Enzyme Catalysis

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Single-Molecule Reactions in Liposomes**

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Single-molecule studies can reveal stochastic behavior that is normally averaged out in bulk experiments. Measurements of the single-molecule activities of lactate dehydrogenase (LDH)[1,2] and of alkaline phosphatase (ALP)[3,4] have been reported. When fluorescent products accumulated from individual enzyme molecules were confined in nL to pL volumes in capillaries or in microfabricated vials, it was found that individual molecules of the enzymes exhibit activities that varied between five-[1] to 20-fold.[3] Several different factors may account for the broad and non-Gaussian distribution of activities. First, glycosylation of proteins[3,5] affects both their flexibility and dynamic stability. Glycoforms and isoforms, however, were not present in the hLDH-H4 samples that were studied.[1,2] Also, glycosylation alone cannot account for the distribution of activities in reference [3], since it has been shown to cause only a fourfold variation in reactivity.[5] Second, proof of the existence of only one molecule in each reaction zone was based solely on statistical analysis and dilution factors. Third, contributions from wall effects cannot be excluded. Herein, we report a determination of single-molecule enzyme activities under conditions such that the presence of only one enzyme molecule and the absence of wall interactions were both confirmed by direct observation.

To mimic biochemical transformations in living systems and to avoid wall effects, reactions were carried out in liposomes[6–11] that also served as ultrasmall containers. Micromanipulation including electrofusion,[6–8] electroinjection,[9,10] and light-induced fusion[12] have been used to initiate reactions in liposomes. Reactions such as intercalation of dye into DNA,[8] catalytic reactions of enzymes,[9,10] and reactions of polymers with metal ions to form hydrogels[13] have been reported. However, previous single-liposome experiments have yet to reach the single-molecule domain.[9,10] In this study, TOTO-3-labeled ALP was confined in one liposome, and fluorescein diphosphate (FDP) was confined in another. The contents of the liposomes were mixed by electrofusion. The enzymatic reaction produced fluorescein (F), which was detected by fluorescence microscopy. The absolute reaction rates were determined by using F-loaded liposomes as calibration standards.

Although originally designed for labeling nucleic acids, TOTO-3 iodide was used to label ALP molecules. As in DNA labeling, the mechanism probably involves charge attraction plus hydrophobic affinity. Such interactions are less likely to affect enzyme activity compared to covalent binding or denaturation, which is required of other protein stains. In control experiments, the difference in activities between labeled and unlabeled ALP was found to be only 4% (Figure 1). This observation confirms that the nonspecific

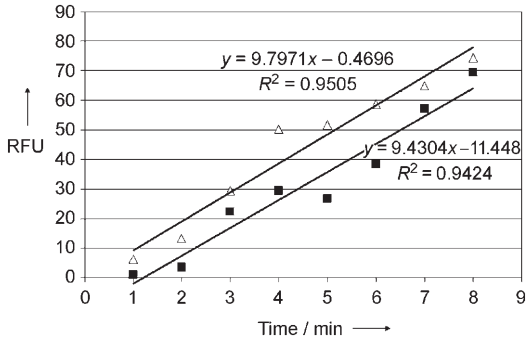

Figure 1. Comparison of the activities of TOTO-3-labeled ALP vs. unlabeled ALP. RFU: relative fluorescence units after background subtraction to account for residual TOTO-3 in the labeling solution. Experimental conditions: 6.6 μM FDP, 10.9 pM ALP. Open triangles: labeled enzyme. Solid rectangles: unlabeled enzyme. The ratio of the slopes indicates that the relative activity is 9.797/9.430 = 1.039, that is, independent of the label. The negative intercept and the displacement between the two data sets resulted from the lower background level when TOTO-3 was not used.

labels are generally distributed on the outer surface of the protein and away from the active site.[14] By comparing the fluorescence intensities of TOTO-3-labeled ALP with those of labeled DNA,[15,16] we estimate that there were roughly 20 labels per protein molecule.

Despite extensive dialysis, there were still a few cell traumas during solution transfer that resulted in the release of enzymes from the liposomes. Fortunately, the short depth of field of the 100× objective helped us distinguish molecules that were inside liposomes from those that were outside or on the lipid membrane. Still, leakage of ALP resulted in a high background when it reacted externally. Thus, we introduced 0.4 μL phosphatase inhibitor to the mixture (20 μL each of ALP-liposome and FDP-liposome solutions) to suppress any external reaction.

Figure 2A shows the optical image of the trapped ALP-liposome. The light source was cut off by a 550-nm short-pass

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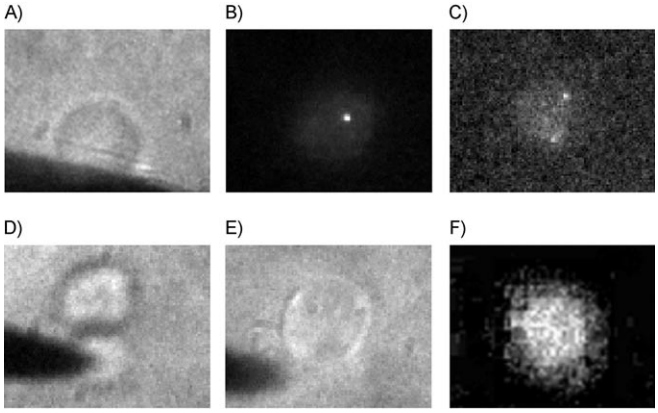

Figure 2. Images of liposomes at different stages of manipulation. A) Bright-field image of the selected ALP-liposome; B) fluorescence image of one labeled ALP in the selected liposome; C) fluorescence image of two labeled ALPs in another liposome (that liposome was not selected); D) bright-field image of the liposomes prior to electrofusion; E) bright-field image of the fused liposome; F) fluorescence image of the fused liposome after incubation. The black object in (A), (D), and (E) is the carbon-fiber electrode. The imaged area is $25 \times 25\ \mu m^2$.

filter to prevent photobleaching of the TOTO-3 labels. To select only those liposomes containing one and only one ALP molecule, we took fluorescence images before electrofusion. The bright spot in Figure 2B depicts one labeled ALP molecule enclosed within the membrane. Another liposome containing two enzyme molecules is shown in Figure 2C. Such liposomes were discarded. Figure 2D shows the optical image of the ALP-liposome and an FDP-liposome subsequently trapped by the laser. Prior to electrofusion, the short-pass filter was replaced by a 670-nm long-pass filter to prevent photobleaching of the products under bright-field illumination. Electrofusion was confirmed by the formation of one continuous membrane, as in Figure 2E. After incubation for 30 min, the amount of product accumulated was probed by fluorescence (Figure 2F).

Figure S1 (in the Supporting Information) was constructed by integrating the fluorescence intensities of individual liposomes that contained different (known) concentrations of fluorescein. The integrated intensity of the incubated liposome can thus be converted into the absolute amount of product and in turn the activity of the enzyme. Not all fused liposomes produced fluorescence upon incubation. The first trapped liposome always contains one ALP molecule (Figure 2B). Similarly, we have made certain that the second trapped liposome does not contain ALP. However, that liposome may not contain FDP either because during synthesis, the ALP-liposomes may contain zero, one, or two molecules, as required by Poisson statistics. The former cannot be distinguished from the FDP-liposomes that are on the same slide and may be inadvertently selected.

Two-dimensional molecular diffusion is given by $\langle x^2 \rangle = 4Dt$, where x is the mean-square displacement, D is the diffusion coefficient, and t is time. Reported D values of FDP and ALP are 4.25×10^{-10} and $6.1 \times 10^{-11}\ m^2 s^{-1}$.[9,17] Thus, the average displacements of FDP and ALP after 1 s are 41 and 16 μm, respectively. Therefore, the contents were well-mixed after several seconds in these liposomes (3.3 to 11 μm in diameter).

Up to 99.5% of the proteins could be lost owing to adsorption to the wall of the glass pipette in electroinjection.[9,18,19] In that case, direct measurement of enzyme concentrations are problematic, and true single-molecule studies are difficult. In contrast, adsorption of proteins to glass surfaces are not an issue in electrofusion, since the labeled proteins, the substrates, and the products were all surrounded only by lipid membranes. Furthermore, it is clear from viewing many sequences of fluorescence images (movies at 1-ms exposure time) that the protein was moving freely inside the liposome and rarely came in contact with the lipid membrane. In 1 ms, the protein moved about 0.49 μm and appeared as a small bright spot in the images (Figure 2B,C).

The catalytic reaction of transforming FDP into fluorescein by ALP involves more than one step. FDP is first digested by the enzyme to become fluorescein monophosphate (FMP), releasing a phosphate ion (P_i). FMP can be further digested by the enzyme to produce fluorescein plus another P_i ion. In addition, there are two other isomers that may be in equilibrium with FMP.[20] Our incubation times were tens of minutes, or many orders of magnitude greater than the characteristic time scale.[9] Consequently, this catalytic reaction can be simplified to one step:

$$\mathrm{FDP} \xrightarrow[\mathrm{ALP}]{} \mathrm{F} + 2\,\mathrm{P_i}. \qquad (1)$$

Furthermore, enzymatic reactions can be treated as pseudo-zeroth-order (reaction rate is independent of substrate concentrations) when the substrate concentrations are large. In this work, the enzyme concentration was 26 pM (single molecule), and the substrate concentration was 10 μM. It is thus reasonable to assume that single ALP molecules quickly reached their maximum reaction velocity (V_{max}) during the incubation time and that the reaction can be treated as a zeroth-order reaction.[3,4]

Figure 3 shows the results of such single-molecule reactions. For 30 ALP molecules, the activity was $272 \pm 206\ s^{-1}$. There was a 20-fold difference from the highest to the lowest

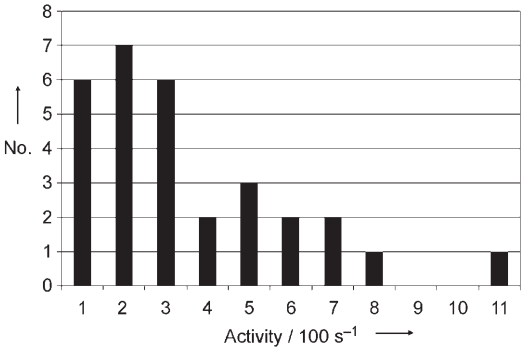

Figure 3. Histogram of activities of 30 individual alkaline phosphatase molecules from bovine intestinal mucosa. Reaction rates were calculated from the reaction times and the accumulated fluorescein concentrations by converting the integrated fluorescence signals according to Figure S1 in the Supporting Information.

value. The broad distribution appears to consist of two maxima centered at 150 and 450 s^{-1} along with a few high-activity molecules. In bulk experiments, we measured the activity to be 168 s^{-1} (Figure S2 in the Supporting Information). The lower bulk value may reflect the loss of enzyme while transferring reagents. Previously, ALP activities were reported to be 108 s^{-1} [3] for single molecules, and 380 s^{-1} [3] and 400 s^{-1} [9] in the bulk. However, since those results were obtained at different buffer conditions, our observations cannot be compared directly.

While the large variation in protein activity in reference [3] may include wall effects, the same bias does not apply here. Recent results from reaction-induced infrared spectroscopy showed that the binding of P_i to the active site of bovine intestinal ALP led to a distortion of the polypeptide carbonyl backbone.[21] Those results also corroborated the well-known fact that phosphate ions are inhibitors of ALPs. We can thus conclude that phosphate-induced structural change and the presence of various glycoforms contributed roughly equally to our observed variation in activities. Clearly, diversity in protein conformations must be considered relative to the time scale of the experiments. Rotational motion on the μs to ps time scale is averaged out within the turnover times of enzymes, and thus do not necessarily affect biological reactions. Even conformational fluctuations in periods of seconds[22] may be unimportant over the measurement times of typical solution kinetics. Herein and in the earlier reports,[1–4] differences in the measured activities can only exist if distinct protein conformations persist over the duration of the experiments, that is, on the order of an hour.

This approach has many potential applications. The use of these biomimetic containers should allow us to measure single-molecule activities under dissimilar intracellular and extracellular conditions. For example, one can incorporate liposome containers with transmembrane molecular/ion channels and create a concentration gradient that is very similar to cell environments to study how biomolecules respond to external changes. In such a system, the kinetics and dynamics of biomolecular reactions can be studied at the lowest possible concentration, that is, the single-molecule limit, without undesirable interferences from other biological species or from the walls of the container.

Experimental Section

Chemicals and materials: Trizma base, alkaline phosphatase from bovine intestinal mucosa, magnesium chloride, and phosphatase inhibitor cocktail 1 were obtained from Sigma-Aldrich. Fluorescein diphosphate, silicone isolator, and TOTO-3 iodide were obtained from Invitrogen. Chloroform and methanol were purchased from Fisher Scientific. Soybean L-α-phosphatidylcholine was obtained from Avanti Polar Lipids. Slide-A-Lyzer analysis cassettes and Halt phosphatase inhibitor cocktail were purchased from Pierce. Amine-coated coverslips were purchased from Telechem International. PVDF membranes (MWCO: 1 000 000) were obtained from Spectrum Labs.

Liposome preparation and protein labelling: Soybean PC liposomes were prepared by the rotary evaporation method.[6–8,11] Typically, soybean PC (15 μL, 133 mM), chloroform (985 μL), and methanol (100 μL) were mixed with aqueous solution (2 mL) containing the molecules of interest. The mixture was heated to 41 °C under reduced pressure, and the organic phase was removed within 2–3 min. The aqueous phase then became cloudy, and liposomes were formed. The cloudy solution was dialyzed extensively to remove reactants outside of the liposomes. The intracellular buffer for both FDP-containing and ALP-containing liposomes was 10 mM trizma base and 1 mM $MgCl_2$ (pH 9.7). The FDP concentration was 10 μM, and the ALP concentration was 25.6 pM for the single-molecule experiments. At pM concentrations, the presence of aggregates for these highly charged proteins can be neglected. TOTO-3 (1 mM solution in DMSO) was first diluted 100 times with the buffer mention above. The 100-fold diluted dye solution (32.6 μL) was mixed with ALP (46.6 μL, 2.2 nM). The intracellular buffer was added to make a final volume of 2 mL, and the mixture was transferred to a round-bottom flask for rotary evaporation. Both of the absorption and emission maxima of TOTO-3 iodide and of fluorescein are separated by about 150 nm. Therefore, the dye-labelled enzymes would not interfere with fluorescence registration of the products.

Microscopy and manipulation: Figure S3 (in the Supporting Information) shows the experimental setup, which consists of an inverted microscope, two 3D translation stages for positioning carbon-fiber microelectrodes, two visible lasers for fluorescence excitation, one near-IR diode laser for optical trapping,[6–8,23] and an intensified-CCD (ICCD) camera. The 488-nm line from an argon ion laser was used to excite fluorescein; the 632.8-nm line from a He–Ne laser was used to excite the TOTO-3-labeled proteins. The 808-nm beam from the near-IR laser diode was collimated and shaped for optical trapping of the liposomes. A polychroic mirror and a dual-notch filter were employed to work with the lasers. An oil-immersion 100× objective was used for imaging and trapping.

Carbon-fiber microelectrodes and optical tweezers were used to manipulate and electrofuse the liposomes.[6–8] Typically, one floating liposome was trapped by optical force and immobilized on the amine-coated surface. Fluorescence images of the TOTO-3-labeled enzyme molecule inside the liposome were then collected by shining the He–Ne laser onto the vesicle. If one was found inside, this liposome (ALP-liposome) was electrofused with another liposome (FDP-liposome) to initiate the enzymatic reaction. Otherwise, a different liposome was trapped. Electrical pulses from a pulse generator ranging from 10 to 30 V with a duration of 2–10 μs were sufficient to achieve electrofusion.

A miniature reaction chamber for electrofusion and incubation was created by placing a silicone spacer onto the amine-coated coverslip. After liposome fusion, the carbon-fiber microelectrodes were removed, and another regular coverslip was placed on top of the spacer. In the chamber, long incubation times (hours) and elevated reaction temperatures were possible without drying the droplet. After incubation, the liposome was exposed to the 488-nm laser, and the fluorescence signal from the product was registered by the ICCD.

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[1] Q. Xue, E. S. Yeung, *Nature* **1995**, *373*, 681.
[2] W. Tan, E. S. Yeung, *Anal. Chem.* **1997**, *69*, 4242.
[3] D. B. Craig, E. A. Arriaga, J. C. Y. Wong, H. Lu, N. J. Dovichi, *J. Am. Chem. Soc.* **1996**, *118*, 5245.
[4] R. Polakowski, D. B. Craig, A. Skelley, N. J. Dovichi, *J. Am. Chem. Soc.* **2000**, *122*, 4853.
[5] P. M. Rudd, H. C. Joao, E. Coghill, P. Fiten, M. R. Saunders, G. Opdenakker, R. A. Dwek, *Biochemistry* **1994**, *33*, 17.

[6] D. T. Chiu, C. Wilson, F. Ryttsen, A. Stromberg, C. Farre, A. Karlsson, S. Nordholm, A. Gaggar, B. P. Modi, A. Moscho, R. A. Garza-Lopez, O. Orwar, R. N. Zare, *Science* **1999**, *283*, 1892.
[7] D. T. Chiu, C. F. Wilson, A. Karlsson, A. Danielsson, A. Lundqvist, A. Stromberg, F. Ryttsen, M. Davidson, S. Nordholm, O. Orwar, R. N. Zare, *Chem. Phys.* **1999**, *247*, 133.
[8] A. Strömberg, A. Karlsson, F. Ryttsén, M. Davidson, D. T. Chiu, O. Orwar, *Anal. Chem.* **2001**, *73*, 126.
[9] A. Karlsson, K. Scott, M. Markström, M. Davidson, Z. Konkoli, O. Orwar, *J. Phys. Chem. B* **2005**, *109*, 1609.
[10] K. Sott, T. Lobovkina, L. Lizana, M. Tokarz, B. Bauer, Z. Konkoli, O. Orwar, *Nano Lett.* **2006**, *6*, 209.
[11] A. Moscho, O. Orwar, D. T. Chiu, B. P. Modi, R. N. Zare, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 11443.
[12] S. K. Kulin, R. Kishore, K. Helmerson, L. Locascio, *Langmuir* **2003**, *19*, 8206.
[13] A. Jesorka, M. Markstrom, O. Orwar, *Langmuir* **2005**, *21*, 1230.
[14] H.-W. Li, E. S. Yeung, *Anal. Chem.* **2005**, *77*, 4374.
[15] M. R. Shortreed, H. Li, W.-H. Huang, E. S. Yeung, *Anal. Chem.* **2000**, *72*, 2879.
[16] Y. Ma, M. R. Shortreed, E. S. Yeung, *Anal. Chem.* **2000**, *72*, 4640.
[17] A. M. Leach, A. R. Wheeler, R. N. Zare, *Anal. Chem.* **2003**, *75*, 967.
[18] S. H. Kang, M. R. Shortreed, E. S. Yeung, *Anal. Chem.* **2001**, *73*, 1091.
[19] S. H. Kang, E. S. Yeung, *Anal. Chem.* **2002**, *74*, 6334.
[20] Z. Huang, Q. Wang, H. D. Ly, A. Gorvindarajan, J. Scheigetz, R. Zamboni, S. Desmarais, C. Ramachandran, *J. Biomol. Screening* **1999**, *4*, 327.
[21] L. Zhang, R. Buchet, G. Azzar, *Biophys. J.* **2004**, *86*, 3873.
[22] H. P. Lu, L. Xun, X. S. Xie, *Science* **1998**, *282*, 1877.
[23] A. Ashkin, J. M. Dziedzic, J. E. Bjorkholm, S. Chu, *Opt. Lett.* **1986**, *11*, 288.

