

## Exploration of Single Molecule Events in a Haloperoxidase and Its Biomimic: Localization of Halogenation Activity

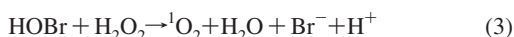
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The spatiotemporal resolution and the ever increasing sensitivity of single-molecule fluorescence spectroscopy (SMFS) give access to insights at the molecular level for a wide range of systems, while such details were previously hidden in ensemble-averaged measurements.<sup>1</sup> In particular, SMFS has provided kinetic and molecular understanding in bio- and chemocatalytic processes thanks to the possibility of performing ultrasensitive real-time dynamic studies under in situ conditions.<sup>2–5</sup> Recently, several strategies have been developed for monitoring catalytic activity using fluorescence microscopy. One of these uses an active site that switches between different fluorescent states during its catalytic cycle.<sup>4</sup> An alternative scheme relies on fluorogenic substrates, thereby bypassing the issue of photobleaching. While a large assortment of fluorogenic probes is available for bulk activity measurements, only a few of them are suitable for SMFS. Exploration of single molecule events with such probes in catalytic systems has thus so far been limited.<sup>3,6,7</sup> This work introduces a simple assay to monitor individual oxidation events at varying distances from a haloperoxidase biocatalyst and its inorganic biomimic.

In the presence of halides and H<sub>2</sub>O<sub>2</sub>, haloperoxidases form oxidizing halonium species which can be used in the synthesis of halogenated organic compounds, for instance in the production of antimicrobial agents, cosmetics, etc.<sup>8,9</sup> As a first catalyst we selected a *Curvularia verruculosa* bromoperoxidase (BPO) containing a vanadate (V) complex at the active site. This vanadate center binds H<sub>2</sub>O<sub>2</sub> resulting in an activated peroxo intermediate, which then oxidizes bromide to hypobromite (reaction 1).<sup>8</sup> This enzyme shares a large structural homology with BPO from *Curvularia inaequalis*, the X-ray fine structure of which has been resolved.<sup>10</sup> Second, as an inorganic haloperoxidase mimic, we used a disk-shaped tungstate-exchanged layered double hydroxide crystal (ø ± 400–500 nm; aspect ratio of ±15) with a composition of Ni<sub>0.64</sub>Al<sub>0.36</sub>(OH)<sub>2</sub>[(WO<sub>4</sub><sup>2–</sup>)<sub>0.045</sub>(NO<sub>3</sub><sup>–</sup>)<sub>0.27</sub>]·0.6H<sub>2</sub>O, further denoted as WO<sub>4</sub><sup>2–</sup>-LDH. Each crystal contains approximately a few million tungstate ions (see Supporting Information (SI)), exchanged mainly at its exterior surface. The immobilized active tungsten is 2 orders of magnitude more active than similar homogeneous tungstate compounds and uses the same mechanism as BPO.<sup>11</sup>



Hypobromite generated by both catalysts either rapidly brominates organic compounds (HA in reaction 2)<sup>12</sup> or decomposes H<sub>2</sub>O<sub>2</sub> into <sup>1</sup>O<sub>2</sub> and water (reaction 3).<sup>13</sup>

HOBr is expected to migrate into the reaction medium where it performs the bromination. However, it cannot be excluded that strong interactions with the catalyst, e.g., via electrostatic interaction of OBr<sup>–</sup> with the LDH or via specific binding with the protein matrix of the enzyme, confine the actual halogenation to the active site's close surroundings. Although under debate, the latter confinement may be the origin of stereo- and regioselective bromination of natural compounds.<sup>14</sup>

We here introduce a nonfluorescent fluorescein derivative, aminophenyl fluorescein or APF (see SI for synthetic procedure) to localize the bromination. This probe reacts with a high specificity and high rate with hypohalites to form the strongly emissive fluorescein (see Figures S1 and S2), with only a limited sensitivity to other reactive oxygen species (ROS).<sup>15</sup> Thus oxidation of APF by H<sub>2</sub>O<sub>2</sub> or <sup>1</sup>O<sub>2</sub> can be neglected.

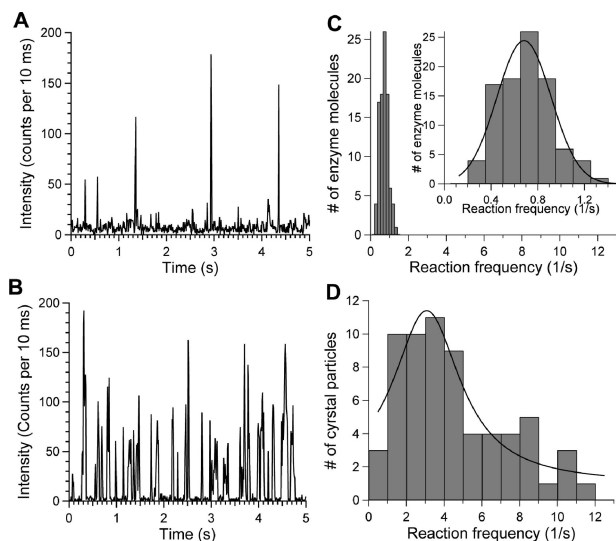
The catalysts were immobilized on a cover glass: the enzymes were entrapped in a dilute agarose polymer (see Supporting Information),<sup>4,7</sup> while the LDH crystals were deposited by spin coating from a dilute suspension directly on the cover glass.<sup>5,16</sup> Subsequently 2 mL of the reactant solution were added, containing H<sub>2</sub>O<sub>2</sub>, the APF probe, and NH<sub>4</sub>Br in MQ-water in concentrations that favor the bromination (2) over the decomposition (3).<sup>13</sup> The samples were mounted on an inverted confocal fluorescence microscope (Figure S3), and the catalyst's positions were identified as fluorescence hotspots in the image obtained by scanning the sample (Figure S4). Subsequently time transients of the fluorescence intensity at these hotspots were recorded to monitor single reaction events (Figures 1A,B and S5).

The signal-to-noise ratio in the intensity–time transient of the *Curvularia verruculosa* enzyme (Figure 1A, S/N > 50; see Figure S6) proves the exceptional sensitivity of the APF probe to monitor hypohalite at the single molecule level. For the first time the turnover frequency of single haloperoxidase enzyme molecules can be estimated. Under the conditions applied (100 mM NH<sub>4</sub>Br, 50 nM APF, 100 nM H<sub>2</sub>O<sub>2</sub>) activity values of 0.7 ± 0.23 s<sup>–1</sup> were obtained (Figure 1C). The narrow spread in activity among individual enzyme molecules, compared to other enzymatic systems often having a spread of more than 1 order of magnitude,<sup>17</sup> accords with the enzyme's rigidity, which is due to the high content of α-helices in the protein's secondary structure.<sup>10,18</sup>

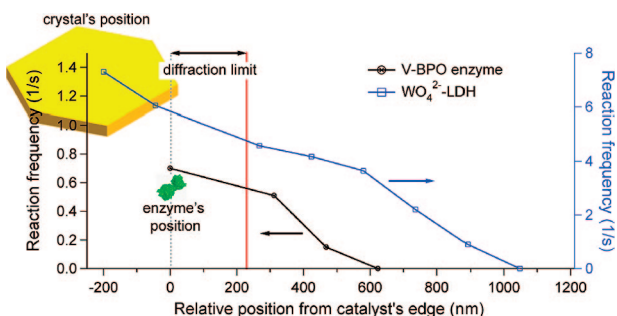
A similar analysis of individual WO<sub>4</sub><sup>2–</sup>-LDH crystals is shown in Figure 1B and D. The number of events per unit of time (between 1 and 12 per second) is 1 order of magnitude larger than the one calculated for the single enzymes due the higher concentration of active sites within the probed area. The broad activity distribution is immediately clear from the histogram and reflects the size distribution of the catalytic crystals.

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**Figure 1.** (A and B) Representative parts of an intensity–time trace for (A) a *Curvularia verruculosa* enzyme and (B) a  $\text{WO}_4^{2-}$ -LDH crystal, measured at a laser power of  $5\ \mu\text{W}$  and binned at 10 ms. (C and D) Histograms of the time-averaged activities in traces of the enzymes,  $n = 94$  (C) and the  $\text{WO}_4^{2-}$ -LDH crystals,  $n = 65$  (D). The inset in (C) shows a zoom of the histogram with a Gaussian fit (solid line;  $\mu = 0.7\ \text{s}^{-1}$  and  $\sigma = 0.23\ \text{s}^{-1}$ ).



**Figure 2.** Time-averaged fluorescein formation rates recorded at various positions with respect to the enzyme (black curve) or the  $\text{WO}_4^{2-}$ -LDH (blue curve). The distances are measured from the enzyme's position (point source of HOBr generation) or from the crystal edge (gray dotted line). The red curve indicates the diffraction limit from this edge.

In addition to single reaction counting, the *in situ* APF assay allows observation in a unique, spatially resolved way of the chemical transformations performed by the halonium species. Trapping with APF is a method used to follow the migration of hypobromite from the catalytic center (enzyme or crystal surface) into the bulk solution. Therefore, intensity–time transients were recorded at different laser positions with respect to the locus of the HOBr generation (see Figures 2 and S8). The activities derived from these traces are plotted as a function of laser position in Figure 2 for both catalytic systems. The spatial resolution of the experiment is determined by the diffraction limit ( $\pm 230\ \text{nm}$ ) indicated by the red vertical line in Figure 2. Only reactions observed outside this region can be assigned unambiguously to reactions of hypobromite in the bulk solution. As can be derived from the plot, both catalytic systems show fluorescein formation in the bulk solution. Indeed, only in very few cases regio- or stereoselectivity was observed for the related *Curvularia inaequalis* haloperoxidase in halogenation of organic compounds,<sup>19</sup> and this is in line with the enzyme's functional roles in its host organism such as the production of free ROS as protective and metabolic agents.<sup>8,18</sup>

The  $\text{WO}_4^{2-}$ -LDH crystal clearly provides a higher hypobromite flux which reaches over 800 nm into the bulk solution in the applied

conditions. Thus, the spatial resolution obtained with this fluorogenic probe based approach allows mapping of the hypobromite flux from such a highly active crystal. The freely diffusing hypobromite can be seen as a vehicle transporting highly activated oxygen generated at the surface from the mild hydrogen peroxide into the bulk solution. There it can freely oxidize or brominate bulky substrates which usually have a poor access to surface-bound reactive species such as peroxo-titanium in silica which are common in oxidation chemistry.<sup>20</sup>

In summary, we present a new fluorescence microscopy based method to study the formation of HOBr using the fluorogenic substrate APF in haloperoxidase type reactions performed by a haloperoxidase enzyme and  $\text{WO}_4^{2-}$ -LDH crystals.

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**Supporting Information Available:** Figures S1–S10, supporting methods, and data analysis. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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