**Supplementary figures:**

**Supplementary figure 1 (related to Figure 2). Crystal structure of JamJ ER.**

**A. Overall structure.** In the ribbon diagram, the putative substrate-binding domain is blue and the NADPH-binding domain is yellow, and the connector helices (α3 and α9-α10) are pink. Cofactor NADPH is shown with atomic colors and the secondary structures are labeled.

**B. NADPH environment.** The stereo view shows electron density for NADPH (2.25-Å $F_o$-$F_c$ contoured at 3σ shown as a green mesh). Hydrogen bonds between the cofactor NADPH and JamJ ER are illustrated as black dashed lines. The acetate ion (ACT) in the active site is shown.

**Supplementary figure 2 (related to Figure 3). Conformational change upon NADPH binding.**

A. Superposition of $NADP^+$-bound CurF ER (cyan), NADPH-bound JamJ ER (blue), apo-CurK ER (yellow) and apo-JamJ ER (green). The cofactor is shown in stick form. Major differences in the β12-α8 and α11-β15 loops are highlighted by arrows. B. Stereo view of the adenine nucleotide region showing how the α11-β15 loop occludes part of the cofactor site in apo-CurK ER. His326 protrudes from the cofactor site in apo JamJ ER, but is not involved in crystal lattice contacts. In the cofactor complexes, JamJ His326 (CurF His328) is H-bonded to the adenosine-5'-phosphate (Fig. 2B, Fig. S1). C. Stereo view of lattice contacts in CurK ER crystals. NADPH is modeled into the yellow molecule to illustrate how the magenta and green neighbors in the crystal lattice are close to the adenine nucleotide end of the cofactor site.

**Supplementary figure 3 (related to Figure 4 and experimental procedures). Wild-type and mutant activity of JamJ ER and CurK ER.**

A. ER and NADPH mediated conversion of crotonyl CoA to butyryl CoA. The HPLC trace on the right panel shows the authentic standards, blue and the red trace correspond to crotonyl CoA (elution 8 min) and butyryl CoA (elution 8.6 min), respectively.

B and C. JamJ ER and CurK ER activity. Activity is represented as the percentage of crotonyl CoA converted to butyryl CoA. The right panel shows the HPLC traces for the wild-type and mutant proteins. Each reaction was performed in duplicate.

**Supplementary figure 4 (related to Figure 2). Protein-ligand interaction in CurF ER and JamJ ER.**

A. NADPH binding site in CurF ER. In the schematic at left, hydrogen bonds are shown as dashed green lines and van der Waals interactions by red shading. The panel on the right shows the cofactor $NADP^+$ in the cleft (white carbon atoms) and the "cyclopropanase loop" (red) with a cyan ER surface (excepting the cyclopropanses loop).

B. NADPH binding site in JamJ ER. The right panel shows the ordered helix α2 and the cofactor NADPH in the cleft.

**Supplementary figure 5 (related to experimental procedures). Characterization of the adduct of NADPH with crotonyl-CoA in CurK ER D272N.**

A) HPLC analysis of the of reaction mix. Six traces shown are; (from bottom) CurK ER wild type, CurK ER D272N, reaction mix without enzyme (negative control), NADPH standard, crotonyl-CoA standard and butyryl-CoA standard. Reactions were performed and subjected to HPLC analysis on a C18 column as described in Material and Methods. The adduct was isolated as a single fraction eluting at 6.4 min.

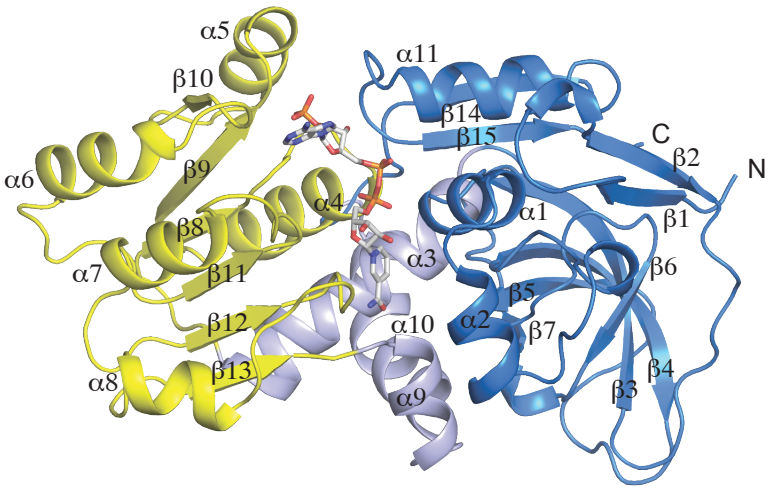
B) LC/MS analysis of the adduct. Ion peaks at m/z 791.95 and 813.95 correspond to $[M+2H]^{2+}$ and $[M+2Na]^{2+}$ ion peak, respectively. The mass is consistent with an adduct of NADPH with crotonyl-CoA (Rosenthal et al., 2014).

C) UV absorbance of the adduct. The absorbance spectrum for the peak eluted at 6.4 min (A) was obtained with the photodiode array detector on the Agilent 1200 HPLC system. No C2-ene adduct with an absorbance maximum at 370 nm was detected.

## References:

Rosenthal, R.G., Ebert, M.O., Kiefer, P., Peter, D.M., Vorholt, J.A., and Erb, T.J. (2014). Direct evidence for a covalent ene adduct intermediate in NAD(P)H-dependent enzymes. Nat Chem Biol *10*, 50-55.

A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C
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α10
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β7
β4
α8
β13
α9
β3

B

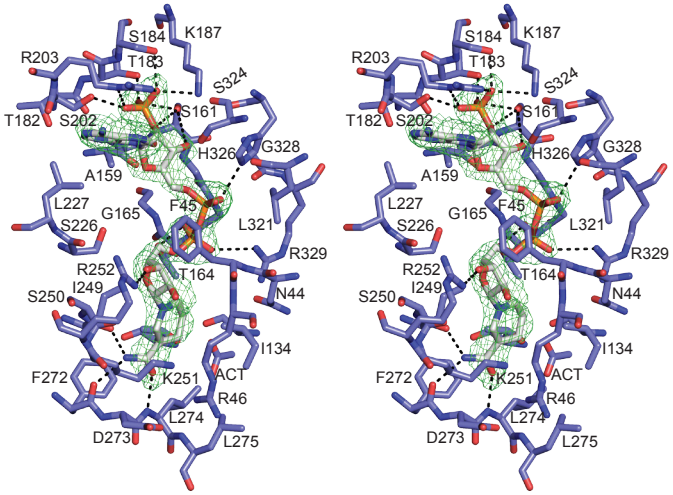
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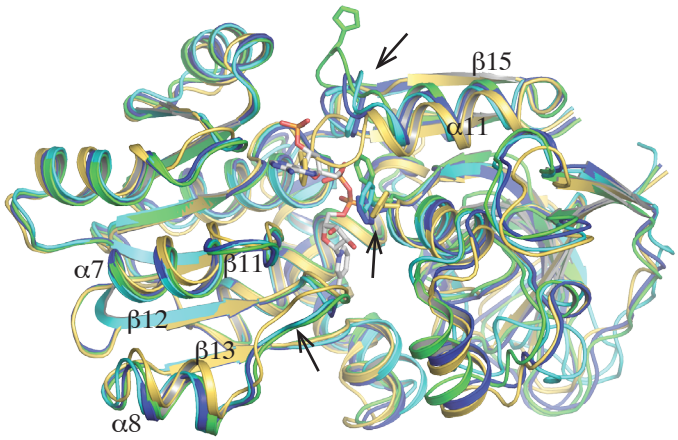
Figure S1

A

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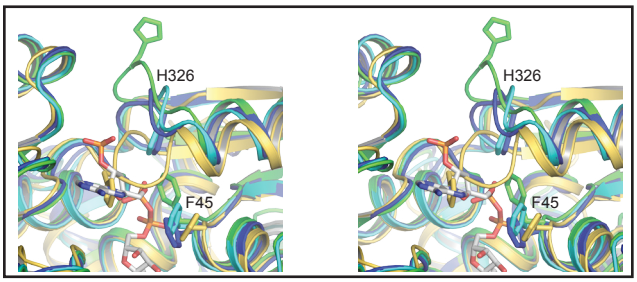


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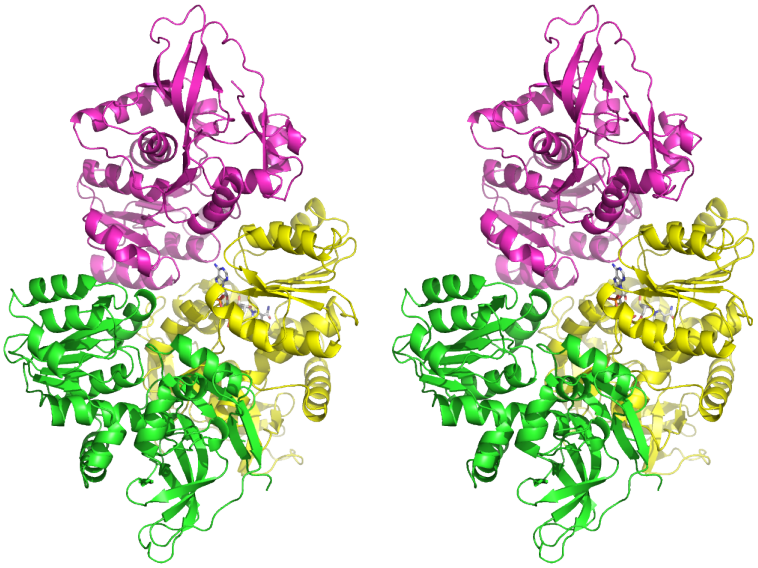
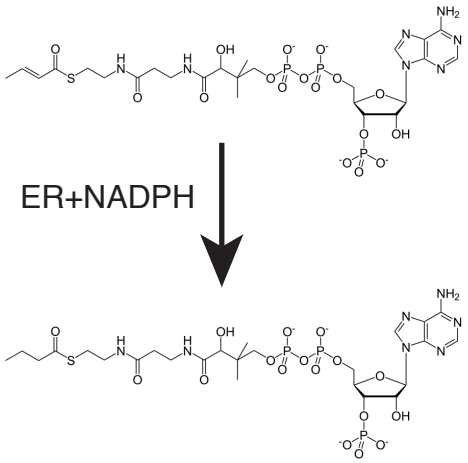
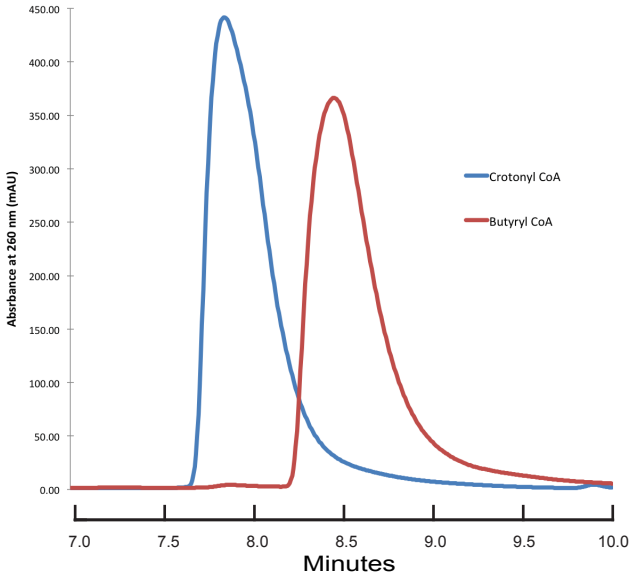


Figure S2

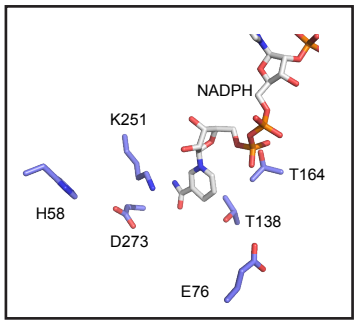
A

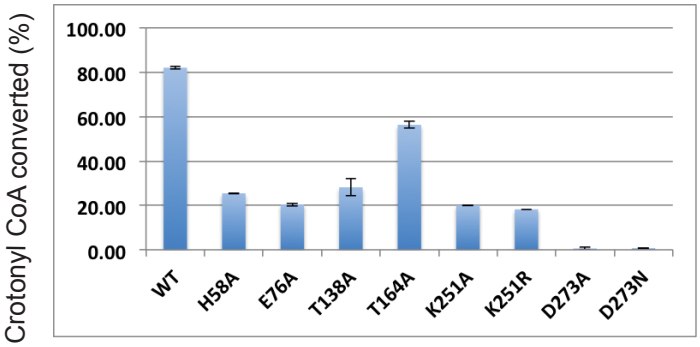


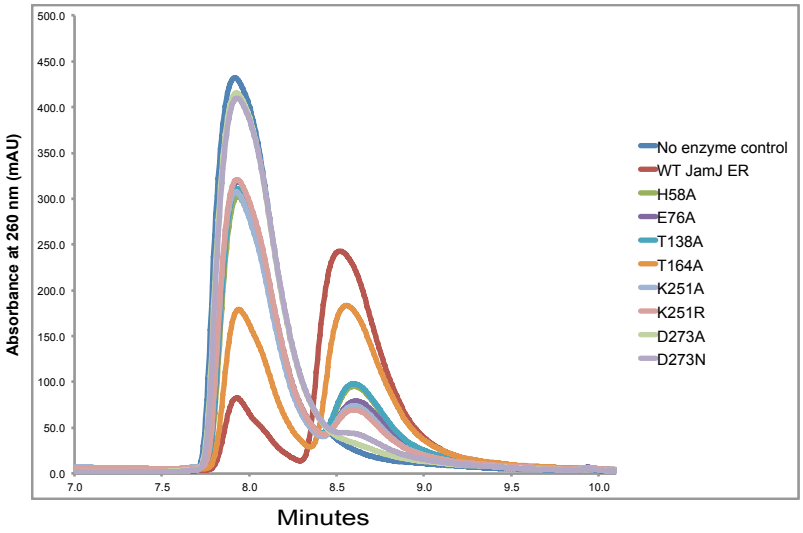




B

**JamJ ER**

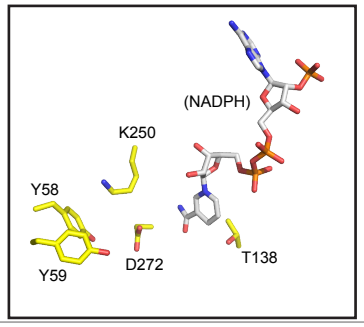


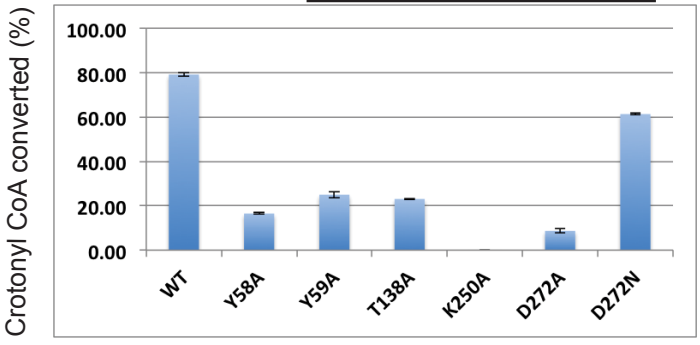




C

**CurK ER**



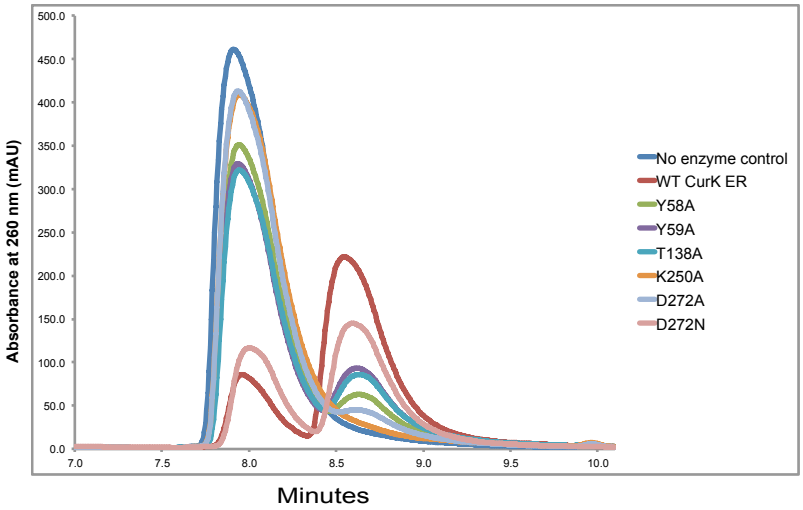
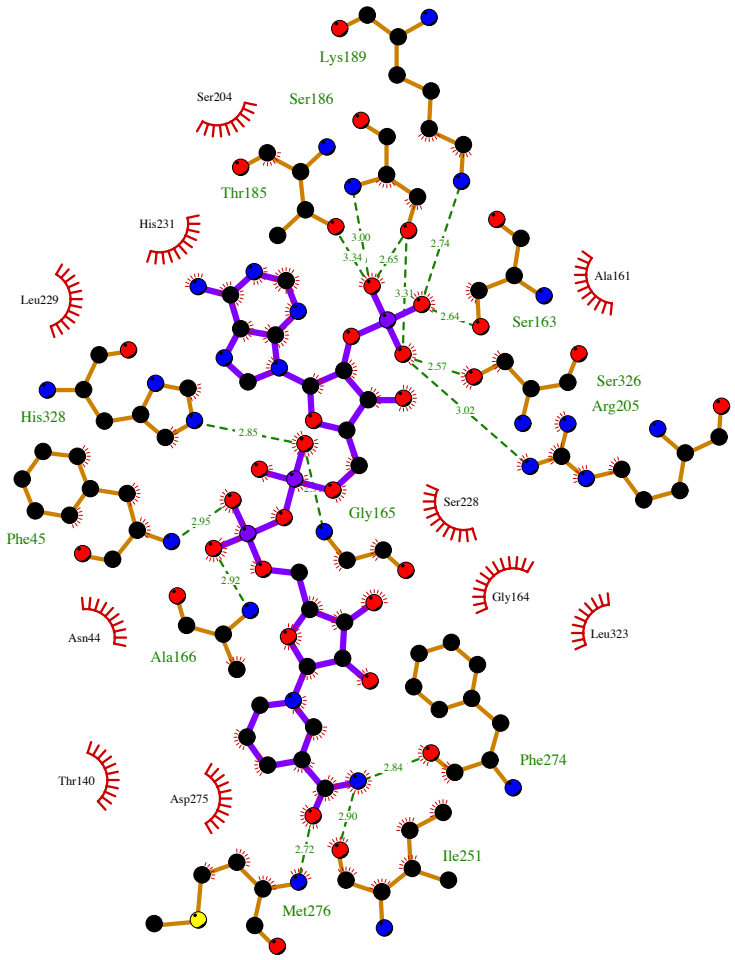
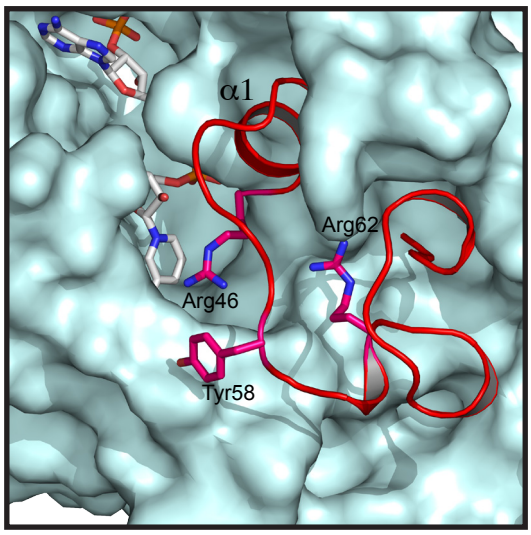




Figure S3

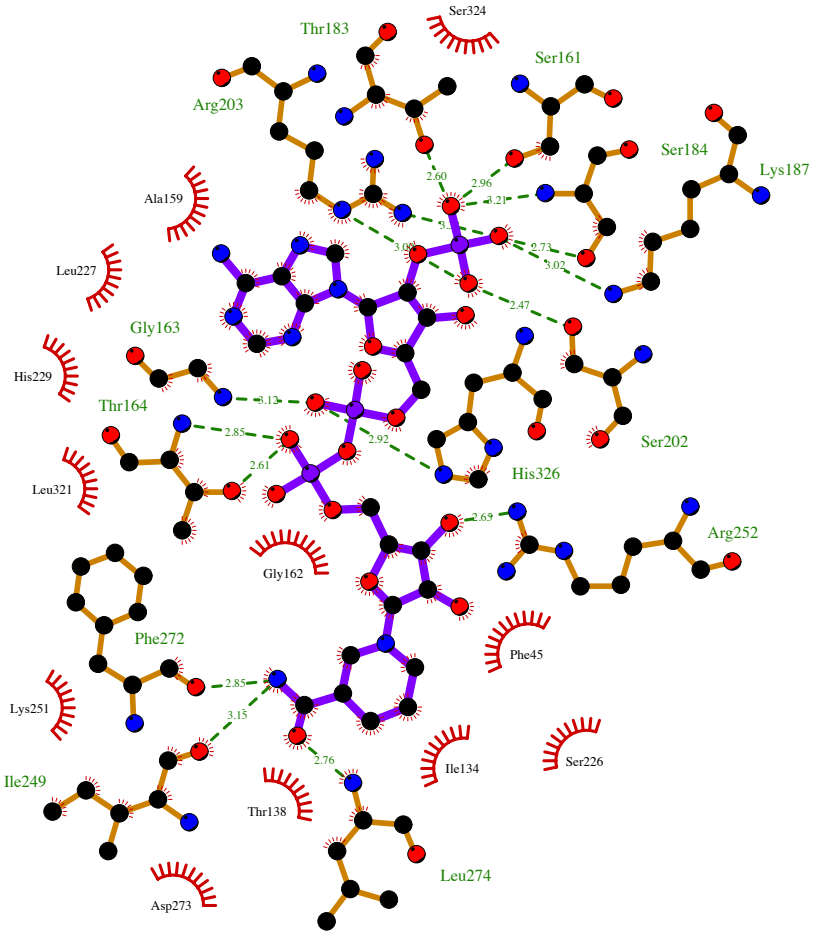
A

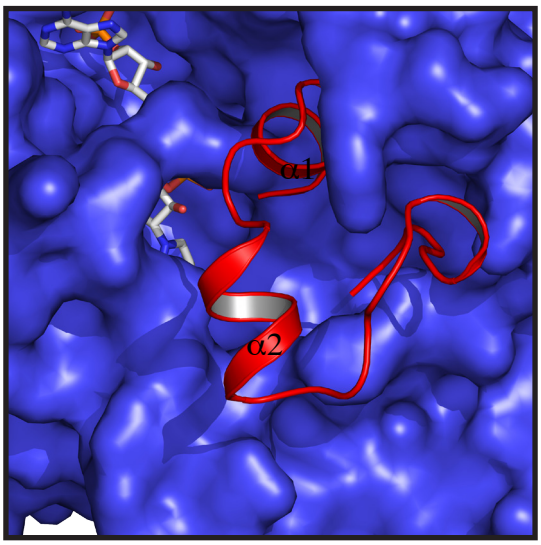



CurF ER

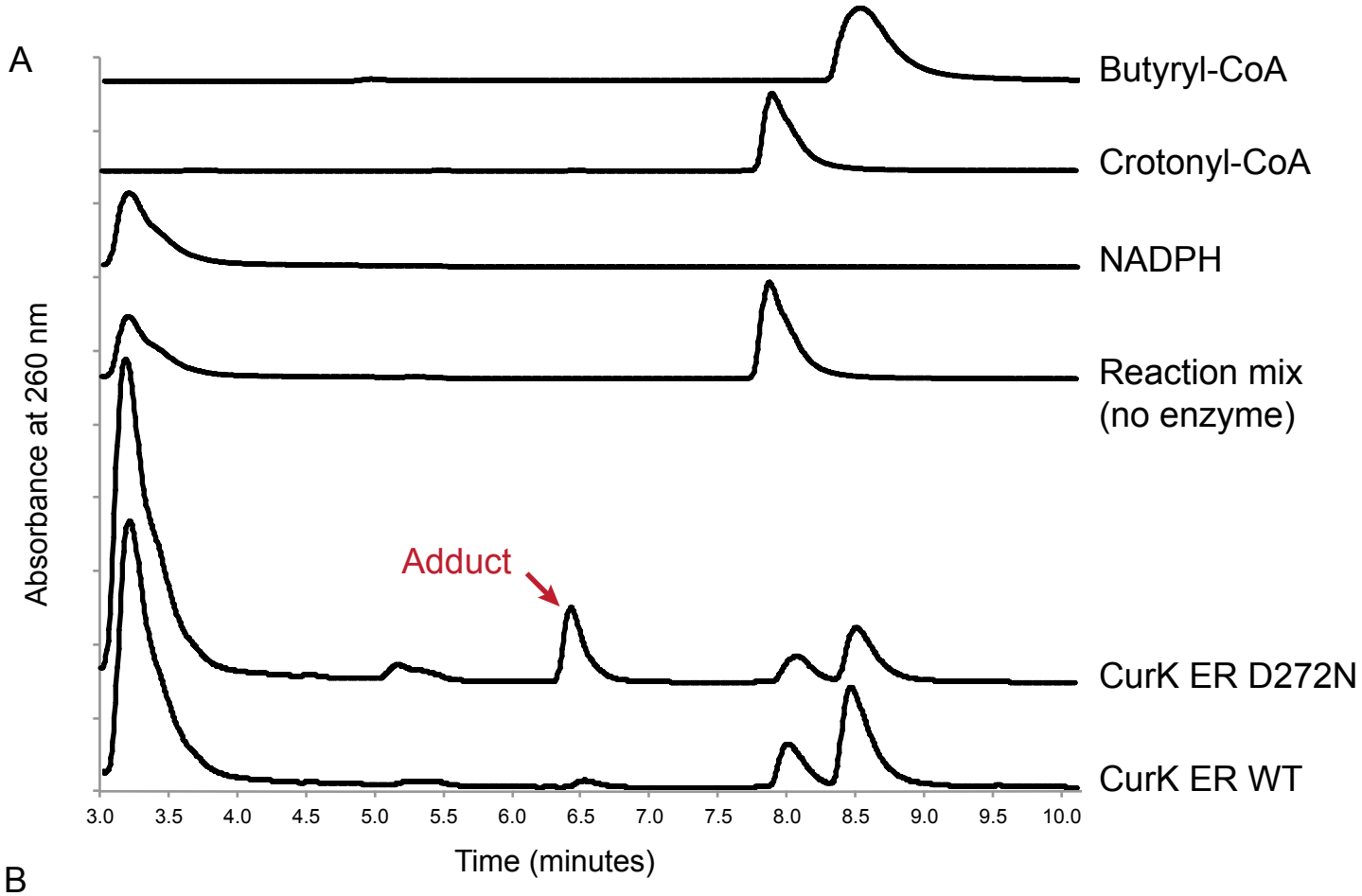
B

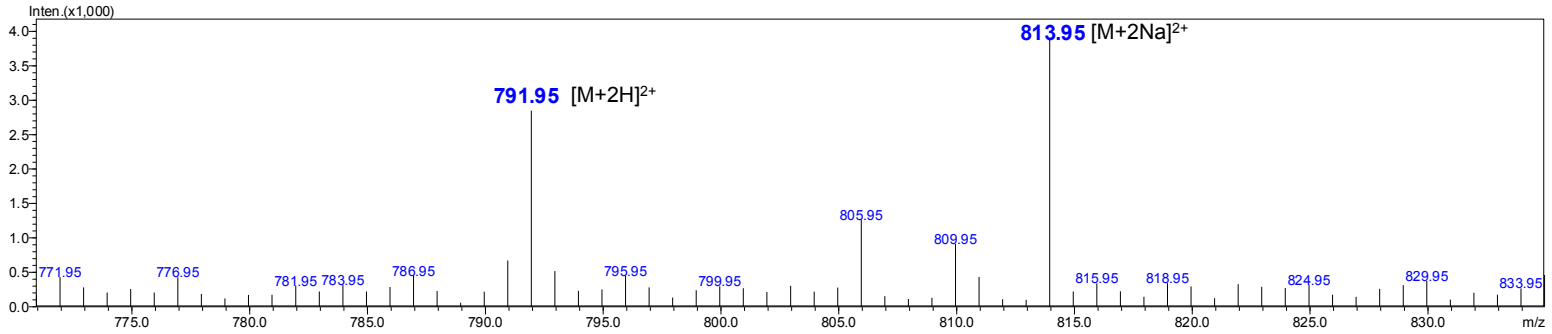


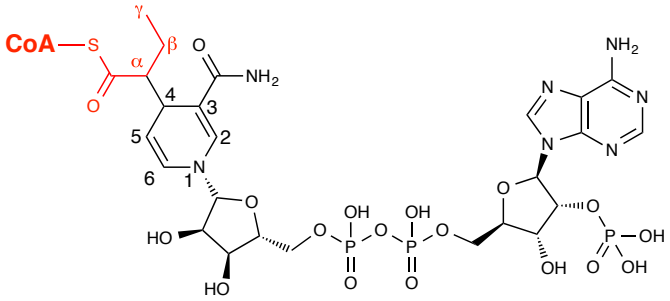




JamJ ER

Figure S4




C4 adduct (calculated mass 1578.21 Da)

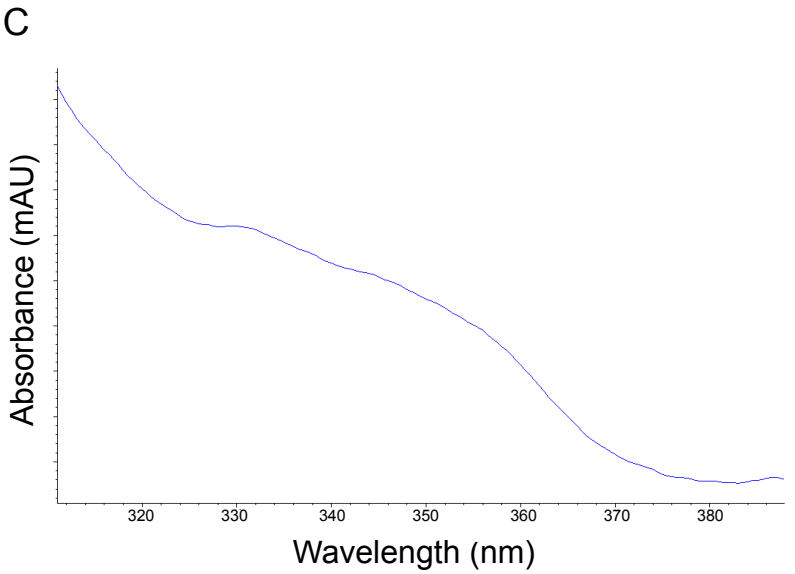



Figure S5