Glycoconj J (2006) 23:371–375
DOI 10.1007/s10719-006-6735-y

A ^{1}H STD NMR spectroscopic investigation of sialylnucleoside mimetics as probes of CMP-Kdn synthetase*

Thomas Haselhorst · Melanie Oschlies · Tareq Abu-Izneid · Milton J. Kiefel · Joe Tiralongo · Anja K. Münster-Kühnel · Rita Gerardy-Schahn · Mark von Itzstein

Received: 17 November 2005 / Revised: 14 December 2005 / Accepted: 21 December 2005

Abstract CMP-Kdn synthetase catalyses the reaction of sialic acids (Sia) and CTP to the corresponding activated sugar nucleotide CMP-Sia and pyrophosphate PP_i. Saturation Transfer Difference (STD) NMR spectroscopy has been employed to investigate the sub-structural requirements of the enzyme's binding domain. Sialylnucleoside mimetics, where the sialic acid moiety has been replaced by a carboxyl group and a hydrophobic moiety, have been used in NMR experiments, to probe the tolerance of the CMP-Kdn synthetase to such replacements. From our data it would appear that unlike another sialylnucleotide-recognising protein, the CMP-Neu5Ac transport protein, either a phosphate group or other functional groups on the sialic acid framework may play important roles in recognition by the synthetase.

Keywords Nucleotide Synthetase · Sialic Acids · Sialic Acid mimetics · NMR spectroscopy

*Dedicated to the memory of Professor Dr Yasuo Inoue

T. Haselhorst · T. Abu-Izneid · M. J. Kiefel · J. Tiralongo · M. von Itzstein (✉)
Institute for Glycomics, Griffith University (Gold Coast Campus), PMB 50 Gold Coast Mail Centre, Queensland 9726, Australia
e-mail: m.vonitzstein@griffith.edu.au
Tel.:+61-7-55527025
Fax:+61-7-55529040

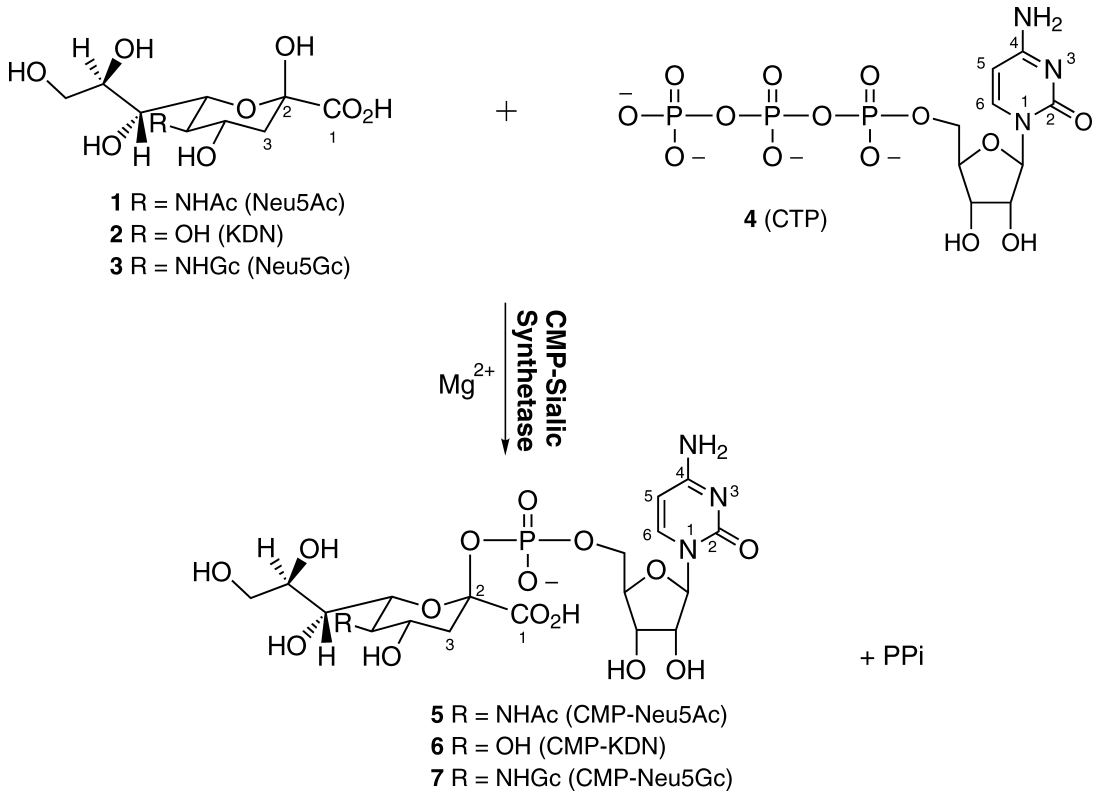
M. Oschlies
Institute for Glycomics, Griffith University (Gold Coast Campus), PMB 50 Gold Coast Mail Centre, Queensland 9726, Australia; Abteilung Zelluläre Chemie, Medizinische Hochschule Hannover (MHH), Carl-Neuberg-Strasse 1, D-30625 Hannover, Germany

A. K. Münster-Kühnel · R. Gerardy-Schahn
Abteilung Zelluläre Chemie, Medizinische Hochschule Hannover (MHH), Carl-Neuberg-Strasse 1, D-30625 Hannover, Germany

Introduction

We have had a longstanding interest [1–3] in the sialic acid family of carbohydrates, as well as sialic acid recognising proteins (SARPS), particularly enzymes, that are involved in their biosynthesis and degradation, and NMR spectroscopic methods for the investigation of enzyme-catalysed reactions [4,5]. It is well known that nucleotide synthetases are essential for the biosynthesis of activated carbohydrates, commonly referred to as glycosyl donors. These glycosyl donors are the key reactive intermediates that are used by the glycosyltransferase machinery for the biosynthesis of various glycoconjugates associated with eukaryotes and prokaryotes [6,7]. The sialic acids (Sia), for example *N*-acetylneuraminic acid (Neu5Ac, **1**), 3-deoxy-D-*glycero*-D-*galacto*-nonulosonic acid (Kdn, **2**) and *N*-glycolylneuraminic acid (Neu5Gc, **3**), are associated with a range of important glycoconjugates and the corresponding cytidine-5′-monophosphate sialic acid (CMP-Sia) is biosynthesised in the nucleus of eukaryotic cells, whereas all other nucleotide sugars are made in the cytoplasm. Our interest in sialic acids has led us to investigate the nucleotide synthetase that is essential for the activation of Kdn (**2**). Kdn (**2**), first discovered in fish egg polysialoglycoprotein [8], has been identified in Kdn-containing glycoconjugates from a large number of organisms ranging from bacteria to mammals [9–11]. The biosynthesis of Kdn-containing glycoconjugates engages several enzyme-catalysed steps. CMP-Kdn synthetase catalyses the activation of Neu5Ac (**1**), Kdn (**2**), Neu5Gc (**3**) using cytidine-5′-triphosphate (CTP) (**4**) to form the corresponding CMP-Sia CMP-Neu5Ac (**5**), CMP-Kdn (**6**) and CMP-Neu5Gc (**7**), respectively (Scheme 1).

A number of NMR spectroscopic-based, protein x-ray crystallographic-based, and biochemical studies of nucleotide synthetases have been reported [12–21]. These



Sch. 1 The biosynthesis of CMP-sialic acids catalysed by CMP-Kdn synthetase

studies have shed some light on key interactions between various nucleotides and the enzyme. NMR spectroscopy has become a valuable tool in the investigation of the elucidation of ligand-biomolecule interactions [22,23]. We have recently reported STD NMR spectroscopic-based binding epitope studies of the cytidine-5′-monophosphate-3-deoxy-D-*glycero*-D-*galacto*-nonulosonic acid synthetase (CMP-Kdn synthetase) in complex with its natural substrate CTP (**4**) and product CMP-Sia [24]. In that study the STD NMR analysis clearly showed that the anomeric proton of the ribose moiety of both CTP (**4**) and CMP-Neu5Ac (**5**) is in close proximity to the protein surface. This led us to conclude that the anomeric region of these compounds is likely to play an important role in the recognition and binding process. Furthermore, both that study and another study also led us to conclude that the Neu5Ac (**1**) *N*-acetyl moiety is involved in some interactions, particularly hydrophobic interactions, with the protein's active site [18,24].

We have previously shown that compounds which mimic sialic acids are useful probes in the identification of substrate or product sub-structural binding requirements of sialic acid-recognising proteins [3,25,26]. Therefore, we thought it of value to investigate such binding requirements of the nucleotide CMP-Kdn synthetase through the evaluation of selected sialylnucleoside mimetics (**8a** and **8b**). In the present work we have employed STD NMR spectroscopy to investigate the recognition of mimetics that have a complete replacement of the sialic acid moiety and phosphate bridge with simple α-linked carboxylic acids that incorporate lipophilic functionalities by the synthetase.

Materials and methods

General

CTP and D_2O (99.9%) were purchased from Sigma Aldrich (Australia). The sialylnucleoside mimetics **8a** and **8b** were prepared in our laboratory [27]. All NMR experiments were performed on a Bruker Avance 600 MHz NMR spectrometer, equipped with a 5 mm TXI probe with triple axis gradient. The measurements were performed at 298 K in 20 mM $MgCl_2$ (D_2O), pH 7.5.

Expression of CMP-Kdn synthetase

The CMP-Kdn synthetase was cloned and expressed in a similar way to that previously described [21]. The enzyme was concentrated and solvent exchanged in an Amicon Ultra-4 Centrifuge filter (Millipore, molecular weight cut-off 10 kDa) to give a final protein concentration of 15 μM.

^{1}H NMR experiments

^{1}H NMR experiments were performed prior to STD NMR experiments. The NMR-based enzyme assay in the presence of sialylnucleoside mimetics **8a** and **8b** as potential inhibitors were performed as described previously [24]. In brief: To a reaction mixture of 1 mM Neu5Ac and 5 mM CTP (**4**) the sialylnucleoside mimetics **8a** and **8b** were added to give a final concentration of 1 mM, 5 mM and 10 mM. In a typical experiment a ^{1}H NMR spectrum was recorded at $t = 0$ min. The synthetase was then added to the mixture and ^{1}H NMR spectra were recorded in 10 min time intervals. The signal of the H3eq proton of the product CMP-Neu5Ac was integrated and relative inhibition [%] was calculated in relation to the H3eq signal integral of the reaction without sialylnucleoside mimetics.

STD NMR experiments

Saturation Transfer Difference (STD) NMR experiments [28,29] were performed at 298 K and pH 7.5. The protein was saturated on-resonance at −0.5 ppm and off-resonance at 33 ppm with a cascade of 40 selective Gaussian shaped pulses, of 50 ms duration with a 100 μsec delay between each pulse in all STD NMR experiments. The total duration of the saturation time was set to 2 s. The sialylnucleoside mimetics **8a** and **8b** were added in a molecular ratio protein:ligand of 1:70 to give a final concentration of 1.05 mM. A total of 512 scans per STD NMR experiment were acquired and a WATERGATE sequence was used to suppress the residual HDO signal. A spin-lock filter with a strength of 5 kHz and a duration of 10 ms was applied to suppress protein background. For the competition STD NMR experiments 1.5 mM of the natural substrate CTP (**4**) was added to the synthethase:sialylnucleoside mimetic (**8a**) mixture to give a molecular ratio synthetase:**8a**:CTP of 1:70:100.

Results and discussion

We have recently reported [24] a preliminary NMR spectroscopic investigation of substrate and product sub-structural recognition and binding requirements of the nucleotide synthetase CMP-Kdn synthetase. In the present study we have investigated the capacity of this synthetase to recognize mimics of CMP-sialic acids in which the main sialic acid pyranose framework is replaced with simple α-linked carboxylic acids and lipophilic functionalities. In a number of sialic acid-recognising protein systems it has been suggested that only the carboxyl group plays a significant role in substrate recognition [3,26]. We have explored two such CMP-sialic acid mimetics, **8a** that contains a phenyl group as the lipophilic moiety, and **8b** that incorporates a methyl group, and their capacity to be recognized by the CMP-Kdn synthetase.

Figure 1a shows the partial ^{1}H NMR spectrum of mimetic **8a** in the presence of 15 μM CMP-Kdn synthetase and the corresponding STD NMR spectrum is shown in Figure 1b (molecular ratio protein:ligand of 1:70). This ^{1}H STD NMR spectrum clearly shows that the phenyl mimetic **8a** is recognized by the synthetase and that the mimetic's phenyl group appears to have the strongest interaction with the protein and as such the phenyl group must be in close proximity to the protein surface. The H1 proton of the ribose moiety in **8a** is also observed in the ^{1}H STD NMR spectrum at 5.75 ppm although the signal is weak in comparison to the phenyl moiety's signal. This is in stark contrast to the results from our preliminary study [24] in which the H1′ signal of the ribose moiety of the substrate and product CTP (**4**) and CMP-Sia such as CMP-Neu5Ac (**5**) respectively, was the strongest in

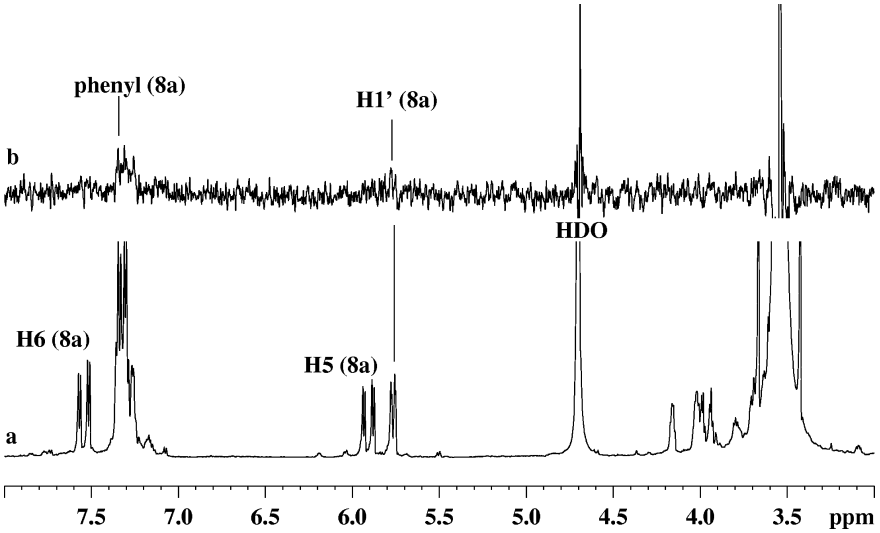


Fig. 1 (a) ^{1}H NMR spectrum and (b) STD NMR spectrum of sialylnucleoside mimetic **8a** (1.05 mM) in the presence of 15 μM CMP-Kdn synthetase (protein:ligand ratio 1:70) with on-resonance saturation at −0.5 ppm and off-resonance at 33 ppm using a Gaussian pulse cascade with a total saturation time of 2 s. All spectra were recorded at 298 K and 600 MHz in 99.9% D_2O containing 20 mM $MgCl_2$ (pH 9.0).

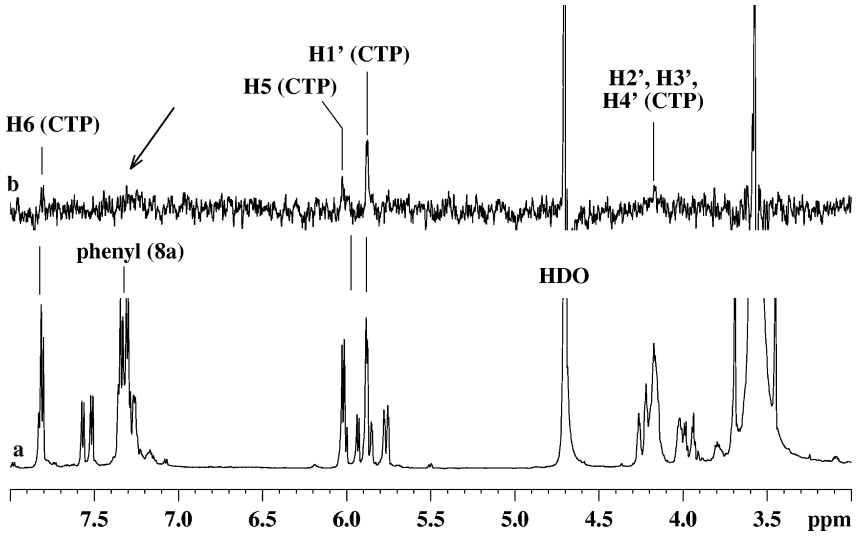
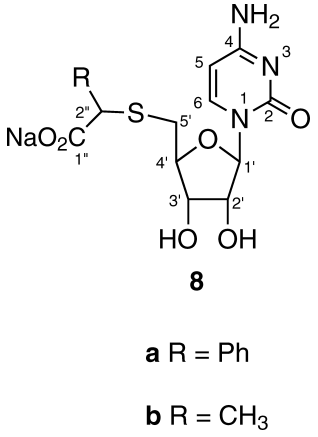


Fig. 2 (a) ^{1}H NMR spectrum and (b) STD NMR spectrum of a mixture of sialylnucleoside mimetic **8a** (1.05 mM) and substrate CTP (**4**) (1.5 mM) in the presence of 15 μM CMP-Kdn synthetase (protein:**8a**:CTP ratio 1:70:100) with on-resonance saturation at −0.5 ppm and off-resonance at 33 ppm using a Gaussian pulse cascade with a total saturation time of 2 s. All spectra were recorded at 298 K and 600 MHz in 99.9% D_2O containing 20 mM $MgCl_2$ (pH 9.0).

the ^{1}H STD NMR spectrum. Little, if any, of the other regions of the mimetic appear to interact with the protein.

To establish if the mimetic was binding in the synthetase active site a competition experiment was performed that sought to investigate the potential displacement of the mimetic **8a** with the known substrate CTP (**4**). Upon addition of CTP (**4**) to the synthetase–**8a** complex, complete loss of the mimetic **8a** signals in the ^{1}H STD NMR spectrum was observed with the concomitant appearance of the expected signals [24] for **4** as shown in Fig. 2. This outcome clearly demonstrates that firstly, the mimetic **8a** is binding in the active site of the synthetase and secondly, that the mimetic **8a** does not compete favourably with the natural substrate CTP (**4**). To determine the level of Kdn-synthetase inhibition by mimetic **8a** we employed a standard NMR-based assay, that we have previously described [24]. As expected no significant inhibition of the synthetase reaction by mimetic **8a** was observed (<5% at a mimetic concentration up to 10 mM, data not shown). Based on these experimental observations we conclude that while the mimetic **8a** is recognized by the synthetase and the phenyl group is not only accommodated but appears to be in close proximity to the active site protein surface, the overall binding efficiency of this mimetic is low.

To explore a less sterically-demanding mimetic we turned our attention to the CMP-sialic acid mimetic **8b**. This mimetic replaces the sialic acid pyranose framework with a carboxyl group and methyl group. Interestingly, using an identical ^{1}H STD NMR protocol to that employed with mimetic **8a**, no STD signals were observed for mimetic **8b** (data not shown). This observation suggests that while the synthetase active site can accommodate a larger aromatic moiety, that appears to facilitate a weak association with the enzyme, the smaller hydrophobic methyl group does not offer the same opportunity.



8

a R = Ph

b R = CH_3

Fig. 3 Cytidine-based sialylnucleoside mimetics.

Conclusions

We[6pc] have explored the capacity of the nucleotide CMP-Kdn synthetase to tolerate wholesale replacement of the sialic acid pyranose framework and phosphate bridge with simple α-linked carboxylic acids that incorporate lipophilic functionalities. From our current data and that previously published [24] it appears that loss of the phosphate bridge and/or other functionality associated with the sialic acid pyranose framework severely reduces recognition of these mimetics by the synthetase.

Acknowledgments MvI gratefully acknowledges the financial support of the Australian Research Council and the award of an Australian Federation Fellowship. MvI also thanks the Alexander von Humboldt Stiftung for the award of a von Humboldt Forschungspreis and the Australian National Health and Medical Research Council for financial support. TA-I thanks the Institute for Glycomics for the award of a Postgraduate Research Scholarship. RG-S gratefully acknowledges the financial support of the Deutsche Forschungsgemeinschaft (DFG).

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