BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **230,** 140–142 (1997)
ARTICLE NO. RC965910

Reversible Inhibition of Lambda Exonuclease with High Pressure

Edwin A. Rudd[1]
BioSeq, Inc., 25 Olympia Avenue, Unit F, Woburn, Massachusetts 01801-6307

Received November 8, 1996

The hydrolytic activity of lambda exonuclease, a highly processive enzyme, is inhibited by high pressure. When assayed at 67 MPa, the activity is 87% of the atmospheric rate; increasing the pressure to 336 MPa causes a greater than 99% inhibition of the enzyme activity. Decreasing the pressure from 336 MPa to 67 MPa at 20°C reverses the inhibition. The use of hydrostatic pressure to control the activity of lambda exonuclease has potential practical application in DNA sample preparation, analysis, and sequencing.

Macgregor reported that a pressure of 200 MPa completely inhibited the endonuclease activity of *Eco*R I at 37°C, and that the inhibition was reversible (1). Since Macgregor's observation, there have been additional reports describing the inhibition of type II restriction endonucleases by high hydrostatic pressure (2-4). Robinson and Sligar reported that *Bam*H I and *Eco*R V were inhibited 21% and 59% respectively by a pressure of 158 MPa, while *Pvu* II was only slightly inhibited at the same pressure (4). Kunugi, using a different substrate and buffer than Macgregor, reported that *Eco*R I was only slightly inhibited at 200 MPa (3). These restriction endonucleases are dimeric proteins. High pressure dissociates subunits of numerous multimeric proteins (5). Lambda exonuclease (E.C.3.1.11.3 *exodeoxyribonuclease*) is a monomeric protein that hydrolyzes DNA in a highly processive manner. Processive enzymes do not dissociate from the DNA substrate after each hydrolysis reaction (6,7). Following the initial enzyme-DNA complex formation, a processive enzyme remains bound to the DNA substrate until the digestion limit is reached. Thus, with processive enzymes the substrate binding step of the normal catalytic cycle is replaced with a translocation step whereby the enzyme moves along the DNA substrate. This report demonstrates that a processive enzyme is inhibited by high hydrostatic pressure, and that this inhibition is reversed when the pressure is subsequently lowered.

MATERIALS AND METHODS

Lambda exonuclease was purchased from Life Technologies, Gaithersburg, MD. The *Hin*d III fragments of lambda DNA were from IBI, New Haven, CT. The high pressure apparatus was from High Pressure Equipment, Erie, PA. The substrates used in these experiments were either a *Hin*d III digest of lambda DNA or linear plasmid pBR322 digested with *Eco*R V.

For these experiments a 5 μl substrate mix and a 5 μl enzyme mix were prepared and chilled on ice. The substrate mix contained 4 μl of assay buffer and 1 μl of DNA stock. The enzyme mix contained 4 μl of assay buffer and 1 μl of lambda exonuclease stock. Assay buffer contained 89 mM glycine-KOH, pH 9.4; 3.3 mM $MgCl_2$; and 50 μg/ml acetylated bovine serum albumin from Promega, Madison, WI.

To start an experiment the enzyme mix was added to the substrate mix on ice. This solution was then loaded into a cold capsule. A small polyethylene capsule was prepared by drawing out the end of a disposable transfer pipette. The capsule was sealed by folding over the reduced ends, which were held in place by crimping a small aluminum band over the folded ends. The capsule was then placed in a 5°C reaction chamber which was sealed and connected to the pressure apparatus. The apparatus was filled with silicone oil as pressurizing fluid. The pressure was then quickly raised to the desired pressure, and the valve to the reaction chamber was closed. The pressurized reaction chamber and valve were then disconnected from the apparatus and placed in a 15°C or 20°C water bath for various time periods. At the end of the incubation the valve and reaction chamber were then placed in a −70° C freezer for 30 minutes. This procedure froze the sample and allowed the capsule to be recovered from the reaction chamber while the sample was still frozen. The sample was recovered from the capsule by cutting off the ends of the capsule, placing the capsule in a microcentrifuge tube containing 4 μl of stop solution, and then briefly centrifuging to transfer the contents of the capsule to the stop buffer. The stop buffer contained 70 mM EDTA, pH 8; 0.35% SDS; 17.5% gycerol and 0.1% xylene cyanol. The total time to load a capsule and place it under pressure, plus the time to release the pressure and recover the sample, is referred to as the "dead-time". The dead-time was 5 to 7 minutes. The dead-time controls were used to determine the extent of the reaction that occured while the samples were not under pressure.

The extent of digestion by lambda exonuclease was determined using the *Hin*d III digest of lambda DNA. At the conclusion of a pressure experiment (described above) the DNA products were separated by electrophoresis on a 0.5% agarose gel containing ethidium

[1] Fax: (617) 932-8705.

0006-291X/97 $25.00

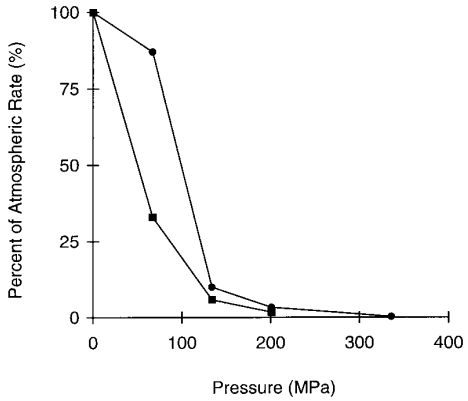

FIG. 1. Relative change in lambda exonuclease rate with pressure. Each 10 μl reaction mixture contained 340 ng of a *Hin*d III digest of lambda DNA and 3.3 units of Lambda exonuclease. Enzyme rates in bases/sec. were determined as described in Materials and Methods. Reaction temperatures, 15°C (■) and 20°C (●).

bromide (0.5 μg/ml). The running buffer was 45 mM Tris-borate, 1mM EDTA, pH 8.0. The high concentration of enzyme used in these experiments permits the digestion to proceed in a relatively synchronous manner. The gels were photographed on a transiluminator with appropriate filters. The migration distance of the DNA bands was determined from the photographs. Migration distance versus log DNA size (in base pairs) standard curves were constructed using Hind III fragments of lambda DNA as standards. The size of unknowns larger that 4,361 base pairs were determined from a linear fit of 9,416, 6,557, and 4,361 base pair standards. For unknowns smaller than 4361 base pairs, a linear fit of the 4,361, 2,322 and 2,027 base pair standards was used. To calculate a rate in bases/second, the size difference between the dead time and experimental bands was determined and divided by the length of time of the incubation.

Reversibility of inhibition was demonstrated using the *Eco*R V digest of pBR322. In these experiments the following pressure temperature profiles were used. The pressure of a 5°C reaction chamber containing a capsule was quickly raised to 336 MPa. The pressurized reaction chamber was then placed in a 20°C water bath for 30 minutes. The reaction chamber was then reconnected to the apparatus. The pressure was quickly reduced to 67 MPa and held there for the desired time period. The pressure was then increased to 201 MPa. Then the reaction chamber was placed in a −70°C freezer for 30 minutes. The sample was then recovered as above.

RESULTS AND DISCUSSION

Lambda exonuclease digests double-stranded DNA from the 5′-phosphorylated ends while releasing deoxyribonucleoside 5′-monophosphates (for a review see 8). The effect of pressure on the relative rate of digestion by lambda exonuclease is shown in figure 1. The average rate of hydrolysis by lambda exonuclease at ambient pressure was 6 bases/sec at 20°C. This rate is in agreement with that calculated (5 bases/sec) from the 20°C data of Little (9) for the hydrolysis of full length T7 phage DNA by lambda exonuclease. At 20°C, the rate of hydrolysis was only slightly inhibited by a pressure of 67 MPa. Increasing the pressure to 134 MPa caused a 90% decrease in the rate compared to the atmospheric rate. Further increases in pressure resulted in increased inhibition. At 336 MPa, the highest pressure tested, the inhibition was greater then 99%. Since the thermodynamic effects of temperature and pressure counteract one another, it was anticipated that decreasing the temperature would increase the inhibition by pressure. As can be seen in figure 1, decreasing the temperature to 15°C increased the inhibition at 67 MPa, resulting in a rate that was 33% of the atmospheric rate, (1.2 bases/sec). The products of the above lambda exonuclease digestions were double-stranded DNA molecules with single-stranded ends, whereas sizing was performed using double-stranded DNA with short (5 or 12 base) 5′ overhangs. Thus, the calculated rates of hydrolysis by lambda exonuclease will tend to be lower than actual. The 5′ ends of lambda DNA contain a 12 base single-stranded region. One of these ends is found in the 4,361 base pair fragment of the *Hin*d III digest. This fragment appeared to be digested from only one end in these experiments. It has been reported that lambda exonuclease is very slow to start digestion from the single-stranded ends of lambda DNA (9).

To determine if the pressure inhibition was reversible, the following experiment was performed. Enzyme and linear pBR322 DNA substrate were equilibrated to 20°C at 336 MPa. The pressure was dropped to 67 MPa in approximately 10 seconds. The enzyme rate at 67 MPa was 87% of the atmospheric rate (see figure 1). After a defined incubation period at 67 MPa the pressure was quickly raised to 201 MPa, and the sample was recovered as described in materials and methods. As can be seen in figure 2, the size of the substrate band decreased as a function of the incubation time at 67 MPa, demonstrating that the inhibition of lambda exonuclease by high hydrostatic pressure was reversible. Incubation at 67 MPa for 3 minutes caused the appearance of two smaller product bands. Size analysis

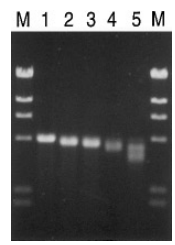

FIG. 2. Electrophoresis on a 0.5% agarose gel of the hydrolysis products of lambda exonuclease; the gel was stained with ethidium bromide. Each 10 μl reaction mix contained 0.25 μg of linear *Eco*R V digested pBR322 DNA and 3.3 units of lambda exonuclease. Lane 1, pBR322 substrate without enzyme, lane 2, exonuclease digestion during the dead-time plus 30 min. at 20°C, 336 MPa, lanes 3-5, extent of digestion during the pressure drop to 67 MPa incubation times of 1, 2, and 3 min. respectively, lanes M, *Hind*III fragments of lambda DNA, 23,130, 9,416, 6,557, 4,361, 2,322, and 2,027 base pairs top to bottom.

of these bands revealed that the lower (faster moving) band had twice as many nucleotides removed as did the upper band. This suggests that the lower band resulted from DNA molecules that were being digested from both 5′ ends, and that the upper band was the result of digestion at only one 5′ end. The reversal of inhibition upon release of pressure with lambda exonuclease is similar to that of *Eco*R I (1). The pressure inhibition of *Eco*R I is attributed to a decrease in the binding constant for enzyme-DNA complex due to electrostriction which destabilizes electrostatic interactions in the complex (10). Electrostriction may also be involved in the inhibition of lambda exonuclease at elevated pressures.

The application and release of high hydrostatic pressure to lambda exonuclease reaction mixtures may enable the generation of very precise deletions from the 5′-ends of linear DNA fragments. If the pressure is released for only a short period of time, then the enzyme will hydrolyze and release only a single mononucleotide during the interval at permissive pressure. This phenomenon could have applications for DNA sample preparation, analysis, and sequencing.

ACKNOWLEDGMENTS

I thank T. Lewis and Dr. K. Gillis for their assistance with data analysis. I also thank Dr. D. Green and J. Laugharn for their support and encouragement.

REFERENCES

1. Macgregor, R. B., Jr. (1990) *Biochem. Biophys. Res. Commun.* **170,** 775–778.
2. Robinson, C. R., and Sligar, S. G. (1994) *Biochemistry* **33,** 3787–3793.
3. Kunugi, S. (1993) *Prog. Polym. Sci.* **18,** 805–838.
4. Robinson, C. R., and Sligar, S. G. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 3444–3448.
5. Silva, J. L., and Weber, G. (1993) *Annu. Rev. Phys. Chem.* **44,** 89–113.
6. Carter, D. M., and Radding, C. M. (1971) *J. Biol. Chem.* **246,** 2502–2512.
7. Thomas, K. R., and Olivera, B. M. (1978) *J. Biol. Chem.* **253,** 424–429.
8. Little, J. W. (1981) *in* Gene Amplification and Analysis (Chirikjian, J. G., and Papas, T. S., Eds.), Vol. 2, pp. 135–145. Elsevier, New York.
9. Little, J. W. (1967) *J. Biol. Chem.* **242,** 679–686.
10. Macgregor, R. B., Jr. (1992) *Biochim. Biophys. Acta* **1129,** 303–308.