Supporting information for

The Dual activity of Quinolinate Synthase: Triose Phosphate Isomerase and dehydration activities play together to form quinolinate

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1- Supporting experimental procedures

Materials

The coupling enzymes rabbit glyceraldehyde-3-phosphate dehydrogenase and rabbit α-glycerol-3-phosphate dehydrogenase, the positive control enzyme rabbit triose phosphate isomerase as well as the substrates dihydroxyacetone phosphate (DHAP) and gylceraldehyde-3-phosphate (G-3P) and the co-substrates NAD^+, NADH, oxaloacetate (OAA) and fumarate were obtained from Sigma Aldrich. Ammonium sulfate (AS) was received from Roth. Sodium arsenate was purchased from Merck. The inhibitor phosphoglycolic acid (PGA) was kindly provided by M. Therisod (ICMMO, Orsay, France).

Plasmids and Strains:

E. coli BL21 (DE3) (Stratagene) cells were used to produce recombinant *E. coli* (NadA-Ec-pT7) and *T. maritima* (NadA-Tm-pT7) NadA proteins as well as *E. coli* NadB (NadB-pT7) as described previously[1,2].

Methods

Expression and purification of NadA:

E. coli competent BL21 (DE3) cells were transformed with NadA-Ec-pT7 or NadA-Tm-pT7 vectors. The expression and purification of NadA-Ec was performed as described before by Rousset et al.[2]. NadA-Tm was expressed, purified and reconstituted as mentioned in reference[1].

Protein and cofactor quantification:

Protein concentrations were measured by the method of Bradford using bovine serum albumin as a standard corrected by factors for NadA and NadB as previously described by Rousset et al.[2]. Protein-bound iron was determined under reducing conditions with bathophenanthroline disulfonate after acid denaturation of the protein [3] and labile sulphide by the method of Beinert [4].

NadA enzymatic assay:

NadA enzymatic activity was assayed under anaerobic conditions, inside a glove box and the time dependent formation of quinolinic acid (QA) was measured directly by HPLC. The standard assay contained in a final volume of 100 µl, buffer A (50 mM Na-HEPES, 100 mM KCl, pH 7.5), 10 µM NadA and 2 mM DHAP/G3-P as well as 25 mM fumarate, 10 mM aspartate and 10 µM NadB for the enzymatic formation of IA. The reaction was started by the addition of NadB and incubated for 3 respectively 20 min at 37°C. For the measurements of NadA-Tm as well as the inhibition assays with PGA (NadA-Ec and NadA-Tm; amounts as indicated) the alternative chemical formation of IA by 5 mM oxaloacetate and 10 mM ammonium sulphate was used[1]. For NadA-Tm the temperature was increased to 45°C and the incubation time was increased to 60 min within the PGA inhibition assay. The reaction was stopped by addition of 0.1 M H_2SO_4 and precipitated protein was removed by centrifugation (15000 rpm, 15 min). The supernatant was diluted 1:2 with buffer B (0.03 % TFA, pH 2.4) and 80 µl were injected onto the HPLC column. The QA produced was analysed and quantified by a HP-1100 HPLC system after injection onto a Tosoh TSK Gel ODS-120T (4.6 mm x 15 cm) column. The column was eluted with buffer B at a flow rate of 0.5 ml/min. QA was detected by its absorbance at 230 nm and was eluted with a retention time of 10-11 min. After 20 min of elution the column was regenerated with a linear

gradient from 0 % to 100 % acetonitrile in 0.03 % TFA at 0.5 ml/min over 10 min and re-equilibrated before injection of the next sample. Commercial quinolinic acid (Sigma, St. Louis, MO) was used to generate a standard curve from 0 to 150 nmol.

Isomerase enzymatic assay:

Standard isomerase enzymatic activity was assayed under aerobic conditions except for the test of iron sulphur cluster dependency which was carried out inside a glove box. To study the formation of dihydroxyacetone phosphate from glyceraldehyde-3-phosphate the reaction mixture contained in a final volume of 250 µl, Buffer C (0.1 M Triethanolamine pH 7.6), 0.24 mM NADH, 2-4 mM glyceraldehyde-3-phosphate, 0.7-1 µM glycerol-3-phosphate dehydrogenase (GPDH) (without EDTA)[5] and for the respective assays the following enzyme concentrations: 5-10 µM NadA-Ec, 20 µM NadA-Tm and 1-5 nM TPI. The reverse reaction (formation of glyceraldehyde-3-phosphate from dihydroxyacetone phosphate) was performed in a final volume of 250 µl with buffer C, 0.7 µM glyceraldehyde dehydrogenase (GAPDH), 1 mM NAD^+, 2-4 mM DHAP, 0.5 mM potassium arsenate and the respective enzyme concentrations as indicated before[5]. For the inhibition assays 1 mM PGA was added to the reaction[5]. The reaction was performed at 37°C for NadA-Ec and TPI, at 50°C for NadA-Tm and at 18°C for the anaerobic/aerobic comparison. With the addition of the assayed enzyme the reaction was initiated. The initial rate was calculated from the change of NADH absorbance at 340 nm which was monitored with a Shimazu spectrophotometer coupled to a water bath for the aerobic measurements and a Bio-Tek spectrophotometer inside a glove box for the anaerobic assays.

Analysis

Protein concentrations were measured by the method of Bradford using bovine serum albumin as a standard[6]. Iron and sulfur quantifications were carried out as previously described [3,4]. UV-visible (UV-Vis) spectra of NadA were recorded on a Shimazu spectrophotometer.

Proteomic analysis

Proteins contained in the fraction containing purified NadA were separated by SDS-PAGE and analysed as previously described[7]. Briefly, the total gel was cut in 3 portions, proteins were in-gel digested with trypsin and resulting peptides analysed by online nanoliquid chromatography coupled to tandem mass spectrometry (UltiMate U3000, Dionex and LTQ-Orbitrap Velos pro, Thermo Scientific). Peptides and proteins were identified through concomitant searches against Uniprot (E. coli Stain BL21/BL21-DE3 taxonomy, September 2014 version), classical contaminants database and the corresponding reversed databases using Mascot (version 2.5.1). The Proline software was used to filter the results (conservation of rank 1 peptides, peptide identification FDR < 1% as calculated on peptide scores by employing the reverse database strategy, minimum peptide score of 25, and minimum of 1 specific peptide per identified protein group). Contaminant proteins were discarded from the list of identified proteins.

2- Supporting data

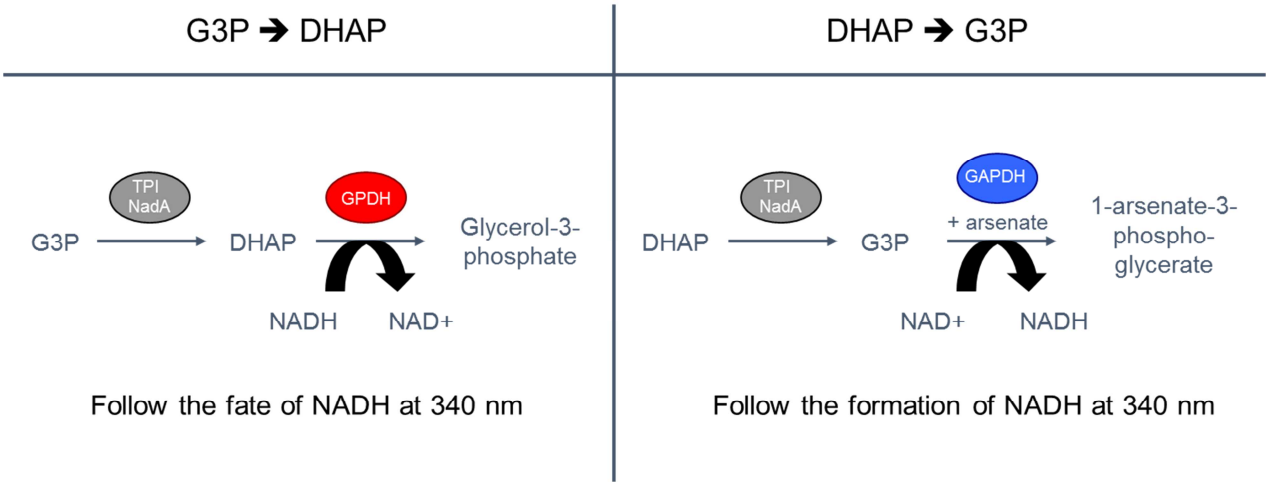


Figure S1: **Measurement of TPI activity**. TPI activity was measured at 37°C under air using spectrophotometric reactions at 340 nm. Left: DHAP formation is monitored by following the NADH-dependent GPDH activity (fate of NADH). Right: G-3P formation monitored by GAPDH activity which generates NADH[5].

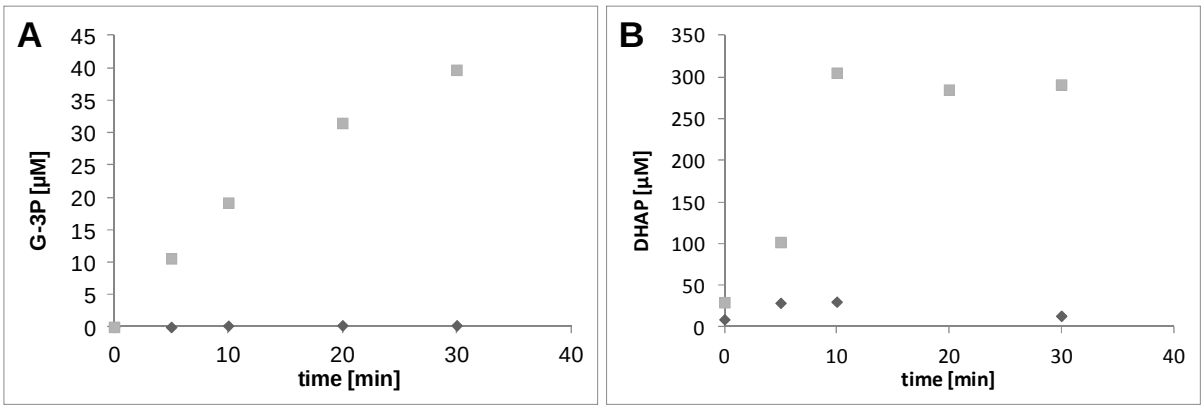


Figure S2: **Protein dependent TPI activity.** (A) Formation kinetics of G-3P from DHAP at 37°C, in the absence (◆) or in the presence (■) of *E. coli* NadA (15 µM). The reaction was performed using 1.2 mM DHAP, 1.8 mM NAD^+, 0.5 mM sodium arsenate and 1 µM GAPDH in Buffer C. The time dependent reaction was started with the addition of NadA and followed with a spectrophotometer at OD_{340}. (B) Formation kinetics of DHAP from G-3P at 37°C, in the absence (◆) or in the presence (■) of *E. coli* NadA (15 µM). The reaction was performed using 1.5 mM G-3P, 1.8 mM NADH and 0.8 µM GPDH in 0.05 M HEPES, 0.1 M KCl, pH 7.5. NadA and the substrate G-3P were incubated for the indicated minutes at 37°C and afterwards transferred to the cuvette in the spectrophotometer containing NADH and GPDH and the amount of DHAP formed was measured at OD_{340}.

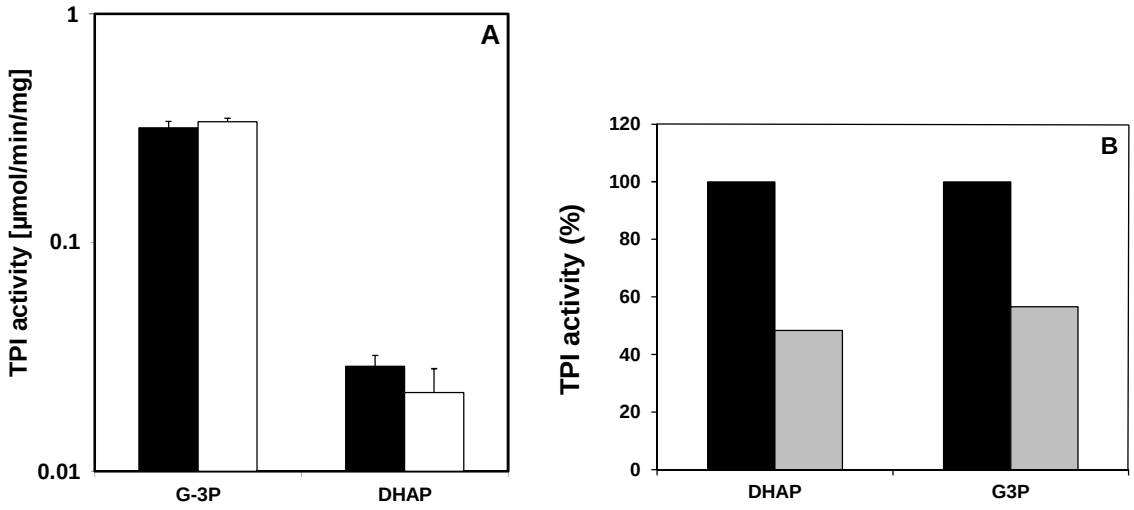


Figure S3. Influence of Fe/S cluster on TPI activity. (A) TPI activity of Ec NadA under aerobic and anaerobic conditions. *E. coli* NadA (5 µM) was added either aerobically (black) or anaerobically (white) in a glove box to NADH (0.24 mM), G-3P (4 mM) and GPDH (1 µM) in Buffer C and the initial rate of the forward reaction has been followed at 340 nm at 18 °C for 100 sec with a spectrophotometer. For the reverse reaction the protein as indicated before was added to NAD^+ (1 mM), potassium arsenate (0.5 mM), DHAP (4 mM) and GAPDH (0.7 µM). (n=2). **(B)** TPI activity of holo and apo *E. coli* NadA. As purified holo *E. coli* NadA (black) or apo (grey) form of NadA (both at 5 µM) generated by treatment with EDTA and DTT overnight and desalting were added under air to NADH (0.24 mM), G-3P (4 mM) and GPDH (1 µM) in Buffer C and the initial rate of the forward reaction has been followed at 340 nm at 37 °C for 100 sec with a spectrophotometer. For the reverse reaction the protein as indicated before was added to NAD^+ (1 mM), potassium arsenate (0.5 mM), DHAP (4 mM) and GAPDH (0.7 µM). (n=1)

For both (A) and (B) DHAP and G-3P mean DHAP and G-3P which were used as substrates.

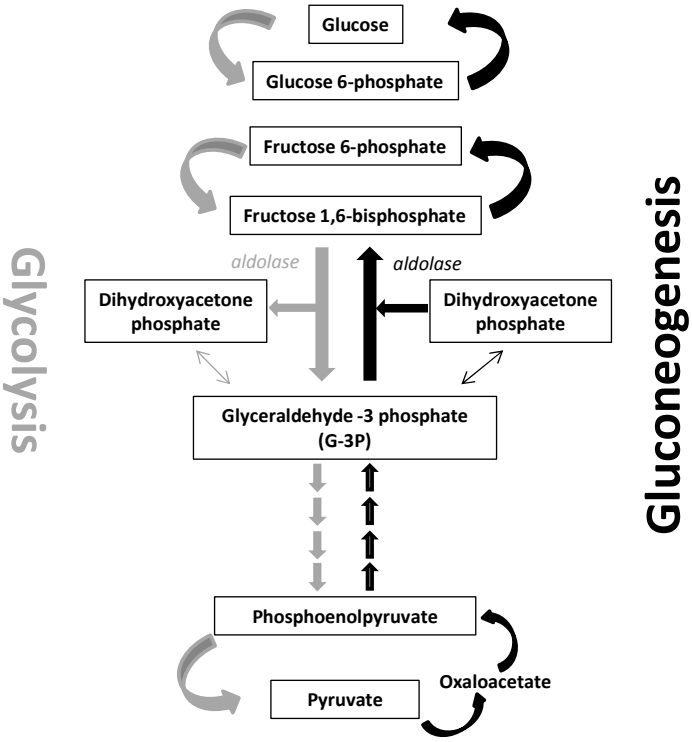


Figure S4. Schematic view of Glycolysis and Gluconeogenesis. Involvement of DHAP and G-3P in Glycolysis and Gluconeogenesis.

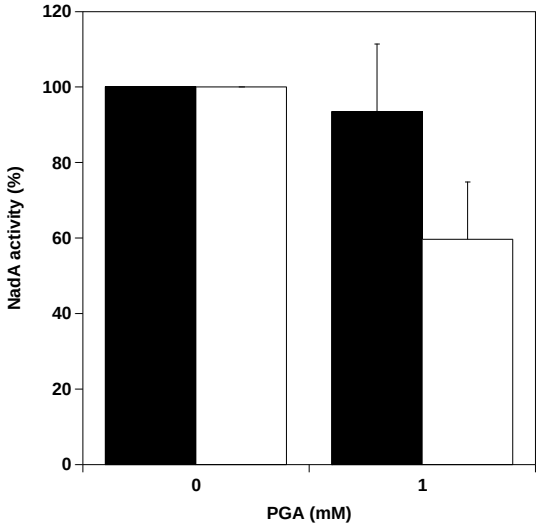


Figure S5. Inhibition of QA formation of *T. maritima* NadA with PGA. NadA (20 µM) was incubated anaerobically for 60 min at 45 °C with DHAP (2 mM) (black) or G-3P (white), OAA (5 mM), AS (10 mM) and in the presence of PGA (1 mM) in Buffer A. The relative amount of formed QA was detected by HPLC and quantified with a standard curve (n=3).

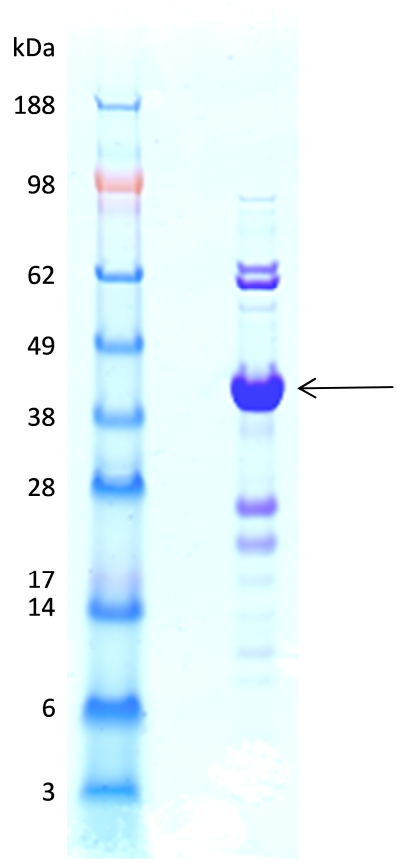
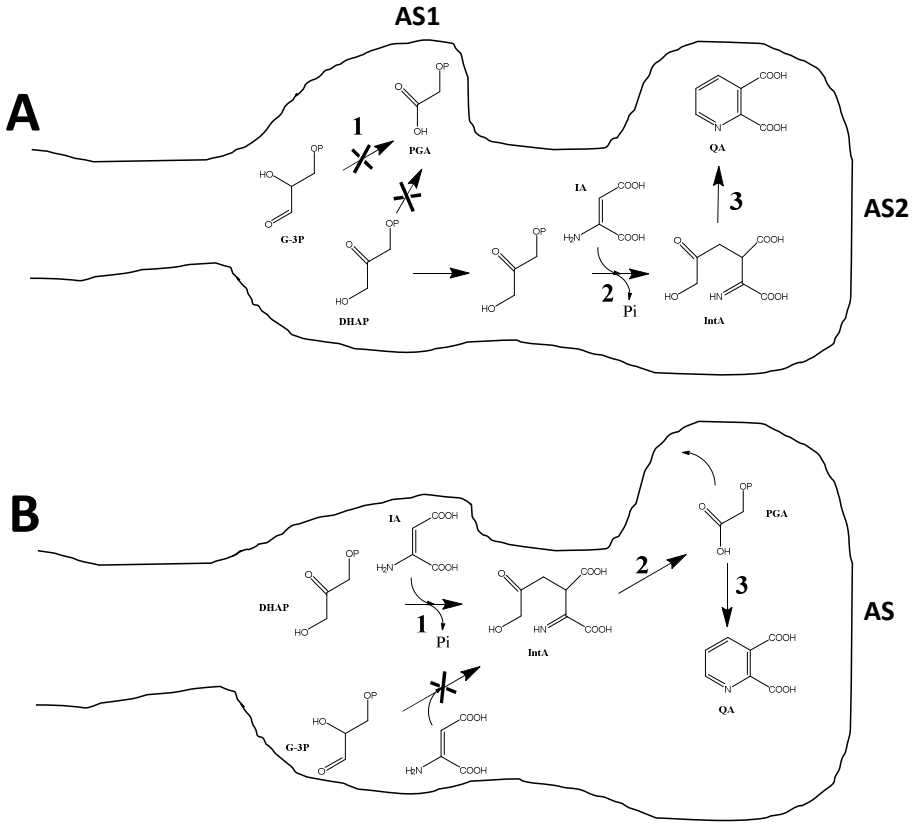


Figure S6. SDS-PAGE analysis of the fraction containing NadA enriched from *E. coli*. After NadA purification, the proteins contained in the fraction were separated by SDS-PAGE (4-12% gradient) and revealed by Coomassie blue staining. The gel was then fractionated into 3 parts (top of the gel to the top of NadA band, NadA band, below NadA band to the bottom of the gel) for proteomic analyses. The arrow shows NadA protein.



Scheme S1. Proposed mechanisms for QA formation in the presence of PGA. (A) TPI and quinolinate synthase activities occur at two different active sites (AS1 and AS2). The PGA molecule blocks G-3P binding and isomerization into DHAP (step 1) hindering QA formation in AS2. In contrast, DHAP, even though it cannot be converted to G-3P at AS1, it can reach quinolinate formation active site (AS2), condense with IA (step 2) and form QA (step 3). **(B)** TPI and quinolinate synthase activities occur at the same active site (AS). Only DHAP can condense with IA (step 1) and condensation product (Intermediate A= Int_A) displaces PGA (step 2) allowing QA formation (step 3).

Table S1: Mass spectrometry-based proteomic analysis of purified *E. coli* NadA enzyme fraction. See Table S1 xls file. Proteomic analyses of the proteins present on the SDS-PAGE gel (the 3 parts described Figure S6).

SI References

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