Regulation of DNA nucleases by molecular crowding

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ABSTRACT
Here, we examined the effects of molecular crowding on the function, structure and stability of nucleases. We found that the hydrolysis of a 29-mer double-stranded DNA by the endonucleases DNase I and S1 nuclease was substantially enhanced by molecular crowding using polyethylene glycol (PEG); however, molecular crowding had little effect on hydrolysis by exo III and exo I exonucleases. Moreover, kinetic analysis showed that the maximum velocity for the reaction of DNase I at 25°C was increased from 0.1 to 2.7 kM/min by molecular crowding with 20% (w/v) PEG, whereas that of exonuclease I at 37°C decreased from 2.2 to 0.4 kM/min. In contrast, molecular crowding did not significantly affect the Michaelis constant of DNase I or exonuclease I. These results indicate that molecular crowding has different effects on the catalytic activities of exonucleases and endonucleases.

INTRODUCTION
Nucleases, which cleave the phosphodiester bonds of nucleic acids, are very important not only for nucleic acid metabolism (1,2) but also for a variety of biotechnologies (3–5). For this reason, many studies have examined their structure, stability and function (6–10). Most of these studies, however, have been carried out under relatively dilute conditions. These are quite different to those inside a cell, where up to 40% of the total volume is taken up by macromolecules (11,12).

As numerous studies have shown, molecular crowding dramatically increases the association between biomolecules and substantially affects biomolecular reaction rates (13–27). For example, Zimmerman and Pheiffer (14) showed that DNA ligase activity is enhanced by molecular crowding conditions. Wenner and Bloomfield (16), in contrast, found that crowding agents have negligible effects on EcoRV activity. Thus, molecular crowding has different effects on different types of nucleic acid metabolizing enzymes. For this reason, systematic studies of the effects of molecular crowding on enzyme activity, structure and stability for a series of nucleases are needed to predict the nuclease behavior in cellular conditions and to understand how molecular crowding affects protein functions in the presence of nucleic acids.

In the current study, we systematically investigated the effects of molecular crowding on DNA hydrolysis by various nucleases. We found that the cleavage of both a large DNA (plasmid) and a short DNA oligonucleotide by DNase I was increased in the presence of polyethylene glycol (PEG). An increase in activity by molecular crowding was found for endonucleases (DNase I and S1 nuclease) but not for exonucleases (exonucleases I and III). Kinetic analyses showed that molecular crowding affected the maximal velocity ($V_{max}$) but not the Michaelis constant ($K_m$) for the nucleases. Overall, our results indicate that molecular crowding has different effects on the catalytic activities of exonucleases and endonucleases.

MATERIALS AND METHODS
Materials
PEG 4000, PEG 8000 and PEG 20000 [average molecular weight = 3000, 8000 and 20000, respectively (28)] were purchased from Wako Pure Chemicals (Osaka, Japan) and used without further purification. DNase I from bovine pancreas was purchased from Invitrogen (Carlsbad, CA, USA). Exonuclease I from Escherichia coli, S1 nuclease from Aspergillus oryzae and exonuclease III from E. coli were purchased from Takara Bio (Tokyo, Japan). Purified DNase I for the circular dichroism (CD) studies was purchased from Roche (Mannheim, Germany). All other reagents were of reagent grade.

Substrate DNAs
Plasmid [pcDNA 3.1(+) ] was purchased from Invitrogen. E. coli strains were grown aerobically in Luria–Bertani medium in the presence of 100 µg/ml ampicillin at 37°C with shaking at 250 r.p.m. E. coli Top 10 cells (Invitrogen, CA, USA) were used for plasmid propagation.

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HPLC-grade oligonucleotide substrates of 29-mer sequences, 5'-ACGATATCTCCCTATAGTGAGTCGTATTA-3' and 5'-TAATACGACGCTACTAGGGAGATATCGT-3', were purchased from Hokkaido System Science (Sapporo, Japan). These sequences were designed using mfold to avoid folding into undesired structures (29). For quantitative analysis of the hydrolysis reaction of nuclease, the sequence 5'-ACGATATCTCCCTATAGTGAGTCGTATTA-3' was labeled with 6-carboxyfluorescein at its 5'-end, although the 6-carboxyfluorescein could affect the rate of cleavage by a nuclease. A 29-mer double-stranded DNA (dsDNA) was prepared by annealing (incubation at 90°C for 5 min, followed by cooling at 1°C/min to the reaction temperature) of 5'-ACGATATCTCCCTATAGTGAGTCGTATTATAGTGAGTCGTATTA-3' with 5'-TAATACGACGCTACTAGGGAGATA TCGT-3'. The 29-mer dsDNA was used as substrate in assays of DNase I and exonuclease III. The sequence 5'-ACGATATCTCCCTATAGTGAGTCGTATTA-3' was used as a single-stranded DNA (ssDNA) substrate in the assays of S1 nuclease and exonuclease I.

**Assay of DNA hydrolysis by nucleases**

Because the optimal condition (cations, pH and temperature) for DNA hydrolysis by each nuclease varies, it is difficult to study all the enzymes using a single-standard assay. Therefore, for DNA hydrolysis by each nuclease, the optimal conditions (cations, pH and temperature) were determined and then used for the assays of S1 nuclease and exonuclease I.

**Exonuclease III** was assayed in a buffer of 5 mM MgCl₂, 80 mM NaCl, 1 mM ZnSO₄ and 30 mM sodium acetate (pH 8.0). The initial rates (v) were estimated from the data for the first 10% of the reaction. The values of v were plotted versus the DNA (substrate) concentration to allow calculations of the kinetic parameters (Kₘ and Vₘₐₓ). The kinetic parameters were calculated from the non-linear best fit of the data to the Michaelis–Menten equation:

\[ v = \frac{V_{\text{max}} [S]}{(K_m + [S])} \]

where \( v \) indicates enzyme activity, \( V_{\text{max}} \) is the maximum rate of the reaction, \( K_m \) is the Michaelis constant, and \([S]\) is the substrate concentration.

**CD measurements**

The structure and stability of DNase I in the absence and presence of PEG was studied by CD analysis. CD spectra were recorded using a Jasco J-820 spectrometer equipped with a Peltier temperature control system (Jasco, Tokyo, Japan). The cuvette-holding chamber was flushed with a constant stream of dry N₂ gas to avoid water condensation on the cuvette exterior. Data were collected from 195 to 255 nm with a 1-s response time and a 1-nm bandwidth using a 0.1-cm quartz cuvette. The CD measurements for 0.5 μM DNase I were carried out in 10 mM Tris–HCl (pH 7.5) in the absence and presence of 20% (w/v) PEG 4000 at 25°C. Each spectrum shown is the average of five individual scans and is corrected for the spectrum of the buffer. For thermal denaturation, the CD signal at 222 nm was monitored for a 1°C/min temperature rise from 5 to 95°C.

**Thermodynamic stability of DNase I and exonuclease I**

The thermodynamic stability of the nucleases was studied by performing stability assays in the absence and presence of PEG. The thermal stability of DNase I was assayed in 10 mM Tris–HCl (pH 7.5) in the absence and presence of PEG 4000, and that for exonuclease I was assayed in 10 mM 2-mercaptoethanol and 67 mM glycine-KOH (pH 9.5) at 37°C. The reactions were performed with 0.5–5 U of exonuclease I and 10 μM ssDNA.

The procedures for the kinetic and thermodynamic stability assays were similar to those described above for the CD studies.
were repeated at least three times. The experimental error was <8%.

RESULTS

Effect of molecular crowding on DNase I activity

DNase I, an endonuclease, can cleave single- and double-stranded DNAs in a non-sequence specific manner (5). Plasmid DNA is widely used as a substrate in studies of DNase I. In the current studies, we examined the effect of molecular crowding on nucleases. To mimic the aqueous cellular environment, we used PEG because it is inert and because it is commercially available in a wide range of molecular weights (11).

We first examined the effect of 20% (w/v) PEG on the hydrolysis of supercoiled plasmid DNA [pcDNA 3.1(+) by DNase I at 25°C. Figure 1A shows the DNA fragments after electrophoresis on a 0.8% (w/v) agarose gel. The two bands in lanes 2 and 3 correspond to supercoiled and open circular plasmid DNA (32). During the reaction, two distinct bands were observed in the absence of PEG 20000 (lanes 3–7). According to previous observations (32), the slower and faster migrating bands correspond to open circular and linear plasmid DNA, respectively. Although open circular and linear plasmid DNAs were observed, diffuse bands corresponding to products degraded by DNase I were not observed. These results show that, in the absence of PEG, 0.01 U of DNase I was not sufficient for hydrolyzing the plasmid DNA within 5 min. In contrast, in the presence of PEG 20000, the plasmid DNA was cleaved rapidly after 1 min (lane 9), indicating that it was already degraded. These results demonstrate for the first time that molecular crowding significantly enhances the hydrolysis of supercoiled plasmid DNA by DNase I.

In addition, the migration of the substrate bands under dilute conditions (lane 2) was slightly different from their migration under crowded conditions (lane 8). We found that the plasmid DNA mobility decreased slightly with increasing concentrations of PEG 20000 (Supplementary Figure S1). This may reflect a slight difference in the conformation of the plasmid DNA induced by PEG 20000 or a slower entry into the gel of DNA molecules from a PEG solution.

To quantitatively evaluate the effect of molecular crowding on DNA hydrolysis by DNase I, we used a short (29-mer) dsDNA oligonucleotide as a substrate. Figure 1B shows native PAGE of the 29-mer dsDNA after reaction with DNase I in the absence and presence of 20% (w/v) PEG 20000 at 25°C. Before the reaction, the migration of the substrate DNA was the same in dilute (lane 1) and crowded conditions (lane 5). This result supports previous studies showing that PEG does not significantly affect the thermodynamic stability of short DNA duplexes (27). We did not observe a substantial change in the migration of the dsDNA before or after the DNase I reaction under dilute conditions (lane 1–4), showing that, in the absence of PEG, 0.01 U of DNase I is not sufficient to hydrolyze the 29-mer dsDNA within 10 min. In contrast, bands migrating faster than that of the substrate DNA are clearly visible in the presence of PEG 20000 (lane 5–8). The result shows that dsDNA hydrolysis by DNase I was greatly enhanced by the addition of 20% (w/v) PEG 20000. Therefore, it appears that molecular crowding increases the cleavage yield of DNase I not only for the large DNA (plasmid) but also for the short (29-mer) DNA oligonucleotide.

In addition, we observed the accumulation of product DNA (lanes 6–8). We found that the size of the product DNA was ~10 bp (Supplementary Figure S2). Suck and Oefner (33) reported that both sides of the DNA double helix at the cleavage point contact DNase I over a span of 10 bp. Thus, 10-bp dsDNA regions on both sides of the cleavage point are required for higher activity. This can explain the accumulation of ~10-bp dsDNAs in our experiments.

Effect of molecular crowding on the hydrolytic activity of various nucleases

We next examined the influence of molecular crowding on the two main types of nucleases (exo and endo), including S1 nuclease, an endonuclease for ssDNA (34); exonuclease III, an exonuclease for dsDNA (35,36); exonuclease I, an exonuclease for ssDNA (37) and DNase I, an endonuclease for both ssDNA and dsDNA (5).

In the case of DNase I, the amounts of residual dsDNA after 10 min in the presence of 20% (w/v) PEG 2000, 4000, 8000 and 20000 was estimated to be 12, 4, 10 and 10%, respectively. Thus, PEG 4000 was the most effective of the tested PEGs at enhancing the hydrolysis reaction (Supplementary Figure S3A). For this reason, we further
investigated the amount of residual 29-mer dsDNA in the presence of 0–20% (w/v) PEG 4000 versus the time using DNase I at 25°C (Figure 2A). In the absence of PEG 4000, the amount of residual dsDNA after 10 min was estimated to be 87%. In contrast, the amount of residual dsDNA after 10 min in the presence of 5, 10, 15 and 20% (w/v) PEG 4000 was estimated to be 37, 12, 4 and 4%, respectively. These results show that hydrolysis of dsDNA by DNase I is greatly enhanced by PEG.

In the case of S1 nuclease, amongst PEG 200, 4000, 8000 and 20000, PEG 4000 was the most effective at enhancing the rate of hydrolysis (Supplementary Figure S3B). Thus, we further investigated the amount of residual 29-mer ssDNA in the presence of 0–20% (w/v) PEG 4000 versus time using S1 nuclease (Figure 2B). Similar to the results for DNase I, PEG 4000 enhanced the hydrolysis of ssDNA by S1 nuclease. Although the substrates for these two nucleases are different, molecular crowding had similar effects, indicating that the effect is not due to changes in substrate structure. Instead, the results indicate that molecular crowding generally enhances DNA hydrolysis by endonucleases regardless of the substrate structure.

Next, we investigated the effects of molecular crowding on DNA hydrolysis by exonucleases. Figure 2C and D show the amount of residual DNAs versus time for exonucleases III and I, respectively, in the presence of 0–20% (w/v) PEG 8000 (Supplementary Figure 3C and D shows the effect of molecular weight of PEG on the activities of exonucleases III and I). There was little hydrolysis of the substrate DNAs by exonucleases III and I at low enzyme concentrations (0.5–1 U) in both the
dilute and molecular crowding conditions. This indicates that the effects of molecular crowding on the hydrolysis reactions of exonucleases were relatively small compared with the effects on the endonuclease reactions. Therefore, to further investigate the effects of molecular crowding on hydrolysis by exonucleases, we increased the amount of enzyme to 10 U for exonuclease III and to 5 U for exonuclease I. Surprisingly, PEG did not enhance the activity of exonuclease III, even at the high enzyme concentration (inset in Figure 2C): after 10 min, the amounts of residual 29-mer dsDNA using 10 U of exonuclease III at 0, 5, 10, 15 and 20% (w/v) PEG 8000 were estimated to be 8, 3, 5, 10 and 13%, respectively. The results show that DNA hydrolysis by exonuclease III is not enhanced by molecular crowding.

Interestingly, DNA hydrolysis by exonuclease I was strongly inhibited by molecular crowding with PEG 8000 (inset in Figure 2D): after 10 min, the amount of residual 29-mer ssDNA at 0, 5, 10, 15 and 20% (w/v) PEG 8000 after 10 min was estimated to be 7, 4, 10, 32 and 41%, respectively. Thus, it appears that molecular crowding generally enhances endonuclease activities but does not affect or inhibit exonuclease activities.

**Thermodynamic stabilities of the endonuclease and exonuclease under dilute and molecular crowding conditions**

Although we found that molecular crowding enhances endonuclease activity rather than exonuclease activity, the origin of the difference in the effects remains unclear. Because we already demonstrated that the structure and stability of the substrate DNAs are not critical factors in the molecular crowding effect, we examined whether molecular crowding affects the thermodynamic stability of the nucleases. We first compared the structure and stability of an endonuclease (DNase I) in dilute and molecular crowding conditions by CD. Figure 3A shows CD spectra of DNase I in the absence and presence of 20% (w/v) PEG 4000 at 25°C. Both CD spectra have negative peaks around 215 and 208 nm and a positive peak around 198 nm, showing that α-helices are the dominant structure in DNase I in dilute and molecular crowding conditions. These results are identical with those reported by Ajitai and Venyaminov (38). In addition, PEG 4000 did not cause a peak shift, indicating that molecular crowding did not affect the secondary structure of DNase I. On the other hand, molecular crowding strongly affected the stability of the DNase I structure. Figure 3B shows melting curves for the α-helical DNase I structure in the absence and presence of 20% (w/v) PEG 4000. The melting temperature ($T_m$) of the DNase I structure in the presence of PEG 4000 was estimated to be 60°C. Surprisingly, the $T_m$ of the DNase I structure was >80°C in the presence of 20% (w/v) PEG 4000. This demonstrates that molecular crowding stabilizes the structure of DNase I.

We did not measure the CD spectra of exonuclease I because purified exonuclease I is not commercially available. Thus, to investigate how molecular crowding affects the stability of exonuclease I, we carried out a heat stability test for both exonuclease I and DNase I in dilute and molecular crowding conditions. In these experiments, DNase I and exonuclease I were incubated for 0–30 min at 60°C in the absence and presence of PEG (PEG 4000 or 8000). After the incubation, we carried out a 10-min hydrolysis reaction. Figure 4 shows the residual DNase I and exonuclease I activities versus time of incubation at 60°C. After a 30-min incubation, the residual activities of DNase I and exonuclease I in the absence of PEG were estimated to be 56 and 26%, respectively. The inactivation of the nucleases by incubation for 30 min at 60°C agrees

![Figure 3.](image)
with the observations of Chow and Resnick (31). On the other hand, the residual activities of DNase I and Exonuclease I in the presence of PEGs (PEG 4000 or 8000, respectively) remained around 100%. These results show that PEG preserves the active structures of DNase I and exonuclease I. The stabilization of DNase I by molecular crowding observed in the heat stability assay agrees with the CD results showing that molecular crowding with PEG increased the $T_m$ of DNase I from 60°C to >80°C. Therefore, these results indicate that the structure of both exonuclease I and DNase I are stabilized by molecular crowding. Although elucidation of the mechanism of the stabilization by molecular crowding is interesting and demands further study, it appears that the difference in the effects of molecular crowding on the endonucleases and exonucleases is not due to effects on their thermodynamic stability.

Kinetic parameters for DNA hydrolysis by DNase I and exonuclease I in dilute and molecular crowding conditions

We further investigated the origin of the different effects of molecular crowding on the endonucleases and the exonucleases by determining the kinetic parameters for the hydrolysis reactions in dilute and molecular crowding conditions. We measured the initial velocity ($v$) of the hydrolysis reaction at various concentrations of DNA substrate in the absence of PEG and in presence of PEG 4000 at 25°C for DNase I or in the presence of PEG 8000 at 37°C for exonuclease I. The value of $v$ for DNase I increased as the concentration of PEG 4000 increased (Figure 5A). In contrast, the value of $v$ for exonuclease I decreased as the concentration of PEG 8000 was increased (Figure 5B). Table 1 lists the $K_m$ and $V_{max}$ of DNA hydrolysis by DNase I and exonuclease I at the various concentrations of PEG. The $K_m$ values of DNase I and exonuclease I for the substrate DNA in the absence of PEG were estimated to be 5.0 and 2.9 μM, respectively, and in the presence of 20% (w/v) PEGs, they were estimated to be 4.5 and 2.3 μM, respectively. The $K_m$ values of DNase I and exonuclease I were not significantly affected by the addition of PEG. On the contrary, the $V_{max}$ of DNase I increased from 0.1 to 2.7 μM/min as the concentration of PEG 4000 was raised from 0 to 20% (w/v), whereas that of exonuclease I decreased from 2.2 to 0.4 μM/min as the concentration of PEG 8000 was raised from 0 to 20% (w/v). These effects of molecular crowding on the kinetic parameters of DNase I and exonuclease I indicate that molecular crowding stabilizes the active structures of these enzymes. However, further investigation of the mechanism of this stabilization is warranted to fully understand the effects of molecular crowding on these enzymes.
on the $V_{\text{max}}$ are consistent with the results of equilibrium analyses of DNase I and exonuclease I (Figure 2A and D). These kinetic parameters reveal that molecular crowding influences the hydrolytic activity of the nucleases by directly affecting catalysis as indicated by the changes in the $V_{\text{max}}$.

**DISCUSSION**

Our equilibrium studies of DNA hydrolysis indicate that molecular crowding enhances the activity of endonucleases but has little effect on exonuclease activity. Moreover, we found that molecular crowding affected neither the thermodynamic stabilities of the nuclease structures nor their binding to substrate DNAs. Kinetic analyses demonstrated that molecular crowding affected catalysis. Therefore, the difference in the reaction mechanism and/or type of reaction (endo or exo) may account for the different effects of molecular crowding on endonucleases and exonucleases.

Because of the importance of nucleases in vivo and in vitro, many studies have examined their reaction mechanisms. For DNase I, Glu75 in the active site accepts a proton from His131, which in turn accepts a proton from a water molecule positioned close to the substrate DNA. Then, the nucleophile attacks the phosphorous of the substrate DNA, cleaving the P-O-3 bond (33). This proton acceptor–donor chain (Glu75–His131–water), which is critical for the catalytic activity, is also found in the active site of exonuclease I as Glu178–His181–water (39). Since the reaction mechanisms for DNase I and exonuclease I are almost identical, it is surprising that molecular crowding had opposite effects on endonucleases and exonucleases. Thus, it is difficult to determine the reason for the opposite effects on the basis of the cleavage mechanisms.

On the other hand, the reaction types mediated by DNase I (endo) and exonuclease I (exo) are distinct. Endonucleases can randomly hydrolyze internal sites in DNA substrates, whereas exonucleases remove terminal nucleotides. We therefore suspect that the origin of the different effects of molecular crowding is the different reaction type (endo versus exo) rather a difference in the reaction mechanism. Wenner and Bloomfield (16) reported that molecular crowding increases the $V_{\text{max}}$ of EcoRV (endo-type). Conversely, the $V_{\text{max}}$ of T7 DNA polymerase activity and the nick-translation reaction of DNA polymerase I (both exo-type) are reduced by molecular crowding (27,40) (Supplementary Table 1). Although the effects of other factors, such as the dielectric constant, viscosity of the solution and flexibility of the protein structure on enzyme activities should be examined, our current findings and these previous results suggest that the different effects of molecular crowding on endonucleases and exonucleases are related to the reaction type. These results and considerations lead us to conclude that molecular crowding generally increases the catalytic activity of endonucleases and decreases or does not affect the catalytic activity of exonucleases. These findings should help clarify in general how molecular crowding affects protein functions.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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