Supplemental Procedures and Data (Ruangchan et al., 2010)

Spectroscopic studies. Spectrophotometric measurements were carried out with a Hewlett Packard diode-array spectrophotometer (HP 8453A), a Shimazu (2501PC) or a Cary300Bio double-beam spectrophotometer. All spectrophotometers were equipped with thermostatic cell compartments. Measurements were normally carried out at 25 ºC.

Enzyme assay for C_2.
An assay method used for measuring C_2 activity during the purification process was based on the protocol described in (1) using a coupled assay with 3,4-dihydroxyphenylacetate oxygenase (DHPAO). DHPAO catalyzes the conversion of DHPA to form 5-carboxymethyl-2-hydroxymuconate semialdehyde (CHS), which is a yellow compound with λ_{max} at 380 nm (ε_{380} = 38 $mM^{-1}cm^{-1}$ at pH 8.0). A typical assay reaction contained 200 μM HPA, 200 μM NADH, ~1-2 nM C_2, excess C_1 (~0.3 μM), 100 μM $(NH_4)_2Fe(SO_4)_2$, and excess DHPAO. At least three different concentrations (1.5, 3 and 5 μM) of DHPAO were used in order to assure that the rates measured were according to the C_2 reaction. The reactions were carried out in 50 mM sodium phosphate buffer, pH 7.5 at 25 °C. Formation of CHS was monitored at 400 nm (ε_{400} = 21.3 $mM^{-1}cm^{-1}$), and one unit of C_2 activity was defined as the amount of C_2 required to form 1 μmole CHS per minute under the above assay condition.

Another assay method based on the protocol previously described, which was modified to eliminate the use of DHPAO in the coupled assay (59), was also employed for the quick measurement of C_2 in chromatographic fractions. As the NADH oxidation (monitored by absorbance decrease at A340 nm) catalyzed by C_1 is increased in the presence of C_2, C_2 activity in each fraction can be measured by monitoring its ability to increase C_1 NADH oxidation (2). A typical assay reaction contained 200 μM NADH, 80 μM HPA and C_1 ~0.3 μM (0.18 unit) in 50 mM sodium phosphate buffer, pH 7.0. This reaction provided a base slope for the C_1 NADH oxidation. When a solution of C_2 was added to the reaction, the slope of the reaction increased. The magnitude of the slope change can be used to represent the concentration of C_2 in the assay reaction.

Rapid Kinetics Measurements. All reactions were performed with a Hi-Tech Scientific Model SF-61DX stopped-flow spectrophotometer in single-mixing mode at 4 ºC. Details for rapid kinetics measurements are described in Supplemental Materials. The optical path-length of the observation cell was 1 cm. The stopped-flow apparatus was made anaerobic by flushing the flow system with an oxygen scrubbing solution containing 400 μM glucose, 1 mg/ml glucose oxidase (15.5 unit/ml), and 4.8 μg/ml catalase in 50 mM sodium phosphate buffer, pH 7.0. The oxygen scrubbing solution was allowed to stand in the flow system overnight and was thoroughly rinsed with an anaerobic buffer prior to performing experiments (3). The methods used for studying the reactions of C_2 with oxygen were similar to the protocols used in the previous report (4) except that dithiotreitol (DTT) was removed from the enzyme solution prior to the experiments. Solutions of C_2 plus oxidized FMN in the presence or absence of HPA were made anaerobic in glass tonometers by several cycles of evacuation and equilibration with nitrogen gas (ultra high purity grade). The solutions were then stoichiometrically reduced with a solution of sodium dithionite (~5 mg/ml in 100 mM potassium phosphate buffer, pH 7.0) delivered from a syringe attached to the tonometer. Buffers used for maintaining various pH values were 100 mM sodium phosphate buffer (pH 6.0-7.5), 100 mM Tris-HCl buffer (pH 8.0-8.5) and 100 mM glycine adjusted with NaOH (pH 9.0-10.0). Stopped-flow experiments were monitored for absorbance changes at 380 nm and 446 nm. For product analysis, about 4 shots from the stopped-flow mixing at each pH were collected after the reaction was complete. HCl was added to the collected sample to a final concentration of 0.08 M, and then passed through an ultrafiltration unit (Microcon) with a molecular weight cut-off of 10 kDa to collect the filtrate. The quantity of 3,4-dihydroxyphenylacetate (DHPA) in each sample was determined using the HPLC analysis as described previously (1,5).

Determination of hydrogen peroxide from single turnover reactions of C_2 using peroxidase coupled assay. To investigate whether the C_2 reaction forms H_2O_2 as a byproduct from the uncoupling pathway, measurements of H_2O_2 were carried out using a horseradish peroxidase assay with ABTS as a substrate (6**)** or an oxygen-electrode to measure the amount of O_2 derived from H_2O_2 (described in the main text). Solutions of C_2 (100 μM), $FMNH^-$ (50 μM), and HPA (2 mM) in 10 mM sodium phosphate buffer pH 7.0 were mixed with air-saturated buffers containing 2 mM HPA at 25 °C and at various pH. The mixings were carried out in closed vessels inside the anaerobic glovebox. Buffers used for maintaining various pH were 100 mM sodium phosphate buffer (pH 6.0 and 7.0), 100 mM Tris-HCl buffer (pH 8.0), and 100 mM Glycine buffer adjusted with NaOH (pH 9.0 and 10.0). All concentrations represent those after mixing conditions. After the reaction completed, the mixture was quenched immediately with HCl (0.15 M). Protein was then removed from the quenched solution using a Centricon YM-10 concentrator. The samples were analyzed for H_2O_2 by measuring final absorbance increases at 420 nm (ε_{420} = 42.3 $mM^{-1}cm^{-1}$), which result from the oxidation of ABTS by H_2O_2 catalyzed by horseradish peroxidase. A typical assay (total volume = 1 mL) contained 50 μL of a sample from the C_2 reaction, 0.5 mM ABTS, and 0.2 unit horseradish peroxidase in 100 mM sodium phosphate buffer pH 7.0 at 25 °C. A standard curve of the H_2O_2 concentration versus the absorbance change was constructed for estimating unknown H_2O_2 concentration. The lower limit of detection based on this method was 0.5 μM H_2O_2 which is equivalent to 1% of H_2O_2 that would be generated from the uncoupling pathway.

FIGURE LEGENDS

FIGURE 1. Kinetics of the reaction of C_2:$FMNH^-$ with oxygen. A solution of the reduced enzyme (C_2:$FMNH^-$) was mixed with 50 mM sodium phosphate buffers with various oxygen concentrations in the stopped-flow spectrophotometer. The final reactions contained C_2 (35 μM), $FMNH^-$ (16 μM), and oxygen (0.13, 0.31, 0.61, and 1.03 mM, from the lower to the upper traces at 380 nm). The reactions were monitored for absorbance changes at 380 and 446 nm.

FIGURE 2. Rate constants for formation of C_2:C4a-hydroperoxy:HPA at pH 6.2 and 8.4. A solution of the reduced enzyme (C_2:$FMNH^-$:HPA) was mixed with buffers containing oxygen plus HPA in the stopped-flow spectrophotometer (The same experiment as in Fig. 4 of the main manuscript). The reactions were monitored for absorbance changes at 380 and 446 nm. After mixing, the reaction contained C_2 (35 μM), $FMNH^-$ (16 μM), HPA (2 mM) and oxygen (0.13, 0.31, 0.61, and 1.03 mM). The plot shows k_{obs} of the second phase (see the main text) versus the oxygen concentrations at pH 6.2 (empty circles) and 8.4 (filled circles), consistent with a bimolecular rate constant of ~4.8×10^4 $M^{-1}s^{-1}$.

FIGURE 3. Analysis of the reaction data of the C_2:$FMNH^-$:HPA complex with oxygen at various pH. A plot shows the observed rate constants from the third phase (FMN oxidation) of A380 traces versus pH.

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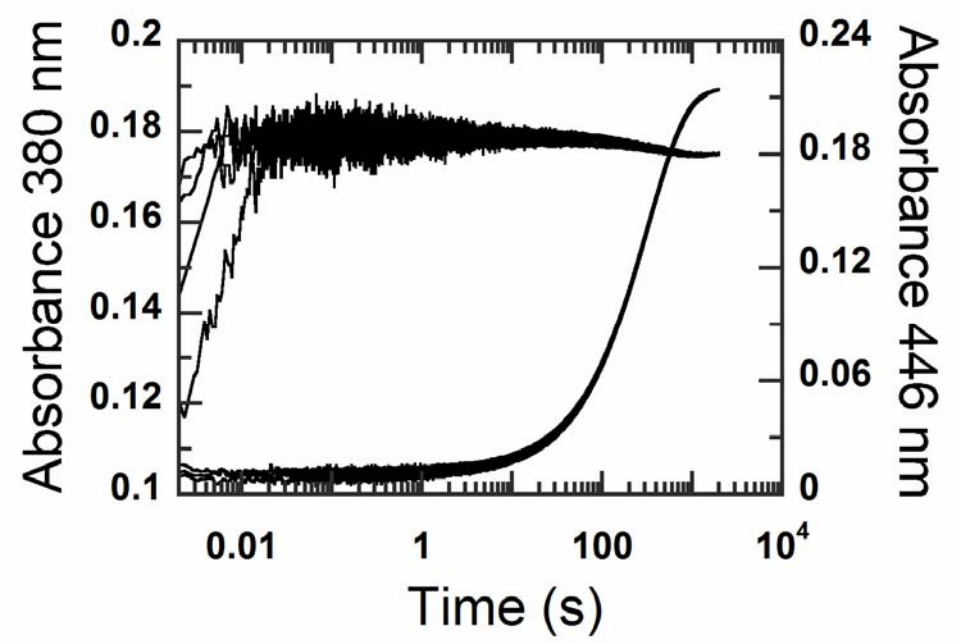
FIGURE 1.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0.2
0.18
0.16
0.14
0.12
0.1
0.24
0.18
0.12
0.06
0
Absorbance 446 nm
0.01
1
100
10^4
Time (s)

FIGURE 2.

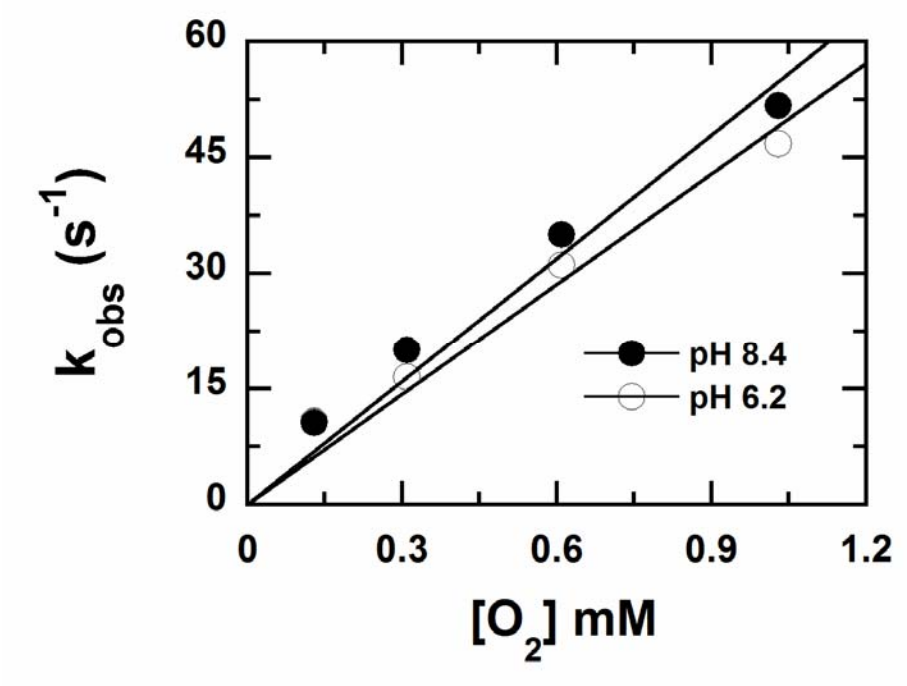

FIGURE 3.