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Supporting Information

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Supporting Information

for

Solid-State NMR Studies of Adenosine 5′-Triphosphate Freeze-Trapped in the Nucleotide Site of Na,K-ATPase

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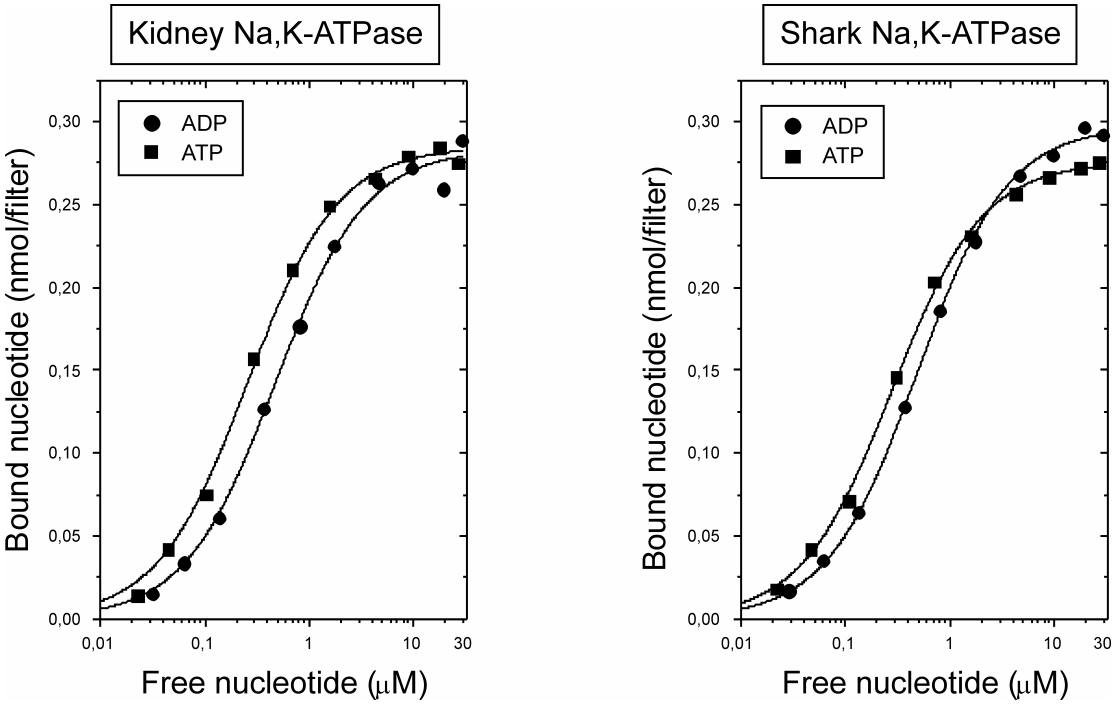

Figure S1. Equilibrium binding of [^{14}C]ADP and [^{14}C]ATP to NKA from pig kidney and shark rectal gland. The amount of [^{14}C]-nucleotide is measured with the filtration technique. Each filter contains 0.095 mg protein with specific NKA activities of 31 units/mg for pig kidney enzyme (left panel) and 29 units/mg for shark rectal gland enzyme (right panel). The full lines represent non-cooperative binding <single hyperbolic functions> with maximal binding capacities of 2.9 - 3.1 nmol/mg protein. The dissociation constant for ATP is 0.244 μM (±0.011, *n*=3) and for ADP it is 0.445 μM (±0.020, *n*=3) for the pig kidney enzyme, and similar values are found for shark rectal gland enzyme (for ATP : 0.252 μM (±0.022, *n*=3) and for ADP: 0.477 μM (±0.016, *n*=3)). The binding capacities are similar for enzymes with similar specific activities: 3.02 ± 0.022 nmol/mg (average of 6 experiments) with an average specific activity of 30 units/mg.

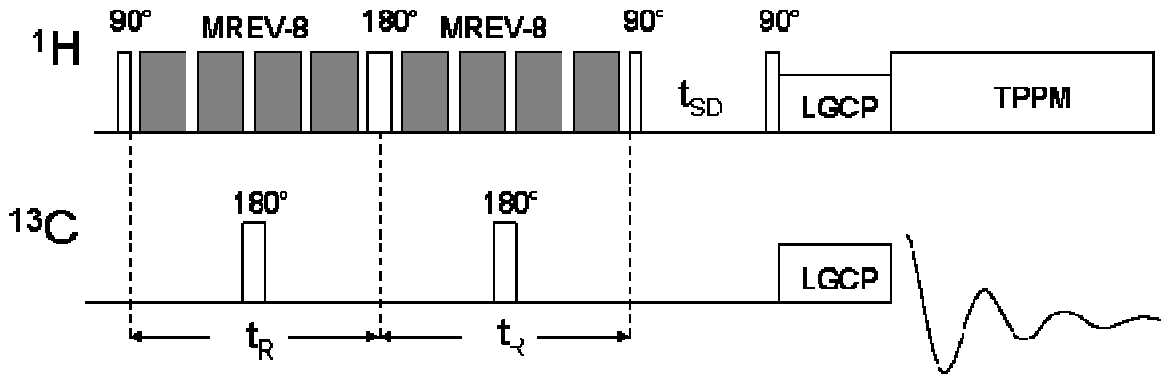

Figure S2. The pulse sequence and experimental conditions for the ^{13}C-detected proton spin diffusion experiment. Measurements were carried out a sample spinning rate of 5102 Hz, corresponding to a rotor period (t_R) of 196 µs. Transverse ^{1}H magnetization evolves for two rotor periods, with the ^{1}H chemical shift interaction refocused by a 7 µs 180° pulse at the end of the first rotor period. Proton homonuclear couplings are suppressed by four 49 µs blocks of MREV-8 decoupling [1] during each rotor period. Simultaneously, ^{1}H,^{13}C heteronuclear dipolar interactions are recoupled by applying an 8 µs 180° pulse to the ^{13}C spins in the centre of each rotor period as in a rotational-echo double resonance (REDOR) experiment[2]. The dipolar modulated ^{1}H magnetization is stored along the *z*-axis by a 3.5 µs 90° pulse for a period t_{SD} during which spin diffusion from the protein protons to the dephased nucleotide protons occurs. The ^{1}H magnetization is returned to the transverse plane and magnetization is transferred to bonded ^{13}C spins via 60 µs Lee-Goldberg cross-polarization [3]. Proton decoupling at 83 kHz with TPPM [4] is applied during ^{13}C signal acquisition. The key to the experiment is to completely silence the ^{1}H magnetization for ATP bound to NKA and then to recover coherent magnetization for protons of ATP in close contact with the protein via spin-diffusion.

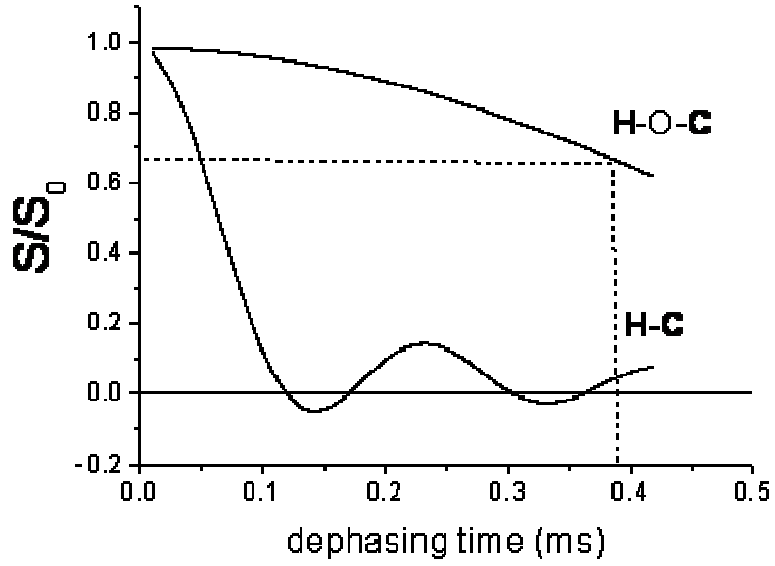

Figure S3. Simulations of the time-dependent dephasing of ^{1}H magnetization by REDOR recoupling of C-H heteronuclear dipolar interactions. Dephasing is measured as the signal intensity obtained with REDOR 180° recoupling pulse s (S) divided by the signal intensity without REDOR pulses (S_0) as described elsewhere[2]. REDOR curves are shown for H-C couplings across one bond and two bonds (e.g. the C-O-H groups in the ribose ring of ATP). The vertical dashed line highlights the dephasing time of 392 µs, which corresponds to $2t_R$ in the ^{13}C-detected proton spin diffusion experiment of Figure S1. The ^{1}H signal intensity for C-H groups will be close to zero at this dephasing time (there will be ± 0.1 deviation from zero depending on non-ideal scaling by MREV-8 and uncertainties in the C-H bond length) and hence the magnetization can be considered to be "silenced". The ^{1}H magnetization for the C-O-H groups of ATP dephase only by approximately 0.3 and the magnetization can only be silenced at much longer (> 1 ms) dephasing times, which compromises the sensitivity of the experiment. Deuterium exchange removes these weak couplings across two bonds.

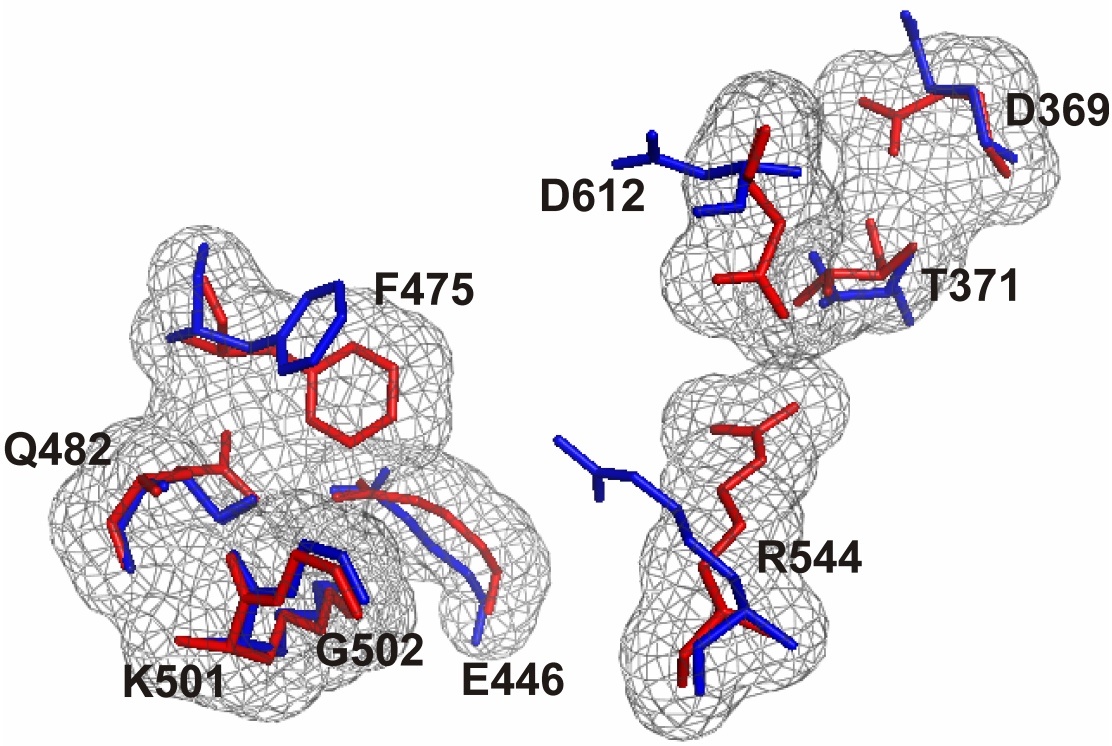

Figure S4. A validity test of the SERCA-NKA sequence alignment used for homology modelling the positions of residues in the nucleotide site of NKA in the E_1 conformation with bound ATP, as shown in Figure 4 of the main text. Using the same sequence alignment, the positions of the same residues in the E_2 conformation of NKA were predicted using the crystal structure of SERCA in the E_2 (thapsigargin-bound) conformation [5] (2C8L) as the template (shown in blue). The relative positions of these residues in the actual crystal structure of NKA in the E_2 conformation [6] (3B8E) are shown in red. Despite some differences in side-chain conformations, the positions of residues in the nucleotide site in our model are in remarkably good agreement with the crystal structure of NKA in the E_2 conformation.

Additional experimental details

Materials: The Li-salt of [U-^{13}C,^{15}N]ATP is obtained from Silantes (Munich, Germany). Li salts of unlabelled ATP and ADP are obtained from Sigma-Aldrich (Denmark). [^{14}C]ATP was from Amersham, and [^{14}C]ADP and [^{3}H]glucose from New England Nuclear.

Enzyme preparations: The specific Na,K-ATPase activities of the purified preparations used in this study are in the range 25-32 units/mg for pig and 29-31 units/mg for shark enzyme. The enzyme preparations contain about 3.0 nmol nucleotide binding sites per mg protein for the preparations with specific activites in the range 29-31 units/mg. The shark enzyme was stored at a protein concentration of about 5 mg/mL in 20 mM histidine and 25% glycerol (pH 7.0), and the kidney enzyme was stored at a protein concentration of about 5 mg/mL in 20 mM histidine, 250 mM sucrose and 1 mM EDTA (pH 7.0). Protein concentrations were determined using the Lowry method[7] and enzymatic activities as described earlier[8].

Nucleotide binding experiments: NKA was determined essentially as described previously [9]. Na,K-ATPase was allowed to equilibrate at 20 ℃ for 10 min in a buffer containing 22 mM NaCl, 11 mM CDTA and 11 mM Tris adjusted to pH 7.0 with NaOH (giving a total [Na^+] of 55 mM). The buffer also contained various concentrations of [^{14}C]ADP, [^{14}C]ATP and [^{3}H]glucose. 0.95 mL of this suspension (0.095 mg protein) 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 nucleotide bound to the protein was calculated by subtracting from the total amount of nucleotide on the filter (bound plus unbound nucleotide) the amount of unbound nucleotide, trapped in the filter together with the wetting fluid; the amount of unbound nucleotide was considered to be proportional to the amount of [^{3}H]glucose in the same filter. The concentration of free ADP or ATP in the suspension was calculated by subtraction of the amount bound to the protein. In the lower filter specific binding of nucleotide was absent, as expected.

The data were fitted to a <single hyperbolic function>. Averages of maximal binding capacities and dissociation constants for three independent binding experiments are given in the text.

Preparation of samples for NMR: Na,K-ATPase membrane stock solution was diluted 10-fold into a buffer containing 22 mM NaCl, 11 mM CDTA and 11 mM Tris adjusted to

pH 7.0 with NaOH (giving a total [Na^+] of 55 mM). The suspension was centrifuged for 2 h at 20000 rpm at 4 ^oC in a Beckman Ti70 rotor. The pellets were transferred to an Eppendorf vial with about 0.25 mL pellet per vial, and the protein concentration was usually about 50 mg/ml. The reaction with [^{13}C]ATP was initiated by addition of a 20-fold concentrated stock solution of Li-salt of the nucleotides. The suspension containing the nucleotide was homogenized in the Eppendorf vial with a Kontes homogenizer, and was kept at about 15 ^oC for 10 min before cooling to 0 ^oC. The suspension was transferred to a 4 mm NMR rotor and frozen in liquid N_2.

H_2O–D_2O exchange experiments are performed with the following protocol. Stock shark enzyme is diluted 10-fold in 20 mM histidine and 25% glycerol (pH 7.0), and stock kidney enzyme is diluted 10-fold in 20 mM histidine, 250 mM sucrose and 1 mM EDTA (pH 7.0) and both are centrifuged for 60 min at 60.000 rpm at 10 ℃. The pel-lets were homogenized in 10 mL of a buffer prepared using 99.5% D_2O instead of H_2O - the buffer contained 22 mM NaCl, 11 mM CDTA and 11 mM Tris adjusted to pH 7.0 with NaOH (giving a total [Na^+] of 55 mM). The protein concentration was about 5 mg/mL. This was allowed to equilibrate for 90 min at 10 ℃. The suspension was subsequently pelletted by centrifugation for 2 h at 40000 rpm at 14 ^oC. The pellet was again taken up in 10 mL of the D_2O buffer and allowed to equilibrate 16 h at 14 ℃. The suspension was again pelletted by centrifugation for 2 h at 40000 rpm at 14 ℃, and the resulting pellets were transferred to Eppendorf tubes and treated with [^{13}C]-ATP as above.

Calculation of bound nucleotide from enzyme concentration: The sample transferred to the NMR rotor contains typically about 45 mg protein/mL. With a binding capacity of 3.0 nmol/mg and a dissociation constant for ATP of 0.25 µM the saturation of binding sites is 99% at a free ATP concentration of 30 µM. The concentration of bound nucleotide in the rotor is therefore 130- 135 µM with about 30 µM unbound ATP in the experiments with 160 µM total ATP.

Calculation of hydrolysis of nucleotide: We have previously observed that the rate of ATP hydrolysis at about 20 ℃ is almost proportiona l to the concentration of ATP (cf. the initial hydrolysis of ATP in Figure 1 in ref. [10]) and that it is temperature dependent[11]. At a free concentration of 30 µM ATP (that of the 160 µM total ATP experiments) the rate of hydrolysis is estimated to be maximally 1 µM per min at 45 mg protein per mL. The free ATP concentration thus decreases maximally from 30 µM to

about 20 μM during the 10 min period from addition of nucleotide to the enzyme to the sample is frozen in liquid N_2. The saturation is 98.5% if the ATP concentration is reduced from 30 μM to 20 μM.

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