Supporting information

# Single-Molecule Enzymatic Conformational Dynamics: Spilling Out the Product Molecules

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HRP C is one of the most abundant isoenzyme of Horse radish peroxides, and HRP C comprises 308 amino acid residues, a prosthetic group (heme), eight carbohydrate side chains and two calcium ions, with the overall molecular mass close to 44,000 Dalton. The three-dimensional structure of the enzyme and its catalytic intermediates, mechanisms of catalysis, and the function of specific amino acid residues have been well studied and reviewed in the literatures[1-3]. Our focus in this paper is about the conformational dynamics of the enzyme in the product releasing process of an enzymatic reaction cycle. To facilitate the overall understanding of the conformational dynamics of the HRP, we highlight the catalytic mechanism and the crystal structure from the previous literatures[1-4].

1. The catalytic mechanism of HRP C

HRP C is able to catalyze the oxidation of a wide variety of substrates, such as aromatic amines, indoles, phenols, and sulfonates, by hydrogen peroxide. The oxidation reactions can be summarized by Eq. (1), in which $AH_2$ represents a reducing substrate, and $AH^{\bullet}$ a free radical product.

$$H_2O_2 + 2AH_2 \rightarrow 2AH^{\bullet} + 2H_2O \qquad (1)$$

There exist 4 intermediate states of horseradish peroxidase during the catalytic cycle (Figure 1). In the first step, the ferric ground state of the enzyme is oxidized by hydrogen peroxide to yield a high oxidation state intermediate known as compound I, which is a oxoferryl porphyrin π-cation radical with two oxidation state equivalents above the ferric ground state. Then compound I can oxide the reducing substrates with a single electron process, meanwhile the compound I has been reduced to a second high oxidation state intermediate known as compound II, which is only one oxidation state equivalent above the ferric ground state. The compound II continue to oxide the substrates with single electron process, and back to the ground state. On the other side, $H_2O_2$ can convert Compound II to an inactive form, Compound III, also called oxyperoxidase, and enhance the reversion of Compound III back to ground-state HRP, which again participates in the peroxidase catalytic cycle.

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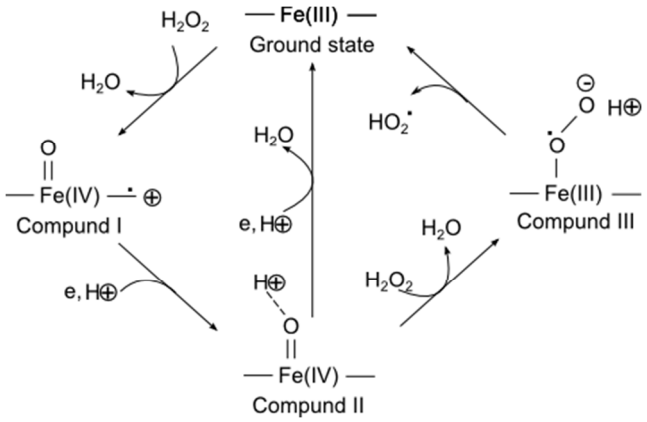


Figure S1 The catalytic cycle of horseradish peroxidase with Hydrogen peroxide

Although the catalysis of HPR with $H_2O_2$ has been extensively studied[2,3,5,6], there are still debates on the electron transfer mechanism between the substrates and the enzymes, i.e. the product is generated through a two-electron-oxidation process discussed above (Figure S1) or through a radical reaction after a one-electron-oxidation.[7-11] Recently, an enzyme-independent dismutation reaction of two radical molecules has been proposed to support the one electron transfer from a single substrate to enzyme, based on the statistics of single HRP molecule reactions in femtoliter chambers[4]. In their proposed mechanism, HRP firstly oxidizes Amplex Red to non-fluorescent radical intermediates, which subsequently undergo an enzyme independent dismutation reaction to form fluorescent resorufin. However, the widely accepted fluoregenic enzymatic reaction mechanism (Figure S1) is in difference from this recently proposed mechanism. We are not in a position to prove one way or another in our current work as it is beyond the

scope of our project. Nevertheless, in our experiments, the products of resorufin molecules are delivered from the enzyme active site one by one as the turnovers shown in Figure 2 of our paper, which is consistent with the HRP-catalyzed oxidation of dhRh123 reaction, the widely accepted two-electron oxidation mechanism (Figure S1). If the product resorufin can only be generated by the enzyme-independent dismutation reaction of two radical molecules, this must be happen outside of the enzyme, since the substrate and the enzyme assemble 1:1 complex[3,5,6]. It is impossible to see the fluorescence turnovers happen at the low concentration of the products, especially at the beginning of the enzymatic reaction. In this assay, the resorufin delivered by HRP may come from the further reaction of Amplex red radical intermediate with the HRP intermediate compound II[9,11], which is still enzyme-dependent. Though we cannot give the reaction rate based on the two-electron transfer process, we can confirm that there exists the two-electron transfer from a single Amplex red to enzyme HRP besides the enzyme-independent dismutation reaction of two radical molecules.

2. The Crystal structures of HRP C

There are two domains in the crystal structure of HRP C, the distal and the proximal, between them the heme group is located (Figure S2). The heme group is attached to the enzyme through a coordinate bond between the His170 side-chain Nε2 atom (located in the proximal domain) and the heme iron atom. Here Asp247 carboxylate side-chain helps to control imidazolate character of His170 ring by an N-H hydrogen bond. Two $Ca^{2+}$ ions, both with seven-coordinate in distal domain and proximal domain respectively, could keep thermal stability and enzymatic activity.

Aromatic substrates form stable, reversible1:1complexes with HRP through both hydrogen-bonded and hydrophobic interactions at the distal side of the heme plane. The amino acid residues Arg38 and His42 play the roles in binding and stabilization of aromatic substrates, meanwhile the residues also play the roles in the formation and stabilization of compound I of the oxidation state intermediate. The oxidation state intermediates may include, for example, that (1) the ferryl oxygen of compound I is hydrogen bonded to Arg38; (2) a water molecule is hydrogen bonded to both Arg38 and His42; and (3) His42 accepts the proton from $H_2O_2$ during the formation of compound I. The oxidations of aromatic substrates happen at the exposed heme edge, a region comprising the heme methyl $C_{18}$ and heme meso $C_{20}$ protons. However, the access of substrate to the oxoferryl centre of HRP C appears to be hindered by the local amino acid residue Phe41. Asn70 of the distal domain maintains basicity of His42 side-chain through hydrogen bond from Asn70 amide oxygen to His42 imidazole NH. The binding site is a relatively mobile

region. The Phe68 side chain operates as a "lid" for the donor molecule binding site, which could re-orientate to accommodate the larger substrate in the complex compared to native enzyme[12].

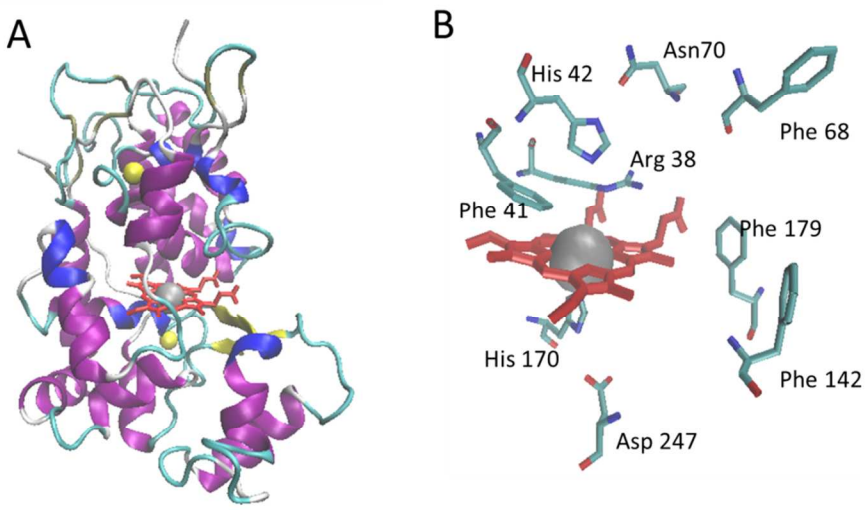


Figure S2 (A) X-ray crystal structure of horseradish peroxidase isoenzyme C (PDB ID 1H5A). The heme group (in red) is located between the distal and proximal domains; calcium atoms are shown as yellow spheres; Fe atom is shown as silver sphere. (B) Key amino acid residues in the heme-binding region of HRP C.

3. Movie of single-molecule fluorogenic turnovers of a single HRP enzyme

Single-molecule enzymatic reaction assay was performed using a Total Internal Reflection Fluorescence Microscopy Imaging-Guided Confocal Fluorescence Spectroscopy (TIRFM-CFS) system built in our lab[13]. The single-molecule tethered enzymes with activity under enzymatic reaction condition on a coverglass are typically first recorded and identified based on the imaging movie from the measurements of total internal reflection fluorescence imaging mode before the enzymatic reaction dynamics and the product releasing dynamics of the identified enzyme is further analyzed by single-molecule photon stamping spectroscopy mode to record the time trajectories of fluorescence intensity, lifetime, and anisotropy simultaneously. The movie was recorded under the condition of the enzymatic reaction in PBS buffer (pH 7.4) solution with 200nM Amplex Red and 2mM $H_2O_2$. In the view field of about 22.5 um X 22.5 um, there is only one enzyme, and the enzyme produced multiple turnover events, releasing fluorogenic enzymatic reaction product, resorufin, one by one.

4. Fluorogenic assay results of tethered single-molecule HRP enzymes

Figure S3 shows additional results of our multiple parameter analysis of the fluorogenic enzymatic reaction turnover events of a single tethered HRP molecule. The correlation distribution (Figure S3 B) of lifetime and anisotropy also shows similar multiple domains in the selected segments (Figure 3S A).

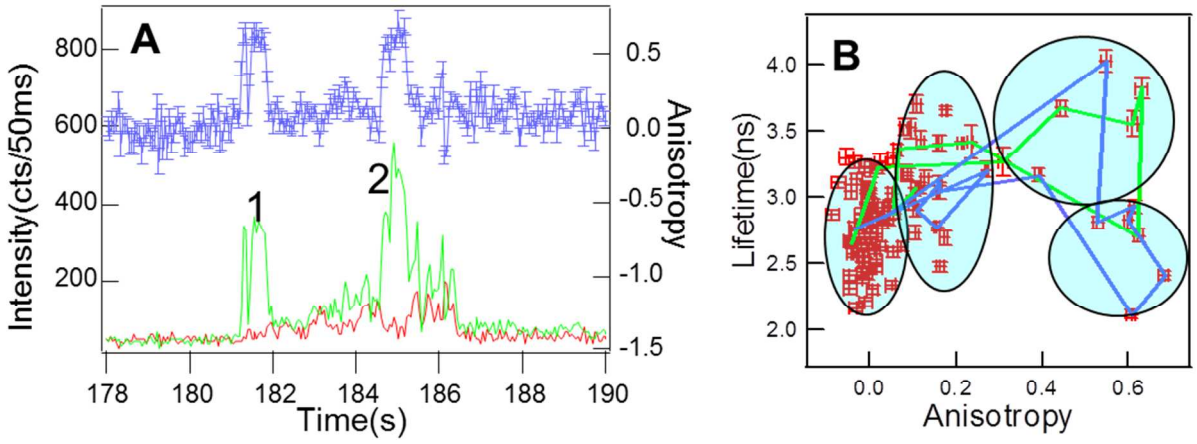


Figure S3 **(A)** Anisotropy and polarization intensity fluctuation trajectories recorded from a single tethered HRP molecule under a fluorogenic enzymatic reaction. Intensity trajectory from the perpendicular component relative to the polarization of excitation (red); Intensity trajectory from the parallel component relative to the polarization of excitation (green); Anisotropy trajectory (blue( deduced from the pair of polarization intensity trajectories. **(B)** Correlation between lifetime and anisotropy of single-molecule enzymatic reaction product turnover and releasing events. The imposed green lines denote a typical burst event at the spike1 in A, and the imposed blue lines denote a typical burst event at the spike2 in A.

**References**


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