Carbohydrate Binding

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Solid-State NMR Spectroscopic Analysis of the Ca^{2+}-Dependent Mannose Binding of Pradimicin A**

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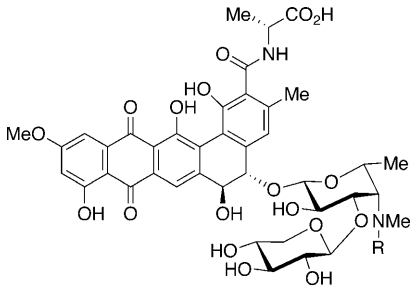
Pradimicins and benanomicins are closely related antibiotics isolated from actinomycetes.[1] These compounds are unique as nonpeptidic natural products with the lectin-like property of being able to recognize D-mannopyranoside (Man) in the presence of Ca^{2+} ions.[2] Recently, pradimicin A (PRM-A, Scheme 1), the most commonmember of this family, has been attracting much attention as a conceptually novel drug candidate for human immunodeficiency virus (HIV).[3] The anti-HIV effect is ascribed to dual modes of action: PRM-A blocks virus entry and triggers the action of the immune system by exposing cryptic immunogenic epitopes on the virus surface. Both of these effects result from the specific binding of PRM-A to Man residues of glycans on the viral envelope.



R = H: Pradimicin A (PRM-A)
R = $^{13}CH_3$: *N*-$^{13}CH_3$-PRM-A

Scheme 1. Structures of pradimicin A (PRM-A) and *N*-$^{13}CH_3$-PRM-A.

There has been rapidly growing interest in small-size "synthetic lectins" since Davis and co-workers demonstrated that synthetic receptors can be used for the selective recognition of carbohydrates in aqueous solution.[4] The biomimetic compounds that they have developed recognize carbohydrates with all-equatorial substitution, including β-D-glucopyranosides and *N*-acetyl-β-D-glucosamines, in a similar manner to lectins and therefore have significant potential as chemical tools in the field of glycomics. Although synthetic receptors for Man are of particular benefit because of the emerging biological significance of high-mannose-type oligosaccharides, especially in protein-quality control,[5] the development of such compounds is challenging and still in its early stages.[6] Under these circumstances, the action of PRM-A is a groundbreaking concept for the design of synthetic receptors for Man.

Given its scientific and therapeutic potential, it is highly desirable to establish the molecular basis of Man recognition by PRM-A. Until now, understanding about how PRM-A recognizes Man in the presence of Ca^{2+} ions has been limited. The essence of the problem lies in the aggregation of the ternary PRM-A/Ca^{2+}/Man complex and the complicated three-component equilibrium, which have frustrated conventional X-ray crystallographic and solution NMR spectroscopic analyses. This situation led us to explore a conceptually novel strategy for the solid-state analysis of the ternary complex. Our strategy benefits from the aggregate-forming propensity of PRM-A and eliminates the equilibrium problem. Herein, we report the interaction analysis of PRM-A with methyl α-D-mannopyranoside (Man-OMe) and Ca^{2+} ions through bipartite solid-state NMR spectroscopic experiments. The results led us to propose an unprecedented Ca^{2+}-mediated binding model of PRM-A with Man.

Although several lines of evidence have indicated that two molecules of PRM-A bind a single Ca^{2+} ion, and the carboxy group of PRM-A was proposed as the putative binding site for the Ca^{2+} ion,[7] clear experimental support for this theory has yet to be provided. Furthermore, it remains unclear whether the role of the Ca^{2+} ion is solely to bridge two PRM-A molecules, or whether it also participates in Man binding. To address these issues, we planned a solid-state ^{113}Cd NMR spectroscopic investigation with $^{113}Cd^{2+}$ ions with a spin of 1/2 as a surrogate probe for Ca^{2+} ions with a spin of 7/2. ^{113}Cd NMR spectroscopy has proven to be an excellent technique for the examination of the Ca^{2+} environments

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present in biological systems because $^{113}Cd^{2+}$ and Ca^{2+} ions have the same formal charge and similar ionic radii.[8] The attractive feature of the ^{113}Cd nucleus is its very broad range of chemical shifts (> 800 ppm), which results in high sensitivity of the chemical shifts to the nature, number, and geometry of ligands coordinated to the $^{113}Cd^{2+}$ ion. Thus, our solid-state analysis started with the investigation of the role of the Ca^{2+} ion on the Man binding of PRM-A by cross-polarization/magic angle spinning (CP/MAS) ^{113}Cd NMR spectroscopy.

As a prerequisite, we confirmed the specific binding of PRM-A to Man-OMe in the presence of Ca^{2+} ions. After preparing the binary PRM-A/Ca^{2+} complex by adding aqueous $CaCl_2$ (1 equiv) to an equimolar mixture of PRM-A and NaOH in water, we examined its coprecipitation with Man-OMe. In the presence of Man-OMe (25 equiv), formation of the ternary complex was observed. We quantified the incorporation of Man-OMe (PRM-A/Man-OMe 1:1.08) by solution ^1H NMR spectroscopic analysis of the mixture after dissociation of the precipitated complex by acid treatment (see the Supporting Information). Methyl α-D-glucopyranoside (Glc-OMe) coprecipitated with the binary PRM-A/Ca^{2+} complex to a negligible extent (PRM-A/Glc-OMe 1:0.08), which indicates that the binary PRM-A/Ca^{2+} complex specifically binds Man-OMe.

Under the complex-forming conditions, the PRM-A/Man-OMe ratio in the ternary PRM-A/Ca^{2+}/Man-OMe complex was estimated to be 1:1 even in the presence of an excess amount of Man-OMe (250 equiv); this result is inconsistent with the 1:2 ratio reported by Ueki et al.[7a] A possible explanation is that PRM-A might possess two Man-binding sites with different affinities. Whereas Ueki et al. determined the PRM-A/Man ratio by the phenol–sulfuric acid method, without washing the aggregate of the ternary PRM-A/Ca^{2+}/Man-OMe complex, our complex-forming procedure included an extensive washing process to eliminate nonspecific binding of Man-OMe; during this washing process, Man-OMe might have been released from the weaker binding site. The existence of two Man-binding sites in PRM-A is supported by a previous spectroscopic study of Fujikawa et al.,[9] who showed that one molecule of PRM-A binds two molecules of Man in two separate steps. They proposed that the binary PRM-A/Ca^{2+} complex initially binds two molecules of Man to form the ternary PRM-A/Ca^{2+}/Man complex with a ratio of 2:1:2, and that another two molecules of Man are then incorporated to form the ultimate ternary complex with a ratio of 2:1:4.

Coprecipitation in the presence of Cd^{2+} ions (1 equiv) and Man-OMe or Glc-OMe (25 equiv) indicated that the binary PRM-A/Cd^{2+} complex also specifically binds Man-OMe (PRM-A/Man-OMe 1:0.46 versus PRM-A/Glc-OMe 1:0.02). The PRM-A/Man-OMe ratio changed to 1:0.88 when 50 equivalents of Man-OMe were used, which indicates that the precipitate formed in the presence of Cd^{2+} ions was a mixture of the ternary PRM-A/Cd^{2+}/Man-OMe and binary PRM-A/Cd^{2+} complexes. This assumption was reinforced by the presence of two signals with almost same area in the CP/MAS ^{113}Cd NMR spectrum of the PRM-A/$^{113}Cd^{2+}$ complex prepared with 25 equivalents of Man-OMe (Figure 1b; see below). The structural similarity of Ca^{2+}- and Cd^{2+}-containing ternary complexes was confirmed by CP/MAS ^{13}C NMR experiments; solid samples of these complexes gave similar spectra (see Supporting Information). Having confirmed that the Cd^{2+} ion serves as a surrogate for the Ca^{2+} ion in the Man binding of PRM-A, we conducted CP/MAS ^{113}Cd NMR spectroscopic experiments with solid samples of the binary PRM-A/$^{113}Cd^{2+}$ and ternary PRM-A/$^{113}Cd^{2+}$/Man-OMe complexes.

The ^{113}Cd NMR spectrum of the binary PRM-A/$^{113}Cd^{2+}$ complex exhibited a broad signal around $\delta = -50$ ppm (Figure 1a). This chemical shift is similar to those

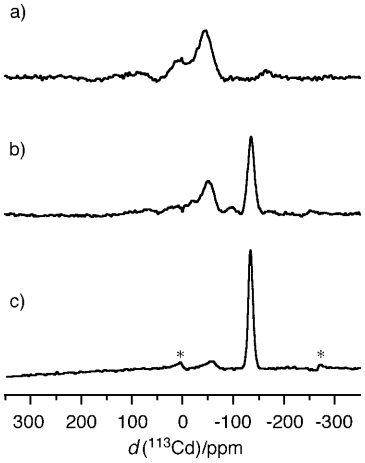
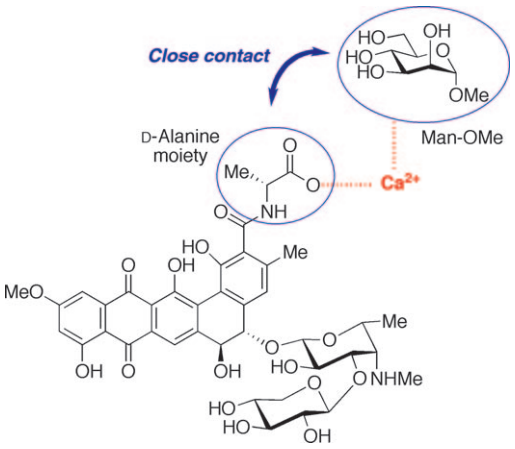


Figure 1. Solid-state CP/MAS ^{113}Cd NMR spectra of PRM-A/$^{113}Cd^{2+}$ complexes prepared a) without Man-OMe, b) with Man-OMe (25 equiv), and c) with Man-OMe (250 equiv). The signals with an asterisk are the spinning side bands of the ^{113}Cd signal at $\delta = -135$ ppm.

reported for solid cadmium compounds with two carboxy groups, such as $Cd(OAc)_2{\cdot}2H_2O$ ($\delta = -46$ ppm), $Cd(O_2CCH_2CH_2CO_2){\cdot}2H_2O$ ($\delta = -52$ ppm), and $[\{Cd(o\text{-}HOC_6H_4CO_2)_2{\cdot}2H_2O\}_2]$ ($\delta = -31$ ppm), but quite different from those reported for $Cd(OH)_2$ ($\delta = 158$ ppm) with hydroxy groups, $[Cd(en)_3Cl_2{\cdot}H_2O]$ ($\delta = 380$ ppm; en = ethylenediamine) with amino groups, $[Na_2Cd(edta)]$ ($\delta = 102$ ppm; EDTA = ethylenediaminetetraacetate) and $Cd(NH_2CH_2CO_2)_2{\cdot}H_2O$ ($\delta = 112$ ppm) with amino/carboxy groups, and $[Cd(glycylglycine)_2{\cdot}2H_2O]$ ($\delta = 169$ ppm) with carboxy/amino/amide groups.[8a,10] It therefore suggests that the $^{113}Cd^{2+}$ ion binds to the carboxy group of PRM-A. This observation supports the putative role of the Ca^{2+} ion to bridge the carboxy groups of two PRM-A molecules.[7b] Considering that chemical exchanges are prohibited in the solid state, and anisotropy effects are minimized by MAS, the broadness of the signal may well be attributed to slightly different chemical environments around the $^{113}Cd^{2+}$ ion owing to structural heterogeneity of the binary PRM-A/$^{113}Cd^{2+}$ complex. On the other hand, the PRM-A/$^{113}Cd^{2+}$ sample prepared in the presence of Man-OMe (25 equiv) exhibited a markedly sharper signal at $\delta = -135$ ppm along with a broad signal almost identical to that observed in the absence of Man-OMe (Figure 1b). The broad signal around $\delta =$

−50 ppm almost disappeared when an excess amount of Man-OMe (250 equiv) was used (Figure 1c), which suggests that the sharp signal at $\delta = -135$ ppm is derived from the ternary PRM-A/$^{113}Cd^{2+}$/Man-OMe complex.[11] The clearly narrower line width of the signal at $\delta = -135$ ppm probably reflects the higher structural homogeneity of the ternary complex. More notable is the marked upfield shift (> 80 ppm) of the signal for the ternary complex with Man-OMe in comparison with that of the binary complex, which does not contain Man-OMe. This observation strongly indicates the occurrence of a change in $^{113}Cd^{2+}$ coordination upon Man-OMe binding. Since ^{113}Cd signals upfield of $\delta = -100$ ppm are observed only for $^{113}Cd^{2+}$ coordinated with more than six oxygen ligands,[8] it is reasonable to assume that Man-OMe coordinates as an additional ligand to the $^{113}Cd^{2+}$ ion in the binary PRM-A/$^{113}Cd^{2+}$ complex. The realistic implication of these results is that the role of the Ca^{2+} ion in the Man-binding process of PRM-A is twofold: it acts as a core to bridge two PRM-A molecules and directly participates in Man binding.

The results of solid-state ^{113}Cd NMR spectroscopic analysis suggested the possibility that Man is located in the proximity of the Ca^{2+}-bound carboxy group of PRM-A in the ternary PRM-A/Ca^{2+}/Man complex (Scheme 2). To obtain



Scheme 2. Model for the Ca^{2+}-mediated binding of PRM-A with Man-OMe. We propose that in the ternary complex (PRM-A/Ca^{2+}/Man-OMe 2:1:2), a bridge structure consisting of the carboxy groups of two PRM-A molecules and the Ca^{2+} ion binds two molecules of Man-OMe in a Ca^{2+}-mediated manner.

more concrete experimental support for this assumption, we performed two-dimensional dipolar-assisted rotational resonance (2D DARR)[12] experiments for detection of the close interaction of Man-OMe with the D-alanine moiety of PRM-A, which contains the Ca^{2+}-bound carboxy group. The DARR method, also known as radiofrequency-assisted diffusion (RAD),[13] has been shown to detect weak ^{13}C–^{13}C coupling in the presence of strong coupling due to directly bound carbon atoms,[14] and dipolar interactions between ^{13}C nuclei that are located within 6 Å of one another can be detected as cross-peaks in the 2D DARR spectrum. Therefore, we began the 2D DARR investigation with the preparation of PRM-A with a ^{13}C-enriched D-alanine moiety.

Although semisynthesis through detachment of the D-alanine moiety followed by the introduction of ^{13}C-enriched D-alanine would be a possible approach to preparation of the target ^{13}C-enriched PRM-A, both cleavage and reconstruction of the amide bond of PRM-A were found to be problematic as a result of steric hindrance around the carboxy group.[15] Thus, we took advantage of the biosynthesis of PRM-A[16] and used *Actinomadura sp.* TP-A0019 for the preparation of PRM-A with a ^{13}C-enriched D-alanine moiety. Exogenous D-[$^{13}C_3$]alanine was successfully incorporated into PRM-A by inhibiting the supply of endogenous D-alanine through the addition of D-cycloserine,[17] an inhibitor of alanine racemase (see the Supporting Information).

We obtained 2D DARR spectra of ^{13}C-enriched PRM-A/Ca^{2+}/[$^{13}C_6$]Man-OMe at mixing times of 20 and 500 ms (Figure 2). Whereas only intramolecular cross-peaks were observed at the mixing time of 20 ms (Figure 2a), the spectrum recorded at the mixing time of 500 ms (Figure 2b) clearly showed intermolecular cross-peaks between carbon signals for the D-alanine moiety of PRM-A ($\delta = 20.0$, 50.8, 179.8 ppm) and those for Man-OMe ($\delta = 63.0$, 68.2, 71–76,

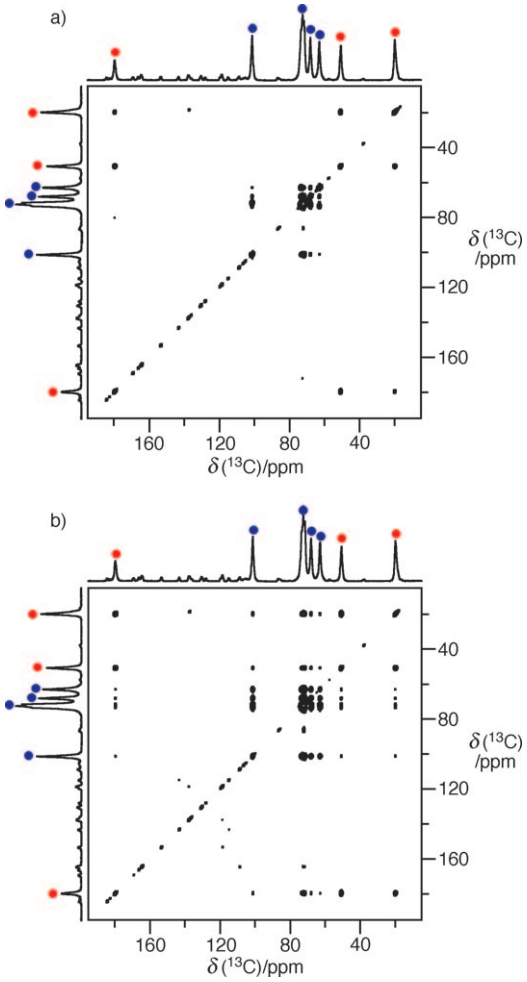


Figure 2. 2D DARR spectra of the ternary ^{13}C-enriched PRM-A/Ca^{2+}/[$^{13}C_6$]Man-OMe complex at mixing times of a) 20 ms and b) 500 ms. Red and blue circles indicate ^{13}C signals derived from ^{13}C-enriched PRM-A and [$^{13}C_6$]Man-OMe, respectively.

101.4 ppm). To eliminate the possibility that these cross-peaks were simply derived from the accidental proximity of PRM-A to Man-OMe in the solid sample, we carried out a control experiment with *N*-$^{13}CH_3$-PRM-A (Scheme 1), which was prepared by the reductive methylation of PRM-A with [^{13}C]formaldehyde.[18] The 2D DARR spectra of the ternary *N*-$^{13}CH_3$-PRM-A/Ca^{2+}/[$^{13}C_6$]Man-OMe complex (Figure 3) showed only intramolecular cross-peaks for [$^{13}C_6$]Man-OMe ($\delta = 64.3$, 68.8, 71–76, 101.3 ppm) and for the $^{13}CH_3$ group of *N*-$^{13}CH_3$-PRM-A ($\delta = 42.8$, 48.6 ppm), which was detected as two signals, probably because of slow inversion at the asymmetric nitrogen center. No intermolecular cross-peak was detectable even at the mixing time of 500 ms. This result indicates that nonspecific binding of PRM-A with Man-OMe is negligible, and the cross-peaks between carbon signals for the D-alanine moiety of PRM-A and Man-OMe truly arises from specific close interactions. Taken together, our results indicate that Man-OMe is located within 6 Å of the D-alanine moiety of PRM-A, which contains the Ca^{2+}-bound carboxy group. They strongly support our proposed binding model in which PRM-A binds Man-OMe through coordination with the Ca^{2+} ion (Scheme 2).

In conclusion, we investigated the Ca^{2+}-dependent mannose binding of PRM-A in the solid state. The analysis strategy based on solid-state NMR spectroscopy enabled us to avoid the problems associated with aggregation and the complicated three-component equilibrium, which have hampered conventional interaction analysis. On the basis of two solid-state NMR spectroscopic experiments, we propose an unprecedented Ca^{2+}-mediated binding model of PRM-A with Man (Scheme 2). It is particularly significant that intermolecular interactions between the D-alanine moiety of PRM-A and Man-OMe were detected by 2D DARR. This result is a rare example of the identification of the ligand-binding region of a receptor by solid-state NMR spectroscopy, and more importantly, the first solid evidence that the D-alanine moiety of PRM-A is the Man-binding region. The present study provides a clue toward the full elucidation of the molecular basis of Man recognition by PRM-A. Further investigations along this line are currently in progress.

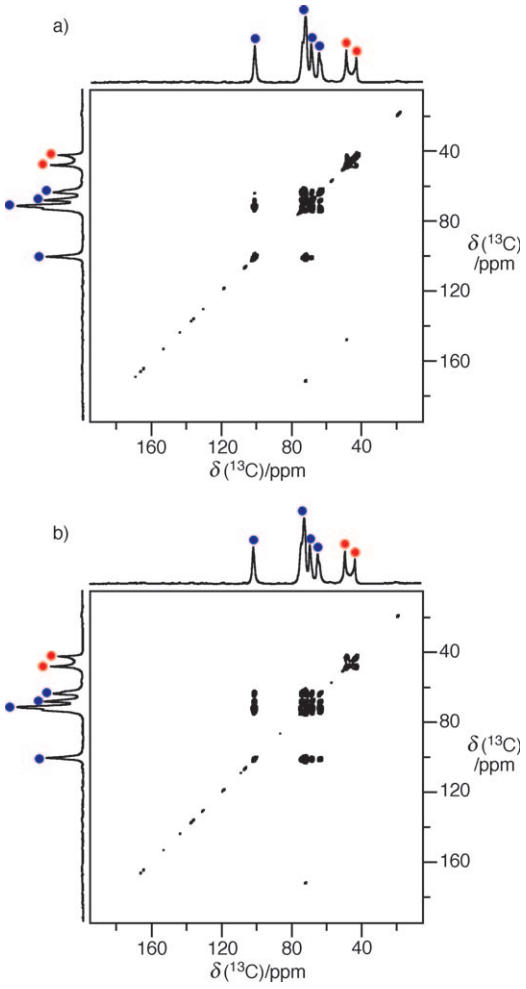


Figure 3. 2D DARR spectra of the ternary *N*-$^{13}CH_3$-PRM-A/Ca^{2+}/[$^{13}C_6$]Man-OMe complex at mixing times of a) 20 ms and b) 500 ms. Red and blue circles indicate ^{13}C signals derived from *N*-$^{13}CH_3$-PRM-A and [$^{13}C_6$]Man-OMe, respectively.

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[11] The presence of the spinning side bands indicates asymmetric charge-density distribution at the $^{113}Cd^{2+}$ ion. The solid-state static ^{113}Cd NMR spectrum and the chemical shift tensors for the ternary PRM-A/$^{113}Cd^{2+}$/Man-OMe complex are shown in the Supporting Information.

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