Studies on Single Alkaline Phosphatase Molecules: Reaction Rate and Activation Energy of a Reaction Catalyzed by a Single Molecule and the Effect of Thermal Denaturation—The Death of an Enzyme

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Abstract: Single molecules of alkaline phosphatase are captured in a capillary filled with a fluorogenic substrate. During incubation, each enzyme molecule creates a pool of fluorescent product. After incubation, the product is swept through a high-sensitivity laser-induced fluorescence detector; the area of the peak provides a precise measure of the activity of each molecule. Three studies are performed on captured enzyme molecules. In the first study, replicate incubations are performed on the same molecule at constant temperature; the amount of product increases linearly with incubation time. Single enzyme molecules show a range of activity; the most active molecules have over a 10-fold higher activity than the least active molecules. In the second study, replicate incubations are performed on the same molecule at successively higher temperatures. The activation energy of the reaction catalyzed by a single molecule is determined with high precision. Single enzyme molecules show a range of activation energy; microheterogeneity extends to thermodynamic properties of catalysis. The average activation energy is within experimental error of the activation energy obtained from analysis of a bulk sample. These results are consistent with the first postulate of statistical thermodynamics: a thermodynamic property obtained from the time average of an individual molecule is identical to that produced by an ensemble average over a large number of molecules. In the third study, the activity of single enzyme molecules is measured after partial heat denaturation. The number of active molecules decreases in proportion to the extent of denaturation. However, the activity of the surviving molecules is experimentally indistinguishable from the activity of control enzyme. Thermal denaturation of alkaline phosphatase is a catastrophic process, wherein the molecule undergoes irreversible conversion to an inactive form.

Introduction

Chemical reactions are usually studied on macroscopic ensembles of molecules. The development of ultrasensitive instrumentation allows the study of individual molecules in order to observe molecular properties that are otherwise obscured by ensemble averaging. Most work has focused on single-molecule detection as the ultimate quantitative analytical tool. Highly fluorescent proteins, multiply labeled polymers, and small molecules have been detected by laser-induced fluorescence in thin films, in neat flowing liquid streams, after separation by capillary electrophoresis, and in levitated droplets.[1−5] More recently, physical characteristics, such as spectra, spring constants, and excited state lifetimes, have been measured on single molecules.[6−8]

Reactions are also being studied at the single-molecule level. In the earliest work, β-galactosidase was detected in individual droplets after a 10−15 h incubation.[9] Individual product molecules have been detected at a microelectrode by electrogenerated chemiluminescence and through redox chemistry.[10,11] More recently, individual myosin molecules were detected through their binding of fluorescently labeled adenosine triphosphate.[12]

As a very interesting example, Xue and Yeung have reported the detection of fluorescent products generated by single molecules of lactate dehydrogenase after a 1 h incubation.[13] The amount of product generated by any single molecule was reproducible and consistent over a long time. However, the amount of product produced by different molecules was variable, with a 5-fold range in activity between the most active and least active molecules. The difference in activity reflects differences in either structure or local environment of the enzyme.

Rate of a Reaction Catalyzed by a Single Molecule. In this paper, we describe three experiments with individual alkaline phosphatase molecules (EC 3.1.3.1). First, we measure the rate of a reaction catalyzed by a single enzyme molecule. A kinetic curve is generated by performing replicate incubations on individual molecules. During each incubation, the single enzyme molecule catalyzes many reactions, and the rate of the

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reaction can be determined by measuring the amount of product generated during the incubation period. We construct a plot of activity versus incubation time for the reaction catalyzed by a single molecule; the activity is constant and independent of incubation time. While the measurement of activity of any one molecule is quite precise, the activity for different enzyme molecules is quite heterogeneous.

Activation Energy of a Reaction Catalyzed by a Single Molecule. Second, we verify the first postulate of statistical mechanics. In 1960, Hill wrote that "the object of statistical mechanics is to provide the molecular theory...of properties of macroscopic systems".[14] Strictly speaking, statistical mechanics refers only to mechanical properties of systems in equilibrium. In 1941, Henry Eyring developed absolute rate theory, which extends statistical mechanics to the properties of chemical reactions.[15] Both theories rely on a fundamental postulate: the long-time average of a single-molecular system is equal to the average over a large ensemble of molecules.

We perform several incubations on a single enzyme molecule. By varying the temperature of the system between incubations, we estimate the activation energy of the reaction catalyzed by the molecule. This value is determined from the time average of many reactions catalyzed by the molecule. These results may be compared with the conventional measurement of activation energy, which is performed with an ensemble of a large number of enzyme molecules. As one complication, the population ensemble for this enzyme is not homogeneous. The heterogeneity of alkaline phosphatase extends to thermodynamic properties. However, the average activation energy of the individual molecules is equal, within experimental error, to the value determined from the ensemble average.

Thermal Denaturation and the Death of an Enzyme. Third, we consider the origin of the variation in activity and activation energy for individual alkaline phosphatase molecules. Xue and Yeung suggest that the variation in activity for lactate dehydrogenase arises because different enzyme molecules are present in different conformations, which are stable on the time scale of the experiment and which generate different activities.[13] These conformations represent a continuum of activity ranging from the most active, native conformation to the inert, denatured form of the enzyme. If this hypothesis is correct, then the different conformations must be separated by energy barriers, which can be crossed given sufficient thermal energy. Thermal denaturation could take a highly active enzyme molecule through a cascade of conformations with decreasing activity, ultimately ending in a denatured conformation. This "Cheshire Cat" model of enzyme denaturation suggests that enzymes undergo a gradual loss of activity during thermal denaturation, which shifts the distribution of activity to lower levels, eventually resulting in a pool of inactive molecules. As an alternative hypothesis, denaturation may be a catastrophic phenomenon. Changes in conformation that occur in portions of the enzyme far from the active site will have no effect on activity; instead, denaturation occurs when the active site of the molecule is damaged. In this catastrophic model of thermal denaturation, thermal denaturation is accompanied by a loss of active molecules but the activity of surviving molecules is unchanged. We measure the activity of individual alkaline phosphatase molecules that have been subjected to partial thermal denaturation; within experimental error, the activity of the surviving molecules is indistinguishable from the activity of control molecules. The loss of activity is due solely to the loss of active molecules. There is no evidence for transitions to conformations with lower, but nonzero, activity for alkaline phosphatase.

Experimental Section

Reagents. AttoPhos, 2′-(2-benzothiazolyl)-6′-hydroxybenzothiazole phosphate, is a weakly fluorescent substrate for alkaline phosphatase marketed by JBL Scientific (San Luis Obispo, CA) and is converted to the highly fluorescent product 2′-(2-benzothiazolyl)-6′-hydroxybenzthiazole. This fluorogenic substrate has been used for sensitive enzyme-based immunoassays and oligonucleotide-probing experiments.[16,17] Boric acid and *p*-nitrophenyl phosphate are from Sigma, and chloroform, diethanolamine, and $MgCl_2$ are from Fisher Scientific.

Substrate Purification. AttoPhos contains small amounts of fluorescent product as an impurity. The product concentration is reduced by double extraction of 10 mM AttoPhos in 100 mM borate buffer (pH 9.5) with equal volumes of chloroform. This extraction is performed on the morning of the assay and generates about a 10-fold decrease in background. It is important to exclude any residual chloroform from entering the reaction capillary; electroosmosis is modified by the organic solvent, which results in data with poor reproducibility. Chloroform is excluded by centrifugation followed by carefully decanting the extracted substrate from the organic solvent.

Enzyme Solutions. The amount of commercial alkaline phosphatase is estimated by monitoring the change in absorbance at 405 nm of approximately 0.01 units of enzyme in 1.5 mL of 1 M diethanolamine (pH 9.8) containing 0.5 mM $MgCl_2$ and 10 mM *p*-nitrophenyl phosphate at 37 °C. The absorbance coefficient of *p*-nitrophenyl phosphate is 18 450 L mol^{-1} cm^{-1} at this wavelength. A molecular weight of 140 kDa and a specific activity of 2000 u/mg under these conditions (Life Technologies, data sheet) are used to determine the enzyme concentration.

The high concentration of diethanolamine in the standard assay leads to poor reproducibility in migration time in the capillary reactor; diethanolamine apparently perturbs electroosmosis in an unpredictable and inconsistent manner. Instead, in the single-molecule assays, enzyme is diluted by at least 8 orders of magnitude in 100 mM borate buffer (pH 9.5) followed by another 2 orders in borate buffer containing 1 mM AttoPhos. The borate buffer is compatible with the capillary reactor, albeit with decreased sensitivity compared to that generated by the manufacturer's suggested buffer.

Solutions are prepared in disposable plasticware. To minimize contamination with exogenous enzyme, solutions are prepared in a clean-air hood. All buffers, vessels, and pipet tips are autoclaved prior to use.

Single Enzyme Molecule Assays. Calf intestinal alkaline phosphatase is obtained from Life Technologies. Enzyme assays are performed in a 70 cm long, 145 μm outer diameter, 10 μm inner diameter fused silica capillary from Polymicro Technology. Contaminating enzymes are removed from the capillary at the start of each experiment by flushing with 100 mM KOH containing 7 M urea.

In the single-incubation assays, alkaline phosphatase is diluted to final concentrations of 1.9×10^{-15} and 9.5×10^{-16} M. Immediately after substrate and enzyme are mixed, a slug of the mixture is electrophoretically injected onto the capillary for 3 min at an electric field of 400 V cm^{-1}. Blanks are identical but with the omission of alkaline phosphatase. Incubation times are chosen arbitrarily in the range 14−30 min. After incubation, the products are driven at an electric field of 400 V cm^{-1} through the fluorescence detector.

In the multiple-incubation assays, alkaline phosphatase concentration is 3.8×10^{-16} M in AttoPhos. On average, one enzyme molecule is captured within the capillary. After an 8 min incubation, an electrophoretic field of 400 V cm^{-1} is applied for 15 s to separate the enzyme and product and move the enzyme into fresh substrate. Incubations of 4, 2, and 1 min are performed with a similar electrophoretic separation

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between each incubation. After the last incubation, the capillary is flushed by the electrophoretic field through the fluorescence detector.

Peak area is converted to activity by estimating the number of fluorescent product molecules contained within each peak and by dividing by the incubation time. To estimate the sensitivity of the detector, a known amount of fluorescent product is injected onto the capillary each day. The peak area generated by the fluorescent product is used to estimate the number of product molecules contained within each peak.

Bulk Capillary Assays. An on-column assay is performed to estimate the turnover number of the bulk alkaline phosphatase from Life Technologies. A 10 μm inner diameter, 73 cm long fused silica capillary is equilibrated with 1 mM AttoPhos in borate buffer. A 3.8 $\times 10^{-12}$ M solution of alkaline phosphatase is injected onto the capillary for 5 s at an electric field of 205 V cm^{-1}. The sample reservoir is then replaced with borate running buffer reservoir, and an electric field of 400 V cm^{-1} is applied to the capillary for 5 min. This field electrophoretically moves the enzyme into the slower moving substrate. The field is removed, and the enzyme is incubated for 16 min on the capillary. Last, electrophoresis is performed at an electric field of 400 V cm^{-1} to drive the sample through the detector. The system is calibrated by injecting known amounts of standard fluorescent product onto the capillary.

Activation Energy Study. Three-quarters of the capillary length, starting at the injection end, is held within a Plexiglas box containing the safety interlock. The temperature of the interior of the box is maintained by a thermostatically controlled heater and a circulating cooling bath. The temperature of the air flowing over the capillary is monitored with a digital thermometer. The air temperature equilibrates within 1 min following a 10 deg increase in the heater setting. Cooling is much slower because of the relatively large thermal mass of the heater.

Stock alkaline phosphatase from Sigma is serially diluted 10 orders of magnitude in 100 mM borate (pH 9.5) and a further 10-fold immediately prior to injection to give a final concentration of 4.6 $\times$ 10^{-16} M enzyme in 100 mM borate (pH 9.5) containing 1 mM AttoPhos. Sample is electrokinetically injected onto the capillary at an electric field of 400 V cm^{-1} for 240 s. The enzyme is incubated for three 15 min periods at different temperatures ranging from 13 to 38 °C. A 15 s injection of 100 mM borate (pH 9.5) at 400 V cm^{-1} is performed between incubation periods to separate the enzyme molecule from substrate. Immediately following the third incubation period the sample is flushed through the detector at 400 V cm^{-1}.

Enzymatic product peaks are produced in the capillary only where both enzyme and substrate are present. Because the substrate has the lower mobility, this volume is set by the injection volume of the AttoPhos, which represents ~15% of the total capillary volume. This portion of the capillary is well within the instrument's Plexiglas box that is temperature controlled. The use of a low-diameter capillary allows for rapid equilibration of internal capillary temperature with that of the box. Photothermal measurements have shown that the capillary interior reaches thermal equilibrium with its surroundings in a few milliseconds.[18] Upon raising the temperature for the second and third incubations, thermal equilibration of the air circulating in the box occurs within the first minute of the 15 min incubations.

Thermal Denaturation Study. A control solution is prepared by diluting alkaline phosphatase (Life Technologies) to 8 $\times$ 10^{-16} M. A second solution of 8 $\times$ 10^{-10} M alkaline phosphatase is heated to 64 °C for 5 min. The sample is immediately diluted by 6 orders of magnitude to quench the denaturation reaction. In both experiments, incubation is for 15 min before the capillary is flushed electrophoretically through the detector.

Instrumentation. Details of the instrument have been published previously.[19−22] A 10 μm i.d., 145 μm o.d., 73 cm long fused silica capillary is used for the reaction. The detection end of the capillary, from which the polyimide coating has been removed by a gentle flame, is placed inside a locally constructed sheath flow cuvette. An argon ion laser beam (λ = 457.9 nm) is focused with a 6.3× microscope objective into the cuvette about 20 μm below the exit of the capillary. Fluorescence is collected at 90° with a 60× (numerical aperature 0.70) microscope objective and selectively transmitted through a slit and a 40 nm band pass filter centered at 580 nm. Light is detected with an R1477 photomultiplier tube. Optimum laser power for the detection of AttoFluor is approximately 5 mW at 457.9 nm.

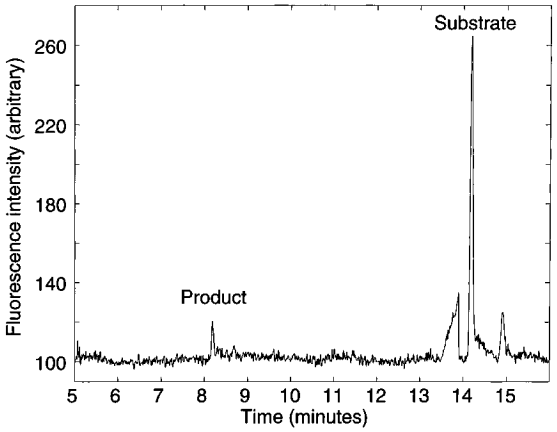

Figure 1. Electrophoretic analysis of AttoPhos and its hydrolysis product.

Results: Heterogeneous Activity

Single Incubations. Several groups have used capillary reactors to study enzyme activity.[23−27] In most cases, these experiments used relatively large amounts of enzyme, although the work by Xue and Yeung extended the procedure to the single-molecule level.[13]

As in Yeung's experiment, we study highly dilute solutions so that only a few enzyme molecules are present in the reactor. A portion of the capillary is filled with a weakly fluorescent substrate mixed with a very dilute solution of enzyme. A single enzyme molecule converts the weakly fluorescent substrate into a pool of highly fluorescent product. After incubation, an electrophoretic current is used to sweep the contents of the capillary through a high-sensitivity fluorescence detector; the pool of product generated by each enzyme molecule produces a Gaussian-shaped peak in the fluorescence detector.

Figure 1 presents the electrophoretic profile generated by the fluorogenic substrate; this substrate slowly undergoes autohydrolysis, generating a small amount of fluorescent product in the period after the cleanup extraction. The slow moving peaks are due to the substrate while the fast moving peak is due to the product generated by autohydrolysis of the substrate. Note that the substrate generates a signal that is at least a factor of 20 larger than the residual product peak. Also note that the peaks associated with the substrate migrate from the capillary at least 5 min after the product peak.

When the entire capillary is filled with the substrate, the strong fluorescence signal from the high-concentration substrate swamps the fluorescence signal from the product generated by individual enzyme molecules. Our single-molecule assay relies on the differences in the electrophoretic properties of the substrate and

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product. We fill a portion of the capillary with a 5 min long slug of substrate and enzyme; all of the peaks in Figure 1 are stretched into plateaus that are 5 min wide. After incubation, electrophoresis separates the product from substrate. There is roughly a 5 min window between the period when the product begins to migrate from the capillary and the beginning of the substrate migration. Single molecules can be detected in this time window. If longer slugs of enzyme and substrate are used, the fluorescent product generated by enzyme molecules will be lost in the huge background signal generated by the very high concentration substrate.

Single-molecule peaks are observed only in regions where both enzyme and substrate are present during the incubation period. If the enzyme and substrate had identical mobility, then the single-molecule peaks would be observed throughout the 5 min window. However, the substrate has a lower mobility than the enzyme. As a result, the single-molecule peaks are observed only in the last ~3.5 min of the observation window.

Figure 2A presents a blank incubation where the first ~15% of the capillary (~10 nL) is filled with the substrate but no enzyme. During mobilization, the product generates a plateau that begins at 8 min, corresponding to the product peak in Figure 1. The height of this plateau depends on the efficiency of the extraction in removing product and the time since extraction; the plateau height is similar to the peak height for the hydrolysis product in Figure 1. A much larger plateau is associated with the substrate; the substrate begins to migrate from the capillary at 13 min and does not interfere in the measurement.

Figure 2B presents the enzyme assay generated with incubation of a slug of nominal 1.9×10^{-15} M enzyme. Superimposed on the background plateau is a set of peaks, including two pairs that overlap. Four replicate assays yielded an average and standard deviation of 11.8 ± 3.5 peaks per assay. On the basis of the nominal enzyme concentration and injection volume, we expect an average of ~11 molecules per run. Peak area is found by nonlinear regression analysis to one or two Gaussian peaks. Area is converted to reaction rate by taking into account the sensitivity of the detector and the incubation time. The mean reaction rate for the 1.9×10^{-15} M solution is 108 s^{-1}, and the standard deviation of the distribution is 70 s^{-1} ($n_1 = 47$ molecules). Finally, note that the peak width increases with migration time. Diffusion during the mobilization process leads to enhanced band broadening for the late migrating peaks.

Figure 2C presents an electropherogram of the enzyme solution that had been diluted by a factor of 2. Four replicates yield an average of 5.3 ± 4.4 enzyme molecules; the number of peaks decreases in proportion to concentration. The mean reaction rate for the 9.5×10^{-16} M solution is 124 s^{-1}, and the standard deviation of the distribution is 97 s^{-1} ($n_2 = 21$ molecules).

The distribution of activity is not Gaussian, see below, with the distribution skewed to higher activity. A rough measure of the overlap of the two distributions comes from a *t*-test.[28] For $n_1 + n_2 - 2 = 66$ degrees of freedom, the *t* value for the two data sets is 0.74. This number is to be compared with the *t* distribution. If the value in the table exceeds the observed *t* value, then the data may be assumed to be from the same population. In our case, there is no difference in the mean for the two concentration data at the 57% confidence limit. Within experimental error, the average activity of the enzymes is independent of enzyme concentration. Of course, these calculations should be treated with caution when applied to non-Gaussian distributions.

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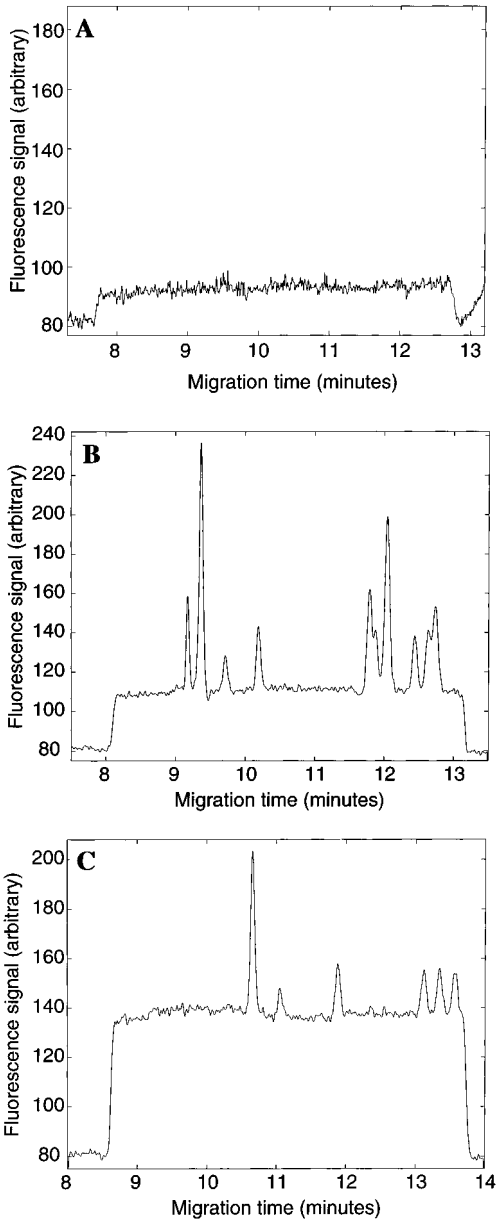

Figure 2. Enzyme assay at the single-molecule level. (A) Blank generated by injection at 400 V/cm for 3 min of 1 mM AttoPhos in 100 mM borate buffer (pH 9.5). Incubation was for 19.25 min. The product was swept through the fluorescence detector at an electric field of 400 V/cm. (B) A 1.9×10^{-15} M mixture of alkaline phosphatase was mixed with 1 mM AttoPhos in 100 mM borate buffer. The mixture was treated as in part A with a 28.5 min incubation. (C) A 9.5×10^{-16} M mixture of alkaline phosphatase was mixed with 1 mM AttoPhos in 100 mM borate buffer. The mixture was treated as in part A with an 18 min incubation.

The standard deviations of the distributions may be compared by use of the *F*-test.[28] The experimental *F* value for 46 and 20 degrees of freedom is 1.9; the standard deviations for the two concentration data are not significantly different at the 97.5% confidence limit.

Replicate Incubations. Figure 3 presents a cartoon for our replicate incubation study. A single enzyme molecule is present within the reaction capillary. After an 8 min incubation, a brief electrophoresis pulse is applied to the capillary. Because the enzyme has higher mobility than the reaction product, the enzyme moves forward into fresh substrate. A second incubation is performed for 4 min and another electrophoretic pulse is applied to move the enzyme into fresh substrate. This process is repeated for 2 and 1 min incubations.

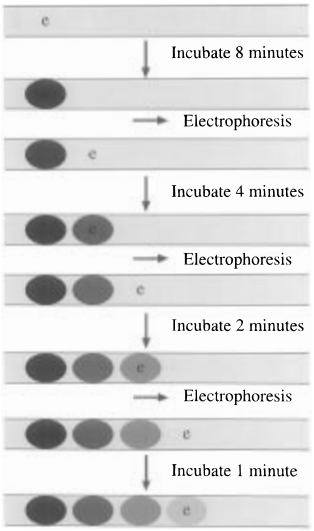

Figure 3. Cartoon of the replicate incubation study. A single enzyme molecule is captured within the capillary. After an 8 min incubation, a brief electrophoretic pulse is applied to the capillary; this pulse separates the enzyme from the surrounding pool of product. The process is repeated for 4, 2, and 1 min incubations. After the last incubation, the contents of the capillary are swept through the fluorescence detector by a 400 V/cm electric field.

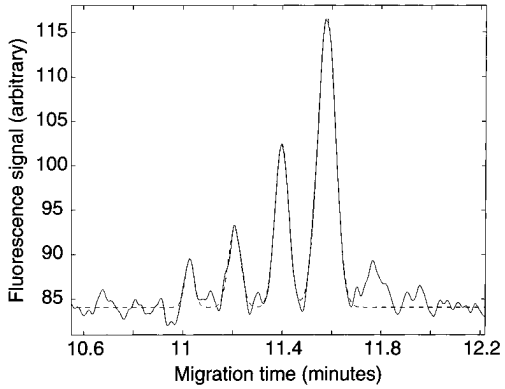

Figure 4. Peaks generated by multiple incubation. The solid line is the data, and the dashed curve is a least-squares fit of four Gaussian peaks to the data.

After the final incubation, the products are swept through the detector. Four peaks are observed with monotonically increasing amplitude, Figure 4; the smooth curve is the least-squares fit of four Gaussian peaks to the data. Peak spacing is uniform, 11.1 ± 0.3 s, which is expected for a single molecule undergoing electrophoretic separation from the fluorescent product. Note that the first peak to migrate from the capillary corresponds to the last incubation time; the enzyme migrates faster than the reaction products. Also, the peak corresponding to a 1 min incubation is detectable above the noise in the background signal.

The peak area for a multiple incubation increases linearly with incubation time, Figure 5. Eight molecules were studied. Within experimental uncertainty, the lines have zero intercept. The average linear correlation coefficient is 0.996 for the four-point activity plot. The mean activity of the molecules is 190 s^{-1}, and the standard deviation of the distribution is 78 s^{-1} (n = 8). This distribution may be biased toward higher mean activity because low-activity molecules may not have generated detectable peaks during the relatively short incubation period used in this experiment.

Activity Distribution. The linear relationship between product concentration and reaction time, Figure 5, confirms that

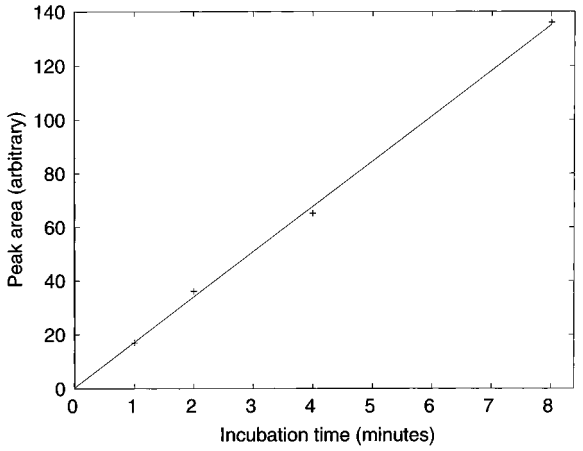

Figure 5. Kinetic plot for a multiple incubation. Peak area is shown by crosses, and the straight line is the least-squares fit to the data.

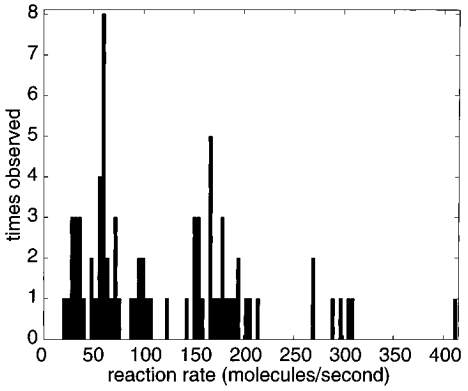

Figure 6. Histogram of reaction rates observed for 83 alkaline phosphatase molecules. Activity was determined from the incubation time and the peak area. Peak area was determined by a nonlinear least-squares fit of either a single or double Gaussian function to the peak.

single-molecule reaction follows pseudo-zeroth-order kinetics. The substrate concentration, 1 mM, is roughly a factor of 10 higher than K_m, 0.15 mM, for this enzyme−substrate combination, determined from a bulk solution assay. The substrate concentration is much higher than the amount consumed during an incubation. It would take several millennia for a single enzyme molecule to consume the substrate contained within the capillary; diffusion replaces substrate faster than the enzyme consumes it. We can be confident that the amount of product produced during an incubation period is proportional to the activity of the enzyme molecule.

The activity of 83 individual alkaline phosphatase molecules is plotted as a histogram, Figure 6. The distribution is quite broad and non-Gaussian, with a 10-fold range in activity. The drop-off in activity below ∼25 s^{-1} appears to be real; our instrument should detect activity of ∼6 s^{-1} in a 20 min incubation. The activity tends to cluster around 60, 160, and 290 s^{-1}; however, it is not clear if this apparent clustering is real or if it is due to the relatively small number of points used to define the distribution. The general shape of the activity distribution is similar to that reported for lactate dehydrogenase.[13] The mean activity for this combined data set is 111 s^{-1}, and the standard deviation of the distribution is 83 s^{-1} (n = 83).

Bulk Assays. Four bulk assays were performed using an on-column reaction.[24] The average activity was 380 ± 108 s^{-1}. The accuracy is dominated by the uncertainty in the enzyme concentration provided by the manufacturer.

Discussion: Heterogeneous Activity

Single-Molecule Detection. There are several pieces of evidence that indicate that these peaks are due to the pools of fluorescent product generated by individual enzyme molecules. First, the average number of peaks is consistent with the concentration of enzyme and the reaction volume. Second, the uncertainty in the number of peaks detected per assay should be dominated by Poisson statistics for detection of discrete events; the standard deviation should be roughly equal to the square root of the mean number of peaks. The standard deviation in the number of peaks is consistent with the signal generated by individual molecules. Third, the number of peaks is proportional to analyte concentration. Fourth, the area of each peak is independent of concentration. Fifth, the peak spacing in the multiple incubation study is constant and consistent with the difference in mobility between product and enzyme.

Rate of a Reaction Catalyzed by a Single Molecule. The slope of the line in Figure 5 produces an unambiguous rate for the reaction catalyzed by a single molecule. In general, several thousand molecules are required to determine a thermodynamic property of a system. In our case, the rate was determined from many thousands of product molecules generated by many reactions catalyzed by the single enzyme molecule. The relative precision in the reaction rate, estimated from the linear least-squares fit to the four-point kinetic plot, ranged from 2 to 10%. The poorest precision was associated with the least active molecules.

The average reaction rate estimated from a large number of single-molecule experiments is roughly a factor of 2 lower than the value determined from bulk measurements. Quite likely, this difference results from uncertainty in the concentration of active enzyme in the bulk sample.

Enzymatic Heterogeneity. While it is possible to estimate with high precision the rate of a reaction catalyzed by a single molecule, the activities of different molecules differ by more than an order of magnitude. This broad distribution of activity can have several causes:

1. The broad range of activity could simply reflect poor experimental precision. However, we are able to estimate reaction rates with a precision of 10% or better in the multiple-incubation studies; similar precision is expected from the longer single-incubation studies.

2. Enzymes could stick to the capillary walls; if the active site of a molecule was partially hidden, then activity would be decreased compared with that of the free protein. However, the replicate incubation study demonstrates that the enzyme molecule migrates faster than the fluorescent product; the capacity factor for adsorption of the enzyme on the capillary wall must be small.

3. Enzymes could denature during the assay; those molecules with low apparent activity would have denatured early in the incubation period. However, the multiple-incubation data showed no evidence for loss of activity during subsequent incubations.

4. Enzyme aggregates would cause heterogeneous activity. However, zone electrophoresis shows that the enzyme migrates as several closely spaced bands with no evidence for aggregates.[29]

5. Heterogeneity could arise from different levels of glycosylation or other post-translational modifications to the enzyme. Mammalian alkaline phosphatases are anchored to the exterior of the cytoplasmic membrane via a phosphatidylinositol glycan moiety.[30] Both calf and rat intestinal alkaline phosphatase generate at least three closely migrating electrophoretic components, each of which consists of a microheterogeneous set of glycoproteins with different levels of glycosylation.[29,31] Variations in glycosylation of enzymes can cause variations in both K_m and V_{max}.[32] Human intestinal alkaline phosphatase has been fractionated. The activity of minor components is much higher than the activity of the main component.[33] Figure 6 is consistent with the last observation, where a large fraction of the enzyme molecules have modest activity and with a smaller fraction with higher activity. The clustering of activity at ~60, 160, and 290 s^{-1} would be consistent with the presence of three different components within the mixture of alkaline phosphatase. Finally, Rudd and co-workers demonstrate that glycoforms modify the dynamic stability and functional activity of an enzyme.[34]

6. It has been suggested that differences in activities between individual molecules can be explained by specific stable conformational arrangements of subunits, making certain sites less accessible than others.[13] Alternatively, tertiary structure may differ within a subunit of the enzyme; there may be more than one active conformation, but not all conformations are equally active. We return to this issue in the last section of this paper.

Results: Activation Energy of a Reaction Catalyzed by a Single Molecule

In these experiments, the first ~15% of the capillary is filled with a slug of very dilute enzyme mixed with the weakly fluorescent substrate. Typically, a single enzyme molecule is captured within the capillary, although occasionally several molecules are present. Each enzyme molecule is incubated three times at successively higher temperatures; application of a short electrophoretic pulse between incubations moves the enzyme molecule into fresh substrate for each incubation. After three incubations, the pools of fluorescent product are swept from the capillary by electrophoresis. This electrophoretic potential also separates the unreacted substrate from the fluorescent product.

Figure 7 presents a result where two enzyme molecules are captured within the capillary. Incubations of 15 min are performed at 16, 24, and 30 °C; other temperatures are used for other incubations. A 15 s electrophoretic pulse is used between incubations to separate the enzyme molecule from the pool of fluorescent product. The enzyme moves faster than the product; thus peaks due to the later incubations migrate first. The plot shows two sets of three peaks, which correspond to fluorescence from the pools of product generated by each enzyme molecule at the three temperatures. Also shown is a least-squares fit of two sets of three Gaussian functions to the data.

The first set of peaks is of higher amplitude than the second set, due to the higher activity of the first molecule. Also note that the peak spacing is larger for the second molecule, which implies a higher mobility for that molecule. Finally, peaks from the earlier incubations are wider because of diffusion during subsequent incubations.

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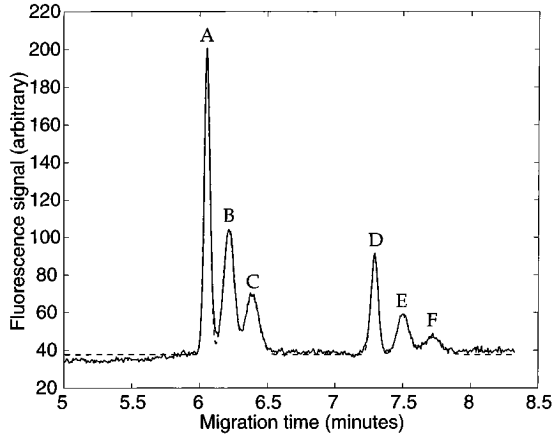


Figure 7. Electropherogram of alkaline phosphatase assay. Alkaline phosphatase (4.6×10^{-16} M) in 100 mM borate (pH 9.5) containing 1 mM AttoPhos was injected onto the capillary. Sample was incubated for three 15 min periods at 16, 24, and 30 °C interrupted by brief periods of high voltage. Following the third incubation the sample was flushed past the detector by CE. A set of three peaks is shown for each of two molecules of enzyme (peaks A, B, and C and peaks D, E, and F). Peaks A and D correspond to the incubation at 16 °C, B and E to 24 °C, and C and F to 30 °C.

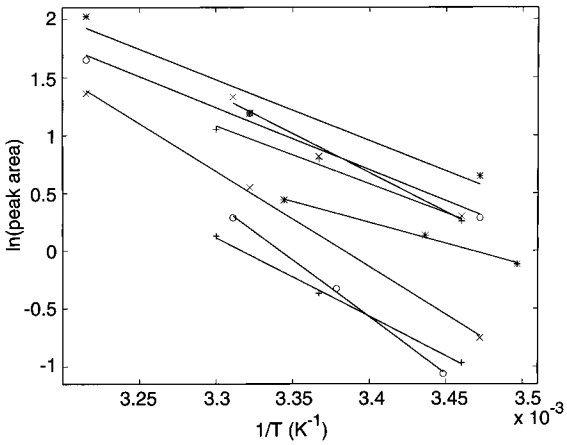


Figure 8. Arrhenius plot for reactions catalyzed by single enzyme molecules. The symbols are the peak areas determined at each temperature, and each straight line is the least-squares fit to the data for one molecule.

Peak area, estimated from the regression parameters, increases with temperature. Eight individual molecules of alkaline phosphatase were assayed for three incubation periods which ranged in temperature from 13 to 38 °C. As demonstrated above, the reaction is zeroth order in substrate concentration; peak area is directly proportional to the activity of the enzyme molecule. A plot of ln(peak area) vs $1/T$ yields a slope of $-E_a/R$, where E_a is the activation energy, Figure 8. A linear least-squares fit to the data is used to estimate the activation energy. The data are linear, with an average correlation coefficient of 0.994. The relative precision of the slope, estimated by the least-squares routine, ranges from 3 to 21%, with an average relative precision of 9%. Activation energies varied from 39 to 91 kJ/mol. The mean activation energy is 53 kJ mol^{-1}, and the standard deviation of the distribution is 16 kJ mol^{-1} ($n = 8$). There is no correlation between activation energy and activity, $r = -0.26$.

To generate an ensemble average, a solution of 4.6×10^{-12} M alkaline phosphatase was incubated at 23 and 32 °C; fluorescent product was diluted by 3 orders of magnitude and analyzed by capillary electrophoresis. The activity was 50 kJ/mol.

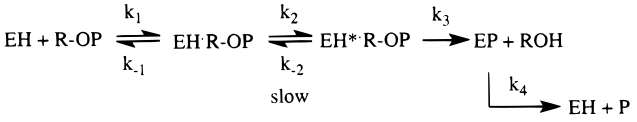
Discussion: Activity of a Reaction Catalyzed by a Single Molecule

The activation energy for a reaction catalyzed by a single alkaline phosphatase molecule is determined with a relative precision of ~10%. This activation energy is determined from many thousands of individual reactions catalyzed by the single enzyme molecule; the value represents a time average of the many reactions catalyzed by that single enzyme molecule.

The activation energy of different enzyme molecules varies by more than a factor of 2. This difference in activation energy is much larger than the experimental uncertainty, and it reflects the heterogeneous nature of this enzyme. As reported above, the activity of single molecules is also highly heterogeneous.[3] We interpret this range in activity and activation energy as being due to structural differences in the enzyme, which result from differences in post-translational modifications. Rudd and coworkers demonstrate that glycoforms modify the dynamic stability and functional activity of an enzyme;[34] presumably activation energy is also changed by glycosylation.

While the activation energy of single molecules is heterogeneous, the activation energy obtained from the time average of the eight molecules is equal at the 90% confidence limit to the ensemble average obtained from $\sim 3 \times 10^7$ molecules. The first postulate of statistical thermodynamics is verified.

Activation energy is uncorrelated with activity, indicating that other and more dominant sources of heterogeneity are present. The dephosphorylation of substrates by alkaline phosphatase has been proposed to occur via the mechanism[35]



where EH is the free enzyme, R−OP is the substrate, EH•R−OP is the enzyme−substrate complex, EH*•R−OP is the activated enzyme−substrate complex, EP is the phosphorylated enzyme, and P is inorganic phosphate. The first step represents ligand binding.[35] The second step represents the catalytic reaction, and it should dominate the activation energy. The third step represents loss of the reaction product. The equilibrium associated with ligand binding may dominate the activity of the enzyme.

Results: Thermal Denaturation and the Death of an Enzyme

Figure 9 presents the results of a 15 min incubation of a nominal 8×10^{-16} M solution of alkaline phosphatase. Five replicates generated an average of 9.8 ± 3.3 molecules/assay; the standard deviation is consistent with a Poisson distribution. The number of peaks is about twice the number observed in the activity study; different enzyme lots were used for the two assays. The mean reaction rate is 138 s^{-1}, and the standard deviation of the distribution is 107 s^{-1} ($n = 48$).

An 8×10^{-10} M solution of the same lot of enzyme was heated at 64 °C for 5 min and then immediately diluted by 6 orders of magnitude before assay, Figure 10. Five replicates yielded an average and standard deviation of 4.4 ± 1.5 molecules/assay; the standard deviation is consistent with Poisson statistics. The mean reaction rate of the surviving molecules is 118 s^{-1}, and the standard deviation of the distribution is 97 s^{-1} ($n = 22$). At the 85% confidence limit, the means of the

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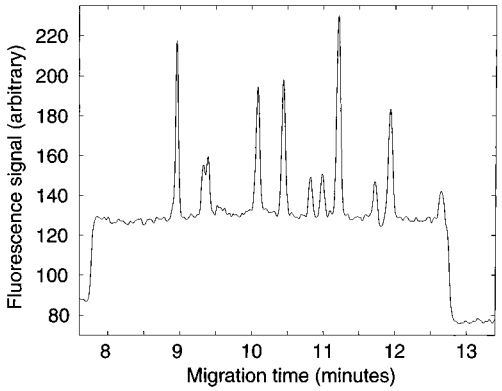

Figure 9. The effect of heat denaturation on alkaline phosphatase molecules: control sample. A nominal 8×10^{-16} M alkaline phosphatase solution was prepared in 1 mM AttoPhos and 100 mM borate buffer, injected onto the capillary for 3 min at 400 V/cm, incubated for 15 min, and then swept through the detector at 400 V/cm.

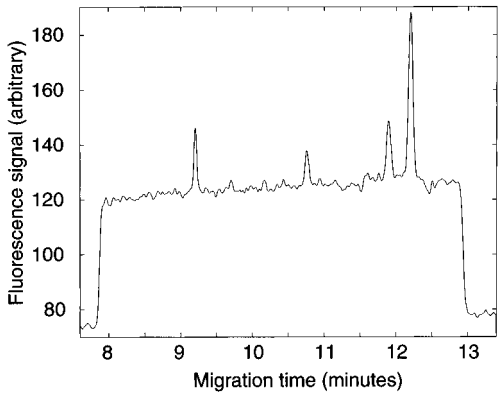

Figure 10. The effect of heat denaturation on alkaline phosphatase molecules: partially heat denatured sample. A nominal 8×10^{-10} M solution of alkaline phosphatase in 100 mM borate buffer was heated to 64 °C for 5 min. The sample was immediately diluted by 6 orders of magnitude in 1 mM AttoPhos and 100 mM borate buffer, injected onto the capillary for 3 min at 400 V/cm, incubated for 15 min, and swept to the detector at 400 V/cm.

distributions are identical, and at the 75% confidence limit, the standard deviations of the distributions are identical.

Discussion: Thermal Denaturation and the Death of an Enzyme

A conventional, bulk assay showed that heating the enzyme at 64 °C for 5 min results in a 50% decrease in activity in bulk solution. Within experimental error, there is no difference between the activities of control enzyme molecules and molecules that have survived thermal denaturation. The loss of activity is due to the loss of active molecules; a 50% loss in bulk activity corresponds to a $45 \pm 9\%$ loss in the number of active molecules.

Alkaline phosphatase apparently undergoes catastrophic loss of activity during thermal denaturation. Catastrophic denaturation is consistent with the results of Castelli and co-workers, who argue that chemical denaturation takes place at or near the active center of this enzyme; modification of the protein far from the active site does not appear to change activity.[36] Spectroscopic evidence suggests that the active site of alkaline phosphatase is quite rigid.[37] Loss of a magnesium ion from the active site would be irreversible in our buffer, which has a low magnesium concentration.

The rigid active site conformation of this enzyme, coupled with the catastrophic loss of activity during thermal denaturation, suggests that there is no transition between several active tertiary structures for calf intestinal alkaline phosphatase during denaturation. Instead, we believe that the microheterogeneity in activity and activation energy is due to heterogeneous glycosylation of the enzyme.[29−31] The range in activity observed for different electrophoretic fractions of human intestinal alkaline phosphatase is consistent with this interpretation.[33] Denaturation of these glycoforms apparently progresses at a uniform rate; there is no significant difference in the distribution of activity of the native and heat-treated enzyme. Presumably, the tertiary structure dominates the thermal denaturation pathway, leading to similar activation energy for denaturation of the different isoforms. Glycosylation instead must produce subtle changes in the conformation near the active site, subtly modifying the enzyme's activity.

As pointed out by a reviewer, thermal denaturation and protein folding may not pass through the same conformation pathways. For example, chaperon proteins can direct the folding of proteins through specific pathways that might not be explored during denaturation. As a result, the thermal denaturation study does not rule out the possibility that conformations exist with a range of activity. However, the study does place a stringent constraint on those conformations; they must undergo thermal denaturation at roughly the same rate.

Conclusion

Measurement of the activity of single enzyme molecules has several applications. As demonstrated here, single-molecule measurements are used to determine enzyme microheterogeneity. Heterogeneity is observed for both activity and activation energy and likely is associated with variations in glycosylation and other post-translational modifications.

These alkaline phosphatase results beg the question of the biological role of heterogeneous glycosylation of an enzyme. Most likely, the variation in glycosylation is a part of the enzyme regulation mechanism, where varying glycosylation patterns are associated with different rates of protease digestion. It will be interesting to explore protease denaturation of alkaline phosphatase at the single-molecule level. However, it will be necessary to perform the experiment with endogenous proteases; those enzymes must be extracted from the same tissue that generates alkaline phosphatase.

For the first time, we are able to study the effects of a physical change of an enzyme at the single-molecule level. Thermal denaturation of alkaline phosphatase leads to a complete loss of activity, with no evidence for intermediate states with lower activity. Of course, our data provide no evidence for the universality of catastrophic denaturation; it will be of interest to replicate these experiments in other enzymatic systems.

Several additional experiments can be considered for this system. For example, the effects of heat shock proteins and chaperonins on thermally denatured molecules can be studied at the single-molecule level; it would be interesting to find if there is any discrimination of the cellular protective machinery toward different isoforms of the enzyme.[38] Similar studies can be performed with other denaturation methods, such as chemical denaturants. Thermal denaturation can be considered in the presence of magnesium to study subsequent renaturation processes. Of course, alkaline phosphatase is a common reporter for both immunoassays and nucleic acid probing experiments;

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it would be interesting to explore these analytical measurements at the single-molecule level.

Ideally, these experiments should be performed with a homogeneous enzyme. We attempted to degylcosylate the enzyme. Unfortunately, severe denaturing conditions are required to open the structure of the enzyme; we have been unsuccessful in renaturing the enzyme into an active form. Homogeneous alkaline phosphatase is produced by bacteria but with much lower activity than the mammalian enzyme;[39] we are improving the sensitivity of our technique to allow assay of the bacterial enzyme.

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