

Supporting Information

Active site mutagenesis of the putative Diels-Alderase macrophomate synthase

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Materials and general methods. Buffers and salts were purchased from Sigma, J.T. Baker, or Fluka and used without further purification. Oligonucleotides were custom-synthesized and purified by MicroSynth (Balgach, Switzerland). Restriction enzymes were from New England BioLabs (Frankfurt, Germany). DNA polymerase was from Stratagene (Amsterdam, the Netherlands). All nucleic acid manipulations were performed according to standard procedures.¹ DNA sequencing was performed on an ABI PRISM 3100-Avant Genetic Analyzer from PE-Applied Biosystems (Foster City, CA) by chain termination chemistry,² using the BigDye Terminator Cycle Sequencing Kit from the same company.

Plasmid construction. The gene for macrophomate synthase (MPS) from *Macrophoma commelinae* was amplified from plasmid pETMPS,³ by PCR using the primers MPSNdeIHis (5'-CGATGTCATATGCACCATCATCATCATCTTCTTCTGGTATGGCAAAGTCG-3', *NdeI*) and MPSrevBamHIStop (5'-AAATGTGGATCCTTACTAAGCCTTGTCTTCGTGCGCA-3', *BamHI*). The *NdeI*/*BamHI* double digested fragment was ligated into a pET22b (+) vector (Novagen) to yield plasmid pETMPS22-2. Gene expression in the resulting construct is under the control of an T7 RNA polymerase promoter and produces the MPS protein with an N-terminal hexahistidine tag for purification. All coding portions of the constructed plasmids were confirmed by DNA sequencing.

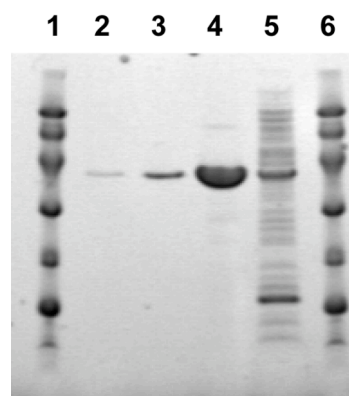
Site-directed mutagenesis. Mutations (bold) were introduced into the MPS gene using the QuickChangeTM site-directed mutagenesis kit (Stratagene)⁴ and the primers listed in Table 1.

Table 1. Mutagenic primers

W68Yforward	5'-ATTTTCGTCT TAT ATCGATGTAGAACAC
W68Yreverse	5'-GTGTTCTACATCGAT ATA GACGAAAT
D70Aforward	5'-CGTCTGGATC GCT GTAGAACA
D70Areverse	5'-TGTTCTAC AGC GATCCAGACG
D70Nforward	5'-TCGTCTGGATC AAT GTAGAACACG
D70Nreverse	5'-CGTGTTCTAC ATT GATCCAGACGA
E72Aforward	5'-ATCGATGT AGC ACACGGCAT
E72Areverse	5'-ATGCCGT GTGCT TACATCGAT
E72Qforward	5'-GATCGATGT CAAC ACGGCATGTT
E72Qreverse	5'-AACATGCCGT GTT GTACATCGATC
H73Aforward	5'-GATCGATGTAGA AGCC GGCATGTT
H73Areverse	5'-AACATGCC GGCT TCTACATCGATC
H73Nforward	5'-GATCGATGTAGAA AAAC GGCATGTTC
H73Nreverse	5'-GAACATGCC GTTTT TCTACATCGATC
R101Aforward	5'-CTCGTCATCGTT GCT GTGCCCAAGC
R101Areverse	5'-GCTTGGGCAC AGCA ACGATGACGAG
R101Kforward	5'-CTCGTCATCGTT AAGG TGCCCAAGC
R101Kreverse	5'-GCTTGGGCAC CTTA ACGATGACGAG
R101Sforward	5'-CGTCATCGTT AGT GTGCCCAAG
R101Sreverse	5'-TTGGGCAC ACTA ACGATGACGAG
H125Aforward	5'-CGTTATTCC AGCT GTTGAAACCGTC
H125Areverse	5'-GACGGTTTCAAC AGCT GGAATAACG
P151Aforward	5'-CCTTCAGC GCCT GGACATTCTC
P151Areverse	5'-GAGAATGTCC AGGC GCTGAAGG
W152Yforward	5'-CTTCAGCCCC TAT ACATTCTCCC
W152Yreverse	5'-GGGAGAAATGT ATAG GGGCTGAAG
Y169Fforward	5'-GACCCG TTC AACGTAGCCA
Y169Freverse	5'-TGGCTACGTT GAAC GGGTC
Q183Aforward	5'-TATCATCCCG GCG ATCGAGTC
Q183Areverse	5'-GACTCGAT CGCC GGGATGATA
Q183Nforward	5'-TATCATCCCG AAT ATCGAGTC
Q183Nreverse	5'-GACTCGAT ATTC GGGATGATA

Protein production and purification. SEM competent BL21 *E. coli* cells were transformed with pETMPS22-2 or the corresponding mutant plasmid. The proteins were overproduced in 250 mL cultures grown at 25 °C in LB medium supplemented with ampicillin (200 mg/L) upon induction with 2.5 mM isopropyl 1-thio- α -D-galactopyranoside (IPTG). Cell pellets were suspended in 7.5 mL loading buffer (50 mM sodium phosphate, 300 mM NaCl, pH 7.0), 2 mg of the protease inhibitor cocktail PEFA-blocTM were added, and the cells were lysed by incubation with 10 mg lysozyme at 4 °C, followed by ultrasonication. Cleared cell lysates were incubated with 2 mL Ni²⁺-NTA-agarose resin (Qiagen), which had been pre-equilibrated with loading buffer containing 10 mM imidazole. After 10 min, this suspension was poured into chromatography columns and then washed with 30 mL aliquots of loading buffer containing 10, 20 and 40 mM imidazole. MPS was then eluted with 12 mL of buffer containing 200 mM imidazole. Protein purity was assessed by SDS-PAGE (Figure S1).

Figure S1. SDS-PAGE analysis of Ni²⁺-NTA affinity purified MPS. Lanes 1 and 6 are low molecular weight markers (Pharmacia); lanes 2, 3 and 4, eluted MPS fractions; lane 5, flow through. MPS runs slightly below the ovalbumin band (43 kDa) of the LMW in agreement with an expected mass of 37.4 kDa.



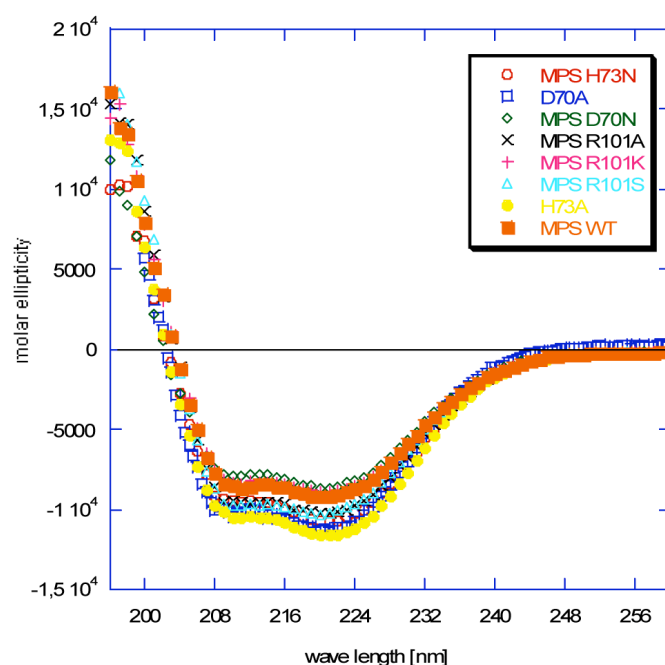
Protein concentration was determined by UV spectroscopy using molar extinction coefficients calculated from the amino acid sequence (Wisconsin Package Version 10.3, Accelrys Inc., San Diego, CA). Similar results (within 10%) were obtained with the Micro BCA protein assay kit (Pierce) using bovine serum albumin as a standard. Typically, a 250 mL culture yielded between 20 and 40 mg of purified protein, depending on the mutant. The proteins were dialyzed into 20 mM potassium phosphate buffer (pH 7.0) at 4 °C, and stored in the cold prior to use.

Mass spectrometry. Proteins were prepared for mass spectrometry by desalting on a NAP-5 size exclusion column (Amersham Pharmacia) that was pre-equilibrated with water. Electrospray ionization (ESI) mass spectrometry was carried out on a Finnigan TSQ 7000

mass spectrometer using a mixture of 70% protein solution containing 0.1% acetic acid and 30% acetonitrile.

Circular Dichroism (CD) Spectroscopy. CD spectra of protein samples (5 μM) were measured in 20 mM potassium phosphate buffer (pH 7.0) on an AVIV Circular Dichroism spectropolarimeter (Model 202, AVIV Instruments, Lakewood, NJ). Data were recorded from 200 to 260 nm in 1 nm steps. Each measurement was done in triplicate and values were averaged. All spectra were corrected for buffer background by subtraction. Figure 2 shows CD spectra of wild type MPS and all inactive mutants. The small differences observed between samples can be attributed to minor inaccuracies in concentration determination and pipetting.

Figure S2. CD spectra of MPS and six inactive mutants



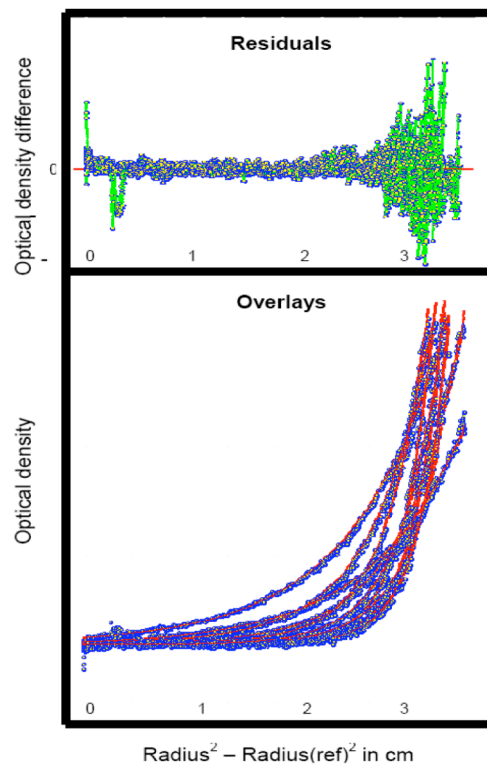
Analytical ultracentrifugation. Sedimentation equilibrium (SE) measurements were performed at 4 $^{\circ}\text{C}$ on a Beckman XL-A analytical ultracentrifuge equipped with an An-60Ti rotor and 2-channel charcoal-epon cells with quartz windows. The cells were sonicated in 2% v/v Hellmanex/distilled water and subsequently rinsed extensively with water (quartz windows subsequently with ethanol) prior to use. Samples at three different concentrations (400, 200 and 100 $\mu\text{g}/\text{mL}$) were measured in parallel in 50 mM PIPES buffer, 50 mM MgCl_2 , pH 7.0. Sample volumes were 120 μL with 30 μL FC43 fluorocarbon oil, whereas reference channels contained 160 μL buffer. SE was performed at rotational speeds of 8k rpm (74h), 10k rpm (50 h), 12k rpm (64 h), and 14k rpm (42 h). The absorbance was monitored at 280

nm. The time to reach equilibrium was estimated using Ultrascan⁵ (University of Texas Health Science Center, San Antonio, TX, USA) and checked by subtracting two scans taken four hours apart. The samples were considered to have reached equilibrium when no difference was observed between the two scans. Upon equilibration, three scans per cell were recorded using 0.001 cm point spacing and averaging 10 readings for each point.

Data analysis was performed using Ultrascan. Each data set was fitted to a single ideal species model (Equation 1), where A_r is the absorbance at radius x , A_0 is the absorbance at a reference radius x_0 (meniscus), M is the molecular weight of a single species, \bar{v} is the partial specific volume of the protein, ρ is the buffer density, ω is the angular velocity of the rotor, R is the universal gas constant, T is the absolute temperature, and B is a baseline correction factor. Buffer density was measured at 20 °C using a DSA48 density and sound analyzer (Anton Paar, Graz, Austria). The value of \bar{v} was calculated using Sednterp.⁶

$$A_r = \left[\ln(A_0) + \left(M(1 - \bar{v}\rho)\omega^2 / 2RT \right) \cdot (x^2 - x_0^2) \right] + B \quad (\text{eq. 1})$$

Figure S3. Sedimentation equilibrium analysis of MPS. Overlays: The data for each sample were collected at 8, 10, 12 and 14 krpm. Triplicate scans for each speed were collected and analyzed. The pink curves were generated by fitting the data for each speed to a single ideal species model. The best fit was provided by a hexameric single ideal species with a hexamer mass of 207 kDa, which agrees well with the calculated mass of 220 kDa. The corresponding residuals for each fit are shown at the top of the plot.



Decarboxylation kinetics. Decarboxylation of oxaloacetate was assayed by monitoring the absorption decrease at 305 nm ($\Delta\epsilon_{305} = 173 \text{ M}^{-1} \text{ cm}^{-1}$) in the absence of 2-pyrone.⁷ Reactions were carried out in 50 mM PIPES buffer, 5 mM MgCl_2 , pH 7.0 at 30 °C. Enzyme concentration varied between 0.10 and 1.6 μM depending on the activity of the respective mutant. After pre-incubating the enzyme solution for 3 min at 30 °C, the reaction was initiated by addition of oxaloacetate (25 μM to 4 mM). Initial velocities were determined by linear regression and the data were fit to the Michaelis-Menten equation $v_0 = k_{\text{cat}}[\text{E}][\text{S}]/(K_m + [\text{S}])$, where v_0 is the initial velocity, $[\text{E}]$ is the total enzyme concentration, and $[\text{S}]$ is the substrate concentration.

Formation of macrophomate from oxaloacetate and 2-pyrone. The MPS-catalyzed reaction between oxaloacetate (1 mM) and 2-pyrone **1** (1 mM) was monitored by reverse phase HPLC.⁷ Reactions were carried out in 50 mM PIPES buffer, 5 mM MgCl_2 , pH 7.0. A solution of enzyme in buffer (750 μL , 0.94 μM) was pre-incubated with 50 μL of a 20 mM solution of **1** in acetonitrile for 5 min at 30 °C. The reaction was initiated by addition of 100 μL of 10 mM oxaloacetate in reaction buffer. The reaction vessel was gently shaken at 30 °C and 100 μL aliquots were removed and quenched with 100 μL of acetonitrile after 2.5, 5, 10, 15, 30, 60 and 240 min. The aliquots were analyzed at 220 nm by analytical reverse phase HPLC (column: Nucleosil 300-5 C_{18} ; Macherey-Nagel, Oensingen, CH) using a gradient starting at 90% water (containing 0.1% trifluoroacetic acid) and 10% acetonitrile (containing 0.05% trifluoroacetic acid), and increasing the amount of acetonitrile by 1% per minute. Typical retention times for the 2-pyrone and macrophomate under these conditions are 13 and 22 minutes, respectively. Initial rates were determined by linear regression of the integrated peak areas.

Formation of macrophomate from pyruvate and 2-pyrone. MPS variants were also tested for their ability to synthesize macrophomate from sodium pyruvate (10 mM) and 2-pyrone **1** (1 mM).³ Enzyme (27 μM) in 750 μL buffer (50 mM PIPES buffer, 5 mM MgCl_2 , pH 7.0) was incubated with 50 μL of a 20 mM solution of **1** in acetonitrile for 5 min at 30 °C. Reaction was initiated by addition of 100 μL of 100 mM sodium pyruvate in the reaction buffer. The reaction vessel was gently shaken at 30 °C for 3 days. An aliquot was removed and analyzed at 220 nm by analytical reverse phase HPLC as described above. Reactions with wild-type MPS and without enzyme were used as positive and negative controls, respectively.

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