J|A|C|S ARTICLES

Published on Web 04/01/2009

Mechanistic Aspects of Horseradish Peroxidase Elucidated through Single-Molecule Studies

Hans H. Gorris† and David R. Walt*

Department of Chemistry, Tufts University, Medford, Massachusetts 02155

Received February 4, 2009; E-mail: david.walt@tufts.edu

Abstract: Many individual horseradish peroxidase (HRP) molecules were isolated and observed simultaneously by fluorescence microscopy in an array of 50 000 femtoliter chambers chemically etched into the surface of a glass optical fiber bundle. The substrate turnovers of hundreds of individual HRP molecules were readily analyzed, and the large number of molecules observed provided excellent statistics. In contrast to other enzymes used for single-molecule studies, the rates of product formation in the femtoliter array were, on average, 10 times lower than in bulk solution. We attribute this phenomenon to the particular redox-reaction mechanism of HRP that involves two separate steps of product formation. HRP first oxidizes fluorogenic substrate molecules like Amplex Red to radical intermediates. Two radical molecules subsequently undergo an enzyme-independent dismutation reaction, the rate of which is decreased when confined to a femtoliter chamber resulting in less product. This two-step reaction mechanism of the widely used Amplex Red, as well as other fluorogenic substrates, is often overlooked. The mechanism not only affects single-molecule studies with HRP but also bulk reactions at low substrate turnover rates.

Introduction

Single-molecule fluorescence microscopy has led to insights into the kinetic behavior of enzymes that cannot be obtained from conventional bulk solution studies. Enclosing a population of hundreds of single enzyme molecules in a large array of femtoliter reaction chambers and observing the individual molecules by fluorescence microscopy is a powerful new technique for fundamental mechanistic studies.[1,2] Using a femtoliter array of microwells on a glass optical fiber array, we have recently analyzed the stochastic inhibition of individual β-galactosidase molecules by D-galactal.[3] Previous studies have shown that single molecules of β-galactosidase,[4,5] lactate dehydrogenase,[6] and chymotrypsin[7] are distinguished by heterogeneous and long-lived catalytic rates. The reaction rates of alkaline phosphatase, however, were only heterogeneous when the mammalian enzyme was used[8] but homogeneous when the highly purified bacterial enzyme was used.[9] Individual enzyme molecules such as β-galactosidase,[10] cholesterol oxidase,[11] lipase,[12] or λ-exonuclease[13] also undergo dynamic substrate turnover fluctuations in sequential catalytic cycles. The static and dynamic heterogeneity of single enzyme molecules, attributed to different conformational states of the enzyme, are obscured in traditional bulk methods.

Horseradish peroxidase (HRP) is a monomeric enzyme, which makes it particularly attractive for single-molecule studies as substrate turnover events originate only from a single catalytic site. HRP—isolated from the roots of *Armoracia rusticana*—is a classic example of an enzyme redox catalytic reaction. HRP is a glycosylated protein of ~44 kDa and can be subdivided into at least seven distinct isoenzymes with similar activities.[14] Although many thousands of papers can be found in the scientific literature,[15] mainly about the most abundant isoenzyme C, only recently have the crystal structure[16] and a high-resolution description of the intermediates in the catalytic cycle of the enzyme[17] been elucidated. HRP catalyzes the oxidation of a broad range of substrates with its prosthetic heme group. Hydrogen peroxide (H_2O_2) initiates the peroxidase catalytic cycle (Figure 1) by a rapid (9×10^6 M^{-1} s^{-1}) two-electron oxidation of the ferric ground-state HRP to Compound I,[18] which is a porphyrin-π-cation radical. Two successive single-

† Present address: Institute of Analytical Chemistry, Chemo- and Biosensors, University of Regensburg, 93040 Regensburg, Germany.

(1) Rondelez, Y.; Tresset, G.; Tabata, K. V.; Arata, H.; Fujita, H.; Takeuchi, S.; Noji, H. *Nat. Biotechnol.* **2005**, *23*, 361–365.
(2) Rissin, D. M.; Walt, D. R. *Nano Lett.* **2006**, *6*, 520–523.
(3) Gorris, H. H.; Rissin, D. M.; Walt, D. R. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 17680–17685.
(4) Rissin, D. M.; Gorris, H. H.; Walt, D. R. *J. Am. Chem. Soc.* **2008**, *130*, 5349–5353.
(5) Craig, D. B.; Nachtigall, J. T.; Ash, H. L.; Shoemaker, G. K.; Dyck, A. C.; Wawrykow, T. M.; Gudbjartson, H. L. *J. Prot. Chem.* **2003**, *22*, 555–561.
(6) Xue, Q.; Yeung, E. S. *Nature (London)* **1995**, *373*, 681–683.
(7) Lee, A. I.; Brody, J. P. *Biophys. J.* **2005**, *88*, 4303–4311.
(8) Craig, D. B.; Arriaga, E. A.; Wong, J. C. Y.; Lu, H.; Dovichi, N. J. *J. Am. Chem. Soc.* **1996**, *118*, 5245–5253.
(9) Polakowski, R.; Craig, D. B.; Skelley, A.; Dovichi, N. J. *J. Am. Chem. Soc.* **2000**, *122*, 4853–4855.
(10) English, B. P.; Min, W.; van Oijen, A. M.; Lee, K. T.; Luo, G.; Sun, H.; Cherayil, B. J.; Kou, S. C.; Xie, X. S. *Nat. Chem. Biol.* **2006**, *2*, 87–94.
(11) Lu, H. P.; Xun, L.; Xie, X. S. *Science* **1998**, *282*, 1877–1882.
(12) Velonia, K.; Flomenbom, O.; Loos, D.; Masuo, S.; Cotlet, M.; Engelborghs, Y.; Hofkens, J.; Rowan, A. E.; Klafter, J.; Nolte, R. J. M.; de Schryver, F. C. *Angew. Chem., Int. Ed.* **2005**, *44*, 560–564.
(13) van Oijen, A. M.; Blainey, P. C.; Crampton, D. J.; Richardson, C. C.; Ellenberger, T.; Xie, X. S. *Science* **2003**, *301*, 1235–1238.
(14) Shannon, L. M.; Kay, E.; Lew, J. Y. *J. Biol. Chem.* **1966**, *241*, 2166–2172.
(15) Veitch, N. C. *Phytochemistry* **2004**, *65*, 249–259.
(16) Gajhede, M.; Schuller, D. J.; Henriksen, A.; Smith, A. T.; Poulos, T. L. *Nat. Struct. Biol.* **1997**, *4*, 1032–1038.
(17) Berglund, G. I.; Carlsson, G. H.; Smith, A. T.; Szoke, H.; Henriksen, A.; Hajdu, J. *Nature (London)* **2002**, *417*, 463–468.

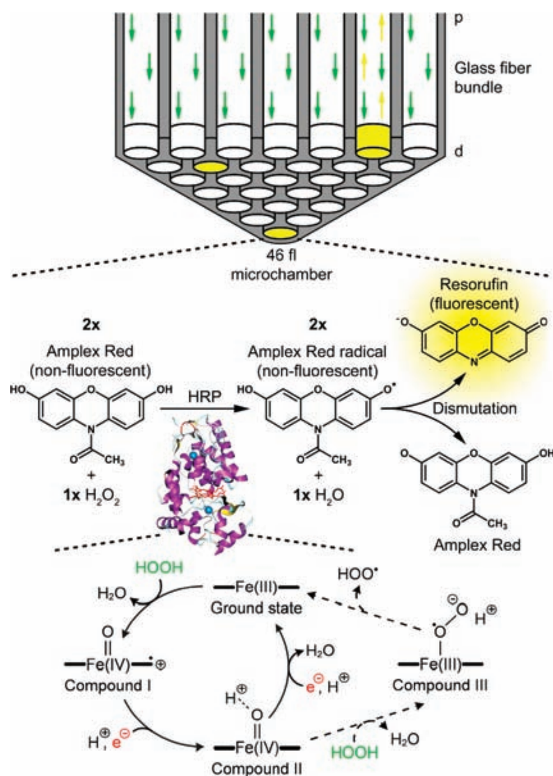

Figure 1. Substrate turnover of single HRP molecules in femtoliter chambers. An array of 50 000 chambers with a volume of 46 fL was etched uniformly into the distal surface (d) of a glass optical fiber bundle. The fiber bundle was mounted on a custom-built upright epifluorescence microscope, and a droplet of enzyme and substrate solution was placed on a silicone gasket. The enzyme concentration in the droplet was calculated according to the Poisson equation such that each chamber would contain either one or zero enzyme molecules. The chambers were sealed by pressing the silicone gasket (not shown) vertically against the distal end of the fiber bundle, and product formation in each chamber was monitored through the proximal end (p) of the fiber bundle. HRP (purple) catalyzes a one-electron oxidation (red) of nonfluorescent Amplex Red to the nonfluorescent Amplex Red radical. The formation of fluorescent resorufin (yellow) from two Amplex Red radicals is an enzyme independent dismutation reaction. The overall reaction stoichiometry between Amplex Red and H_2O_2 (green) is 1:1[25] only if all Amplex Red radicals are converted to resorufin.

electron transfers from donor molecules reduce Compound I first to Compound II and then back to the ground state. Both of these electron transfer steps yield free radical products that may undergo subsequent reactions.[19] The rate of the peroxidase catalytic cycle depends on the nature of the electron donor and the reduction of Compound II to ground-state HRP is rate-limiting.[18] H_2O_2, however, is not only a substrate of HRP but at high concentration converts Compound II to an inactive form, Compound III,[20] also called oxyperoxidase.[21] H_2O_2 can also enhance the reversion of Compound III back to ground-state HRP,[22] which again participates in the peroxidase catalytic cycle. The intermediate steps in the versatile catalytic cycle of HRP were analyzed by transition state kinetics. The different oxidation states of the heme group were first characterized by observing distinct absorption bands in the visible and near-ultraviolet (Soret) region.[20] Later, HRP was investigated with many additional spectroscopic techniques.[23]

In order to observe the individual activity of hundreds of single HRP molecules simultaneously in a femtoliter chamber array, we performed a steady-state experiment based on the HRP-catalyzed oxidation of the nonfluorescent reducing substrate 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) to fluorescent resorufin. The Amplex Red assay has been widely employed in recent years as the most stable and sensitive reporter for H_2O_2 production of many different substrate oxidations, although the underlying kinetic mechanism of Amplex Red oxidation remains poorly understood.[24] The initial presumption was that the oxidation of Amplex Red was a single two-electron transfer reaction to explain why Amplex Red reacts with H_2O_2 in a 1:1 ratio.[24,25] Although two-electron transfer reactions of HRP were reported in a few cases,[26] most reducing substrates transfer only a single electron to HRP by the general mechanism[18,27,28] outlined in Figure 1. This mechanism holds for phenol[29,30] and other phenolic substrates such as Amplex Red. A one-electron transfer from phenol leads to radical formation. The phenoxy radicals must further react to yield the stable oxidation product biphenol. As two phenoxy radicals are produced per H_2O_2 molecule in two successive one-electron transfer steps, the formation of biphenol proceeds in a 1:1 ratio with H_2O_2, just as the formation of resorufin from Amplex Red, although in both cases product formation involves two separate steps. The only difference lies in the enzyme-independent reaction of the radicals. While biphenol is formed by the combination of two phenoxy radicals, two Amplex Red radicals undergo a dismutation reaction to form one molecule of resorufin and one molecule of Amplex Red. Furthermore, biphenol is also a substrate for HRP,[31] such that radical formation can initiate a polymerization process.[32] Under certain conditions, resorufin can be further oxidized and polymerized[25] in a manner analogous to phenol. In this study, we observe the oxidation of Amplex Red with single-molecule resolution and demonstrate the broader implications of intermediate radical formation during HRP turnover. Although single-molecule experiments with HRP have been reported[1,33−35] the enzyme independent step of product formation has not been considered.

Experimental Section

Materials. HRP (93% isoenzyme C) was purchased from Roche (Indianapolis, IN) and analyzed by MALDI (Applied Biosystems

(18) Chance, B. *Arch. Biochem. Biophys.* **1952**, *41*, 416–424.
(19) Rodriguez-Lopez, J. N.; Gilabert, M. A.; Tudela, J.; Thorneley, R. N. F.; Garcia-Canovas, F. *Biochemistry* **2000**, *39*, 13201–13209.
(20) Keilin, D.; Mann, T. *Proc. R. Soc. London B: Biol. Sci.* **1937**, *122*, 119–133.
(21) Wittenberg, J. B.; Noble, R. W.; Wittenberg, B. A.; Antonini, E.; Brunori, M.; Wyman, J. *J. Biol. Chem.* **1967**, *242*, 626–634.
(22) Chen, S. X.; Schopfer, P. *Eur. J. Biochem.* **1999**, *260*, 726–735.
(23) Dunford, H. B.; Wiley: New York, 1999.
(24) Towne, V.; Will, M.; Oswald, B.; Zhao, Q. J. *Anal. Biochem.* **2004**, *334*, 290–296.
(25) Zhou, M. J.; Diwu, Z. J.; Panchuk-Voloshina, N.; Haugland, R. P. *Anal. Biochem.* **1997**, *253*, 162–168.
(26) Harris, R. Z.; Newmyer, S. L.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1993**, *268*, 1637–1645.
(27) George, P. *Nature (London)* **1952**, *169*, 612–613.
(28) George, P. *Biochem. J.* **1953**, *54*, 267–276.
(29) Hewson, W. D.; Dunford, H. B. *J. Biol. Chem.* **1976**, *251*, 6036–6042.
(30) Hewson, W. D.; Dunford, H. B. *J. Biol. Chem.* **1976**, *251*, 6043–6052.
(31) Danner, D. J.; Brignac, P. J., Jr.; Arceneaux, D.; Patel, V. *Arch. Biochem. Biophys.* **1973**, *156*, 759–763.
(32) Zou, H. X.; Taylor, K. E. *Chemosphere* **1994**, *28*, 1807–1817.
(33) Edman, L.; Földes-Papp, Z.; Wennmalm, S.; Rigler, R. *Chem. Phys.* **1999**, *247*, 11–22.
(34) Hassler, K.; Rigler, P.; Blom, H.; Rigler, R.; Widengren, J.; Lasser, T. *Opt. Express* **2007**, *15*, 5366–5375.
(35) Comellas-Aragones, M.; Engelkamp, H.; Claessen, V. I.; Sommerdijk, N. A. J. M.; Rowan, A. E.; Christianen, P. C. M.; Maan, J. C.; Verduin, B. J. M.; Cornelissen, J. J. L. M.; Nolte, R. J. M. *Nat. Nanotechnol.* **2007**, *2*, 635–639.

Voyager DE-PRO) (Supporting Information, Figure 1). Phosphate buffered saline (PBS) (2.7 mM KCl, 1.5 mM KH_2PO_4, 136 mM NaCl, 8.1 mM Na_2HPO_4, pH 7.3) was used as the buffer system. HRP was reconstituted to 100 μM in 50:50 PBS/glycerol and stored at −20 °C. Glycerol (Invitrogen, ≥99.5% pure) was added to prevent freezing of the stock solution. For the single molecule experiments, the glycerol concentration—together with HRP—was diluted in PBS more than 10^6-fold, so that any effects associated with glycerol viscosity are prevented. Stock solutions of 100 mM Amplex Red (Invitrogen, Carlsbad, CA) in dimethylsulfoxide (DMSO), and of 180 mM resorufin, sodium salt, (Invitrogen) in DMSO were aliquoted and stored at −20 °C. The hydrogen peroxide (Sigma-Aldrich) concentration was determined spectrophotometrically at 240 nm using a molar extinction coefficient of 43.6.[36] All experiments were conducted in PBS with 0.05 mg/mL bovine serum albumin (BSA) (Sigma-Aldrich, Fraction V) and 1 mM ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich) at room temperature. The enzyme stock solution was diluted just prior to experimentation.

Femtoliter Array Fabrication. Bundles of 50 000 individually clad 4.5-μm glass fibers were purchased from Schott North America (Elmsford, NY). The 2 mm diameter bundles were cut to ~4.5 cm length and polished on both sides with an automated fiber polisher (MultiPrep, Rancho Dominguez, CA), using lapping films with grit sizes of 30, 15, 6, 3, 1, and 0.5 μm (Diamond Lapping Film, Rancho Dominguez, CA). The cladding and core material of the fiber bundle are composed of differently doped silica such that the core could be selectively etched.[37] Etching one end of the polished fiber bundle with 0.025 M HCl for 115 s with stirring created an array of 50 000 2.9-μm-deep chambers with volumes of 46 fL (μm^3).

Sealing Single Enzyme Molecules in the Femtoliter Array. A 1 × 1 cm^2 piece of 0.01-in. nonreinforced gloss silicone sheeting material (Specialty Manufacturing, Saginaw, MI) was cleaned with soapy water followed by deionized water. The silicone sheet, adhered to an equally sized cleaned microscope slide, was used as a gasket. The femtoliter array on the fiber bundle was fixed in a custom-built stage of an upright epifluorescence microscope. A 20 μL substrate/enzyme solution was placed on the silicone gasket. The gasket was put on a mechanical platform under the fiber bundle. The gasket on the mechanical platform was moved up and down repeatedly to fill the femtoliter chambers with solution. The chambers were sealed tightly by pressing the gasket vertically against the fiber bundle. The probability $P(x)$ that exactly x enzyme molecules are enclosed in a particular chamber is given by the Poisson distribution $P_\mu(x) = e^{-\mu}\mu^x/x!$, where μ is the mean number of enzyme molecules per chamber. 3.6 pM enzyme in a volume of 46 fL results in a probability of only one HRP molecule in every 10 chambers.[2] After each experiment, the femtoliter array was cleaned with deionized water under ultrasonication for 20 s.

Optical Detection of Single Enzyme Molecules. An upright Olympus BX61 microscope with a short arc mercury lamp (Ushio, Tokyo, Japan), a filter set with $\lambda_{ex} = 571$ nm and $\lambda_{em} = 584$ nm (Chroma Technology, Rockingham, VT), and a CCD camera (Cooke: Sensicam QE, Romulus, MI) was used to take images every 2 min with low illumination (neutral density filter with 10% transmission) and an exposure time of 200 ms. Light is totally internally reflected in the cores of the fiber bundle due to the higher refractive index of the core material compared to the cladding material, which results in an NA of 0.7 according to the manufacturer. The emission light from the femtoliter chambers is focused on the proximal face of the fiber bundle. A 20× objective with an NA of 0.75 was used to collect the emission light efficiently. To monitor a larger part of the array, a 0.33× demagnification lens was used in front of the CCD camera. IPLab software (Scanalytics, Fairfax, VA) was used to analyze the fluorescence signals and process the images. The fluorescence signal increases over the initial 2 min of the experiment were converted to apparent substrate turnover rates by calibrating the fluorescence intensities in the femtoliter chambers with resorufin standard solutions.

Bulk Enzyme Experiments. HRP (36 pM, corresponding to the concentration of, on average, a single enzyme molecule in every 46-fL chamber) was incubated with 250 μM Amplex Red and various concentrations of H_2O_2 in a volume of 200 μL in a nontransparent microtiter plate. In addition, various HRP concentrations were used with 250 μM Amplex Red and 1 mM H_2O_2. Measurements were taken ($\lambda_{ex} = 558$ nm and $\lambda_{em} = 590$ nm) every minute using an Infinite M200 microtiter plate reader (Tecan AG, Switzerland), and the initial fluorescence signal increase was converted to apparent substrate turnover rates by comparison to resorufin standards.

Results and Discussion

Measurement of Single HRP Molecules. Single HRP molecules were enclosed with the fluorogenic substrate Amplex Red and H_2O_2 in an array of 50 000 femtoliter-sized chambers (Figure 1).[2,38−40] A droplet of 3.6 pM HRP, 250 μM Amplex Red and various concentrations of H_2O_2 was loaded in the chambers and sealed tightly between the distal face of an etched optical fiber bundle and a silicone gasket. This method maintains the natural state of the enzyme and circumvents any problems that may arise due to surface attachment of the enzyme. According to the Poisson distribution, a 3.6 pM enzyme solution spread into volumes of 46 fL should result in a 1:10-ratio of HRP molecules to femtoliter chambers. The experimentally observed ratio, however, was 1:20 (±6), which can be explained by the presence of some inactive enzyme molecules in the solution. All the reaction components were sealed in the 46 fL chambers, thereby preventing evaporation.[1,2] By isolating many individual enzyme molecules, the substrate turnover of a large population of molecules could be determined accurately by monitoring the fluorescent signals from resorufin as the reaction proceeded. Monitoring was accomplished by introducing excitation light into the array's proximal end, thereby using the individual fibers in contact with each chamber to excite resorufin, capture the emitted fluorescence light, and deliver it to the CCD detector. In this manner, the optical fibers were used to interrogate each chamber. The concentration of the substrate Amplex Red used for the femtoliter experiments was constrained by both the detection limit of the developing product and by its solubility. As the maximum solubility of Amplex Red is 300 μM at pH 7.4,[25] we used a concentration of 250 μM in all experiments. Amplex Red can be oxidized by unspecific reactions with metal ions. To minimize these undesirable reactions, we added 1 mM EDTA to scavenge any divalent metal ions that could leach from the microwell surface into the solution and lead to unspecific oxidation of Amplex Red. We confirmed in a bulk experiment that the activity of HRP was not influenced by EDTA (Supporting Information, Figure 2). Furthermore, strong excitation light led to an enzyme independent exponential background increase in the array (Supporting Information, Figure 3). We attribute this unspecific reaction to an autocatalytically enhanced photooxidation of Amplex Red to resorufin. However, when five measurements were taken over 10 min with reduced excitation light we observed only a low linear background increase that could be easily corrected. A

(36) Noble, R. W.; Gibson, Q. H. *J. Biol. Chem.* **1970**, *245*, 2409–2413.
(37) Pantano, P.; Walt, D. R. *Chem. Mater.* **1996**, *8*, 2832–2835.
(38) Gorris, H. H.; Blicharz, T. M.; Walt, D. R. *FEBS J* **2007**, *274*, 5462–5470.
(39) Rissin, D. M.; Walt, D. R. *J. Am. Chem. Soc.* **2006**, *128*, 6286–6287.
(40) Li, Z.; Hayman, R. B.; Walt, D. R. *J. Am. Chem. Soc.* **2008**, *130*, 12622–12623.

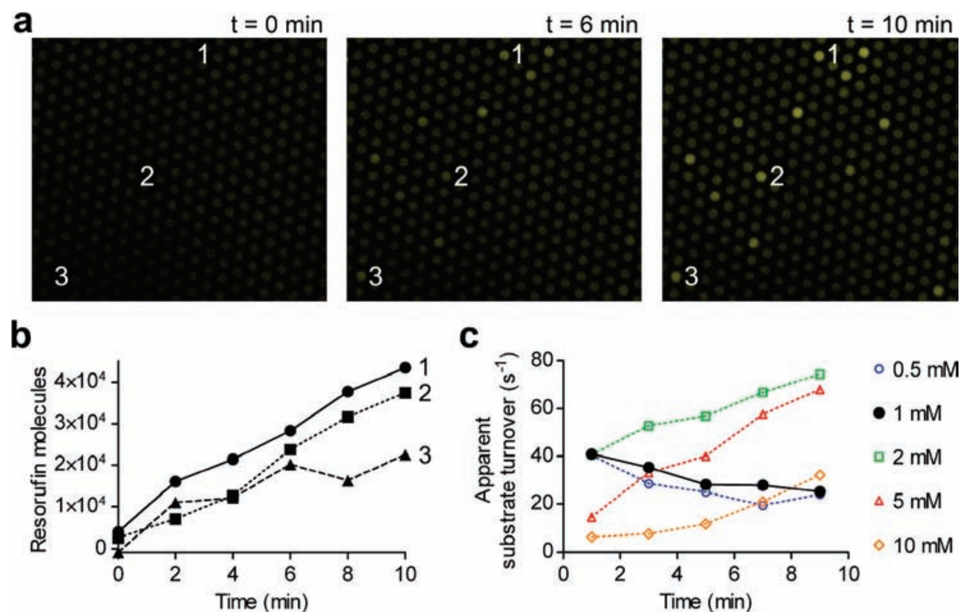

Figure 2. Apparent substrate turnover rates of individual HRP molecules in the femtoliter array. (a) HRP (3.6 pM) was enclosed with 250 μM Amplex Red and 1 mM H_2O_2 in 46-fL chambers, and sequential fluorescence images were taken every 2 min with low excitation illumination and an exposure time of 200 ms (Supporting Information, Movie 1). Three images are shown—directly after closing the chambers, after 6 min, and after 10 min. (b) Background corrected fluorescence trajectories in the indicated chambers diverge. The noncorrected trajectories and the background trajectories are shown in Supporting Information, Figure 5. (c) Apparent substrate turnover from an ensemble of ~1000 single HRP molecules calculated as the average of $(R_t - R_{t-120s})/120$ s (black line). The ensemble substrate turnovers at other H_2O_2 concentrations are shown in different colors.

further advantage of using low intensity and less frequent excitation is the negligible amount of resorufin photobleaching.[4] When we enclosed 5 μM resorufin in the array under these excitation conditions, the signal decreased only by 1.4% over 10 min (Supporting Information, Figure 4). For the calculation of exact turnover rates it is necessary that the substrate concentration remains constant over time. At a concentration of 250 μM, each femtoliter chamber contained ~7 million Amplex Red molecules and the maximum substrate turnover observed was 710 s^{-1}. Thus, less than 10% of the substrate molecules are depleted during a 10-min experiment. Consequently, while a 46-fL chamber is small enough to isolate single enzyme molecules and accumulate a detectable concentration of fluorophores, it is also large enough to hold a sufficient number of substrate molecules to prevent substrate depletion.

Single HRP molecules showed the highest initial resorufin increase in the femtoliter chambers at H_2O_2 concentrations between 0.5 and 2 mM (Figure 2), while in control experiments without HRP, no substrate turnover was observable under these conditions. It is important to recognize that the signal increase reflects the substrate turnover of single HRP molecules only indirectly, as two nonfluorescent Amplex Red radicals form one fluorescent resorufin molecule in an enzyme independent process. Hence, we refer in Figure 2c to "apparent substrate turnover rates". Due to the mild excitation conditions and the limited number of images taken in each experiment, it was only possible to obtain low-intensity noisy fluorescence signals that could not be smoothed with a digital filter. Consequently, instead of calculating the substrate turnover along the fluorescence trajectory for each individual enzyme molecule, the average apparent substrate turnover from an ensemble of ~1000 single enzyme molecules was determined. Figure 2c shows that the average substrate turnover rates were not constant over time. The substrate turnover changes may be explained by the accumulation of HRP reaction byproduct that can subsequently react with the Amplex Red or the Amplex Red radicals in the femtoliter chamber. Interestingly, the substrate turnover changes are dependent on the H_2O_2 concentration. At a concentration of 1 mM H_2O_2 or lower, the signal decreases over time while at a concentration of 2 mM or higher the signal increases with time. The strongest apparent substrate turnover increase over 10 min was observed with 10 mM H_2O_2. These results indicate that H_2O_2 is not only a substrate of HRP but is also involved in other enzyme independent reactions.

To minimize these complicating reactions in further analyses, we did not observe the entire 10-min trajectories but only the initial rates during the first 2 min, which are shown as single enzyme molecule activity distribution histograms in Figure 3a. The activity follows a Gaussian distribution with the highest mean activity at 1 mM H_2O_2. While fluctuations in the low fluorescence signal time traces lead to overall high coefficients of variation, Figure 3b shows that the low signal intensity is probably not the only reason for the broad activity distribution because the lowest variation is found at the lowest H_2O_2 concentration, which does not yield the highest fluorescence signals. Furthermore, there is a direct linear relationship between the H_2O_2 concentration and the coefficient of variation. We conclude therefore that H_2O_2 somehow contributes to the broad activity distribution in the femtoliter chambers.

HRP in Bulk Solution. For a direct comparison between the single-molecule and bulk rates, we performed microtiter plate experiments to analyze the bulk turnover of 250 μM Amplex Red at concentrations from 1 μM to 100 mM H_2O_2 using 36 pM HRP (Figure 4a). Thirty six picomolar corresponds to the effective concentration of a single HRP molecule in a volume of 46 fL. The substrate turnovers (black bars) plotted versus the H_2O_2 concentrations do not yield a typical Michaelis−Menten saturation plot, as high concentrations of H_2O_2 inhibit HRP. These results are consistent with an earlier report that provided only relative enzyme activities.[24] In our experiments, the highest bulk activity is obtained with 1 mM H_2O_2 which coincides with the single-molecule experiments (red bars). At lower and higher

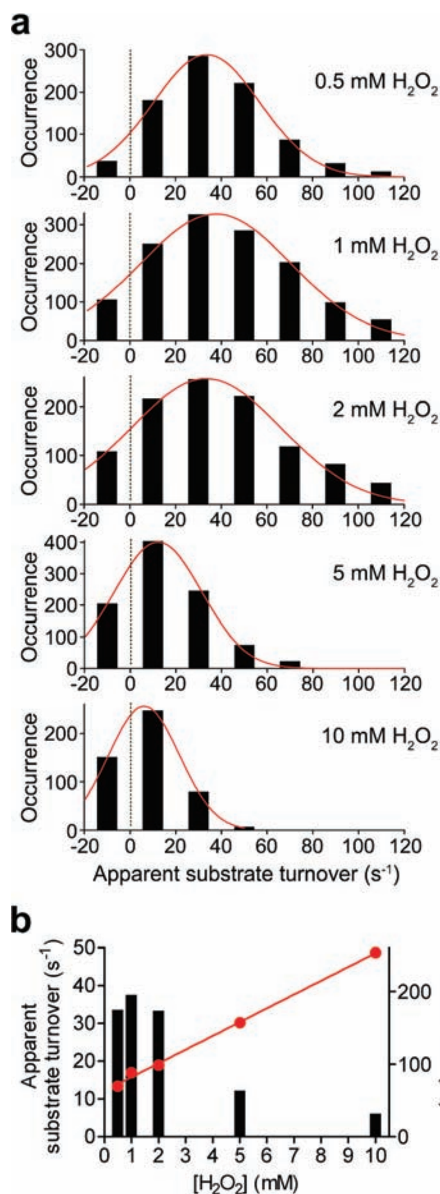

Figure 3. Initial activities of single HRP molecules at various H_2O_2 concentrations. (a) Activity distribution histograms of several hundred single enzyme molecules at 0.5, 1, 2, 5, and 10 mM H_2O_2. Activity in the femtoliter chambers was identified from time traces of 10 min but to minimize unspecific reactions, only the initial activity over the first 2 min is plotted. Due to fluctuations in the time traces, some enzymes have an apparent activity lower than zero as indicated by a dashed line. The red curve is a Gaussian fit to the histograms. (b) Summary of the mean substrate turnovers (black bars) and the coefficients of variation (red curve) of the Gaussian distributions.

H_2O_2 concentrations, the rates decrease in bulk and in the femtoliter chambers. In the femtoliter chambers, however, the apparent substrate turnover rates are about 10 times lower than in bulk (38 vs 440 s^{-1} at 1 mM H_2O_2). This result is in contrast to our previous experiments with β-galactosidase where the bulk rates were in good agreement with the single-molecule rates.[4] The experiments with β-galactosidase suggest that enzyme inactivation in the femtoliter chambers is not the reason for the lower rates. After an experiment with HRP, there was no enzyme activity left when the array was incubated with the substrates only, which indicates that HRP did not bind to the surface of the femtoliter chambers. Additionally, the hydrolytic reaction of β-galactosidase yielded the same fluorescent product, re-

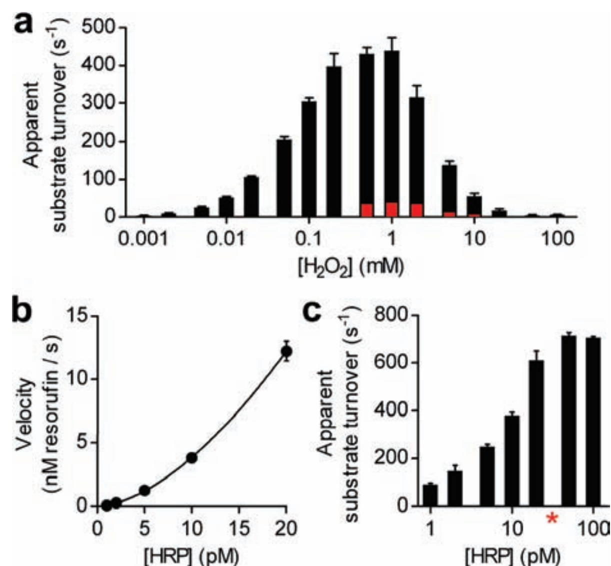

Figure 4. Bulk experiments. (a) Histogram of the apparent Amplex Red turnover rates at concentrations from 0.001 to 100 mM H_2O_2 in a microtiter plate (black bars) compared to the ensemble rates of single HRP molecules enclosed in the femtoliter chambers (red bars, taken from Figure 3 b). For both experiments, the same reaction conditions were chosen—36 pM HRP, which is equivalent to a single molecule in a 46 fL-chamber, 250 μM and 1 mM H_2O_2. (b) The velocity of resorufin formation plotted against HRP concentration shows a fractional reaction order of 1.7 up to a concentration of 20 pM HRP. (c) Apparent substrate turnover of Amplex Red derived from (b). From 1 to 50 pM HRP, the apparent substrate turnover increases 8-fold. High HRP concentrations yield a plateau of 710 s^{-1}. The red asterisk indicates the bulk HRP concentration corresponding to the single-molecule experiments and Figure 4a.

sorufin, showing that the resorufin product is stable (also see Supporting Information, Figure 4) and can be detected quantitatively in the femtoliter array.

Another possible explanation for the dramatically lower rates of product formation in the femtoliter array is the enzyme independent formation of resorufin from two Amplex Red radicals. It has previously been reported that metabolites interfere with Amplex-Red-coupled fluorescence assays such that an Amplex Red radical formation was inferred.[41] One possibility is that Amplex Red radicals react unspecifically on surfaces. The surface to volume ratio in a femtoliter chamber is 2000 times larger than in a microtiter plate well. Thus, Amplex Red radicals confined in a femtoliter chamber have a much higher probability of encountering the chamber surface before forming a resorufin molecule. BSA is added to the reaction buffer for surface passivation to prevent the enzymes from adsorbing to the surface but the glass surface may still facilitate the reaction of highly reactive radical species. Therefore, the surface reactions of the Amplex Red radicals likely lead to the formation of nonfluorescent side products. The formation of resorufin from two Amplex Red radicals, however, should be a concentration-dependent process—in the femtoliter chambers and in bulk solution. Low HRP concentrations would produce only low concentrations of Amplex Red radicals that should be unable to form resorufin quickly enough before they react via a different route. Therefore, we investigated the formation of resorufin at low (pM) HRP concentrations with 250 μM Amplex Red and 1 mM H_2O_2 in a microtiter plate (Figure 4b). A concentration

(41) Leon, D.; Marin-Garcia, P.; Sanchez-Nogueiro, J.; de la O, F. O.; Garcia-Carmona, F.; Miras-Portugal, M. T *Anal. Biochem.* **2007**, *367*, 140–142.

range from 1 to 20 pM HRP did not yield a linear or first-order relationship for the rate of resorufin formation, as would be expected if resorufin were formed directly from Amplex Red, but it exhibited a nonlinear relationship with a fractional order of 1.7. This fractional order is more consistent with second order kinetics, indicating the likely reaction between two Amplex Red radical molecules. At HRP concentrations higher than 20 pM, however, a linear relationship of the HRP concentration against the velocity of resorufin formation was observed. Figure 4c shows the substrate turnover derived from the velocities. The apparent substrate turnover increases 8-fold as the HRP concentration increases from 1 to 50 pM. A plateau at more than 50 pM HRP indicates that all Amplex Red radicals contribute to the formation of resorufin with substrate turnover rates of 710 s^{-1}. Thus, only these higher turnover rates reflect the "real" rather than "apparent" turnover of Amplex Red. These results suggest that there is a minimum concentration of Amplex Red radicals required to form resorufin efficiently. A single HRP molecule in a 46-fL chamber corresponds to a concentration of 36 pM, indicated with a red asterisk in Figure 4c, which even in bulk is not at the plateau. Thus, there are Amplex Red radicals that react via a different route before they react with a second Amplex Red radical.

General Implications for the Detection of Single HRP Molecules. The nonenzymatic step in the reaction mechanism of the widely used Amplex Red, as well as other fluorogenic substrates such as dihydrorhodamine, has general implications for bulk reactions at low substrate turnover rates and especially for single-molecule studies. First, caution is required before postulating a fluorescent enzyme−product complex of HRP and resorufin. Second, the high local Amplex Red radical concentration necessary for resorufin formation requires confinement of the Amplex Red radicals in the chambers etched in the fiber bundle or molded in PDMS.[1] Another elegant way to enclose single HRP molecules and constrain the diffusion of fluorogenic radicals is to use virus capsids.[35] If single HRP molecules are attached to a surface to observe the substrate turnover of these sparsely distributed HRP molecules,[33] however, the fluorogenic radicals would diffuse into the bulk solution before they combine to form the fluorescent product. This difficulty with immobilized single redox enzymes and freely diffusible product molecules has been circumvented by using flavoenzymes like cholesterol oxidase.[11] This redox enzyme has a tightly bound FAD as a cofactor, which is fluorescent in its oxidized form but nonfluorescent in its reduced form. Since FAD is involved in the redox catalysis, it toggles between on- and off-states with each catalytic cycle.

Conclusion

We investigated hundreds of individual HRP molecules concurrently in an array of 50 000 femtoliter chambers on the surface of a glass optical fiber bundle. The rates of product formation of single HRP molecules confined in femtoliter chambers were proportional to the reaction rates of HRP in bulk solution but, in general, were 10 times lower. The lower apparent substrate turnover rates can be explained by the redox reaction mechanism of HRP that involves two separate steps of product formation. HRP first catalyzes a one-electron oxidation of the fluorogenic substrate Amplex Red to a radical intermediate. The second step is an enzyme-independent dismutation reaction of two Amplex Red radicals to fluorescent resorufin, which can be disrupted by side reactions. We conclude that the large surface to volume ratio in the femtoliter chambers results in side reactions on the chamber surfaces and thereby reduces the formation of resorufin by a factor of 10. The results reported here have general implications for the design of single molecule experiments with HRP.

Acknowledgment. We thank Quanterix Corporation for generous financial support of this work.

Note Added after ASAP Publication. After ASAP publication on April 1, 2009, the name of the product formed was corrected in the concluding statement. The corrected version was published April 8, 2009.

Supporting Information Available: One movie and five figures supporting the Experimental and Results and Discussion sections. This material is available free of charge via the Internet at http://pubs.acs.org.

JA9008858