Supporting Information

Enzymatic One-Step Reduction of Carboxylates to Aldehydes with Cell-Free Regeneration of ATP and NADPH

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# Table of contents

Table of contents ...................................................................................................................... 1

1) Experimental procedures ..................................................................................................... 2

   1.1. General ............................................................................................................................. 2

   1.2. Preparation of enzymes ................................................................................................... 2

       1.2.1. Carboxylate reductases ......................................................................................... 2

       1.2.2. Polyphosphate kinases .......................................................................................... 2

       1.2.3. Pyrophosphatase ...................................................................................................... 2

   1.3. Analytical Methods and Preparation of Reference Materials .............................................. 3

       1.3.1. HPLC-analysis of AMP, ADP and ATP (Method_1) .................................................. 3

       1.3.2. HPLC-analysis of carboxylic acids 1a-l, aldehydes 2a-l and alcohols 3a-l (Method_2) ... 3

       1.3.3. GC-FID-analysis of carboxylic acids 1m-o, aldehydes 2m-o and alcohols 3m-o (Method_3) 4

   1.4. Enzyme-catalyzed reactions ............................................................................................. 4

       1.4.1. Activity tests with PPK enzymes (protocol 1) ............................................................. 4

       1.4.2. Investigation of the regeneration of ATP (Figure 1 in the main manuscript) .................. 5

       1.4.3. Influence of polyphosphate and different buffer systems on NcCAR-catalyzed carboxylate reductions of piperonylic acid 1a (Table 1 in the main manuscript) ................................................................................. 5

       1.4.4. Investigation of the in vitro carboxylate reduction (Table 2 in the main manuscript) .......... 6

       1.4.5. Investigation of the polyP amounts required for the in vitro carboxylate reduction (Figure 2 in the main manuscript) ................................................................................................................. 7

       1.4.6. ATP feeding .................................................................................................................. 7

2) Results and Discussion .............................................................................................................. 8

3) NMR data ................................................................................................................................. 10

4) References ................................................................................................................................. 11

5) Author Contributions ................................................................................................................ 11
1) Experimental procedures

1.1. General

ATP, ADP and AMP were obtained from Aldrich, Roche Diagnostics and Carl Roth (Karlsruhe, Germany). NADP, NADPH and MES were purchased from Carl Roth, IPTG from Serva, and MgCl₂ from Merck. HPLC-MS grade acetonitrile was purchased from J.T.Baker/Avantor Performance Materials (Deventer, The Netherlands) and VWR (Vienna, Austria). Sodium polyphosphate was obtained from Merck (Darmstadt, Germany); order no. 1.065.291.001, Lot K46879329603; 69.0% concentration based on P₂O₅, medium chain length n = 25) and concentrations mentioned in this work are based on ortho-phosphate units. Analysis data on purity and water content provided by the vendors was used to adjust the amounts of reagents used in all experiments appropriately. Glucose dehydrogenase GDH-105 was obtained from Codexis (Redwood City, CA, USA). All other chemicals were obtained from Sigma-Aldrich/Fluka or Carl Roth and used without further purification.

E. coli cells were cultivated in an RS 306 shaker (Infors, Bottmingen, Switzerland) and Multitron shakers (Infors AG), and the cells were harvested with an Avanti J-20 centrifuge (Beckman Coulter). Cell pellets were disrupted using a 102C converter with a Sonifier 250 (Branson, Danbury, CT), and the cell-free extracts were obtained by centrifugation in an Ultracentrifuge Optima LE80K (Beckman). Enzymes were purified using an ÄKTApure 25 with a fraction collector F9-C (Unicorn 7.3 software; GE Healthcare) or alternatively purified using the gravity flow protocol. Desalting was performed with an ÄKTAPrime (PrimeView 5.0 software; GE Healthcare) or by repeated centrifugation. Small scale reactions were performed in 1.5 mL-polypropylene tubes (Eppendorf, Hamburg, Germany) on a Thermomixer comfort (Eppendorf).

Flash column chromatography was performed on silica gel 0.035-0.070 mm, 60 Å (product no. 240360300; Acros Organics, Geel, Belgium). ¹H and ¹³C NMR spectra were recorded on a Bruker AVANCE III 300 spectrometer (1H: 300.36 MHz; 13C: 75.53 MHz) and chemical shifts are referenced to residual protonated solvent signals as internal standard.

1.2. Preparation of enzymes

1.2.1. Carboxylate reductases

Codon optimized synthetic genes coding for Nocardia iowensis CAR [1] (Q6RK81.1; NCAR) and Neurospora crassa CAR [3] (XP_955820.1; NcCAR) were expressed with N-terminal TEV-cleavable HIS-tag from the multiple cloning site (MCS) 2 of the pETDUET1 vector. For the essential post-translational modification of CAR enzymes, [4] E. coli phosphopantetheinyl transferase (NCBI accession code CAQ31055.1) was expressed from MCS1 simultaneously. E. coli K-12 MG1655 RARE [5] served as the expression host and the cells were cultivated under autoinduction conditions. [6] E. coli cells were disrupted by sonication. Purification was performed under standard conditions (pH 7.4) for immobilized metal affinity chromatography using Ni-sepharose as the stationary phase. Protein samples were analyzed with 4-12% NuPAGE Bis-Tris Gel (Invitrogen). Protein containing fractions were pooled and re-buffered into 100 mM phosphate buffer, pH 7.0, containing 10 mM MgCl₂, 100 mM NaCl, 1 mM EDTA, and 1 mM DTT either using a HiTrap™ 26/10 Desalting column or VivaSpin 20 tubes (30,000 MWCO, Sartorius). Protein concentrations were determined with the BCA assay according to the manufacturer’s protocol using bovine serum albumin as the standard. Aliquots of concentrated protein preparations were shock frozen in liquid nitrogen and stored at -80°C. Activity of CAR preparations was assessed using a spectrophotometric NADPH depletion assay as published with 5 mM piperonylic acid as the substrate. [7]

1.2.2. Polyphosphate kinases

HIS-tagged polyphosphate kinases from Sinorhizobium meliloti (SmPPK) and Acinetobacter johnsonii (A)PPK were expressed from the pET28+ vector constructed previously by Mordhorst et al. [8] Meiothermus ruber polyphosphate kinase (MrPPK) was used similarly as described previously by Parnell et al. [9] E. coli BL21 (DE3) Gold served as the expression host. Cells were grown in LB medium and PPK expression was induced by addition of 0.5 mM IPTG at 28°C. Cell disruption and purification was performed as described for CARs. PPKs were stored in 20 mM Tris/HCl buffer (pH 7.4, 0.2 M NaCl) at -80°C. Protein concentrations were determined with the Bradford assay according to the manufacturer’s protocol. Polyphosphate kinase activity was assessed using an HPLC based protocol.

1.2.3. Pyrophosphatase

Strep-tagged pyrophosphatase from Escherichia coli (EcPPase) was expressed from the pETSTREP3 vector as constructed previously by Pfeiffer et al. [10] E. coli BL21 (DE3) served as the expression host and was cultivated in LB medium at 37°C. EcPPase expression was induced by addition of 0.5 mM IPTG. Purification was performed under standard conditions (pH 7.4) using a gravity flow strep
tactin sepharose column as the stationary phase. Protein concentrations were determined with the Bradford assay according to the manufacturers protocol. EcPPase was stored in 20 mM Tris/HCl buffer (pH 7.4, 0.2 M NaCl) at -80°C. Activities of pyrophosphatase preparations were assessed using an assay described by Sigma Aldrich for the characterization of inorganic phosphatase.[11]

1.3. Analytical Methods and Preparation of Reference Materials

1.3.1. HPLC-analysis of AMP, ADP and ATP (Method_1)

Instrument: Agilent 1100 system consisting of a G1322A degasser, G1311A quaternary pump, G1313A autosampler, G1316A thermostatted column compartment and a G1315A DAD detector
Column: Poroshell 120 SB-C18 100 x 3 mm, 2.7 µm (Agilent, PN 685975-302, SN USCFC01161)
Analysis conditions: eluent A: 20 mM potassium phosphate buffer pH 5.90 containing 40 mM tetrabutylammonium bromide (TBAB); eluent B: MeCN; 0.65 mL/min flow rate; 30°C;
A gradient was used: 0-1 min: A/B = 95:5; 6-8min: A/B = 45:55; 5 µL injection volume; UV detection at 260 nm.
Retention times: AMP: 2.56 min, ADP: 4.97 min, ATP: 6.53 min.

1.3.2. HPLC-analysis of carboxylic acids 1a-l, aldehydes 2a-l and alcohols 3a-l (Method_2)

Instrument: Shimadzu Nexera UHPLC consisting of following modules: DGU-20A5 Prominence degasser, LC-30AD pumps, SIL-30AC Nexera autosampler, CTO-20AC Prominence column oven, SPD-M20A Prominence photodiode array detector, LCMS-2020 single quadrupole mass spectrometer equipped with an ESI ionization source and a CBM-20A communications bus module.
Column: Poroshell 120 EC-C18 100 x 3 mm, 2.7µm (Agilent, PN 695975-302; SN USCFX01317);
Analysis conditions: eluent A: H2O containing 0.02 vol% TFA; eluent B: MeCN; 0.70 mL/min flow rate; 35°C;
A stepwise gradient was used: 0 min: A/B=85:15; 9 min: A/B=40:60; 9.01-9.5min: A/B=20:80; 2 µL injection volume; UV detection at 210 nm;
The calculation of conversions was done by applying corrections with response factors for each compound.

Table S1. List of retention times of analytes 1, 2, and 3 in rp-HPLC analysis.

| Alcohol 3 | Carboxylic acid 1 | Aldehyde 2 |
|-----------|------------------|------------|
| a         | 2.76             | 3.59       | 4.25 |
| b         | 2.73             | 3.63       | 4.35 |
| c         | 3.29             | 3.08       | 4.96 |
| d         | 3.07             | 4.10       | 5.17 |
| e         | 2.85             | 3.89       | 4.64 |
| f         | 4.40             | 4.08       | 6.51 |
| g         | 4.67             | 5.43       | 6.55 |
| h         | 4.65             | 5.53       | 6.42 |
| i         | 3.34             | 4.01       | 4.61 |
| j         | 2.21             | 2.80       | 3.06 |
| k         | 4.71             | 5.02       | 5.74 |
| l         | 4.87             | 4.89       | 5.74 |

[a] Analyzed by HPLC Method_2.

Preparation of alcohol reference materials for analyses
Alcohols 3c-j and 3l-o were prepared in situ by adding sodium borohydride (1.5 mg, 40 µmol, 20 eq) to a solution of the respective aldehyde in methanol (2.0 mM; 1.0 mL volume) under stirring at r.t.
1.3.3. GC-FID-analysis of carboxylic acids 1m-o, aldehydes 2m-o and alcohols 3m-o (Method_3)

Preparation of etheral diazoethane solution: 10 M NaOH (1.0 mL, 10.0 mmol) was added to a scratch-free glass vessel containing H₂O (500 µL) and ether (5 mL). The mixture was cooled to 0°C with the aid of an ice bath. While stirring, N-ethyl-N-nitrosourea (40 mg, 342 µmol) was added in three portions to the bi-phasic mixture. After 30 min, the diazoethane formation was complete and the yellow-colored ether solution was used for the esterification reaction.

Analysis method 3: Analysis of carboxylic acids 1m-o, aldehydes 2m-o and alcohols 3m-o

Instrument: Agilent 6890N GC system equipped with a FID detector, a G2614A sample tray and a G2613A injector;

Column: Agilent DB-1701 (part no. 1220732; SN: US3143946H), capill. 30 m × 0.25 mm, 0.25 µm film;

Analysis conditions: inlet temperature: 260°C; detector temperature: 260°C; 1 mL/min const. N₂ flow; 1 µL injection volume, split ratio 10:1; 60°C hold 1 min; 10°C/min to 200°C; 20°C/min to 240°C hold 1 min.

Different responses of the compounds were compensated based on their number of carbon atoms, assuming a linear dependency of the FID signal on the carbon fraction.

Table S2. List of retention times of analytes 1m-o, 2m-o, and 3m-o in GC analyses using method 3.

| Alcohol 3 | Carboxylic acid 1m-o | Aldehyde 2 |
|----------|----------------------|------------|
| m        | 4.93                 | 5.95       | 3.49       |
| n        | 8.58                 | 8.89       | 6.36       |
| o        | 11.16                | 12.83      | 11.11      |

[a] Analyzed as ethyl after derivatization with diazoethane.

1.4. Enzyme-catalyzed reactions

1.4.1. Activity tests with PPK enzymes (protocol 1)

The concentrations in the reactions were as follows: AMP or ADP (2.0 mM), potassium phosphate buffer (100 mM, pH 7.50), MgCl₂ (10.0 mM), sodium polyphosphate (150 mM) and PPK (100 or 400 µg/mL).

Reagent specifications according to the vendor (Aldrich): AMP (PN 01930, Lot BCBN8574V): 100 % purity, 25 % water content; ADP (PN A2754, Lot SLBJ8914V): 97 % purity, 9 % water content.

Concentrations of purified enzyme preparations used: ΣPPK: 5.75 mg/mL, MrPPK: 6.61 mg/mL, SmPPK: 3.45 mg/mL

All reactions were shaken at 30°C at 700 rpm in an Eppendorf thermomixer. 50 µL samples were taken from the reaction solutions after certain reaction times, quenched and diluted by the addition of 150 µL methanol and analyzed by rp-HPLC (Method_1) after prior centrifugation.

Table S3. Preparation of stock solution for the activity tests of PPK enzymes (protocol 1).

|                        | Amount       | Concentration in stock solution |
|------------------------|--------------|---------------------------------|
| AMP or ADP             | 6.7 mg AMP or 6.1 mg ADP | 3.2 mM                          |
| MgCl₂                  | 6.1 mg       | 16.0 mM                         |
| 0.60 M sodium polyphosphate pH 7.50 | 1600 µL      | 240 mM                         |
| 1.0 M potassium phosphate pH 7.50 | 640 µL       | 160 mM                         |
| Water                  | 1760 µL      |                                 |

1) Prepared by dissolving sodium polyphosphate (308.6 mg, 3.00 mmol) in water (5.00 mL total volume) and adjustment of the pH to 7.50 with 2.0 M NaOH.
**1.4.2. Investigation of the regeneration of ATP (Figure 1 in the main manuscript)**

The concentrations in the reactions were as follows: AMP (2.0 mM), buffer (100 mM containing 50 mM NaCl, pH 7.5, types: HEPES, imidazole-HCl, MOPS, PIPES, potassium phosphate and Tris-HCl), MgCl₂ (10, 20, 30 or 40 mM), sodium polyphosphate (60 mM), MrPPK (20 µg/mL) and SmPPK (4.0 µg/mL).

All reactions were shaken at 30°C at 700 rpm in an Eppendorf thermomixer. 25 µL samples were taken from the reaction solutions after certain reaction times, quenched and diluted by the addition of 75 µL methanol and analyzed by rp-HPLC (Method_1) after prior centrifugation.

### Table S5. PPK/polyP stock solution for the investigation of the ATP regeneration.

| Substance                        | Amount | Concentration in stock solution |
|----------------------------------|--------|---------------------------------|
| MrPPK                            | 22.70 µL | 83.3 µg/mL                     |
| SmPPK                            | 8.70 µL  | 16.7 µg/mL                     |
| 0.80 M sodium polyphosphate pH 7.50 | 563.0 µL | 250 mM                      |
| Water                            | 1206 µL   |                                |

1) Prepared by dissolving sodium polyphosphate (164.6 mg, 1.60 mmol) in water (2.00 mL total volume) and adjustment of the pH to 7.50 with 2.0 M NaOH.

### Table S6. PPK/polyP stock solution for the investigation of the ATP regeneration.

| MgCl₂ concentration | MrPPK stock solution | SmPPK stock solution | MgCl₂ stock solution | MgCl₂ stock solution |
|----------------------|----------------------|----------------------|----------------------|----------------------|
| 250 mM MgCl₂ (if)    | 60.0 µL              | 60.0 µL              | 60.0 µL              | 60.0 µL              |
| 200 mM buffer (if)   | 5.0 µL               | 10.0 µL              | 15.0 µL              | 20.0 µL              |
| 25.0 mM AMP (if)     | 40.0 µL              | 35.0 µL              | 30.0 µL              | 25.0 µL              |

1) see Table S5; 2) prepared by dissolving MgCl₂ (52.4 mg, 550 µmol) in water (1100 µL); 3) all buffers contain 100 mM NaCl; 4) prepared by dissolving AMP (7.2 mg) in water (550 µL); reactions started by the addition of AMP stock solution.

### Scheme 1. Two-step regeneration sequence of ATP from AMP via ADP.

1.4.3. Influence of polyphosphate and different buffer systems on NcCAR-catalyzed carboxylate reductions of piperonylic acid 1a (Table 1 in the main manuscript)

All reactions were shaken at 30°C at 700 rpm in an Eppendorf thermomixer. After 2 h, 50 µL samples were taken from the reaction solutions, diluted by the addition of 150 µL methanol/formic acid 19:1 to stop the reaction. These solutions were analyzed by rp-HPLC (Method_2) after prior centrifugation.

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1) see Table S3.
Table S7. Composition of reaction mixtures used for the investigation of the influence of polyP and various buffer systems on the NcCAR-catalyzed reduction of 1a.

| Reaction mixtures used for the investigation of the influence of polyP and various buffer systems on the NcCAR-catal. reduction of 1a. | Reactions without polyP | Reactions with 100 mM polyP |
|---|---|---|
| 100 mM ATP stock solution\(^1\) | 20 µL | 20 µL |
| 75 mM NADPH stock solution\(^2\) | 20 µL | 20 µL |
| 200 mM buffer pH 7.50\(^3\) | 100 µL | 100 µL |
| 0.80 M sodium polyphosphate pH 7.50\(^4\) | - | 25 µL |
| Piperonylic acid/MgCl\(_2\) stock \(^5\) | 15 µL | 15 µL |
| NcCAR stock (500 µg/mL)\(^6\) | 20 µL | 20 µL |
| Water | 25 µL | - |
| Total reaction volume | 200 µL | 200 µL |

\(^1\) ATP (25.1 mg, 42.0 µmol; Aldrich, PN A26209, Lot MKBQ5256V) + water (420 µL); \(^2\) NADPH (28.4 mg, 31.5 µmol; Carl Roth, PN AE14.3, Lot 267262462); \(^3\) all buffers contain 100 mM NaCl; \(^4\) prepared by dissolving sodium polyphosphate (164.6 mg, 1.60 mmol) in water (2.0 mL total volume) and adjustment of the pH to 7.50 with 2.0 M NaOH; \(^5\) piperonylic acid (4.98 mg, 30.0 µmol, 66.7 mM in stock solution) + MgCl\(_2\) (5.71 mg, 60.0 µmol, 133.3 mM in stock solution) + NaHCO\(_3\) (aq) (30.0 µL, 1.0 M, 1 eq) + water (420 µL); \(^6\) NcCAR (35 µL, 4.0 mg/mL) + water (245 µL).

1.4.4. Investigation of the in vitro carboxylate reduction (Table 2 in the main manuscript)

The concentrations in the reactions were as follows: carboxylic acid (10 mM), ATP (1.0 mM), NADPH (0.5 mM), imidazole-HCl buffer (100 mM, pH 7.5), MgCl\(_2\) (70 mM), β-D-glucose (100 mM), sodium polyphosphate (140 mM), CAR (50 µg/mL), MrPPK (100 µg/mL), SmPPK (40 µg/mL), EcPPase (25 µg/mL) and GDH-105 (0.2 U/mL).

Concentrations of purified enzyme preparations used:
- NcCAR: 4.00 mg/mL,
- NiCAR: 3.00 mg/mL,
- MrPPK: 6.61 mg/mL,
- SmPPK: 3.45 mg/mL,
- EcPPase: 10.82 mg/mL.

All reactions were shaken at 30°C at 700 rpm in an Eppendorf thermomixer. After 18 h, 1000 µL of methanol/formic acid 19:1 was added to stop the reaction and these solutions were analyzed by rp-HPLC (Method_2) after prior centrifugation. In the case of reactions with the carboxylic acids 1m-o, 150 µL 2.0 M potassium hydrogensulfate was added, then the mixture was extracted twice with 400 µL ethyl acetate each and subsequently, the combined extracts were dried over sodium sulfate. 200 µL of the dried extract was then mixed with 200 µL ethereal diazoethane solution (ca. 60-70 mM) to convert unreacted carboxylic acids into the corresponding ethyl esters and these samples were analyzed by GC-FID (Method_3).

Table S8. Enzyme stock solution used for the investigation of the in vitro carboxylate reduction.

| Enzyme stock solution used for the investigation of the in vitro carboxylate reduction. | NcCAR\(^1\) | NiCAR\(^1\) |
|---|---|---|
| CAR | 62.5 µL | 83.3 µL |
| MrPPK | 75.6 µL | 75.6 µL |
| SmPPK | 58.0 µL | 58.0 µL |
| EcPPase | 11.6 µL | 11.6 µL |
| GDH-105 (50 U/mL) | 20.0 µL | 20.0 µL |
| Water | 172.3 µL | 151.5 µL |

\(^1\) Concentrations in stock solution: NcCAR or NiCAR (625 µg/mL), MrPPK (1250 µg/mL), SmPPK (500 µg/mL), EcPPase (313 µg/mL), GDH-105 (1.00 U).

Table S9. Reaction mixture used for the investigation of the in vitro carboxylate reduction.

| Used in reactions | Used in reactions |
|---|---|
| MgCl\(_2\)/β-D-glucose/buffer\(^1\) | 125.0 µL |
| ATP/ NADPH stock solution\(^2\) | 20.0 µL |
| Enzyme stock\(^3\) | 20.0 µL |
| 2.00 M sodium polyphosphate pH 7.50\(^4\) | 17.5 µL |
| 250 mM carboxylic acid stock\(^5\) | 10.0 µL |
| Water | 57.5 µL |
| Total reaction volume | 250.0 µL |

\(^1\) MgCl\(_2\) (133.3 mg, 1.40 mmol, 140 mM in stock solution) + β-D-glucose (360.3 mg, 2.00 mmol, 200 mM in stock solution) + 200 mM imidazole-HCl buffer (10.0 mM); \(^2\) ATP (11.9 mg, 20.0 µmol, 12.5 mM in stock solution) + NADPH (9.0 mg, 10.0 µmol, 6.25 mM in stock solution) + water (1600 µL); \(^3\) see Table S8; \(^4\) prepared by dissolving sodium polyphosphate (617.1 mg, 6.00 mmol) in water (3.0 mL total volume) and adjustment of the pH to 7.50 with 2.0 M NaOH; \(^5\) carboxylic acids dissolved in 250 mM NaOH.
1.4.5. Investigation of the polyP amounts required for the in vitro carboxylate reduction (Figure 2 in the main manuscript)

The concentrations in the reactions were as follows: piperonylic acid (10 mM), ATP (1.0 mM), NADPH (0.5 mM), imidazole-HCl buffer (100 mM, pH 7.5), MgCl₂ (70 mM), β-D-glucose (100 mM), sodium polyphosphate (140 mM), CAR (50 µg/mL), MrPPK (100 µg/mL), SmPPK (40 µg/mL), EcPPase (25 µg/mL), and GDH-105 (0.2 U/mL).

All reactions were shaken at 30°C at 700 rpm in an Eppendorf thermomixer. After 20 h, 1000 µL of methanol/formic acid 19:1 was added to stop the reactions and these solutions were analyzed by rp-HPLC (Method_2) after prior centrifugation.

Table S10. Stock solution used for the investigation of the polyP amounts required for the in vitro carboxylate reduction of 1a.

| Amounts used¹  |
|----------------|
| 200 mM imidazole-HCl buffer pH 7.50 | 2000 µL |
| 250 mM piperonylic acid² | 160.0 µL |
| MgCl₂ | 26.7 mg |
| β-D-glucose | 72.1 mg |
| NcCAR | 50.0 µL |
| MrPPK | 60.5 µL |
| SmPPK | 46.4 µL |
| EcPPase | 9.2 µL |
| GDH-105 (50 U/mL) | 16.0 µL |
| Water | 17.9 µL |

¹ Piperonylic acid (33.2 mg, 200.0 µmol) dissolved in NaOH (800 µL, 250 mM); ² prepared by dissolving 77.1 mg (750 µmol) sodium polyphosphate in a total volume of 3.0 mL water and adjustment of the pH to 7.50 with 2.0 M NaOH.

Table S11. Reaction mixtures used for the investigation of the polyP amounts required for the in vitro carboxylate reduction of 1a.

| Conc. of polyP in reaction | Stock solution 250 mM sodium polyphosphate pH 7.50³ | Water |
|--------------------------|-----------------------------------------------|--------|
| 100.0 mM | 150.0 µL | 100.0 µL | 0.0 µL |
| 80.0 mM | 150.0 µL | 80.0 µL | 20.0 µL |
| 60.0 mM | 150.0 µL | 60.0 µL | 40.0 µL |
| 55.0 mM | 150.0 µL | 55.0 µL | 45.0 µL |
| 50.0 mM | 150.0 µL | 50.0 µL | 50.0 µL |
| 45.0 mM | 150.0 µL | 45.0 µL | 55.0 µL |
| 40.0 mM | 150.0 µL | 40.0 µL | 60.0 µL |
| 35.0 mM | 150.0 µL | 35.0 µL | 65.0 µL |
| 30.0 mM | 150.0 µL | 30.0 µL | 70.0 µL |
| 25.0 mM | 150.0 µL | 25.0 µL | 75.0 µL |
| 20.0 mM | 150.0 µL | 20.0 µL | 80.0 µL |
| 15.0 mM | 150.0 µL | 15.0 µL | 85.0 µL |
| 10.0 mM | 150.0 µL | 10.0 µL | 90.0 µL |
| 5.0 mM | 150.0 µL | 5.0 µL | 95.0 µL |
| 0.0 mM | 150.0 µL | 0.0 µL | 100.0 µL |

¹ see Table S10; ³ prepared by dissolving 77.1 mg (750 µmol) sodium polyphosphate in a total volume of 3.0 mL water and adjustment of the pH to 7.50 with 2.0 M NaOH.

1.4.6. ATP feeding

The concentrations in the reactions were as described in 1.4.5, with the following modifications: MOPS (100 mM, pH 7.5, 50mM NaCl) was used instead of imidazole buffer. Bromothymol blue was used to monitor the pH. For ATP feed, 1.0 mM of ATP was added at each sampling time shown in...
2) Results and Discussion

The activities of enzyme preparations for the selected PPK’s in the stepwise regeneration of ATP were checked as described in protocol 1.

Table S12. Conversions in PPK-catalyzed reactions found.

| PPK     | PPK concentration / µg/mL | Conversion of AMP to ADP after 1h / % | Conversion of ADP to ATP after 1h / % |
|---------|---------------------------|--------------------------------------|-------------------------------------|
| AjPPK   | 400                       | 1                                    | 0                                   |
| MPPK    | 400                       | 10                                   | <1[a]                               |
| SmPPK   | 100                       | 0                                    | 57                                  |

[a] Additional formation of 4% AMP.
Table S13. Full list of conversions of AMP to ADP and ATP for Figure 1.

| Entry | MgCl2 / mM | 100 mM Buffer pH 7.5 + 50 mM NaCl | Conversion to ADP% / % | Conversion to ATP% / % | Formal specific activity of \( \text{MPPK}^\text{[a]} \) / U/mg prot. | Formal specific activity of \( \text{SmPPK}^\text{[a]} \) / U/mg prot. |
|-------|------------|----------------------------------|------------------------|------------------------|---------------------------------|---------------------------------|
| 1     | 10         | HEPES                             | 20.7                   | 27.2                   | 0.80                           | 2.27                           |
| 2     | 20         | HEPES                             | 36.4                   | 51.4                   | 1.46                           | 4.28                           |
| 3     | 30         | HEPES                             | 30.7                   | 60.1                   | 1.51                           | 5.01                           |
| 4     | 40         | HEPES                             | 29.6                   | 63.1                   | 1.54                           | 5.26                           |
| 5     | 10         | Imidazole-HCl                     | 13.7                   | 14.9                   | 0.48                           | 1.24                           |
| 6     | 20         | Imidazole-HCl                     | 28.2                   | 38.8                   | 1.12                           | 3.23                           |
| 7     | 30         | Imidazole-HCl                     | 33.2                   | 58.4                   | 1.53                           | 4.87                           |
| 8     | 40         | Imidazole-HCl                     | 27.1                   | 64.0                   | 1.52                           | 5.33                           |
| 9     | 10         | MOPS                              | 25.9                   | 23.9                   | 0.83                           | 1.99                           |
| 10    | 20         | MOPS                              | 40.2                   | 44.7                   | 1.42                           | 3.73                           |
| 11    | 30         | MOPS                              | 39.8                   | 50.3                   | 1.50                           | 4.19                           |
| 12    | 40         | MOPS                              | 32.9                   | 58.7                   | 1.53                           | 4.89                           |
| 13    | 10         | PIPES                             | 13.3                   | 19.0                   | 0.54                           | 1.59                           |
| 14    | 20         | PIPES                             | 25.5                   | 45.4                   | 1.18                           | 3.79                           |
| 15    | 30         | PIPES                             | 30.8                   | 52.0                   | 1.38                           | 4.34                           |
| 16    | 40         | PIPES                             | 30.5                   | 56.2                   | 1.44                           | 4.68                           |
| 17    | 10         | Potassium phosphate              | 6.4                    | 13.3                   | 0.33                           | 1.10                           |
| 18    | 20         | Potassium phosphate              | 13.3                   | 28.5                   | 0.70                           | 2.37                           |
| 19    | 30         | Potassium phosphate              | 15.0                   | 39.1                   | 0.90                           | 3.25                           |
| 20    | 40         | Potassium phosphate              | 19.5                   | 42.2                   | 1.03                           | 3.52                           |
| 21    | 10         | Tris-HCl                          | 10.6                   | 21.3                   | 0.53                           | 1.77                           |
| 22    | 20         | Tris-HCl                          | 18.7                   | 64.3                   | 1.38                           | 5.36                           |
| 23    | 30         | Tris-HCl                          | 18.1                   | 71.5                   | 1.49                           | 5.96                           |
| 24    | 40         | Tris-HCl                          | 23.4                   | 65.4                   | 1.48                           | 5.45                           |

[a] Determined by rp-HPLC (Method 1) after 1 h at 30°C. [b] Average specific activity of the PPK’s per milligram protein calculated based on the assumption that \( \text{MPPK} \) catalyzes the formation of ADP from AMP but the subsequent step towards ATP only to a negligible extend. The total conversion contributed by \( \text{MPPK} \) catalysis is therefore the sum of the conversion from AMP to ADP and ADP to ATP. \( \text{SmPPK} \), as already shown, can only catalyze the formation of ATP from ADP. The values presented here do not reflect any maximum rates used to describe enzyme activities and kinetics based on literature models. One U(nit) is defined as the amount of the enzyme that catalyzes the conversion of 1 mmol of substrate per minute.

These results shown in Table S12 clearly indicate that an efficient recycling of ATP requires the simultaneous use of two different PPKs, ideally \( \text{MPPK} \) and \( \text{SmPPK} \), to proceed in an acceptable time frame. Starting from these initial results, the reaction conditions were optimized as described in the following part of the supporting information (Table S13) and in the main manuscript (Figure 1).

The data presented in Table S13 was primarily obtained to determine suitable buffer systems and appropriate Mg\(^{2+}\) concentrations to enable a fast ATP regeneration. Most conversions were far too high to calculate initial enzyme activities. Moreover, \( \text{MPPK} \) operates in a cascade coupled with \( \text{SmPPK} \). However, the latter marks the final enzyme in the cascade and additionally faces a possibly close to equilibrium situation in some of the examples shown (Table S13). The data was used to estimate average specific activities from the conversions obtained after 1 h. This information is not dedicated to characterize enzymes according to often used theoretical models but serves as rough estimation to define a suitable ratio of PPK enzymes to be used. Based on the average specific activities calculated, the \( \text{SmPPK} \) is approximately three- to four-times more active than the same mass of \( \text{MPPK} \) under the given conditions. Taking this as a lead, a protein mass ratio of 2.5:1 (\( \text{MPPK}/\text{SmPPK} \)) instead of 5:1 as used in initial experiments was used in the following investigation of the \textit{in vitro} carboxylate reduction, thereby setting the second phosphorylation step intentionally as little rate limiting.
3) NMR data

$^1$H-NMR (300 MHz, CDCl$_3$) of 2a

$^{13}$C-NMR APT (75 MHz, CDCl$_3$) of 2a
SUPPORTING INFORMATION

4) References

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5) Author Contributions

G. A. Strohmeier: investigation, methodology, data curation, formal analysis, validation, visualization, reaction design, writing of original draft

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M. Winkler: research design, conceptualization, funding acquisition, project administration – writing, review & editing