SUPPORTING INFORMATION FOR

Mechanism and Specificity of an Acyltransferase Domain from a Modular Polyketide Synthase

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Materials and Methods

Reagents and Chemicals

Methylmalonyl CoA, malonyl CoA, propionyl CoA, β-NAD^+, NADH, α-ketoglutarate dehydrogenase (porcine heart) (αKGDH), α-ketoglutaric acid, thiamine pyrophosphate (TPP), and EDTA were purchased from Sigma. TCEP was from CalBioChem. BSA was from New England Biosciences. IPTG was from Gold Biotechnology. Ni-NTA affinity resin was from Qiagen and SDS-PAGE gradient gels were from Bio-Rad. The Hi-Trap Q anion exchange column was from GE Healthcare.

DNA Constructs

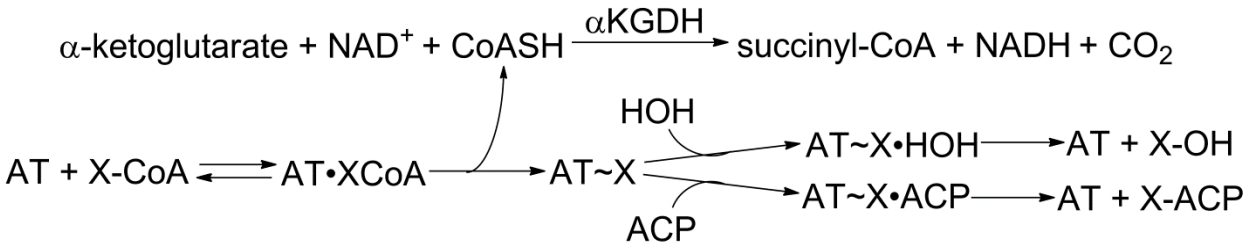
DEBS AT3, ACP3, and ACP6 were expressed from plasmids pAYC47[1], pVYA05[2], and pFW55[3] respectively. Point mutants of DEBS AT3 were created using the Quikchange Site-directed Mutagenesis Kit (Agilent). The Q652L mutant (pBD35) was created with primer 5'-GTCGTGGGGCACTCG**CTC**GGCGAGATCGCCGCC-3' and its antiparallel complement using pAYC47 as the template. The Y751H/S753F mutant (pBD38) was created in two steps. The Y751H mutant (pBD43) was created with primer 5'-GACATCGACGTGGAC**CAC**GCCTCGCACTCGCCG-3'and its antiparallel complement using pAYC47 as the template. pBD38 was then created using primer 5'-GACGTGGAC**CAC**GCC**TTC**CACTCGCCGCAGATC-3' and its antiparallel complement using pBD43 as the template. All mutations were confirmed by sequencing.

Protein Expression and Purification

All plasmids were electrically transformed into *E.coli* BAP1[4] cells for protein expression except in the case of apo-ACP3, which was produced in *E.coli* BL21. Proteins were expressed and purified as described previously[5] with the addition of a second elution step from the Ni-NTA resin (2 resin volumes; 150 mM sodium phosphate, pH 7.6, 50 mM sodium chloride, 8% glycerol, 450 mM imidazole). Eluted

protein fractions were combined and applied to the HiTrap Q anion exchange column. Phosphopantetheinylation of ACP proteins was confirmed by MALDI-TOF mass spectrometry.

Fluorometric Assays



Scheme S1. Coupling of AT catalysis to αKGDH activity. AT-catalyzed release of free Coenzyme A (CoASH) is coupled to the formation of NADH in the α-KGDH-catalyzed conversion of α-ketoglutarate to succinyl-CoA, thereby allowing continuous and sensitive fluorometric monitoring of AT activity.

Assays were adapted from a malonyl-CoA:acyl carrier protein transacylase assay described previously.[6] Specifically, assays were run in 96-well microtiter plates (black polystyrene, flat bottom, half area, non-binding surface, Corning). NADH fluorescence was monitored using a Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek). Samples were illuminated with a tungsten light source and a 360 nm filter, and fluorescence emission was monitored using a 400 nm dichroic mirror with a 460 nm filter. All measurements were made with a sensitivity setting of 85. Reactions were run for five minutes using the minimum interval between measurements.

Assay components were prepared in three different solutions: solution (1) contained the acyl carrier protein (ACP), αKGDH, NAD+, TPP, and α-ketoglutaric acid at four times their final concentration; solution (2) contained the acyl-CoA substrate prepared at four times its final concentration; and solution (3) contained the acyltransferase (AT) domain prepared at twice its final concentration. All solutions were prepared in 50 mM sodium phosphate buffer, pH 7.6, 10% glycerol, 1 mM TCEP, and 1 mM EDTA. The 2X AT solution also contained 0.1 mg/mL BSA. Solutions were added to the wells in the following order:

25 μL of solution (1), 25 μL of solution (2), and 50 μL of solution (3), which initiated the reaction. Combination of solutions (1) and (2) prior to the start of measurement allowed any free Coenzyme A in the acyl-CoA stock to be consumed, therefore avoiding its interference with the detection of AT-mediated Coenzyme A production. Final assay concentrations were: 50 mM sodium phosphate, pH 7.6, 10% glycerol, 1 mM TCEP, 1 mM EDTA, 0.4 mU/μL αKGDH, 0.4 mM NAD^+, 0.4 mM TPP, 2 mM α-ketoglutaric acid, and 0.05 mg/mL BSA. AT, ACP, and acyl-CoA concentrations were varied as described below.

DEBS AT3-mediated substrate hydrolysis: Reactions were run with 200 nM DEBS AT3 in the absence of *holo*-ACP (except when the inactive *apo*-ACP3 surrogate was used), over methylmalonyl-CoA, malonyl-CoA, and propionyl-CoA concentrations of zero to 100 μM. When the *apo*-ACP3 surrogate was used (Figure 1), reactions were run over methylmalonyl-CoA concentrations of zero to 100 μM. The *apo*-ACP3 concentration was 100 μM. 0 μM AT3 controls were run in parallel. All data points were obtained in triplicate.

Acyl-CoA saturation kinetics for DEBS AT3: Reactions were run with 200 nM DEBS AT3 and 100 μM DEBS *holo*-ACP3 over methylmalonyl-CoA, malonyl-CoA, and propionyl-CoA concentrations of zero to 200 μM. 0 μM AT3 controls were run in parallel. All data points were obtained in triplicate.

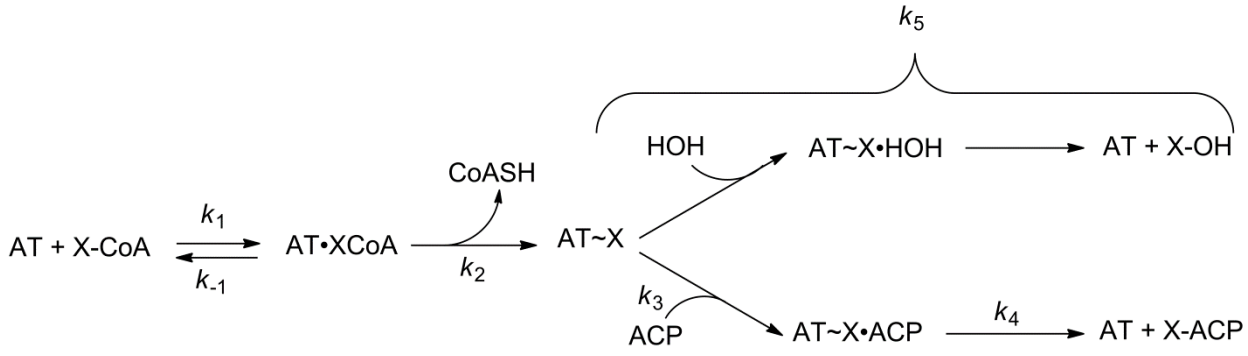
ACP saturation kinetics for DEBS AT3: DEBS ACP3 reactions were run with 200 nM DEBS AT3 and 150 μM methylmalonyl-CoA over *holo*-ACP3 concentrations of zero to 200 μM. DEBS ACP6 reactions were run with 2 μM DEBS AT3 over *holo*-ACP6 concentrations of zero to 200 μM. 0 μM AT3 controls were run in parallel. All data points were obtained in triplicate.

Mutant DEBS AT3 kinetics: Reactions were run with 10 μM BD35 and BD38 in the presence of 100 μM DEBS *holo*-ACP3 and 150 μM methylmalonyl-CoA or malonyl-CoA. 0 μM AT3 controls were run in parallel. All reactions were run in triplicate.

Data Analysis

Initial velocities were defined as the linear portion of the product concentration vs. time curve over consumption of 20% or less of the limiting substrate, comprising at least two enzymatic turnovers. Initial rates of the no-AT control reactions were subtracted from their enzyme-containing counterparts. Initial velocity vs. concentration curves were fit to the Michaelis-Menten equation using the curve fitting tool in MATLAB to define kinetic parameters. The *holo*-ACP titration curves were fit to a slightly altered form of the Michaelis-Menten equation that included the addition of a constant to account for the zero *holo*-ACP hydrolysis rate.

Kinetic Analysis of AT Catalysis



Scheme S2. Ping-pong mechanism of the AT-catalyzed transacylation of a (methyl)malonyl-CoA (XCoA) substrate to a (methyl)malonyl-ACP (X-ACP)with defined rate constants. Transacylation requires *holo*-ACP, which contains the nucleophilic phosphopantetheine prosthetic group (see Figure S2). Non-productive partitioning of the acyl enzyme intermediate by competing hydrolysis is shown in the top branch of the mechanism.

Derivation of Kinetic Parameters Describing α-carboxyacyl-CoA-limited AT Catalysis

Rate constants are as defined in Scheme S2. Note that the value of the specificity constant, k_{cat}/K_M, for AT-catalyzed hydrolysis is identical to that for AT-catalyzed transacylation and that in both cases this parameter is independent of the rate of breakdown of the (methyl)malonyl-enzyme intermediate.

Hydrolysis:

$$\boxed{k_{cat}/K_M = \frac{k_1 k_2}{k_{-1}+k_2} \qquad k_{cat} = \frac{k_2 k_5}{k_2+k_5}}$$

$$\frac{d[X\text{-}OH]}{dt} = k_5[AT\text{-}X]$$

$$\frac{d[AT\text{-}X]}{dt} = k_2[AT\cdot XCoA] - k_5[AT\text{-}X]$$

$$\frac{d[AT\cdot XCoA]}{dt} = k_1[AT][XCoA] - k_{-1}[AT\cdot XCoA] - k_2[AT\cdot XCoA] \cong 0$$

$$[AT\cdot XCoA] = \frac{k_1}{k_{-1}+k_2}[AT][XCoA]$$

$$[AT] = [AT]_o - [AT\cdot XCoA] - [AT\text{-}X] - [AT\text{-}X\cdot OH]$$

Neglecting [AT-X·OH],

$$[AT\cdot XCoA] = \frac{k_1}{k_{-1}+k_2}([AT]_o - [AT\cdot XCoA] - [AT\text{-}X])[XCoA]$$

$$[AT\cdot XCoA] = \frac{\frac{k_1[XCoA]}{k_{-1}+k_2}([AT]_o - [AT\text{-}X])}{1+\frac{k_1[XCoA]}{k_{-1}+k_2}}$$

$$\frac{d[AT\text{-}X]}{dt} = \frac{\frac{k_1 k_2[XCoA]}{k_{-1}+k_2}([AT]_o - [AT\text{-}X])}{1+\frac{k_1[XCoA]}{k_{-1}+k_2}} - k_5[AT\text{-}X] \cong 0$$

$$[AT\text{-}X] = \frac{\frac{k_1 k_2}{k_{-1}+k_2}[XCoA][AT]_o}{k_5 + [XCoA]\frac{k_1 k_5 + k_1 k_2}{k_{-1}+k_2}} = \frac{\frac{k_2}{k_2+k_5}[XCoA][AT]_o}{\frac{k_5(k_{-1}+k_2)}{k_1(k_2+k_5)} + [XCoA]}$$

$$\frac{d[\text{X-OH}]}{dt}=\frac{\frac{k_2k_5}{k_2+k_5}[\text{XCoA}][\text{AT}]_o}{\frac{k_5(k_{-1}+k_2)}{k_1(k_2+k_5)}+[\text{XCoA}]}$$

Thus,

$$k_{cat}=\frac{k_2k_5}{k_2+k_5} \qquad K_M=\frac{k_5(k_{-1}+k_2)}{k_1(k_2+k_5)} \qquad {k_{cat}}/{K_M}=\frac{k_1k_2}{k_{-1}+k_2}$$

Transacylation:

$${k_{cat}}/{K_M}=\frac{k_1k_2}{k_{-1}+k_2} \qquad k_{cat}=\frac{k_2k_3[\text{ACP}]k_4}{k_2k_3[\text{ACP}]+k_2k_4+k_3[\text{ACP}]k_4}$$

$$\frac{d[\text{X-ACP}]}{dt}=k_4[\text{AT-X}\cdot\text{ACP}]$$

$$\frac{d[\text{AT-X}\cdot\text{ACP}]}{dt}=k_3[\text{AT-X}][\text{ACP}]-k_4[\text{AT-X}\cdot\text{ACP}]$$

$$\frac{d[\text{AT-X}]}{dt}=k_2[\text{AT}\cdot\text{XCoA}]-k_3[\text{ACP}][\text{AT-X}]$$

As above,

$$[\text{AT}\cdot\text{XCoA}]=\frac{\frac{k_1[\text{XCoA}]}{k_{-1}+k_2}([\text{AT}]_o-[\text{AT-X}]-[\text{AT-X}\cdot\text{ACP}])}{1+\frac{k_1[\text{XCoA}]}{k_{-1}+k_2}}$$

$$\frac{d[\text{AT-X}]}{dt}=\frac{\frac{k_1k_2[\text{XCoA}]}{k_{-1}+k_2}([\text{AT}]_o-[\text{AT-X}]-[\text{AT-X}\cdot\text{ACP}])}{1+\frac{k_1[\text{XCoA}]}{k_{-1}+k_2}}-k_3[\text{ACP}][\text{AT-X}]\cong 0$$

$$[\text{AT-X}]=\frac{k_1k_2[\text{XCoA}]([\text{AT}]_o-[\text{AT-X}\cdot\text{ACP}])}{k_1k_2[\text{XCoA}]+(k_{-1}+k_2)k_3[\text{ACP}]+k_1k_3[\text{ACP}][\text{XCoA}]}$$

$$\frac{d[\text{AT-X}\cdot\text{ACP}]}{dt}=k_3[\text{ACP}]\frac{k_1k_2[\text{XCoA}]([\text{AT}]_o-[\text{AT-X}\cdot\text{ACP}])}{k_1k_2[\text{XCoA}]+(k_{-1}+k_2)k_3[\text{ACP}]+k_1k_3[\text{ACP}][\text{XCoA}]}-k_4[\text{AT-X}\cdot\text{ACP}]\cong 0$$

$$[\text{AT-X}\cdot\text{ACP}]=\frac{k_1k_2k_3[\text{ACP}][\text{XCoA}][\text{AT}]_o}{[\text{XCoA}](k_1k_2k_3[\text{ACP}]+k_1k_2k_4+k_1k_3[\text{ACP}]k_4)+k_3[\text{ACP}]k_4(k_{-1}+k_2)}$$

$$\frac{d[\text{X-ACP}]}{dt}=\frac{\frac{k_2k_3[\text{ACP}]k_4[\text{XCoA}][\text{AT}]_o}{(k_2k_3[\text{ACP}]+k_2k_4+k_3[\text{ACP}]k_4)}}{[\text{XCoA}]+\frac{k_3[\text{ACP}]k_4(k_{-1}+k_2)}{(k_1k_2k_3[\text{ACP}]+k_1k_2k_4+k_1k_3[\text{ACP}]k_4)}}$$

Thus,

$$k_{cat}=\frac{k_2k_3[\text{ACP}]k_4}{k_2k_3[\text{ACP}]+k_2k_4+k_3[\text{ACP}]k_4} \qquad K_M=\frac{k_3[\text{ACP}]k_4(k_{-1}+k_2)}{(k_1k_2k_3[\text{ACP}]+k_1k_2k_4+k_1k_3[\text{ACP}]k_4)} \qquad k_{cat}/K_M=\frac{k_1k_2}{k_{-1}+k_2}$$

Supplemental Figures

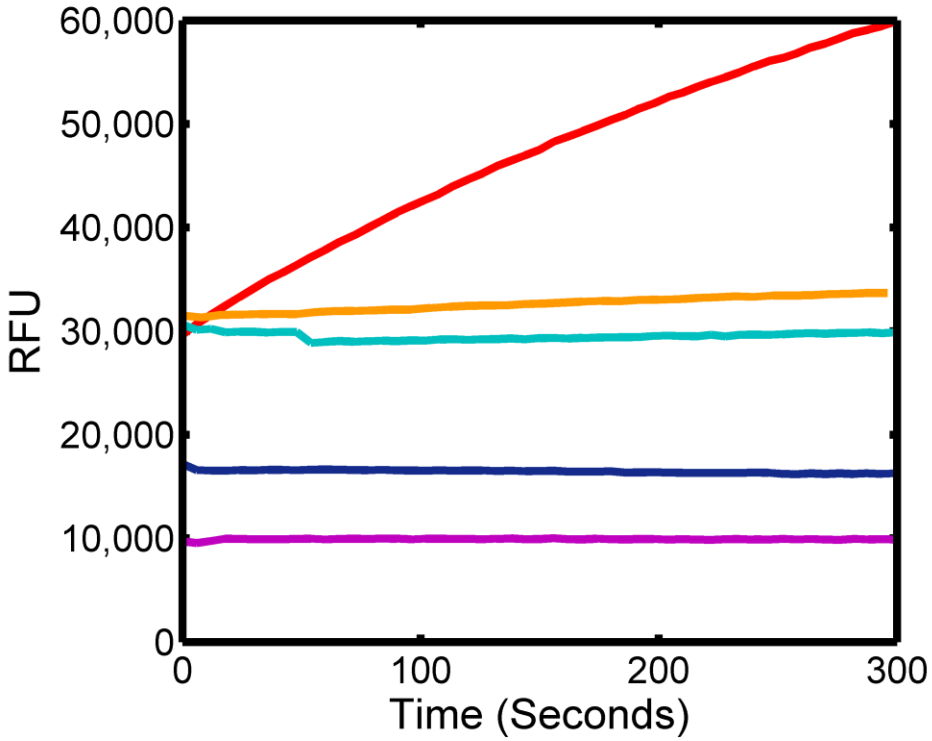


Figure S1. Example progress curves for the coupled reaction and relevant controls: the complete DEBS AT3/ACP3/MMCoA/αKGDH reaction (red), without AT (orange), without MMCoA (cyan), without NAD+ (blue), and without αKGDH (purple). Reactions were run as described above. RFU: Relative Fluorescence Units.

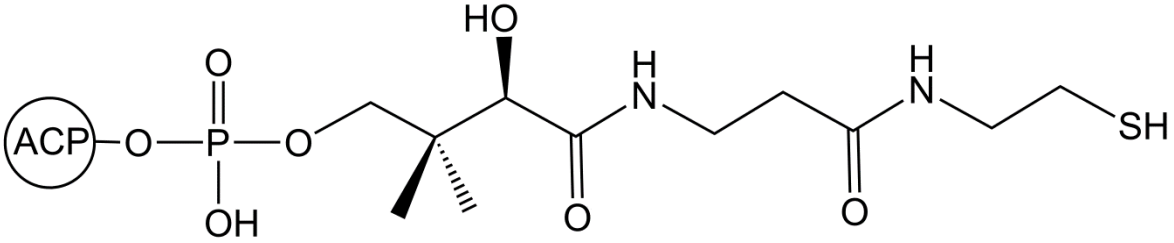


Figure S2. Catalytically active, phosphopantetheinylated *holo*-ACP. The nucleophilic thiol residue is responsible for attack of the acyl enzyme intermediate during transacylation. *Apo*-ACP is not phosphopantetheinylated and is thus not catalytically active.

References

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