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Citation	Biophysical Journal (2006), 90(3): 993-999
Issue Date	2006-02
URL	<a href="http://hdl.handle.net/2433/49159">http://hdl.handle.net/2433/49159</a>
Right	This un-edited manuscript has been accepted for publication in Biophysical Journal and is freely available on BioFast at <a href="http://www.biophysj.org">www.biophysj.org</a> . The final copyedited version of the paper may be found at <a href="http://www.biophysj.org">www.biophysj.org</a> .
Type	Journal Article
Textversion	author

**Protective effect of vitamin C against double-strand breaks in reconstituted chromatin visualized by single-molecule observation**

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Condensed running title: Effect of vitamin C on DNA lesion

Keywords: ascorbic acid, fluorescence microscopy, DNA damage, reactive oxygen

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**ABSTRACT**

Direct attack to genomic DNA by reactive oxygen species causes various types of lesions, including base modifications and strand breaks. The most significant lesion is considered to be an unrepaired double-strand break that can lead to fatal cell damage. We directly observed double-strand breaks of DNA in reconstituted chromatin stained by a fluorescent cyanine dye, YOYO, in solution, where YOYO is known to have the ability to photo-cleave DNAs by generating reactive oxygen species. Reconstituted chromatin was assembled from large circular DNA (106 kbp) with core histone proteins. We also investigated the effect of vitamin C (ascorbic acid) on preventing photo-induced double-strand breaks in a quantitative manner. We found that DNA is protected against double-strand breaks by the addition of ascorbic acid, and this protective effect is dose-dependent. The effective kinetic constant of the breakage reaction in the presence of 5 mM ascorbic acid is 20 times lower than that in the absence of ascorbic acid. This protective effect of ascorbic acid in reconstituted chromatin is discussed in relation to the highly compacted polynucleosomal structure. The results highlight the fact that single-molecule observation is a useful tool for studying double-strand breaks in giant DNA and chromatin.

## **INTRODUCTION**

It is widely accepted that oxidative damage to genomic DNA by reactive oxygen species induces various types of DNA lesions, including base and sugar modifications, and strand breaks (1-3). Among these types of damage, a double-strand break is considered to be the most significant lesion in genomic DNA, and can lead to chromosomal rearrangements that are lethal to eukaryotes (3,4). Although a large number of in vitro studies concerning double-strand breaks have been carried out at short DNA molecules or oligomer level (see Ref. (5) for a review), studies on the double-lesion of genomic giant DNA remain at a primitive stage. This may be due to a lack of a suitable methodology for studying lesions of genomic giant DNA. We recently performed direct observations of photo-induced double-strand breaks in giant DNAs stained by a cyanine dye, YOYO, under intense illumination ( $\lambda = 450 - 490 \text{ nm}$ ) in solution (6). YOYO is known to have the ability to photo-cleave DNAs by generating reactive oxygen species (7). We reported that a quantitative kinetic analysis of the double-strand breakage of giant DNA can be successfully performed by using single-molecule observation (6).

As the next step, in the present study we observed photo-induced double-strand breaks in individual single reconstituted chromatin using fluorescence microscopy. In a eukaryotic nucleus, a long duplex DNA is complexed with histone proteins to form a highly folded chromatin. Therefore, it is important to understand the relationship between the higher-order structure of compacted chromatin and the susceptibility to oxidative damage. A sensitive and reliable technique for studying such lesions would be a useful tool in genotoxicity and antioxidative sensitivity testing. For these studies, we used a polynucleosomal assembly consisting of a large circular DNA (106 kbp) and core histone proteins as reconstituted chromatin.

We also examined the ability of ascorbic acid (vitamin C) to protect against double-strand breaks. Vitamin C is essential for many enzymatic reactions and also acts as a free-radical scavenger. However, the role of vitamin C in protecting against oxidative DNA damage is controversial (8-13). Numerous studies have demonstrated the antioxidant effects of vitamin C (14-20). On the other hand, both in vivo and in vitro studies often show that vitamin C acts as a pro-oxidant (10,21,22). Thus, in the present study, we performed a quantitative analysis of breakage reactions in the presence of ascorbic acid through single-molecule observation.

## **MATERIALS AND METHODS**

### **Purification of histone octamer**

Core histones were purified from HeLa cells essentially as described (23) with slight

modifications (24,25). The cells were harvested, washed with phosphate-buffered saline (PBS) and then lysed with L buffer (140 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.5% Triton-X-100). Nuclei were isolated by low-speed centrifugation and washed three times with W buffer (350 mM NaCl, 10 mM Tris-HCl, pH 7.5). The nuclei were then treated with micrococcal nuclease (40 U/mg of DNA) in D-buffer (10 mM Tris-HCl, pH 7.5, 1.5 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 0.25 M sucrose, 0.1 mM phenylmethyl sulfonylfluoride (PMSF)) at 37°C for 15 min. The reaction was stopped by an addition of ethylene glycol-bis (β-aminoethylether)-N,N,N',N'- tetraacetic acid (EGTA) to a final concentration of 2 mM, and the nuclei were pelleted by centrifugation at 10,000g for 5 min. The pellet was resuspended in N buffer (10 mM Tris-Cl, pH 6.8, 5 mM ethylenediaminetetraacetic acid [EDTA], 0.1 mM PMSF), and dialyzed against N buffer overnight at 4°C. The sample was centrifuged at 10,000 g for 10 min and the soluble chromatin supernatant was redialyzed against HA-buffer (0.1 M NaPO<sub>4</sub>, pH 6.7, 0.63 M NaCl), and mixed with hydroxyapatite resin (Bio-Rad). After batch-binding at 4°C for 1 h, the resin was packed into a column and washed with five volumes of HA buffer. The core histones were eluted by E buffer (0.1 M NaPO<sub>4</sub>, pH 6.7, 2 M NaCl). The eluate was applied to a gel-filtration column (Amersham Biosciences, HiPrep 16/60 S-200) to separate the octamer from the H3/H4 tetramer, H2A/H2B dimer, and other contaminants.

### **Preparation of DNA template and chromatin reconstitution**

DNA (100 kbp) composed of tandem repeats with a 171 bp unit of alphoid DNA was a kind gift from Dr. Ikeno at Fujita Health University. The 100 kbp DNA was subcloned into a bacterial artificial chromosome, pBAC-108L (6 kbp), to obtain a 106 kbp circular DNA as the reconstituted chromatin template.

To reconstitute the chromatin structure, equal amounts (0.5 μg) of the purified DNA template and the histone octamer were first mixed in Hi buffer (10 mM Tris-HCl, pH 7.5, 2 M NaCl, 1 mM EDTA, 0.05% NP-40, 5 mM 2-mercaptoethanol) and then were put into a dialysis tube (total volume, 50 μl). Dialysis was started with 150 ml of Hi buffer with stirring at 4°C. Lo buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA, 0.05% NP-40, 5 mM 2-mercaptoethanol) was added to the dialysis buffer at 0.46 ml/min and simultaneously the dialysis buffer was pumped out at the same speed with a peristaltic pump, so that the dialysis buffer contained 50 mM NaCl after 20 h. The sample was collected from the dialysis tube and stored at 4°C.

### **AFM imaging**

Reconstituted chromatin samples were diluted with Di-buffer (10 mM HEPES-NaOH,

pH 7.5, 20 mM NaCl). The diluted sample was fixed with 0.3% glutaraldehyde for 30 min at 25°C. For atomic force microscopy (AFM) imaging, the fixed sample was applied to a freshly cleaved mica surface that had been pretreated with 10 mM spermidine unless otherwise stated. After 10 min, the mica was gently washed with water and dried under nitrogen gas. AFM imaging was performed with Nanoscope IIIa (Digital Instruments) with a type E scanner under tapping mode in air at room temperature. AFM probes made of a single silicon crystal with a cantilever length of 129  $\mu\text{m}$  and a spring constant of 33–62 N/m (Olympus) were used. Images were collected in the height mode and stored in a 512  $\times$  512 pixel format. The images obtained were then plane-fitted and analyzed by the computer programs that accompanied the imaging module.

### **Fluorescence microscopic observations**

For fluorescence microscopic measurements, reconstituted chromatin and naked DNA were dissolved in 10 mM Tris-HCl buffer solution with 0.1  $\mu\text{M}$  YOYO (trade name YOYO-1: Molecular Probes, Inc., Oregon, USA) and 20 mM NaCl at pH 7.4. To avoid intermolecular DNA aggregation, measurements were conducted at a low DNA concentration: 0.3  $\mu\text{M}$  in nucleotide units. The possible effect of ascorbic acid on the reduction of breakage was evaluated by adding 0.2 mM to 5 mM L-ascorbic acid to the DNA solution. Illumination with 450–490 nm light was performed with an optical excitation filter, and fluorescence was observed at 510 nm. To reduce photocleavage to a level suitable for real-time observation, 4 % (v/v) 2-mercaptoethanol was added to samples prior to optical imaging. Fluorescent DNA images were obtained using a microscope (Axiovert 135 TV, Carl Zeiss, Germany) equipped with a 100  $\times$  oil-immersion objective lens and a highly sensitive Hamamatsu SIT TV camera, which allowed us to record images on video tapes. The video images were analyzed with an image processor (Argus 20, Hamamatsu Photonics, Hamamatsu, Japan).

## **RESULTS**

### **Single-molecule observation of double-strand breaks**

Figure 1 shows a naked circular DNA and a reconstituted chromatin observed by AFM. The chromatin reconstitution was carried out by the salt-dialysis method as described in MATERIALS AND METHODS. The AFM image in Fig. 1 *b* clearly shows a beads-on-a-string structure. Figure 2 exemplifies the real-time measurement of the breakage of a naked DNA (Fig. 2 *a*) and a reconstituted chromatin (Fig. 2 *b*) in solution by fluorescence microscopy. In naked DNA, the double-strand breaks occur in a

successive manner, from a circular into a linear conformation (Step I) and then from a linear conformation into a pair of fragments (Step II). Similar changes in conformation are encountered for the reconstituted chromatin as shown in Fig. 2 *b*. Thus, fluorescence microscopy makes it possible to monitor the process of double-strand breaks in individual molecules. The actual time of the change from a circular into a linear conformation (Step I) can be recognized only for specimens that show a rather extended conformation during thermal agitation. Thus, the time of the second breakage (Step II), i.e., the timing of the fragmentation from a single DNA molecule, can be clearly judged from the video images. The protective effect of ascorbic acid against photo-induced double-strand breaks is summarized as the time distribution in Fig. 3, where Step II in Fig. 2 is given.

Figure 3 shows a histogram of the breakage time distribution, where the breakage time is taken at the moment of fragmentation (Step II in Fig. 2). For naked DNA, more than 90% of the DNA molecules are damaged into fragments within 20 sec in the absence of ascorbic acid. On the other hand, the addition of ascorbic acid prolongs the breakage time significantly, indicating that ascorbic acid protects against DNA breakage. At 5 mM ascorbic acid, the breakage time becomes more than 30 sec for most of the naked DNA molecules. The breakage time in reconstituted chromatin is apparently greater than that in naked DNA. For reconstituted chromatin, most of the DNA remains without breakage even after 100 sec with a higher concentration of ascorbic acid (5 mM).

Figure 4 shows the time course of the increase in damaged DNA molecules, indicating that breakage in reconstituted chromatin is slower than that in naked DNA, together with the protective effect of ascorbic acid. Besides the significant protective effect of ascorbic acid, the time profile shows a unique characteristic; i.e., the fragmentation starts after a certain induction period.

### **Kinetic analysis on the double-strand break in the circular DNA**

To evaluate the time-dependent change in the relative ratio of double-strand breaks as shown in Fig. 4, we can deduce the kinetic equation by considering the stepwise process of the oxidative damage of circular DNA. Under the experimental conditions for individual DNA observation, the process of breaking from a circular to linear structure, i.e., the first stage of the double-strand break in DNA, is difficult to detect. In contrast, the second step of the double-strand break is easier to detect because this process is observed as the fragmentation of a single fluorescence object. The frequency of single-strand breakage caused by photo-illumination in the presence of YOYO is

considered to be proportional to the intensity of light,  $I$ . For example, Martens and Clayton (26) studied the effect of an intercalator on the single-strand breakage or nick formation under visible light irradiation, and found that the number of nicks was proportional to the total light intensity. By denoting the number of nicks per DNA molecule as  $n$ , the rate of the increase in nicks can be written as,

$$dn/dt = \alpha I \quad (1)$$

where  $\alpha$  is a constant. For simplicity, we assume that variation in the base composition along the DNA chain has a negligible effect on the possibility of breakage. As the initial condition, we take  $n = 0$  when  $t = 0$ . Thus, eq. (1) is integrated as,

$$n = \alpha I t \quad (2)$$

The first double-strand break on circular DNA induces ring-opening into a linear conformation, where the precise distinction between circular and linear DNAs is practically difficult with fluorescence microscopic measurement. Thus, to avoid complexity in the data analysis, we would like to consider the probability of the second double-strand break, by omitting the apparent inclusion of the first double-strand break from the equation. We consider  $x$  as the number of DNA molecules that survive fragmentation. By introducing a rate constant  $k$ ,  $dx/dt$  is given as,

$$dx/dt = -knx = -k\alpha I t x \quad (3)$$

After integration of eq. (3) under the initial condition of  $x = x_0$  at  $t = 0$ ,

$$\ln(x/x_0) = -(1/2)k\alpha I t^2 \quad (4)$$

By introducing an effective kinetic constant,  $A = (1/2)k\alpha I$ , eq. (4) becomes

$$\ln(x/x_0) = -A t^2 \quad (5)$$

Equation (5) implies that the logarithm of the ratio of surviving DNA molecules is proportional to the kinetic constant of double-strand break. The constant  $A$  is also linearly correlated to the light intensity and to the kinetic constant of photo-induced single-strand break.

Based on the above theoretical consideration, in Fig. 5, we plotted the square of time with respect to the logarithm of the relative ratio of the surviving DNA, which includes both the circular and linear forms without fragmentation. A linear relationship is seen for all of the experimental data with both naked DNA and reconstituted chromatin, indicating that the method used for the kinetic analysis is adequate. From the relative slope, the apparent kinetic constant of the double-strand break can be deduced.

Table 1 shows the kinetic constants  $A$  deduced from eq. (5). In the first column, the relative kinetic constant  $A_1$  is given for where the breakage of DNA without histone



proteins is adopted as a standard. It is clear that the breakage reaction is largely suppressed with an increase in ascorbic acid concentration. When we compare the breaks between naked DNA and reconstituted chromatin in the absence of ascorbic acid, the breakage rate, i.e., the relative kinetic constant  $A_1$  of reconstituted chromatin is much smaller, ca. one fourth, than that of naked DNA. A similar protective effect for nucleosomal DNA has been found with regard to iron-mediated damage (27). It is also obvious that the efficiency of the protection is enhanced with the addition of ascorbic acid in reconstituted chromatin. In the second column of Table 1, the relative kinetic constant  $A_2$  of the breakage reaction in reconstituted chromatin is shown, where the rate of reconstituted chromatin without ascorbic acid is taken as unity. The protective effect of ascorbic acid shows a similar trend in naked DNA and reconstituted chromatin, i.e., in naked DNA the relative kinetic constants ( $A_1$ ) are 0.25 and 0.05 for 1 and 5 mM ascorbic acid, respectively, while in reconstituted chromatin the relative kinetic constants ( $A_2$ ) are 0.28 and 0.05. This means that the degree of the protective action of ascorbic acid for naked DNA is similar to that for reconstituted chromatin.

## DISCUSSION

In the present study, we showed that double-strand breaks can be monitored at the level of individual DNA molecules, and that the kinetics of the breakage reaction can be deduced from such single-molecule observation.

We obtained useful information on the protective effects of the chromatin structure compared to naked DNA, together with the effect of ascorbic acid, through individual DNA observation. In relation to our observation, several recent *in vivo* studies have suggested that the organization of DNA into a highly compacted chromatin structure helps to protect against double-strand breaks (27-29). Irvine et al. (30) reported that poorly compacted abnormal sperm chromatin frequently contains DNA strand breaks. Our results clearly indicate the protective effect of the polynucleosomal structure against oxidative damage. Furthermore, this protection was enhanced by the addition of ascorbic acid.

An additional important implication of our results is that ascorbic acid above millimolar concentrations exhibits a marked protective effect on double-strand breaks. Generally, the protective effect of ascorbic acid can be explained by scavenging reactive oxygen species due to its property as an antioxidant. Another potential explanation for the protective effect is the direct interaction of ascorbic acid with DNA. This hypothesis is associated with the result of our previous study, which indicated that ascorbic acid in millimolar concentrations induces condensation in the higher-order structure of giant

DNA (31). Thus, it is expected that the change in the higher-order structure of DNA induced by ascorbic acid may be closely associated with its ability to protect against double-strand breaks. It is known that ascorbic acid reaches millimolar concentrations in human circulating immune cells, such as neutrophils, monocytes and lymphocytes (32), which suggests that the ascorbic acid concentration used in this study is of physiological significance. It may be useful to examine such a possible effect of ascorbic acid in the future.

Currently, it is thought that double-strand breaks have a fatal effect in living cells (3). However, it has been rather difficult to measure double-strand breaks, especially at very low damage conditions. Our experimental system does not require a large amount of DNA fragments for the detection of DNA damage. Thus, the experimental methodology of single-molecule observation is expected to serve as a useful tool for studying double-strand breaks in giant DNA and chromatin.

#### **ACKNOWLEDGEMENTS**

This work was supported in part by a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science.

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FIGURE 1 Representative atomic force microscopy (AFM) images. (a) Naked circular DNA (106 kbp). (b) Reconstituted chromatin. The chromatin fiber was reconstituted from the circular DNA and histone octamers by a salt-dialysis method and observed by AFM under tapping mode in air.

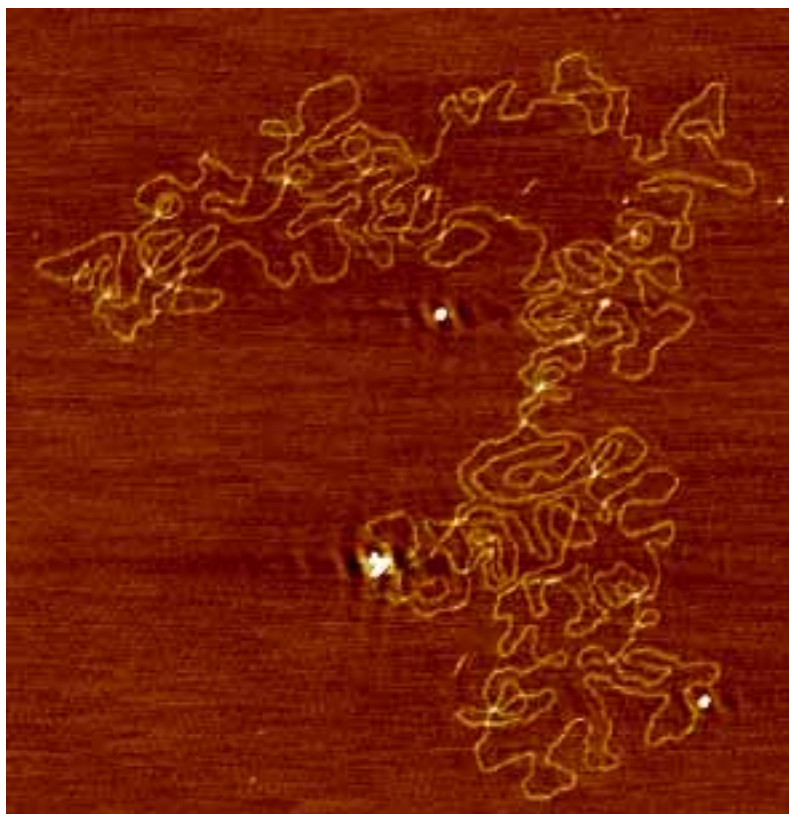
FIGURE 2 Fluorescence microscopic observations of photo-induced double-strand breaks in (a) naked circular DNA and (b) reconstituted chromatin stained by YOYO. (c) Schematic illustration for double-strand breaks. Individual DNAs undergo structural changes from a circular to a linear conformation (Step I), and then to a pair of fragments (Step II).

FIGURE 3 Histograms of the breakage time distribution depending on ascorbic acid (AsA) concentration as monitored by fluorescence microscopy. The breakage time is taken at the moment of fragmentation (Step II in Fig. 2). Left; naked circular DNA. Right; reconstituted chromatin. The black bins ( $100<$ ) indicate the percentages of surviving DNA molecules even after 100 sec under intense light illumination.

FIGURE 4 Time-dependence of the percentage of damaged DNA molecules deduced from the summation of the probability in the histograms of Fig. 3. Left; naked circular DNA. Right; reconstituted chromatin.

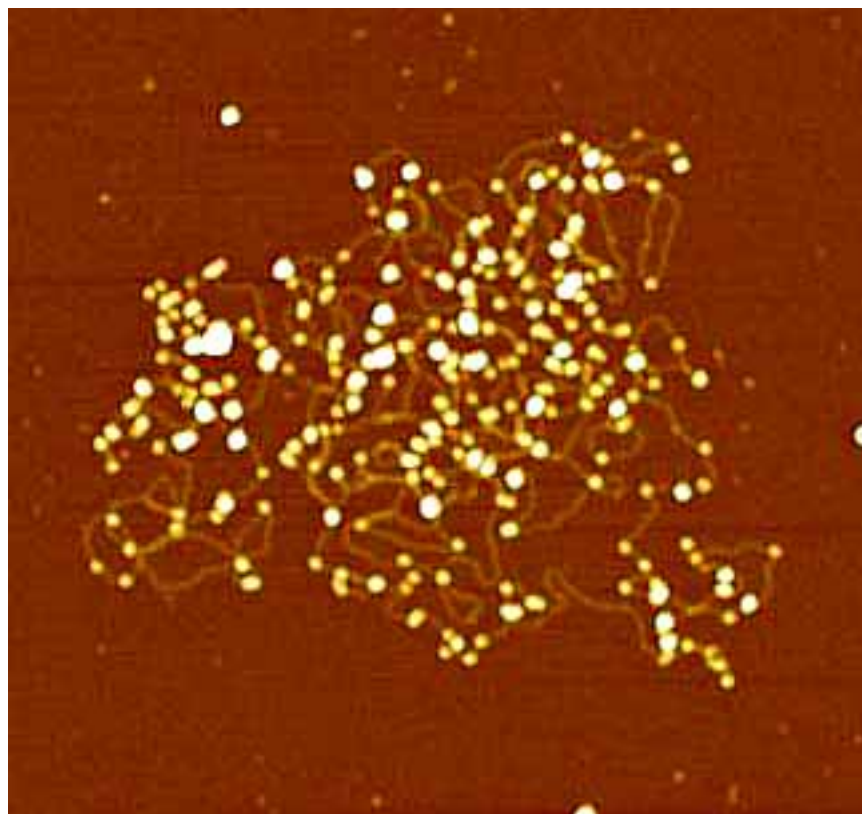
FIGURE 5 Linear relationships between  $t^2$  and  $\ln x$ , where  $x$  is the percentage of surviving DNA molecules and is calculated as  $100\% - [\text{percentage of damaged DNA}]$ .

a)



0.5  $\mu\text{m}$

b)



0.5  $\mu\text{m}$

Fig. 1

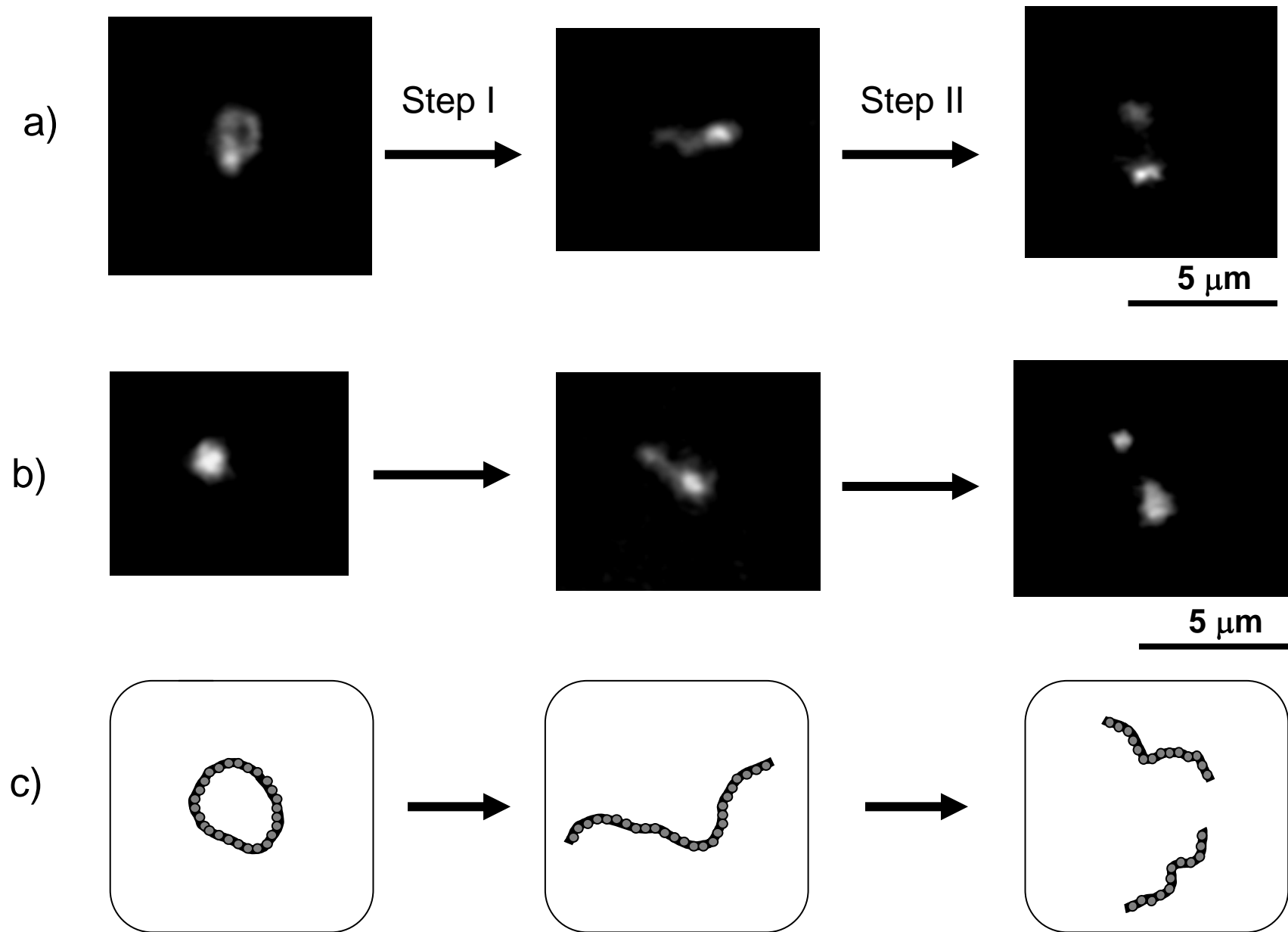


Fig. 2



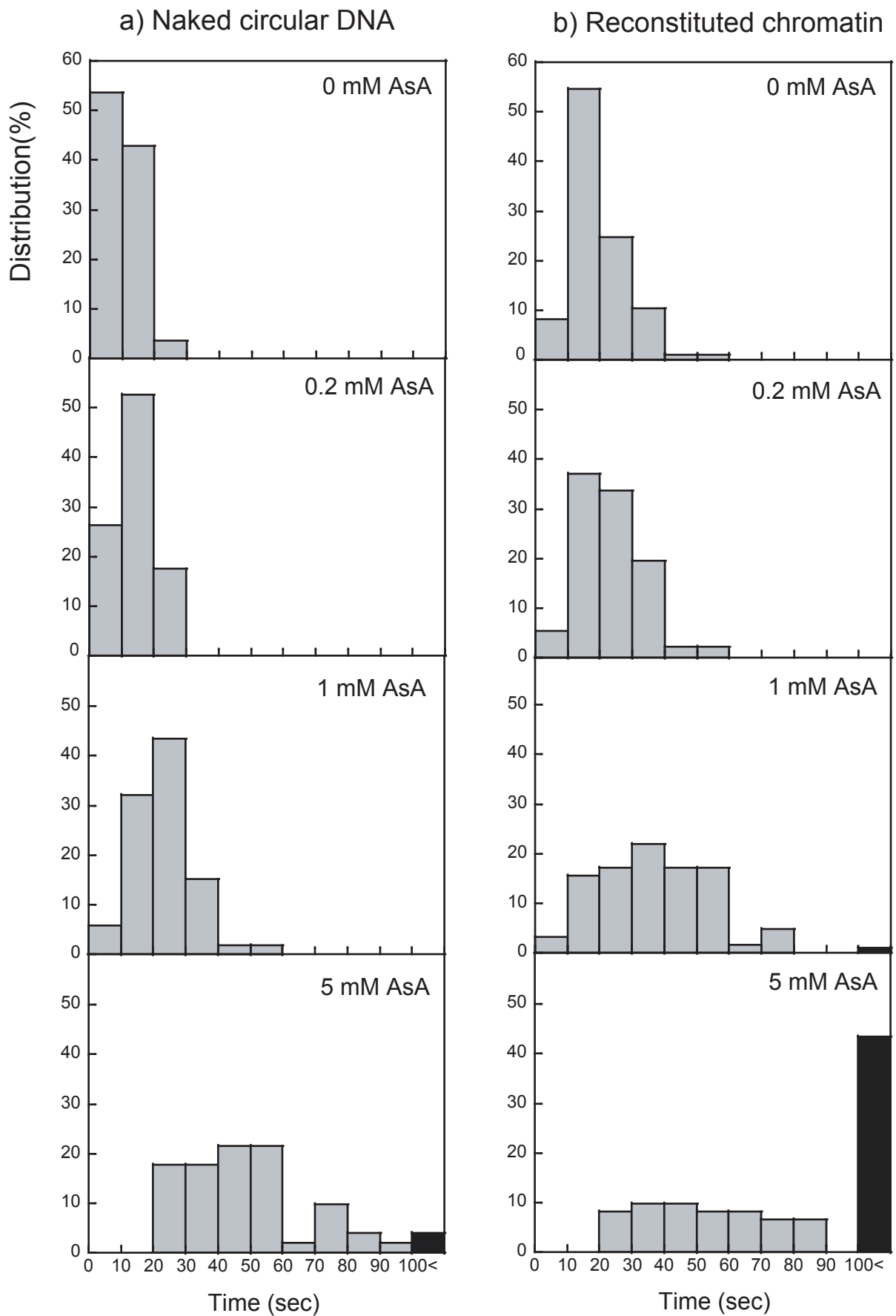
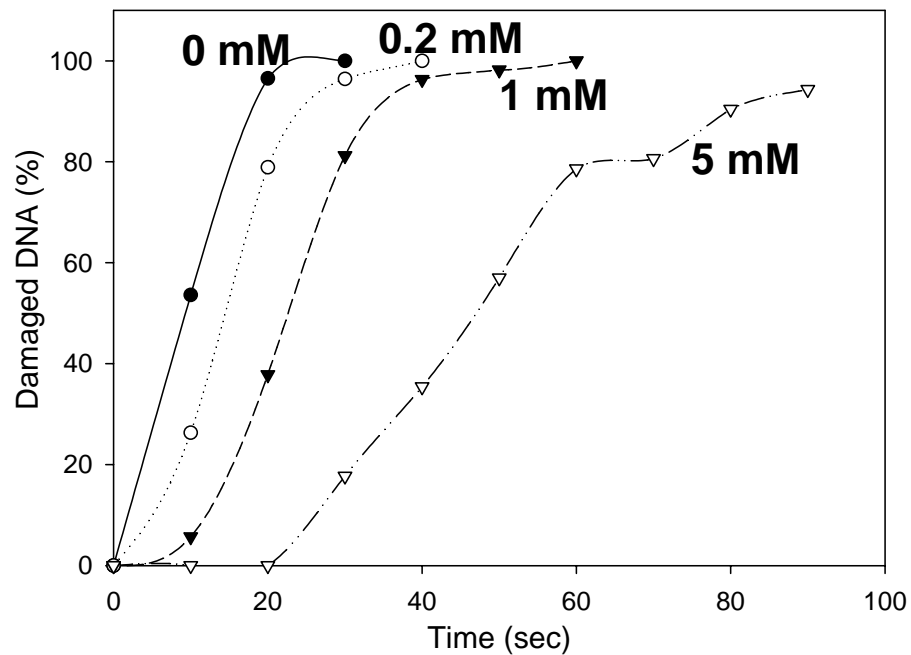


Fig. 3

a) Naked circular DNA



b) Reconstituted chromatin

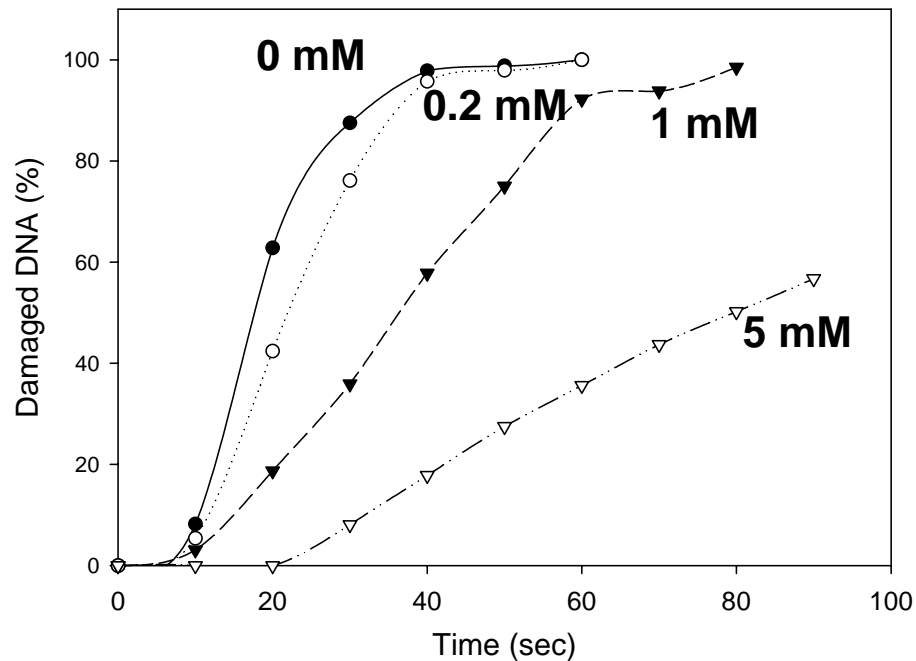
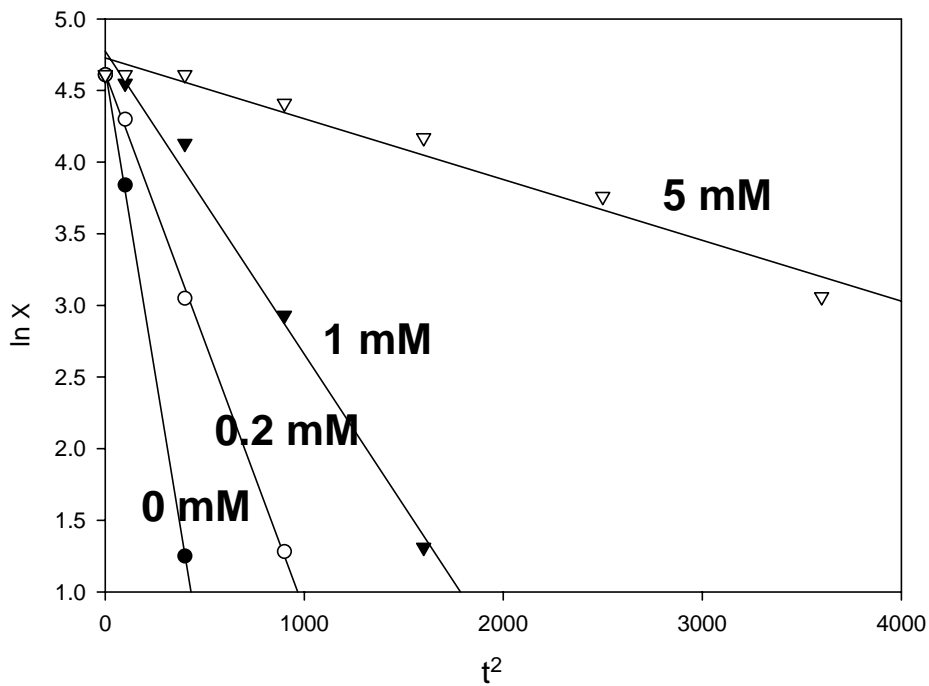


Fig. 4

a) Naked circular DNA



b) Reconstituted chromatin

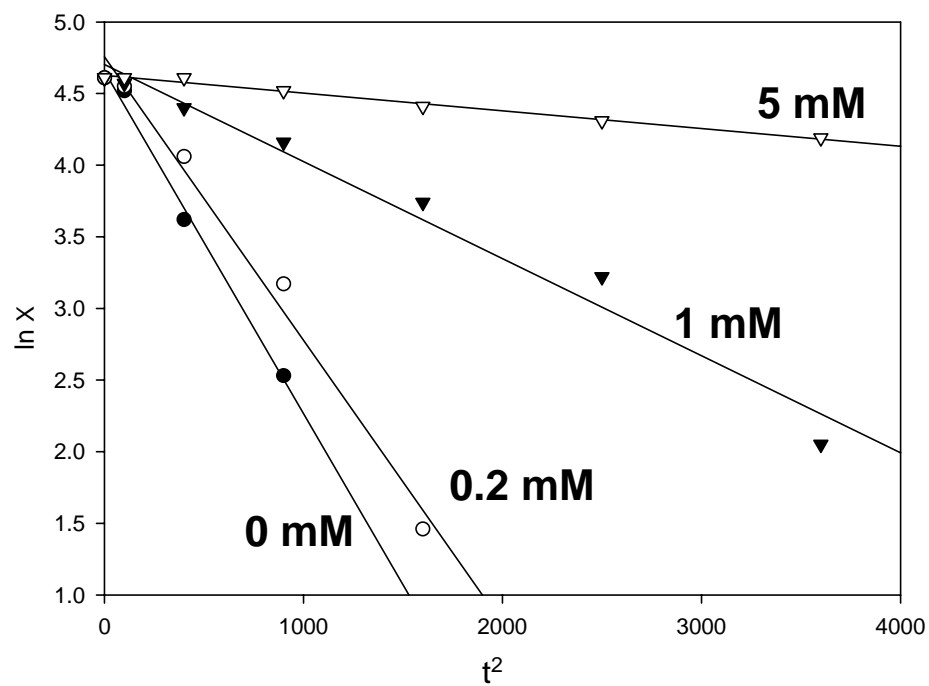


Fig. 5

TABLE 1 Relative kinetic constants of the breakage reaction, where  $A_1$  and  $A_2$  are normalized to be unity in the control experiment on naked circular DNA and reconstituted chromatin, respectively.

Sample	Relative kinetic constant $A_1$	Relative kinetic constant $A_2$
Naked circular DNA	1.00	-
+ 0.2 mM AsA	0.44	-
+ 1 mM AsA	0.25	-
+ 5 mM AsA	0.05	-
Reconstituted chromatin	0.28	1.00
+ 0.2 mM AsA	0.23	0.83
+ 1 mM AsA	0.08	0.28
+ 5 mM AsA	0.01	0.05

AsA: Ascorbic acid