

## Supporting Information

### Structure and Catalysis in the *Escherichia coli* Hotdog-fold Thioesterase

#### Paralogs YdiI and YbdB

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## MATERIALS & METHODS

### *Synthesis of 2,4-dihydroxyphenacyl-CoA and phenacyl-CoA*

2,4-Dihydroxyphenacyl-CoA and phenacyl-CoA were synthesized according to the following procedure. Under a N<sub>2</sub> atmosphere, 45 mg of 2-bromo-2',4'-dihydroxyacetophenone or 2-bromoacetophenone were added drop-wise over a 1 h period to 30 mg of CoA in 1.5 mL of H<sub>2</sub>O/THF (1:1). The solution pH was monitored and maintained within a range of 7.2-8.0. Following overnight incubation, the reaction mixture was extracted twice with ethyl acetate. The crude product obtained after lyophilization of the aqueous phase was chromatographed on a G-15 Sephadex column. The fractions were monitored by HPLC (dual wavelengths 280 nm/260 nm). The desired fractions combined, concentrated and subjected to <sup>1</sup>H-NMR spectral analysis for structure confirmation (NMR facilities, the Department of Chemistry, UNM).

### *Synthesis of [<sup>14</sup>C]-benzoyl-CoA*

[<sup>14</sup>C]-benzoyl-CoA was synthesized enzymatically by using 4-hydroxybenzoic acid ligase (4HBAL) from *Rhodospseudomonas palustris* hydroxybenzoate ligase (Uniprot accession code Q53005). The 4HBAL-pET3a was used to transform competent *E. coli* BL21 (DE3) cells (Invitrogen). A single colony was used to inoculate 10 mL LB media containing 50 µg/ml ampicillin. The culture was scaled, the gene expression induced, the cells were harvested described above. The cells were resuspended in 50 mM K<sup>+</sup>HEPES (pH 7.5) lysis buffer then lysed as described above. The cleared supernatant was fractioned by ammonium sulfate induced precipitation. The protein precipitated with 0-35 % ammonium sulfate and was harvested by centrifugation at 20,000 rpm for 15 min. The protein pellet was resuspended in 10X its weight of 50 mM K<sup>+</sup>HEPES buffer pH 7.5 containing 1 M ammonium sulfate (Buffer A) and loaded onto a FPLC attached 30 x 1 cm butyl sepharose column

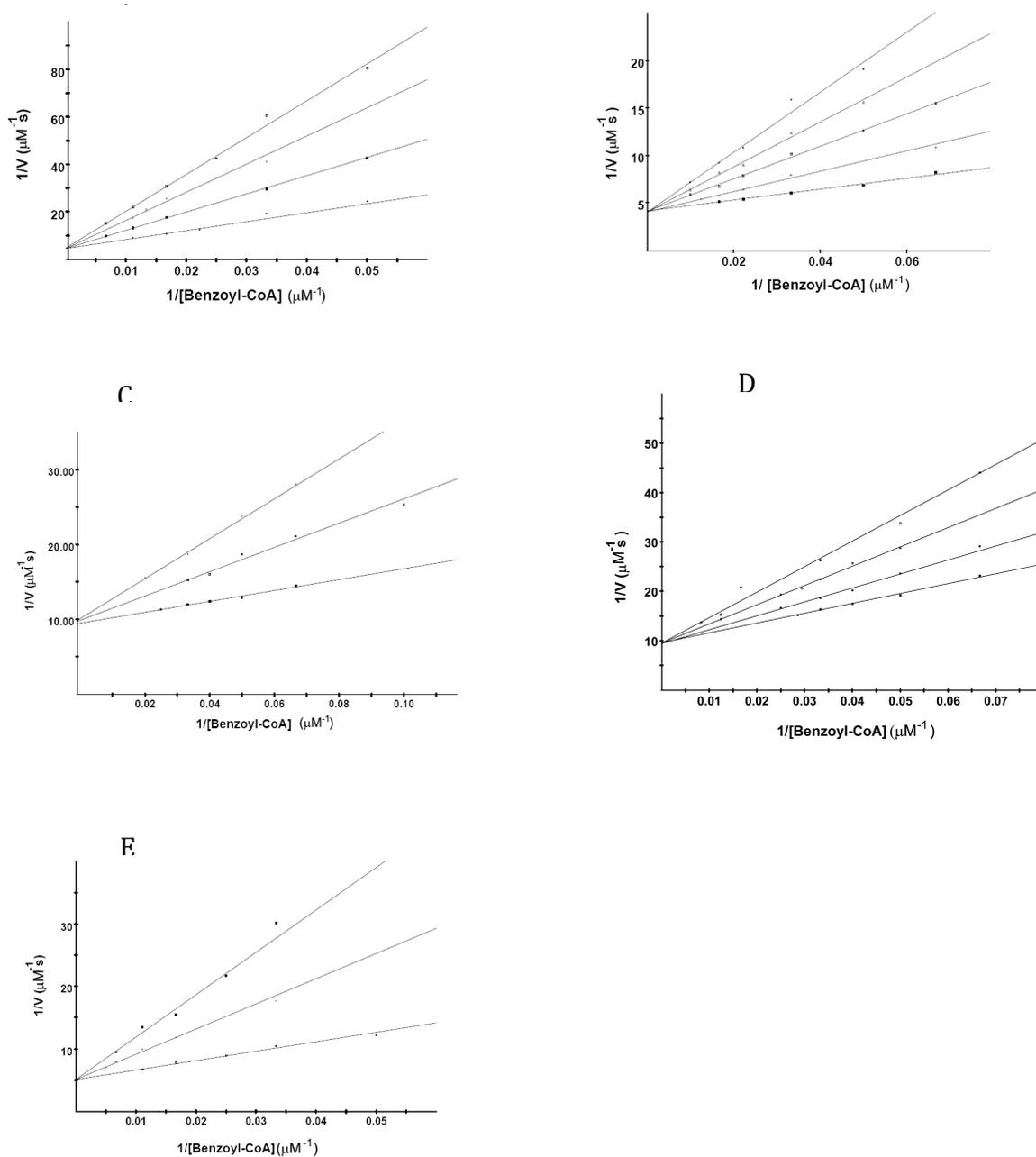
equilibrated with 5 CV of Buffer A. The column was washed until the  $A_{280}$  reached baseline and the protein was eluted with a gradient of 0-50% 50 mM  $K^+$ HEPES pH 7.5 (Buffer B). The protein eluted at 35% Buffer B and the fractions were combined, concentrated and dialyzed 3 times against Buffer B.

$[^{14}C]$ -Benzoyl-CoA (specific activity of 56 mCi/mol) was synthesized in a 1 mL reaction solution containing 100 mM  $K^+$ HEPES, 5 mM  $MgCl_2$ , 13 mM CoA, 13 mM ATP, and 5  $\mu$ M 4HBAL and 9 mM  $[^{14}C]$ -benzoate (American Radiolabeled Chemicals Inc., St. Louis, Mo; the supplied acid form was converted to the sodium salt prior to addition to the reaction mixture). The reaction solution was allowed to incubate at room temperature overnight and then quenched by the addition of 1 M HCl. The precipitated protein was harvested by centrifugation.  $[^{14}C]$ -Benzoyl-CoA was purified from the crude reaction mixture by using a semi-prep Ultra Aqueous C-18 reverse phase HPLC column (RESTEK) eluted with a 50% linear gradient of 20 mM  $KH_2PO_4$  (pH 6.1) (Buffer A) and 80%  $CH_3CN$  (Buffer B) over a 35 min period. The desired compound eluted at  $\sim$  14 min. The fractions containing the final compound were combined and lyophilized to powder.

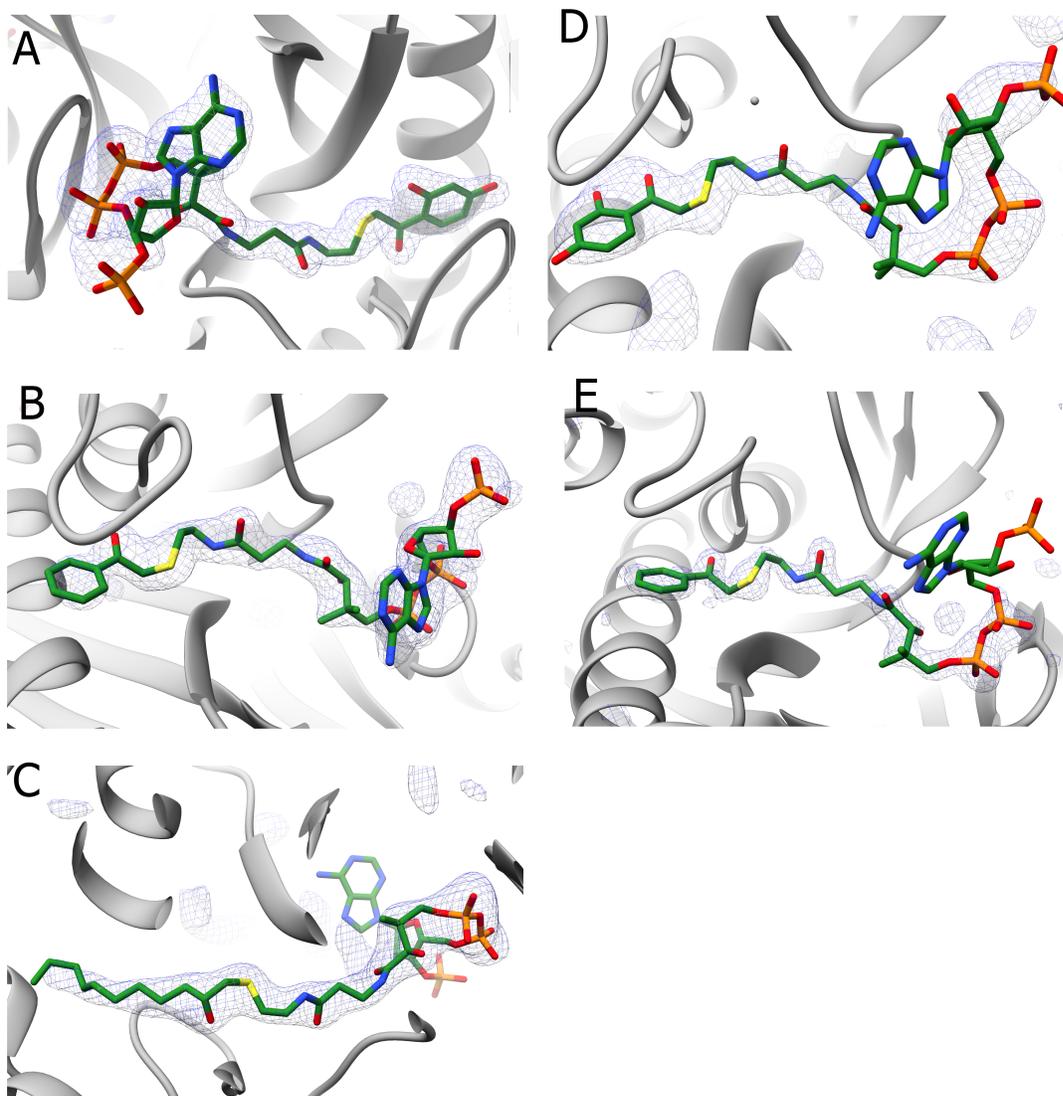
#### *Purification of native YbdB and YdiI*

The supernatant of the lysed cells was loaded onto a DEAE-Sepharose column, which was then washed with a linear gradient of NaCl from 0.05 to 1 M at a flow rate of 2 mL/min. The fractions were analyzed by SDS-PAGE, selectively pooled, and then further purified using a Butyl-Sepharose column. Native YbdB and YdiI were eluted from the respective columns by applying a linear gradient of ammonium sulfate (1 M to 0.1 M). The homogeneous YbdB or YdiI-containing fractions were combined, dialyzed at 4 °C against and 50 mM NaCl/20 mM HEPES (pH 8.2) overnight, and then concentrated to 20 mg/mL for storage at -80°C. Yield: 15 mg YbdB/gm wet cell paste and 12 mg YdiI/gm wet cell paste.

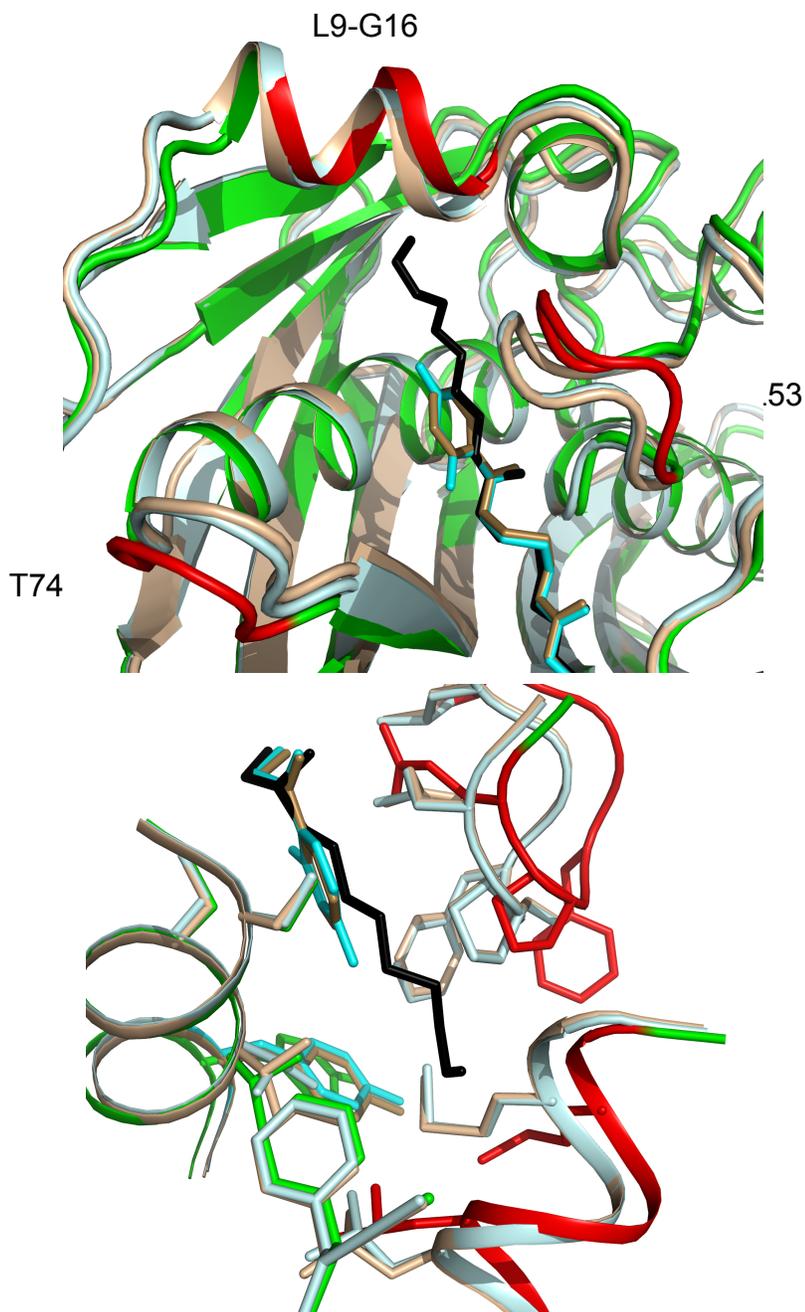
## RESULTS



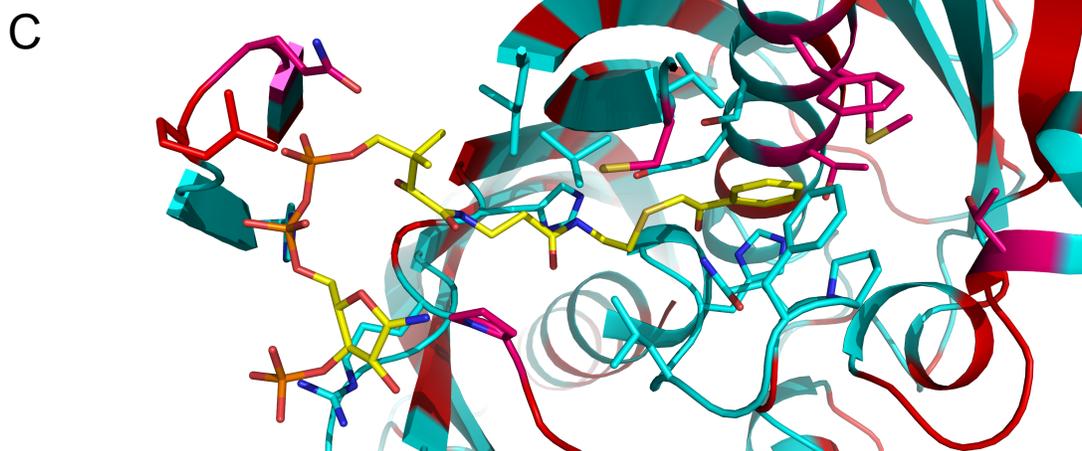
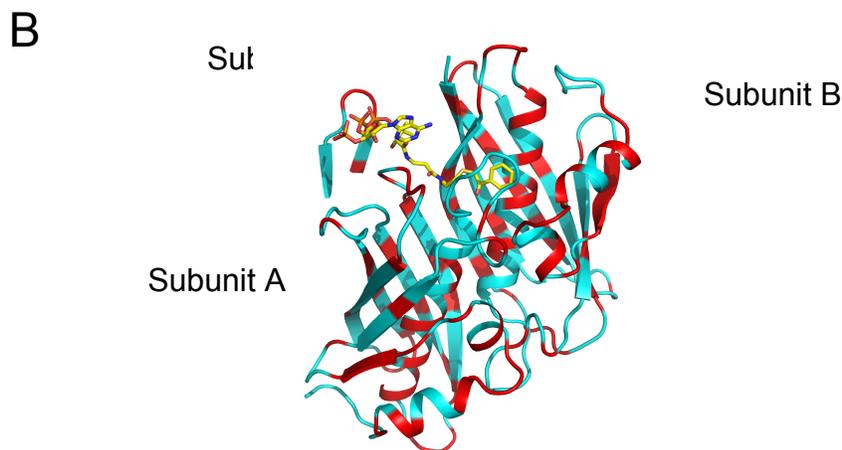
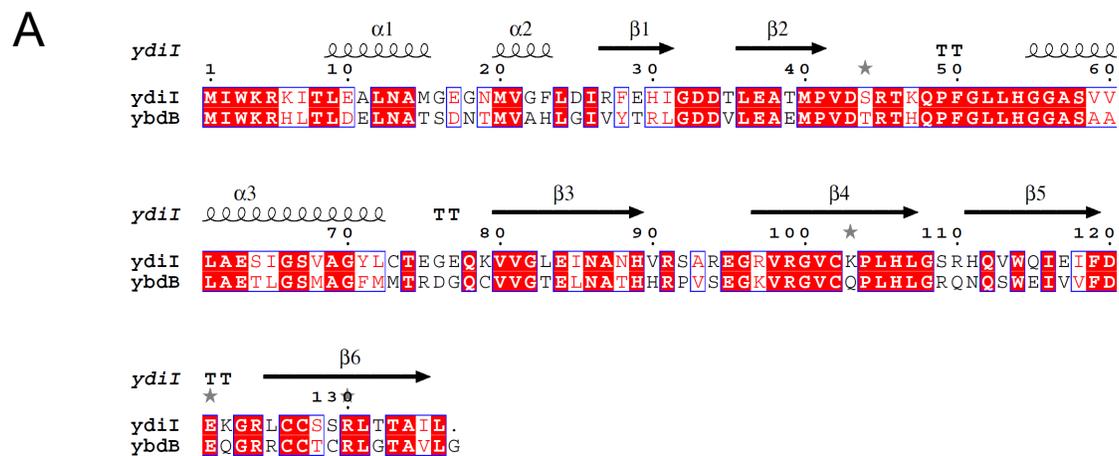
**Figure S11.** Lineweaver-Burk plots for inhibition of YdiI and YbdB by inert substrate analogs. YdiI or YbdB-catalyzed hydrolysis of benzoyl-CoA (15 to 200  $\mu M$ ) was monitored using DTNB (1 mM) as the coupling agent. Reaction solutions contained 0.07  $\mu M$  YdiI (A-C) or YbdB (D and E). (A) (5 -20  $\mu M$ ) phenacyl-CoA, (B) (2.5-10  $\mu M$ ) 2,4-dihydroxyphenacyl-CoA (C), (15-30  $\mu M$ ) undecan-2-one-CoA, (D) 35-105  $\mu M$  phenacyl-CoA and (E) 5-10  $\mu M$  2,4-dihydroxyphenacyl-CoA.



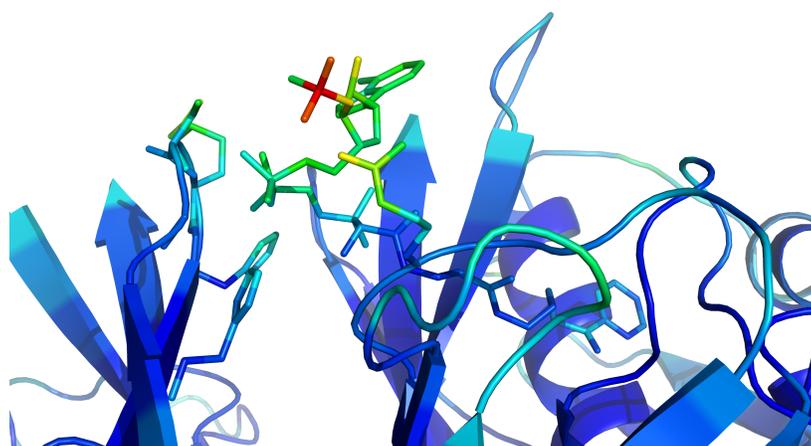
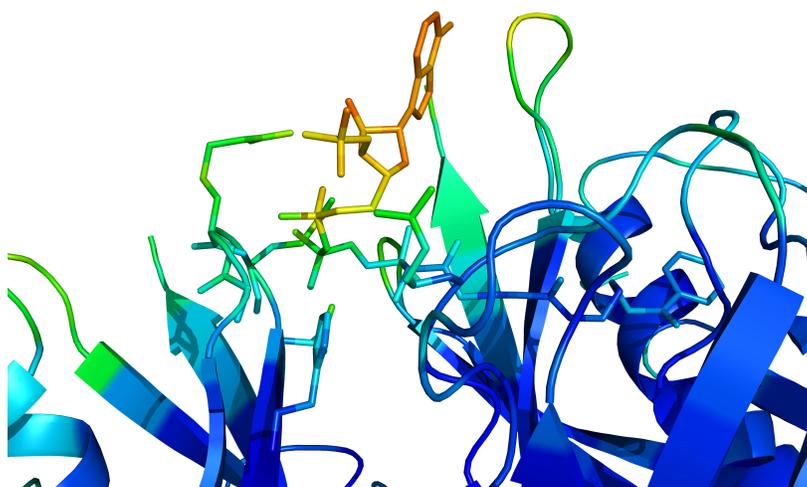
**Figure S12.** Simulated annealing omit electron density maps for bound ligands. Electron density maps of ydiI bound to **(A)** 2,4-dihydroxyphenacyl-CoA, **(B)** phenacyl-CoA and **(C)** undecan-2-one-CoA ligands. Electron density maps of ybdB bound to **(D)** 2,4-dihydroxyphenacyl-CoA and **(E)** phenacyl-CoA. The maps are contoured at the following: (A)  $4\sigma$ , (B)  $4\sigma$ , (C)  $3\sigma$ , (D)  $2.5\sigma$ , and (E)  $4\sigma$ .



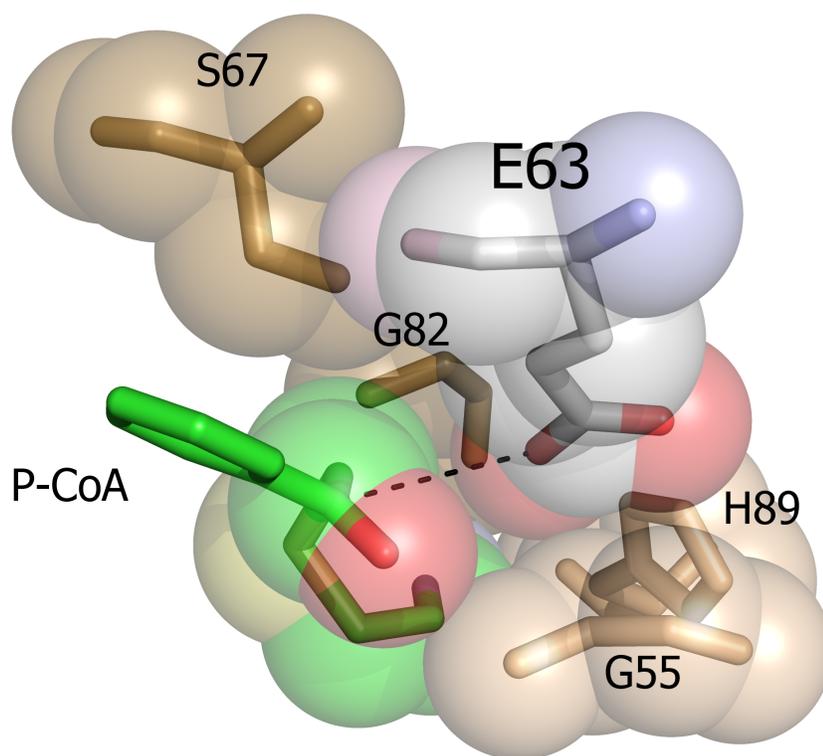
**Figure S13.** YdiI·(P-CoA) (wheat), YdiI·(2,4-DHPCoA) (cyan) and YdiI·(UDO-CoA) green and red backbone. The truncated UDO-CoA ligand is colored black, the truncated P-CoA ligand wheat and the truncated 2,4-DHP ligand cyan. **(top)** The altered secondary structural elements observed in the YdiI·(UHO-CoA) complex highlighted in red color. **(bottom)** A zoom-in depiction at a different orientation which includes the side chains that are moved in the expansion of the active site.



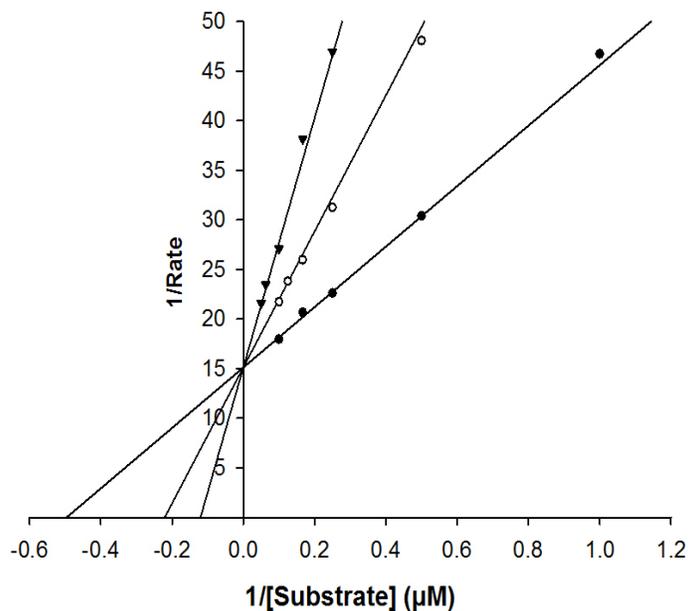
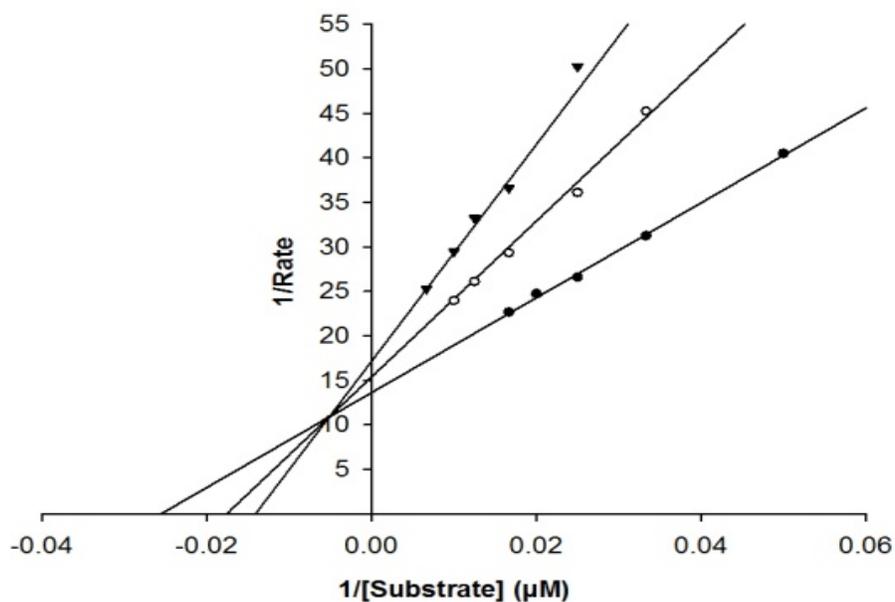
**Figure SI4.** (A) ESPrict display of the alignment of *E. coli* YdiI and YdbB amino acid sequences in the context of units of regular secondary structure. Sequence identities are shown in the red background, similarities are shown with red font. (B) YdbB(P-CoA) complex with residues that differ from those of YdiI colored red, conserved residues cyan; P-CoA carbon atoms yellow, nitrogen atoms blue, oxygen atoms red and sulfur atoms mustard. (C) Zoom-in on the ligand binding site with the inclusion of side chains represented in “stick” to illustrate the concentration of conserved residues (carbon atoms colored cyan; variant carbon atoms colored red or magenta) surrounding the benzoyl-pantetheine unit



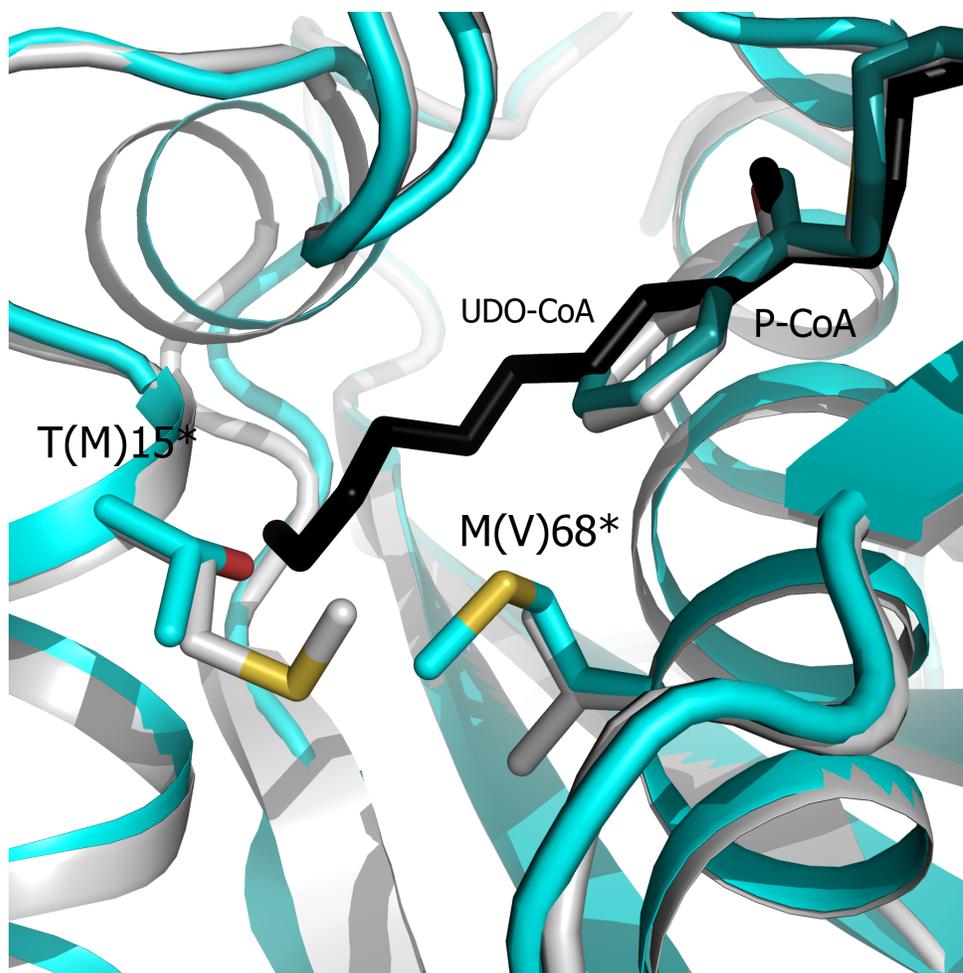
**Figure SI5.** Depiction of the relative degree of atomic motion within the crystalline YdbB(phenacyl-CoA) complex (top) and the crystalline YdiI(phenacyl-CoA) complex (bottom) as represented by color ramping of the crystallographic B-factors (from lowest motion shown in blue to highest motion shown in red). The phenacyl-CoA ligands are shown in stick as are the side chains of the residues that form the pyrophosphate binding loop in subunit C.



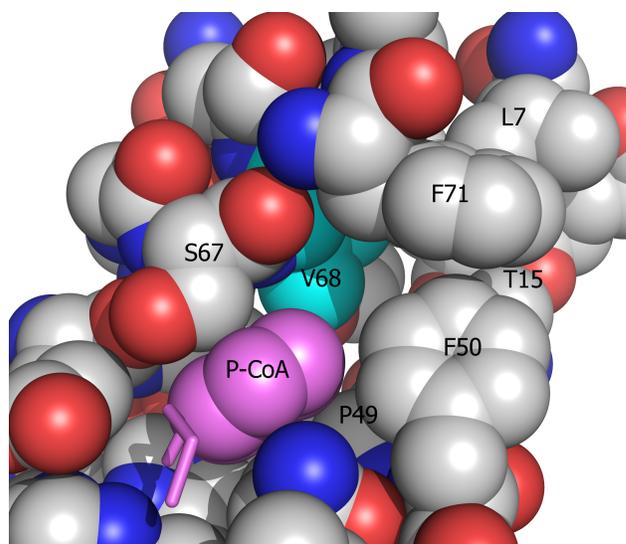
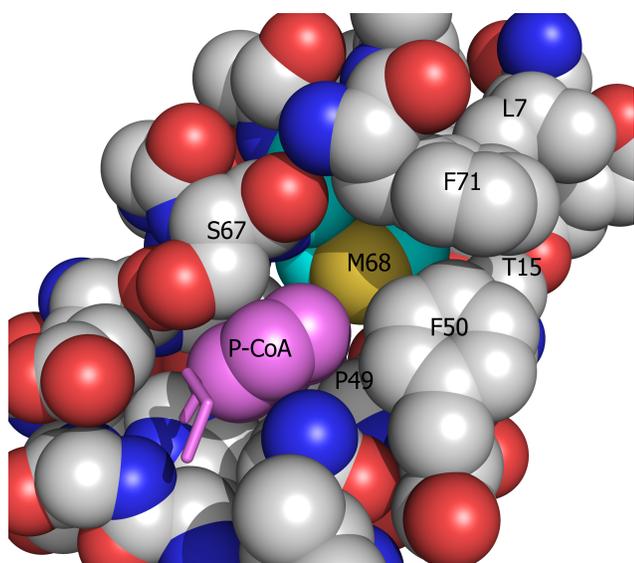
**Figure SI6.** Pymol representation of the closely packed residues that surround the carbonyl group of the YdiI in complex with phenacyl-CoA. There is no open space in the region of the Glu63 carboxylate group for a water molecule to bind for Glu63 assisted nucleophilic attack .



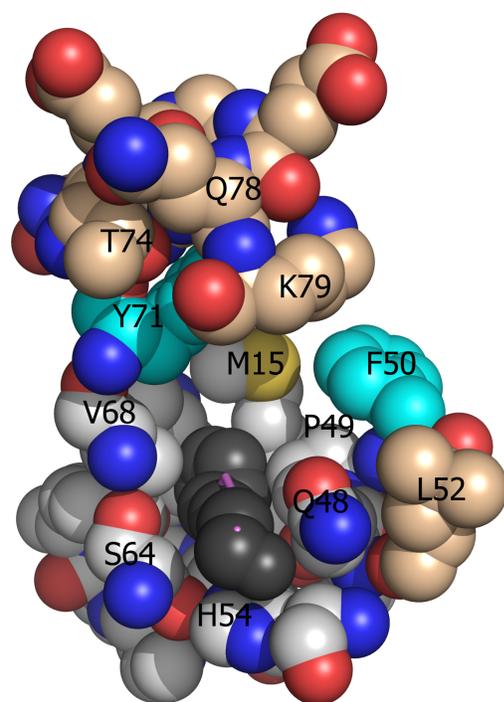
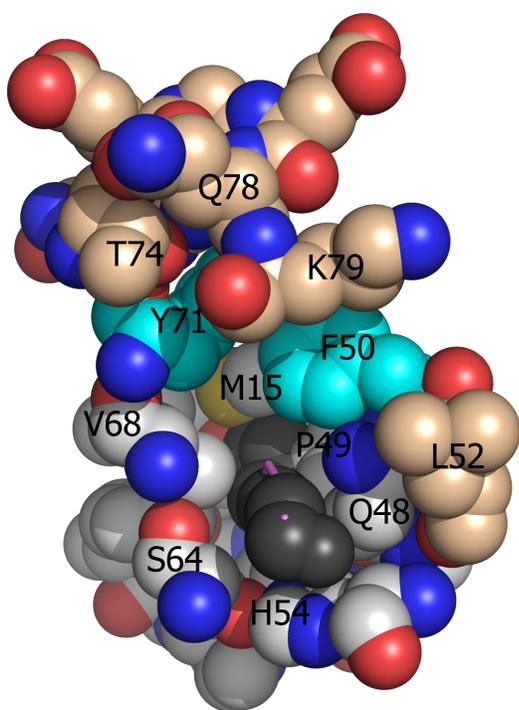
**Figure SI7.** Lineweaver-Burk plot of the initial velocities of (A) YbdB-catalyzed 4-HB-CoA hydrolysis measured as a function of 4-HB-CoA concentration at changing, fixed CoA (product inhibitor) concentration (0, 200, 400  $\mu\text{M}$ ). (B) YdiI-catalyzed 4-HB-CoA hydrolysis measured as a function of 4-HB-CoA concentration at changing, fixed CoA (product inhibitor) concentration (0, 250, 500  $\mu\text{M}$ ).



**Figure SI8.** Superposition of YdbB'(P-CoA) with protein carbon atoms colored cyan with truncated ligand atoms colored teal, YdiI'(P-CoA) with protein carbon atoms and all ligand atoms colored white and YdiI'(UDO-CoA) protein atoms not shown and truncated ligand atoms colored black. Residue 15 and 68 side chains shown in stick with sulfur atoms colored yellow and oxygen atoms red.



**D**



**Figure SI9.** **A.** Wild-type YdbB(P-CoA). **B.** Wild-type YdbB(P-CoA) mutated *in silico* to Val68 **C.** Wild-type YdiI(P-CoA) shown with UDO-CoA of superimposed wild-type YdiI(undecane-2-one-CoA) complex. **D.** Wild-type YdiI(undecane-2-one-CoA) complex shown with P-CoA of superimposed YdiI(P-CoA). P-CoA (pink stick) and UDO-CoA (dark gray spheres) are truncated up to phenacyl or undecan-2-one moieties, respectively.