Single Molecules of Highly Purified Bacterial Alkaline Phosphatase Have Identical Activity

Robert Polakowski, Doug B. Craig,† Alison Skelley, and Norman J. Dovichi*

Contribution from the Department of Chemistry, University of Alberta, Edmonton, Alberta T6G 2G2, Canada

Received December 23, 1999. Revised Manuscript Received March 28, 2000

Abstract: The central paradigm of chemistry is that molecular structure determines molecular function. Details of this paradigm can be tested with single-molecule enzymology, where the activity of individual molecules is studied. In all cases reported thus far, there is a large molecule-to-molecule heterogeneity in activity and activation energy. This heterogeneity must arise from differences in structure. Replicate incubations on the same molecule yield consistent results; the structural heterogeneity must be stable over the time period of the experiment, which can extend over several hours. In this paper, we demonstrate that highly purified molecules of bacterial alkaline phosphatase generate identical activity; structurally identical molecules behave identically. In contrast, the glycosylated mammalian enzyme demonstrates a complex isoelectric focusing pattern and has a dramatic molecule-to-molecule variation in activity and activation energy. Glycosylation affects both the kinetics and energetics of this enzymatically catalyzed reaction.

Introduction

The enzymatic activity of single molecules of lactate dehydrogenase, calf intestinal alkaline phosphatase, β-galactosidase, and cholesterol oxidase has been reported.[1−8] In most cases, the molecule-to-molecule activity was clearly heterogeneous. While there was a wide range in activity between molecules, the activity of any single molecule was stable over periods ranging from minutes to hours. The molecule-to-molecule heterogeneity also extends to activation energy, which varied by a factor of 2.5 for calf intestinal alkaline phosphatase.[3]

There are two explanations for this heterogeneity in molecular properties. In the energy landscape model, the molecule can exist in a number of different conformations that are separated by energy barriers of varying heights.[9] Yeung argued that the molecule-to-molecule heterogeneity in enzymatic properties of lactate dehydrogenase arises from molecules with identical primary structure that are trapped in different long-lived conformational substates; heterogeneity in molecular function is due to differences in the tertiary structure of the molecule.[1] In contrast, we have argued that molecule-to-molecule differences in the behavior of calf intestinal alkaline phosphatase reflect differences in the posttranslational modification of the molecule; heterogeneity in molecular function reflects heterogeneity in the primary structure of the molecule.[3]

In this paper, we demonstrate that the activities of single molecules of highly purified *Escherichia coli* alkaline phosphatase are identical. *E. coli* alkaline phosphatase is a nonglycosylated protein that is coded by a single *PhoA* gene. The enzyme exists in three forms, designated isoenzyme 1, 2, and 3. The isoenzymes differ by an N-terminus arginine that is present in isoenzyme 1 but absent in isoenzyme 3. Isoenzyme 2 represents a heterodimer of isoenzyme 1 and 3 monomers.[10−11] We used isoelectric focusing to purify each isoenzyme and laser-induced fluorescence to monitor the activity of individual enzyme molecules.

Experimental Section

Reagents. AttoPhos, 2′-[2-benzthiazole]-6′-hydroxybenzthiazole 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-benzthiazole]-6′-hydroxybenzthiazole. AttoPhos was purified as described previously.[3]

***E. coli* Alkaline Phosphatase Purification.** The purification is described in detail in the Supporting Information section of this paper. Briefly, samples were separated by isoelectric focusing and visualized with AttoPhos. Bands corresponding to isoenzymes were excised from the gel. Enzymes were purified by centrifugation through an appropriate ultrafilter. To avoid proteolytic digestion of the enzyme, procedures were performed in a cold-room and the purified enzyme was protected with a commercial protease inhibitor cocktail.

Single-Molecule Activity. The analysis was performed in a 10-μm i.d., 145-μm o.d., 72.5-cm long capillary; most of the capillary was coiled in a Peltier-controlled heating chamber. To perform the single-enzyme experiments, a 3×10^{-9} M solution of standard fluorescent product was injected onto the capillary by application of a 400 V/cm electric field for 2 s; the peak from this injection was used to calibrate

* Corresponding author. E-mail: norm.dovichi@ualberta.ca. Phone: 780-492-2845.

† Present address: Department of Chemistry, University of Winnipeg, Winnipeg, Manitoba R3B 2E9, Canada.

(1) Xue, Q.; Yeung, E. S. *Nature* **1995**, *274*, 681−682.
(2) Tan, W.; Yeung, E. S. *Anal. Chem.* **1997**, *69*, 4242−4248.
(3) Craig, D. B.; Arriaga, E. A.; Wong, J. C. Y.; Lu, H.; Dovichi, N. J. *J. Am. Chem. Soc.* **1996**, *118*, 5245−5253.
(4) Craig, D. B.; Arriaga, E. A.; Wong, J. C. Y.; Lu, H.; Dovichi, N. J. *Anal. Chem.* **1998**, *70*, 39A−43A.
(5) Craig, D. B.; Dovichi, N. J. *Can. J. Chem.* **1998**, *76*, 623−626.
(6) Lu, H. P.; Xun, L.; Xie, X. S. *Science* **1998**, *282*, 1877−1882.
(7) Xie, X. S.; Lu, H. P. *J. Biol. Chem.* **1999**, 274, 15967−15970.
(8) Edman, L.; Földes-Papp, Z.; Wennmalm, S.; Rigler, R. *Chem. Phys.* **1999**, *247*, 11−22.
(9) Frauenfelder, H.; Sligar, S. G.; Wolynes, P. G. *Science* **1991**, *254*, 1598−1603.
(10) Bradshaw, R. A.; Cancedda, F.; Ericsson, L. H.; Neumann, P. A.; Piccoli, S. P.; Schlesinger, M. J.; Shrieffer, K.; Walkh, K. A. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 3473−3477.
(11) Schlesinger, M. J.; Andersen, L. *Ann. N.Y. Acad. Sci.* **1968**, *151*, 159−170.

10.1021/ja994488j CCC: $19.00
Published on Web 05/05/2000

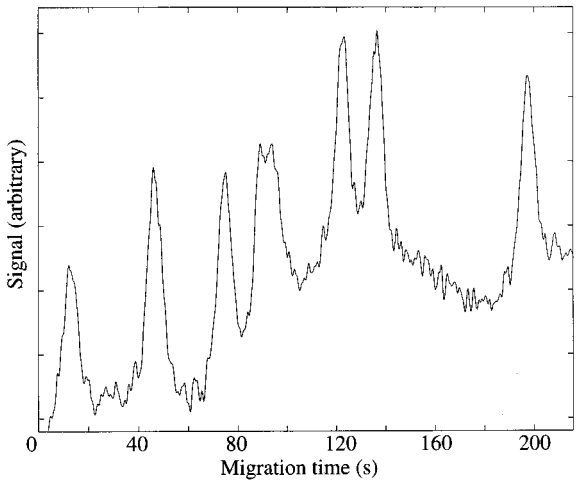

Figure 1. Single-molecule enzyme measurement on highly purified *E. coli* alkaline phosphatase. The mixture was incubated for 30 min at 40 °C. Only the portion of the electropherogram corresponding to the single-molecule peaks is shown.

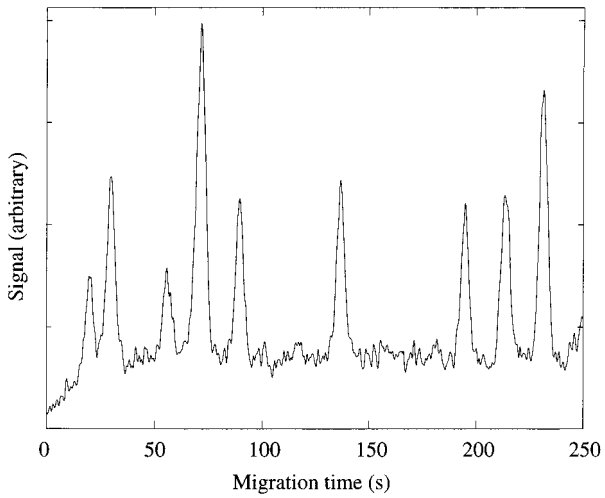

Figure 2. Single-molecule enzyme measurement on degraded *E. coli* alkaline phosphatase. The enzyme solution was stored for several weeks at 4 °C before analysis. Incubations were performed for 60 min at room temperature.

the response of the instrument. After the capillary and electrode were carefully rinsed and placed in a vial containing running buffer, electrophoresis was performed for 60 s at 400 V/cm to drive the standard into the capillary. Next, a nominal 10^{-15} M solution of enzyme was mixed with 1 mM AttoPhos. The mixture was injected for 360 s at 400 V/cm. Electrophoresis was applied for 120 s to drive the enzyme/substrate mixture into the heated region of the capillary. Last, the reaction products were separated by capillary electrophoresis at 400 V/cm and detected by laser-induced fluorescence in a sheath-flow cuvette.[3]

Results

Single-Molecule Enzymology of Highly Purified *E. coli* Alkaline Phosphatase. In single-molecule enzymology, a highly dilute solution of enzyme is mixed with a high concentration substrate and loaded into a capillary. Each molecule converts the weakly fluorescent substrate into a pool of fluorescent product. After incubation, an electric field is applied across the capillary. The pools of fluorescent product are swept through a high-sensitivity fluorescence detector, generating peaks in the fluorescence signal.

Figure 1 presents the result of a typical single-molecule incubation of purified enzyme from the high pI band of *E. coli* alkaline phosphatase. A set of eight peaks was detected; the fourth and fifth peaks were barely resolved. Peak area, which is proportional to enzyme activity, was determined by nonlinear regression analysis. Absolute activity was estimated by calibrating the response of the instrument with known amounts of fluorescent product. For the data of Figure 1, the molecule-to-molecule activity was $30 \pm 5\ s^{-1}$ ($n = 8$).

To investigate this residual molecule-to-molecule variation in activity, a synthetic data set was prepared. First, a Gaussian peak was constructed with the same height and width as the average peak in Figure 1. Then, seven of these peaks were added at random time points to the electropherogram generated by a blank. The synthetic data were subjected to the same regression analysis and generated similar uncertainty in activity as was observed from the single-molecule data. The minor molecule-to-molecule variation in activity is dominated by experimental precision.

Data were also generated from the two other isoforms of the enzyme, along with the commercial mixture. Within experimental error, the molecule-to-molecule activities of the isoenzymes and the commercial mixture were identical, with an average activity of $33 \pm 10\ s^{-1}$ ($n = 43$). There is a ~25% uncertainty day-to-day in calibrating the turnover number, which accounts for the increase in uncertainty of this average compared to the average obtained from a single run.

Single-Molecule Enzymology of Degraded *E. coli* Alkaline Phosphatase. There is a potential pitfall in single-molecule enzyme measurements. Samples of *E. coli* alkaline phosphatase were stored at 4 °C for several days in the absence of protease inhibitor. During storage, the enzyme was degraded by endogenous proteases. An isoelectric focusing analysis of these samples revealed up to 12 fractions of enzyme that retained their phosphatase activity. Figure 2 presents a single-molecule analysis for such a sample. The molecule-to-molecule activity in this sample varied by a factor of 5, which reflects the heterogeneous population produced during degradation.

Care is required to minimize proteolysis. A commercial protease inhibitor was added to the reagents used to generate Figure 1. Samples were also stored on-ice while awaiting incubation and operators wore latex gloves to prevent contamination of the sample.

Isoelectric Focusing Analysis of Other Enzymes. We have reported that calf intestinal alkaline phosphatase generates a broad molecule-to-molecule distribution in both activity and activation energy.[3] As expected because of its well-known posttranslational modifications, calf intestinal alkaline phosphatase produced a broad smear from pH 5 to 6 during isoelectric focusing. The highly heterogeneous molecule-to-molecule activity and activation energy undoubtedly arose from posttranslational modifications of the enzyme.

We have reported a wide range of activity for β-galactosidase.[5] Isoelectric focusing of this enzyme generated a smear from pH 4.6 to 5. The molecule-to-molecule variation in enzymatic activity likely reflects differences in structure due to proteolysis of this enzyme.

Xie reported the activity of single cholesterol oxidase molecules.[6] The observed rate constant was measured for 32 different molecules and varied by a factor of 5. The authors purified this enzyme by gel filtration chromatography and generated a single band by SDS-PAGE. We obtained a sample of this enzyme from the manufacturer; it generated a set of two bands under isoelectric focusing from pH 8.2 to 9.6, which straddles the pI value of 8.9 predicted by the ProtParam page

at the ExPASy web-site.[12] Unfortunately, our isoelectric focusing ampholytes did a poor job of resolving components of this high pI enzyme; most of the protein sample generated a broad band at the top of the gel.

Discussion

Molecular Heterogeneity. Single-molecule enzymology has been performed on a number of enzymes and in a number of laboratories. For each type of enzyme studied, there has been reported a wide range of behavior from molecule to molecule. This difference in behavior reflects differences in molecular structure. It has been proposed that the difference in behavior reflects transient structural differences, albeit structural differences that are stable on time periods of an hour.[1] However, all earlier studies were performed with commercial enzymes that had undergone relatively crude purification.

The data in Figure 1 demonstrate that molecules of highly purified *E. coli* alkaline phosphatase behave identically, at least when their activity is averaged over a 15-min incubation period. If there are any transient structural changes in the molecule, those changes are well averaged over the 15-min time period of our experiment.

Within experimental error, the three isoforms of this molecule have identical activity. These isoenzymes differ by a single amino acid deletion at the N-terminus, which is far removed from the active site of this enzyme. Minor structural changes do not change the activity of single molecules of *E. coli* alkaline phosphatase.

The data of Figure 2 demonstrate that proteolytic digestion of the enzyme leads to 5-fold differences in the activity of different molecules. These differences are correlated with the appearance of new bands upon isoelectric focusing analysis. Gross differences in primary structure are associated with significant molecule-to-molecule differences in activity of *E. coli* alkaline phosphatase.

Similarly, complex isoelectric focusing patterns are associated with the molecule-to-molecule differences in activity reported for calf intestinal alkaline phosphatase, β-galactosidase, and cholesterol oxidase. These isoelectric focusing patterns are caused by glycosylation, proteolysis, or other posttranslational modifications. The molecule-to-molecule variation in activity and activation energy reflects these gross structural differences. The energy landscape model is not required to explain molecule-to-molecule differences in enzyme activity reported for these crude enzyme preparations.

Effect of Posttranslational Modifications on Enzymatic Kinetics and Energetics. Molecules of the nonglycosylated *E. coli* alkaline phosphatase had identical kinetic behavior. In contrast, molecules of the highly glycosylated calf intestinal alkaline phosphatase had a 10-fold variation in activity and a 2.5-fold variation in activation energy. Two glycosylation sites are predicted in the mature mammalian enzyme,[13−14] and microheterogeneity in glycosylation patterns undoubtedly affects the kinetic and energetic properties of the molecule. On one hand, this observation is not surprising: changes in an enzyme's glycosylation cause increased sensitivity to proteases, changes in aggregation properties, shifts in pH optima, changes in both K_m and V_{max}, and complete loss of catalytic activity.[15] On the other hand, these molecule-to-molecule variations must be due to subtle interactions between the carbohydrate and the enzyme's active site.

Dynamic Variation in Activity. In addition to these molecule-to-molecule variations in activity, both Xie and Rigler have described a short-term fluctuation in the activity of an individual enzyme molecule.[6,8] These fluctuations arise from the molecule switching between active and inactive states with a rate of ~ 1 s^{-1}. This dynamic variation in activity cannot be explained by posttranslational modifications and instead must be due to short-term fluctuations in the molecule's structure.

In principle, our molecule-to-molecule measurement of enzyme activity can set limits on dynamic fluctuations in enzyme activity. Assuming the dynamic fluctuations are governed by Poisson statistics, the relative variance of the molecule-to-molecule enzyme activity will have a contribution that is equal to the inverse of the number of fluctuations that occur during the experiment. If there were any dynamic fluctuations in our experiment, there must have been more than ~ 30 fluctuations during the 30-min incubation; a smaller number of fluctuations would have made a detectable contribution to the molecule-to-molecule distribution in enzyme activity. If this enzyme fluctuates between active and inactive states, those fluctuations must occur with a rate greater than ~ 0.02 s^{-1}.

Conclusions

Single-molecule studies on highly purified *E. coli* alkaline phosphatase reveal no experimental difference in their properties. Similar studies on mishandled commercial enzymes generated large differences in properties. The long-term, molecule-to-molecule differences in activity and activation energy reported earlier for calf intestinal alkaline phosphatase are correlated with complex isoelectric focusing patterns.

Heterogeneous molecule-to-molecule enzyme activity is likely an artifact associated with posttranslational modifications, such as proteolysis and glycosylation, which interact with the active site of the enzyme. There is no need to invoke the energy landscape model to explain molecule-to-molecule variation in enzyme activity.

Glycosylation and proteolysis result in dramatic changes in the kinetic and energetic behavior of an enzyme. These changes are associated with subtle interaction between the active site of the enzyme and the posttranslational modification.

Acknowledgment. This work was supported by a research grant from the Natural Sciences and Engineering Research Council.

Supporting Information Available: Experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA994488J

(12) Wilkins, M. R.; Gasteiger, E.; Bairoch, A.; Sanchez, J.-C.; Williams, K. L., Appel, R. D.; Hochstrasser, D. F. In *2-D Proteome Analysis Protocols*; Link, A. J., Ed.; Humana Press: New Jersey, 1998.

(13) Culp, J. S.; Hermodson, M.; Butler, L. G. *Biochim. Biophys. Acta* **1985**, *831*, 330−334.

(14) Barker, W. C.; Garavelli, J. S.; McGarvey, P. B.; Marzec, C. R.; Orcutt, B. C.; Srinivasarao, G. Y.; Yeh, L.-S. L.; Ledley, R. S.; Mewes, H.-W.; Pfeiffer, F.; Tsugita, A.; Wu, C. *Nucleic Acids Res.* **1999**, *27*, 39−43.

(15) Varki, A. *Glycobiology* **1993**, *2*, 97−130.