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Electrophoretic mobility, catalytic rate, and activation energy of catalysis of single molecules of the enzyme β-glucuronidase from *Escherichia coli*

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ABSTRACT

Single molecule assays were performed on the enzyme *E. coli* β-glucuronidase using a capillary electrophoresis-based protocol. Electrophoretic mobility, catalytic rate and activation energy of catalysis were all found to be heterogeneous. The average mobility at 22 °C was $-1.1 \times 10^{-8} \pm 0.1\ m^2V^{-1}\ s^{-1}$ (N = 49) with a total range of −0.6 to $-1.3 \times 10^{-8}\ m^2V^{-1}\ s^{-1}$. The range in electrophoretic mobility suggests that the differences in shape or charge of the individual molecules underlying the heterogeneity are likely minimal. The average catalytic rate at 22 °C was 37,000 ± 19,000 min^{-1} (N = 49) with a total range of 14,000 to 130,000 min^{-1}. Both of these properties were measured simultaneously for each of the molecules. There was a weak correlation ($r^2 = 0.43$) between mobility and rate with the molecules with a less negative mobility having a tendency to have a higher rate. The average activation energy of catalysis, as determined by comparing rates at 22 and 35 °C, was found to be 48 ± 18 kJ mol^{-1} (N = 7) with a total range of 18–66 kJ mol^{-1}.

1. Introduction

Single molecule assays have been performed on a variety of different enzymes using different experimental approaches [1–7]. Multiple measurements on a given molecule have demonstrated that the measurement of catalytic rate is reproducible to typically within 10%, but the rates differ between different individual molecules by up to 10-fold or more [8]. Furthermore, activity of an individual molecule has also been found to vary over time [9], and after a brief incubation period at elevated temperature [10]. Variation between individual molecules has been termed 'static' heterogeneity and variation in the activity of a single molecule over time has been dubbed 'dynamic' heterogeneity [11]. Dynamic and static heterogeneity have been considered as two facets of the same phenomenon and have been attributed to conversion between different active conformations [12]. Activation energy of catalysis has also been found to be heterogeneous, although measurements at the single molecule level have only been performed with alkaline phosphatase [8] and β-galactosidase [13]. Additionally, electrophoretic mobility has been found to show both static [14] and dynamic [15] heterogeneity, although only the enzyme β-galactosidase has thus far had this measurement made for a single molecule. Mobility measurements for a given molecule were found to be reproducible to within 0.8% but differ by up to 17% between different individual molecules [14].

Chemical dogma states that differences in properties must be due to differences in structure. It is difficult to infer what these structural differences might be from measurements of the variation in activity. This is because a very subtle structural difference near the active site might cause a large difference in activity. Conversely, a relative large structural difference far from the active site could conceivably have a minimal effect on activity [14]. Electrophoretic theory relates the effect of charge, size and shape to the mobility of a protein. From the difference in mobility of the different individual molecules, it is possible to determine the magnitude of differences between individual molecules with respect to charge or shape that are required to account for the observed heterogeneity in mobility [16].

E. coli β-glucuronidase (EC 3.2.1.31) is a homotetrameric enzyme with a mass of 273 kDa. The 3-dimensional structure has been determined with high resolution [17]. The enzyme has been shown to be catalytically heterogeneous with a 4-fold range of

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activity [18]. This study was undertaken to determine the distribution of electrophoretic mobilities of β-glucuronidase in order to gain insight into the magnitude of the differences in charge or shape associated with the enzyme heterogeneity. Catalytic rate and activation energy of catalysis were also measured at the single molecule level.

2. Methods and materials

2.1. Materials

Escherichia coli β-glucuronidase (EC 3.2.1.31) was obtained from Sigma (product number G-8420). Resorufin-β-D-glucuronide (res-gluc) and resorufin standard were obtained from Invitrogen/Molecular Probes. All other reagents were obtained from Sigma.

2.2. Assay

The substrate res-gluc contains some of the product resorufin as an impurity, which results in an increased background signal. This signal was reduced by washing the substrate immediately prior to use. 5 μL of 10 mM res-gluc in DMSO was diluted with 95 μL of 10 mM sodium citrate adjusted to pH 5.0 with HCl. This solution was washed 3 times with 400 μL of chloroform. To 50 μL of the washed substrate was added 448 μL of 25 mM KH_2PO_4 adjusted to pH 7.2 with NaOH and 2 μL of enzyme diluted in the same buffer. Final enzyme dilution was 250 million-fold into 22.5 mM KH_2PO_4 (pH 7.2) containing 1 mM citrate and 50 μM substrate. The enzyme concentration was approximately 1 molecule per capillary volume of 1.3 nL (2 μm inner diameter, 40 cm length), which corresponds to approximately 1 fM. Samples were injected continuously at an electric field of 100 V cm^{-1} (injection end positive).

2.3. Capillary electrophoresis (CE) instrument

Assays were performed using an in-laboratory constructed CE instrument which utilizes post-column laser-induced fluorescence (LIF) detection within a sheath flow cuvette. This instrument has been described previously [19]. The injection end of the uncoated fused silica capillary (Polymicro Technologies) and a 0.5 mm diameter platinum wire connected to a high voltage power supply (Spellman model CZE 2000) were placed into a buffer-containing vessel in the injection carousel and held at a positive potential. The detection end of the capillary, from which approximately 1 mm of the external polyimide coating was removed by flame, was inserted into a quartz sheath flow cuvette containing a 250 × 250 μm inner bore (Hellma). The capillary was grounded through the sheath flow buffer within the cuvette. The 1 mW output at 543.5 nm of a HeNe laser (Melles Griot) was focused using a 6.3 X, 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 60X, N.A. 0.7 microscope objective (Universe Kogaku), passed through a 580DF40 optical filter (Omega Optical) and a slit and onto a photomultiplier tube (Hamamatsu model 1477). The analog 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). The same board was used to control the electrophoresis voltage and the PMT bias, which was 1100 V. Sheath and running buffers were both 25 mM KH_2PO_4 adjusted to pH 7.2 with NaOH.

During the assay, the sample vial was placed in a plastic insert which sat within an aluminum box through which water at 1 °C was continuously passed via a recirculating heater/cooler unit. In the experiments where a portion of the capillary was incubated above or below room temperature, the portion of capillary was affixed to a glass tube through which water of the given temperature was passed via a second recirculating heater/cooler unit.

The data was analyzed using IgorPro (Wavemetrics) software. Raw data was smoothed 500 times using a binomial algorithm.

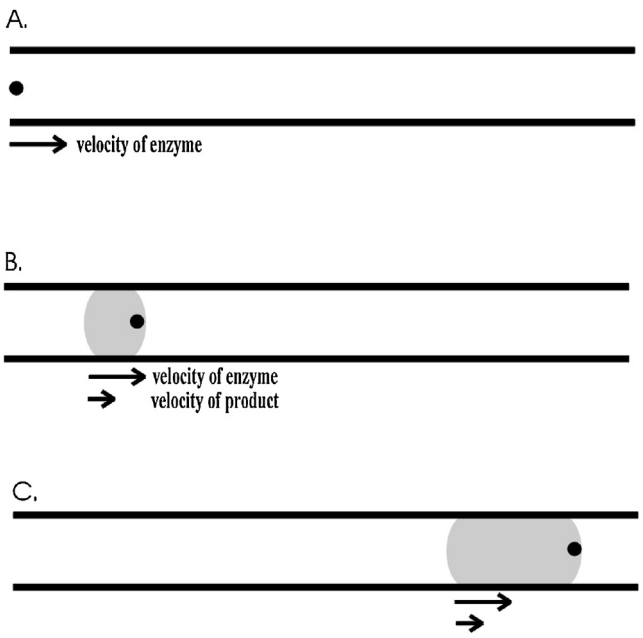

Fig 1. Depiction of the assay. Buffer containing substrate and dilute enzyme is mobilized through the capillary. Enzyme concentration is such that approximately 1 enzyme molecule is present per capillary volume (top). As the enzyme travels the length of the capillary it forms product. Since the enzyme molecule has a higher net mobility than the product, it moves ahead of the product molecules it is continuously forming (middle). This results in the formation of a smear of product. As this product smear exits the capillary and passes the detector it results in the presence of a box-shaped peak in the resulting electropherogram (bottom).

3. Results and discussion

3.1. Single enzyme molecule assay

The assay monitors the formation of the highly fluorescent product resorufin from the weakly fluorescent substrate res-gluc by the actions of the *E. coli* enzyme β-glucuronidase. In this assay, dubbed the continuous flow assay, sample containing substrate and very dilute enzyme is continuously and electrophoretically mobilized from a sample vial into and through a narrow bore capillary (Fig. 1). The enzyme concentration is such that approximately 1 enzyme molecule will be present per capillary volume. The enzyme molecule has a higher net mobility than the product it forms. As it travels the length of the capillary it continuously moves ahead of the product it forms, resulting in the formation of a smear of product, with the width of the smear reflecting the difference in the net mobility of the enzyme molecule and the product it forms. As the product smear exits the capillary and passes the ultra-sensitive laser-induced fluorescence detector it results in the formation of a wide box-shaped peak in the resultant electropherogram. Differences in the width of the different peaks reflect differences in the mobility of the individual enzyme molecules. The area of each peak represents the catalytic rate with differences in the areas reflecting differences in this rate for the individual molecules. If the enzyme molecules have a higher net mobility than the product, the height of the leading edge of each peak will represent the catalytic rate as the enzyme molecule exited the capillary, the trailing edge the rate as it entered, and the height at every point in between the rate at the corresponding time between entering and exiting. Variation in