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

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The Na,K-ATPase (NKA) maintains the cellular potentials of all animal cells by driving the vectorial transport of Na^+ and K^+ across the plasma membrane through two different enzyme conformations (E_1 and E_2).[1] NKA exhibits high affinity towards the ATP substrate when sufficient Na^+ is present to induce the E_1 conformation.[2,3] More than fifty years after the discovery of NKA, the details of how ATP interacts with the enzyme in the E_1 conformation remain unknown, and the only X-ray crystal structure of NKA is for the E_2 conformation in the absence of nucleotide.[4] Solid-state NMR spectroscopy (SSNMR) is a valuable alternative to X-ray diffraction for providing atomistic details of ligands bound to receptors within their native membranes.[5–8] SSNMR studies of bound nucleotide in membranous preparations of NKA from pig kidney and shark salt glands are hampered by a contaminating enzyme activity that rapidly hydrolyses ATP, ADP and AMP in the absence and presence of Mg^{2+}.[9,10] Hydrolysis is not inhibited by ouabain, vanadate or adenylate kinase inhibitor. This problem is overcome here by including a Mg^{2+} chelator and rapidly freeze-trapping uniformly $^{13}C/^{15}N$-labelled ATP ([U-^{13}C,^{15}N]ATP) complexed with NKA in membrane preparations. SSNMR spectroscopy experiments at −25 °C detect ^{13}C signals from bound ATP and provide preliminary insights into the nucleotide contacts with the high-affinity site.

A critical requirement for the SSNMR experiments is to saturate the high-affinity nucleotide site with ATP whilst avoiding excess free or unspecifically bound nucleotide, because SSNMR spectroscopy cannot readily distinguish these from specifically bound nucleotide. Biochemical measurements of radiolabelled nucleotide binding were therefore carried out to establish such a regime. Equilibrium binding curves show nucleotide binding to a single site with dissociation constants in the range of 0.2–0.5 μM, and a capacity of about 2.9 nmol mg^{-1} protein for both shark and pig NKA in the buffer used for SSNMR experiments (Figure S1 in the Supporting Information). Samples for SSNMR analysis contain 130 μM NKA, so we can safely assume that the nucleotide site is saturated by adding 160 μM [U-^{13}C,^{15}N]ATP to the membranes; this gives a free nucleotide concentration of about 30 μM. The binding assay also demonstrates the absence of additional unspecific binding to NKA. We thus confirm that at these concentrations more than 80% of the ^{13}C NMR signal for the nucleotide will correspond to ATP bound specifically to shark or kidney enzyme.

Figure 1 A shows ^{13}C cross-polarisation magic-angle spinning (CP-MAS) SSNMR spectra at −25 °C for a control shark NKA

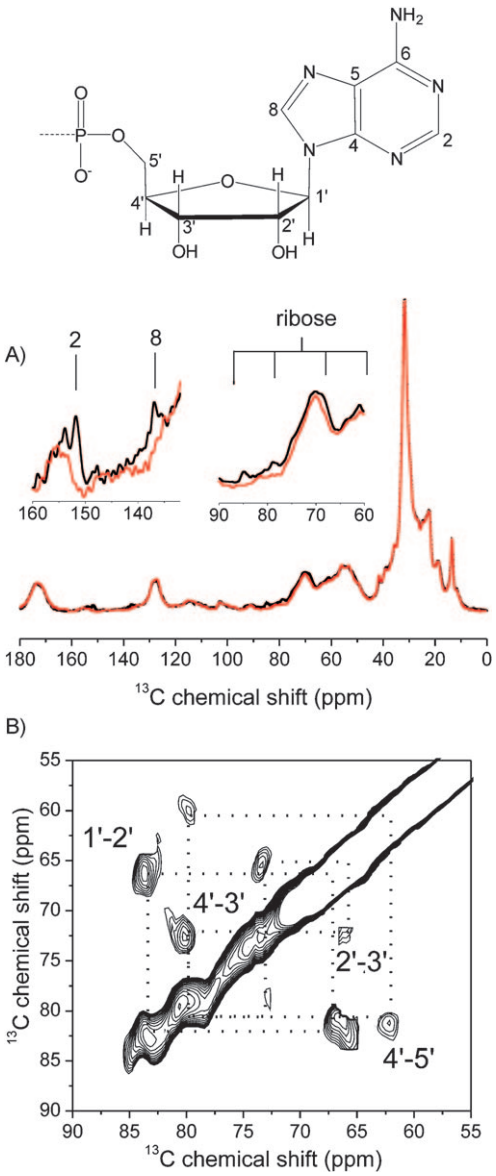

Figure 1. ^{13}C SSNMR spectra for [U-^{13}C,^{15}N]ATP complexed with shark NKA. A) CP-MAS spectrum (at −25 °C) of hydrated shark NKA membranes before (red lines) and after addition of [U-^{13}C,^{15}N]ATP to a concentration of 160 μM (black lines). The insets show specific regions where nucleotide resonance peaks are observed above the natural abundance ^{13}C signals from lipids and protein. B) Region of a two-dimensional DARR spectrum of lyophilised membranes highlighting cross-peaks for the ribose spin system of [U-^{13}C,^{15}N]ATP. The chemical structure of ATP with the numbering convention is shown at the top.

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membrane sample in the absence of nucleotide (red line). After freeze-trapping the [U-^{13}C,^{15}N]ATP–NKA complex (Figure 1A, black line), several new peaks can be seen for the ~16 nmol of nucleotide (i.e., 160 μM in a 100 μL volume) bound predominantly to NKA. Conspicuous peaks include those for C2 and C8 of the adenine ring at 151.4 and 136.7 ppm, respectively, and peaks for the ribose moiety occur in the region from 60.0–90.0 ppm, although the latter overlap the natural abundance signals from the membranes (Figure 1A). Assignment of the nucleotide peaks to specific positions in the ribose rings was assisted by our previous assignments for bound nucleotide at 4 °C[10] and with a two-dimensional ^{13}C dipolar assisted rotational resonance (DARR) NMR spectrum (Figure 1B). The spectrum shows several cross-peaks correlating the ribose ring system, but interactions between the ribose and adenine rings are not detected above the noise.

Peaks for the nucleotide ribose moiety are observed more clearly in difference ^{13}C CP-MAS spectra of the freeze-trapped complex, in which the background natural abundance ^{13}C signals have been eliminated by subtracting control spectra of NKA membranes obtained under identical conditions but excluding nucleotide (Figure 2A). Peaks for [U-^{13}C,^{15}N]ATP complexed with shark enzyme in an approximately equimolar concentration (Figure 2A) occur at virtually identical chemical shifts to the peaks for [U-^{13}C,^{15}N]ATP in complex with kidney NKA enzyme (Figure 2B); this suggests that the nucleotide-binding site and conformation are similar in both cases. This is not unexpected since the α subunits of shark and kidney enzymes share 93% sequence homology and the residues defining the high-affinity nucleotide site are highly conserved across all species. The lines are rather narrow; this suggests that the nucleotide adopts a well-defined conformation in the high-affinity site. By contrast, a difference spectrum of [U-^{13}C,^{15}N]ATP at a 25 molar excess over the nucleotide-binding sites shows substantially broader lines; this is consistent with multiple environments and/or conformations (Figure 2C). Peaks for [U-^{13}C,^{15}N]ATP in aqueous solution (Figure 2D and E) lie within the broad signals for the nucleotide in excess over NKA (Figure 2C), but several of them appear at markedly different positions to the peaks for the nucleotide in equimolar complex with NKA (Figure 2A and B). Such differences were seen in an earlier SSNMR study of nucleotide binding to NKA at 4 °C[10] and indicate that the microenvironment or conformation of the nucleotide within the high-affinity site is different to that in the bulk aqueous or membrane phases. The peaks for C4 (149 ppm), C5 (118 ppm) and C6 (156 ppm), which are visible in the direct polarisation spectrum of 4 mM ATP (Figure 2E), are not detected in the CP-MAS spectra (Figure 2A–D) because they lack bonded protons that provide the mechanism for efficient cross-polarisation. The ^{13}C chemical shift values for [U-^{13}C,^{15}N]ATP are summarised in Table 1.

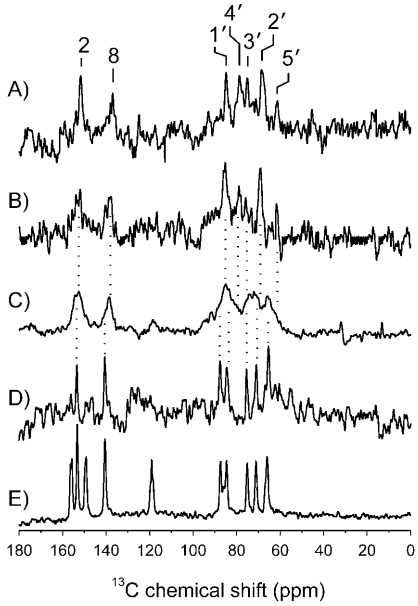

Figure 2. Comparison of ^{13}C CP-MAS NMR spectra for [U-^{13}C,^{15}N]ATP in different environments. A) Difference spectrum of nucleotide (160 μM) in shark NKA membrane suspensions (130 μM NKA). B) Difference spectrum of nucleotide (160 μM) in a kidney NKA membrane suspension (130 μM NKA). C) Difference spectrum of nucleotide (4 mM) in kidney NKA membranes. D) Spectrum of nucleotide (160 μM) in aqueous solution. E) Direct polarisation ^{13}C spectrum of nucleotide (4 mM) in aqueous solution. All spectra were collected from hydrated samples at −25 °C and are the result of averaging 64000 transients.

Table 1. Summary of ^{13}C chemical shift values (in ppm relative to an external adamantane standard) for [U-^{13}C,^{15}N]ATP in different environments at −25 °C.

Site[a]	Shark NKA[b]	Kidney NKA[b]	Kidney NKA[c]	Aqueous[c]
C2	151.4	151.9	152.5	153.2
C8	136.7	136.8	137.9	140.2
C1′	84.4	84.9	~85.0	87.9
C4′	78.5	79.1	~85.0	84.9
C3′	74.4	75.6	~73.8	75.0
C2′	67.9	68.1	~65.6	70.3
C5′	60.9	61.5	~65.6	65.6

[a] Peaks for sites C4, C5 and C6 were not detected reliably by SSNMR spectroscopy and are not shown; [b] for 160 μM ATP; [c] for 4 mM ATP.

A ^{13}C-detected proton spin diffusion (PSD) experiment was used to detect contacts between [U-^{13}C,^{15}N]ATP and the nucleotide binding site of kidney NKA. The aim of the experiment was to identify those nucleotide sites that are in closest proximity to the binding pocket. The general strategy is described in the following, and further details of the NMR pulse scheme are given in Figure S2 in the Supporting Information. First the ^{1}H magnetisation for bound [U-^{13}C,^{15}N]ATP was crushed by recoupling dipolar interactions to the bonded ^{13}C spins over a defined period.[11] The ^{1}H magnetisation for NKA remained largely unperturbed because the vast majority of the enzyme protons are bonded to ^{12}C at natural abundance and, therefore, are not silenced by ^{13}C spins. To improve the efficiency of nucleotide signal silencing at the shortest possible dephasing times, and thereby avoid signal losses by other mechanisms, the enzyme was washed and suspended in D_2O prior to com-

plex formation. This additional measure was taken to exchange ATP –OH and $-NH_2$ protons for deuterium, thereby removing weak two-bond C–H couplings that could otherwise be silenced only with long dephasing times (Figure S3 in the Supporting Information). Proton magnetisation is then returned to the nucleotide via spin diffusion pathways from the protein. The period of spin diffusion (t_{SD}) is optimised so that ^{13}C peak intensities for nucleotide sites in close contact with NKA recover more rapidly than peak intensities for sites oriented away from the binding site (Figure 3A). Finally the recovered ^{1}H magnetisation for the nucleotide is transferred to the ^{13}C spins and the ^{13}C spectrum is collected. Figure 3B shows PSD difference spectra at different spin-diffusion periods after removing natural abundance signal. Even with short dephasing times, each experiment requires approximately 50 h of data collection, although much longer measurements would be required in the absence of D_2O. No signals were detected at a t_{SD} of zero (Figure 3B, i); this confirms that the ATP signal is completely silenced without spin diffusion from the enzyme. After a spin-diffusion period of 500 μs, signals from C2 and C8 of the adenine moiety begin to recover (Figure 3B, ii); this confirms that spin diffusion occurs from NKA to the nucleotide. Signals from the ribose moiety are not evident above the noise at this stage. At longer spin-diffusion periods the signal from the ribose moiety also recovers (Figure 3B, iii). The recovery of peak intensities for the nucleotide follow the order C2 > C8 > ribose; this is consistent with the adenine ring of ATP being in contact with the binding site and the ribose ring being relatively exposed.

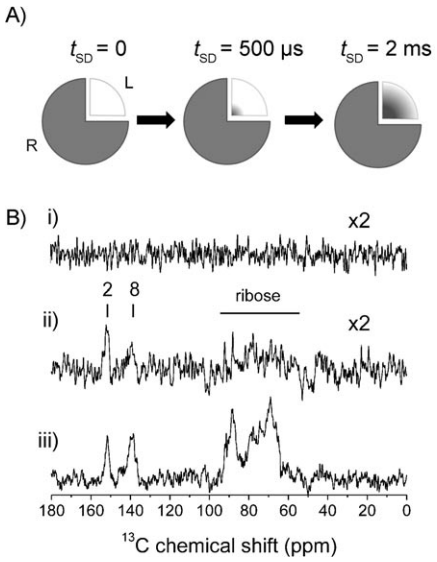

Figure 3. A ^{13}C-detected PSD SSNMR experiment to detect bound nucleotide sites in closest contact with NKA. A) A simplified scheme of the procedure for the general case of a ligand (L) in a receptor site (R). An initial state is created in which the ligand magnetisation is crushed. As the spin-diffusion period (t_{SD}) increases, ligand magnetisation recovers most rapidly at sites in closest contact with the receptor. B) Experimental spectra for [U-^{13}C,^{15}N]ATP (160 μM) bound to kidney NKA (130 μM). Natural abundance signal was removed by subtracting control spectra of membranes in the absence of labelled nucleotide. Spectra correspond to spin-diffusion periods (t_{SD}) of: i) zero, ii) 500 μs, and iii) 2 ms. Each spectrum is the result of averaging 170000 transients.

Alternative structural information for ATP in the nucleotide site of NKA is not available, and so it was not possible to cross-validate our PSD results with reference to independent experimental data. We instead modelled the nucleotide site of NKA by comparison with a crystal structure of the homologous rabbit sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) in the E_1 conformation. A test for the reliability of the sequence alignment and our homology model is given in Figure S4 in the Supporting Information. Figure 4A shows the structure of the nucleotide site of the NKA homologue SERCA in the E_1 conformation with a bound ATP analogue AMPPCP;[12,13] this highlights amino acids situated within a 4 Å radius of the nucleotide. Notably, the purine ring and phosphate groups of AMPPCP are surrounded by several residues, including F489 and G516, whereas the ribose ring is relatively exposed to an aqueous chamber. Based on this structure, a homology model of ATP in the NKA nucleotide site indicates that the adenine ring is buried within a binding pocket defined by E446, F475, Q482, K501 and G502, with C2 facing toward the pocket, and the terminal phosphate group (Pγ) is situated close to the phosphorylation site D369 (Figure 4B). The model supports the conclusions of the SSNMR spectroscopy measurements and shows that the ribose group is somewhat exposed compared to the adenine ring, as in the SERCA structure, with only one residue (R544) situated within 4 Å of the ribose ring. The

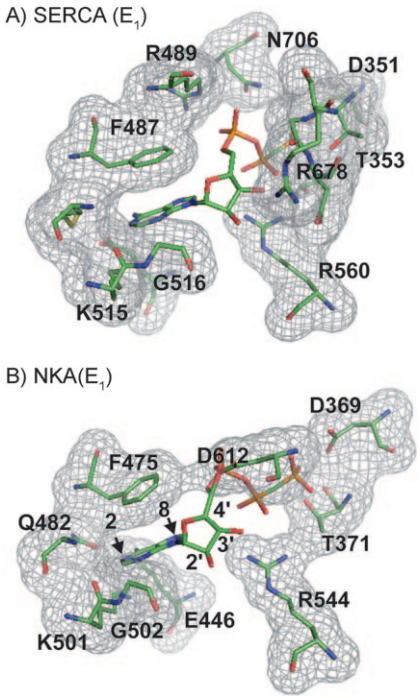

Figure 4. Representations of the nucleotide-binding sites of P-type ATPases. A) AMPPCP in the nucleotide-binding site of SERCA in the E_1 conformation, showing residues less than 4 Å from the bound nucleotide. B) A model of ATP in the NKA nucleotide-binding site in the E_1 conformation obtained by sequence alignment with SERCA and mapping onto the coordinates for the structure shown in A).

models suggest structural similarities in the modes of interaction between ATP and SERCA and NKA, and that the ATP is restrained by a binding pocket possibly involving π–π interactions with F475.

In summary, it is shown that, with careful biochemical analysis and by exploiting solid-state NMR spectroscopy at low temperatures, we have been able to detect ATP in the nucleotide site of NKA in native membranes, and overcome difficulties associated with ATP-hydrolysing contaminants in the membrane preparation.[9,10] In so doing we provide insights into the contact surface for ATP within the high-affinity nucleotide site and provide the foundation for further SSNMR studies of bound nucleotide conformation exploiting ^{13}C–^{31}P distance measurements, for example.

Experimental Section

NKA was purified from pig kidney and shark rectal gland as described previously.[14,15] Equilibrium binding of [^{14}C]ADP and [^{14}C]ATP to NKA was determined by measuring the amount of [^{14}C]-nucleotide with the filtration technique essentially as described previously.[16] Each filter contained protein (0.095 mg) with specific NKA activities of 31 units mg^{-1} for pig kidney enzyme and 29 units mg^{-1} for shark rectal gland enzyme. For SSNMR spectroscopy measurements, NKA membranes were homogenised with [U-^{13}C,^{15}N]ATP, transferred to a 4 mm diameter magic-angle spinning sample rotor and immediately frozen in liquid nitrogen. The entire procedure took less than 10 min, during which less than 10 μM ATP hydrolysed out of the 160 μM present; this maintained a concentration that virtually saturated all nucleotide sites. Further details are given in the Supporting Information. The rotor held 100 μL of membrane sample containing NKA (13 nmoles) and nucleotide (16 nmoles). Spectra were obtained by using a Bruker Avance 400 instrument operating at a frequency of 100.13 MHz for ^{13}C. Membrane samples were spun at a MAS rate of 5.1 kHz for one-dimensional (1D) experiments and 8 kHz for the DARR experiment, and 1D spectra were obtained by averaging no more than 170 000 transients. Conditions common to all experiments were a 4.0 μs ^{1}H excitation pulse, two-pulse phase modulated (TPPM) proton decoupling[17] at a field of 85 kHz during signal acquisition and a 1 s recycle delay pulse. For CP-MAS and DARR experiments cross-polarisation was achieved with a 2 ms contact time at a proton field of 65 kHz. PSD experiments used a 1D adaptation of an experiment described previously.[18] The ^{1}H-^{13}C magnetisation transfer was via 60 μs Lee–Goldburg cross-polarisation. All other conditions for the PSD experiment are described in the legend to Figure S2 in the Supporting Information. Two-dimensional DARR NMR spectra[19] were recorded with 32 hypercomplex points in the indirect dimension with a mixing time of 50 ms during which the proton field was adjusted to the spinning frequency of 5 kHz. The spectrum was obtained by averaging 16 000 transients per t_1 increment and processed with complex linear prediction in t_1. Models were generated by using Modeller[20] based on the coordinates for PDB ID: 1VFP[12] and with the sequence alignment proposed by Hebert et al.[21] An alternative structure (PDB ID: 1T5S) could also have been used.[13] Model refinement was performed with energy minimisation and simulated annealing, and visualised by using PyMOL (DeLano Scientific LLC).

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