MODULATING FATTY ACID EPOXIDATION *vs* HYDROXYLATION IN A FUNGAL PEROXYGENASE

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Unspecific peroxygenases (UPOs) are fungal counterparts of the cytochrome P450 monooxygenases. Both enzyme types share the ability to perform selective oxygenation reactions, but UPOs present the advantages of using H_2O_2 as co-substrate and are very stable thanks to their secreted nature. Moreover, the *Marasmius rotula* UPO (*Mro*UPO) catalyzes reactions of interest compared with the previously described UPOs, including formation of reactive epoxy fatty acids. To investigate substrate epoxidation, the most frequent positions of oleic acid at the *Mro*UPO heme channel were predicted using binding and molecular dynamics simulations. Then, mutations in neighbor residues were designed aiming at modulating the enzyme epoxidation vs hydroxylation ratio. Both the native (wild-type recombinant) *Mro*UPO and the mutated variants were expressed in *Escherichia coli* as active enzymes, and their action on oleic and other fatty acids was investigated by gas chromatography–mass spectrometry in combination with kinetic analyses.

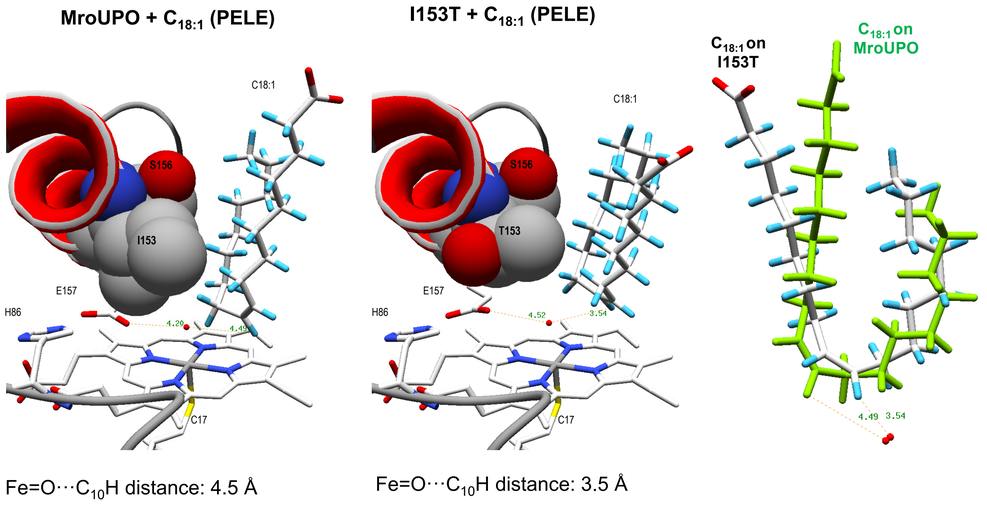

Figure 1. Estimated distances from C10 of oleic acid to the heme iron in *wild-type Mro*UPO and its I153T variant after substrate docking using PELE. The configuration that oleic acid adopts inside the active sites is also compared.

Computational simulations suggested that the positioning of the substrate inside the active site was crucial for the oxygenation of the fatty acid. In fact, oleic acid must bend at the cavity so that the unsaturation may approach the heme cofactor in order to be epoxidized. **Figure 1** illustrates the theoretical positioning of oleic acid in the *wild-type Mro*UPO as well as in its I153T mutated variant. Substitution of Ile by Thr enlarges the channel connecting the solvent and the active site of the enzyme (**Figure 2B**) in such a way that the double bond of oleic acid is closer to the heme than in the *wild-type* enzyme. This would allow better epoxidation and, consequently, would diminish the production of hydroxylated products. In contrast, the introduction of two phenylalanines in the I153F/S156F variant partially occludes the entrance to the active site (**Figure 2C**) thus preventing the bending of the substrate. As a result, this variant, according to the simulations would not epoxidize the oleic acid, but would hydroxylate it.

Experimental work was carried out in order to confirm the hypotheses of the computational simulations. Mutated variants were produced and their activity on oleic acid was tested under several conditions. The products of their reactions were analyzed by gas chromatography coupled to mass spectrometry. The *wild-type* enzyme produced a mixture of epoxidized and hydroxylated products from oleic acid (**Figure 3A**). The variant I153T, in which the substrate was theoretically able to better approach the cofactor, did produce fewer hydroxylated products (**Figure3B**). This meant that this variant is more selective towards the production of epoxides than its *wild-type* counterpart and that the hypotheses derived from the simulations were right. The second mutated variant, I153F/S156F was not able of producing any epoxidized products. On the contrary, it gave rise to a mixture of keto, hydroxy and carboxylic compounds as a result of the hydroxylation of oleic acid (**Figure 3C)**. It is remarkable that, opposite to the other two enzymes, only terminal and subterminal positions were oxygenated. This is in agreement with the computational simulations which anticipated that the oleic acid would not be able to bend inside the enzyme and only the terminal positions would attain catalytically relevant positions.

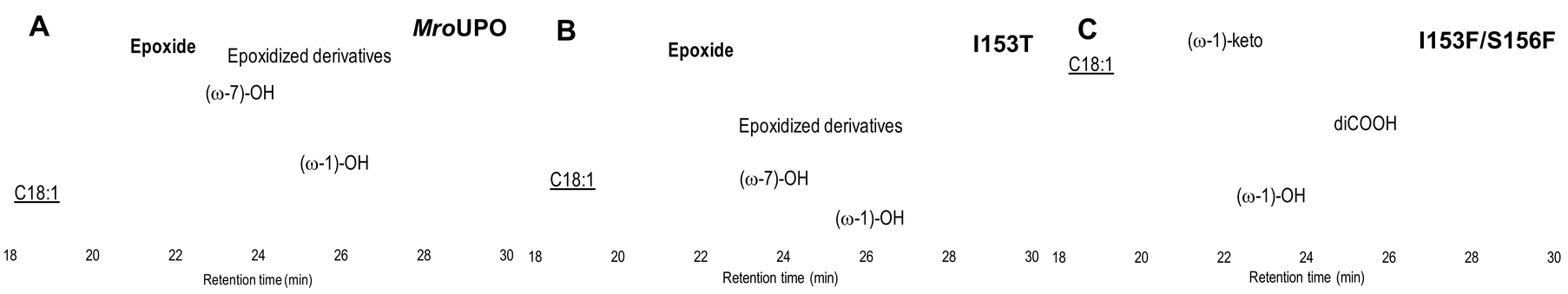

Figure 3. GC-MS analysis of oleic acid (C18:1, underlined) reactions with native *Mro*UPO (**A**) and its I153T (**B**) and I153F/S156F (**C**) variants.

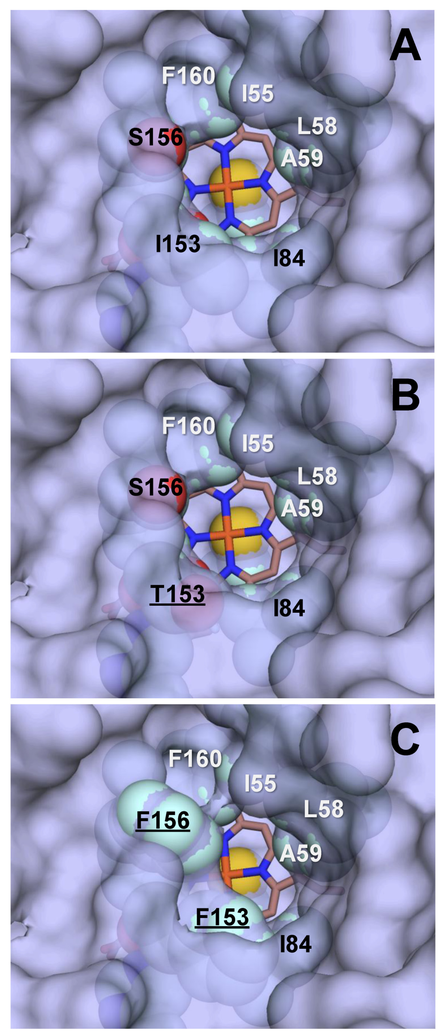

Figure 2. Heme channels after *in silico* mutations. **A** wild-type *Mro*UPO; **B**, I153T variant; **C**, I153F/S156F variant

Conclusions: Based on computational simulations of oleic acid diffusion on the *Mro*UPO crystal structure, some mutations were designed to modulate the epoxidation *vs* hydroxylation activities of the enzyme, an important aspect to produce reactive compounds of industrial interest. Then, *E. coli* expression was used for the first time to produce UPO variants (bearing the previously designed mutations) which were obtained in soluble and active form. Finally, GC-MS analyses of unsaturated fatty-acid reactions with the purified mutated variants confirmed their new/improved catalytic properties. Using this combined approach, we show that the *Mro*UPO epoxidation activity on oleic acid can be removed by the double I153F/S156F mutation and that the epoxidation selectivity of the enzyme can be improved by the I153T mutation reducing the distance between the oleic double bond and the C-I oxygen atom to be transferred to the substrate.

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