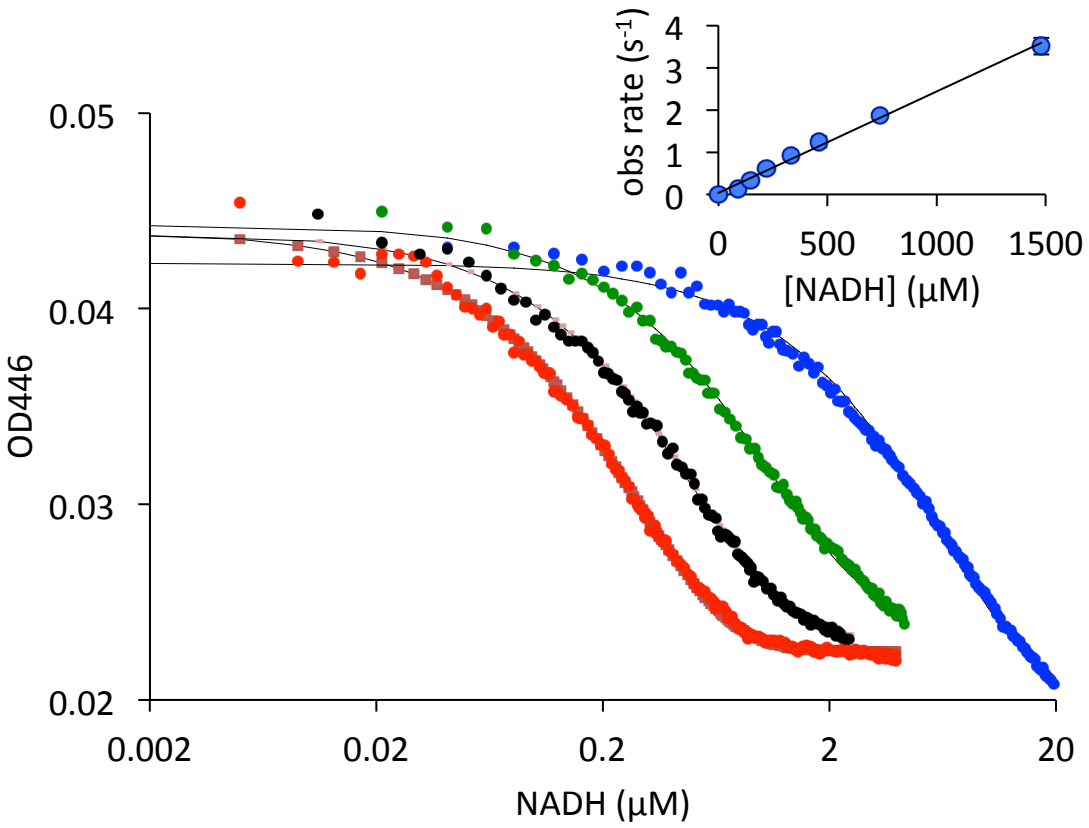
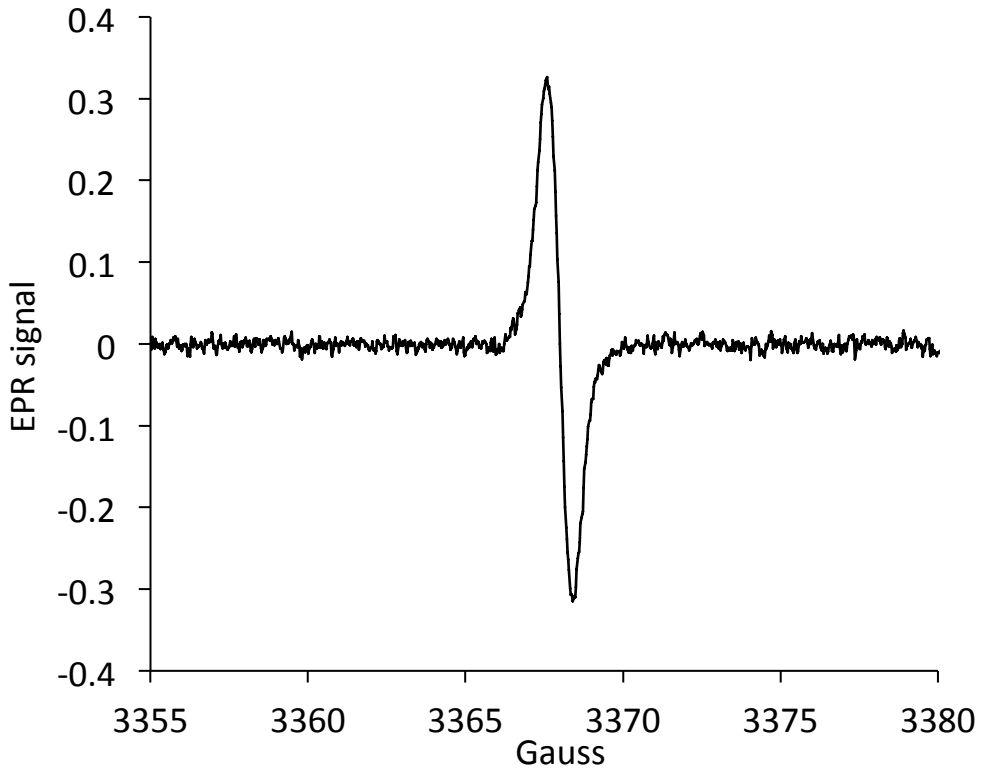
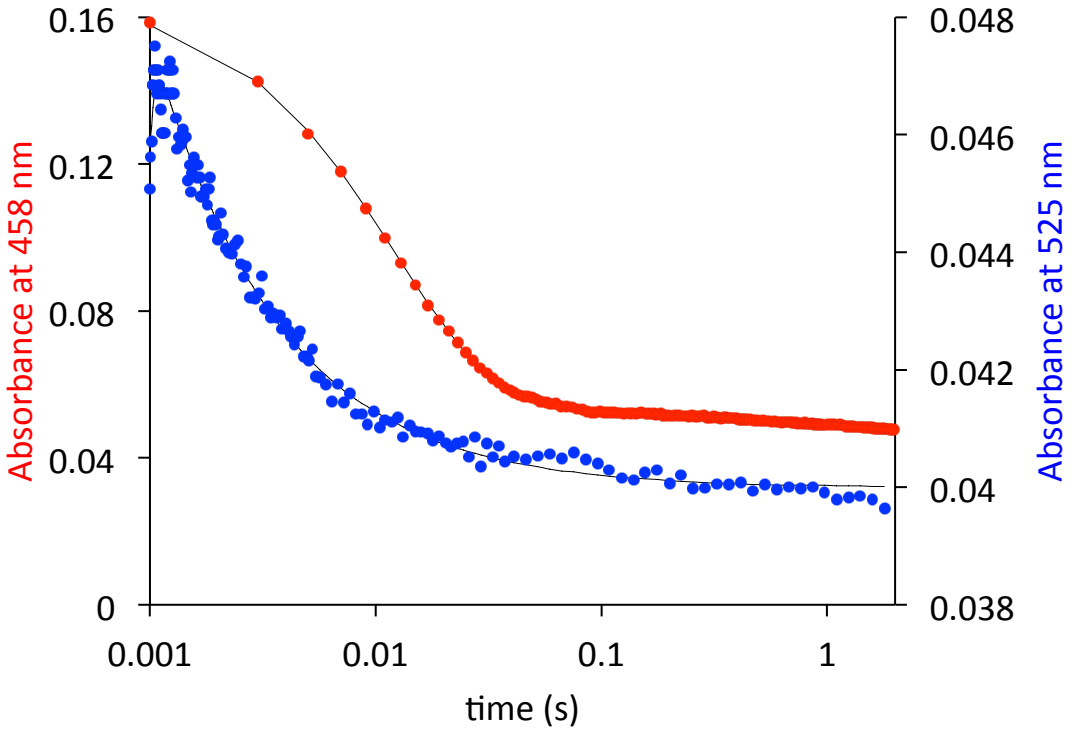
Supplementary Information



Supplementary Figure 1. Oxidized PCP hydroxylase (20 μM) in the presence of PCP (25 μM) was mixed with varying concentrations of NADH (0.2 – 8 mM) under anoxic conditions in a stopped flow. Representative datasets collected at 446 nm are shown. Symbols represent observed data points and the lines are derived from a best-fit using a single exponential. Final NADH concentrations were 0.18 mM (blue), 0.66 mM (green), 1.5 mM (black), and 3.0 mM (red). Replots derived from more extensive data sets, including replicates, are shown in the inset.

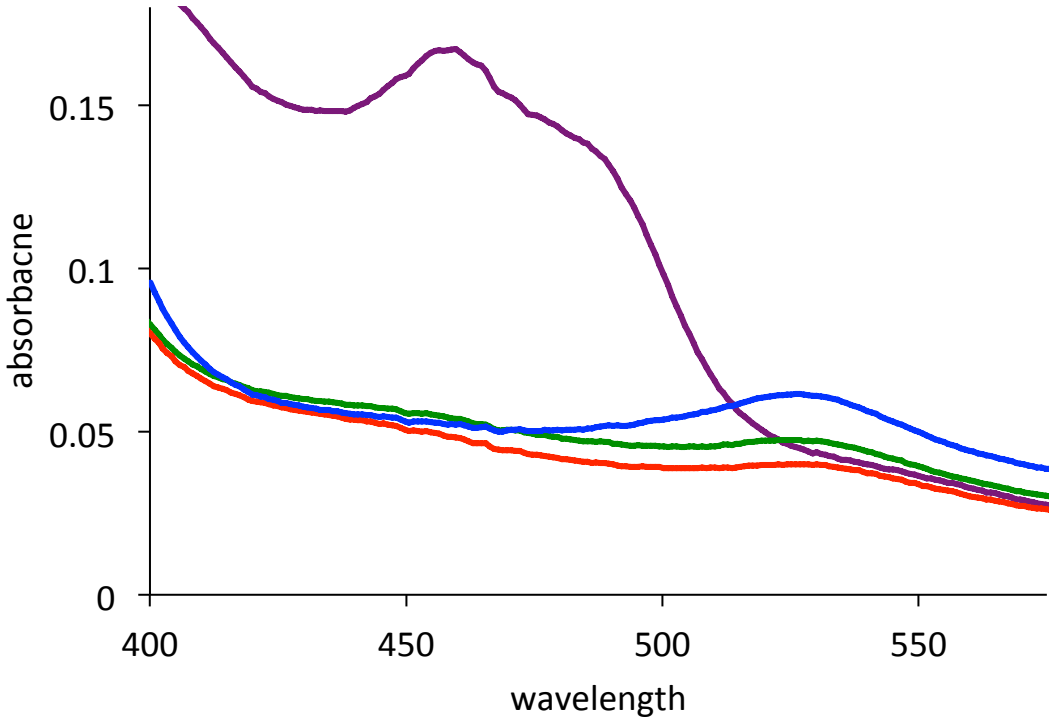


Supplementary Figure 2. EPR spectrum of TCSQ at 298 K. TCSQ was prepared from TCBQ and TCHQ as described in the Experimental Procedures section.

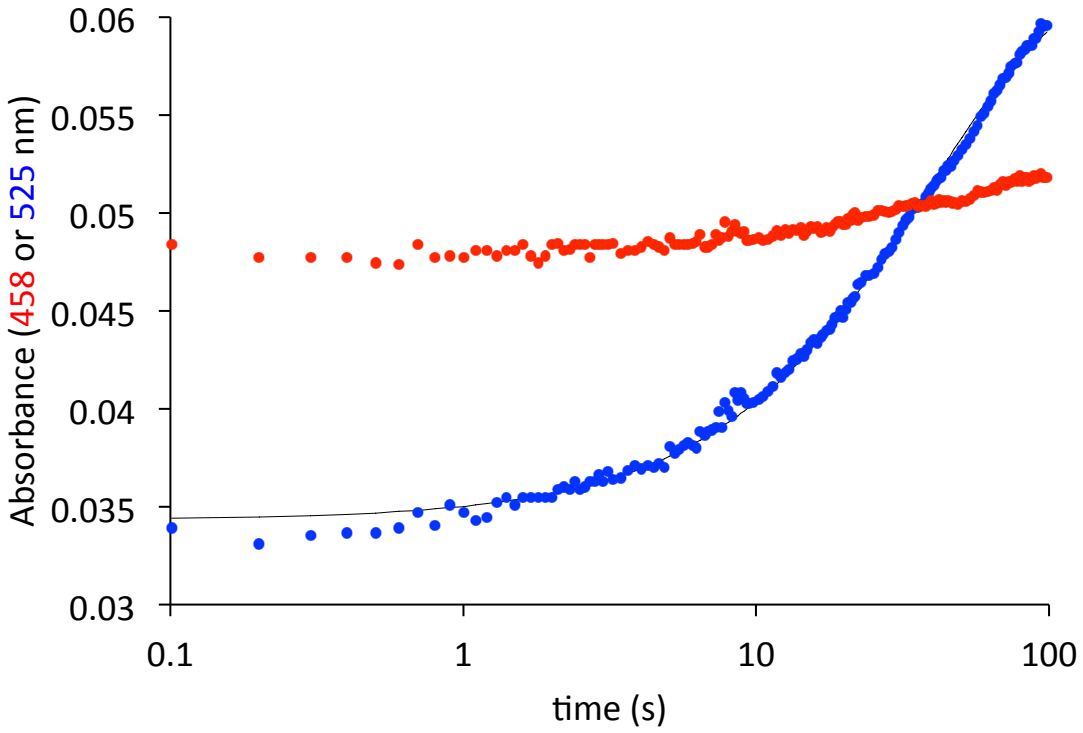


Supplementary Figure 3. TCBQ reductase (27 μM) was mixed with NADH at saturating concentrations (100 – 200 μM, equal to 9 – 18 x K_m) under anoxic conditions in the stopped-flow instrument. Representative time-courses at 458 and 525 nm are shown in blue and red, respectively, with the lines showing the fits to the data using two rate constants. The rate constant for the initial reduction and immediate formation of the FMN_{SQ} – Fe_2S_{2red} form of the enzyme (k_{1D} = 79 s^{-1}) is determined from the transient increase in absorbance at 525 nm (blue) and the decrease in absorbance at 458 nm (red). The second rate constant of k_{2D} = 2.7 s^{-1} corresponds to the disappearance of the FMN_{SQ} – FeS_{red} form of the enzyme (decrease at 525 nm) in a disproportionation reaction between two FMN_{SQ}-Fe_2S_{2red} active sites to generate FMN_{red}-Fe_2S_{2red} and FMN_{ox}-Fe_2S_{2red}. The FMN_{ox} – Fe_2S_{2red} form of the enzyme does not accumulate to a detectable level, indicating

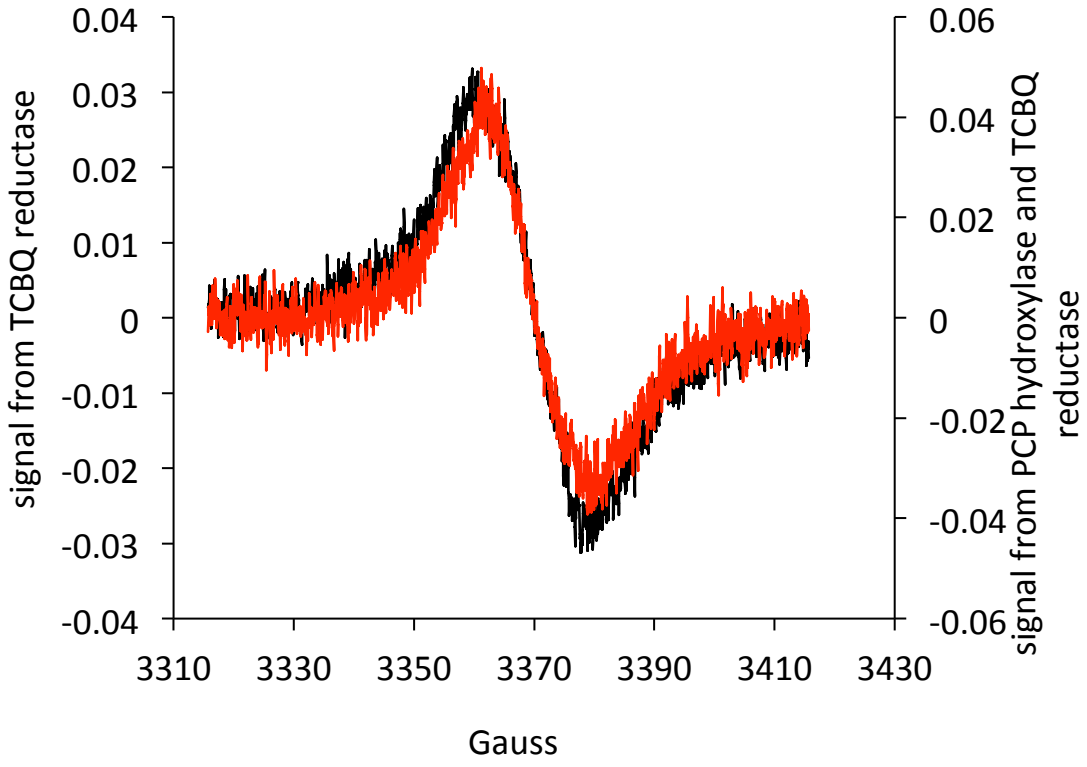
that the rate of reduction of the $FMN_{ox} – Fe_2S_{2red}$ form by NADH must be significantly greater than 2.7 s^{-1} under these conditions. Consequently, the absorbance at 458 nm typical of oxidized flavins does not increase. Notably, the disproportionation of the $FMN_{SQ} – Fe_2S_{2red}$ form is significantly faster for TCBQ reductase (complete in 2 sec) than it is in phthalate dioxygenase reductase (complete in 7 min), presumably because it can occur at least in part via intramolecular electron transfer in the trimeric TCBQ reductase, whereas disproportionation requires collision between two molecules of the monomeric phthalate dioxygenase reductase.



Supplementary Figure 4. Spectral intermediates observed during the reduction and re-oxidation of TCBQ reductase. The initial spectrum (purple) of the enzyme shows a maximum absorbance at 458 nm that is indicative of the oxidized FMN at the active site. The flavin absorbance decreases when the enzyme is reduced by NADH to generate the FMN_{red} – Fe_2S_{2ox} form, which rapidly undergoes electron transfer from the flavin to the iron-sulfur cluster to yield the FMN_{SQ} – Fe_2S_{2red} form, which has a characteristic absorbance at 525 nm (green). The spectrum of the final three-electron reduced form of TCBQ reductase (FMN_{red}-Fe_2S_{2red}) is shown in red. The blue curve shows the spectrum of the FMN_{SQ}-Fe_2S_{2red} (two-electron reduced) form of TCBQ reductase produced by transfer of an electron from pre-reduced TCBQ reductase to O_2; the assignment of this species as the FMN_{SQ}-Fe_2S_{2red} form was confirmed by EPR (see Supp. Fig. 6).



Supplementary Figure 5. Reduced TCBQ reductase (13.5 uM) prepared under anoxic conditions in the presence of excess NADH (100 μM) was mixed with oxygenated buffer (90 μM) in a stopped-flow apparatus. Representative traces at 458 and 525 nm are shown in red and blue, respectively. The line through the data represents fitting with a single exponential with (0.025 s^{-1}). Notably, the FMN_{SQ}-Fe_2S_{2red} form of the protein accumulates slowly (as indicated by the increase in absorbance at 525 nm), but complete re-oxidation of the flavin (as indicated by the increase in absorbance at 458 nm) does not occur to a significant extent over 100 s.



Supplementary Figure 6. The flavin semiquinone radical on TCBQ reductase (FMN_{red}-Fe_2S_{2red}) generated during oxidation of TCBQ reductase alone (black) or during the PCP hydroxylase reaction in the presence of TCBQ reductase (red). The radical on TCBQ reductase alone was generated by pre-reducing enzyme (130 μM) with NADH (300 μM) under anoxic conditions followed by exposure to buffer containing O_2 for 100 s and then freezing by immersion in liquid nitrogen. A typical flavin radical signal consisting of 0.24 equivalents (per enzyme-bound flavin) was observed with g = 2.0041 and ΔH = 19.1 G. The radical formed during the PCP hydroxylase catalyzed reaction of PCP in the presence of one equivalent of TCBQ reductase was obtained by pre-reducing both enzymes (130 μM each) with NAD(P)H (300 μM each) under anoxic conditions at 4°C (to slow the reaction). The reaction was initiated by addition of one volume equivalent of oxygenated buffer and quenched after 20 sec by freezing in liquid nitrogen.