Supplementary Information

Monitoring Enzymatic ATP Hydrolysis by EPR Spectroscopy

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Figure S2: RP-HPLC traces of probe 1 (400 μM) after incubation with SVPD (1.2 U/μL) and magnesium chloride (10 mM) in the presence (red line) or absence (black line) of 20 mM EDTA for 30 minutes at 30 °C.
Figure S3: X-band cw-EPR spectrum of a mixture of 60% cleaved and 40% non-cleaved probe 1 (black), recorded at -50°C, and its fit (red) by a linear combination of the EPR spectra of the cleaved and non-cleaved probe 1. The residuum (violet) shows only minor deviations.
Figure S4: Mean ± standard deviation of triplicates of double integrals, normalized to the mean of the double integrals, of the EPR spectra of the time-course of SVPD reaction with 0.30 U/μL SVPD and an initial concentration of 200 μM 1.
<table>
<thead>
<tr>
<th>c(SVPD)</th>
<th>c(probe 1)</th>
<th>$A_0$</th>
<th>$t_d$</th>
<th>$y$</th>
</tr>
</thead>
<tbody>
<tr>
<td>in U/μL</td>
<td>in μM</td>
<td>in %</td>
<td>in min</td>
<td>in %</td>
</tr>
<tr>
<td>0.15</td>
<td>200</td>
<td>89(2)</td>
<td>128(7)</td>
<td>5(2)</td>
</tr>
<tr>
<td>0.30</td>
<td>200</td>
<td>87.6(8)</td>
<td>62(2)</td>
<td>8.6(7)</td>
</tr>
<tr>
<td>0.30</td>
<td>400</td>
<td>86(1)</td>
<td>61(2)</td>
<td>11(1)</td>
</tr>
<tr>
<td>0.60</td>
<td>200</td>
<td>90(1)</td>
<td>16.3(6)</td>
<td>3.9(4)</td>
</tr>
</tbody>
</table>

**Table S1:** Parameters obtained from fits with exponential decays with the general equation $A(t) = A_0 \exp(-t/t_d) + y$. At time zero the decay does not start with 100 % but with the parameter $A_0+y$, because of a time delay between mixing and first measurements.
Supplementary Methods

General experimental procedures:
All reagents are commercially available and used without further purification. Solvents are dried over molecular sieves as needed and used directly without further purification. Reactions were conducted under exclusion of air and moisture as needed. Reversed phase high pressure liquid chromatography (RP-HPLC) was performed using a Shimadzu Prominence system having preparative LC-20AP pumps. For the purification of nucleotides a VP 250/21 NUCLEODUR C18 HTec, 5 μm (Macherey-Nagel) column and a linear gradient of 5% to 100% acetonitrile in 50 mM triethylammonium acetate buffer (TEAA, pH 7.0) were used. 1.0 M TEAA was made by adding 1 mol triethylamine to a solution of 1 mol acetic acid in 1 L water and adjusting the pH to 7.0. The buffer was diluted to 50 mM as needed. Analytical HPLC was performed on a Shimadzu Prominence system having analytical LC-20AT pumps using an EC 250/4 NUCLEODUR 100-5 C18 ec (Macherey-Nagel) column and a linear gradient of acetonitrile in 50 mM TEAA buffer. NMR spectra were recorded on a Bruker Avance III 400 MHz spectrometer. 1H and 13C chemical shifts are reported relative to the residual solvent peak and are given in ppm (δ). s: singlet, d: duplet, t: triplet, q: quartet, bs: broad signal, m: multiplet. HRMS was measured on a Bruker Daltronics micrTOF-Q II ESI-Q-TOF. The reported yield refers to the analytically pure substance and is not optimized. All temperatures quoted are uncorrected. One unit (U) SVPD is defined to hydrolyze one micromole of p-nitrophenyl thymidine-5’-monophosphate per minute at a concentration of 500 μM at 25°C in buffer containing 0.1 M Tris (pH 8.9), 0.1 M NaCl and 15 mM MgCl2.

DEER experiment:
The EPR DEER experiment was performed in X-band using an Elexsys E580 spectrometer (Bruker Biospin) equipped with a helium gas flow system (CF935, Oxford Instruments). The four-pulse, dead time free DEER sequence is given by: π/2_{obs} − τ_{1} − π_{obs} − t − π_{pump} − (τ_{1}+τ_{2}−t) − π_{obs} − τ_{2} − echo. The pump pulse (12 ns corresponding to a π-pulse) was set to the maximum of the nitroxide spectrum and the observer pulse was set 67 MHz higher; π/2 and π pulses at observer frequency were of 12 ns and 24 ns length, respectively. The sample was measured at τ_{2} = 3 μs with nuclear modulation averaging to avoid artifacts from the proton nuclear modulation. The accumulation time was 18 hours at 50 K.

Analysis of DEER data:
Processing and distance distribution analysis of the DEER time trace was performed using the DeerAnalysis2013 software. The distance distribution was extracted with the model-free Tikhonov regularization method with a regularization parameter of 100.
cw-EPR measurement procedures:
Continuous wave (cw-) EPR spectra were recorded at -50°C on an X-band MiniScope spectrometer (MS200, magnettech GmbH) equipped with a variable temperature unit (Temperature Controller TC-H02, magnettech GmbH). Samples were loaded into glass capillaries (Blaubrand, outer diameter 1 mm) with typical sample volumes of 30 µl. Spectra were obtained with a sweep width of typically 200 G, a modulation amplitude of 1000 mG, and microwave attenuation of 22 dB. The signal-to-noise ratio was improved by accumulation of 2 spectra featuring 100 s scan time each, except for the reference spectra, which were averaged 10 times. The spectra were fitted as described below. Signal intensities were determined via the double integral of the first derivative EPR spectrum. The spectra were corrected for frequency drifts for visualization and fitting.

Fitting of cw-EPR spectra:
The cw-EPR spectra were analyzed using Matlab R2013a (The MatWorks, Inc.). The spectra were fitted by a linear combination $S_{\text{sup}} = a S_{\text{cc}} + (1 - a) S_{\text{nc}}$ of the experimental spectra of completely cleaved $S_{\text{cc}}$ and non-cleaved $S_{\text{nc}}$ probe 1 to obtain the fraction $a$ of cleaved probe 1 using the Matlab function `fmincon`.

SVPD cleavage of probe 1:
400 µM probe 1 and 1.2 U/µL SVPD were incubated in a buffer containing 10 mM MgCl$_2$, 100 mM NaCl and 50 mM Tris-HCl (pH 7.9) for 30 min at 30 °C. The reaction was stopped by addition of 20 mM EDTA. As a negative control the same reaction was set up with addition of EDTA before addition of SVPD. The samples were analyzed by RP-HPLC and peaks were identified by HR-ESI-MS.

cw-EPR experiments of probe 1:
Enzymatic reactions were performed as described above and the samples were diluted with a glycerol/water solution to yield a probe concentration of 200 µM and a glycerol concentration of 20%. EPR was measured as described above. To measure the cw-EPR spectra of different ratios of cleaved and non-cleaved probe 1, the corresponding amounts of the two reaction mixtures were mixed and monitored by cw-EPR in the same manner.

Time-course experiment by EPR:
200 µM probe 1 and the indicated amount of SVPD were dissolved in a buffer containing 20% glycerol, 10 mM MgCl$_2$, 100 mM NaCl and 50 mM Tris-HCl (pH 7.9). The sample was immediately frozen on dry ice or put into the pre-cooled spectrometer. The cw-EPR spectrum was measured as described above. The solution was warmed to 20 °C and incubated for the indicated time. The solution was frozen again inside the spectrometer and the cw-EPR spectrum was measured. This process was repeated for all following time-points. The obtained data for each time-
point was fitted as described. The fraction of cleaved probe 1 over time was fitted using an exponential decay.
Chemical Synthesis:

Scheme S1: Synthesis of EPR probe 1. Conditions: a) 25% ammonia, water, 8 h, r.t., 80%; b) TCEP-HCl, water, methanol, triethylamine, 12 h, r.t., 88%; c) 2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl-3-carboxylic acid NHS ester, 0.1 M NaHCO₃ (pH 8.7), DMF, 12 h, r.t., 42%.

N₆-(6-Aminohexyl)-δ-(6-azidoheptyl)-adenosine-O₅'-tetraphosphate 3:
N₆-(6-Trifluoroacetamidohexyl)-δ-(6-azidoheptyl)-adenosine-O₅'-tetraphosphate 2[^3] (34.7 μmol) was dissolved in 5 mL water and 10 mL 25% ammonia in water were added. The reaction mixture was stirred at room temperature for 8 hours. The solvent was evaporated. Purification by RP-HPLC gave N₆-(6-aminohexyl)-δ-(6-azidoheptyl)-adenosine-O₅'-tetraphosphate 3 (27.7 μmol, 80%) as colorless oil.

[^1]H-NMR (d₄-methanol, 400 MHz): δ 8.63 (s, 1H, H-8), 8.18 (s, 1H, H-2), 6.11 (d, J = 4.8 Hz, 1H, H-1'), 4.78 – 4.65 (m, 1H, H-2'), 4.65 – 4.56 (m, 1H, H-3'), 4.36 – 4.17 (m, 3H, H-4', H-5'a, H-5'b), 3.99 (q, J = 6.5 Hz, 2H, δP-O-C₃H₂), 3.55 (bs, 2H, N₆-C₃H₂), 3.26 (t, J = 6.8 Hz, 2H, N₃-C₃H₂), 2.96 – 2.80 (m, 2H, NH₂-C₄H₂), 1.78 – 1.50 (m, 8H, 4x C₄H₂-linker), 1.47 – 1.32 (m, 8H, 4x CH₂-linker).

[^3]P-NMR (d₄-methanol, 162 MHz): δ -10.64 - -11.11 (m, 1P), -11.26 - -11.70 (m, 1P), -22.33 - -23.29 (m, 2P).
HR-ESI-MS: found: 810.1544; calculated: 810.1538 (M-H⁺, C₂₂H₄₀N₉O₁₆P₄⁺); deviation: 0.7 ppm.

N₆-(6-Aminohexyl)-δ-(6-aminohexyl)-adenosine-O₅'-tetraphosphate 4:
N₆-(6-Aminohexyl)-δ-(6-azidoheptyl)-adenosine-O₅'-tetraphosphate 3 (8.3 μmol) was dissolved in 2.5 mL water, 2.5 mL methanol and 1.25 mL triethylamine. TCEP hydrochloride (13 mg, 41.5 μmol, 5 equiv.) was added. The reaction mixture was stirred at room temperature overnight. The solvents were evaporated. Purification by RP-HPLC gave N₆-(6-aminohexyl)-δ-(6-aminohexyl)-adenosine-O₅'-tetraphosphate 4 (7.3 μmol, 88%) as colorless oil.

[^1]H-NMR (d₄-methanol, 400 MHz): δ 8.55 (s, 1H, H-8), 8.25 (s, 1H, H-2), 6.14 (d, J = 5.9 Hz, 1H, H-1'), 4.85 – 4.80 (m, 1H, H-2'), 4.63 – 4.59 (m, 1H, H-3'), 4.45 – 4.39 (m, 1H, H-4'), 4.35 – 4.21 (m, 2H, H-5'a, H-5'b), 3.96 (q, J = 6.5 Hz, 2H, δP-O-CH₂),
3.57 (bs, 2H, N6-CH₂), 3.04 – 2.94 (m, 4H, 2x NH₂-CH₂), 1.77 – 1.53 (m, 8H, 4x CH₂-linker), 1.50 – 1.33 (m, 8H, 4x CH₂-linker).

³¹P-NMR (d₄-methanol, 162 MHz): δ -10.45 - -11.12 (m, 1P), -11.21 - -11.72 (m, 1P), -22.86 - -24.11 (m, 2P).

HR-ESI-MS: found: 784.1608; calculated: 784.1633 (M-H⁺, C₂₂H₄₂N₇O₁₆P₄⁻); deviation: 3.2 ppm.

**EPR probe 1:**

N6-(6-aminohexyl)-δ-(6-aminohexyl)-adenosine-O5'-tetraphosphate 4 (7.3 μmol) was dissolved in 2 mL 0.1 M NaHCO₃ solution (pH 8.7). 2,2,5,5-Tetramethyl-3-pyrrolin-1-oxyl-3-carboxylic acid NHS ester (5 mg, 18.3 μmol, 2.5 equiv.) was added and the solution was stirred at room temperature overnight. The solvents were evaporated. Purification by RP-HPLC gave EPR probe 1 (3.1 μmol, 42%) as colorless oil.

HR-ESI-MS: found: 557.6645; calculated: 557.6643 (M-2H⁺, C₄₀H₆₅N₉O₂₀P₄²⁻); deviation: 0.4 ppm.
N6-(6-Aminohexyl)-δ-(6-azidoheptyl)-adenosine-O5'-tetraphosphate 3:

$^1$H-NMR

$^{31}$P-NMR
N6-(6-Aminohexyl)-δ-(6-aminohexyl)-adenosine-O5'-tetraphosphate 4:

$^1$H-NMR

$^{31}$P-NMR
Supplementary References

