# The Catalytic Mechanism of the Hotdog-fold Enzyme Superfamily *Arthrobacter sp*. strain SU 4-Hydroxybenzoyl-CoA Thioesterase[+]

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Fourteen pages total which includes Experimental for the preparation of thioesterase mutants, Table SI1 X-ray Data Collection and Least-Squares Refinement Statistics, Figures SI1-3, SI5 and SI7-9 which are graphic representations of the thioesterase mutant active sites and Figures SI4 and SI6 which are stopped-flow UV-*vis* traces of thioesterase mutant catalyzed 4-HB-CoA hydrolysis.

## Experimental

*Preparation of Arthrobacter sp strain SU 4-HBA-CoA thioesterase site-directed mutants*

Site-directed mutagenesis was carried out using the Quikchange site-directed mutagenesis kit (Stratagene), the wild-type-Arthio/pET-23b plasmid DNA as template, and commercial primers. The PCR protocol employed 16 cycles of 30 s denaturation at 95 °C, 1 min annealing at 55 °C, and 12 min elongation at 68 °C. The PCR product was digested with *DpnI* endonuclease, and then used to transform competent *E. coli* JM109 cells (Promega). The plasmids were purified by using the QIAprep Spin Miniprep Kit (Qiagen) and the thioesterase gene was verified by DNA sequencing. The plasmid was used to transform competent *E. coli* BL21(DE3) cells (Novagen), which were grown at 37°C in LB media containing 50 μg/mL carbenicillin. Protein production was induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) *ca*. 10 h post inoculation (cell density had reached $OD_{600}$ 1.0). After a 5 h induction period, the cells were harvested by centrifugation (5000 g for 10 min) and then resuspended in 100 mL of buffer (50 mM $K^+$HEPES, 1 mM DTT, 0.1 mM PMSF, pH 7.5) at 0 °C. The cell suspension was passed through a French press at 1,200 psi twice, before centrifugation at 48,000 g and 4 °C for 60 min. The supernatant was applied to a (5 × 40 cm) DEAE-Sepharose (Amersham Pharmacia Biotech) column and eluted at 4 °C with a 2 L gradient of 0- 0.5 M KCl in 50 mM HEPES/1 mM DTT (pH 7.5). The thioesterase-containing fractions (eluted at ~ 0.2 M KCl) were identified by SDS-PAGE analysis. The desired fractions were combined, and then treated with ammonium sulfate. The 0~60% ammonium sulfate (w/v) protein precipitant was dissolved in 10 mM HEPES /0.2 M KCl/1 mM DTT (pH 7.5), applied to a (2 × 100 cm) Sephacryl S-200 column (Amersham Pharmacia Biotech) and eluted at 4 °C with the same buffer. The thioesterase-containing fractions were pooled and concentrated with a 10 kD Macrosep centricon (Pall Filtron). The mutant proteins were shown to be homogeneous by SDS-PAGE gel analysis. The yields of the pure proteins in mg/g of wet cells

were as follows: E73A (90), E73Q (80), E73D (90), T77A (40) and T77S (8), Q58A (100), Q58E (38), Q58D (28), Q58D/E73D (25), Q58E/E73Q (18), Q58E/E73A (25), H64A (80), H64Q (60), E78A (10), D31A (14), D31N (15), R150A (60), R102A (24), S120A (44) and T121A (21).

Table SI1. X-ray Data Collection and Least-Squares Refinement Statistics.

| mutant | E73A | E73D | E73D | E73Q | E73Q | Q58A | Q58A | H64A | T77S | T77A | Q58E/E73A |
|---|---|---|---|---|---|---|---|---|---|---|---|
| PDB code | 3R32 | 3R34 | 3R35 | 3R36 | 3R37 | 3R3A | 3R3B | 3R3C | 3R3D | 3R3F | 3TEA |
| ligand[a] | 4-HP-CoA | CoASH | 4-HP-CoA | 4-HB | 4-HP-CoA | 4-HB, CoASH | 4-HP-CoA | 4-HP-CoA | 4-HP-CoA | 4-HP-CoA | 4-HP-CoA |
| unit cell dimensions (Å) [b] | 111.3, 62.7 | 112.7, 59.9 | 112.6, 60.8 | 112.9, 59.9 | 112.6, 60.6 | 112.6, 60.0 | 112.5, 59.5 | 112.6, 60.6 | 112.4, 60.4 | 112.4, 60.4 | 112.8, 60.9 |
| resolution | 100-1.80 (1.85-1.80) [c] | 100-1.80 (1.85-1.80) [c] | 100-1.80 (1.85-1.80) [c] | 30.0-1.95 (2.0-1.95) [c] | 30.0-1.80 (1.85-1.80) [c] | 100-1.80 (1.85-1.80) [c] | 100-1.80 (1.85-1.80) [c] | 30.0-1.80 (1.85-1.80) [c] | 30.0-1.75 (1.80-1.75) [c] | 30.0-1.75 (1.80-1.75) [c] | 30.0-1.80 (1.85-1.80) [c] |
| independent reflecions | 36909 (1957) | 37116 (2243) | 37534 (2255) | 29543 (2032) | 37313 (2519) | 36932 (2340) | 36193 (2347) | 36908 (2356) | 40925 (2262) | 40350 (2037) | 37991 (2669) |
| completeness (%) | 90.0 (65.0) | 97.6 (78.6) | 95.4 (78.4) | 96.2 (90.5) | 95.2 (83.7) | 95.2 (80.9) | 94.2 (83.3) | 94.2 (82.0) | 96.8 (73.5) | 95.5 (66.4) | 96.2 (92.6) |
| redundancy | 3.9 (2.2) | 4.2 (2.7) | 3.4 (1.9) | 3.7 (2.1) | 4.1 (2.4) | 4.8 (2.1) | 4.6 (2.4) | 3.9 (2.2) | 3.8 (1.7) | 3.7 (1.6) | 4.1 (2.5) |
| avg I/avg σ(I) | 29.5 (3.4) | 32.1 (2.8) | 28.1 (1.9) | 24.1 (1.8) | 20.1 (1.7) | 35.1 (2.2) | 34.9 (3.1) | 27.9 (2.0) | 25.5 (2.1) | 24.1 (1.7) | 30.1 (2.1) |
| $R_{sym}$ (%)[d] | 6.5 (28.2) | 7.1 (26.4) | 7.3 (29.5) | 6.7 (28.4) | 7.2 (29.9) | 5.9 (26.1) | 5.8 (25.1) | 6.4 (29.7) | 6.6 (28.7) | 6.9 (30.1) | 7.8 (33.4) |
| R-factor (overall) % [e] /no. reflections | 18.3 / 36909 | 19.1 / 37116 | 20.0 / 37534 | 19.6 / 29543 | 20.2 / 37313 | 18.6 / 36932 | 18.5 / 36193 | 19.6 / 36908 | 19.5 / 40925 | 19.6 / 40350 | 20.7 / 37991 |
| R-factor (working) %/ no. reflections | 18.1 / 34950 | 18.9 / 35156 | 19.8 / 35560 | 19.4 / 27983 | 20.1 / 35348 | 18.4 / 34972 | 18.3 / 34294 | 19.4 / 34952 | 19.3 / 39748 | 19.4 / 38199 | 20.5 / 35983 |
| R-factor (free) %/ no. reflections | 21.6 / 1959 | 21.7 / 1960 | 22.2 / 1974 | 23.5 / 1560 | 22.8 / 1965 | 21.2 / 1960 | 22.1 / 1899 | 23.2 / 1956 | 23.1 / 2177 | 23.3 / 2151 | 24.4 / 2008 |
| number protein atoms | 2154[f] | 2158[h] | 2155[j] | 214[l] | 2156[n] | 2168[p] | 2164[r] | 2140[t] | 2144[v] | 2140[x] | 2135[z] |
| number hetertoatoms | 265[g] | 324[i] | 308[k] | 325[m] | 347[o] | 385[q] | 374[s] | 338[u] | 378[w] | 386[y] | 3299[*] |
| avg. B protein ($Å^2$) | 31.3 | 36.1 | 37.5 | 29.6 | 29.0 | 25.8 | 26.3 | 30.5 | 22.5 | 22.1 | 33.0 |
| avg. B ligands ($Å^2$) | 38.5 | 46.3 | 44.4 | 33.9 | 34.7 | 41.6 | 30.5 | 35.9 | 29.7 | 31.8 | 39.3 |
| avg. B solvent ($Å^2$) | 53.0 | 51.6 | 56.1 | 48.2 | 45.8 | 43.2 | 43.1 | 46.9 | 38.6 | 39.4 | 46.0 |
| RMS bond lengths (Å) | 0.015 | 0.014 | 0.014 | 0.012 | 0.012 | 0.012 | 0.014 | 0.012 | 0.012 | 0.012 | 0.012 |
| RMS bond angles (°) | 2.14 | 2.12 | 1.97 | 2.24 | 2.39 | 2.18 | 2.03 | 2.18 | 2.00 | 2.06 | 2.16 |
| RMS general planes (Å) | 0.011 | 0.011 | 0.010 | 0.010 | 0.012 | 0.011 | 0.010 | 0.011 | 0.009 | 0.011 | 0.012 |

[a]4-HP-CoA = 4-hydroxyphenacyl-coenzyme A, 4-HB = 4-hydrozybenzoate, CoASH = coenzyme A
[b]all complexes crystallized in the space group P3(2)21, *a* and *c* lattice lengths are given
[c]Statistics for highest resolution bin.
[d]$R_{sym} = (\sum| I - \overline{I} |/ \sum I) \times 100$
[e]*R*-factor = $(\Sigma|F_o - F_c| / \Sigma|F_o|)$
[f]These include multiple conformations for Asp39 and Arg110 in subunit A and Asn14 and Asp39 in subunit B
[g]Heteroatoms include 2 4-hydroxyphenacyl coenzyme A molecules and 149 waters
[h]These include multiple conformations for Asp17 and Glu27 in subunit A and Glu44 and Ser120 in subunit B
[i]Heteroatoms include 2 coenzyme A molecules, one 1,2-ethanediol, and 224 waters
[j]These include multiple conformations for Glu27 and Ser128 in subunit A and Asp39 and Ser120 in subunit B
[k]Heteroatoms include 2 4-hydroxyphenacyl coenzyme A molecules and 192 waters
[l]These include multiple conformations for Asp17 in subunit A
[m]Heteroatoms include 2 4-hydroxybenzoate molecules and 305 waters
[n]These include multiple conformations for Val114, Phe124, and Ser128 in subunit A and Asp39, Ser120, and Ser128 in subunit B
[o]Heteroatoms include 2 4-hydroxyphenacyl coenzyme A molecules and 231 waters
[p]These include multiple conformations for Asp17 and Glu27 in subunit A and Asp17, Glu27, Asp39, Leu62, Gln94, and Ser120 in subunit B
[q]Heteroatoms include 2 coenzyme A molecules, 2 4-hydroxybenzoate molecules, and 269 waters
[r]These include multiple conformations for Asp17, Leu30, Arg110, Ser140, and Ser143 in subunit A and Asp17, Asp39, and Ser140 in subunit B
[s]Heteroatoms include 2 4-hydroxyphenacyl coenzyme A molecules and 258 waters
[t]These include multiple conformations for Asp17 and Ser128 in subunit A and Ser20, Asp39, and Ser128 in subunit B
[u]Heteroatoms include 2 4-hydroxyphenacyl coenzyme A molecules and 222 waters
[v]These include multiple conformations for Asn14, Asp39, Ser128, and Ser140 in subunit B
[w]Heteroatoms include 2 4-hydroxyphenacyl coenzyme A molecules and 262 waters
[x]These include multiple conformations for Ser140 in subunit A and Asp39, Ser128, and Ser140 in subunit B
[y]Heteroatoms include 2 4-hydroxyphenacyl coenzyme A molecules and 270 waters
[z]These include multiple conformations for Phe124 in subunit A
[*]Heteroatoms include 2 4-hydroxyphenacyl coenzyme A molecules and 183 waters

**Figure SI1.** Double reciprocal plots of the initial velocity of thioesterase-catalyzed hydrolysis of 4-HB-CoA measured at varying substrate concentration and changing fixed inhibitor concentration. **A.** Wild-type thioesterase inhibition by 4-HB. **B.** Wild-type thioesterase inhibition by CoA.

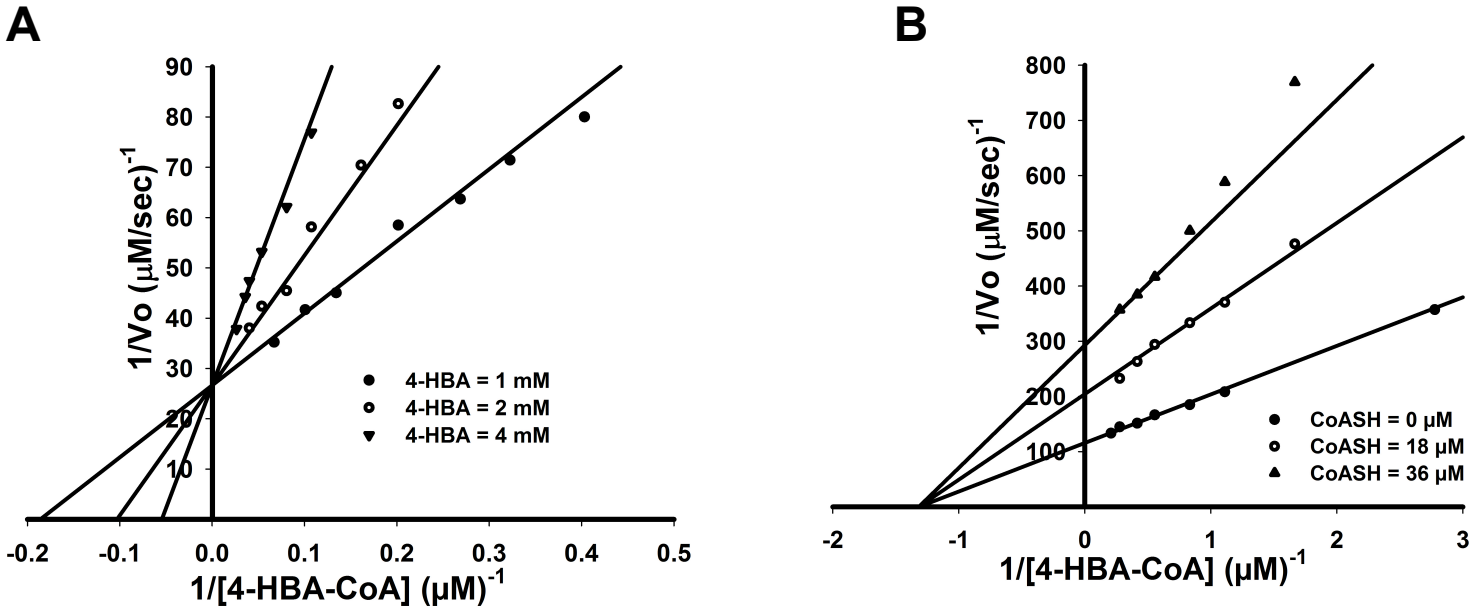

**Figure SI2**. Superposition of the X-ray structure of the wild-type thioesterase(4-HP-CoA) complex with that of the E73A thioesterase(4-HP-CoA) complex with zoom-in on the catalytic site. The nitrogen atoms are colored blue, the oxygen atoms red, the sulfur atoms yellow and the carbon atoms are colored gray for the wild-type thioesterase and cyan for the E73A thioesterase. The dashed lines represent hydrogen bonds.

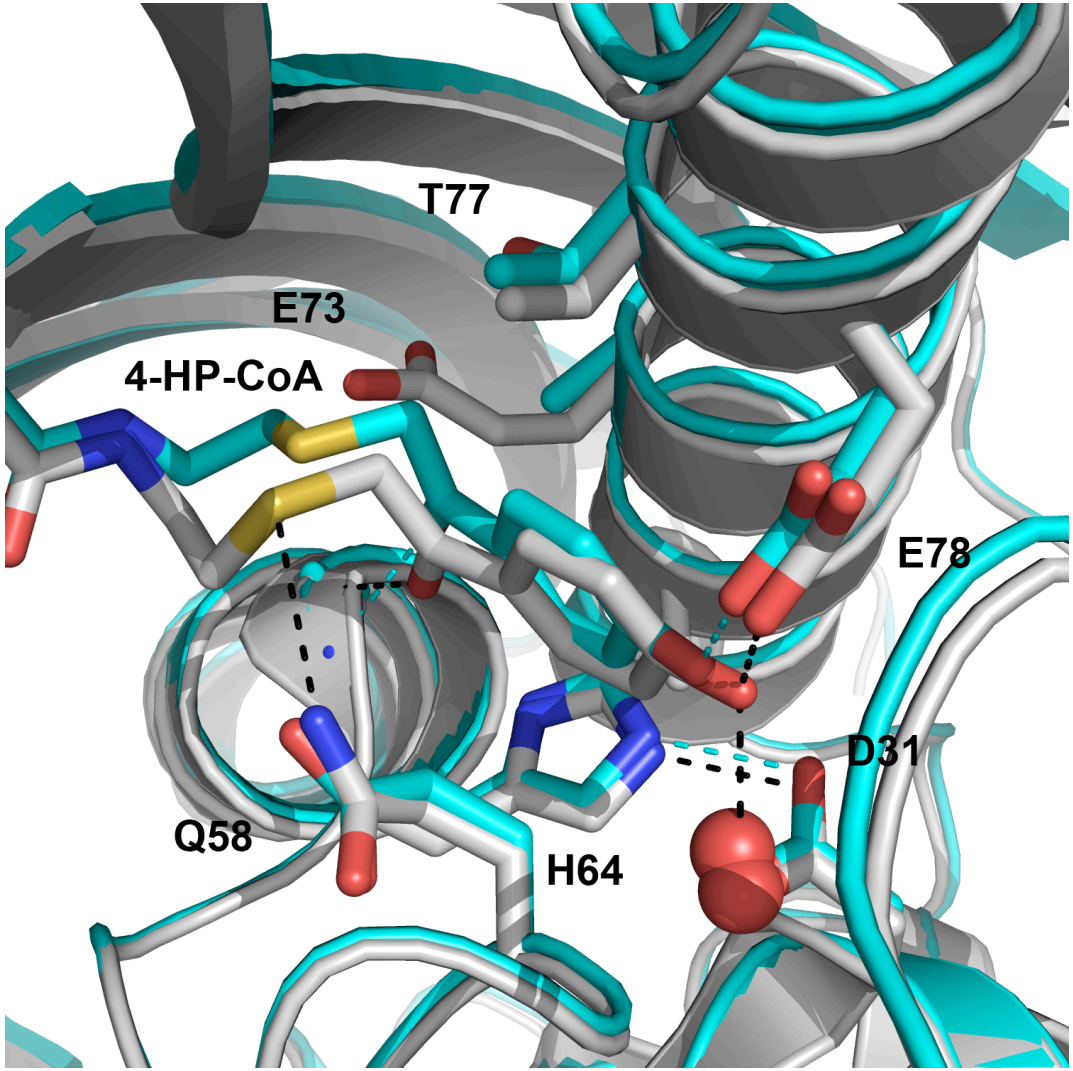

**Figure SI3.** Superposition of the X-ray structure of the wild-type thioesterase(4-HP-CoA) complex with that of the E73D thioesterase(4-HP-CoA) complex with zoom-in on the catalytic site. The nitrogen atoms are colored blue, the oxygen atoms red, the sulfur atoms yellow and the carbon atoms are colored gray for the wild-type thioesterase and cyan for the E73D thioesterase. Only the backbone of the wild-type thioesterase is shown. The dashed lines represent hydrogen bonds.

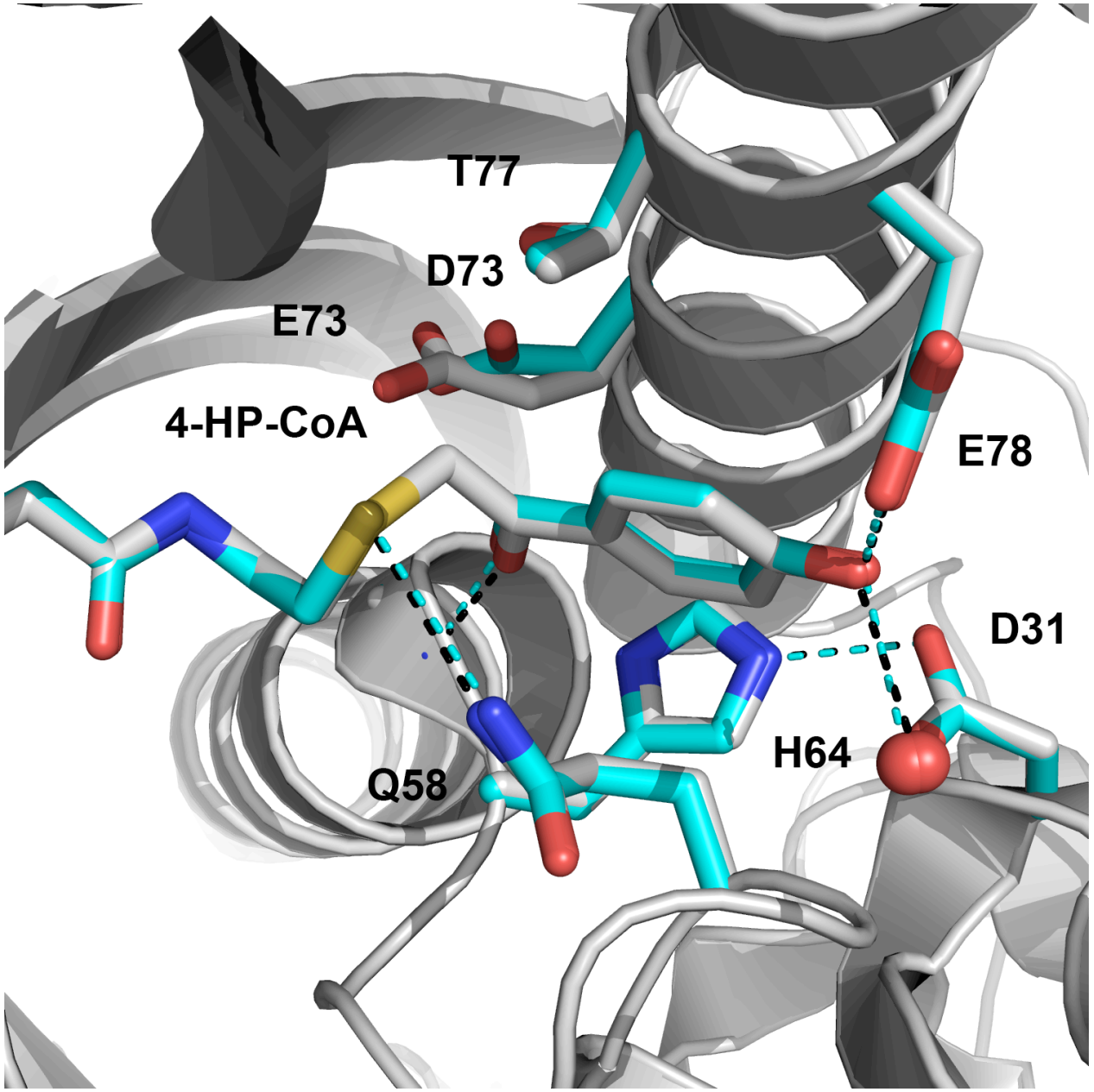

**Figure SI4.** Superposition of the X-ray structure of the wild-type thioesterase(4-HP-CoA) complex with that of the E73Q thioesterase(4-HP-CoA) complex with zoom-in on the catalytic site. The nitrogen atoms are colored blue, the oxygen atoms red, the sulfur atoms yellow and the carbon atoms are colored gray for the wild-type thioesterase and cyan for the E73Q thioesterase. The dashed lines represent hydrogen bonds.

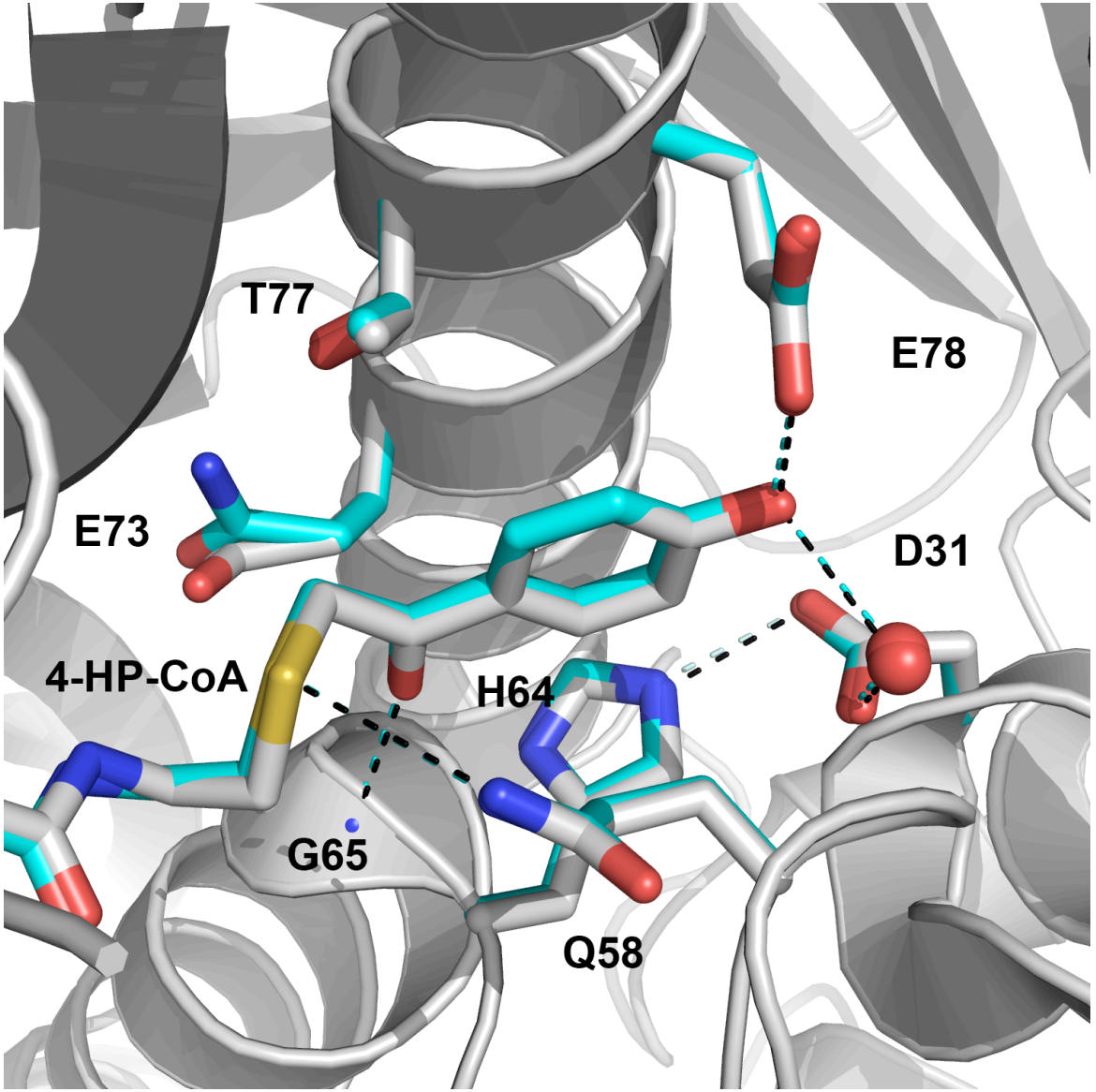

**Figure SI5**. **A.** Stopped-flow UV-*vis* trace absorbance of (30 μM) T77S thioesterase-catalyzed hydrolysis of 4-HB-CoA (100 μM) carried-out under multiple turnover conditions and monitored at 300 nm. The trace was fitted with a double exponential equation. **B.** Stopped-flow UV-*vis* absorbance trace of (20 μM) T77V catalyzed thioesterase hydrolysis of 4-HB-CoA (80 μM) carried-out under multiple turnover conditions and monitored at 300 nm. The trace was fitted with a single exponential equation **C.** Stopped-flow UV-*vis* absorbance trace of (20 μM) T77D catalyzed thioesterase hydrolysis of 4-HB-CoA (80 μM) carried-out under multiple turnover conditions and monitored at 300 nm. The trace was fitted with a single exponential equation. **D.** Stopped-flow UV-*vis* absorbance trace of (40 μM) T77A thioesterase-catalyzed hydrolysis of 4-HB-CoA (40 μM) carried-out under single turnover conditions and monitored at 300 nm. The trace was fitted with a single exponential equation. **E.** Time course for the single turnover reaction of 5 μM [$^{14}$C]4-HB-CoA and 50 μM T77A thioesterase monitored using rapid quench/HPLC techniques. The data were fitted to a single exponential equation. **F.** Stopped-flow UV-*vis* absorbance trace of (40 μM) T77S thioesterase-catalyzed hydrolysis of 4-HB-CoA (20 μM) carried-out under single turnover conditions and monitored at 300 nm. The trace was fitted with a single exponential equation. **G.** Stopped-flow UV-*vis* absorbance trace of (20 μM) T77A/E73D thioesterase- catalyzed hydrolysis of 4-HB-CoA (80 μM) carried-out under multiple turnover conditions and monitored at 300 nm. The trace was fitted with a single exponential equation. **G.** Stopped-flow UV-*vis* absorbance trace of (20 μM) E73D/T77S thioesterase-catalyzed hydrolysis of 4-HB-CoA (80 μM) carried-out under single turnover conditions and monitored at 300 nm. The trace was fitted with a single exponential equation.

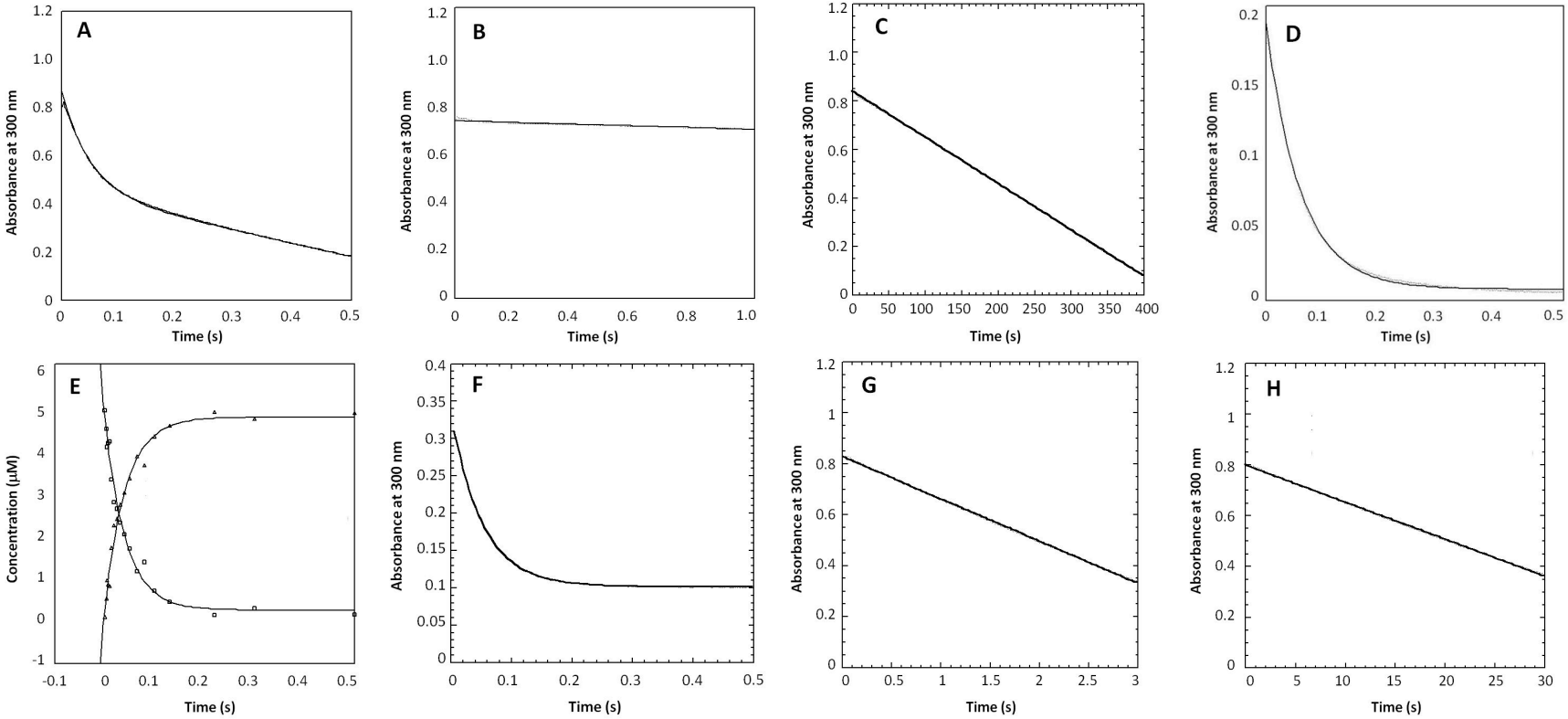

**Figure SI6.** The superposition of the structures of the wild-type thioesterase(4-HP-CoA) and T77S thioesterase(4-HP-CoA) complexes with zoom-in on the catalytic site. The nitrogen atoms are colored blue, the oxygen atoms red, the sulfur atoms yellow and the carbon atoms are colored gray for the wild-type thioesterase and cyan for the T77S thioesterase.

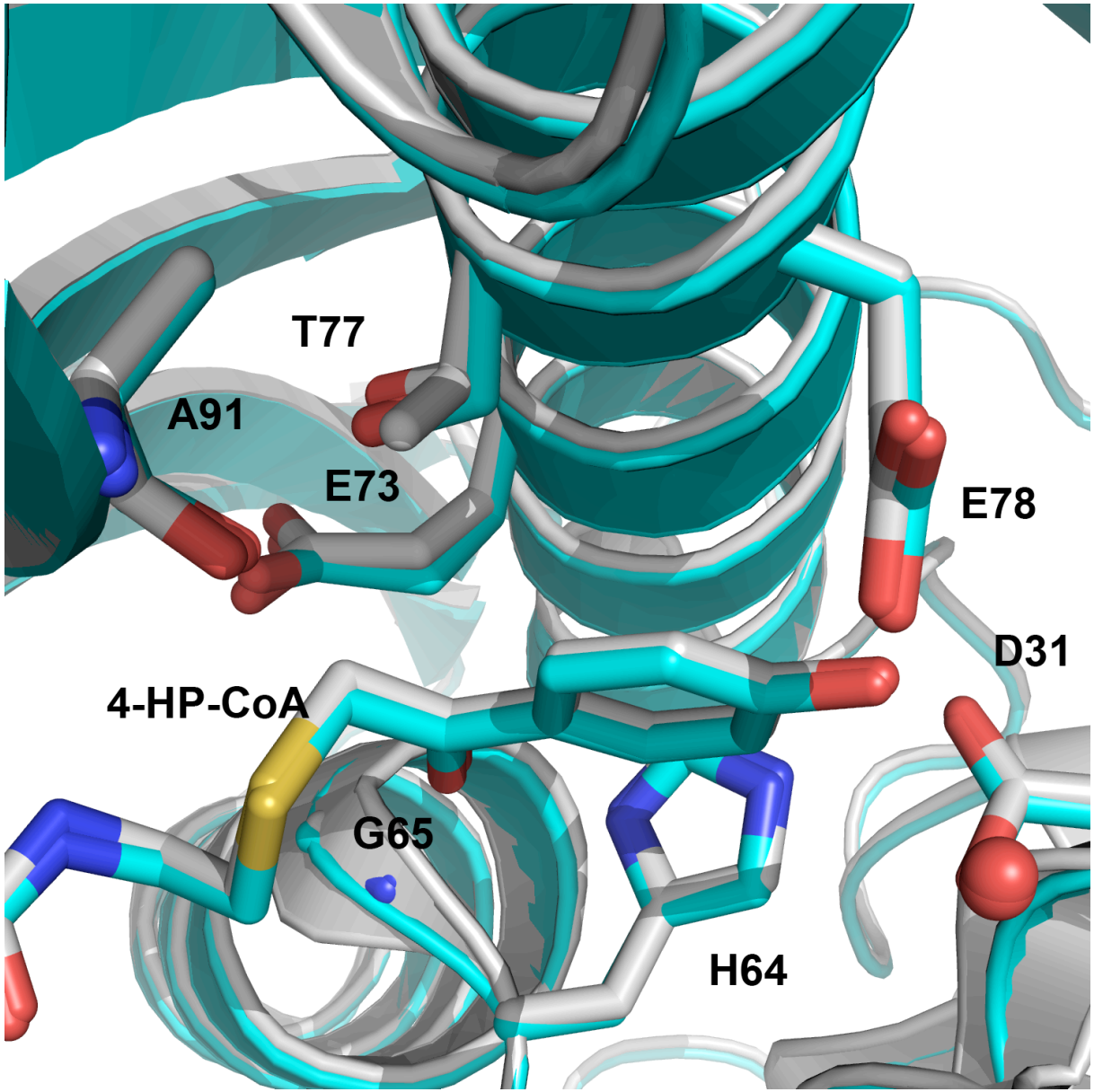

**Figure SI7. A.** Stopped-flow UV-*vis* absorbance trace of (20 μM) mutant thioesterase-catalyzed hydrolysis of 4-HB-CoA (80 μM) carried-out under multiple turnover conditions and monitored at 300 nm. Each trace was fitted with a single exponential equation. **A.** Q58A thioesterase. **B.** D31A thioesterase. **C**. D31N thioesterase. **D**. H64A thioesterase. **E**. H64Q thioesterase. **F**. E78A thioesterase.

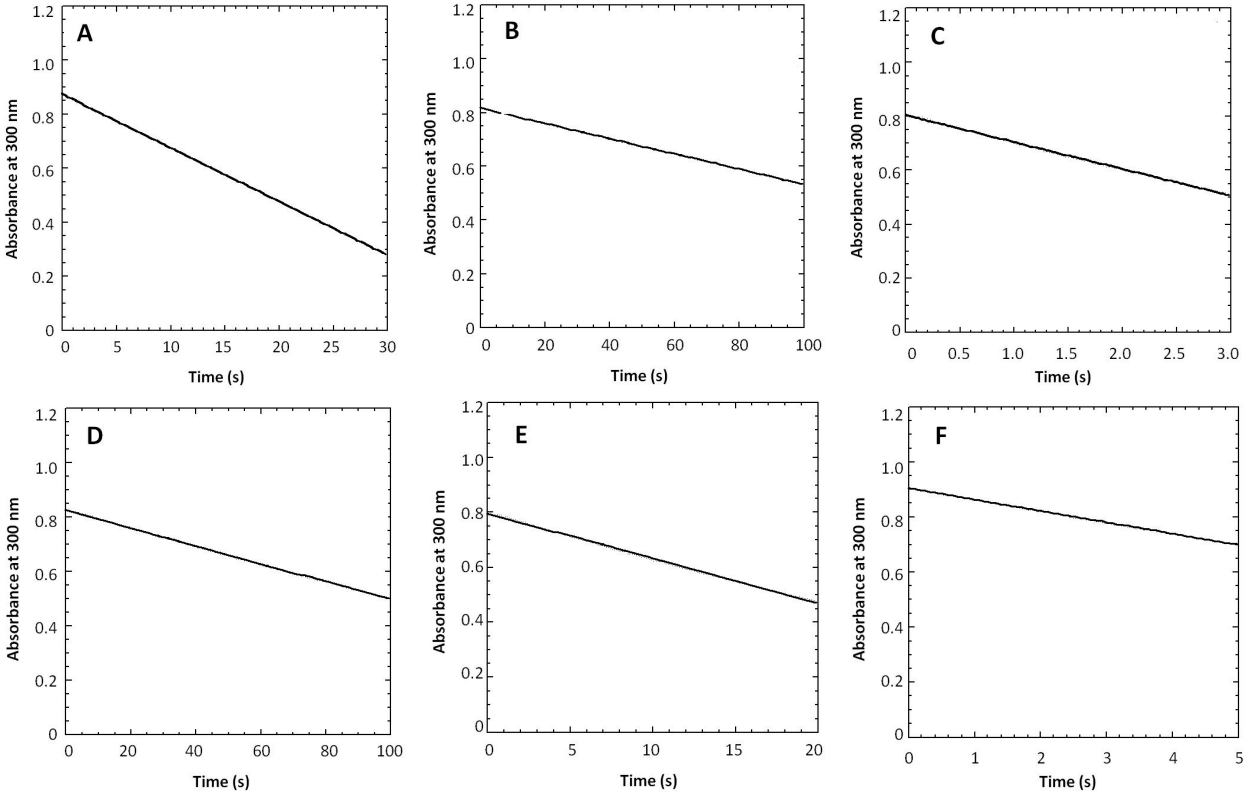

**Figure SI8. A.** Superposition of the X-ray structure of Q58A thioesterase (4-HB)(CoA) complex with that of the wild-type(4-HB)(CoA) complex. **B.** Superposition of the X-ray structure of Q58A thioesterase (4-HP-CoA) complex with that of the wild-type(4-HP-CoA) complex. Zoom-in on the respective catalytic sites. The nitrogen atoms are colored blue, the oxygen atoms red, the sulfur atoms yellow and the carbon atoms are colored gray for the wild-type thioesterase and cyan for the mutant thioesterase.

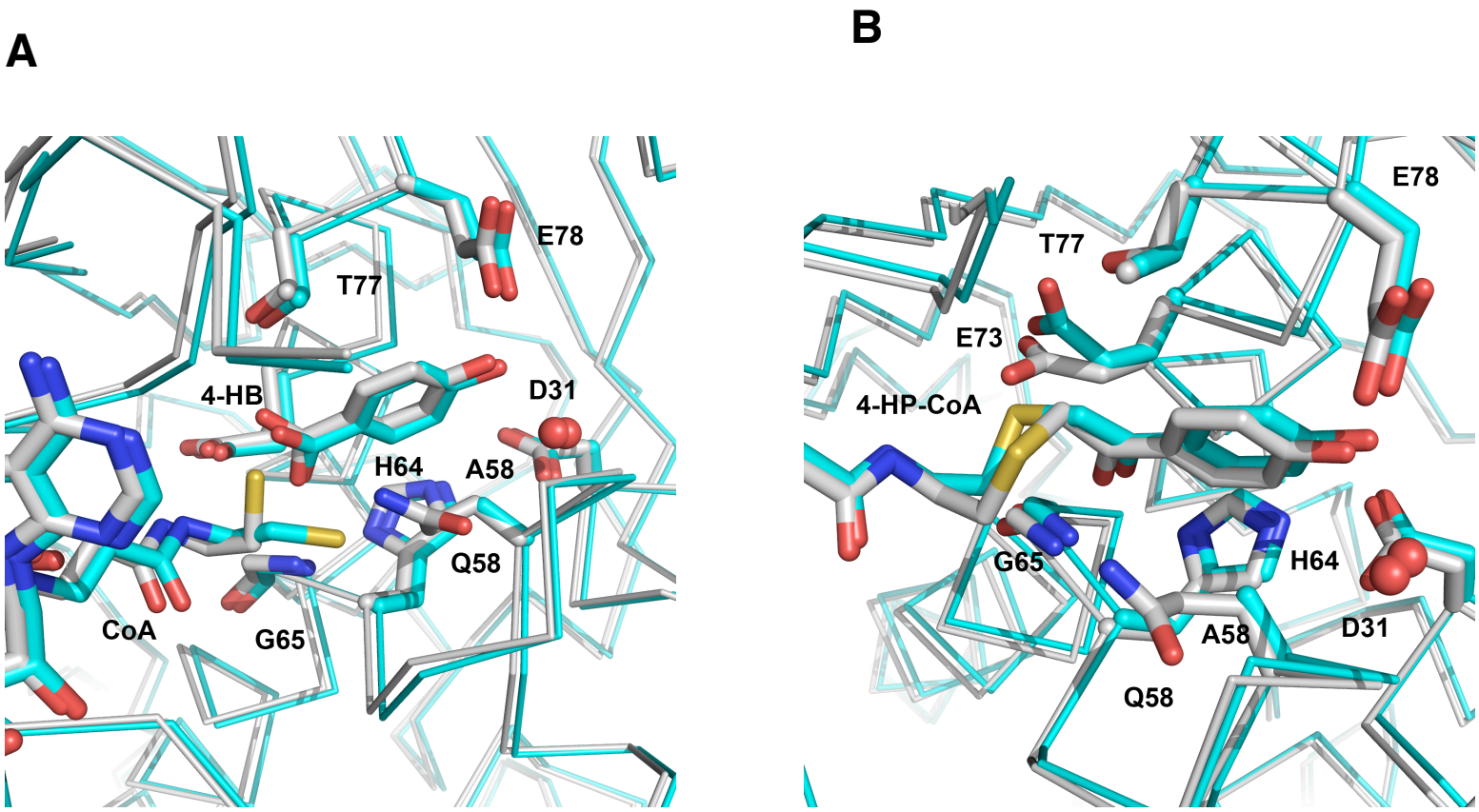

**Figure SI9.** Superposition of the wild-type thioesterase complexes of 4-HP-CoA and 4-hydroxybenzyl-CoA complexes. The ligand (stick) and main chain atoms are colored according to B factor. Color ramping is from blue (low B factor) to red (high B factor). One active site of the dimer is shown.