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Ligand orientation in a membrane-embedded receptor site revealed by solid-state NMR with paramagnetic relaxation enhancement.

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Abstract

NMR relaxation enhancement by paramagnetic metals provides powerful restraints on the three-dimensional structures of proteins in solution, and this approach has recently been utilized in several NMR structural investigations of proteins in the solid-state. Here we utilize paramagnetic relaxation enhancement (PRE) by Mn^{2+} with cross-polarization magic-angle spinning (CP-MAS) solid-state NMR to investigate the interaction of a membrane-embedded protein the Na,K-ATPase (NKA) with a cardiotonic steroid inhibitor. The inhibitor, a diacetonide derivate of the cardiac glycoside ouabain, with ^{13}C labelled acetonide groups in the rhamnose sugar and steroid moieties ([$^{13}C_2$]ODA), is 1000-fold less potent than the parent compound. It is shown that the ^{13}C CP-MAS solid-state NMR spectra of the NKA-[$^{13}C_2$]ODA complex exhibit distinct signals for the two ^{13}C labels of the inhibitor when bound to the ouabain site of membrane-embedded NKA. Recent crystal structures of NKA indicate that the catalytic α-subunit binds a single Mn^{2+} in a transmembrane site close to the high-affinity ouabain site. Here, complexation of NKA with Mn^{2+} broadens the resonance line from the rhamnose group substantially more than the steroid peak, indicating that the rhamnose group is closer to the Mn^{2+} site than is the steroid group. These observations agree with computational molecular docking simulations and are consistent with ODA adopting an inverted orientation compared to ouabain in the cardiac glycoside site, with the modified rhamnose group drawn toward the transmembrane centre of the protein. This work demonstrates that PRE can provide unique information on the positions and orientations of ligands within their binding pockets of transmembrane proteins.

Key words: Magic-angle spinning NMR; manganese; Na,K-ATPase, ouabain.

Magic-angle spinning (MAS) solid-state NMR (SSNMR) spectroscopy is a powerful technique for examining the molecular details of protein complexes and assemblies whose size, solubility or lack of long-range order renders their structures inaccessible to solution-state NMR or X-ray diffraction. SSNMR measurements of ^{13}C and ^{15}N chemical shifts and dipolar couplings have provided unique details on the structures of fibrillar and microcrystalline proteins, and proteins embedded in lipid bilayers. Paramagnetic relaxation enhancement (PRE) has been utilised recently in SSNMR to obtain additional, long-range restraints on the structures and interactions of proteins possessing native or engineered paramagnetic metal coordination sites.[1-4] The unpaired electron paramagnetic centre, such as Mn^{2+}, Cu^{2+} or a nitroxide radical, within the protein gives rise to long-range electron-nuclear spin couplings over tens of Ångstroms, which are observed as distance-dependent line broadening effects and pseudocontact shifts. When applied to membrane-embedded proteins, metal ion-induced PRE has been used to probe the proximity of protein sites from the membrane surface and to determine monomer-monomer interface in an oligomeric protein complex, for example.[5,6] Here we report the use of PRE solid-state NMR to investigate how a small molecule inhibitor interacts with Na,K-ATPase (NKA), the membrane-embedded protein responsible for maintaining ionic potentials across the plasma membranes of all animal cells and the target for the heart failure drug digoxin.

NKA hydrolyses 1 ATP to outwardly transport 3 Na^+ and inwardly transport 2 extracellular K^+, via the magnesium-catalysed formation of a phosphorylated aspartic acid (Asp369) intermediate in the cytoplasmic domain. Cardiac glycoside (CG) compounds derived from digitalis plants, including digoxin, and digitalis-like compounds such as ouabain (g-strophanthin; Scheme 1, left) are highly potent (K_D < 5 nM) inhibitors of NKA that recognise an extracellular site of the α-subunit in its phosphorylated, K^+-sensitive E_2-P conformation. Recent X-ray structures reveal a high-affinity coordination site for Mg^{2+} comprising Asn776, Glu779 and Asp804 within the transmembrane region close to ouabain, and this Mg^{2+} is replaced by a single Mn^{2+} when protein crystals are soaked in 20 mM $MnCl_2$,[7] providing a convenient relaxation enhancing probe of the inhibitor environment.

Cardiac glycosides are widely used therapies for chronic heart failure because of their ability to increase the force of contraction of the heart (positive inotropic effect), and digoxin is cited on the World Health Organisation's list of essential medicines. Recently this class of compounds has shown antiproliferative properties against human tumour cells and a lower mortality rate was reported for breast cancer patients receiving digoxin,[18] but their high inhibitory potency give rises to a narrow margin between efficacy and toxicity.[17] We previously reported the preparation of ^{13}C-, ^{2}H- and ^{19}F- labelled diacetonide derivatives of ouabain (ODA) with acetonide groups modifying the steroid and rhamnose sugar moieties, with a reduced inhibitory potency of 15-30 μM.[8] Structural analysis of the labelled ODA molecules bound

to NKA in purified membrane preparations using magic-angle spinning solid-state NMR (SSNMR) methods provided restraints on the molecular conformation and dynamics of the inhibitor, but a convincing docking model of the enzyme-inhibitor complex could not be obtained because of the lack of a high-resolution structure of NKA at that time. Here we conduct Mn^{2+}-induced PRE ^{13}C SSNMR measurements on an ODA-NKA-Mn^{2+} complex and interpret the results in the light of crystal structures of NKA with ouabain occupying the CG site and Mn^{2+} in the transmembrane site.[7,9,10] For the NMR investigation, ODA was ^{13}C-labelled at the quaternary carbons of the two acetonide groups ([$^{13}C_2$]ODA; Scheme 1, right).

[$^{13}C_2$]ODA inhibits NKA with an IC_{50} of 29.2 μM in the presence of 3 mM $MgCl_2$ and an IC_{50} of around 40 μM when Mg^{2+} is replaced by 0.2 mM $MnCl_2$ or 0.5 mM $MnCl_2$ (Figure S1, Supporting Information). Low concentrations of Mn^{2+} are therefore sufficient, in the absence of magnesium, to form the phosphoenzyme complex and induce the E_2-P conformation required for ODA binding and inhibition of the catalytic cycle. Figure 1 shows ^{13}C cross-polarization magic-angle spinning (CP-MAS) SSNMR spectra of [$^{13}C_2$]ODA in kidney membrane preparations containing 130 μM NKA, recorded at -20°C to minimise molecular dynamics and prevent sample degradation over the long (7 day) measurement times for each spectrum. Such long measurement times were required even with ^{13}C labelled ODA because of the low amount of inhibitor in the sample (130 nmoles). The inhibitor concentration added (390 μM) was calculated to occupy >90 % of the ~130 μM CG binding sites provided that K_D is 30 μM or lower. A ^{13}C NMR spectrum of [$^{13}C_2$]ODA in aqueous solution shows single resonance lines at 101.6 ppm and 110.2 ppm from the steroid and rhamnose sites, respectively (Figure 1A,(i)). In the spectrum of the NKA preparation (Figure 1A, (ii)), the rhamnose acetonide gives rise to two resonance lines at 110.2 ppm and 111.9 ppm (peaks I and II, respectively), as observed previously.[8] That two distinct resonance frequencies are observed for the rhamnose group suggests that the sugar adopts two different conformations and/or experiences different local environments. We have shown for other membrane protein-ligand complexes that only signals from the bound inhibitor are detected by ^{13}C CP-MAS NMR when there is a large excess of free, unbound ligand, because of differences in molecular dynamics in the free and bound states even at -20°C.[11,12] In the present case there is ~260 μM free [$^{13}C_2$]ODA and so to confirm whether the detected signal originates only from [$^{13}C_2$]ODA in the CG site, a further spectrum was obtained for an enzyme sample pre-incubated for 1 h with 500 μM ouabain before adding [$^{13}C_2$]ODA. Ouabain abolishes all three NMR resonances from the labelled inhibitor and the spectrum is virtually identical to the spectrum of NKA alone (Figure 1A, (iii) and (vi), and Figure 1B). The loss of the inhibitor signal indicates that ouabain excludes [$^{13}C_2$]ODA from the CG site and consequently all the [$^{13}C_2$]ODA in the sample is unbound and not detected because the increased molecular dynamics prevent cross-polarization. Ouabain inserts into

a transmembrane pocket, with residues of helices M4, M5 interacting with the apolar α-surface of the inhibitor and polar residues of helices M1, M2 and M6 stabilising the hydroxyl groups of the inhibitor β-surface.[7]

Molecular docking simulations of ODA to NKA were performed using the software GOLD, which has previously been used to accurately predict ligand interactions with membrane proteins.[13,14] To test the reliability of the procedure for predicting inhibitor binding to NKA, the ouabain coordinates were removed from the crystal structure of the complex (PDB ID 3N23) and simulations were performed to ascertain whether the inhibitor could be docked into the enzyme in the original position. A 20 Å search radius was defined from the approximate mid-point of the known ouabain site and the orientations of the docked inhibitors in an arbitrary axis system (chosen to be the principal axes of inertia of NKA) were defined by angles θ and ϕ (Figure 2A). In the top 100 ranked docking predictions, ouabain overlaps substantially with the X-ray determined site and with a narrow distribution of molecular orientations (θ = 45° +/- 15°; Figure 2B, black circles) that are very close to the crystal structure, with the rhamnose group oriented toward the extracellular space. Values of the rotational angle ϕ within the ~30° range allow the β-surface of ouabain to contact polar residues in transmembrane helices M1, M2 and M6. For ODA the inhibitor occupied the ouabain site, with approximately 50 % of the predictions showing ODA adopting an "ouabain-like" orientation. However, a second ensemble of orientations were predicted ([θ,ϕ] angles centred at [~140°, 15°]) in which the rhamnose group faces down into the centre of the protein, with the β-surface of the inverted steroid skeleton rotated to maintain contact with M1, M2 and M6 (Figure 2, red circles). The calculated docking energies for both orientations were very similar to each other. In both predicted orientations, ODA is situated close to the transmembrane Mg^{2+}/Mn^{2+} coordination site (Figure 2C). In the predicted inverse orientation, the ^{13}C labelled site of the rhamnose group lies within 7-10 Å from the Mn^{2+} centre (Figure 2D, filled circles) and the steroid ^{13}C site is some 15-18 Å from Mn^{2+} (Figure 2D, open circles). For the ouabain-like orientation the Mn^{2+}-steroid and Mn^{2+}-rhamnose distances are 15-18 Å and 25-27 Å, respectively.

PRE SSNMR measurements were carried out to test the prediction that ODA can adopt an inverted orientation within the CG site, by exploiting the proximity of the paramagnetic Mn^{2+} site to the inhibitor binding pocket. Figure 3 shows spectra of [$^{13}C_2$]ODA-NKA complex in the presence or absence of $MnCl_2$ and $MgCl_2$. In the absence of Mg^{2+}, addition of as little as 0.2 mM Mn^{2+} apparently abolishes the signal assigned to the ODA rhamnose peak I, whereas the intensities of rhamnose peak II and the steroid peak appear relatively unchanged (Figure 3, left). At 0.5 mM Mn^{2+} rhamnose peak II and the steroid peak remain clearly visible above the noise, although the steroid peak appears to broaden somewhat. These observations are consistent with Mn^{2+} saturating the high-affinity transmembrane coordination site at

concentrations below 0.5 mM. In the presence of 3 mM Mg^{2+}, a similar trend is observed, although higher concentrations of Mn^{2+} are required to abolish rhamnose peak I (Figure 3, right). The resonance frequencies remain constant throughout indicating no pseudocontact shift, as expected for an ion such as Mn^{2+} with isotropic magnetic susceptibility. The loss of peak II is consistent with enhanced ^{1}H (and ^{13}C) $T_{1\rho}$ relaxation during cross-polarization and/or line broadening as a result of enhanced ^{13}C transverse relaxation at the rhamnose carbon centre. In the presence of Mg^{2+} competition with Mn^{2+} reduces the transmembrane site occupancy by the paramagnetic ion at low concentrations, and so higher concentrations of Mn^{2+} are required to invoke the PRE effects. Indeed, quantitative studies of Mn^{2+} binding in the presence and absence of Mg^{2+} confirm that the two ions bind competitively (Figures S2 and S3).

Qualitatively, the PRE effects indicate that ODA adopts an orientation in the CG site in which the rhamnose group is closer to the Mn^{2+} transmembrane site than is the steroid diacetonide bridge. Such an orientation is consistent with the predictions made in the docking simulations. The spectra in Figure 3 were compared with simulated line shapes corresponding to transverse relaxation rates calculated from the Mn^{2+}-^{13}C distances in the ensemble of inverted ODA structure represented in Figure 2D. The closest correspondence with the experimental data was obtained for distances of 7.5 Å and 15.5 Å from Mn^{2+} to the rhamnose and steroid ^{13}C sites, respectively, with T_{1e} taken to be 20 ns. For spectra in the absence of Mg^{2+}, Mn^{2+} site occupancy was assumed to be 100 % above 0.2 mM Mn^{2+} (the approximate concentration of ODA sites being approximately 0.13 mM). For spectra in the presence of 3 mM Mg^{2+}, fractional site occupancies (F^{Mn}) of 0.35, 0.5 and 1.0 were assumed at Mn^{2+} concentrations of 0, 1, 3.4 and 5.3 mM (details of calculations given in the supplementary). For T_{1e} values of less than 10 ns, which have been measured for Mn^{2+} in biological complexes,[15] the distance from the paramagnetic centre to the rhamnose ^{13}C site must necessarily be shorter than 7.5 Å to give rise to the observed PRE. By contrast, no combination of T_{1e} values and site occupancy values gave simulated spectra for the ouabain-like orientation (data not presented). Hence the PRE measurements were found to be consistent with ODA adopting an inverted orientation within the CG site. Interestingly, the resonance line Rh_{II} is insensitive to Mn^{2+}, suggesting that in the second co-existing population of ODA the rhamnose ^{13}C site is situated too far away from the paramagnetic centre to give rise to observable relaxation enhancement. A population of ODA with an ouabain-like orientation would account for the observed insensitivity of the rhamnose site and agree with the docking predictions, but this can only be speculated upon at this stage.

Cardiac glycosides with reduced inhibitory potency against NKA may have beneficial anti-cancer properties without the cardiotoxicity resulting from their positive inotropic effect, although much more research into their therapeutic mechanism is required to confirm this. Here, PRE measurements reveal

that a reduced-potency ouabain derivative adopts an alternative orientation in the cardiac glycoside site, in which the modified rhamnose group faces toward the transmembrane centre of the protein α-subunit and the furanone group is situated closer to the surface of the protein. Interestingly, a similar inverted orientation was recently observed experimentally for a fluorescent derivative of ouabain with a BODIPY dye group attached to the sugar.[16] Hence, PRE solid-state NMR can provide unique and valuable information on the structure-activity relationship of NKA inhibitors that could guide the design of new cardiac and cancer therapies with a higher therapeutic index. We envisage that with site-directed engineering of metal coordinating or nitroxide reactive residues, this technique could be applied more widely to membrane proteins, which represent over 50 % of drug targets.

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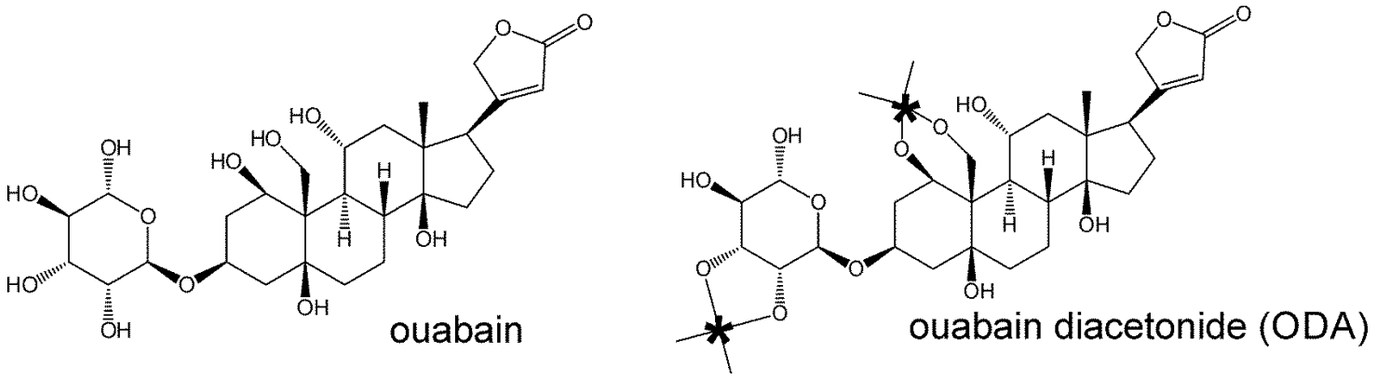

Scheme 1. Chemical structures of ouabain and ouabain diacetonide showing the ^{13}C labelled sites (asterisks).

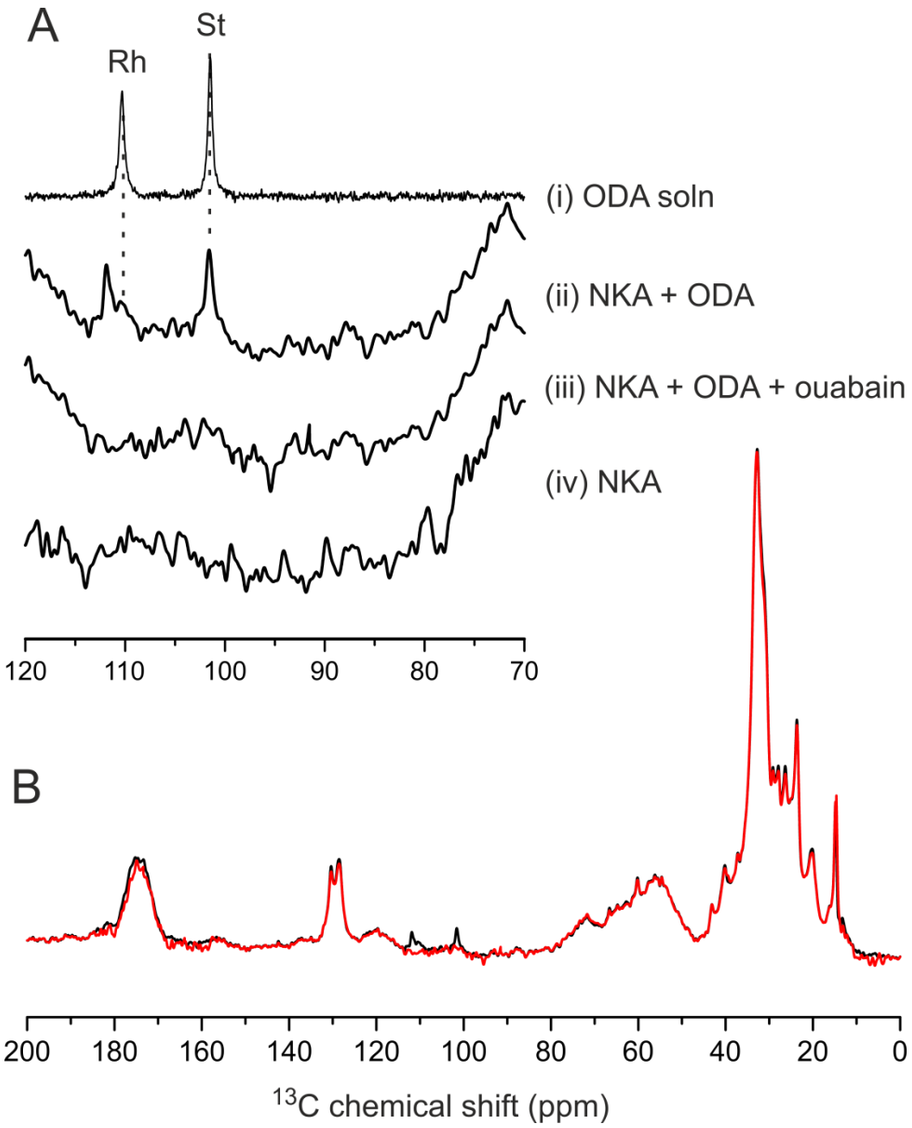

Figure 1. ^{13}C NMR spectra (at 9.4 T) of ODA and NKA in membrane preparations. (A) (i): 390 μM [$^{13}C_2$]ODA in frozen aqueous solution; From (ii)-(iv) CP-MAS SSNMR of NKA (at 130 μM) with 390 μM [$^{13}C_2$]ODA added; 390 μM [$^{13}C_2$]ODA added to NKA preincubated with 500 μM ouabain; NKA membranes alone. (B) Full spectra of A(ii) (black) and A(iii) (red), superimposed to illustrate the reproducibility of the background signals from lipid and protein ^{13}C at natural abundance.

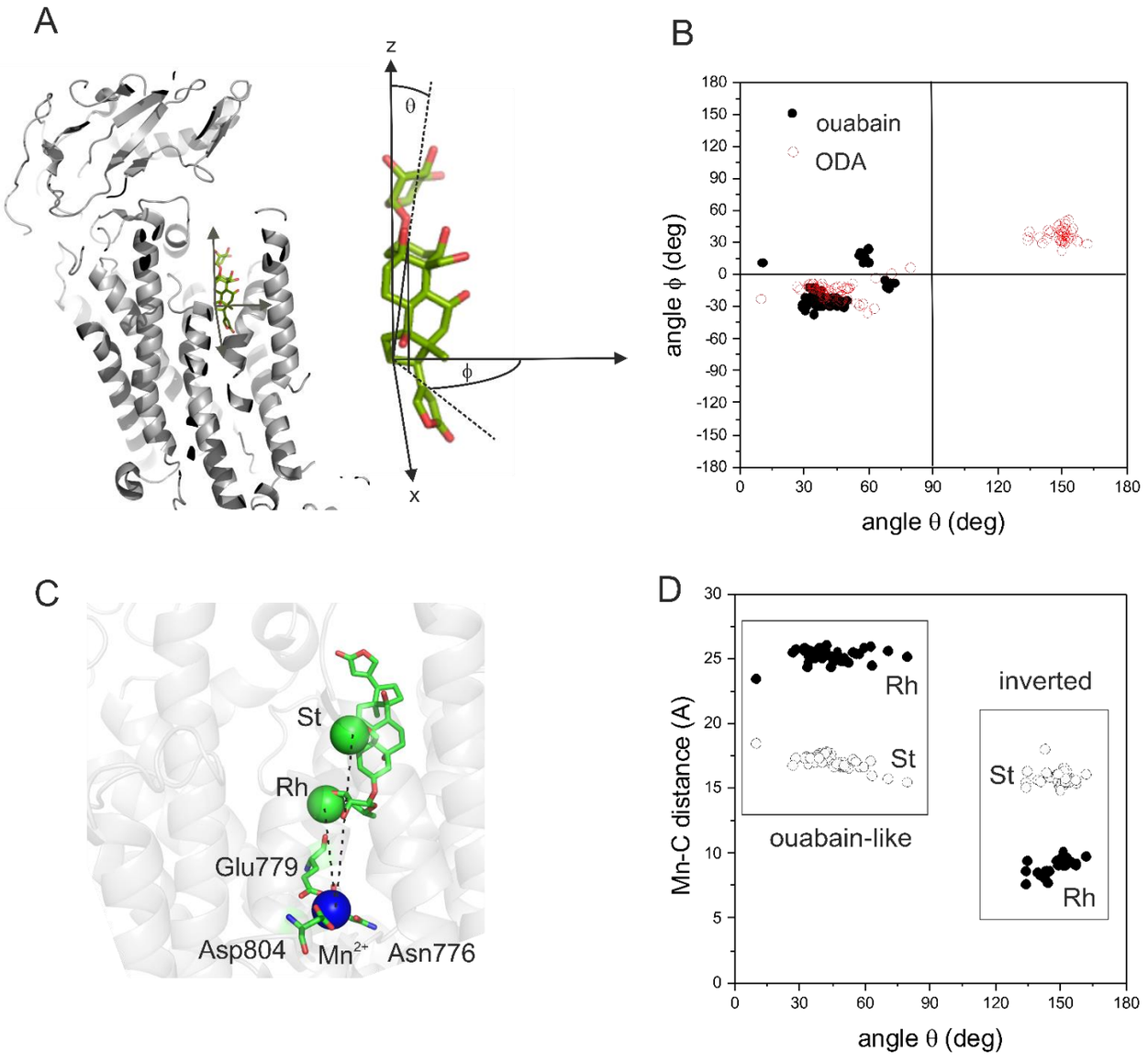

Figure 2. Molecular docking simulations of ouabain and ODA around the cardiac glycoside site of NKA. (A) Expanded view of the ouabain-NKA complex (taken from Ref (7)) in an axis system defined by the principal axes of inertia of the protein. The orientation of ouabain in this reference frame (right) is defined by angles [θ,ϕ], which are calculated from the crystal structure to be 28° and -41°, respectively. (B) A plot of [θ,ϕ] angles calculated from the 100 top ranked docking structures of the NKA-ouabain complex (black) and ODA-NKA complex (red). (C) Expanded view of the ouabain site with ODA docked in one of the inverted orientations predicted by the docking simulations. The relative distances between the Mn^{2+} site and the rhamnose (Rh) and steroid (St) are highlighted by dashed lines. (D) Through-space distances between Mn^{2+} and the rhamnose and steroid ^{13}C labels of [$^{13}C_2$]ODA in the two ensembles of predicted orientations, shown as a function of tilt angle θ.

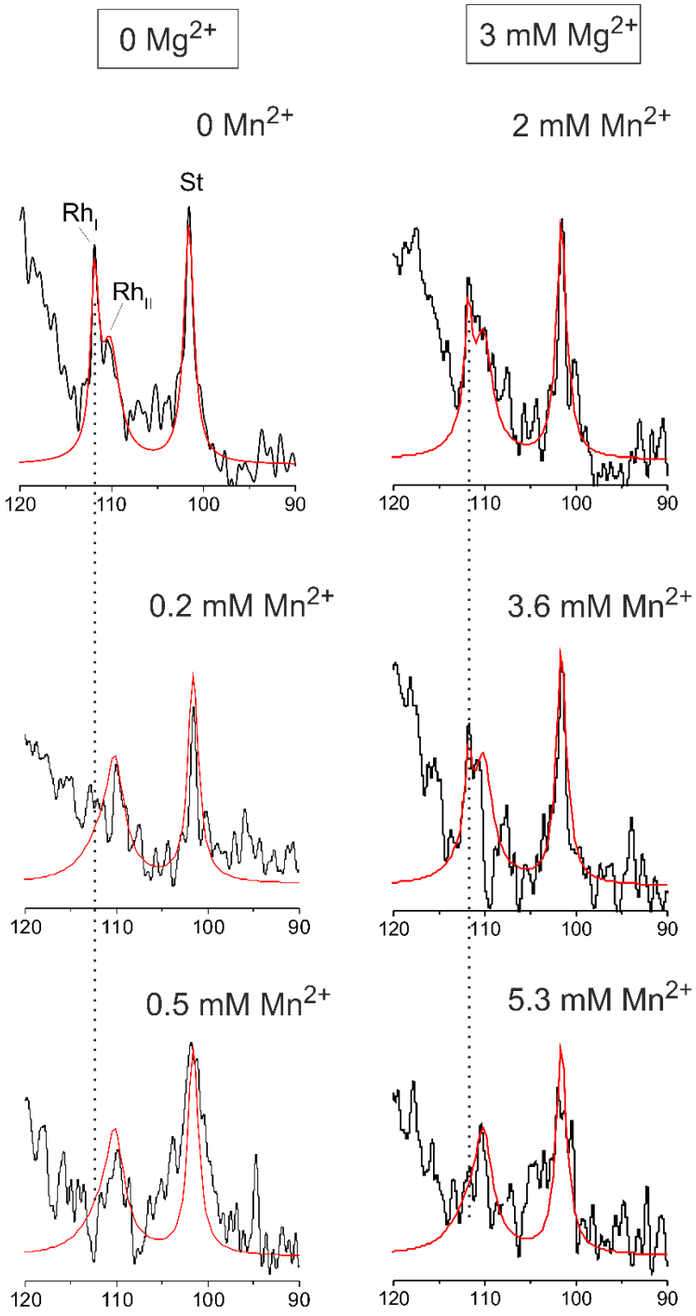

Figure 3. Measurement of Mn^{2+}-induced PRE of the rhamnose (Rh) and steroid (St) ^{13}C spins of [$^{13}C_2$]ODA. ^{13}C CP-MAS SSNMR spectra of membrane prepared in the absence of $MgCl_2$ (left) and with 3 mM $MgCl_2$ (right) at the $MnCl_2$ concentrations shown. Red lines are simulated spectra calculated for the inverted ODA orientation as described in the text. All spectra are normalized to the intensity of the phospholipid terminal methyl signal at ~13 ppm, which is virtually unaffected by Mn^{2+} at the concentrations used here.