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**Biocatalytic Conversion of Ethylene to Ethylene Oxide using an Engineered Toluene o-xylene Monooxygenase**

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Mutants of toluene o-xylene monooxygenase are demonstrated to oxidize ethylene to ethylene oxide *in vivo* at yields of >99%. While the native enzyme is not observed to carry out the reaction, several mutants increase activity by >8500-fold and are able to specifically drive the reaction to completion in less than 4 hours. This is the first report of a recombinant enzyme capable of carrying out this industrially significant chemical conversion.

Ethylene oxide is an industrially important feedstock chemical with more than 20 million tons produced annually. Currently ethylene oxide is produced through the oxidation of ethylene by a silver catalyst in high-pressure, high-temperature reactors, with a yield of up to 75% [Dever]. In order to develop a renewable alternative to this important transformation, it will be critical to develop methods that increase yields, are carried out under ambient temperatures and pressures, and do not generate toxic waste.

Enzymes are biology’s catalysts and are renowned for their ability to reduce the activation energy of chemical reactions, allowing mild reaction conditions. In nature, a class of enzymes called alkene monooxygenases (AOs) are known to oxidize alkenes, including ethylene [CITE]. The organisms where AOs are naturally found further utilize the oxidized alkenes, such as ethylene oxide, for growth and are also not readily amenable to industrial processes. Therefore, for the development of an industrial biocatalyst to convert ethylene into ethylene oxide the recombinant production of AOs in an industrial organism, such as *E. coli*, would be ideal. However, expression of recombinant AOs in *E. coli* has proven elusive [CITE].

Toluene o-xylene monooxygenase (TOM) is an enzyme closely related to AOs, and has been shown to oxidize aromatic compounds such as toluene via an epoxide intermediate [Whited]. Furthermore, TOM is established to be recombinantly produced in *E. coli*. In addition, TOM has been previously engineered to oxidize the related compound trichloroethylene. Therefore, we hypothesized that either TOM, or variants of TOM, would be capable of oxidizing ethylene to ethylene oxide (Figure 1).

We obtained TOM and a panel of TOM mutants from a previous effort by Wood and colleagues [Iwashita; Rui]. These mutants were derived from a series of libraries to reengineer TOM to oxidize trichloroethylene. Specifically, we focused on mutants found in these previous studies where small amino acids had been changed to large amino acids. The one exception was V106A, which was previously identified as the best mutant for oxidation of trichloroethylene.

The panel of 12 mutants were each grown in E*. coli* TG1 as overnight cultures, and then resuspended in pH 7.4 phosphate buffered saline at an OD of 10. In gas-tight 2 mL vials 0.5 mL of cells were added, and the headspace was purged with 1.5% ethylene in air. The cells were incubated at 37 C while mixing for 24 hours, after which the headspace was analyzed using GC-FID to detect the fraction of ethylene remaining and if ethylene oxide was produced. As illustrated in Figure 2, while the native TOM did not utilize ethylene there were several mutants that had utilized >99% of the ethylene present. Furthermore, the major product detected in these biotranformations was ethylene oxide.

The most active mutants, A113F and V106F, were further characterized for ethylene oxide production. The equivalent procedure as described was carried out, however headspace was measured from a series of samples produced in parallel at 30, 100, and 220 minutes. As illustrated in Figure 3, both mutants quantitatively converted >99% of ethylene into ethylene oxide in less than 4 hours. This corresponds a rate of >30,000 nM/min for the A113F mutant. Using the native TOM no ethylene oxide was produced or ethylene oxidized over a 24-hour period. Based on analytical standards as little as 5 μM ethylene oxide in a whole cell biotransformation over a period of 24 hours could have been detected, which would correspond to a conversion rate of 3.5 nM/min. Therefore, the single mutant A113F enhanced activity by >8500-fold.

To better understand why these mutations had a significant effect on activity we generated molecular models of each. While a crystal structure of TOM is not available, we generated a molecular model of the native enzyme as well as each mutant using standard Rosetta-CM protocols [Song]. Three templates (PDB-ID 3U52, 2INN, and 2INP) were used, all of which were ~65% identical in sequence to TOM and the mutants. Once the models were made we used RosettaDock [Kaufmann] with functional constraints to model in the placement of the coordinated iron residues observed in the template crystal structures used as templates for the modeling. In the resulting models the conformation and geometry of the iron coordinating residues in the models was consistent to what is observed in the crystal structure templates.

The models revealed a pocket around the open coordination sites of the diiron center in TOM (Figure 4). In the mutants A113F and V113F, the pocket is partially occluded by the side chain of phenylalanine. This indicates a relatively simple explanation for the increase in activity whereby the aromatic ring in the active site promotes a catalytically productive binding mode for ethylene.

Conclusions

To the best of our knowledge this is the first report of an engineered biocatalyst capable of converting ethylene to ethylene oxide. This engineered protein was obtained from a repurposed directed evolution library originally targeted at the oxidation of the related compound trichloroethylene. Interestingly, the best mutant on trichloroethylene was not the best mutant on ethylene. This suggests that there may be a fundamentally different mechanism by which the two are oxidized. In addition, it highlights the importance of rescreening libraries of mutants against new target substrates.

The system with the highest activity in this study was able to quantitatively convert >99% of ethylene to ethylene oxide in less than 4 hours at a relatively low temperature and pressure, in mild aqueous conditions, from which there was no toxic by-products are produced. As the most active mutant was not explicitly developed for ethylene oxide production, further efforts to engineer the mutant TOM discovered here can likely improve activity. This novel renewable catalyst has the potential to transform the production of the industrially important chemical ethylene oxide.

Notes and references

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/c000000x/

1 Citations here in the format A. Name, B. Name and C. Name, Journal Title, 2000, 35, 3523

1 Dever, J. P., K. F. George, W. C. Hoffman, and H. Soo. "Ethylene oxide." *Kirk-Othmer encyclopedia of chemical technology*,2006

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Figure 1. Reaction scheme showing the native reaction of toluene to a p-cresol intermediate and the novel reaction of ethylene to ethylene oxide.

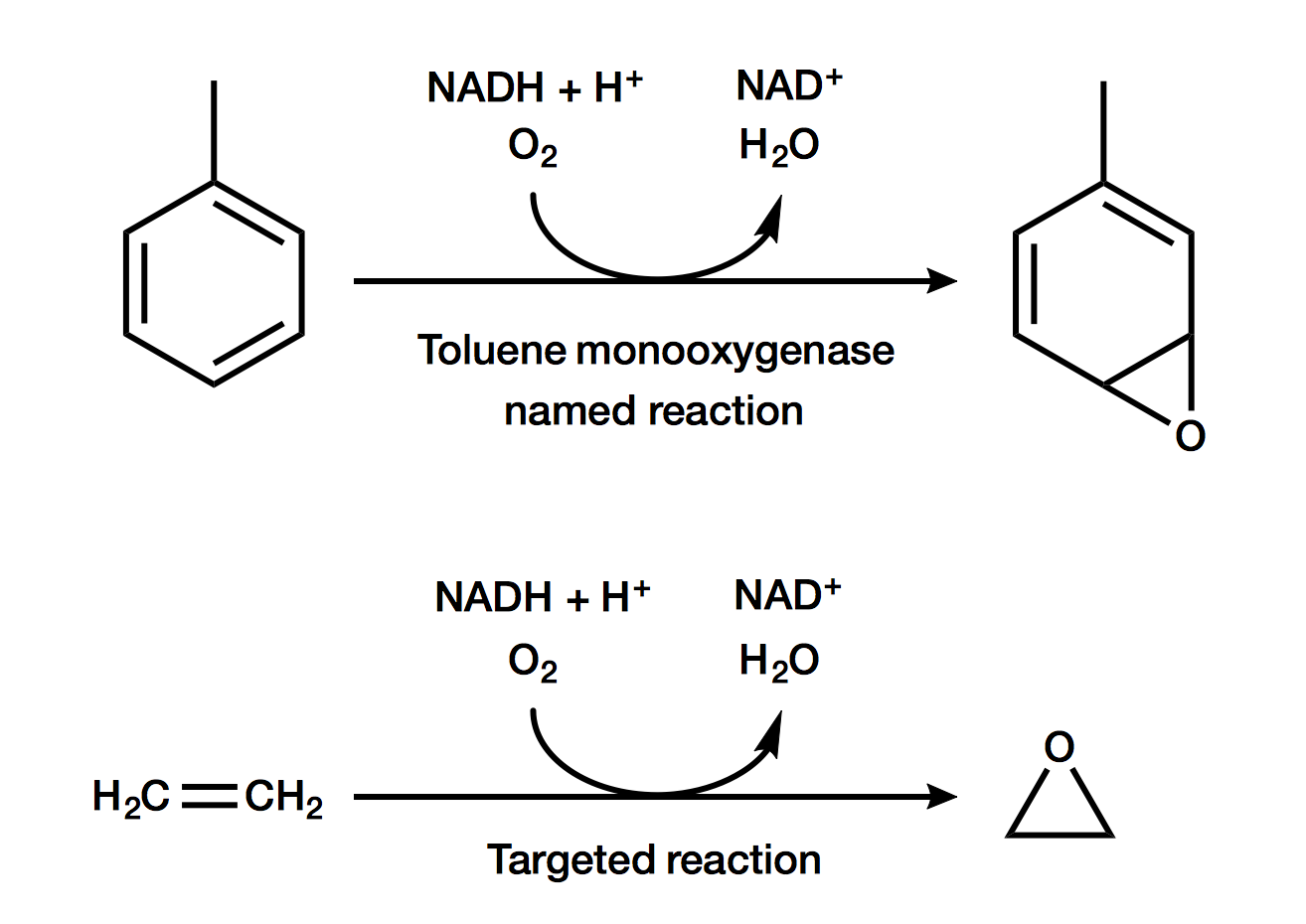


Figure 2. Ethylene remaining after 24 hours of contact with whole cells expressing variants mutants of TOM. Cells suspended in phosphate-buffered saline were sealed in glass vials under 1.5% ethylene atmosphere. After 24 hours, headspace was assayed for ethylene and ethylene oxide by GC-FID.

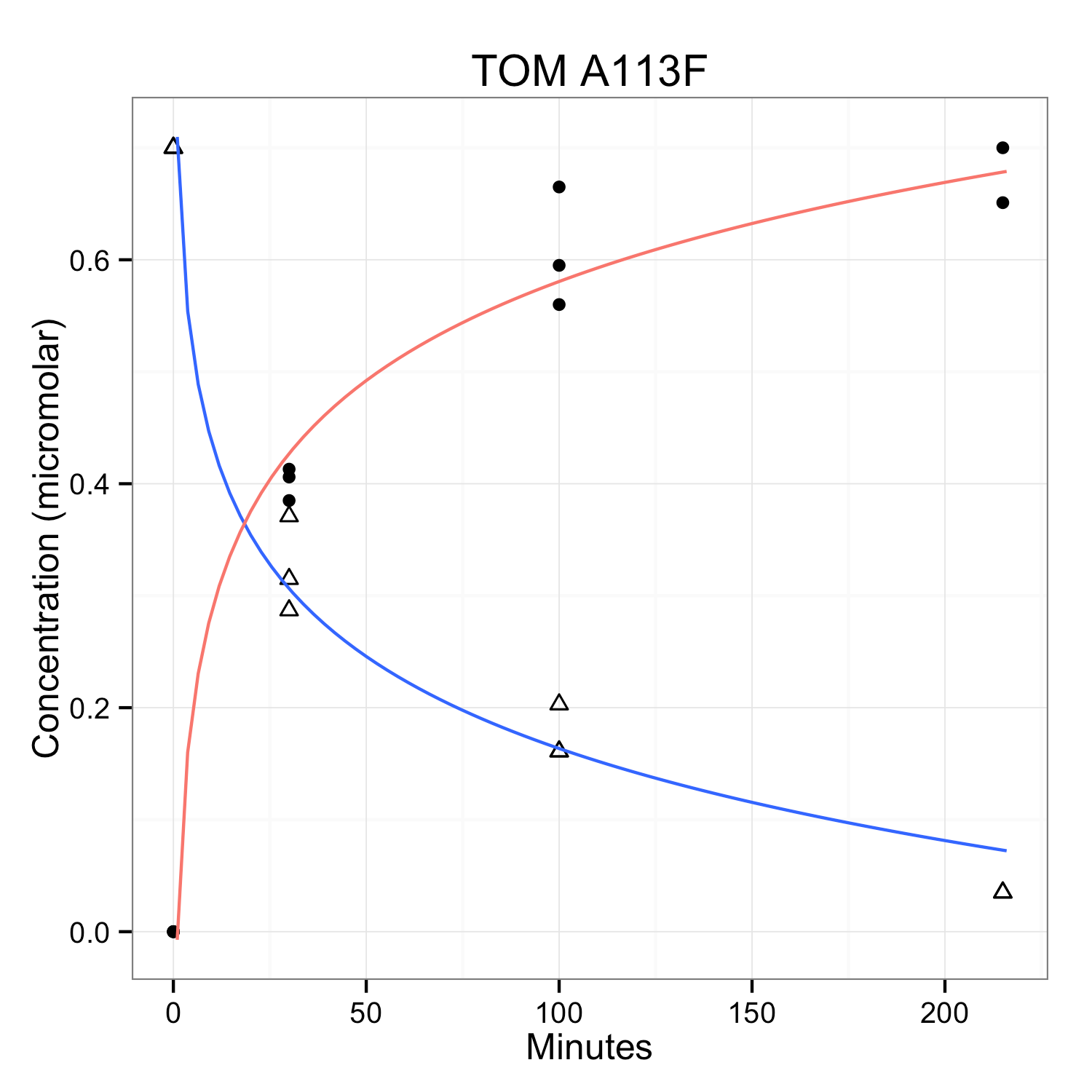
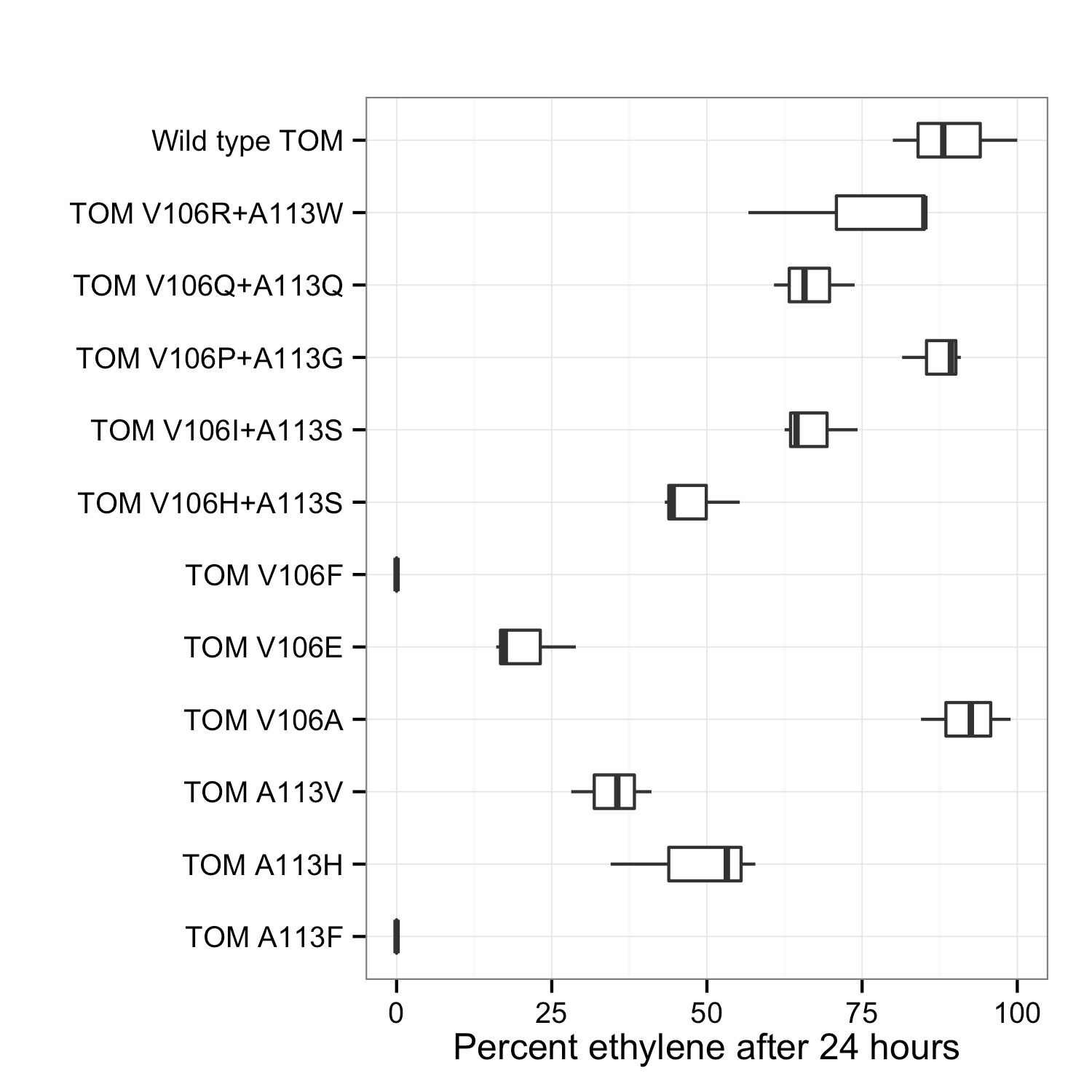


Figure 3. Further characterization of mutant A113F showing the complete conversion of 0.7 mM ethylene to ethylene oxide in under 4 hours. The concentration of ethylene is shown in blue with triangles and the concentration of ethylene oxide is shown in red with dots.

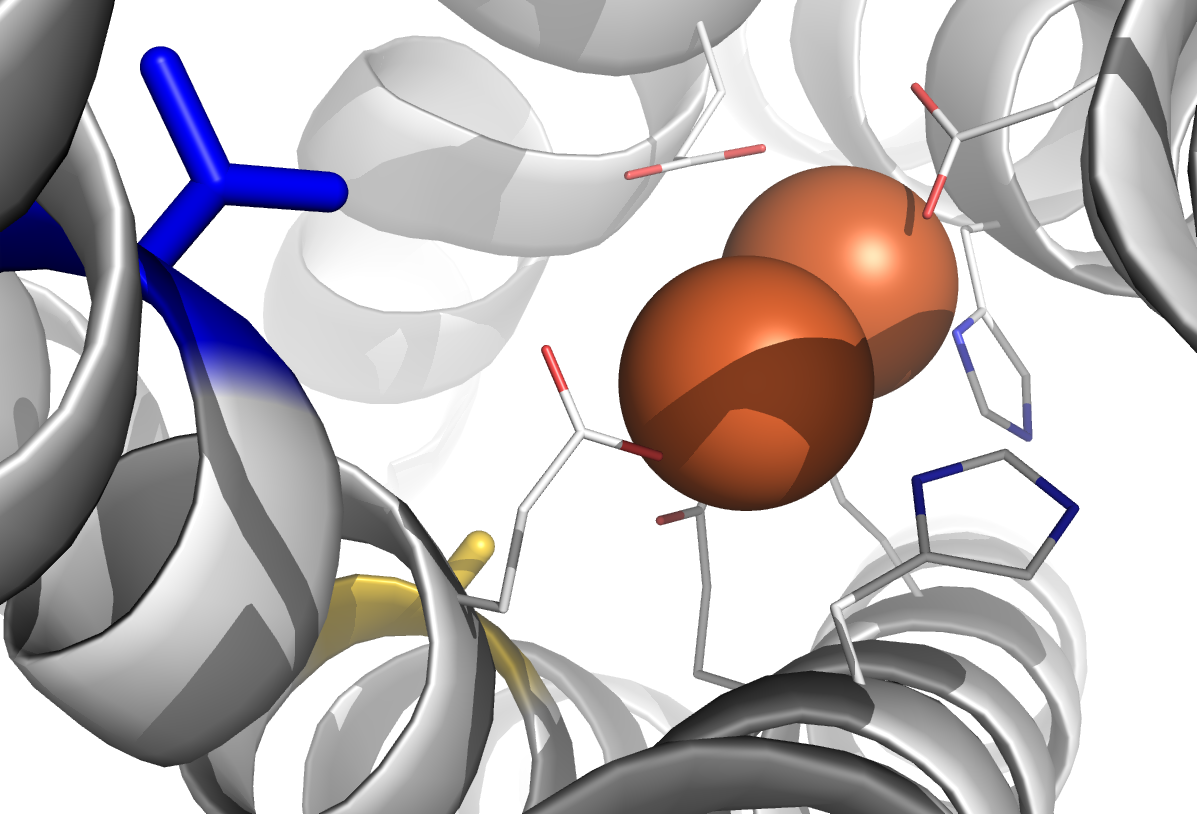
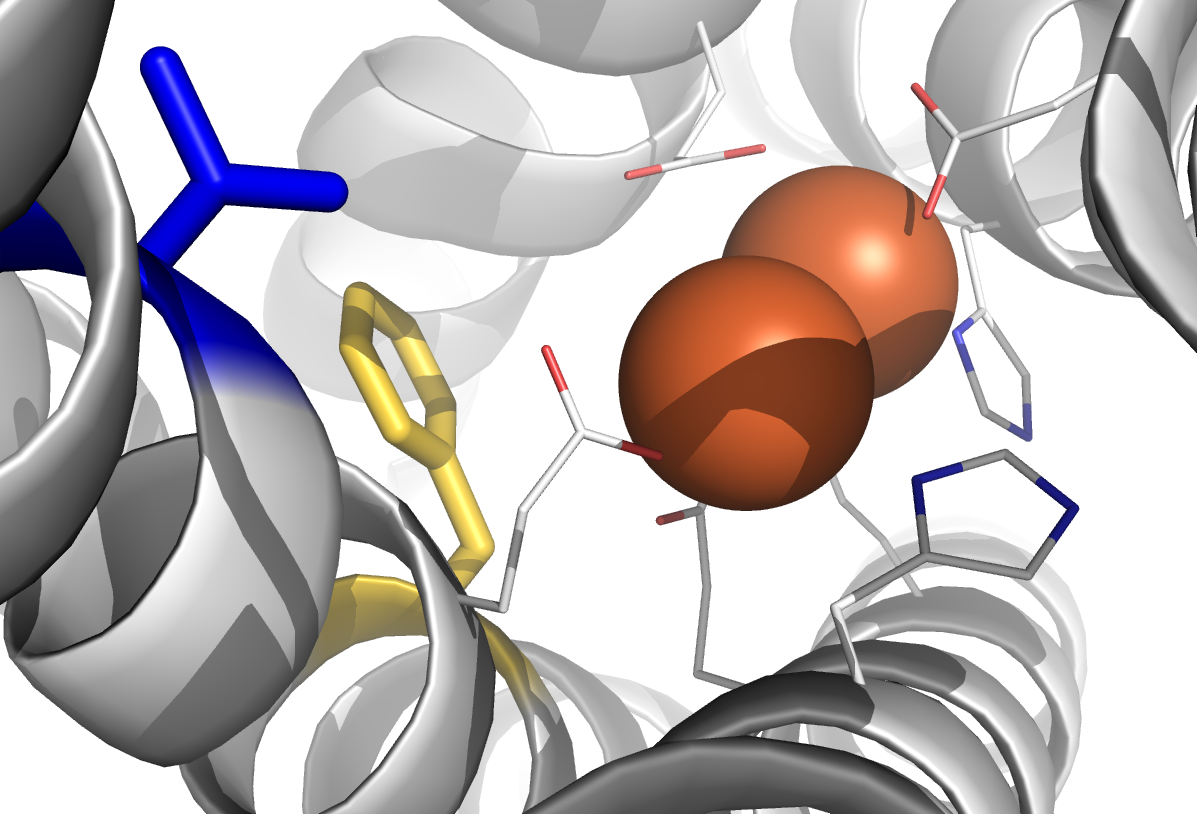
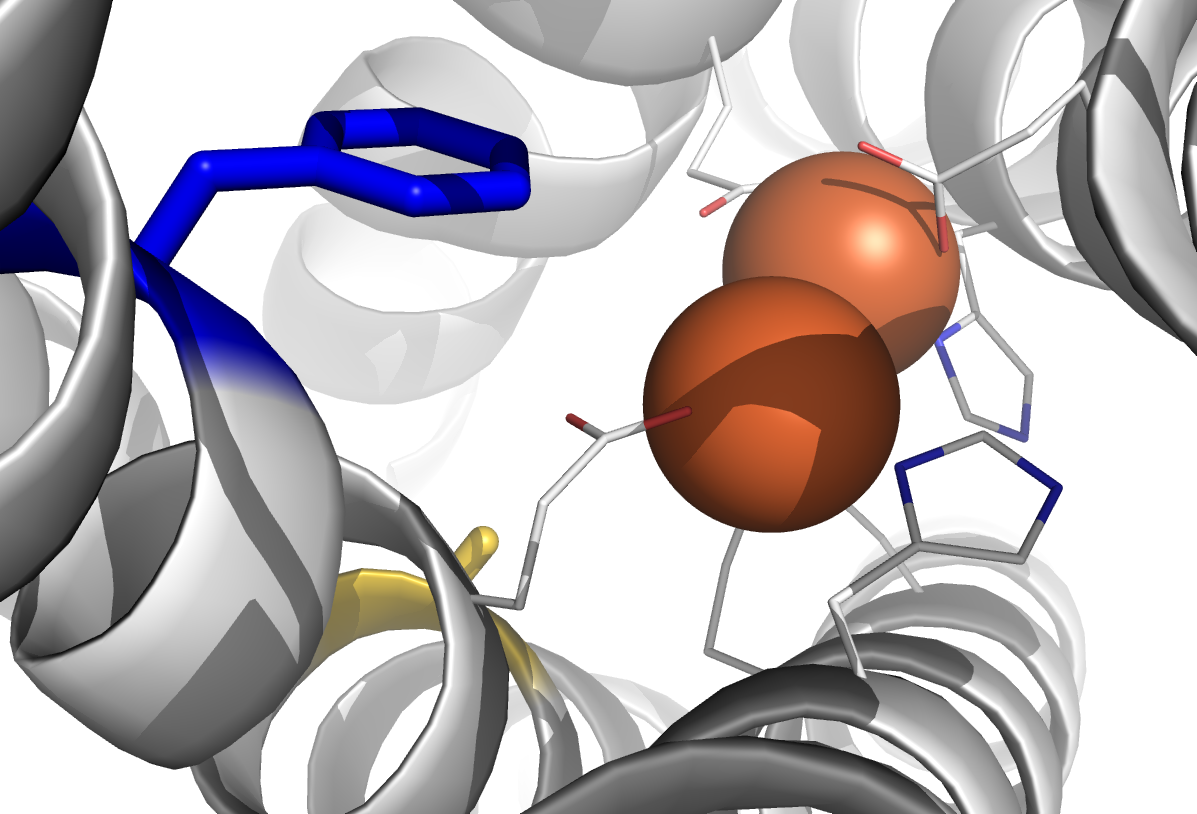


Figure 4. Rosetta comparative models of TOM (left), mutant V106F (center), and mutant A113F (right). Irons (spheres) are shown with coordinating residues. Mutated residues are colored blue (106) and gold (113).