

Crystallization of β -Galactosidase Does Not Reduce the Range of Activity of Individual Molecules[†]

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ABSTRACT: By use of a capillary electrophoresis-based procedure, it is possible to measure the activity of individual molecules of β -galactosidase. Molecules from the crystallized enzyme as well as the original enzyme preparation used to grow the crystals both displayed a range of activity of 20-fold or greater. β -Galactosidase molecules obtained from two different crystals had indistinguishable activity distributions of $31\,600 \pm 1100$ and $31\,800 \pm 1100$ reactions min^{-1} (enzyme molecule)⁻¹. This activity was found to be significantly different from that of the enzyme used to grow the crystals, which showed an activity distribution of $38\,500 \pm 900$ reactions min^{-1} (enzyme molecule)⁻¹.

Capillary electrophoresis utilizing laser-induced fluorescence detection (CE-LIF) has been used to measure the activity of individual molecules of lactate dehydrogenase (1), β -galactosidase (2), and alkaline phosphatase (3). Single lactate dehydrogenase molecule activities have also been detected by fluorescence microscopy (4), as have peroxidase (5) and cholesterol oxidase (6). In each case the activities of individual molecules have been found to differ widely. In most cases the range of activities was greater than 10-fold.

Crystallization is a commonly used purification method, particularly in organic and inorganic chemistry. Protein crystallization is also used as a prelude to structure determination by X-ray crystallography. Nevertheless, it is unclear whether enzyme molecules from a single crystal are heterogeneous or whether the range of activities differs among crystals. It is also not known whether the activity of a crystalline protein differs from the original enzyme preparation used in the crystallization process. We here address these questions.

MATERIALS AND METHODS

Chemicals. Resorufin and resorufin β -D-galactopyranoside were purchased from Molecular Probes (Eugene, OR). Poly(vinylpyrrolidone) (PVP, average molecular weight of 1.3 MDa) was from Aldrich (Milwaukee, WI). Poly(ethylene glycol) (PEG) 8000 was from Hampton Research (Laguna Niguel, CA), Ni-NTA agarose was from Qiagen (Hilden, Germany), and thrombin protease was from Amersham Pharmacia Biotech (Piscataway, NJ). All other chemicals were supplied by Sigma (St. Louis, MO).

β -Galactosidase Purification and Crystallization. *Escherichia coli* β -galactosidase was purified and crystals in

space group $P2_12_12_1$ were grown as previously described (7), with the exception that the anion-exchange step (after His tag cleavage) was omitted. The crystals were stored in 100 mM Bis-tris (pH 6.5), 200 mM MgCl_2 , 100 mM NaCl buffer, 10 mM DTT,¹ and 10% (w/v) PEG 8000.

CE Instrumentation. Assays were performed on a non-commercial capillary electrophoresis instrument that utilizes postcolumn laser-induced fluorescence detection in a sheath flow cuvette. Details of this instrument have been published (8). A 40 cm long, 10 μm internal diameter fused silica capillary was used in this study. Excitation was with the 1 mW output at 543.5 nm of a HeNe laser. Emission was detected with a photomultiplier tube after passage through a slit and a 580df40 optical filter.

Single Enzyme Molecule Assay. Pipet tips, vessels, and nonprotein solutions were autoclaved prior to use. At the start of each day the capillary was cleaned by flushing by pressure injection with 100 mM NaOH followed by water.

Sheath and running buffers were 50 mM HEPES (pH 7.3) containing 1 mM MgCl_2 , 0.0005% (w/v) BSA, and 0.02% (w/v) PVP. The capillary was coated by pressure injection with 50 mM HEPES (pH 7.3) containing 1 mM MgCl_2 and 2.5% (w/v) PVP for 20 min. The coating solution was removed by flushing by pressure injection with running buffer for 20 min. Resorufin standards were run in triplicate daily, and peak areas were compared to that produced in the single-molecule assays for the purpose of determining catalytic rates. Peak areas were determined by fitting to Gaussian curves followed by integration with PeakFit software.

Two 0.5 μL aliquots of the buffer in which the crystals were stored, each containing one β -galactosidase crystal, were suspended in 100 mM HEPES (pH 7.3) containing 50% (v/v) glycerol, 1 mM MgCl_2 , and 1% (v/v) Sigma protease

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¹ Abbreviations: DTT, dithiothreitol; PEG, poly(ethylene glycol); PVP, poly(vinylpyrrolidone); BSA, bovine serum albumin; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid.

inhibitor cocktail (contained no metal chelators) and the crystals were dissolved. Samples were stored unfrozen at -20°C . One $0.5\ \mu\text{L}$ aliquot of the original β -galactosidase preparation used to grow the crystals was also suspended in the HEPES buffer.

Stored samples were diluted from 7.1×10^6 - to 63×10^6 -fold to produce an assay solution containing approximately 1 fM β -galactosidase in 50 mM HEPES (pH 7.3), 1 mM MgCl_2 , 0.0005% (w/v) BSA, 0.02% (w/v) PVP, 1 mM citrate, and 200 μM resorufin β -D-galactopyranoside.

The assay solution was pressure-injected into the capillary for 5 min and then allowed to incubate for 15 min. During this incubation period there was no mobility of any of the components of the assay mixture other than diffusion. This allowed product to accumulate around the individual stationary enzyme molecules, forming localized pools. Following the incubation, an electric field of $400\ \text{V cm}^{-1}$ (injection end negative) was used to migrate the resorufin product past the detector.

To reduce the effect of any possible systematic bias, an equal or near-equal number of runs were performed daily on each sample and samples were assayed in an arbitrary order.

Statistics. The effects of treatments on enzyme activity were examined by analysis of variance. The Student Newman–Kuels multiple range test was used to test for differences between means. Homogeneity of variance between the different treatments was compared by Levene's test (9). Differences at the 95% or higher confidence level were considered significant.

RESULTS AND DISCUSSION

Enzyme Purity. During purification, enzyme purity was assessed by use of Coomassie-stained polyacrylamide gels. Insofar as possible, the presence of higher-order oligomers was kept to a minimum since this seems critical for crystallization. Enzyme purity prior to crystallization was approximately 90%, as determined by SDS capillary electrophoresis.

Substrate and Standard Purity. Resorufin β -D-galactopyranoside purity ranged from 96% to 98%, with the impurity largely consisting of resorufin (Molecular Probes, product information). To reduce the amount of resorufin impurity present, stock solutions of substrate dissolved in citrate (pH 4.8) were extracted three times with chloroform immediately prior to use. The resorufin used as a standard had a purity of 96–98% (Molecular Probes, product information) and yielded a single peak in the electropherograms.

Single-Molecule Assay. The single-molecule β -galactosidase assay was similar to that reported previously (2) except that the polymer was replaced with PVP and the assay medium contained Mg^{2+} and BSA. The assay relied on the conversion of the weakly fluorescent substrate resorufin β -D-galactopyranoside into the highly fluorescent product resorufin. The individual enzyme molecules were not detected. It was the product that produced the observed signal.

The capillary electrophoresis instrument used in this study employed an exquisitely sensitive fluorescence detector. Detection limits range from several thousand to single dye molecules, depending on the fluorophore (10). Individual β -galactosidase molecules can produce several thousand

product molecules per minute, and an incubation of a few minutes was sufficient to provide a detectable signal. The enzyme concentration was such that approximately 20 molecules were present in the 40 cm long capillary. These molecules were randomly distributed and on average about 2 cm apart. Assuming a diffusion constant of $5 \times 10^{-10}\ \text{m}^2\ \text{s}^{-1}$, which is typical for a molecule the size of resorufin, the 15 min incubation time allowed the product molecules to diffuse a distance of approximately 1 mm. The expected linear diffusion for β -galactosidase was approximately a third of this distance. Since the predicted diffusion distances are much less than the average distance between protein molecules, in general, product formed by one enzyme molecule did not have sufficient time to mix with product from another. Rather, localized pools of product were formed surrounding each enzyme molecule. Postincubation, electrophoretic mobilization of these pools past the detector resulted in the formation of peaks in the electropherogram.

In addition to the 15 min incubation period, the enzyme and substrate were in contact during the 5 min pressure injection and the 10 min electroelution periods. During injection, both substrate and product were in motion relative to one another due to turbulent flow in the injection assembly (data not shown) and product did not accumulate locally. The product and enzyme were also moving at different rates during elution due to differences in electrophoretic mobilities. It was only during the 15 min incubation that localized pools of product were formed.

The substrate, although weakly fluorescent, would have produced sufficient signal at 200 μM to ensure that the background signal would have been unmanageably high. For this reason a PVP-coated capillary was used with a negative potential at the injection end. Under these conditions the net mobility of the neutral substrate was equal to that of the residual electroosmotic flow, which was away from the detector. The product, however, being negatively charged, moved toward the detector. Thus it was only the product that produced signal.

When aliquots of identical volume are removed from a given solution, the expected number of analyte molecules present can be estimated from Poisson statistics. In the present case a 31 nL capillary volume of a 1 fM enzyme is expected to contain 18.7 ± 4.3 molecules per run. The exact position of the enzyme molecules within the capillary will be random. Therefore one expects the exact number and position of the peaks to vary from run to run.

Assays performed with the proprietary polymers Genescan (2) and Polymer 6 (11) (PE Applied Biosystems, Foster City, CA) to coat the capillary showed enzyme heterogeneity similar to that reported here, as did assays with an uncoated capillary (data not shown). Heterogeneity has also been observed with the fluorogenic substrate fluorescein β -D-digalactopyranoside (Molecular Probes, Eugene, OR) (unpublished data).

Reproducibility of the Measurements. Reproducibility of the activity measurements can be estimated by repeated incubations of a single molecule (1, 3). After an initial incubation period, an electric field is applied to the capillary. This causes the enzyme molecule to move out of the product pool it had just formed and into fresh substrate where a second incubation can be carried out. Comparison of the

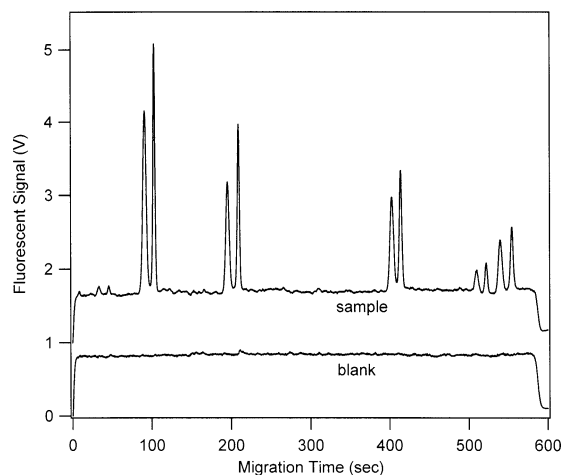


FIGURE 1: Electropherogram resulting from multiple incubations of individual β -galactosidase molecules. A very dilute solution of enzyme was incubated for 15 min and then subjected to an electric field of 400 V cm^{-1} for 60 s followed by a second 15 min incubation. Postincubation the sample was electrokinetically swept past a laser-induced fluorescence detector, producing the electropherogram shown (upper trace). The lower trace is that of a sample which was identical but contained no enzyme.

measured magnitudes of the pools gives an estimate of the reproducibility of the method, which was found to be 9% (3).

Figure 1 shows the resultant electropherogram from a double 15 min incubation of β -galactosidase. Each pair of peaks was formed by a single enzyme molecule. The first peak is shorter and wider than the second. This is because it resulted from the first incubation and had more time to broaden due to diffusion. Additionally, later-eluting peaks are generally wider and lower. This is because the product present in the early peaks was formed closer to the detector and thus had a smaller distance to travel, resulting in less broadening.

Integration of each pair of peaks shows that the area of the second peak is, on average, $85\% \pm 16\%$ that of the first. If, however, a double incubation is performed in the absence of Mg^{2+} , the second peak disappears completely. This suggests that the application of high voltage between the first and second incubation separates the metal ion from the enzyme and the enzyme unfolds or loses activity. Possibly the application of the high voltage even in the presence of Mg^{2+} causes some loss in activity. In any event, the multiple incubation experiment suggests that the activity measurements for β -galactosidase are reproducible to within about 15%.

Activity of the Crystallized Enzyme. Removal of individual crystals was achieved by suctioning a $0.5 \mu\text{L}$ volume of buffer containing a single crystal. The sample contained not only enzyme from the crystal but also any enzyme that might have been dissolved in the surrounding buffer. To quantitate the latter, $0.5 \mu\text{L}$ aliquots containing no crystals were also assayed and found to contain an average of less than 0.1% of the active enzyme molecules present in the aliquots that contained a crystal. Therefore, of the 300–400 molecules assayed from the crystallized enzyme, one would predict very few if any of these molecules came from the surrounding buffer.

Figure 2 depicts a resultant electropherogram for β -galactosidase from a single crystal. It is apparent that the

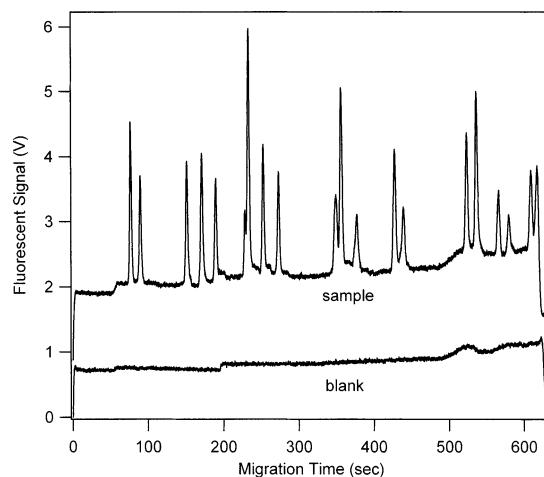


FIGURE 2: Electropherogram resulting from single-molecule assay of crystallized β -galactosidase. A very dilute solution of enzyme was incubated for 15 min and then electrokinetically swept past a laser-induced fluorescence detector, producing the electropherogram shown (upper trace). The lower trace is that of a sample which was identical but contained no enzyme.

individual molecules did not all produce the same amount of product during the 15 min incubation period. Thus the molecules from crystalline β -galactosidase showed the same heterogeneity in catalytic rate that has been observed in all enzymes assayed in this manner to date.

Fourteen runs were performed on each of the samples containing crystallized enzyme, resulting in the detection of 22.4 ± 3.8 and 22.0 ± 5.2 peaks, for a total of 313 and 308 molecules assayed, respectively. In the two samples the average single-molecule activities were nearly identical at $31\,600 \pm 1100$ and $31\,800 \pm 1100$ reactions/min. The range is reported as the standard error of the mean. Fourteen assays of the enzyme from the original preparation used to make the crystals were performed, producing an average of 25.6 ± 4.6 peaks for a total of 359 molecules assayed. The average single-molecule activity was found to be $38\,500 \pm 900$ reactions/min. The observed activity difference between the most and least active molecule observed was 52- and 50-fold for the two crystallized enzyme samples and 20-fold for the noncrystallized enzyme.

Figure 3 depicts the distribution of single-molecule activities of the samples assayed. There were significant differences in the enzyme activity from the different treatments. The noncrystallized enzyme had significantly higher activity than either of the crystallized enzymes but the activity of the two crystallized enzymes did not differ from each other. Levene's test showed that the variance in the response was not significantly different between treatment groups, indicating that the degrees of heterogeneity of the crystallized and noncrystallized samples were indistinguishable.

As has been found previously with enzymes that were not crystallized, individual enzyme molecules obtained from crystals of β -galactosidase are heterogeneous with respect to activity. This heterogeneity in activity must reflect heterogeneity in structure. It is important to note that in the single-molecule assays no direct measurements were made on the enzyme molecules. Therefore the data do not permit one to determine the structural basis for the observed differences in activities. Several sources of structural heterogeneity can, however, be envisaged, such as differences

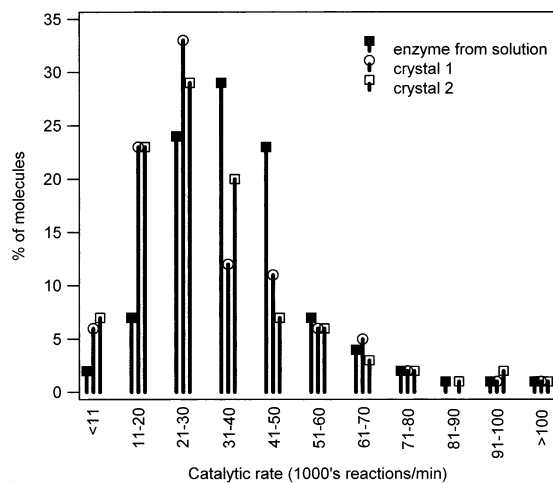


FIGURE 3: Distribution of activities of individual β -galactosidase molecules obtained from two different crystals and from the enzyme solution used to grow the crystals.

in the oxidation of cysteine or methionine residues, deamidation of glutamine or asparagine residues, differential phosphorylation, or modifications leading to alterations in amino acid sequence, including partial proteolysis. Regardless, the apparent structural or chemical differences do not prevent crystallization, indicating that the differences are likely minor. The finding that the enzyme molecules within a crystal do contain likely minor structural differences does imply that the protein structure determined from such crystals by X-ray crystallography represents an average structure of the heterogeneous population. However, since the distribution of activities did not vary between individual crystals, one would expect no deviation from the derived three-dimensional protein structure due to differences in the particular crystals used to obtain the structure.

Protein structures determined by X-ray crystallography may have poorly defined regions. This is typically attributed to mobility of that particular region of the structure within the crystal or to differences in the conformation of the region between individual molecules. Enzyme heterogeneity represents another possible explanation. If the structural basis for the observed heterogeneity in enzyme activity was localized to a specific region of the protein, one might expect this to appear as a region of poorly defined structure.

The enzyme from all crystals had an identical distribution of activities and this distribution was significantly different from that of the enzyme preparation used to grow the crystals. There are three possibilities that might account for this difference. The buffer used to store the crystals was different from the buffer used to store the enzyme solution. However, since the enzyme molecules were dramatically diluted into the same buffer prior to assay, differences due to buffer composition seem unlikely. The protein crystals grew and remained at room temperature for about a week prior to being dissolved and stored at -20°C . During this time the protein from the solution used to make the crystals remained frozen at -80°C . This might have contributed to the difference in mean activity, although the pattern of bands obtained by isoelectric focusing of β -galactosidase has been found to not

be affected by processes such as repeated freezing and thawing or prolonged cold storage (12). A more likely possibility is that crystallization may be selective. One possible basis for selection could be against higher weight oligomers. β -Galactosidase is known to form octamers and higher molecular weight aggregates (13), and minimization of the presence of such oligomers has been found to be critical for crystallization. The noncrystallized enzyme showed an average single molecule activity that was 20% greater than that obtained from the crystals. This could, in principle, be explained if approximately a fifth of the noncrystallized enzyme consisted of octamers with double the average activity of the tetramers. On the other hand, there is little evidence that the purified enzyme contains such a high proportion of octamers. Also, under the extremely low concentration of enzyme used in the assay (1 fM), any higher oligomers would tend to dissociate to tetramers. Thus the basis for selection by crystallization remains unclear.

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