

Supplemental Information

Materials and Methods

Synthesis of Hydroxyphenylacetate Substrates - To a stirred solution of the benzoic acid or phenylacetic acid derivative (470 μmol) in dry tetrahydrofuran (THF) (4 mL), ethyl chloroformate (40 μL , 418 μmol) and triethylamine (50 μL , 360 μmol) were added at 25 $^{\circ}\text{C}$ under N_2 . A white precipitate, which formed over a period of 1 h, was removed by filtration. The resulting clear solution was added dropwise to a N_2 -purged solution of 50 mg coenzyme A lithium salt (65 μmol) in 5 mL of 50% THF-water. The pH of the solution was maintained between 7.5 and 8.0 by the addition of 0.1 M LiOH. After stirring for 1 h under N_2 , the solution was acidified to pH 4 with 1 M HCl. The precipitate was removed by centrifugation (15 min) and the resulting supernatant was adjusted to pH 10. After 1.5 h (for hydrolysis of the side-product), the pH was adjusted to 7 with 1 M HCl, then lyophilized. The residue was dissolved in deionized water, then chromatographed on 120 cm \times 2.5 cm Sephadex-G25 (Amersham Pharmacia) column at 4 $^{\circ}\text{C}$ using deionized water as eluant with elution rate 0.5 mL/min. The yield was 51% for 4-hydroxyphenylacetyl-CoA, 49% for 3-hydroxyphenylacetyl-CoA, 63% for 3,4-dihydroxyphenylacetyl-CoA and 21% for 3,5-dihydroxyphenylacetyl-CoA. ^1H NMR of 4-hydroxyphenylacetyl-CoA (D_2O , pH 6.0): δ 0.53 (s, 3H), 0.68 (s, 3H), 2.07 (m, 2H), 2.80 (t, 2H, J = 6 Hz), 3.13 (m, 2H), 3.36 (m, 1H), 3.59 (s, 2H, phenylacetyl methylene H), 3.64 (m, 1H), 3.82 (s, 1H), 4.06 (d, 2H, J = 3.0 Hz), 4.39 (s, 1H), 5.97 (d, 1H, J = 7.0 Hz), 6.61 (d, 2H, J = 8.5 Hz, aromatic H), 6.92 (d, 2H, J = 8.5 Hz, aromatic H), 8.03 (s, 1H), 8.35 (s, 1H). ^1H NMR of 3-hydroxyphenylacetyl-CoA (D_2O , pH 6.0): δ 0.56 (s, 3H), 0.71 (s, 3H), 2.09 (t, 2H, J = 6.5 Hz), 2.83 (t, 2H, J = 6 Hz), 3.15 (m, 2H), 3.39 (m, 1H), 3.66 (s, 2H, phenylacetyl methylene H), 3.68 (m, 1H), 3.84 (s, 1H), 4.08 (d, 2H, J = 3.0 Hz), 4.41 (s, 1H), 5.99 (d, 1H, J = 7.0 Hz), 6.61 (m, 3H, aromatic H), 7.03 (t, 1H, J = 7.0 Hz, aromatic H), 8.06 (s, 1H), 8.37 (s, 1H). ^1H NMR of 3,4-dihydroxyphenylacetyl-CoA (D_2O , pH 6.0): δ 0.56

(s, 3H), 0.71 (s, 3H), 2.09 (t, 2H, J = 6.5 Hz), 2.83 (t, 2H, J = 6 Hz), 3.15 (m, 2H), 3.39 (m, 1H), 3.56 (s, 2H, phenylacetyl methylene H), 3.68 (m, 1H), 3.85 (s, 1H), 4.08 (d, 2H, J = 3.0 Hz), 4.41 (s, 1H), 5.99 (d, 1H, J = 7.0 Hz), 6.48 (m, 1H, aromatic H), 6.62 (s, 1H, aromatic H), 6.68 (m, 1H, aromatic H), 8.06 (s, 1H), 8.37 (s, 1H). ^1H NMR of 3,5-dihydroxyphenylacetyl-CoA (D_2O , pH 6.0): δ 0.61 (s, 3H), 0.76 (s, 3H), 2.14 (t, 2H), 2.87 (t, 2H), 3.19 (m, 2H), 3.43 (m, 1H), 3.60 (s, 2H, phenylacetyl methylene H), 3.70 (m, 1H), 3.88 (s, 1H), 4.15 (d, 2H, J = 3.0 Hz), 4.45 (s, 1H), 6.02 (d, 1H, J = 7.0 Hz), 6.15 (s, 1H, aromatic H), 6.17 (s, 2H, aromatic H), 8.09 (s, 1H), 8.38 (s, 1H).

Cloning and Purification of His-tagged E. coli PaaI for Structural Characterization - The gene for *paaI* was cloned and expressed at Structural GenomiX, Inc (www.nysgsrc.org). To facilitate purification, *paaI* was amplified by PCR using Taq polymerase, cloned into a pET vector modified for topoisomerase directed cloning (Invitrogen) that was designed to express the protein of interest followed by a C-terminal hexahistidine tag. In addition to the C-terminal hexahistidine tag, cloning introduced three additional N-terminal amino acids (MSL) in place of the native methionine. Clones containing *paaI* were transformed into the *E. coli* B834 (DE3) expression strain to facilitate isotopic labeling with selenomethionine (54). A 3 L culture was grown by fermentation at 37 $^{\circ}\text{C}$ to an OD_{600} of 0.8, adjusted to 21 $^{\circ}\text{C}$ and induced to express PaaI by addition of 0.4 mM isopropyl- β -D-thiogalactopyranoside (IPTG) and incubated overnight by shaking. Cells were harvested by centrifugation and resuspended in lysis buffer (35 mL/10 g) containing 50 μL of protease inhibitor cocktail (Sigma) and 5 μL benzonase (Novagen) and subjected to repeated sonication with intervals of cooling. After insoluble material was removed by centrifugation, PaaI-His₆ was purified by metal-affinity and gel filtration chromatography (Superdex 200). PaaI was concentrated, snap-frozen in liquid nitrogen, and stored at -80 $^{\circ}\text{C}$. Mass spectral analysis showed that the N-terminal Met had been removed by posttranslational modification.

Substrate Specificities of A. evansii and E. coli PaaI Proteins - The *A. evansii* PaaI reactions were monitored at 25 °C by using a 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) based assay in which the absorbance of 5-thio-2-nitrobenzoate at 412 nm ($\Delta\epsilon = 13.6 \text{ mM}^{-1}\cdot\text{cm}^{-1}$) was measured. The 5-thio-2-nitrobenzoate was produced by the reaction of DTNB with the CoA liberated from the acyl-CoA substrate upon hydrolysis. The 0.2 mL reaction solutions contained enzyme (PaaI: 0.02 μM for 3,4-dihydroxyphenylacetyl-CoA, 4-hydroxyphenylacetyl-CoA and 3-hydroxyphenylacetyl-CoA, 0.08 μM for 3,5-dihydroxyphenylacetyl-CoA, 0.12 μM for phenylacetyl-CoA, 55 μM for 4-hydroxybenzoyl-CoA, 3-hydroxybenzoyl-CoA, 4-hydroxybenzyl-CoA, 4-hydroxyphenacyl-CoA, 3-hydroxyphenacyl-CoA, 4-chlorobenzoyl-CoA, 4-methoxybenzoyl-CoA, crotonyl-CoA and phenylpyruvyl-CoA, 22 μM for acetyl-CoA and n-hexanoyl-CoA, 3 μM for n-butyryl-CoA, iso-butyryl-CoA, β -hydroxybutyryl-CoA and methylmalonyl-CoA), various concentrations of substrate ($0.5 - 5 \times K_m$), DTNB (1 mM), KCl (0.2 M) and 50 mM K^+Hepes (pH 7.5, 25 °C) in a quartz cuvette of 1 cm light path-length.

For *E. coli* PaaI, which is rapidly inactivated by DTNB under assay conditions, a continuous spectrophotometric assay was employed. Accordingly, the decrease in solution absorbance at 236 nm was monitored for the reaction mixtures of 4-hydroxyphenylacetyl-CoA ($\Delta\epsilon = 4.2 \text{ mM}^{-1}\text{cm}^{-1}$), 3-hydroxyphenylacetyl-CoA ($\Delta\epsilon = 3.6 \text{ mM}^{-1}\text{cm}^{-1}$) and 3,4-dihydroxyphenylacetyl-CoA ($\Delta\epsilon = 3.2 \text{ mM}^{-1}\text{cm}^{-1}$). Reactions were carried out in 0.2 mL of 50 mM Tris buffer (pH 8.1) with 1 mM DTT, containing PaaI (0.8 nM for 3,4-dihydroxyphenylacetyl-CoA, and 2 nM for 4-hydroxyphenylacetyl-CoA and 3-hydroxyphenylacetyl-CoA, and 96 nM for 3,5-dihydroxyphenylacetyl-CoA) and proper amount of substrate (0.5 to $5 K_m$) in a quartz cuvette of 1 cm light path-length.

For the phenylacetyl-CoA substrates, a fixed-time, HPLC-based assay was used. Substrate ($0.5 \sim 5 K_m$) was incubated with 480 nM of PaaI in 200 μL 10 mM Tris (pH 8.1) with 1 mM DTT for a specified time period and then mixed with 10 μL of 0.3 M HCl to terminate the reaction within 20 % conversion. The hydrolysis of phenylacetyl-

CoA by the *E. coli* PaaI protein was analyzed using an HPLC (Rainin Dynamax) equipped with Beckman Ultrasphere analytical reversed-phase C_{18} column. A linear gradient (20% - 68% solvent B, 2 to 6.4 min and 68% - 20% solvent B, 6.4 to 9 min) was employed to elute the column at a flow rate of 1 mL/min. Solvent A was 20 mM ammonium phosphate (adjusted to pH 6.7 with H_3PO_4), and solvent B was 16:9 (v/v) CH_3CN : H_2O and 20 mM ammonium phosphate (adjusted to pH 6.7 with H_3PO_4). The absorbance of the eluant was monitored at 260 nm. The retention times of CoASH and phenylacetyl-CoA were 2.7, 7.3 min respectively.

The initial velocity was calculated from the concentration of product formed divided by the reaction time. The initial velocity data, measured as a function of substrate concentration, were analyzed using equation (1) and the computer program KinetAsyst (IntelliKinetics, PA).

$$V = V_{\max} [S] / ([S] + K_m) \quad (1)$$

V = initial velocity, V_{\max} = maximum velocity, $[S]$ = substrate concentration, and K_m = Michaelis constant. The k_{cat} was calculated from $V_{\max} / [E]$ where $[E]$ is the total enzyme concentration.

Steady-State Kinetics of PaaI Mutants - 3,4-Dihydroxyphenylacetyl-CoA hydrolysis catalyzed by the *A. evansii* PaaI mutants (37.5 μM for D75A, 9.4 μM for D75N 1.9 μM for N60A and 0.66 μM for N60D) was monitored using the DTNB assay described above. The hydrolyses of 3,4-dihydroxyphenylacetyl-CoA, 3-hydroxyphenylacetyl-CoA and 4-hydroxyphenylacetyl-CoA catalyzed by *E. coli* PaaI mutants (24 nM for E14A, 16 nM for N15A, 5 μM for D16A, 12 μM for G23P, 6 μM for N46A, 0.3 μM for H52A and 16 μM for D61A) were monitored by measuring the change at 236 nm absorbance as described above. For the hydrolysis of 3,4-dihydroxyphenylacetyl-CoA catalyzed *A. evansii* PaaI D75A and *E. coli* PaaI D61A, only the V_{\max} was determined.

Inhibition of A. evansii PaaI Thioesterase - Reactions were monitored using a continuous spectrophotometric assay. For *A. evansii* PaaI, reactions were monitored at 25 °C by using a DTNB based assay in which the absorbance of 5-thio-2-nitrobenzoate at 412 nm was measured. The assay solution contained PaaI (2 nM with 4-hydroxyphenacyl-CoA, 4 nM with 3-

hydroxyphenacyl-CoA and 8 nM with 4-hydroxybenzoyl-CoA or 4-hydroxybenzyl-CoA), varying amounts of amount of substrate 3,4-dihydroxyphenylacetyl-CoA (0.5 to $5 \times K_m$) and varying amount of inhibitors (0, 64, 128 μ M for 4-hydroxyphenacyl-CoA, 0, 25, 50 μ M for 3-hydroxyphenacyl-CoA, 0, 1350 μ M for 4-hydroxybenzyl-CoA and 0, 283, 565 μ M for 4-hydroxybenzoyl-CoA) in 0.2 mL of 50 mM K^+ Hepes (pH 7.5). The inhibition constant K_i was obtained by fitting the initial rates to equation (2).

$$V = V_{\max} [S] / [K_m (1 + [I]/K_i) + [S]] \quad (2)$$

[I] = concentration of inhibitor, K_i = inhibition constant.

Determination of the pH Optimum for A. evansii and E. coli PaaI Enzymes - Catalyzed Hydrolysis of 3,4-Dihydroxyphenylacetyl-CoA - For *A. evansii* PaaI, the initial-velocity data were measured as a function of the reaction pH by using the following buffers at the indicated pH values: 50 mM potassium acetate (KAc) and 2-(N-morpholino)ethanesulfonate (MES) (pH 4.5-5.5), 50 mM MES and 50 mM N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonate (HEPES) (pH 6.0-8.0), 50 mM HEPES and 50 mM N-Tris(hydroxymethyl)methyl-3-aminopropane sulfonate (TAPS) (pH 8.5), 50 mM TAPS and 50 mM of 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonate (CAPSO) (pH 9.0-9.5), and 50 mM of CAPSO and 50 mM of 3-(cyclohexylamino)-1-propanesulfonate (CAPS) (pH 10.0-10.5) with 0.2 M KCl. The k_{cat} and K_m values were determined from initial velocity data measured using a continuous spectrophotometric assay in which the absorption change at 236 nm was monitored. The hydrolysis of 3,4-dihydroxyphenylacetyl-CoA resulted in the absorption decrease at 236 nm with $\Delta\epsilon = 3.2 \text{ mM}^{-1}$

cm^{-1} for pH 4.5-10.5. For the range pH 4.5-10.0, a 2 mm light path length cuvette was used to contain substrate (up to $5 K_m$) and PaaI (46 nM for pH 4.5, 18 nM for pH 5.0, 5.5, and 10-9 nM for pH 6.0-9.5) in 200 μ L of assay buffer. The reaction at pH 10.5 was carried out using a 1 mm light path length cuvette containing 92 nM PaaI and varying substrate (up to $5 K_m$) in 0.1 mL of assay buffer. For the *E. coli* PaaI, the buffer and assay method were the same as used with *A. evansii* PaaI. Reactions were carried out in 0.2 mL assay buffer containing PaaI (7 nM for pH 6.0, 6.5 and 10.0, 4 nM for pH 7.0, and 3 nM for pH 7.5 to 9.5) and varying concentrations of substrate (0.5 to $5 K_m$).

The $\log k_{\text{cat}}$ and $\log(k_{\text{cat}}/K_m)$ values were fitted using equation (3) and the computer program KaleidaGraph.

$$\log Y = \log(C / (1 + [H]/K_a + K_b/[H])) \quad (3)$$

where Y is k_{cat} or k_{cat}/K_m , [H] is the hydrogen ion concentration, C is the k_{cat} or k_{cat}/K_m value where it does not change with pH, K_a is the acid dissociation constant and K_b is the base dissociation constant.

The stability of the *A. evansii* (0.37 μ M) and *E. coli* (0.65 μ M) PaaIs under the assay conditions used above were determined by incubating the enzyme in the buffer solution for 2 min and then assaying the remaining enzyme activity. The *A. evansii* PaaI (9 nM) activity was measured at 412 nm ($\Delta\epsilon$ of $13.6 \text{ mM}^{-1} \text{ cm}^{-1}$) using a 200 μ L assay solution of 1 mM DTNB, 490 μ M 3,4-dihydroxyphenylacetyl-CoA and 50 mM/0.2 M KCl Hepes (pH 7.5, at 25 °C). The *E. coli* PaaI (17 nM) activity was monitored 236 nm using a 200 μ L assay solution of 400 μ M 3,4-dihydroxyphenylacetyl-CoA, 0.05 M KCl, 1 mM DTT and 10 mM TRIS (pH 8.1, 25 °C).