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Single enzyme molecule assay with time resolution using capillary electrophoresis

Abstract: The continuous flow assay is a capillary electrophoresis-based methodology for the simultaneous measurement of the catalytic rate and electrophoretic mobility of individual molecules of the enzyme β-galactosidase. The method also provides time resolution. This method was used to measure the distribution of single-molecule activities and mobilities of a population of the wild-type *Escherichia coli* β-galactosidase. The catalytic rate was found to vary over time at an elevated temperature, suggesting a switching between different conformations. The successive incubation of individual molecules at 27°C, 45°C, and again at 27°C was found to convert molecules from one form with a stable catalytic rate to a different form with a different stable catalytic rate. Incubation at higher temperatures was found to cause a sudden and catastrophic loss in activity, which was consistent with denaturation. Increasing the incubation temperature over time was used to generate an Arrhenius plot for a single enzyme molecule. Finally, assaying the enzyme as the slow-release inhibitor D-galactal dissociated allowed for the measurement of the activity of individual subunits within a single molecule.

Keywords: β-galactosidase; capillary electrophoresis; heterogeneity; laser-induced fluorescence; single molecule.

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Introduction

The first report regarding the measurement of the catalytic rate of individual enzyme molecules was by Rotman (1961) using fluorescence microscopy on oil-coated microdroplets containing β-galactosidase. The study of single-molecule enzymology re-emerged in 1995 with Yeung's study of lactate dehydrogenase using a capillary electrophoresis (CE)-based protocol. This study showed that individual molecules of a given enzyme support different catalytic rates (Xue and Yeung 1995). In a later study, using fluorescence microscopy, Yeung found that catalytic rate varies over time for individual molecules of lactate dehydrogenase, but not for reactions catalyzed by single metal ions, suggesting that different conformational states might be a cause of the heterogeneity (Tan and Yeung 1997). Further studies, using CE (Craig et al. 1996, Shoemaker et al. 2003) and fluorescence microscopy (Gorris and Walt 2007), have consistently showed, using different enzymes, that catalytic rates vary between different individual molecules and over time for a given molecule. Such heterogeneity is not limited to catalytic rate. Activation energy of catalysis and V_{max} (Craig et al. 1996), K_m (Craig et al. 2010), electrophoretic mobility (Nichols and Craig 2008), and dependence upon co-factors to maintain activity (Craig et al. 2000) have also been found to be heterogeneous properties. Random errors in translation (Nichols and Craig 2008), differential posttranslational modifications (Craig et al. 1996), and conformational differences (Xue and Yeung 1995) have all been proposed as structural bases for the observed heterogeneity, with the last having the strongest experimental support (Craig et al. 2012a).

In one CE-based methodology (Craig and Nichols 2008), the catalytic rate, its variation over time and with changing temperature, as well as the electrophoretic mobility, of the individual β-galactosidase was measured. In this system, measurements were made as the molecules traversed the length of the capillary and, hence, termed the 'continuous flow assay'.

Capillary electrophoresis instrument

The continuous flow assays were performed using an in-laboratory-constructed CE instrument equipped with a postcolumn laser-induced fluorescence detection system. Fused silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of both 2- and 5-μm inner diameters and 40- or 50-cm lengths were used. The injection end of the capillary, along with a platinum electrode connected to a high voltage supply (Spellman model CZE 2000, Hauppauge, NY, USA), was placed into a buffer-filled vessel. Electric fields of 200–400 V cm^{-1} (injection end positive) were used. The detection end of the capillary, from which a 1-mm length of the external polyimide coating was removed by flame, was inserted into a quartz sheath flow cuvette with a 250- by 250-μm inner bore (Hellma, Concorde, ON, Canada). The system was grounded through the sheath flow buffer within the

cuvette. A 633-nm, 10-mW HeNe laser (Melles Griot, Nepean, ON, Canada) was used for excitation. Light from the laser was focused with a 6.3×, N.A. 0.2 microscope objective (Melles Griot) approximately 10 μm below the detection end of the capillary. Emission was collected at 90° using a 60×, N.A. 0.7 microscope objective (Universe Kogaku, Oyster Bay, NY, USA), passed through a 670DF40 or a 660AF10 optical filter (Omega Optical, Brattleboro, VT, USA) and a slit and onto a photomultiplier tube (PMT), Hamamatsu model 1477, Bridgewater, NJ, USA. The analog PMT signal was collected at 10 Hz and digitized using a Pentium 4 computer through a PCI-MIO-16XE I/O board utilizing LabView software (National Instruments, Austin, TX, USA).

β-Galactosidase (E.C. 3.2.1.23)

Escherichia coli β-galactosidase is a homotetramer of 1023-amino acid subunits and has a mass of 464 kDa (Jacobson et al. 1994). The enzyme catalyzes the hydrolysis of lactose into galactose and glucose. It has a high specificity for the galactose unit and the β(1 →4) linkage but not for the glucose moiety. As a consequence, the glucose can be substituted for a range of different chromophores or fluorophores, which is the basis of many of the different substrates used in its assay (Nichols et al. 2007). One such substrate, which was the one used in the assays discussed here, is 9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl)-β-D-galactopyranoside (DDAO-gal, Molecular Probes, Eugene, OR, USA), which is converted into the product 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one) (DDAO, Molecular Probes). DDAO-gal has no net charge, and DDAO is anionic. The removal of the galactose unit from the DDAO-gal results in a large shift in the spectral properties of the fluorophore. As a result, excitation at 633 nm provides a very strong fluorescent signal at 660 nm for the product DDAO but very little signal for DDAO-gal.

Sample preparation

DDAO-gal contains a small amount of DDAO, which is present as an impurity. Furthermore, upon its dissolution in water, additional DDAO is continuously formed, albeit at a slow rate, due to non-enzymatic hydrolysis. This DDAO results in a substantially increased baseline signal and must, therefore, be removed immediately prior to each assay. This is efficiently achieved by washing the substrate dissolved in assay buffer with toluene.

Single-molecule β-galactosidase assays have shown that measurements made on crude homogenates are indistinguishable from those from commercial preparations and redissolved high-quality crystals (Nichols and Craig 2008). As such, the purification of the enzyme prior to the assay is not necessary.

Double static incubation assay and the effect of citrate

In the double static incubation assay (Shoemaker et al. 2003), a capillary was filled with the buffer containing the substrate and very dilute enzyme. The enzyme concentration was such that 5–10 molecules were present within the capillary. The sample was incubated without mobilization for 10–20 min. As the enzyme molecules were on average several centimeters apart, and the incubation time was short, there was not sufficient time for the product formed by one enzyme molecule to diffuse to and mix with that from another. Rather, a distinct pool of product was formed in the immediate vicinity of each enzyme molecule. Following the incubation period, a brief period of electrophoresis was used to separate the enzyme molecules from their newly formed product pools and into the fresh substrate. During a second incubation period, a second product pool was formed for each enzyme molecule. After the second incubation, the capillary contents were mobilized past the detector. For each enzyme molecule, a set of two peaks was observed, one corresponding to each of the product pools formed during each incubation. For each set, the peak from the first incubation, having more time for diffusion to occur, was shorter and wider than that from the second.

In a double static incubation assay, where the buffer contained citrate, the wider peak of the pair exited the capillary first (Figure 1, lower trace). This indicates that during the separation period between the incubations, the newly formed product pool migrated ahead of the enzyme molecule, demonstrating that the enzyme had a more negative electrophoretic mobility than did DDAO. In the absence of citrate, the narrower and taller peaks exited first (Figure 1, top trace). This indicates that in the absence of citrate in the buffer, it is the electrophoretic mobility of the product that is more negative than the enzyme. The change in mobility is presumably due to the citrate binding to the enzyme, causing an increase in the negative charge of the enzyme (Craig et al. 2012c).

Multiple static incubations using up to four incubations have been reported. The areas of the peaks in a given set were within 10% of one another, despite the activities of the different molecules varying by 10-fold or greater, demonstrating the reproducibility of the activity measurement. The spacings between each successive peak in each set can be used to

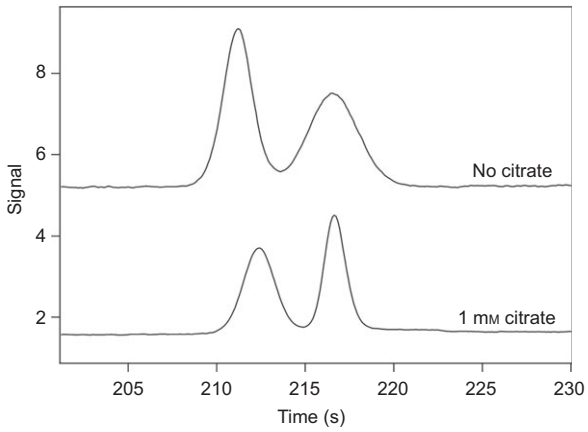

Figure 1 Double static incubation assay. The resultant electropherograms from the double static incubations of one molecule of β-galactosidase with 50 μM DDAO-gal in 10 mM HEPES buffer containing 1 mM $MgCl_2$ and the presence (lower trace) and absence (upper trace) of 1 mM citrate are shown.

calculate the electrophoretic mobility, yielding three values per enzyme molecule. The average variation for a given molecule was <1%. The reproducibility in both rate and mobility for any given enzyme molecule suggests that β-galactosidase is not interacting with the capillary walls, as the differential absorption occurring at the different locations along the capillary length would be expected to have a notable effect. Furthermore, the average and range of the determined electrophoretic mobilities were indistinguishable for the enzyme when measured in uncoated capillaries or capillaries coated with either polyvinylpyrrolidone or Genescan polymer 6 (PE Applied Biosystems). Finally, β-galactosidase has a calculated net average charge of -146 at pH 7.3 and is, therefore, expected to be repelled by the highly anionic capillary surface (Craig et al. 1996, Nichols and Craig 2008).

Continuous flow assay

In the CE, continuous-flow single enzyme molecule assay buffer containing a fluorogenic substrate and very dilute enzyme was continuously mobilized through a narrow-bore capillary tube. Enzyme concentration was such that, on average, approximately two to five enzyme molecules were traveling within the capillary at any given moment. As each enzyme molecule traversed the capillary, it was continuously forming a product. In the initial study (Craig and Nichols 2008), a 2-μm inner diameter capillary was used. In all but the last of the studies described in this review, the running buffer was 50 mM HEPES, 1 mM $MgCl_2$, 1 mM citrate (pH 7.3) containing 100 μM DDAO-gal. As the buffer contained citrate, the electrophoretic mobility of the product, DDAO, was less negative than that of the enzyme, β-galactosidase. As such, the product had a higher net mobility than did the enzyme. This resulted in newly formed product continuously advancing ahead of the enzyme molecule, forming a smear of the product (Figure 2). This produced a box-shaped peak in the resulting electropherogram (Figure 3). The height of the leading edge of the peak represents the activity of the enzyme molecule as it entered the capillary, the height at the trailing end, the activity as it exited the capillary, and the height at any point in between, the activity at the corresponding time between entering and exiting. The differences in the areas of the peaks indicate the differences in the catalytic rates of the individual enzyme molecules, and the differences in the widths represent the differences in their electrophoretic mobilities.

Determination of mobilities and catalytic rates

At the start of each run, the capillary was electrophoretically filled with the buffer containing the substrate

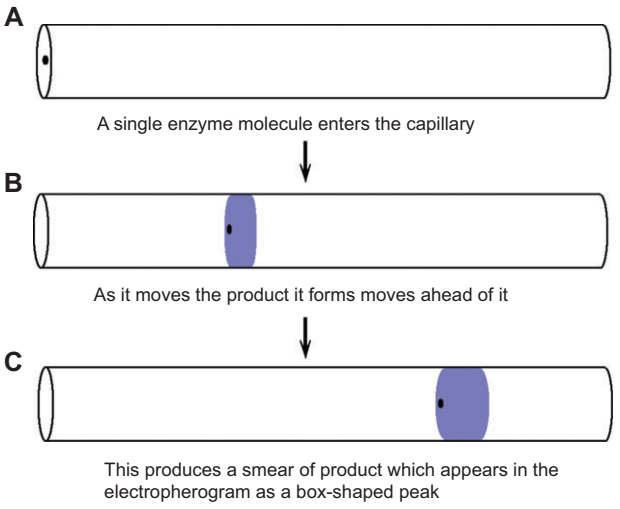

Figure 2 Continuous flow assay protocol. A capillary was continuously electrophoretically flushed with substrate containing very dilute enzyme. (A) An enzyme molecule enters the capillary. Enzyme concentration is such that individual molecules will enter the capillary, on average, several centimeters apart. (B) The enzyme molecule continuously forms the product, DDAO, from the substrate. DDAO has a higher net velocity than the enzyme molecule that produced it. As such, the newly formed DDAO molecules move ahead of the enzyme molecule. This starts to produce a smear of DDAO in advance of the enzyme molecule. (C) Once the enzyme molecule has reached the end of the capillary, a relatively wide smear of DDAO has formed. The peak widths are used to determine the electrophoretic mobilities of each enzyme molecule and the peak areas to determine the catalytic rate.

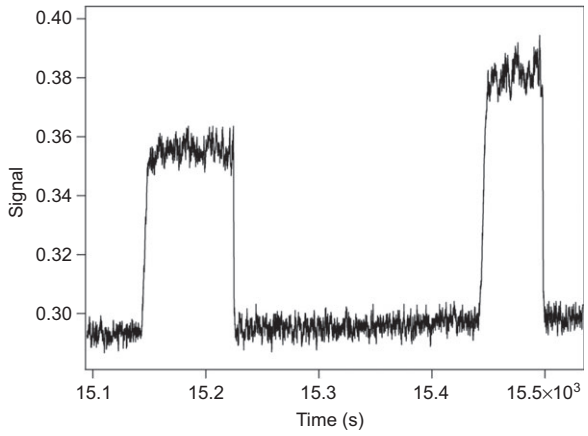

Figure 3 Electropherogram resulting from the assay of β-galactosidase at 24°C. The buffer containing β-galactosidase and DDAO-gal was continuously electrophoretically mobilized at 300 V cm^{-1} through a 60-cm-long 2-μm inner diameter capillary at 24°C. The buffer was 50 mM HEPES (pH 7.3) containing 1 mM $MgCl_2$ and 1 mM citrate. The substrate concentration was 100 μM. The signal obtained from two enzyme molecules is shown.

and very dilute enzyme. The substrate, being neutral in charge, moved with the same mobility of the electroosmotic flow (EOF). Because of its weak fluorescence, there was a slight shift in the background signal as the substrate began to elute. The time of this shift was used to determine the mobility of the EOF (μ_{EOF}) according to:

$$\mu_{EOF}=L/t_{m,DDAO\text{-}gal}E$$

where L is the capillary length, $t_{m,DDAO\text{-}gal}$ is the migration time of the substrate, and E is the electric field.

The substrate contained a small amount of the negatively charged product, DDAO, which was present as an impurity. Washing with toluene reduced the amount present but did not eliminate it. Because of this, and due to the continual formation of DDAO from DDAO-gal even in the absence of enzyme due to non-enzymatic hydrolysis, there was a second shift in the baseline as the product began to elute. The time of this shift was used to calculate the electrophoretic mobility of the DDAO (μ_{DDAO}) according to:

$$\mu_{DDAO}=(L/t_{m,DDAO}E)-\mu_{EOF}$$

where $t_{m,DDAO}$ is the migration time of the DDAO.

The width of each box-shaped peak represents the difference in the mobility of the enzyme molecule and the product formed. The electrophoretic mobility of each enzyme molecule (μ_{enzyme}) was determined by:

$$\mu_{enzyme}=[L/(t_{m,DDAO}+w)E]-\mu_{EOF}$$

where w is the width of each box-shaped peak.

The time each enzyme molecule spent traversing the capillary (t), which is the incubation period, was calculated using:

$$t=L/(\mu_{EOF}+\mu_{enzyme})E$$

The peak areas were compared to that of the DDAO standards run daily and divided by the incubation time in order to determine the catalytic rates.

The β-galactosidase molecules from a commercially obtained preparation were assayed and the catalytic rate and electrophoretic mobility determined for each of the 69 molecules. The average determined catalytic rate was 29,000±11,000 min^{-1} with a total range of 9200–53,000 min^{-1}. Static multiple incubation assays have consistently shown that the single enzyme molecule rates can be determined to within a reproducibility of 10% (Shoemaker et al. 2003). As such, the range of activities given reflects more the heterogeneity of the molecules than the reproducibility of the measurement. The rates obtained were similar to those reported elsewhere using the CE-based static incubation assay, although care must be taken in comparing the values from the different studies as the average catalytic rates of the enzyme from the different wild-type strains of *Escherichia coli* have been found to differ (Craig et al. 2003). The average catalytic rate determined for the individual molecules of β-galactosidase has been found to be similar to that of the large ensembles of the enzyme assayed under similar conditions in bulk solution (Rissen et al. 2008).

The electrophoretic mobilities of the 69 enzyme molecules were determined to be $-1.74\pm0.04\times10^{-4}$ cm^2 V^{-1} s^{-1} with a total range of -1.65 to -1.94×10^{-4} cm^2 V^{-1} s^{-1}. The negative sign indicates that the electrophoretic mobility was directed toward the injection end of the capillary. These values were similar to that obtained using the CE-based static incubation assays. The average and range of the electrophoretic mobilities of the individual β-galactosidase molecules have been found to be indistinguishable from that of the large ensembles of the enzyme (Craig and Nichols 2008).

Varying incubation temperatures

The capillary temperature was controlled by sandwiching it between an aluminum block, which contained an internal coil, through which water from a recirculating heating/cooling water bath was passed, and a piece of insulating foam. In the setup used, the entire length of the capillary could not be placed within the temperature control device. Rather, the approximately five to

seven terminal centimeters at both the injection and detection ends remained at ambient room temperature. Figure 4 shows the electropherogram from the assaying of 2 β-galactosidase molecules at 40°C (Craig and Nichols 2008). At the leading and trailing ends of each peak, there is a small shelf formed as the enzyme molecules traveled the end lengths of the capillary that were at the ambient room temperature of 27°C. The signal height varied substantially across the middle section of each peak, which corresponds to the travel across the heated portion of the capillary. This variation in height, which was not observed at lower temperatures (Figure 3), suggests that the catalytic rates of the enzyme molecules varied over time at the elevated temperatures. The variation in rate for a given molecule, referred to at a dynamic heterogeneity, has been observed for the different enzyme molecules across a wide range of time scales. In one study, this variation was noted to occur with the enzyme lactate dehydrogenase, but not for the rate of reaction catalyzed by the single metal ions, suggesting that the conversion between the different conformational states over time may be a cause (Tan and Yeung 1997).

Figure 5 shows the peak formed from a single β-galactosidase molecule in a continuous flow assay using a 60-cm-long capillary, where the length of the capillary is 7 cm from the injection end to 25 cm before the detection end was maintained at 37°C, and the remainder was at the room temperature of 28°C (Craig and Nichols 2008). There is a small shelf at the leading end of the peak formed as the enzyme crossed the initial unheated portion of the capillary. Upon entering the region at 37°C, the signal increased and varied over time, as observed in Figure 4. After exiting the heated region and returning to the unheated region of the capillary, the peak height decreased and remained nearly constant over time, as observed in Figure 3. A substantially larger fluctuation in signal over time at the elevated temperature than at room temperature was observed for all the enzyme molecules assayed (n=25). The shift in signal upon the shift in temperature reflects the change in the catalytic rate with the temperature. This, too, was found to vary between the individual molecules, suggesting that the activation energy of catalysis is also a heterogeneous property. This was observed previously using multiple static incubation assays of the enzyme alkaline phosphatase (Craig et al. 1996). On average, the height of the plateau at 37°C was found to be 2.1±0.5 times that at 28°C, with a total range of 1.7- to 3.7-fold. Figure 5 shows that for a single enzyme molecule, the catalytic rate, how rate varies over time, how rate varies with temperature, and electrophoretic mobility can all be assessed simultaneously using the continuous flow assay.

Figure 6 shows a variation on the previous experiment. In this case, the central 20 cm of a 60-cm-long capillary was held at 45°C with the remainder at 27°C (Craig and Nichols 2008). As the enzyme crossed the first region at 27°C, the catalytic rate remained approximately constant

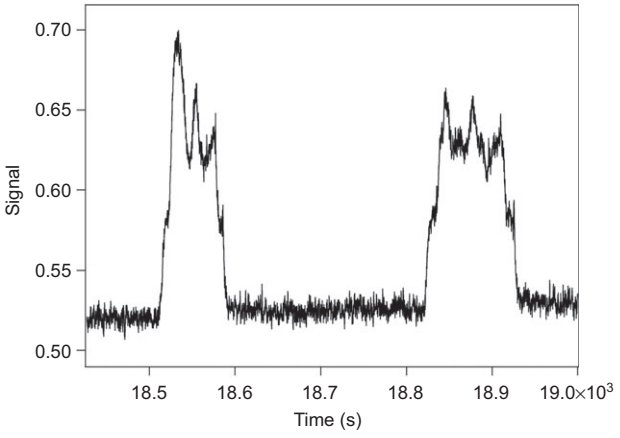

Figure 4 Electropherogram resulting from the assay of β-galactosidase at 40°C. The buffer containing β-galactosidase and DDAO-gal was continuously electrophoretically mobilized at 300 V cm^{-1} through a 60-cm-long 2-μm inner diameter capillary where the middle 46 cm of the capillary was at 40°C, and the remaining 7 cm on both ends was at 27°C. The buffer was 50 mM HEPES (pH 7.3) containing 1 mM $MgCl_2$ and 1 mM citrate. The substrate concentration was 100 μM. The signal obtained from two enzyme molecules is shown.

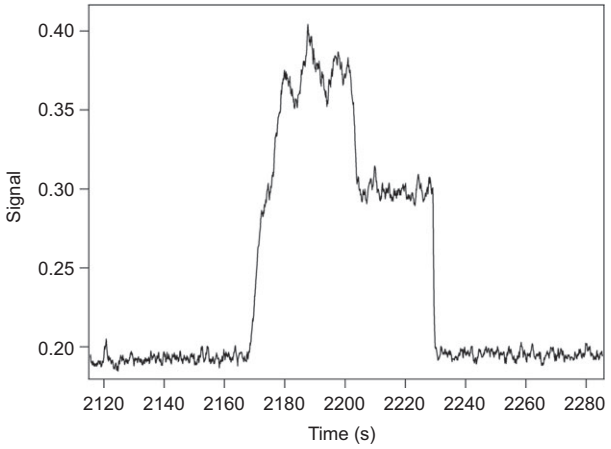

Figure 5 Electropherogram resulting from the assay of β-galactosidase at 28°C and 37°C. The buffer containing β-galactosidase and DDAO-gal was continuously electrophoretically mobilized at 300 V cm^{-1} through a 60-cm-long 2-μm inner diameter capillary where the capillary 7 cm from the injection end to 25 cm from the detector end was at 37°C and the remainder at 28°C. The buffer was 50 mM HEPES (pH 7.3) containing 1 mM $MgCl_2$ and 1 mM citrate. The substrate concentration was 100 μM. The signal obtained from one enzyme molecule is shown.

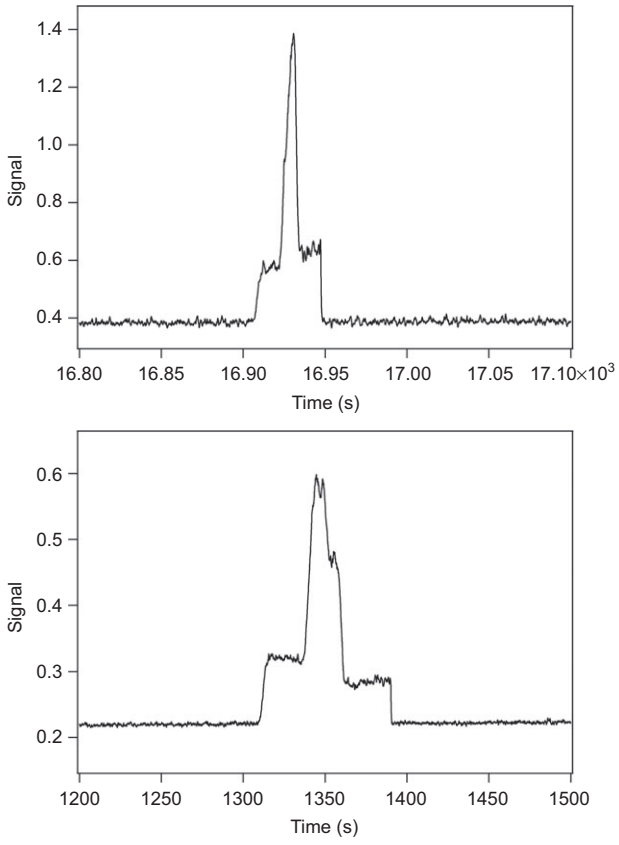

Figure 6 Electropherogram resulting from the assay of β-galactosidase at 27°C, 45°C, and again 27°C. The buffer containing β-galactosidase and DDAO-gal was continuously electrophoretically mobilized at 300 V cm^{-1} through a 60-cm-long 2-μm inner diameter capillary where the middle 20 cm was at 45°C and the remainder at 27°C. The buffer was 50 mM HEPES (pH 7.3) containing 1 mM $MgCl_2$ and 1 mM citrate. The substrate concentration was 100 μM. The signal obtained from one enzyme molecule is shown. The upper trace shows an enzyme molecule, which was thermally converted to a form with a higher activity and the lower trace one that was converted to a less active form.

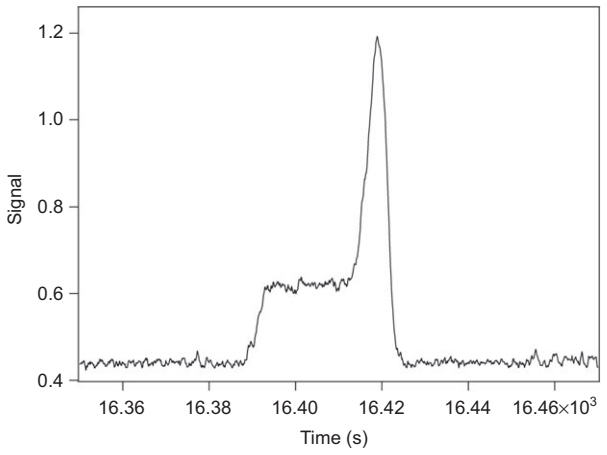

Figure 7 Electropherogram resulting from the assay of β-galactosidase at 27°C, 50°C, and again 27°C. The buffer containing β-galactosidase and DDAO-gal was continuously electrophoretically mobilized at 300 V cm^{-1} through a 60-cm-long 2-μm inner diameter capillary where the middle 20 cm was at 50°C and the remainder at 27°C. The buffer was 50 mM HEPES (pH 7.3) containing 1 mM $MgCl_2$ and 1 mM citrate. Substrate concentration was 100 μM. The signal obtained from 1 enzyme molecule that denatured during the assay is shown.

over time. Once reaching the region at 45°C, the rate increased and again varied substantially over time. Upon returning to 27°C, the rate again became approximately constant over time, although often, the rate was different than it was initially. Figure 6 shows the electropherogram for an enzyme molecule, which was converted into a more active form as well as that for one which was converted into a less active form. When the temperature of the central portion of the capillary was increased to 50°C (Figure 7), there was a rapid loss in signal after the enzyme molecule entered the heated region of the capillary and no return of the signal upon return to 27°C. This suggested a rapid and unrecoverable loss in activity at the higher temperature. This was consistent with denaturation. Of the 82 molecules assayed at 27°C/45°C/27°C, 13 became denatured. Of the 69 that remained active, the activity during the second 27°C incubation was, on average, 91±22% that of the first, with a total range of 42–130%. Incubation at the elevated temperature resulted in the conversion of the enzyme molecules into either more or less active conformation, with conversion to a less active form more common. Fifty-three enzyme molecules were assayed at 27°C/50°C/27°C. Of these, 45 became denatured, and of the 8 that retained activity, the activity during the second 27°C incubation was 56±10% of the first, with a total range of 43–69%. At the higher temperature, all the surviving molecules were converted to a form with a lower activity.

In a different study (Craig and Chase 2012), the central 40 cm of a 50-cm-long, 5-μm internal diameter capillary was placed in a temperature control unit at 27°C. The ambient room temperature was 23°C. During the course of a continuous flow assay, the temperature of the water in the recirculating heater/cooler was increased in a linear manner from 27°C to 37°C over the course of approximately 5 min. The time for an enzyme molecule to traverse the length of the capillary was approximately 10 min. Figure 8 shows the electropherogram resulting from a single enzyme molecule entering the capillary prior to the start of the temperature increase and exiting shortly afterwards. Initially, there is a shelf that corresponds to the portion of the capillary that was at room temperature. The enzyme molecule then entered the heated region, which was initially at 27°C, which resulted in an increase in the

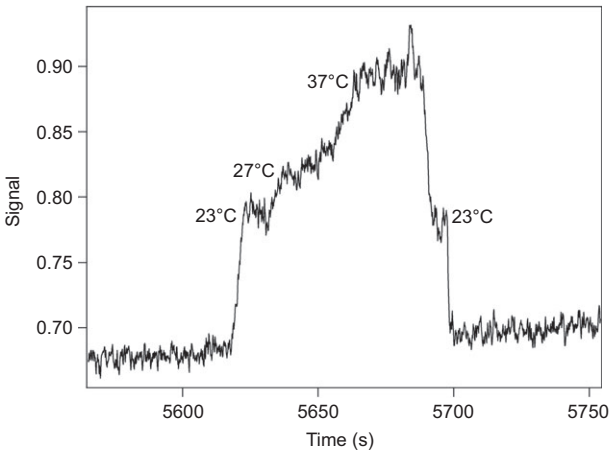

Figure 8 Electropherogram of the signal obtained by a single enzyme molecule in the presence of a 27°C–37°C temperature gradient. A molecule of *Escherichia coli* β-galactosidase was mobilized through a 50-cm-long, 5-μm internal diameter capillary at an electric field of 200 V cm^{-1} in the presence of the substrate DDAO-gal. As it traversed the capillary, the incubation temperature was increased in a linear manner from 27°C to 37°C. The buffer was 50 mM HEPES (pH 7.3) containing 1 mM $MgCl_2$ and 1 mM citrate. The substrate concentration was 50 μM. The resultant electropherogram is shown.

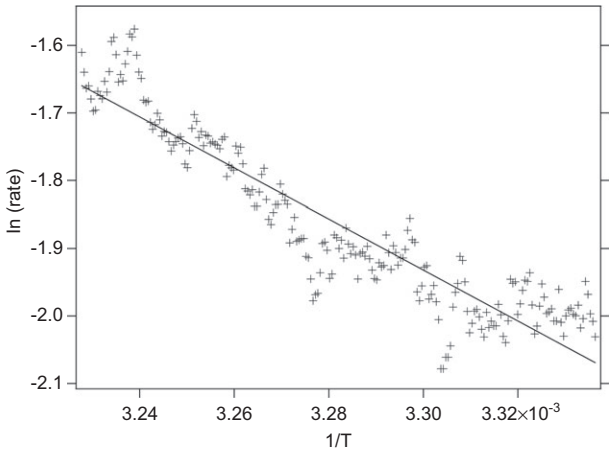

Figure 9 Single molecule Arrhenius plot. An Arrhenius plot of the change in signal with temperature for the reaction catalyzed by a single molecule of *Escherichia coli* β-galactosidase is shown.

plateau height. Shortly after entering the heated region, the temperature was increased linearly to 37°C. During this time, the signal obtained increased until the temperature reached and was maintained at 37°C, at which point the signal leveled off. As the enzyme molecule left the heated region of the capillary, there was a shift in the signal back down to approximately where it had been upon the enzyme entering the capillary. The region of the peak corresponding to the temperature gradient was fitted to an Arrhenius plot (Figure 9). The slope of the plot was -3800 (r^2=0.861), which corresponds to an activation energy of 31 $kJmol^{-1}$. Previous studies that measured the single molecule rates of β-galactosidase at two temperatures as per that shown in Figure 4 yielded activation energies ranging from 33 to 63 $kJmol^{-1}$ (Craig 2010). The single alkaline phosphatase molecule activation energies have also been measured and the average value found to be indistinguishable from that determined from large ensembles of the enzyme in bulk solution under identical conditions (Craig et al. 1996).

Inhibition by D-galactal

D-Galactal is a nonclassical competitive inhibitor of β-galactosidase (Lee 1969). It shows slow binding ($k=2.7\times10^2$ M^{-1} s^{-1}) and release rates ($k=4.6\times10^{-3}$ s^{-1}) and a K_i of 14 μM (Wentworth and Wolfenden 1974). As one D-galactal molecule binds per subunit, as the inhibitor slowly dissociates, one might predict a stepwise increase in the activity of a given β-galactosidase molecule as each individual subunit becomes active. Alternatively, if the inhibitor binding or dissociation was highly cooperative, all-or-none binding, resulting in the presence of only the fully active or fully inhibited states, might be expected.

In one study, β-galactosidase was incubated with D-galactal at a concentration that was 70 times its K_i for 30 min in order to saturate the enzyme. The sample was then diluted 20,000-fold to decrease the inhibitor concentration to K_i/280, causing dissociation to occur. The sample was subjected to a continuous flow assay as the inhibitor dissociated. This study used a different buffer system from that employed in the previously described continuous flow assays. Here, the buffer used contained no citrate, which reverses the relative mobilities of the enzyme and the DDAO product. It is for this reason that the box-shaped peaks in the electropherograms are reversed compared to the earlier figures. The leading edges of the peaks represent the activities of the enzyme molecules as they exited the capillary and the trailing edges of the activities as they entered.

Figure 10 shows the resultant electropherogram from a continuous flow assay of the individual β-galactosidase molecules as the inhibitor D-galactal dissociated (Craig et al. 2012b). In a similar study, although not using a CE-based methodology, Walt proposed that the dissociation of bound D-galactal from a given individual β-galactosidase molecule occurred simultaneously at all four subunits (Gorris et al. 2007). No stepwise increase in activity as inhibitor molecules dissociated one at a time from a given enzyme molecule was noted. The continuous flow assay

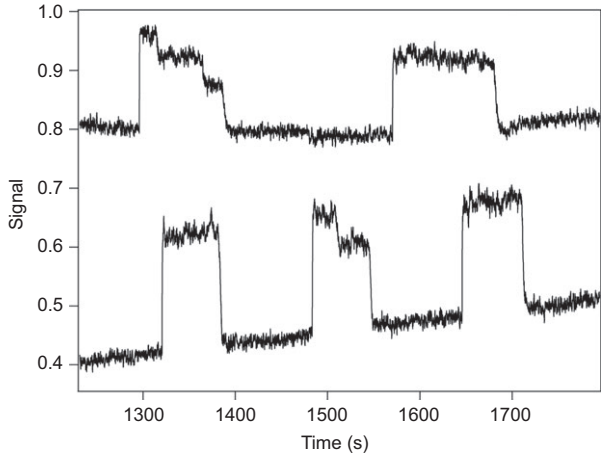

Figure 10 Assay of individual molecules of β-galactosidase as the inhibitor D-galactal dissociates. Portions of the resultant electropherograms from single molecule assays of β-galactosidase as the slow-release inhibitor D-galactal dissociates are shown. A 40-cm-long, 5-μm inner diameter capillary was used along with an electric field of 400 V cm^{-1}. The buffer was 10 mM HEPES (pH 7.3) containing 1.3 mM KCl and 68 mM NaCl. In the lower trace, box-shaped peaks for three individual molecules are observed. In the middle peak, there is a shift in the height of the peak due to the activation of a single subunit. In the upper trace, two peaks are observed. In the earlier eluting peak, there are two stepwise changes in the peak height due to the sequential activation of two individual subunits.

showed a similar observation for the large majority of the enzyme molecules. In the lower trace of Figure 10, the first and last peaks show no stepwise increase in activity. However, in the middle peak, a single stepwise increase in activity is observed. Of the 89 molecules assayed, 16 showed a single-step increase in activity. In the upper trace of Figure 10, the first peak shows two stepwise increases in activity. This pattern was observed in three instances. A peak with a maximum of three shifts in plateau height is possible for the tetrameric enzyme. No such peaks were observed. However, for those peaks in which two shifts were observed, at least one must represent the activation of a single subunit.

In the instances where a single shift in height was observed, the relative heights of the signal change from the baseline to the first plateau, and the signal shift from the lower to the higher plateau, were 1:1, 2:1, or 3:1. This pattern can most easily be explained in terms of tetramers with subunits of indistinguishable activity where a single subunit activates on a molecule with one, two, or three already active subunits. However, in one instance, the observed ratio was 5:1. This is not consistent with the subunits of equal activity. In two of the instances where there were three shifts in the activity, the ratios of the shifts in signal were 1:1:1 and 2:1:1, which is again consistent with the subunits of indistinguishable activity. However, in one instance, neither pattern was observed. In no instance was there an observed shift to a lower activity.

Single molecule assays of β-galactosidase have shown that the range of activities exceeds 10-fold (Shoemaker et al. 2003). The D-galactal results can be most easily explained in the large majority of instances by β-galactosidase molecules being made of subunits of indistinguishable activity. Other studies suggest that the differences in activity of the different molecules are at least largely due to conformational differences (Craig et al. 2012a). If the differences in the activities are indeed conformational, that individual molecules show a wide range of different activities and yet the subunits for a given molecule appear to have similar activity is consistent with the symmetry model of multisubunit enzymes (Monod et al. 1965). That is, there is more than one possible conformation, each with different activities, but for a given molecule, all subunits must be of the same conformation.

Summary

In the CE-based continuous flow assay, the individual molecules of the enzyme β-galactosidase traveled the length of a capillary filled with the buffer and fluorogenic substrate. As the enzyme molecules moved, they continuously converted the substrate into a highly fluorescent product. As the enzyme and product have different net mobilities, the product formed continuously moved away from the individual enzyme molecules, producing smears. As the smears passed an ultrasensitive detector, the box-shaped peaks were observed. The height of each peak at its leading edge represents the activity of the given enzyme molecule as it entered the capillary, the height at the trailing end, the activity as it exited, and the height at each point in between, the activity at the corresponding time between entering and exiting. This provided time resolution in the activity measurement. The width and height of each peak can be used to simultaneously measure the electrophoretic mobility and catalytic rate of the individual β-galactosidase molecules and the variation in peak height along its length, the variation in activity over time.

The control of the capillary temperature was achieved using a heating block. The catalytic rate of the individual enzyme molecules was found to vary over time at an elevated temperature but not at room temperature, suggesting the thermal interconversion between the conformations with the different activities. Furthermore, it was found that a brief incubation at an elevated temperature could convert the enzyme from one conformation with a stable activity to a different conformation with a

different stable activity. The conversion to a more or less active conformation was observed with the latter occurring more often. The maintenance of the different regions of the capillary at different temperatures was also used to determine the change in catalytic rate with temperature. Furthermore, the change in the catalytic rate was measured continuously for a single molecule as it traversed the capillary, while the capillary temperature was slowly increased. This was used to generate an Arrhenius plot for a reaction catalyzed by a single enzyme molecule.

In a final experiment, the catalytic rates of the individual molecules were monitored as the slow-release inhibitor D-galactal dissociated. In some instances, stepwise increases in the activity were observed as the single subunits on the individual molecules became active. The relative changes in the activity upon the dissociation of the D-galactal suggested that the enzyme consists of subunits of indistinguishable activity.

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