Type-2 Isopentenyl Diphosphate Isomerase. Mechanistic Studies with Cyclopropyl and Epoxy Analogs

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Supporting Information

CONTENTS

I. Materials. The synthesis of oIPP and cIPP will be reported elsewhere. IPP was produced from isopentenol using published procedures.[1] *Thermus thermophilus* IDI-2 was expressed in *E. coli* and purified as described.[2]

II. Enzymatic assays were performed using the acid lability procedure of Satterwhite,[3] with modifications as described previously.[4]

III. Inactivation kinetics. Time-dependent inhibition of IDI-2 by cIPP and oIPP was measured as follows. Assay solutions, with a final volume of 50 μL and final concentrations of 40 nM IDI-2, 40 μM FMN, 2 mM NADPH, 2 mM $MgCl_2$, 200 mM Tris (pH 7.0) after addition of the analogs, were equilibrated for 10 min at 37 °C. The analogs were added and the samples were incubated for varying periods of time. A 5 μL portion of the assay solutions were then diluted ten-fold into assay buffer containing 50 μM IPP (10 μCi/μmol) and assayed by the acid lability assay according to the protocol described above. Percent residual activity was determined by comparing enzyme pre-incubated with and without analog. Plots of natural log (ln) percent residual activity versus time were used to determine K_I and k_{inact} values as described by Muehlbacher and Poulter.[5]

IV. Large Scale Inactivation Experiments. Flavin-bound IDI-2 (50 μM) and oIPP (100 μM) were incubated at 37 °C for 2 h in 200 mM Tris buffer, pH 7.8, containing 2 mM $MgCl_2$, and 4 mM NADPH. The sample was washed five times under aerobic conditions with 10 mM NH_4HCO_3, pH 7.8, at 4 °C using a Microcon™ centrifugal filter (molecular weight cut off of 30 KDa).

Parallel large scale inactivation experiments were also carried out in D_2O and H_2O buffers. IDI-2 was washed extensively against 200 mM Tris D_2O buffer, pD 7.8, containing 2 mM $MgCl_2$ by membrane filtration. The final mixtures contained 50 μM IDI-2 (FMN-bound) and 100 μM oIPP in 200 mM Tris buffer, pH 7.8 (H_2O) or pD 7.8 (99% D_2O), containing 2 mM $MgCl_2$ and 5 mM NADPH. The mixtures were preincubated without inhibitor for 5 min to reduce bound FMN before addition of oIPP or IPP and then were incubated for 2 h at 37 °C. Following the incubations, the samples were washed under aerobic conditions in H_2O and analyzed in protic solvents.

UV-visible spectra were recorded on an Agilent 8453 diode array spectrophotometer. For RP-HPLC studies the samples were denatured in 10 mM Tris pH 7.4 containing, 8 M guanidine chloride; protein was removed by membrane filtration; and the filtrate was chromatographed on a Phenomenex Prodigy™ ODS column, with a 5 mM ammonium acetate (pH 6.0): methanol (90:10) mobile phase. LC-MS analyses were performed with a Waters Alliance 2695 HPLC connected to a Micromass Quattro II triple quadrupole mass spectrometer, and analyzed by negative ion ESI-MS. Samples for direct infusion studies were diluted into 50:50:0.05 (H_2O:acetonitrile:formic acid) and analyzed on the Micromass Quattro II triple quadrupole mass spectrometer by negative ion ESI-MS for the flavin adducts and by positive ion ESI-MS for the protein adducts.

V. NMR assay. The NMR assay conditions were as follows: 12 μM IDI-2, 2.5 mM cIPP, 20 μM FMN, 2 mM NADPH, 10 mM $MgCl_2$, 0.1 mg/ml BSA, 5 mM DSS, 50 mM sodium phosphate (pD 7.2, 99% D_2O). Reactions were prepared in H_2O and exchanged with D_2O. Stock solutions of FMN and NADPH were prepared directly in D_2O. Enzyme was buffer exchanged with 50 mM phosphate buffer (pD 7.2, 99% D_2O) using five cycles of ultrafiltration. The concentration and activity of buffer-exchanged enzyme was measured using the BCA and acid-lability assays, respectively. Data for each time point was the average of 16 scans (3.13 min per time point). The reaction was monitored for 16 h at 37 °C. Integrals are reported in ppm relative to an internal DSS standard.

VI. Physical data

3-Cyclopropyl-but-3-en-1-yl bis-ammonium diphosphate (cIPP)
^{1}H NMR (D_2O) δ 0.45-0.48 (m, 2H), 0.61-0.65 (m, 2H), 1.33-1.37 (m, 1H), 2.36 (t, 2H, *J* = 6.9 Hz), 4.06 (q, 2H, *J* = 6.9 Hz); ^{13}C NMR (D_2O) δ 6.30, 16.25, 36.53, 65.29, 108.47, 149.51; ^{31}P NMR (D_2O) δ -6.45 (d, *J* = 22.0 Hz), -10.42 (d, *J* = 22.0 Hz); HRMS (CI) calcd for $C_7H_{14}O_7P_2$ (M-H) 271.0127, found 271.0137.

3-Oxiranyl-but-3-en-1-yl bis-ammonium diphosphate (oIPP)
^{1}H NMR (DMSO-d_6) δ 2.18 (br s, 2H), 2.65-2.67 (m, 1H), 2.83-2.86 (m, 1H), 3.39 (br s, 1H), 3.84 (br s, 2H), 5.06 (s, 1H), 5.18 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 31.2, 47.2, 53.2, 62.0, 114.1, 142.6; ^{31}P NMR (DMSO-d_6) δ -8.45 (br s), -9.90 (br s); HRMS (MALDI) calcd for $C_6H_{12}O_8P_2$ (M-H) 272.9929, found 272.9943.

2-Cyclopropyl-but-3-en-2-ol (solvolyzed cIPP)
^{1}H NMR (D_2O) δ 0.23-0.42 (m, 4H), 0.96-1.05 (m, 1H), 1.20 (s, 3H), 5.06 (dd, 1H, *J* = 1.5, 10.8 Hz), 5.17 (dd, 1H, *J* = 1.5, 17.5 Hz), 5.81 (dd, 1H, *J* = 10.8, 17.5 Hz).

VII. References

(1) (a) Davisson, V. J.; Woodside, A. B.; Poulter, C.D. *Methods Enzymol.* **1985**, 110, 130-144. (b) Davisson, V. J.; Woodside, A. B.; Neal, T. R.; Stremler, K. E.; Muehlbacher, M.; Poulter, C. D. *J. Org. Chem.* **1986**, 51, 4768-4779.

(2) de Ruyck, J.; Rothman, S. C.; Poulter, C. D.; Wouters, J. *Biochem. Biophys. Res. Commun.* **2005**, 338, 1515-1518.

(3) Satterwhite, D. M. *Methods Enzymol.* **1985**, 110, 92-99.

(4) Rothman, S.C.; Helm, T.R.; Poulter, C.D. *Biochemistry*, in press.

(5) Muehlbacher, M.; Poulter, C.D. *Biochemistry* **1988**, 27, 7315-7328.

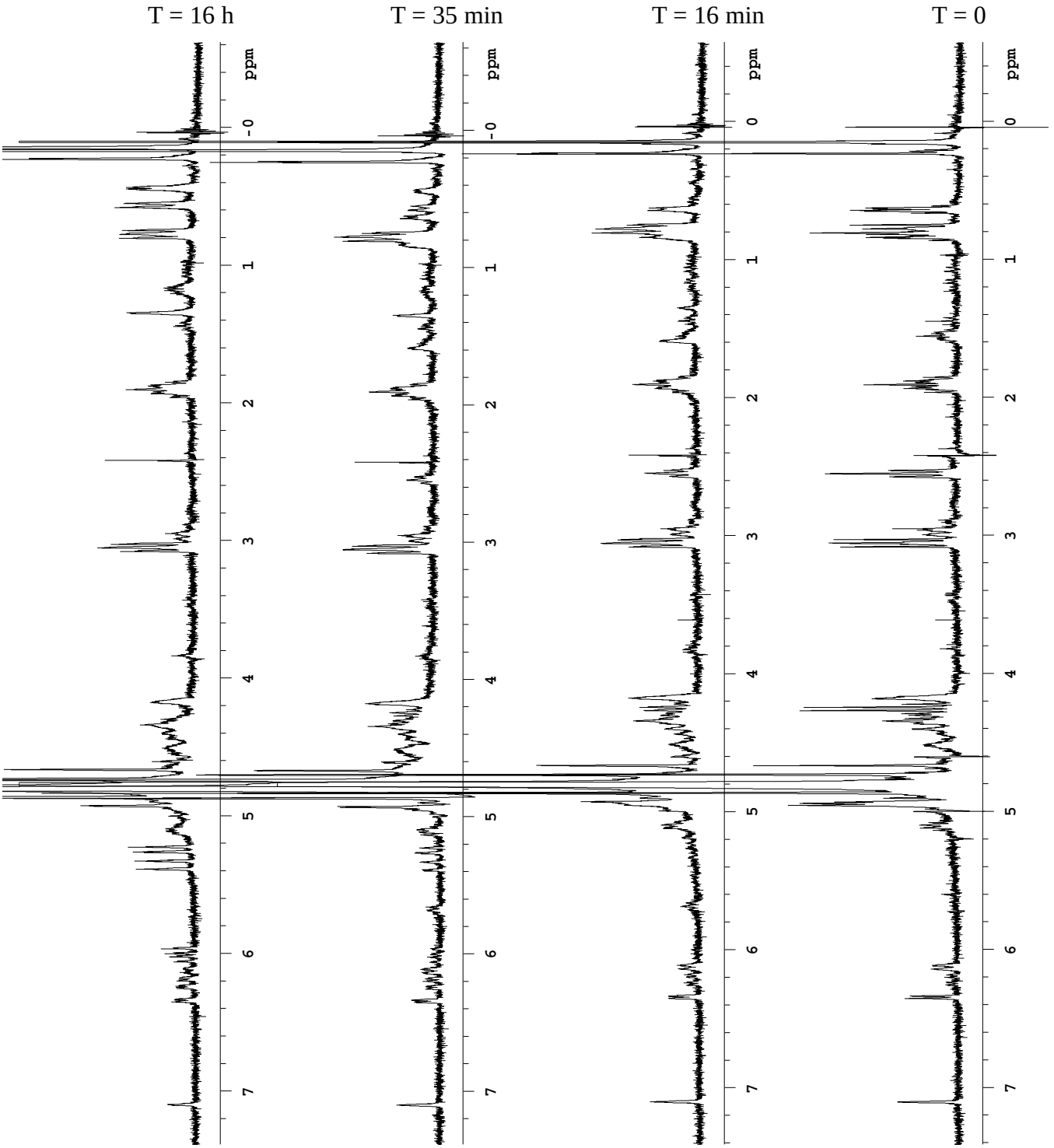

Figure S1. Representative NMR spectra for conversion of cIPP to cDMAPP. Reaction conditions were 50 mM $NaHPO_4$, pD 7.2, containing 12 μM enzyme, 2.5 mM cIPP, 10 mM $MgCl_2$, 20 μM FMN, 2 mM NADPH, 0.1 mg/ml BSA, 5 mM DSS, 900 μL final volume D_2O. NMR spectra we recorded at 37 °C on a Varian Inova 500 spectrometer at 500 MHz.

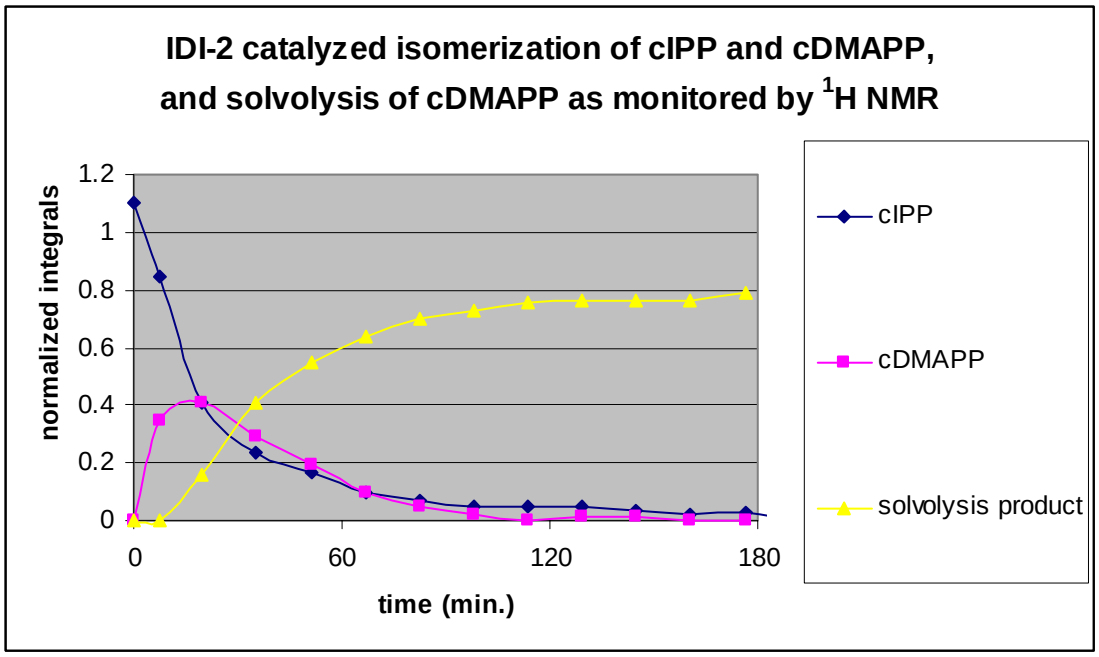

Figure S2. Time course for the interconversion of cIPP and cDMAPP, with concomitant solvolysis of cDMAPP, at 37 °C monitored by ^{1}H NMR spectroscopy at 500 MHz. Ratios of substrate and products were determined from integration of the following ^{1}H signals: cIPP (2.41 ppm), cDMAPP (1.45 ppm), solvolysis product (1.20 ppm), and DSS internal standard (0 ppm). Each spectrum was integrated, normalized relative to the internal standard and plotted as a function of time.

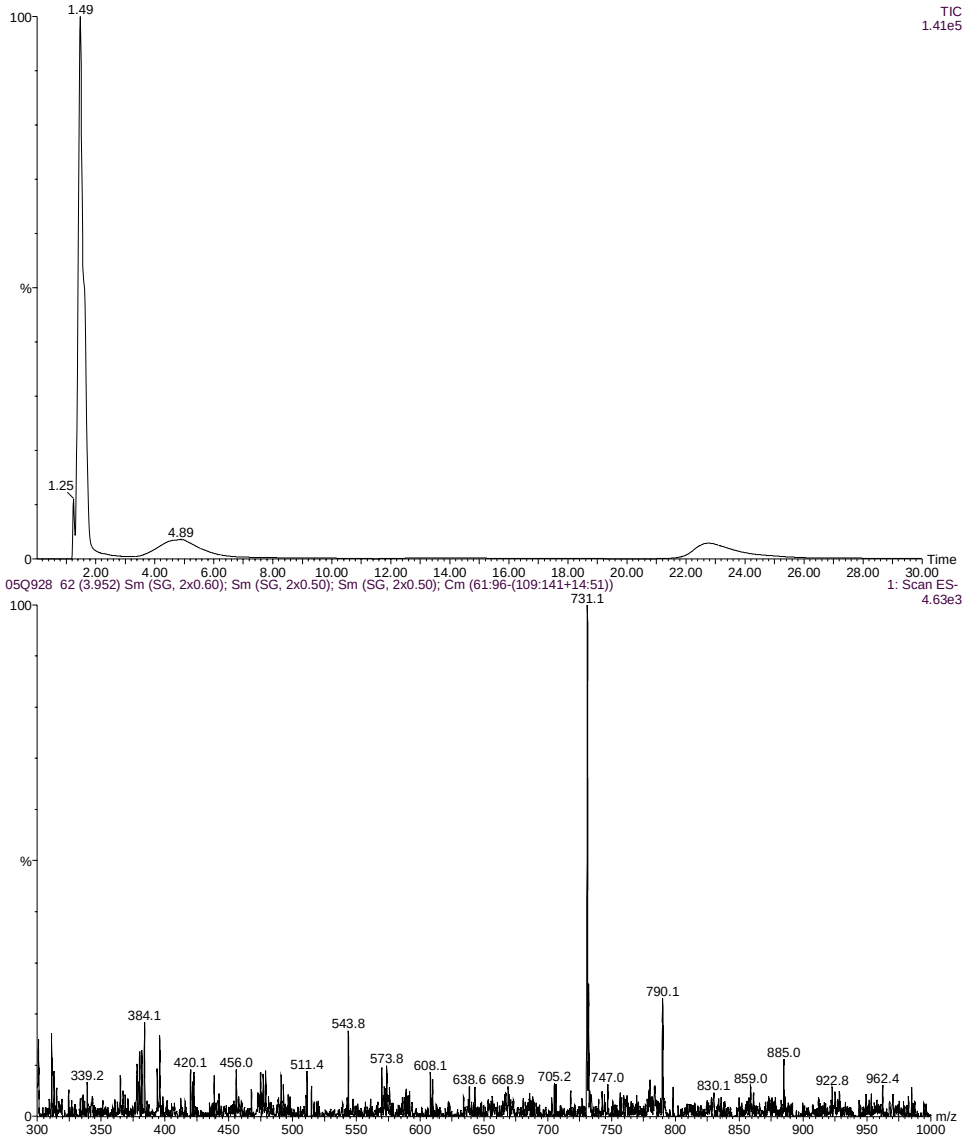

Figure S3. LC-MS analysis of the guanidinium-released cofactor for oIPP inactivated enzyme. A. RP-HPLC chromatrogram at 270 nm. B. Negative Ion-ESI spectrum for the peak at 4.89 min.

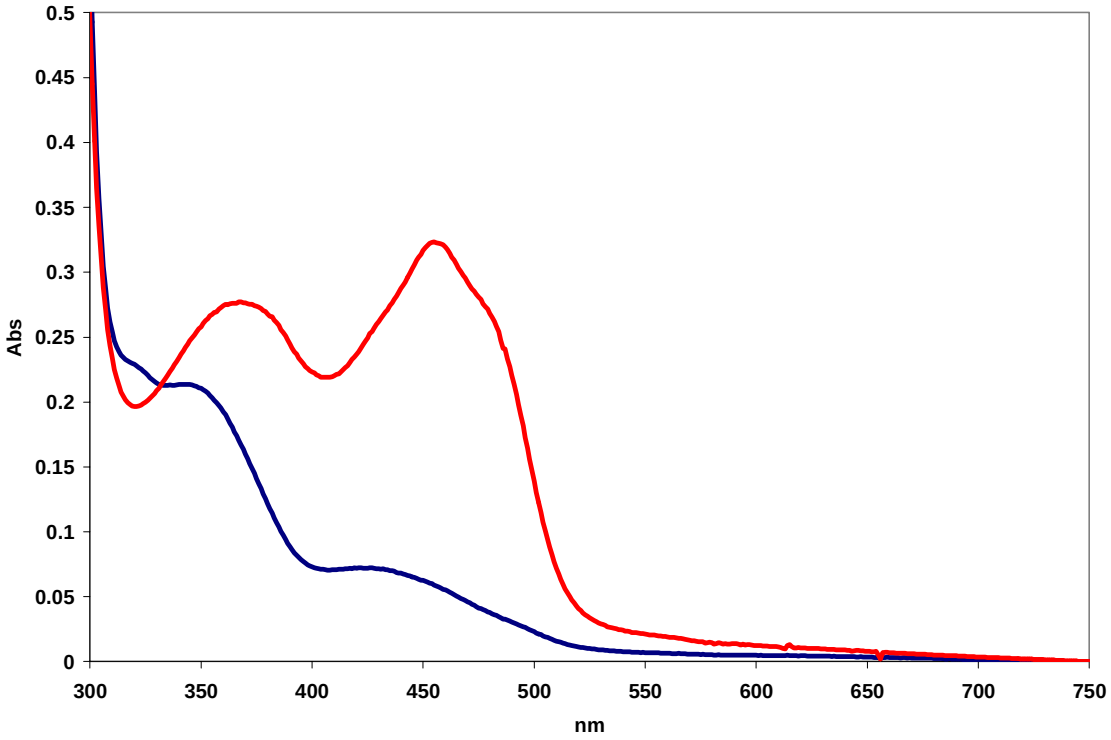

Figure S4. UV-visible spectra of enzyme-flavin complexes following 2 h incubation under assay conditions and subsequent aerobic washes. Shown are spectra for enzyme incubated with IPP (–) and with oIPP (–). The failure of oIPP inactivated enzyme to regenerate oxidized FMN is consistent with covalent modification of the flavin.

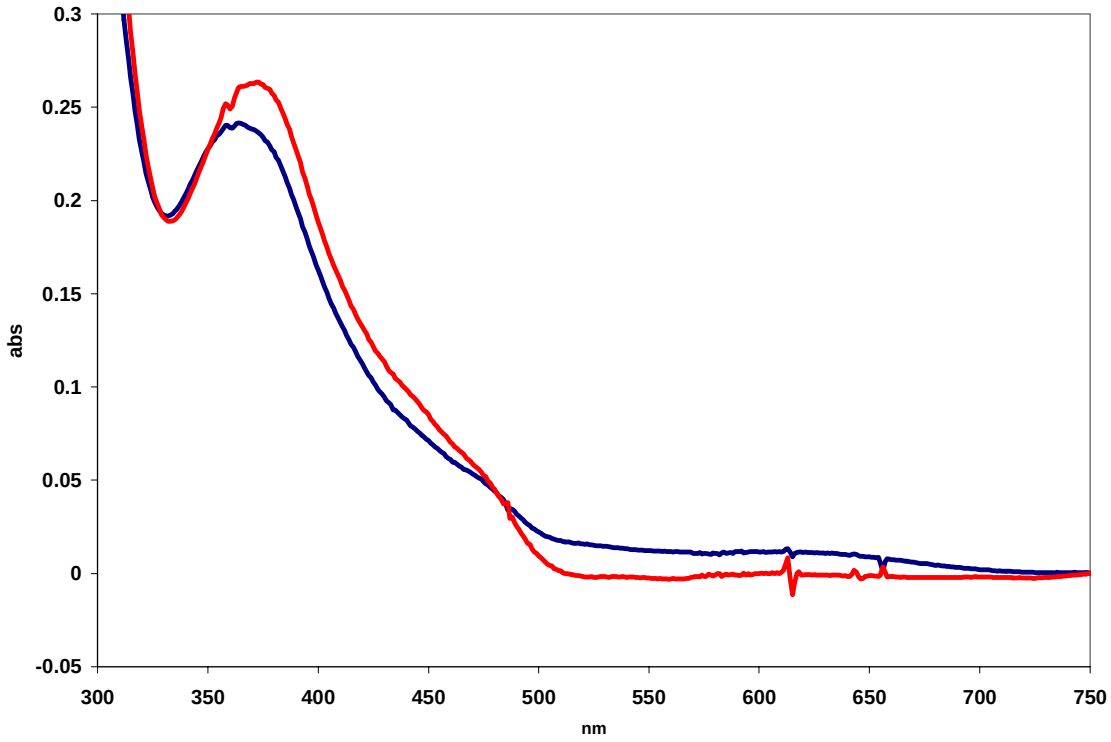

Figure S5. UV-visible spectrum of guanidinium-released flavin for oIPP inactivated enzyme. Shown are the spectra after initial denaturation (–) and following extended incubation (–).

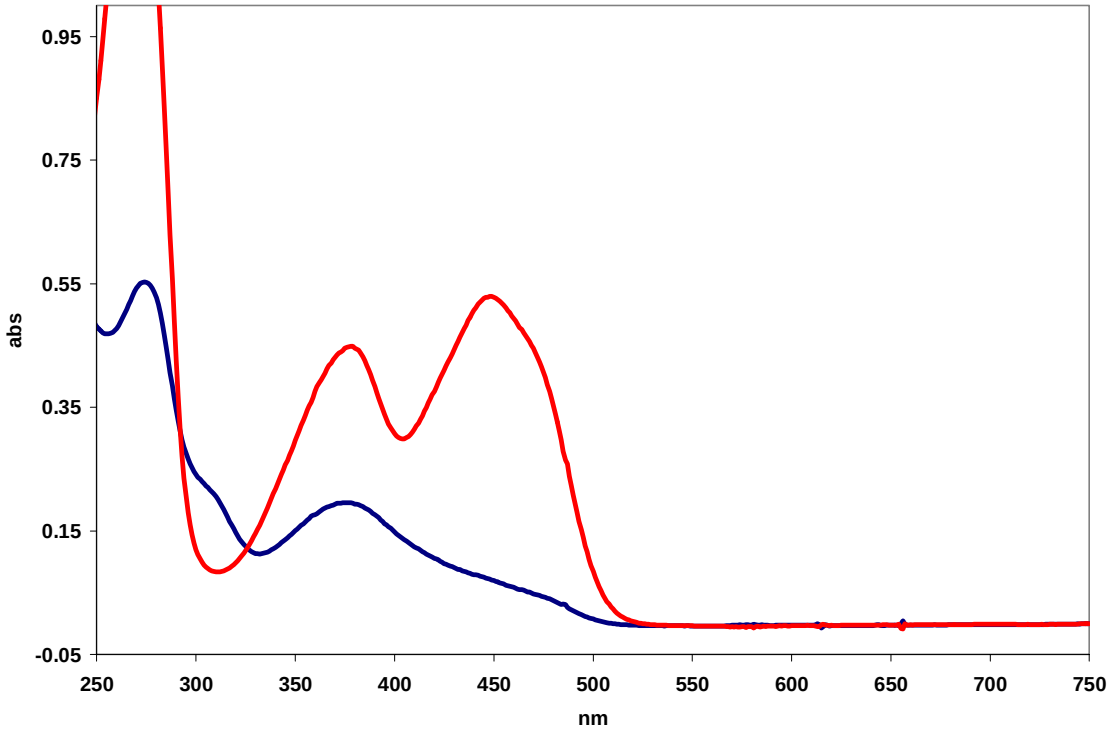

Figure S6. UV-visible spectra of guanidinium-released flavins following ultrafiltration. Shown are spectra for enzyme incubated with IPP (⁻) and with oIPP (⁻).

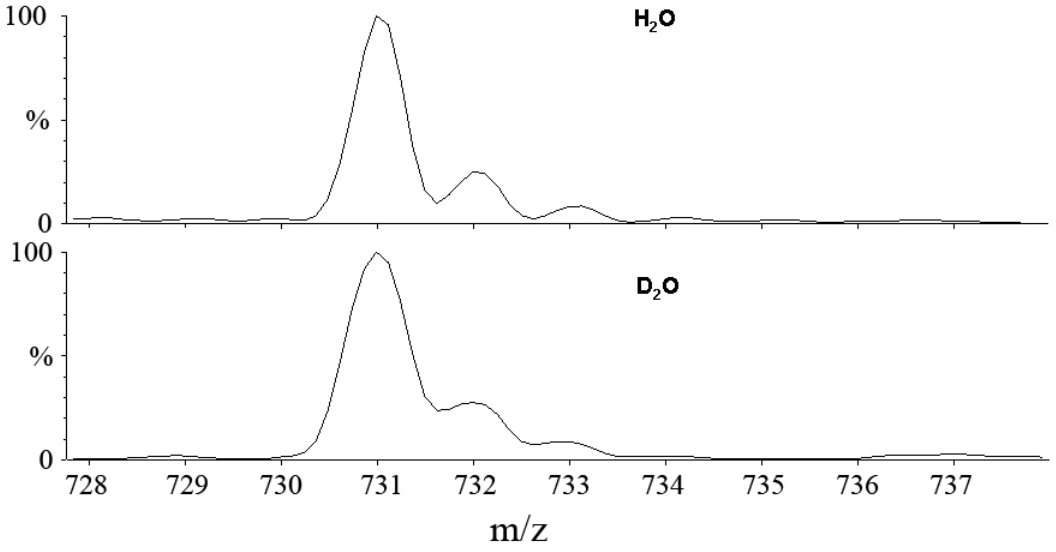

Figure S7. Negative ion electrospray mass spectra of large-scale inactivation reactions carried out in H_2O (top) and D_2O (bottom). Peak at 731 Da. corresponds to an [oIPP -$FMNH_2$]$^{M-H}$ adduct. Theoretical mass for $C_{23}H_{34}N_4O_{17}P_3^-$ = 731.1 Da., observed mass = 731.1 Da.