Induction of Single Strand Scission in ϕ X174 RFI DNA by D-Isoglucosamine

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The mechanism of the DNA breaking activity of 1-amino-1-deoxy-D-fructose (D-isoglucosamine) in double-stranded replicative form I DNA (RFI DNA) of bacteriophage ϕ X174 was investigated by agarose gel electrophoresis under various conditions. Cu^{2+} was indispensable for DNA strand scission. Induction of scission by D-isoglucosamine was proportional to incubation time and concentration, and was inhibited by the addition of chelating agents (EDTA, DETAPAC), superoxide dismutase (SOD), catalase, and some other free radical scavengers. These results suggested that DNA strand scission by D-isoglucosamine was caused by oxygen radicals generated during the autoxidation of D-isoglucosamine in the presence of Cu^{2+} .

Recently, attention has been paid to the finding that active oxygen radicals generated by carcinogenic and anti-carcinogenic agents cause strand scission in nucleic acids. Some of these substances have a reductone in their molecular structure, and the high reducibility of reductone expedites autoxidation by oxygen in the molecular state.

Some mono-^{1~5)} and polysaccharide⁶⁾ derivatives inactivate various kinds of bacteriophages, and this inactivation is caused by single strand scission of virus DNA in the phage virions. This scission is caused by oxygen radicals generated during the autoxidation of these sugars.^{1,2)} We reported⁸⁾ that amino-hexoses induce DNA strand scission more effectively than their corresponding hexose 6-phosphates, and that 1-amino-1-deoxy-D-fructose (D-isoglucosamine) had the highest DNA breaking activity in the reducing sugars reported so far. Reducing sugar phosphate^{5,6)} that induces the DNA strand scission, and 2-amino-2-deoxy-D-glucose⁷⁾ and its 6-phosphate ester³⁾ have phage-inactivating effects, so D-isoglucosamine is also expected to have anti-

virus activity. It is of interest to know if the mechanism of DNA strand scission by D-isoglucosamine is different from that by reducing sugar phosphates,^{1,2)} or other reductones such as ascorbic acid,^{9~11)} and antibiotics such as mitomycin C.^{12~16)}

In this paper, we report on the DNA strand scission by D-isoglucosamine in the presence of Cu^{2+} .

MATERIALS AND METHODS

Preparation of bacteriophage ϕ X174 RFI DNA. Double-stranded replicative form I DNA (RFI DNA; supercoiled, covalently closed, circular duplex DNA) of bacteriophage ϕ X174 was prepared by the procedure described previously.¹²⁾

Chemicals and enzymes. D-Isoglucosamine synthesized according to a previously described procedure¹⁷⁾ had a mp of 114~115°C (in ref.,¹⁸⁾ mp was 114~116°C). Superoxide dismutase (EC 1.15.1.1; from bovine blood Type I, 2750 units/mg protein), catalase (EC 1.11.1.6; from bovine liver, 2250 units/mg protein), and diethylenetriaminepentaacetic acid (DETAPAC) were purchased from the Sigma Chemical, Co. Other chemicals were obtained from Nakarai Chemicals, Ltd., and were used

without further purification.

Reaction of D-isoglucosamine with ϕ X174 RFI DNA. The standard reaction mixture (20 μ l) contained 300 ng of ϕ X174 RFI DNA, 1 mM of D-isoglucosamine, and 50 μ M of CuCl_2 in 50 mM Tris-HCl buffer (pH 7.1), unless otherwise noted. The reaction was carried out for 3 hr at 37°C, and stopped by the addition of 5 μ l of 0.1 M EDTA solution containing 50% (w/v) sucrose and 0.1% (w/v) bromophenol blue. The sample, with a final volume of 25 μ l, was directly analyzed by agarose gel electrophoresis.^{1,12)}

Agarose gel electrophoresis. The sample was layered onto 1.2% agarose slab gels. Electrophoresis was conducted at 3.8 V/cm for 4 hr in 40 mM Tris-acetate buffer (pH 8.2) containing 5 mM sodium acetate and 1 mM EDTA. Experiments involving relaxed RFI DNA were conducted with 1 μ g/ml ethidium bromide in the gel, in order to separate the relaxed RFI DNA from RFII DNA. RFI DNA migrated faster than RFIII DNA (linear duplex DNA), which in turn migrated faster

than RFII DNA (nicked, open circular duplex DNA)¹⁹⁾ under the conditions described above.

After electrophoresis, the gels were stained for 30 min in the dark in ethidium bromide solution (1 μ g/ml). The stained DNA bands were visualized with an ultraviolet lamp (253 nm) and photographed on Kodak Polaroid film through a Kenko red filter.

Quantitative analysis of duplex DNA bands on the gels. The photographic negatives were scanned with a Shimadzu dual-wavelength TLC-scanner CS-900. Absorbance intensity was recorded as a function of distance along the negatives, and the areas under the DNA bands were measured. Thirteen samples (1~500 ng) each of RFI DNA, RFII DNA prepared by DNaseI, and RFIII DNA prepared by *Xho*I form RFI DNA were also subjected to electrophoresis, staining, and scanning as described above. Under these conditions, the integral absorbance intensity of RFI, RFII, and RFIII DNA was a linear function of the DNA mass in the range of 60~400 ng per band (Fig. 1). The mass of these topological ϕ X174 duplex DNA separated with agarose gel electrophoresis from the reaction solution were measured from the calibration curve,²⁰⁾ and the degree of DNA strand scission was determined.

RESULTS

Induction of DNA strand scission by D-isoglucosamine

D-Isoglucosamine induced strand scission in ϕ X174 RFI DNA and converted it to RFII and RFIII DNA in the presence of Cu^{2+} (Fig. 2).

Figure 2 shows the effect of D-isoglucosamine concentration on the induction of ϕ X174 RFI DNA strand scission. Induction was proportional to the concentration. The amount of RFII DNA generated by single strand scission in RFI DNA increased in proportion to the concentration of D-isoglucosamine (0.01~0.5 mM). RFIII DNA was observed when RFII DNA was generated (0.5~4 mM), but the relative amount of RFIII DNA did not increase by more than 27.6% of the initial concentration of RFI DNA. When the concentration of D-isoglucosamine was over 1.5 mM, ϕ X174 duplex DNA molecules were degraded to smaller fragments.

Figure 3 shows the effect of incubation time with D-isoglucosamine on the induction of DNA strand scission. RFI DNA was incu-

FIG. 1. Calibration Curve of ϕ X174 Duplex DNA.

Thirteen samples (1~500 ng) each of RFI, RFII, and RFIII DNA were subjected to electrophoresis, and quantified as described in MATERIALS AND METHODS. ○—○, RFI DNA; △—△, RFII DNA. RFIII and RFI DNA had similar curves. The integral absorbance intensity of RFI, RFII, and RFIII DNA was a linear function of DNA mass in the range of 60~400 ng. When this intensity was not a linear function of DNA mass, mass was calculated from the calibration curve directly.

FIG. 2. Effect of D-Isoglucosamine Concentration on Induction of ϕ X174 RFI DNA Strand Scission in the Presence of CuCl_2 ($50 \mu\text{M}$).

1, drug-free control; 2, CuCl_2 control; 3, D-isoglucosamine (4 mM) control; 4, 4 mM; 5, 3 mM; 6, 2.5 mM; 7, 2 mM; 8, 1.5 mM; 9, 1 mM; 10, 0.5 mM; 11, 0.1 mM; 12, 0.05 mM; 13, 0.001 mM.

FIG. 3. Effect of Incubation Time with D-Isoglucosamine (1 mM) on Induction of ϕ X174 RFI DNA Strand Scission in the Presence of CuCl_2 ($50 \mu\text{M}$).

1, D-isoglucosamine control; 2, 0 min; 3, 5 min; 4, 10 min; 5, 20 min; 6, 30 min; 7, 45 min; 8, 1 hr; 9, 90 min; 10, 2 hr; 11, 150 min; 12, 3 hr; 13, 4 hr; 14, 5 hr; 15, 7 hr; 16, 9 hr; 17, 24 hr; 18, REIII DNA (linear duplex DNA) converted by *Xho*I from RFI DNA.

bated with D-isoglucosamine in the presence of CuCl_2 ($50 \mu\text{M}$) for the times indicated, and the reaction products were analysed by agarose gel electrophoresis. The conversion of RFI DNA to RFII DNA was proportional to incubation time (0~90 min). The amount of RFII DNA increased with decreasing amounts of RFI DNA. RFIII DNA was observed clearly after RFI DNA disappeared, and increased with decreasing amounts of RFII DNA (90~180 min). However, the relative amount of RFIII DNA did not increase by more than 22.8% of the initial concentration of RFI DNA. When incubation was more than 3 hr, ϕ X174 duplex DNA molecules were degraded to smaller fragments.

Effects of metal ions and chelating agents

The effects of metal ions and chelating agents on the DNA breaking activity of D-isoglucosamine were investigated. ϕ X174 RFI DNA was treated with 1 mM D-isoglucosamine in the presence of $50 \mu\text{M}$ metal ion (Mg^{2+} , Zn^{2+} , Mn^{2+} , Fe^{3+} , Co^{2+} or Cu^{2+}). Induction of DNA strand scission was observed only in the presence of Cu^{2+} (Table I), and depended upon the Cu^{2+} concentration being higher than $5 \mu\text{M}$ (Fig. 4). Other transition metal ions were not effective at the concentration used.

More than $10 \mu\text{M}$ of chelating agents (EDTA or DETAPAC) inhibited DNA strand scission by D-isoglucosamine in the presence of Cu^{2+} ($10 \mu\text{M}$), and the degree of inhibition depended

TABLE I. EFFECTS OF METAL IONS ON STRAND SCISSION IN ϕ X174 RFI DNA BY D-ISOGLUCOSAMINE

Metals* (50 μ M)	D-Isoglucosamine (1 mM)	Relative amount of DNA (%)			
		RFI	RFII	RFIII	Others
Control	—	94.1	4.7	0	1.2
Control	+	93.9	5.9	0	0.2
MgCl ₂	+	93.3	6.2	0	0.5
ZnCl ₂	+	86.8	11.4	0	1.8
MnCl ₂	+	93.1	6.6	0	0.3
FeCl ₃	+	91.5	7.5	0	1.0
CoCl ₂	+	91.3	7.6	0	1.1
CuCl ₂	+	0	85.2	13.7	1.1
CuSO ₄	+	0	90.1	8.4	1.5

* No metal ion at this concentration induced scission in the absence of D-isoglucosamine.

FIG. 4. Effect of CuCl₂ Concentration on Induction of ϕ X174 RFI DNA Strand Scission by D-Isoglucosamine.

1, drug-free control; 2, CuCl₂ (50 μ M) control; 3, D-isoglucosamine (1 mM); 4, 3 + CuCl₂ (50 μ M); 5, 3 + CuCl₂ (40 μ M); 6, 3 + CuCl₂ (30 μ M); 7, 3 + CuCl₂ (25 μ M); 8, 3 + CuCl₂ (15 μ M); 9, 3 + CuCl₂ (10 μ M); 10, 3 + CuCl₂ (5 μ M); 11, 3 + CuCl₂ (1 μ M); 12, 3 + CuCl₂ (0.1 μ M).

TABLE II. EFFECTS OF CHELATING AGENTS ON STRAND SCISSION IN ϕ X174 RFI DNA BY D-ISOGLUCOSAMINE IN THE PRESENCE OF CuCl₂ (10 μ M)

Chelating* agents	D-Isoglucosamine (1 mM)	Relative amount of DNA (%)			
		RFI	RFII	RFIII	Others
Control (— CuCl ₂)	—	93.7	6.1	0	0.2
	+	93.3	6.0	0	0.7
Control (+ CuCl ₂)	—	92.2	7.5	0	0.3
	+	0.9	95.1	3.1	0.9
EDTA (100 μ M)	+	93.4	5.9	0	0.7
EDTA (10 μ M)	+	93.0	6.5	0	0.5
EDTA (1 μ M)	+	0.2	95.1	3.4	1.3
EDTA (0.1 μ M)	+	0	95.2	4.5	0.3
DETAPAC** (100 μ M)	+	92.7	6.0	0	1.3
DETAPAC (10 μ M)	+	78.1	20.1	0.4	1.4
DETAPAC (1 μ M)	+	2.4	94.1	2.0	1.5
DETAPAC (0.1 μ M)	+	0.4	95.5	3.3	1.2

* Neither chelating agent at these concentrations induced scission in the absence of D-isoglucosamine.

** DETAPAC, Diethylenetriaminepentaacetic acid.

TABLE. III. EFFECTS OF CATALASE AND SUPEROXIDE DISMUTASE (SOD) ON STRAND SCISSION IN ϕ X174 RFI DNA BY D-ISOGLUCOSAMINE (1 mM) IN THE PRESENCE OF CuCl_2 (10 μM)

Enzymes	Concentration (units/ml)	Relative amount of DNA (%)			
		RFI	RFII	RFIII	Others
Control (– CuCl_2)	—	94.1	5.2	0	0.7
Control (+ CuCl_2)	—	0.8	94.6	3.5	1.1
Heat inactivated catalase*	22.50	2.1	94.7	2.2	1.0
Catalase	22.50	0	30.2	18.5	1.5
Catalase	11.25	0	85.3	14.0	0.7
Catalase	9.00	0	92.0	7.1	0.9
Catalase	6.75	1.5	93.1	4.2	1.2
Catalase	4.50	3.5	91.2	4.5	0.8
Catalase	2.25	42.3	53.5	3.2	1.0
Catalase	0.025	3.1	94.8	1.4	0.7
Heat inactivated SOD*	68.75	2.4	94.1	1.7	1.8
SOD	68.75	11.7	86.4	1.1	0.8
SOD	27.50	4.1	93.7	1.2	1.0
SOD	2.75	1.9	95.2	1.8	1.1
SOD	0.275	1.7	94.3	2.5	1.5
Catalase (2.25 units/ml) + SOD	137.50	11.7	83.2	3.7	1.4
Catalase (2.25 units/ml) + SOD	68.75	25.8	71.0	2.4	0.8
Catalase (2.25 units/ml) + SOD	27.50	16.3	80.2	2.3	1.2
Catalase (2.25 units/ml) + SOD	2.75	18.1	75.2	5.4	1.3
Catalase (2.25 units/ml) + SOD	0.275	16.1	81.5	2.1	0.3

* The enzyme was inactivated by heating for 15 min. at 100°C.

upon the concentration of these chelating agents (Table II). These results mean that DNA strand scission by D-isoglucosamine was catalyzed by Cu^{2+} .

Effects of enzymes and radical scavengers

The results described above suggest that induction of DNA strand scission by D-isoglucosamine is due to generation of free oxygen or other free radicals during the auto-oxidation of D-isoglucosamine in the presence of Cu^{2+} . So, we investigated the inhibiting effects of enzymes and several radical scavengers on the DNA strand scission by D-isoglucosamine in the presence of CuCl_2 (10 μM). Superoxide dismutase (SOD), which removes superoxide anion, and catalase, which removes hydrogen peroxide, inhibited scission (Table III). 1,2-Dihydroxybenzene-3,5-disulfonic acid (tiron),²¹⁾ a scavenger for superoxide anion, completely inhibited the DNA breaking activity of D-isoglucosamine. 1,4-Diazabicyclo[2,2,2]octane (DABCO),²²⁾ a

scavenger for singlet oxygen, inhibited DNA breaking activity weakly (Table IV). Some hydroxyl radical scavengers, such as KI, and 2-aminoethyl-isothiuroniumbromide-HBr (AET)²³⁾ inhibited scission almost completely, but the effects of other hydroxyl radical scavengers, such as sodium benzoate, 2-mercaptoethylamine-HCl (cysteamine), D-mannitol, ethanol, isopropanol, and dimethylsulfoxide (DMSO) were weak (Table IV).

These results suggest that oxygen radicals and other free radicals participate in the breaking of ϕ X174 RFI DNA by D-isoglucosamine and Cu^{2+} .

DISCUSSION

Various kinds of bacteriophages were inactivated by D-isoglucosamine effectively (data not shown). This phage inactivation might be caused by the accumulation of genetic lesions due to DNA strand scission.

D-Isoglucosamine induced strand scission in

TABLE IV. EFFECTS OF RADICAL SCAVENGERS ON STRAND SCISSION IN ϕ X174 RFI DNA BY D-ISOGLUCOSAMINE IN THE PRESENCE OF CuCl_2 (10 μM)

Scavengers*	D-Isoglucosamine (1 mM)	Relative amount of DNA (%)			
		RF I	RF II	RF III	Others
Control	—	94.5	5.1	0	0.4
Control	+	0	94.2	5.0	0.8
a) Tiron (1 mM)	+	94.6	5.0	0	0.4
Tiron (100 μM)	+	78.1	21.3	0	0.6
Tiron (10 μM)	+	3.6	93.9	1.4	1.1
Tiron (1 μM)	+	0.7	96.4	2.4	0.5
KI (100 mM)	+	91.1	8.2	0	0.7
KI (10 mM)	+	12.2	86.0	0.6	1.2
KI (1 mM)	+	0.5	97.0	2.0	0.5
Sodium benzoate (100 mM)	+	7.6	91.1	0.5	0.8
Sodium benzoate (10 mM)	+	2.3	95.5	1.9	0.3
Sodium benzoate (1 mM)	+	0	94.4	5.1	0.5
b) DABCO (100 mM)	+	2.2	95.1	2.2	0.5
DABCO (10 mM)	+	0	95.6	4.0	0.4
DABCO (1 mM)	+	1.0	97.2	1.3	0.5
c) AET (10 mM)	+	79.1	20.2	0	0.7
d) Cysteamine (100 mM)	+	10.8	87.0	1.6	0.6
D-Mannitol (100 mM)	+	0.5	96.5	2.8	0.2
Isopropanol (1 M)	+	2.1	94.0	2.8	1.1
DMSO (100 mM)	+	1.4	96.1	1.5	1.0
Ethanol (1 M)	+	2.3	94.5	2.7	0.5

* No radical scavenger at these concentrations induced scission in the absence of D-isoglucosamine.

- a) Tiron, 1,2-dihydroxybenzene-3,5-disulfonic acid.
 b) DABCO, 1,4-diazabicyclo[2,2,2]octane.
 c) AET, 2-aminoethyl-isothiuroniumbromide-HBr.
 d) Cysteamine, 2-mercaptoethylamine-HCl.

ϕ X174 RFI DNA, causing conversion to RFII and RFIII DNA more effectively than other DNA-breaking sugars. Induction of strand scission in ϕ X174 RFI DNA to produce RFII DNA was proportional to the concentration of D-isoglucosamine (Fig. 2) and to incubation time (Fig. 3). Cu^{2+} was indispensable for DNA strand scission (Table I and Fig. 4). RFIII DNA was not generated by double-strand scission in RFI DNA directly, but by single strand scission in RFII DNA.

Induction of DNA strand scission by D-isoglucosamine was inhibited by chelating agents (Table II). These results indicate that D-isoglucosamine must be autoxidized by catalysis of Cu^{2+} in the presence of molecular oxygen; oxygen radicals are generated, and induce the DNA strand scission. It was reported that copper acted as a catalyst in the

reduction of Cu^{2+} to Cu^+ by dithiol and in the reoxidation of Cu^+ to Cu^{2+} by oxygen,²⁴⁾ and Cu^{2+} stimulated the autoxidation of hydroxylamine.²⁵⁾ The 1,10-phenanthroline-cuprous complex is known to cause oxygen-dependent cleavage of DNA.^{26,27)} 1,10-Phenanthroline coordinated to copper ion cleaves DNA in a reaction requiring hydrogen peroxide and thiol.²⁸⁾ This DNA cleavage is due to oxidative destruction of the deoxyribose by hydroxyl radicals produced by the oxidation of DNA-bound Cu^+ by H_2O_2 .²⁸⁾ The 1,10-phenanthroline-copper complex cleaved three general helical forms of DNA at different rates.²⁹⁾ D-Isoglucosamine is oxidized by catalysis of Cu^{2+} , and an intermediate containing amino reductone structure may be generated. This intermediate is autoxidized by catalysis of Cu^{2+} . During this autoxidation

process with oxygen, a D-isoglucosamine-copper complex may be generated.

Enzymes and some radical scavengers inhibited DNA strand scission by D-isoglucosamine (Tables III and IV). These results indicate that oxygen radicals are generated in the process of the autoxidation of D-isoglucosamine and induce the scission. The main radical species responsible for DNA strand scission could not be specified. Tiron, a scavenger for superoxide anion, and KI, a scavenger for hydroxyl radical, inhibited the scission by D-isoglucosamine completely. These results suggest that superoxide anion and hydroxyl radical might be intermediates of active oxygen radicals reactive to DNA. Catalase inhibited the DNA strand scission most effectively at the concentration of 2.25 units/ml. SOD inhibited DNA strand scission more weakly than catalase, but in the presence of catalase (2.25 units/ml), SOD inhibited more effectively in proportion to the concentration of SOD. This result shows that H_2O_2 participates in the scission. Mechanisms for the production of hydroxyl radical by the metal-catalyzed interaction of O_2^- with H_2O_2 have been proposed.³⁰⁾ However, it is not certain whether DNA strand scission by D-isoglucosamine is caused indirectly by oxygen radicals generated during the autoxidation of D-isoglucosamine, or whether the D-isoglucosamine-copper complex binds to DNA directly to form a DNA-D-isoglucosamine-copper complex, where oxygen radicals generated would cause DNA strand scission.

The induction of strand scission in ϕ X174 RFI DNA by sugar phosphate has been described,¹⁾ and the characteristics of sugar phosphate against DNA were almost the same as those of D-isoglucosamine under our experimental conditions. Although the detailed mechanism of DNA strand scission is not yet known, it is probable that DNA strand scission by D-isoglucosamine and sugar phosphate are similar.

Damage to nucleic acids such as DNA strand scission introduces genetic lesions. We

are interested in whether D-isoglucosamine causes breakage of the genetic material in organisms and wish to know whether biological activities based on the chemical properties of D-isoglucosamine are effective *in vivo* systems.

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