Supporting Information

Thiamine pyrophosphate stimulates acetone activation by *Desulfococcus biacutus* as monitored by a fluorogenic ATP analogue

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General experimental details

All temperatures quoted are uncorrected. All reagents were commercially available and used without further purification. All solvents were dried over molecular sieves and used directly without further purification. All reactions were conducted under exclusion of air and moisture. For medium pressure liquid chromatography (MPLC), a Büchi unit with a Büchi controller C-620, two pumps C-605, a UV monitor C-630 (λ = 254 nm) and fraction collector C-660 was used. For the purification of nucleosides and nucleotides, a 310-25 LiChroprep® RP-18 ready-to-use column (Merck, 40-63 mm) with a linear gradient (5 to 100%) of acetonitrile in 50 mM aqueous triethylammonium acetate (TEAA buffer, pH 7.0) was used. Reversed phase high pressure liquid chromatography (RP-HPLC) was performed using a Shimadzu unit. For the purification of nucleotides a EC 250/4 NUCLEODUR 100-5 C18 ec (Macherey-Nagel), VP 250/10 NUCLEODUR 100-5 C18 ec (Macherey-Nagel) or VP 250/21 NUCLEODUR C18 HTec, 5μm (Macherey-Nagel) column and a linear gradient (5 to 100%) of acetonitrile in 50 mM TEAA buffer (pH 7.0) was used. NMR spectra: Bruker Avance III 400 MHz spectrometer and Bruker Avance III 600 MHz spectrometer. ^{1}H and ^{13}C chemical shifts are reported relative to the residual solvent peak and are given in ppm (δ). A BBFOplus probe with actively shielded z-gradient was used with its inner (BB-) coil tuned to ^{19}F and ^{31}P, respectively. Flash chromatography: Merck silica gel G60. TLC: Merck precoated plates (silica gel 60 F_{254}). ESI-IT: Bruker Esquire 3000 plus. HRMS: Bruker Daltronics micrOTOF-Q II ESI-Qq-TOF. The reported yield refers to the analytically pure substance and has not been optimized. Compound **2** was synthesized according to the corresponding literature. Snake venom phosphodiesterase (SVPD) was purchased from

Worthington Biochemical Corporation. For fluorescent measurements, a Perkin Elmer Luminescence Spectrometer LS50 was used.

Synthesis of Compounds

γ-(6-Azidohexyl)-2-(5-aminopent-1-yn-1-yl)-adenosine-triphosphate 2

Nucleotide **2** was synthesized according to Hardt *et. al.*.[1]

γ-(6-Azidohexyl)-2-(5-eclipse-amidopent-1-yn-1-yl)-adenosine-triphosphate 3

Compound **2** (30.0 μmol, 1 eq.) was dissolved in 5 mL 0.1M $NaHCO_3$ (pH 8.7) and eclipse-NHS (50.0 mg, 102.1 μmol, 3.4 eq.), dissolved in dry DMF (1.9 mL), was added and stirred at room temperature overnight. The solvents were evaporated under reduced pressure. The compound was purified by MPLC. Fractions containing the product were evaporated and further purified by RP-HPLC. The solvent was evaporated and the product repeatedly freeze-dried from water to give 7.7 μmol (26%) of compound **3**.

^{1}H NMR (MeOD-d_4, 600 MHz): δ 8.65 (s, 1H, H8), 8.35 (d, *J* = 2.3 Hz, 1H, H-Ar_2-eclipse), 8.16 (dd, *J* = 8.9, 2.3 Hz, 1H, H-Ar_2-eclipse), 7.87 (d, *J* = 9.1 Hz, 2H, H-Ar_1-eclipse), 7.76 (d, *J* = 8.9 Hz, 1H, H-Ar_2-eclipse), 6.83 (d, *J* = 9.2 Hz, 2H, H-Ar_1-eclipse), 6.05 (d, *J* = 5.2 Hz, 1H, H1'), 4.62 (t, *J* = 5.0 Hz, 1H, H2'), 4.54 (t, *J* = 4.3 Hz, 1H, H3'), 4.36 – 4.30 (m, 1H, H5'a), 4.30 – 4.22 (m, 2H, H4', H5'b), 4.01 (q, *J* = 6.1 Hz, 2H, C**H**$_2$-linker), 3.52 (t, *J* = 7.5 Hz, 2H, C**H**$_2$-linker), 3.36 (t, *J* = 6.6 Hz, 2H, C**H**$_2$-linker), 3.23 (t, *J* = 6.9 Hz, 2H, C**H**$_2$-linker), 3.09 (s, 3H, CH_3-eclipse), 2.50 (t, *J* = 7.0 Hz, 2H, C**H**$_2$-linker), 2.32 (t, *J* = 7.1 Hz, 2H, C**H**$_2$-linker), 2.02 – 1.91 (m, 2H, C**H**$_2$-linker), 1.84 (p, *J* = 6.8 Hz, 2H, C**H**$_2$-linker), 1.68 – 1.57 (m, 2H, C**H**$_2$-linker), 1.58 – 1.48 (m, 2H, C**H**$_2$-linker), 1.46 – 1.32 (m, 4H, 2x C**H**$_2$-linker).

^{13}C NMR (MeOD-d_4, 151 MHz): δ 175.41, 156.20, 154.69, 154.34, 150.72, 148.46, 145.47, 142.11, 134.64, 127.93, 126.78, 123.87, 119.28, 119.06 112.68, 89.05, 85.56 (d, $J_{C\text{-}P}$ = 9.2 Hz), 80.76, 76.49, 71.63, 67.27 (d, $J_{C\text{-}P}$ = 6.1 Hz), 66.41 (d, $J_{C\text{-}P}$ = 6.4 Hz), 52.67, 52.40, 39.82, 38.77, 33.86, 31.63 (d, $J_{C\text{-}P}$ = 8.1 Hz), 29.86, 28.81, 27.55, 26.43, 24.07, 17.56.

^{31}P NMR (MeOD-d_4, 162 MHz): δ -11.13 (d, *J* = 17.4 Hz, 1P), -11.45 (d, *J* = 19.0 Hz, 1P), -22.94 (t, *J* = 17.9 Hz, 1P).

HR-ESI-MS: found: 1070.2238; calculated: 1070.2238 (M-H^+, $C_{38}H_{48}ClN_{13}O_{16}P_3^-$); deviation: 0.1 ppm.

γ-(6-Aminohexyl)-2-(5-eclipse-amidopent-1-yn-1-yl)-adenosine-triphosphate 4

Compound **3** (7.7 μmol, 1 eq.) was dissolved in 5 mL water/methanol/triethylamine (0.5:1.0:0.5) and tris(2-carboxyethyl)phosphine (25.0 mg, 99.9 μmol, 13.0 eq.), dissolved in water (0.5 mL), was added rapidly and stirred at room temperature for 4 hours. The solvents were evaporated under reduced pressure. The compound was purified by RP-HPLC. Fractions containing the product were evaporated and the product repeatedly freeze dried from water to give 4.4 μmol (57%) of compound **4**.

^{1}H NMR (MeOD-d_4, 600 MHz): δ 8.63 (s, 1H, H8), 8.33 (d, *J* = 2.4 Hz, 1H, H-Ar_2-eclipse), 8.14 (dd, *J* = 8.9, 2.4 Hz, 1H, H-Ar_2-eclipse), 7.85 (d, *J* = 9.2 Hz, 2H, H-Ar_1-eclipse), 7.74 (d, *J* = 8.9 Hz, 1H, H-Ar_2-eclipse), 6.82 (d, *J* = 9.2 Hz, 2H, H-Ar_1-eclipse), 6.03 (d, *J* = 4.0 Hz, 1H, H1'), 4.59 – 4.55 (m, 2H, H2', H3'), 4.34 – 4.29 (m, 1H, H5'a), 4.29 – 4.19 (m, 2H, H4', H5'b), 4.03 – 3.93 (m, 2H, C**H**$_2$-linker), 3.55 – 3.48 (m, 2H, C**H**$_2$-linker), 3.35 (t, *J* = 6.7 Hz, 2H, C**H**$_2$-linker), 3.09 (s, 3H, CH_3-eclipse), 2.96 (t, *J* = 6.9 Hz, 2H, C**H**$_2$-linker), 2.48 (t, *J* = 7.0 Hz, 2H, C**H**$_2$-linker), 2.31 (t, *J* = 7.1 Hz, 2H, C**H**$_2$-linker), 2.00 – 1.92 (m, 2H, C**H**$_2$-linker), 1.83 (p, *J* = 6.9 Hz,

2H, C**H**$_2$-linker), 1.72 – 1.64 (m, 2H, C**H**$_2$-linker), 1.61 – 1.54 (m, 2H, C**H**$_2$-linker), 1.46 – 1.40 (m, 4H, 2x C**H**$_2$-linker).

^{13}C NMR (MeOD-d$_4$, 151 MHz): δ 175.36, 156.96, 154.67, 154.32, 150.75, 148.44, 147.68, 145.46, 141.69, 134.65, 127.90, 126.77, 123.83, 119.49, 119.02 112.65, 89.27, 87.48, 85.30 (d, J_{C-P} = 9.2 Hz), 81.59, 76.54, 71.22, 66.18 (d, J_{C-P} = 6.4 Hz), 66.11 (d, J_{C-P} = 4.5 Hz), 52.65, 40.12, 39.83, 38.77, 33.85, 30.61 (d, J_{C-P} = 7.6 Hz), 28.90, 28.03, 25.88, 25.58, 24.07, 17.52.

^{31}P NMR (MeOD-d$_4$, 162 MHz): δ -10.63 (d, *J* = 19.0 Hz, 1P), -11.34 (d, *J* = 18.9 Hz, 1P), -22.02 – -22.96 (m, 1P).

HR-ESI-MS: found: 1044.2354; calculated: 1044.2333 (M-H$^+$, $C_{38}H_{50}ClN_{11}O_{16}P_3^-$); deviation: 2.0 ppm.

γ-(6-Sulfo-cyanine3-amidohexyl)-2-(5-eclipse-amidopent-1-yn-1-yl)-adenosine-triphosphate 1

Compound **4** (4.4 μmol, 1 eq.) was dissolved in 1 mL 0.1 M $NaHCO_3$ (pH 8.7) and sulfo-cyanine3-NHS (25.0 mg, 34.4 μmol, 7.8 eq.), dissolved in dry DMF (1.0 mL), was added and stirred at room temperature overnight. The solvents were evaporated under reduced pressure. The compound was purified by MPLC. Fractions containing the product were evaporated and further purified by RP-HPLC. The solvent was evaporated and the product repeatedly freeze-dried from water to give 3.5 μmol (80%) of compound **1**.

^{1}H NMR (MeOD-d$_4$, 600 MHz): δ 8.68 (s, 1H, H8), 8.46 (t, *J* = 13.5 Hz, 1H, H-β-Cy3), 8.36 (d, *J* = 2.3 Hz, 1H, H-Ar$_2$-eclipse), 8.16 (dd, *J* = 8.9, 2.4 Hz, 1H, H-Ar$_2$-eclipse), 7.98 – 7.89 (m, 4H, H-Ar-Cy3), 7.86 (d, *J* = 9.1 Hz, 2H, H-Ar$_1$-eclipse), 7.76 (d, *J* = 8.9 Hz, 1H, H-Ar$_2$-eclipse), 7.40 (d, *J* = 8.7 Hz, 1H, H-Ar-Cy3), 7.39 (d, *J* = 8.7 Hz,

1H, H-Ar-Cy3), 6.84 (d, *J* = 9.2 Hz, 2H, H-Ar_1-eclipse), 6.46 (d, *J* = 13.4 Hz, 1H, H-α-Cy3), 6.42 (d, *J* = 13.4 Hz, 1H, H-α'-Cy3), 6.08 (d, *J* = 6.0 Hz, 1H, H1'), 4.85 – 4.70 (m, 1H, H-2'), 4.58 – 4.50 (m, 1H, H3'), 4.35 – 4.28 (m, 1H, H5'a), 4.27 – 4.23 (m, 1H, H4'), 4.23 – 4.16 (m, 3H, H5'b, C**H**$_2$-linker), 4.16 – 4.08 (m, 2H, C**H**$_2$-linker), 4.04 – 3.92 (m, 2H, C**H**$_2$-linker), 3.55 – 3.48 (m, 2H, C**H**$_2$-linker), 3.34 – 3.28 (m, 2H, C**H**$_2$-linker), 3.13 (t, *J* = 6.7 Hz, 2H, C**H**$_2$-linker), 3.09 (s, 3H, CH_3-eclipse), 2.38 (t, *J* = 7.0 Hz, 2H, C**H**$_2$-linker), 2.31 (t, *J* = 7.1 Hz, 2H, C**H**$_2$-linker), 2.22 (t, *J* = 7.4 Hz, 2H, C**H**$_2$-linker), 1.97 – 1.92 (m, 2H, C**H**$_2$-linker), 1.83 – 1.80 (m, 2H, C**H**$_2$-linker), 1,78 – 1.72 (m, 12H, 4x CH_3-Cy3), 1.71 -1.63 m, 2H, C**H**$_2$-linker), 1.62 – 1.53 (m, 2H, C**H**$_2$-linker), 1.52 – 1.43 (m, 4H, 2x C**H**$_2$-linker), 1.39 (t, *J* = 7.2 Hz, 3H, CH_3-ethyl), 1.36 – 1.27 (m, 6H, 3x C**H**$_2$-linker).

^{13}C NMR (MeOD-d_4, 151 MHz): δ 176.57, 176.36, 175.80, 175.34, 156.94, 154.71, 154.30, 152.73, 151.20 148.43, 147.66, 145.42, 144.64, 144.22, 144.04, 143.99, 142.38, 142.23, 134.64, 128.27, 127.96, 126.84, 123.92, 121.47, 121.42, 119.42, 119.05, 112.74, 112.21, 112.03, 104.63, 104.49, 88.51, 87.41, 81.64, 76.20, 67.00, 52.68, 50.70, 50.64, 45.51, 40.76, 40.30, 39.77, 38.86, 36.82, 33.84, 31.46 (d, $J_{C\text{-}P}$ = 7.7 Hz), 30.17, 28.29, 28.99, 28.26, 28.16, 27.53, 27.42, 26.69, 26.40, 24.12, 17.48, 12.73.

^{31}P NMR (MeOD-d_4, 162 MHz): δ -10.52 (d, *J* = 17.4 Hz, 1P), -11.03 (d, *J* = 17.0 Hz, 1P), -20.56 – -21.40 (m, 1P).

HR-ESI-MS: found: 827.7091; calculated: 827.7107 (M-2H$^+$, $C_{69}H_{85}ClN_{13}O_{23}P_3S_2^{2-}$); deviation: 1.9 ppm.

General procedures

SVPD cleavage: 2.5 μL (1 mM) of the corresponding doubly dye-labeled ATP analogue and 3.13 μL SVPD (0.2 mg mL^{-1}) were processed in a total volume of 25 μL of NEB buffer 3 (100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9) for 30 min at 30°C. Negative controls were performed in the absence of SVPD.

Fluorescence spectra: For fluorescence measurements the SVPD reactions were diluted with 3 mL 1x PBS buffer and the fluorescence spectra of the crude reaction solution was measured. Excitation of sulfo-Cy3 was performed using a wavelength of 532 nm.

HPLC analysis: For HPLC analysis the SVPD reactions were diluted with 275 μL of H_2O and separated by analytical RP-HPLC (a linear gradient of acetonitrile in 50 mM aqueous triethylammonium acetate (TEAA buffer, pH 7.0) was used). RP-HPLC analytics were visualized using 2D-plots. Fractions were collected and further characterized by HRMS.

HRMS characterisation of probe 1 and its cleavage products 5 and 6 after SVPD cleavage: Control without SVPD: HR-ESI-MS **1**: found: 827.7125; calculated: 827.7107 ($M\text{-}2H^+$, $C_{69}H_{85}ClN_{13}O_{23}P_3S_2^{2-}$); deviation: 2.2 ppm. Reaction with SVPD: HR-ESI-MS **5**: found: 443.6127; calculated: 443.6138 ($M\text{-}H^{2+}$, $C_{37}H_{52}N_3O_{14}P_2S_2^{2-}$); deviation: 2.5 ppm. HR-ESI-MS **6**: found: 785.1978; calculated: 785.1958 ($M\text{-}H^+$, $C_{32}H_{35}ClN_{10}O_{10}P^-$); deviation: 2.5 ppm.

Cell cultures: The sulfate-reducing bacterium *Desulfococcus biacutus* strain KMRActS was grown in freshwater mineral medium as described before. [2, 3] The medium was reduced with 1 mM sulfide, buffered with CO_2-bicarbonate, and adjusted to a final pH of 7.2. Cells were cultured in 1 L flasks with medium supplemented with 5 mM acetone as sole carbon source and 10 mM sulfate as the electron acceptor. Cultures were incubated under a strictly anoxic N_2-CO_2 (80/20) atmosphere at 30°C in the dark.

Preparation of cell extract: Cells of *D. biacutus* were harvested in the late exponential growth phase at an optical density of 0.3 (OD_{600}). All experiments with cell extract were done under strictly anoxic conditions. Cells were centrifuged at 6,000 x g at 4°C and the pellet was washed at least twice with 50 mM potassium phosphate (KP) buffer, pH 7.2, supplemented with 3 mM dithioerythritol as reducing agent. The cell pellet was resuspended in the same buffer plus 0.5 mg DNase mL^{-1} and 1 mg mL^{-1} of complete protease inhibitor cocktail (Complete Mini, EDTA-free protease inhibitor cocktail tablets, Roche Diagnostics GmbH). Cells were disrupted by passing them twice through a cooled French pressure cell at 100 MPa. Cell debris and un-opened cells were removed by centrifugation at 27,000 x g for 20 min at 4°C.

Reaction of probe 1 in cell extract: Cell extract of *D. biacutus* prepared after growth on acetone was used to test the carbonylation reaction. All enzyme assays were carried out under strictly anoxic conditions. The buffer solution was the same as described for washing the cells pellet, and contained 1 g L^{-1} NaCl, 0.6 g L^{-1} $MgCl_2$ H_2O and 3 mM dithioerythritol. The enzyme reaction was tested in Eppendorf tubes sealed with a rubber stopper. Concentrations of substrates were 1 mM acetone, 0.5 mM ATP analogue **1**, 2 mM TPP and 10% CO in the headspace. The reaction was

incubated for 1 hour at 30°C. For the time course experiments, samples were taken at different time intervals with syringes previously flushed with N_2. Addition of 2 mM CoA and 4mM NAD^+ to the same reaction system was also tested. Before testing the effect of the addition of those cofactors, cell extract was pretreated for removal of small molecules by passing freshly prepared sample protein by gravity through a Sephadex™ G-25 column (illustra NAP-25 Columns; GE Healthcare). The same KP buffer described avobe was used for column equilibration and protein elution. All reactions were stopped with 2 mM EDTA solution. Each reaction condition was run in triplicate.

Fluorescent read out: From each sample, 10 μL aliquots were uniformly mixed with 90 μL Milli Q water and placed onto a dark 96 well plate for fluorescence measurement. Dilutions were done as it was required. The procedure was run three times for each triplicate. Standards of sulfo-Cy3 were prepared in order to quantify the rate and specific activity of hydrolysis of ATP probe **1**.

HPLC analysis of the assay mixtures: Protein was precipitated by the addition of 10 μL of 2 M H_2SO_4, and removed by centrifugation at 10,000 x g. The supernatant was analysed by analytical RP-HPLC as described above. Fractions containing ATP fragments were collected and further characterized by HRMS.

HRMS characterisation of probe 1 and its cleavage products after incubation in cell extract: Fractions were collected during RP-HPLC separation and analysed by HRMS. Control under nitrogen: HR-ESI-MS **1**: found: 827.7091; calculated: 827.7107 ($M\text{-}2H^+$, $C_{69}H_{85}ClN_{13}O_{23}P_3S_2^{2-}$); deviation: 1.9 ppm. Reaction under CO: HR-ESI-MS

5: found: 888.2377; calculated: 888.2360 (M-H^+, $C_{37}H_{53}N_3O_{14}P_2S_2^-$); deviation: 1.9 ppm.

Literature

[1] (a) Hacker, S. M., Hardt, N., Buntru, A., Pagliarini, D., Mockel, M., Mayer, T. U., Scheffner, M., Hauck, C. R., and Marx, A. (2013) Fingerprinting differential active site constraints of ATPases. *Chemical Science* 4, 1588-1596.

(b) Hardt, N., Hacker, S. M., and Marx, A. (2013) Synthesis and fluorescence characteristics of ATP-based FRET probes, *Organic & biomolecular chemistry* 11, 8298-8305.

[2] Platen, H., Temmes, A., and Schink, B. (1990) Anaerobic degradation of acetone by *Desulfococcus biacutus* spec. nov., *Archives Microbiology* 154, 355-361.

[3] Gutierrez Acosta, O. B., Hardt, N., and Schink, B. (2013) Carbonylation as a key reaction in anaerobic acetone activation by *Desulfococcus biacutus*, *Applied and Environmental Microbiology* 79, 6228-6235.