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Supporting Information for

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

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Methods

Solid-state NMR. Membrane samples were packed into a 4 mm zirconium MAS rotor, with an internal volume of approximately 70 μl containing approximately 130 nmoles protein. CP-MAS NMR experiments were performed at -20 °C using a Bruker Avance 400 spectrometer equipped with a double-resonance 4 mm MAS probe tuned to 100.13 MHz for ^{13}C and 400.1 MHz for ^{1}H. A sample spinning frequency of 5.5 kHz was used throughout. A proton spin-lock field of 63 kHz was used for Hartmann-Hahn cross-polarisation at a contact time of 0.5 ms. Protons were decoupled at 85 kHz using TPPM during the acquisition period. Each spectrum was the result of accumulating 403200 transients with a recycle delay of 1.5 s.

^{54}Mn Equilibrium Binding Experiments. Equilibrium binding of Mn^{2+} was measured in double-labeling filtration experiments essentially as previously described for ADP binding experiments.[1,2] Na,K-ATPase was allowed to equilibrate at 20 °C for 10 min in 10 mM $Tris^+$-buffer (pH 7.0). The buffer also contained various concentrations of $^{54}Mn^{2+}$ (obtained from Eckert & Ziegler Isotope Products, Berlin, Germany, as a $MnCl_2$ solution at a radioactive concentration of 342 kBq/ml) and [^{3}H]glucose (from New England Nuclear) and in some experiments also additional $Tris^+$ or Mg^{2+}. One mL of this suspension (usually about 0.1 mg protein/mL) was loaded on two stacked Millipore HAWP 0.45 μm filters. Then, without rinsing, filters were separately counted in 4 mL Packard Filtercount scintillation fluid. The amount of Mn^{2+} bound to the protein was calculated by subtracting from the total amount of Mn^{2+} on the (top) filter (bound plus unbound Mn^{2+}) the amount of unbound Mn^{2+}, trapped in the filter together with the wetting fluid; the amount of unbound Mn^{2+} was considered to be proportional to the amount of [^{3}H]glucose in the same filter. The concentration of free Mn^{2+} in the suspension was calculated by subtraction of the amount bound to the protein. The lower filter served as a control. There was no binding of Mn^{2+} to this filter, indicating that all the protein was trapped on the top filter.

Simulation of paramagnetic relaxation enhanced line shapes. Enhancement of the transverse relaxation rate Γ_2 of nuclear spin *I* by the unpaired electron spin *S* (see Ref 3 for a recent review) is given by:

$$\Gamma_2 = R_2^p - R_2^d \qquad [1]$$

where R_2^p is the transverse relaxation rate of the paramagnetic molecule and R_2^d is the transverse relaxation rate of a diamagnetic control (i.e., here the NKA preparation with Mg^{2+} in the transmembrane coordination site). Γ_2 and the enhanced relaxation in the rotating frame, $\Gamma_{1\rho}$, can be estimated according to the Solomon-Bloembergen equation:

$$\Gamma_2 = \Gamma_{1\rho} = \approx \frac{1}{15}\left(\frac{\mu_0}{4\pi}\right)^2 \frac{\gamma_I^2 g_e^2 \beta_e^2 S(S+1)}{r^6}\left(4\tau_C + \frac{3\tau_C}{1+\omega_I^2\tau_C^2} + \frac{13\tau_C}{1+\omega_e^2\tau_C^2}\right) \quad [2]$$

where r is the electron-nuclear distance, ω_I and ω_e are the nuclear and electron Larmor frequencies respectively (here at an applied magnetic field of 9.4 T), β_e is the Bohr magneton and all other terms have their usual meanings. The correlation time τ_C is given by:

$$\tau_C^{-1} = \tau_R^{-1} + T_{1e}^{-1} \quad [3]$$

where τ_R is the rotational correlation time and T_{1e} is the electron relaxation constant. In the solid-state, in which molecular tumbling does not occur (i.e., $\tau_R \to \infty$), equation [3] simplifies to $\tau_C = T_{1e}$. Electron relaxation times for Mn^{2+} are typically 1-100 ns (ref 3) and the electron spin can give rise to a sizeable effect on ^{13}C relaxation rates when the paramagnetic ion is situated less than 15 Å from the carbon centre. The enhancement of ^{1}H and ^{13}C rotating frame relaxation by Mn^{2+} at distances of >7 Å from the nuclear spin sites is expected to have minimal effect on signal intensities at the relatively short (0.5 ms) contact times used here. We propose that the observed effects arise from line broadening as a result of enhanced transverse relaxation rates.

The three peaks (i = 1, 2, 3) for [$^{13}C_2$]ODA at each Mn^{2+} concentration (Figure 3) were fitted by the sum of the Lorentzian functions:

$$P_i = \sum_{i=1}^{3} P_i(Mn) + P_i(Mg) \quad [4]$$

where

$$P_i(Mn) = F^{Mn}\left(\frac{A_i^{Mn}}{2\pi}\right)\left(\frac{w_i^{Mn}}{\left(x-\delta_i^{Mn}\right)^2 + \left(w_i^{Mn}\right)^2}\right) \quad [5]$$

and

$$P_i(Mg) = (1-F^{Mn})\left(\frac{A_i^{Mg}}{2\pi}\right)\left(\frac{w_i^{Mg}}{\left(x-\delta_i^{Mg}\right)^2 + \left(w_i^{Mg}\right)^2}\right) \quad [6]$$

Here, F^{Mn} is the fraction occupancy ($F^{Mn} \leq 1$) of the transmembrane coordination site by Mn^{2+} and was the only variable calculated by non-linear least squares fitting. All other parameters were known or calculated from the GOLD docking models. A_i^{Mg},w_i^{Mg} and δ_i^{Mg} are the areas, widths at half height and chemical shifts of peak *i* measured from the spectrum in the

absence of Mn^{2+} and presence of 3 mM Mg^{2+}. A_i^{Mn},w_i^{Mn} and δ_i^{Mn} are the areas, widths and chemical shifts calculated from the predicted Mn-^{13}C distances given in Figure 2D. The chemical shifts were not affected by Mn^{2+} (zero pseudocontact effect) and it was assumed that $\delta_i^{Mn} = \delta_i^{Mg}$. Measurements of cross-polarization build-ups of signal intensities in the absence of Mn^{2+} indicated that ^{13}C and ^{1}H $T_{1\rho}$ relaxation times exceeded 50 ms. Calculations suggested that, at the predicted Mn-^{13}C distances, the enhancement of the ^{13}C and ^{1}H spin lattice rotating frame relaxation rates $\Gamma_{1\rho}$ during Hartmann-Hahn cross polarization had little effect on the signal intensities at the relatively short (0.5 ms) contact time used, and it was assumed that $A_i^{Mg} = A_i^{Mn}$. Consequently the dominant effect of Mn on the spectrum is to broaden the lines and reduce peak heights. For full Mn^{2+} co-ordination site occupancy calculations indicated that Mn-^{13}C distances of < 8 Å would be sufficient to broaden the peaks beyond detection above the noise. Peak widths were calculated according to:

$$w_i = \frac{\Gamma_2(i)}{\pi} \qquad [7]$$

Additional Results

Manganese Binding. Equilibrium binding was done at room temperature. The binding capacity was determined with $^{54}Mn^{2+}$ using established methods for Ca^{++} or nucleotide binding analysis. Data in Figure S2 were analysed with a simple hyperbolic function :

Bound = B_{max}●[Mn^{2+}]/(K_{Diss} + [Mn^{2+}])

With B_{max} = 6.4 µM and a protein concentration of 0.095 mg/ml we obtain about 67 nmol Mn^{2+} bound/mg protein. The nucleotide binding capacity (one site per enzyme molecule) is about 2.9 nmol/mg. We thus have a maximal Mn^{2+} binding capacity of 23 manganese ions per enzyme molecule.

The effect of mono- or di-valent cations is shown in Figure S3. $Tris^{+}$ is used to show the decrease in Mn^{2+} binding induced by a monovalent cation (Na^{+} or K^{+} give essentially the same result). Mg^{2+} is used as an example of a divalent cation (Ca^{2+} has the same effect). Clearly divalent cations are more efficient in displacing the bound Mn^{2+}.

Analysis of manganese binding. In the NMR rotor we have 130 µM Na,K-ATPase. Using the hyperbolic fit of the data in the absence of Mg^{2+} we thus have a maximal binding capacity of 2.99 mM Mn^{2+} (23 times 130 µM) = B_{max}. K_D was 6.6 µM. We can calculate the concentration of enzyme with Mn^{2+} bound (B) using the equation:

B = 0.5•{B_{max} + K_{Diss} + Mn_{TOT} - squareroot [(B_{max} + K_{Diss} + Mn_{TOT})^2 - 4•B_{max}•Mn_{TOT}]}

where Mn_{TOT} is the added (total) Mn^{2+} in the sample.

For an experiment with 0.5 mM Mn^{2+} we can calculate the free Mn^{2+} concentration to 1.2 µM (and the bound to 498,8 µM). Under the conditions of the NMR experiment there is about 40 mM Tris in the NMR rotor, and the dissociation constant could be 4 times larger (see Figure 2S, upper panel). This lead to a small increase in free Mn^{2+}, to 5 µM. The bound Mn^{2+} is then 495 µM, i.e. 99% of the added Mn^{2+} is bound.

With 3.6 mM total Mn^{2+} (Figure 3 right) and 3 mM Mg^{2+} we can take K_{Diss} as increased 10 fold by Mg^{2+} to K_{Diss} = 0.06 mM (see Figure S2, lower panel,). We then have - again with B_{max} =2.99 mM - a bound concentration of 2.79 mM Mn^{2+}, a 93% saturation of the total number of Mn^{2+} sites.

In conclusion it is reasonable to assume that the specific Mn^{2+} site near the ODA site is saturated for all practical purposes under all the conditions of our experiments.

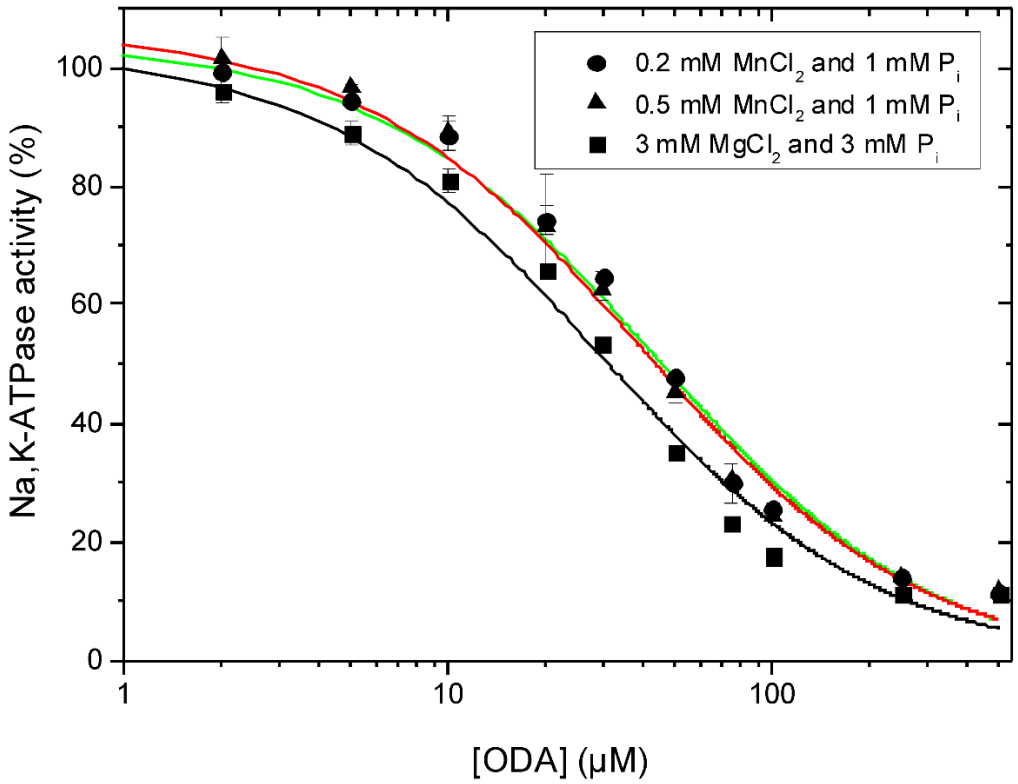

Figure S1. Inhibition of NKA in kidney membrane preparations by ODA in the presence of 3 mM $MgCl_2$ and no $MnCl_2$ (squares), 0.2 mM $MnCl_2$ and no $MgCl_2$ (circles) and 0.5 mM $MnCl_2$ and no $MgCl_2$ (triangles). Hyperbolic fits correspond to ■ K_{Diss} 29.2 μM (black line), ● K_{Diss} 42.8 μM (green line) and ▲ K_{Diss} 39.1 μM (red line).

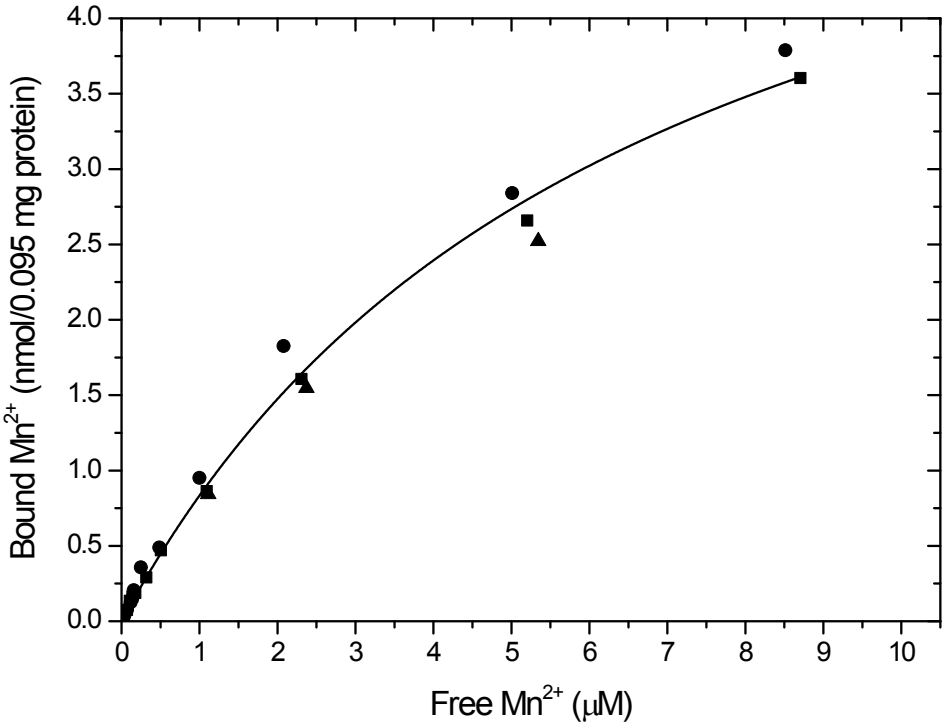

Figure S2. Equilibrium binding of ^{54}Mn to NKA in pig kidney membranes. The amount of bound Mn^{2+} is measured with the filtration technique (see experimental section). The results of three separate experiments 20°C for 10 min in 10 mM $Tris^+$-buffer (pH 7.0) are shown. The hyperbolic fit of all data yields an equilibrium dissociation constant for Mn^{2+} binding K_{Diss} = 6.6 μM, and the maximal binding capacity is B_{max} = 6.4 μM.

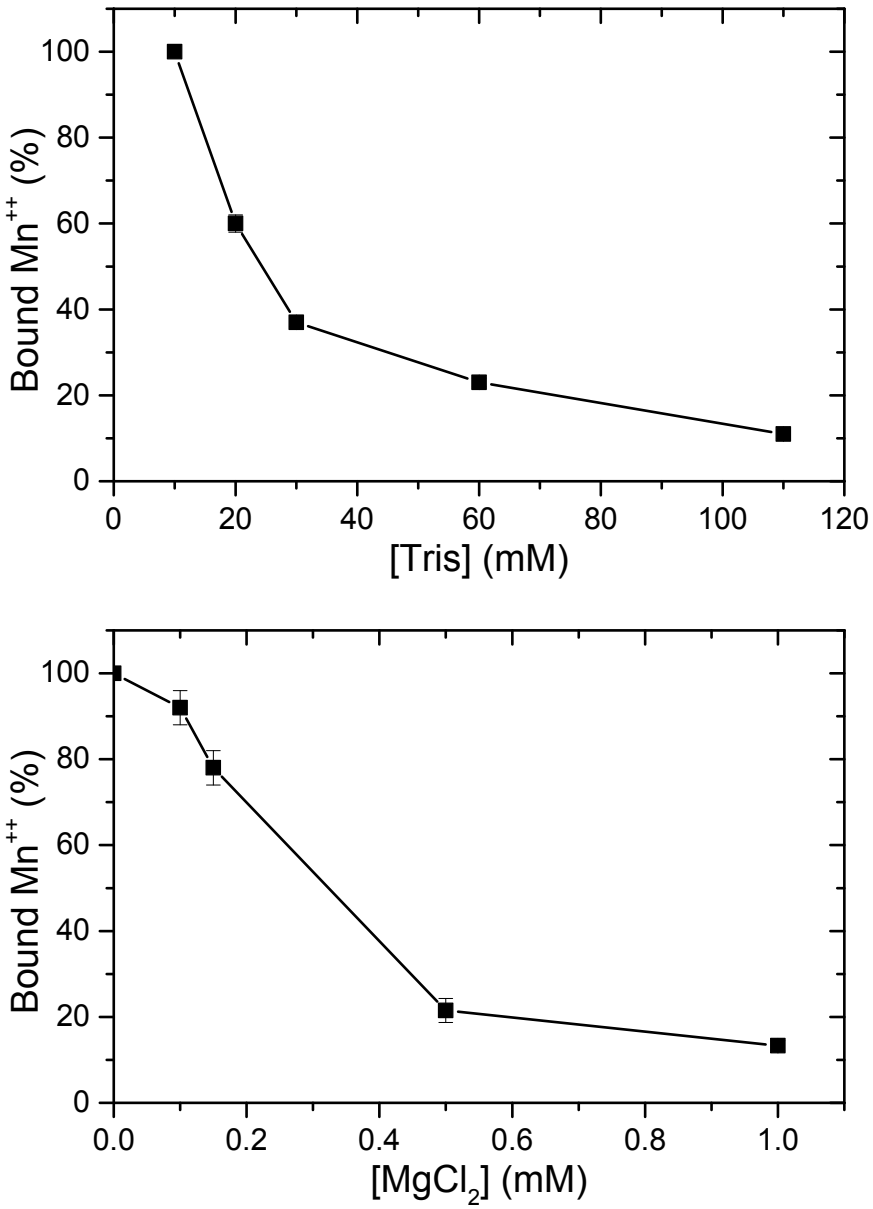

Figure S3. Equilibrium binding of ^{54}Mn to NKA in pig kidney membranes in $Tris^+$ or Mg^{2+}. The amount of bound Mn^{2+} is measured with the filtration technique (see experimental section). The upper panel gives the Mn^{2+} binding in $Tris^+$-buffer concentrations between 10 and 110 mM, taking the binding at 10 mM as 100%. The lower panel gives the Mn^{2+} binding in 10 mM $Tris^+$ and $MgCl_2$ concentrations between 0 and 1 mM, taking the value in 10 mM $Tris^+$ (no $MgCl_2$) as 100%.

References

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