needle and syringe. In addition to the analogue of interest, the loading buffers contained 0.25 M (8.3%) sucrose, 150 mM KCl, 10 mM Tris-HCl (pH 7.6), 1 mM $MgCl_2$, 0.5 mM 2,3-dimercaptopropanol, 0.5 mM 5′-adenylyl imidodiphosphate, and 3 mM disodium ethylenediaminetetraacetate. These samples were then centrifuged at 178000*g* for 5 min at 4 °C. The pellet was resuspended in 20 μL of the supernatant using a 50 μL Hamilton syringe. Portions of this preloaded vesicle suspension (2 μL) were used in the CMP-sialic acid transport assay described above. Because the preloaded suspensions contributed a small amount of extravesicular analogue to the reaction medium, these transport assays were compared with control reactions containing the same final concentration of extravesicular analogue. The protein concentration was individually determined for each preloaded Golgi sample and used to calculate the corresponding CMP-sialic acid import rate.

RESULTS

Synthesis of [^{32}P]CMP-Sialic Acid and Transport into the Golgi Apparatus. We used CMP-*N*-acetylneuraminic acid synthetase to generate [^{32}P]CMP-sialic acid from [α-^{32}P]CTP and sialic acid (see Experimental Procedures). This rapid synthesis typically gave >90% yield of [^{32}P]CMP-sialic acid with >95% radiochemical purity. The resulting [^{32}P]CMP-sialic acid has a specific activity (3000 Ci/mmol) much greater than that of the commercially available CMP-[^{3}H]-sialic acid (20−60 Ci/mmol) and is much less expensive.

We initially performed several control experiments to verify that a change in the type and location of the isotope used would not affect the kinetic parameters of CMP-sialic acid transport by Golgi membranes. Under initial velocity conditions (0−90 s), Golgi membranes transported both [^{32}P]-CMP-sialic acid and CMP-[^{3}H]sialic acid similarly.[2] In each case, import remained linear with time for at least 90 s. Importantly, the steady-state parameters for [^{32}P]CMP-sialic acid [$V_{max} = 2.0 \pm 0.3$ pmol/(mg·min), $K_m = 1.0 \pm 0.1$ μM, $V_{max}/K_m = 2.0 \pm 0.6$ pmol/(mg·min·μM)] and CMP-[^{3}H]sialic acid [$V_{max} = 2.5 \pm 0.3$ pmol/(mg·min), $K_m = 1.5 \pm 0.3$ μM, $V_{max}/K_m = 1.7 \pm 0.6$ pmol/(mg·min·μM)] were similar, indicating that the CMP-sialic acid transporter does not differentiate between these two compounds as substrates for transport.

Investigation of the Binding Specificity of the CMP-Sialic Acid Transporter. To better understand how the CMP-sialic acid transporter binds NMPs, we examined the ability of a series of NMPs to inhibit [^{32}P]CMP-sialic acid import into Golgi membranes. Only pyrimidine analogues were tested because previous work established that the CMP-sialic acid transporter strongly discriminates against purine NMPs (*31*). Since a pyrimidine monophosphate is a normal substrate for the enzyme, we anticipated that these analogues would competitively inhibit CMP-sialic acid import. Indeed, detailed kinetic analysis showed that four NMPs of quite different structure and potency of inhibition [CMP, AZTMP, 5-ethyl-UMP, and araCMP, $K_i = 1.1-31$ μM (Table 1)] all competitively inhibited CMP-sialic acid transport (data not shown). Thus, it appears likely that NMPs will generally exhibit competitive inhibition of CMP-sialic acid transport. For the remainder of the compounds, we measured IC_{50} values at 1 μM CMP-sialic acid and calculated the corresponding K_I values (Table 1).

Interactions between the Base and the Transporter. We examined a series of pyrimidine monophosphate analogues to identify contacts between the base and the transporter (Figure 2). The addition of a small group at C5 (chlorine, methyl, and ethyl groups) resulted in either little effect (compare 5-chloro-3′-fluoro-ddUMP with 3′-fluoro-3′-deoxy-

[2] At very long time points, import of [^{32}P]CMP-sialic acid appeared slower than that of CMP-[^{3}H]sialic acid. This likely resulted from hydrolysis of the CMP-sialic acid inside the vesicles. In the case of [^{32}P]CMP-sialic acid, the resulting [^{32}P]CMP can be exported from the Golgi membranes, while in the case of CMP-[^{3}H]sialic acid, the resulting [^{3}H]sialic acid is not exported [data not shown (*31*)].

Table 1: Inhibition of CMP-Sialic Acid Transport by NMP Analogues[a]

compound	$K_I \pm$ SE (μM)	R_1	R_2	R_3	R_4	R_5	R_6	R_7
CMP	1.1 ± 0.4	H	NH_2	N	O	H	OH	OH
TMP	7.0 ± 0.6	CH_3	O	NH	O	H	H	OH
UMP	20 ± 3	H	O	NH	O	H	OH	OH
5-methylUMP	31 ± 6	CH_3	O	NH	O	H	OH	OH
5-ethylUMP	11 ± 2	CH_2CH_3	O	NH	O	H	OH	OH
2-hydroxypyridine ribofuranoside MP	10 ± 1	H	H	CH	O	H	OH	OH
2-hydroxypyrimidine ribofuranoside MP	16 ± 2	H	H	N	O	H	OH	OH
2,4-dihydroxypyridine ribofuranoside MP	18 ± 3	H	OH	CH	O	H	OH	OH
4-hydroxypyrimidine ribofuranoside MP	≫100	H	O	N	H	H	OH	OH
3-*N*-methyl-3′-azido-2′-deoxyUMP	≫150	H	O	NCH_3	O	H	H	N_3
3-*N*-methyl-3′-azidoTMP	≫100	CH_3	O	NCH_3	O	H	H	N_3
AZTMP	4.8 ± 0.3	CH_3	O	NH	O	H	H	N_3
3′-deoxyTMP	12 ± 1	CH_3	O	NH	O	H	H	H
3′-fluoro-3′-deoxyTMP	5.4 ± 1.4	CH_3	O	NH	O	H	H	F
5-chloro-3′-fluoro-ddUMP	7.9 ± 0.4	Cl	O	NH	O	H	H	F
3′-urea-3′-deoxyTMP	29 ± 4	CH_3	O	NH	O	H	H	urea
2′-deoxyCMP	2.4 ± 0.9	H	NH_2	N	O	H	H	OH
2′-deoxyUMP	21 ± 3	H	O	NH	O	H	H	OH
3′-azido-2′-deoxyUMP	52 ± 5	H	O	NH	O	H	H	N_3
araCMP	31 ± 3	H	NH_2	N	O	OH	H	OH
2′-methyl(ara)CMP	34 ± 2	H	NH_2	N	O	CH_3	OH	OH
2′-fluoro(ara)TMP	53 ± 3	CH_3	O	NH	O	F	H	OH
acycloTMP	≫500	CH_3	O	NH	O			

[a] Assays contained 1 μM CMP-sialic acid and were performed as described under Experimental Procedures. IC_{50} values were determined from a linear fit of [I] vs $1/v$, and these values were then used to calculate the corresponding K_I value. Each compound was assayed a minimum of two times.

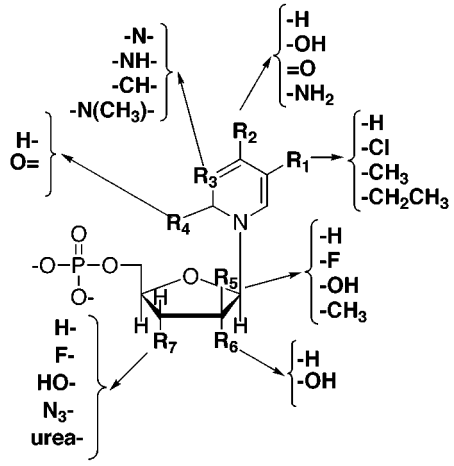

FIGURE 2: NMP diagram highlighting the structural features that were modified in order to determine their importance for binding to the CMP-sialic acid transporter.

TMP and UMP with 5-methylUMP and 5-ethylUMP) or slightly enhanced binding (compare TMP with 2′-deoxyUMP). The importance of the C4 amino group was assessed by converting it to either a hydrogen, a hydroxyl, or a carbonyl group. Replacing the C4 amino group with a hydrogen significantly decreased inhibition (compare CMP with 2-hydroxypyrimidine ribofuranoside MP), indicating that the exocyclic amino group interacts with the enzyme. Likewise, replacing the C4 amino group with a hydroxyl (compare 2-hydroxypyridine ribofuranoside MP with 2,4-dihydroxypyridine ribofuranoside MP) or a carbonyl (compare CMP with UMP) resulted in compounds that bound much more weakly to the transporter. Since changing the nature of the group at C4 will change the hydrogen-bonding characteristics of N3, we examined the effect of converting N3 to a CH to minimize the H-bonding potential of this position. Unlike the large effects observed upon altering the exocyclic group at C4, converting N3 to a methylene (compare 2-hydroxypyrimidine ribofuranoside MP with 2-hydroxypyridine ribofuranoside MP) did not appreciably affect binding of the NMP to the enzyme. Although the transporter readily tolerated the replacement of N3 with CH, two groups of similar size, it strongly discriminated against increasing the size of the exocyclic group at N3 (compare 3-methylAZTMP and 3-methyl-3′-azido-2′-deoxyUMP with AZTMP and 3′-azido-2′-deoxyUMP, respectively), suggesting that the enzyme makes a close contact at N3. We also examined the importance of the C2 carbonyl (compare UMP with 4-hydroxypyrimidine ribofuranoside MP) and found that the transporter requires this carbonyl for tight binding. Together, these data indicate that while the enzyme makes important contacts with the exocyclic groups at C2 and C4, it will tolerate modification at C5 and N3.

Interactions between the Sugar and the Transporter. To bind the CMP-sialic acid transporter, an intact ribose ring is clearly required, since acycloTMP gave no measurable inhibition at concentrations as high as 225 μM. Replacing the 3′-hydroxyl with either hydrogen, fluorine, azide, or urea minimally affected inhibition, indicating that the enzyme does not make a critical contact with the 3′-hydroxyl and will accommodate substitutions of very different sizes and chemical properties. Likewise, the enzyme does not interact significantly with the 2′-ribo hydroxyl, since replacing this group with hydrogen had little effect on inhibition. In contrast, the 2′-ara hydrogen makes an important contact, as replacing this hydrogen with either a methyl, a hydroxyl, or a fluorine results in compounds that bind very poorly to the transporter.

Transport of NMP Analogues by the CMP-Sialic Acid Transporter. In addition to determining the structural features necessary for transporter binding, we addressed the question of whether the nucleotide analogues also served as substrates. As described in the introduction, NMPs that can be trans-

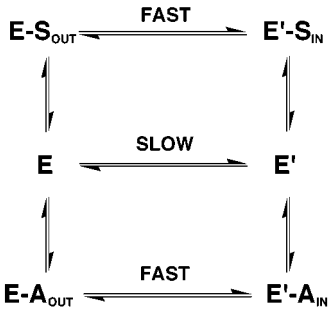

FIGURE 3: Single-site model for a nucleotide−sugar translocator that accounts for trans stimulation.[3] After the translocator imports a substrate, completion of the cycle requires the enzyme to reset. This can occur slowly in the absence of ligand binding or rapidly upon ligand binding and export of this ligand (bottom). E = transporter ready to import (binding site facing cytosol); E′ = transporter after import (binding site facing lumen); S_{out}, A_{out} = substrate in cytosol; S_{in}, A_{in} = substrate in lumen.

ported by the CMP-sialic acid transporter will trans stimulate CMP-sialic acid import when present in the lumen of the membranes. While the actual mechanism of the reaction remains unclear, a model such as that shown in Figure 3 accommodates the kinetic properties of the transporter.[3] When only CMP-sialic acid is present, the rate of transport is limited by a slow step after import of a CMP-sialic acid that "resets" the transporter into a state capable of importing another CMP-sialic acid molecule. However, when the lumen of the membrane contains a NMP that is also transported, this slow step is avoided and the rate of CMP-sialic acid import increases. Thus, we measured NMP transport indirectly by determining the ability of NMPs to trans stimulate the import of CMP-sialic acid into Golgi vesicles.[4]

We measured trans stimulation with a diverse subset of the analogues described earlier. The extent of trans stimulation is given by the rate of CMP-sialic acid transport into Golgi vesicles that contain the NMP of interest divided by the rate of transport into control Golgi vesicles that do not contain the NMP. Vesicles were preloaded with either 1 mM NMP or only buffer (control vesicles), and the rate of CMP-sialic acid import was measured under initial velocity conditions. If the export rate of the preloaded analogue exceeds the rate of empty transporter conversion (the slow step in Figure 3), trans stimulation of CMP-sialic acid import will occur. Alternatively, the analogue could bind the lumenal active site and not be transported. In this case, the active site could no longer reset to the cytosolic face of the membrane such that inhibition of CMP-sialic acid import would result. Finally, the analogue could be transported at the same rate as an empty site, thus not affecting CMP-sialic acid import.

[3] One can also construct an analogous two-site model that accounts for trans stimulation. In this more complicated model, the two sites would transport substrate in the opposite direction relative to the membrane, and transport could occur with either one or both sites occupied. However, the rate of transport with ligand bound to only one site must be slower than when both sites are occupied.

[4] This could have been approached directly by generating a radiolabeled version of each analogue for use in the transport assay. However, this not only would have been excessively time-consuming but would not conclusively report activity of the CMP-sialic acid transporter as the Golgi membranes contain other nucleotide−sugar transporters that could also transport these analogues.

Table 2: Trans Stimulation of CMP-Sialic Acid Import into Golgi Vesicles by NMP Analogues[a]

analogue tested	K_I (μM)[b]	% of control (preloading with 1 mM NMP)
CMP	1.1	650 ± 40
AZTMP	4.8	400 ± 80
TMP	7.0	320 ± 90
2-hydroxypyridine ribofuranoside MP	9.8	340 (n = 1)
2,4-dihydroxypyridine ribofuranoside MP	18	210 ± 100
UMP	20	250 ± 20
3′-ureaTMP	29	160 ± 30
araCMP	31	120 ± 60
3′-azido-2′-deoxyUMP	52	120 ± 20
4-hydroxypyrimidine ribofuranoside MP	≫100	70 ± 10
3-*N*-methyl-3′-azido-2′-deoxyUMP	≫100	65 ± 2
3-*N*-methyl-3′-azidoTMP	≫150	110 ± 40

[a] Import was measured in assays containing 1 μM CMP-sialic acid and Golgi vesicles that had been mechanically preloaded with either the analogue of interest (1 mM) or only buffer. The data are reported as the rate of import into analogue-loaded vesicles divided by the rate of import into buffer-loaded vesicles (×100%) and are the average of at least two independent experiments unless otherwise noted. [b] From Table 1.

As expected from previous studies (*15*, *32*), lumenal CMP significantly enhanced CMP-sialic acid import (6.5-fold; Table 2). UMP similarly stimulated CMP-sialic acid import, even though it binds the transporter much less tightly than CMP, indicating that this transporter uses UMP as a substrate. Likewise, several of the unnatural NMPs resulted in significant trans stimulation, indicating that the transporter also recognized them as substrates (Table 2). Interestingly, some of the poorly bound analogues had either unmeasurable or slightly inhibitory effects on CMP-sialic acid import in the trans stimulation assays, suggesting that tight binding and translocation by the CMP-sialic acid transporter are related.

DISCUSSION

We have previously shown that AZTMP inhibits the import of CMP-sialic acid into Golgi membranes and that this inhibition likely accounts for the decreased incorporation of sialic acid into glycoproteins and glycolipids when cultured cells are treated with AZT (*20*). Here we examined a diverse set of NMP analogues to help to define those portions of an NMP with which the CMP-sialic acid transporter makes important interactions. We found that the transporter is tolerant of alterations in several positions, with notable exceptions for the 2′-ara hydrogen of the ribose ring and the exocyclic groups at C2, N3, and C4 of the base.

Specificity of the CMP-Sialic Acid Transporter. The CMP-sialic acid transporter's binding specificity shares significant similarities with that of the UDP-*N*-acetylglucosamine transporter, including the necessity of an intact ribose ring, neutral or slightly positive effect of a small exocyclic group at C5, tolerance of large modifications at the 2′- and 3′-ribopositions, and an important contact with the 2′-ara hydrogen (*33*). In contrast to the similarities in sugar binding, these two transporters clearly differ with regard to binding of the base. Whereas the UDP-*N*-acetylglucosamine trans-

Table 3: Cytosolic Concentrations of Nucleotide−Sugar, UMP, and CMP[a]

compound	cellular concn (μM)
CMP-sialic acid	450[b]
UDP-glucose	170[b]
UDP-*N*-acetylglucosamine	530[c]
UDP-galactose	60[b]
UDP-*N*-acetylgalactosamine	180[c]
UMP	80[d]
CMP	8[d]

[a] Measured cytosolic concentrations of several nucleotide−sugars, CMP, and UMP as reported in the literature. [b] Data determined as nanomoles per milligram of cell protein in Chinese hamster ovary cells (*34*). Corresponding concentrations were calculated using 0.695 mg of cell protein/10^6 cells (average value across eight mammalian cell lines) and 2.5 μL/10^6 cells [a commonly used estimate (*19*)]. [c] Data determined as micromoles per 4.5×10^8 HeLa cells (*35*). Concentrations calculated as above. [d] Data determined as picomoles per 10^6 cells in chicken embryo fibroblasts (*19*). Concentrations calculated as above.

porter bound CMP, UMP, and TMP with similar affinities, the CMP-sialic acid transporter strongly discriminated against UMP and TMP. The lowered binding affinity of these two pyrimidines could be attributed to the direct change at C4 and also the indirect change in hydrogen-bonding character simultaneously caused at N3. However, comparison of UMP with 2,4-dihydroxypyridine ribofuranoside MP and 2-hydroxypyrimidine ribofuranoside MP with 2-hydroxypyridine ribofuranoside MP showed no significant change in the K_I. Since these comparisons involved substitution of an N3−H and N3, respectively, with C3−H, a change that minimized the possibility of H-bonding at this position but had little effect on the size of this position (N−H = 3.136 Å, C−H = 3.364 Å), changes in H-bonding capacity of N3 cannot account for the enzyme's ability to discriminate between CMP and UMP/TMP. Thus, this reduced affinity is likely due in large part to the alteration of the exocyclic C4-amino group.

Biologically, the ability of the CMP-sialic acid transporter to strongly discriminate against UMP may be essential for efficient sialylation, whereas discrimination between UMP and CMP by the UDP-GlcNAc transporter is probably not required for efficient GlcNAc-ylation. The cellular CMP-sialic acid concentration is substantially lower than that measured for UDP-GlcNAc (Table 3), whereas the concentration of UMP is much higher than that of CMP (*19*). Hence, in the absence of any discrimination against UMP, the cellular levels of UMP would likely inhibit CMP-sialic acid import more severely than UDP-GlcNAc import. Discrimination against UMP may also help to reduce the lumenal concentration of CMP, thereby minimizing the amount of inhibition of sialyltransferases by CMP. Previous studies of various sialyltransferases have shown that, in some cases, elevated CMP concentrations significantly inhibit these enzymes (*36*−*39*). For example, in assays containing 1 mM CMP-sialic acid, 1 mM CMP inhibited G_{D3} synthase by approximately 61% and G_{D1a} synthase by only approximately 21% (*36*). While the lumenal concentrations of CMP and UMP are unknown, the much higher cellular concentration of UMP suggests that the lumenal concentration of UMP will likewise exceed that of CMP. Thus, the ability of the CMP-sialic acid translocator to discriminate against UMP may enable it to maintain a very low lumenal concentration of CMP in the presence of much higher levels of UMP and, consequently, minimize effects of CMP on different sialyltransferases.

NMP-derived glycosylation inhibitors, such as AZTMP, can interfere with cellular RNA and DNA polymerases when metabolized to their triphosphate forms. Although the polymerases likely do not directly contact the Watson−Crick hydrogen-bonding face of the base (*43*), the hydrogen-bonding groups provide critical information to both DNA and RNA polymerases. Correct hydrogen bonding between the incoming (d)NTP and the next template base to be read results in rapid phosphodiester bond formation, whereas improper base pairing results in much slower (d)NTP polymerization (*40*−*42*).[5] In contrast to nucleotide polymerases, the CMP-sialic acid transporter readily binds and transports nucleotide analogues that have altered hydrogen-bonding characteristics in the base. Most notably, the ring nitrogen at position 3 can be replaced by carbon with no detectable effects on binding/transport and the C4 amine replaced with either hydrogen or a hydroxyl, resulting in only moderate decreases in binding. Hence, it should be possible to synthesize nucleoside analogues that will potently inhibit nucleotide−sugar transport as the monophosphate, but if converted to the triphosphate, will not interfere with other nucleotide-utilizing biological pathways. Experiments to test this hypothesis are in progress.

The 2′-ara hydrogen likely interacts with a hydrophobic side chain in the active site of the CMP-sialic acid transporter. Replacing this hydrogen with a considerably larger hydrophilic (OH) or hydrophobic (CH_3) group significantly decreased binding, consistent with the 2′-ara H interacting closely with the transporter. Replacement of this H with F, which is only slightly larger than H but is much more electronegative, also greatly decreased binding, suggesting that the interacting group from the enzyme is likely hydrophobic. Two alternative possibilities for the effects of modifying the 2′-ara H on binding include changes in either the sugar pucker or the pK_a of the 3′-OH. Changes in sugar pucker of the free NMP likely do not account for decreased binding because NMR studies have shown that araC and cytidine have similar sugar pucker in solution [approximately 50% 2′-endo and 50% 3′-endo (*44*)]. The differential effects of substituents in the ribo and arabino configurations cannot be due to effects on the pK_a of the 3′-OH since the translocator does not require a 3′-OH group for tight binding. While only the 2′-ara F and 2′-ara OH modifications were tested with the UDP-GlcNAc transporter, the tremendous similarity between these two transporters with respect to the effects of modifying the ribose ring suggests that the interaction between the UDP-GlcNAc transporter and the 2′-ara H also likely involves a hydrophobic side chain.

Transport of NMPs by the CMP-Sialic Acid Transporter. Consistent with previous studies (*15*, *29*), we found that the CMP-sialic acid translocator transported its cognate NMP (CMP), and preloading the lumen of the Golgi membranes with CMP greatly stimulated CMP-sialic acid transport.

[5] While nucleotides incapable of Watson−Crick hydrogen bonding, such as the thymine analogue difluorotoluene, can be specifically incorporated by the Klenow fragment across from their appropriate partners in templates, they are utilized much less efficiently than the endogenous substrates (*43*).

Additionally, we found that this transporter readily translocated a number of nucleoside analogues, including AZTMP. Thus, the effects of AZTMP on cellular sialylation reactions likely result from AZTMP serving as an alternate substrate for the CMP-sialic acid transporter and consequent depletion of CMP-sialic acid from the lumen of the Golgi membrane. While the imported AZTMP could potentially inhibit sialyl transferases, previous studies found no detectable inhibition of these enzymes (*21*).

Even though the CMP-sialic acid transporter used a variety of NMP analogues as substrates, the rate of transport of these analogues appeared slower than for the cognate CMP. Whereas preloading Golgi membranes with 1 mM CMP increased CMP-sialic acid import 6.5-fold, preloading the membranes with the other analogues tested resulted in significantly less stimulation of import. Potentially, this lower level of trans stimulation could have resulted from either a lower rate of transport across the membrane as compared to CMP or much weaker binding to the transporter such that 1 mM NMP did not saturate the active site. This latter explanation seems unlikely since the membranes contained 1 mM NMP, and the measured K_is for those analogues that were clearly transported ranged from 4.8 μM (AZTMP) to 20.1 μM (UMP). While it is conceivable that NMPs interact less efficiently with the lumenal side of the translocator, this seems unlikely since, physiologically, the translocator serves to transport the normal NMP byproducts of the glycosylation reactions from the lumen of the Golgi to the cytosol.

Most of the NMPs tested gave significant trans stimulation of CMP-sialic acid import; however, either no effect or slight inhibition resulted from preloading the membranes with several of the weakly binding NMPs (Table 2). Since, for reasons noted above, the transporter was likely saturated with 2′-deoxy-3′-azidoUMP, araCMP, and 3′-ureaTMP (K_i = 52, 31, and 29 μM, respectively) under these conditions, these data indicate that the translocator transports these compounds but at a rate similar to that at which an empty active site moves across the membrane. For example, if the transporter had bound araCMP but was then unable to transport it across the membrane, this would have resulted in significant inhibition of CMP-sialic acid transport import. In the cases of 4-hydroxypyrimidine ribofuranoside MP, 3-*N*-methyl-AZTMP, and 3-*N*-methyl-3′-azido-2′-dUMP, the extent of transporter binding on the lumenal side of the Golgi membrane cannot be assessed due to the very weak binding of these NMPs. Again, however, high lumenal concentrations of these compounds resulted in almost undetectable effects on CMP-sialic acid transport. Together, these data suggest that it may be difficult to design NMPs that bind to the translocator and completely prevent the enzyme from transporting the NMP to the other side of the membrane.

These studies provide a detailed map of the structural preferences of the CMP-sialic acid transporter for tight binding and transport of NMPs. The wide variety of modifications that the enzyme will tolerate suggests that one can design novel and perhaps more specific nucleoside-based glycosylation inhibitors. Furthermore, the similarities between the UDP-GlcNAc and CMP-sialic acid transporters indicate that these two enzymes have structurally similar NMP binding sites.

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