Supporting Information for Oxygen Reactivity in Flavoenzymes: Context Matters

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Materials and Methods

Creation of the Lys276Met Fructosamine Oxidase (FAOX) Mutant and its Purification. The Lys276Met FAOX mutant was created using the Stratagene QuickChange II XL mutagenesis kit. It was expressed and purified as described [1].

Creation of Dihydroorotate dehydrogenase (DHOD) Mutants and their Purification. The Class 2 DHOD encoded by the *pyrD* gene from *E. coli* in plasmid *pAG1* was mutated using the Stratagene QuickChange II XL mutagenesis kit. The Class 1A DHOD encoded by the *pyrDa* gene from *L. lactis* in plasmid *pFN1* was mutated using the Stratagene QuickChange II XL mutagenesis kit. Mutant plasmids were transformed into SØ6645 *E. coli* cells, grown, expressed, and purified as described [2 3].

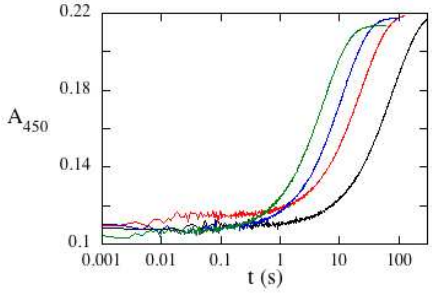
Oxidative Half-Reactions. Enzyme solutions (~40 μM) were made anaerobic in an anaerobic tonometers by repeatedly replacing the atmosphere with argon [4]. Dithionite in a syringe connected to the tonometer was used to titrate the FAOX and DHOD mutants until the enzymes were completely reduced, as judged spectrophotometrically. The wild-type FAOX was reduced using 1 equivalent of substrate as described [5].

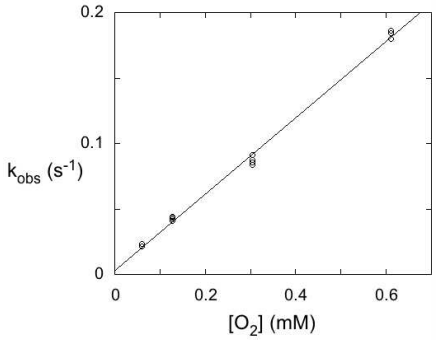
Reduced enzymes were loaded into a Hi-Tech Scientific KinetAsyst SF-61 DX2 stopped-flow spectrophotometer at 4 °C. Solutions of 0.1 M Tris pH 8.0 were bubbled at 25 °C with different concentration of oxygen (5 – 100 %). For higher oxygen concentrations, buffer was bubbled at 4 °C. Oxygen solutions were mixed with anaerobic enzyme and the

flavin absorbance was recorded at 450 nm. Traces at 590 nm were also recorded to determine if semiquinone was present, however none was observed.

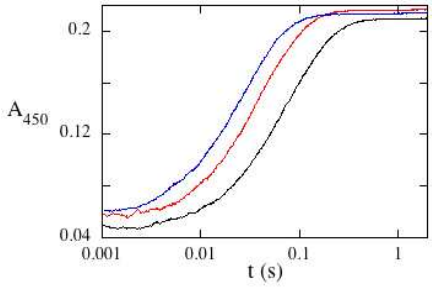
Kinetic traces were fit to a single exponential using Kaleidagraph (Synergy, Inc.). The observed rate constants for flavin oxidation were plotted vs. O_2 concentration and fit to a straight line to obtain the bimolecular rate constant k_{ox}.

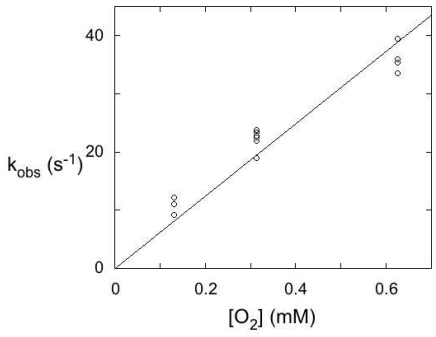
Fructosamine Oxidase Lys276Met



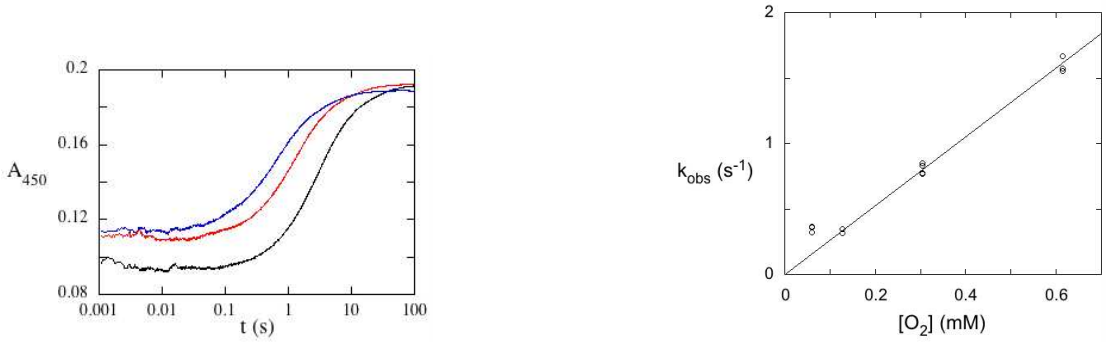


DHOD Class2 Lys66Met

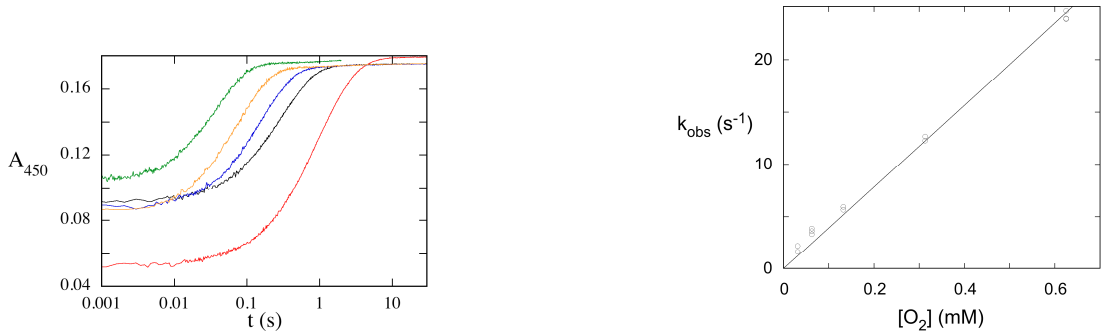




DHOD Class1A Lys43Met



DHOD Class2 His19Asn



DHOD Class2 Arg102Met

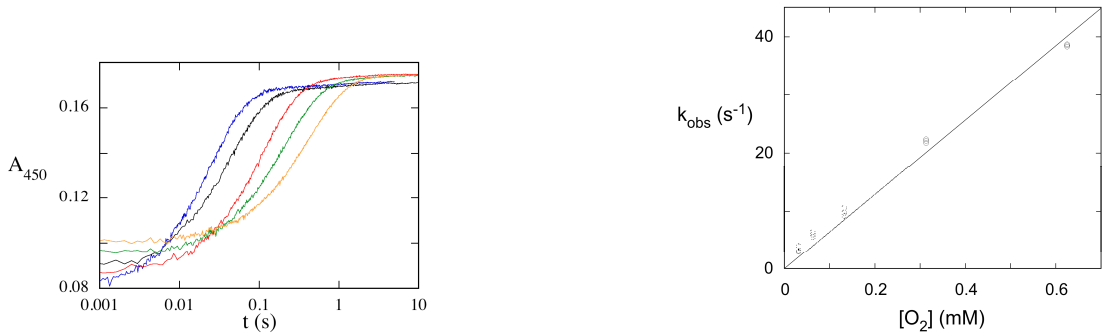


Figure S1. Stopped-flow traces and concentration dependencies of the mutant enzymes.

Superoxide assay. Enzymes were made anaerobic as described previously [4] and reduced with dithionite. The enzymes (~40 μM) in 50 mM phosphate buffer, pH 7.0, were loaded

into a stopped-flow instrument and mixed with anaerobic or aerobic cytochrome C (1 mg/mL) at 4 °C. Anaerobic experiments were done in order to determine the rate of electron transfer directly between the flavoenzyme and the cytochrome. Subsequently including oxygen in the cytochrome syringe allowed the flavoprotein to either make superoxide which could rapidly reduce cytochrome C or to directly form H_2O_2. To monitor the reduction of cytochrome C traces at 550 nm were recorded. The inclusion of O_2 slowed the reduction of cytochrome C for all enzymes indicating that superoxide was not formed. To validate that superoxide was not present, assays were also performed in the presence of superoxide dismutase; this had no effect.

References

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4. Palfey, B. A., Time Resloved Spectral Analysis. In *Kinetic Analysis of Macromolecules*, Johnson, K. A., Ed. Oxford University Press: New York, 2003; pp 203-227.

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