Exploration of single molecule fluorescence in haloperoxidase-like chemistry: hypobromite as the active vehicle of active oxygen

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Introduction

The spatio-temporal resolution and high sensitivity of single-molecule fluorescence spectroscopy (SMFS) provides insight to the deeper secrets in a wide range of systems at the molecular level, previously hidden in ensemble measurements.[1] In particular, SMFS has contributed to the molecular insight of bio- and chemocatalytic processes thanks to the ultrasensitive real–time dynamic studies under in situ conditions.[2] We here adopt the sensitive fluorogenesis strategy for investigating at the molecular level the catalytic action of individual sites of haloperoxidases and its inorganic solid biomimick.

Materials and Methods

The tested catalysts were immobilized on cover glasses; the enzymes were entrapped in a dilute agarose polymer, while the LDH crystals were deposited by spin coating. Subsequently 2 mL of the reactant solution, containing H_2O_2, the APF probe and NH_4Br in MQ-water in concentrations that favor reaction was applied on top of the catalyst-loaded cover glasses and the sample was mounted on an inverted confocal fluorescence microscope.

Results and Discussion

In the presence of halides and H_2O_2 haloperoxidase catalysts are capable of smoothly halogenating unsaturates in benign conditions. When carried out selectively and safely, the reaction is of great appeal in the production of antimicrobial agents, cosmetics, etc. Firstly we studied a *Curvularia verruculosa* bromoperoxidase (BPO) containing a single vanadate (V) active center. Mechanistically, the vanadate binds H_2O_2 yielding an activated peroxovanadate intermediate which oxidizes bromide to hypobromite. Secondly, tungstate-exchanged layered double hydroxides (WO_4^{2-}-LDH), containing a manifold of active surface sites, were studied.[3] As a result of its exchange on the positively charged clay-like surfaces, the tungstate centers exhibit a more than 100-fold activation compared to their homogeneous tungstate analogues, making them a unique biomimick for bromoperoxidase enzymes. Similarly, upon exposure to H_2O_2, WO_4^{2-} forms a peroxo intermediate, which reacts with Br^- to yield HOBr (reaction [1]).

$$H_2O_2 + H^+ + Br^- \rightarrow HOBr + H_2O \quad [1]$$

$$HOBr + HA \rightarrow ABr + H_2O \quad [2]$$

The HOBr generated by both catalysts oxidizes organic compounds (HA in reaction [2]). HOBr is expected to be released in the reaction medium where it can perform its oxidizing activity. However, strong interaction with the catalyst via electrostatic forces with the LDH or specific binding to the enzyme limits the secondary reaction to the catalyst's close surroundings. In the particular case of the enzyme this might give rise to regioselective bromination, which occurs in Nature. To localize the HOBr activity, we made use of a non-fluorescent fluorescein derivative, aminophenyl fluorescein (APF). This probe specifically reacts with hypohalites to form the highly emissive fluorescein with only a limited sensitivity towards other reactive oxygen species. Figure 1 illustrates a fluorescence intensity time trace of a *Curvularia verruculosa* enzyme.[4] Such trace compiles signals corresponding to the formation of single fluorescein molecules. The high signal-to-noise ratio in our single molecule traces is exceptionally high in comparison to literature examples, as will be shown.[1,2] From these data, a time averaged activity can be calculated. Typically, the activity amounts to ~ 1.0 turnovers per second. While for other enzymatic systems, static disorder over several orders of magnitude has been reported due to enzyme dynamics[1], the activity of this BPO population seems to be relatively homogeneous. This is explained by the high stability and robustness of the α-helix rich protein backbone. The large heterogeneity in size and number of immobilized active sites in the WO_4^{2-}-LDH catalysts is reflected in a broad distribution of the time-averaged activity.

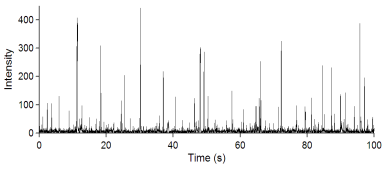

Figure 1. Representative part of intensity time trace for a *Curvularia verruculosa* enzyme.

Even more important than pure turnover counting, is the spatially resolved localization of the oxidation activity - a key issue in haloperoxidase systems: there is a debate whether the active HOBr remains localized on the catalyst or is released in solution. By focusing the laser at various distances of the catalyst, we were able to monitor activity up to at least 1 μm from the crystal surface/enzyme, proving HOBr, after being formed at the catalytic site, diffuses in the bulk of the solution.

Significance

Where such evolution of HOBr has important protective purposes for microorganisms in Nature, it is useful in organic synthesis as well. Indeed, as there is no need for the organic molecule to adsorb at the active site, the catalytic system is free of mass transfer issues allowing the oxidation of bulk molecules. Moreover, oxidation chemistry with HOBr affords interesting stereochemistry that is often opposite to classic oxidation protocols with *e.g.*, peroxide/metal or peracid. Applications in the furan, terpene and phenol chemistry[5] will illustrate the uniqueness of the haloperoxidase system.

References

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