Supporting Information: The Substrate-Bound Crystal Structure of a Baeyer–Villiger Monooxygenase Exhibits a Criegee-like Conformation

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EXPERIMENTAL SECTION

Subcloning the chnB1 gene into the pJW234 expression vector

The chnB1 gene was amplified using Pwo DNA polymerase (Roche) from the pSDRmchnB1 plasmid.† PCR primers with NsiI and EcoRI restriction sites were used to amplify the gene (restriction sites are underlined), as shown in Table S2. The desired band was purified using the QiAprep Gel Extraction Kit (Qiagen) and subcloned into the PCR-Script Cam vector (Stratagene) using the PCR-Script Cam Cloning Kit (Stratagene). Plasmid DNA from positive clones was prepared using the QiAprep Spin MiniPrep kit (Qiagen), and then digested using NsiI and EcoRI (New England BioLabs). The pJW234 expression vector (obtained from Dr. Mirek Cygler, University Saskatchewan, Saskatoon, Canada) was digested with the same enzymes. The desired bands from both digests were purified using gel extraction (MO BIO Laboratories UltraClean 15 DNA Purification Kit). The chnB1 insert was ligated into the pJW234 vector, and this plasmid was used to transform E. coli DH5α cells. Plasmid DNA from positive clones was prepared using the QiAprep Spin MiniPrep kit (Qiagen), and then digested using NsiI here. The irradiation power was (1/2π)B1= 3000 Hz was used to eliminate background protein signals. Spectra were recorded with 32k points, a sweep width of 16 ppm, and 128 transients. The experiments were repeated the next day in order to ensure that the samples were stable. The intensity of each peak in the STD spectrum was divided by the intensity in the reference spectrum. In order to determine the percent STD effect, this ratio was divided by the same ratio for
the largest peak in the STD spectra, the A2 proton of the adenine ring. In those cases where multiplets were observed for a single proton, the percent STD effect was averaged for each peak in the multiplet. The data from the two replicates were averaged to obtain a final value for each proton.

RESULTS

The following figures and table are described in the text of main article.

Figure S1. The percent STD effect for the nicotinamide (left) and adenine (right) protons, using A2 as a reference, for the wild-type and the three mutant enzymes. Error bars show the standard deviation. The structure of NADP⁺ is shown inset, with the positions of each proton indicated.

Figure S2. The uncoupling ratio is compared to $k_{cat}/K_{M,NADPH}$ and $k_{cat}/K_{M,cyclohexanone}$. All values are normalized to the corresponding wild-type values. Error bars indicate the standard deviations for each value. The standard deviation of the wild-type uncoupling ratio is included in the $k_{cat}/K_{M}$ error bars in order to account for error in scaling $k_{cat}/K_{M}$ values to the wild-type uncoupling ratio.

Figure S3. The NADP⁺ conformations in the CHMO_Closed (blue) and CHMO_Rotated (green) structures are compared to the rotated NADP⁺ conformation of mFMO (purple, PDB ID 2XLR). The structures were superimposed using the FAD molecule, which is also shown.

Figure S4. Comparison of the "back" of CHMO (panel A, green, CHMO_Rotated conformation) and PAMO (panel B, wheat, PDB ID 2YLT). The protein is shown as a surface, and the ligands (FAD, NADP⁺, cyclohexanone, and MES) are shown as sticks. The large tunnel seen in PAMO is obstructed in CHMO, obscuring the view of the FAD, NADP⁺, and cyclohexanone in the CHMO structure.
As described in the main article, the uncoupling ratio data are consistent with the interpretation that the mutants are less effective in stabilizing the peroxyanion intermediate than the wild-type enzyme. The uncoupling ratio represents the ratio between the enzyme catalyzing the full BV reaction (BV activity), and a short-circuited reaction in which NADPH is merely oxidized (NADPH oxidase activity). In the wild-type enzyme, the full BV reaction is dramatically favored, as indicated by a ratio of 114. The three mutants are all less efficient in favoring the BV reaction, as shown with their uncoupling ratios of 20 or less. This implies that the mutants are either defective in catalyzing the full BV reaction, enhanced in the short-circuited NADPH oxidation reaction, or both. The steady-state kinetics data indicate that the mutants do have compromised BV activity. To access whether the mutations also enhanced NADPH oxidase activity, we compared both the normalized $k_{cat}/K_m(NADPH)$ and the normalized $k_{cat}/K_m(cyclohexanone)$ to the normalized uncoupling ratio (with wild-type values set to 100%). This analysis provides insights into how efficiently each substrate is used by the enzymes (see Figure S2). Of course, the mutants are less efficient catalysts both with respect to NADPH and cyclohexane. Intriguingly, using the drop in uncoupling ratios as a base for the extent of anticipated efficiency loss, the mutants are actually using NADPH more effectively than predicted. Conversely, the mutants use cyclohexanone as a substrate much less efficiently than would be anticipated. This observation implies that the mutations have differential impact on the BV and NADPH oxidase reactions. Given that the only unique step in the NADPH oxidase pathway, as compared to the BV activity pathway, is the unproductive collapse of the peroxyanion intermediate without formation of the Criegee intermediate, we conclude that the mutants destabilize this peroxyanion intermediate. In so doing, the peroxyanion intermediate is more likely to collapse unproductively prior to the arrival of the ketone substrate. The CHMO$_{NADP^+}$ crystal structure readily rationalizes this property of the mutants. In contrast to the Closed conformation, NADP$^+$ cannot play a role in stabilizing the peroxyanion intermediate in the Rotated conformation.

**REFERENCES**

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**Table S1.** Enzyme kinetics data. Errors are the standard errors determined by regression, or standard deviations for the Uncoupling Ratio.

|            | Wild-type | L145N | L145D | F507Y |
|------------|-----------|-------|-------|-------|
| $K_m$ (M)  |           |       |       |       |
| NADPH      | (6.19±0.70) $\times 10^4$ | (3.53±0.34) $\times 10^4$ | (4.46±0.47) $\times 10^4$ | (3.34±0.63) $\times 10^4$ |
| Cyclo-hexanone | (2.95±0.72) $\times 10^7$ | (2.46±0.26) $\times 10^6$ | (4.93±1.95) $\times 10^7$ | (1.14±0.11) $\times 10^7$ |
| $k_{cat}$ (s$^{-1}$) | 12.47±0.47 | 1.77±0.05 | 0.67±0.02 | 0.79±0.04 |
| Cyclo-hexanone | 8.86±0.47 | 1.45±0.05 | 0.80±0.004 | 0.36±0.01 |
| $k_{cat}/K_m$ (M$^{-1}$s$^{-1}$) | (2.01±0.24) $\times 10^6$ | (5.02±0.51) $\times 10^5$ | (1.51±0.17) $\times 10^5$ | (2.37±0.46) $\times 10^6$ |
| Cyclo-hexanone | (3.00±0.75) $\times 10^7$ | (5.89±0.66) $\times 10^6$ | (1.63±0.65) $\times 10^6$ | (3.16±0.31) $\times 10^7$ |
| Uncoupling Ratio | 114±16 | 20±2 | 4±1 | 5±1 |
| $K$ (M) NADPH | (1.65±0.19) $\times 10^4$ | (4.20±0.75) $\times 10^4$ | N.D. | (1.92±0.38) $\times 10^4$ |

**DISCUSSION**

In order to estimate the affinity of NADP$^+$, the $K$ values of the wild-type and the mutants were determined using NADP$^+$ as the inhibitor and varying NADPH as the substrate (Table S1). As expected, NADP$^+$ appeared to behave as a competitive inhibitor for CHMO. NADP$^+$ proved to be only a weak inhibitor of $Rm$CHMO, in contrast to AcCHMO$^2$ and PAMO$^3$, which have $K$ values in the low micromolar range, but similar to 4-hydroxyacetophenone monooxygenase (HAPMO)$^4$. In comparison to the $Rm$CHMO wild-type enzyme, the L145N mutant is more sensitive to inhibition by NADP$^+$. The L145D mutant also shows this same trend, but an accurate $K$ could not be determined due to the low activity of this mutant. The F507Y mutant reveals that it is similar to the wild-type enzyme, in this respect.

The NADP$^+$ inhibition studies imply that the affinity of NADP$^+$ is much weaker than would have been suggested by the values for $K_m(NADPH)$; however, as NADP$^+$ is known to stay bound to the enzyme throughout the catalytic mechanism (see Figure S5), and readily co-crystallizes with $Rm$CHMO, it must be concluded that this apparent weak affinity is at least partially caused by complex binding kinetics. Despite this puzzling aspect, the mutants display $K$ values that are less or statistically equal to that of the wild-type enzyme, mirroring the trend in affinity seen by monitoring $K_m(NADPH)$.  

Inhibition of CHMO and Mutants by NADP$^+$

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Table S2. PCR primers for subcloning and site-directed mutagenesis. Restriction sites are double underlined and mutated bases are single underlined.

| Construct             | Primer       | Sequence                                             |
|----------------------|--------------|------------------------------------------------------|
| His8-TEV-ChnB1       | Sense        | 5'-CAGGAAACAATGCATATGACCGCACAG-3'                    |
|                      | Antisense    | 5'-CAGTACAGAATTCTAGACCCTGACCCTCTCT-3'                |
| His8-TEV-ChnB1-L145N | Sense        | 5'-GTGTACCCGTGGGGTAATACAGTACGCTCCGCTCGAC-3'          |
|                      | Antisense    | 5'-GAAAGTTTGATCAGCCTGTGGGAGAGATTGCCCACGCCGACGAC-3'  |
| His8-TEV-ChnB1-L145D | Sense        | 5'-GTCGACCCGCTGGGGAGAGATTGCCCACGCCGACGAC-3'          |
|                      | Antisense    | 5'-GAAAGTTTGATCAGCCTGTGGGAGAGATTGCCCACGCCGACGAC-3'  |
| His8-TEV-ChnB1-F507Y | Sense        | 5'-GCCAGCGTACTTGATATTACCTGGGCGCTG-3'                 |
|                      | Antisense    | 5'-CAGGCCGCCAGGTAATACAGTGCTCGGC-3'                   |