Possible Involvement of Dihydrofructosazine in the DNA Breaking Activity of D-Glucosamine

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Dehydrochlorination of D-glucosamine (2-amino-2-deoxy-D-glucose) hydrochloride with an anion exchange resin made its DNA breaking activity in plasmid pBR322 much higher, especially in the presence of Cu^{2+} . The sample of anion exchanger-treated D-glucosamine hydrochloride, *i.e.*, HCl-free D-glucosamine sample, showed an absorption maximum at 274 nm on the UV absorption spectrum in water as seen in the case of fructosazine [2,5-bis(D-arabino-tetrahydroxybutyl)pyrazine], one of the dimers of D-glucosamine. On a positive-ion fast atom bombardment (FAB) mass spectrum, the sample showed an ion peak at m/z 323 as a base peak, corresponding to dihydrofructosazine [2,5-bis(D-arabino-tetrahydroxybutyl) dihydropyrazine], which was a precursor of fructosazine, as well as those of D-glucosamine itself (m/z 180) and fructosazine (m/z 321). The DNA strand breaking activity of HCl-free D-glucosamine sample was directly proportional to the peak intensity of m/z 323 ion, while the DNA breaking activity of fructosazine was much weaker than that of HCl-free D-glucosamine sample. 2,5-Dihydro-3,6-dimethylpyrazine and 2,3-dihydro-5,6-dimethylpyrazine having a dihydropyrazine skeleton the same as dihydrofructosazine showed the same extent of DNA strand breaking activity as did the HCl-free D-glucosamine sample. These results indicated that dihydrofructosazine produced during the dehydrochlorination is closely involved in the DNA breaking activity of HCl-free D-glucosamine sample.

Key words D-glucosamine; dihydrofructosazine; dihydropyrazine skeleton; DNA breaking activity; cupric ion; FAB mass

Some carbohydrate derivatives have been shown to break DNA, especially in the presence of Cu^{2+} and, in some cases the oxygen radicals generated in the carbohydrate solutions have been demonstrated to be involved in the DNA strand breaking.¹⁾ The elucidation of the specific features of these DNA-breaking agents, particularly through comparison with those of DNA-alkylating or -intercalating compounds, may help to clarify the mechanism of such biological phenomena as carcinogenesis, carcinostasis, mutagenesis, virus induction, virus inactivation, and aging.

Previously, we found that some amino sugars such as D-glucosamine (2-amino-2-deoxy-D-glucose) hydrochloride caused phage PL-1 inactivation²⁾ and plasmid pBR322 breakage,^{3,4)} and that the active oxygen molecules, especially hydroxyl radical ($\cdot\text{OH}$), generated during autoxidation of these amino sugars in their aqueous solutions were involved in some of these phenomena.⁵⁾ Thereafter, during the course of developing more active compounds by chemical modification of these amino sugars, the dehydrochlorinated product of D-glucosamine hydrochloride, which had been obtained by treating D-glucosamine hydrochloride with an anion exchange resin, was found to show much higher activity than the original D-glucosamine hydrochloride. In general, amino sugars themselves, for example, D-glucosamine having a free amino group in the moiety, are unstable in aqueous solutions at physiological pH. It has been reported that D-glucosamine undergoes autoxidative rearrangement and degradation with a formation of fructosazine [2,5-bis(D-arabino-tetrahydroxybutyl)pyrazine], D-arabinose, 5-(hydroxymethyl)-2-furaldehyde and other unidentified products.⁶⁾ However, there have been few reports on the physiological activities of the degradation products of

amino sugars. These sugars, which have an aminoreductone structure, are widely present in organisms, foods and sometimes in drugs such as antitumor antibiotics. Therefore, it is important to investigate in detail the characteristics of the interaction between nucleic acids and amino sugars.

In this paper, we describe that the dehydrochlorinated product of D-glucosamine hydrochloride, *i.e.*, HCl-free D-glucosamine sample, shows much higher DNA breaking activity than the original D-glucosamine hydrochloride in plasmid pBR322 DNA, and that dihydrofructosazine [2,5-bis(D-arabino-tetrahydroxybutyl)dihydropyrazine] generated by the condensation of two molecules of D-glucosamine during dehydrochlorination is mainly involved in its higher activity.

MATERIALS AND METHODS

Plasmid DNA and Chemicals Covalently closed circular duplex DNA (ccc-DNA) of plasmid pBR322 was isolated from *Escherichia coli* w3350/pBR322 as described by Kupersztoch-Portnoy *et al.*⁷⁾ Each batch of pBR322 DNA was analyzed for integrity and purity by agarose gel electrophoresis, and only the DNA samples that contained more than 85% covalently closed circular (ccc)-DNA were used. Amberlite® IRA-402 was purchased from Organo Co. 2,5-Bis(D-arabino-tetrahydroxybutyl)pyrazine (fructosazine) was purchased from Sigma Chemical Co. D-Glucosamine (2-amino-2-deoxy-D-glucose) hydrochloride and the other chemicals were purchased from Wako Pure Chemical Industries, Ltd.

Dehydrochlorination of D-Glucosamine Hydrochloride (Preparation of HCl-Free D-Glucosamine Sample) D-Glucosamine hydrochloride dissolved in water, pH 3.5,

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was treated with anion-exchange resin (Amberlite® IRA-402, OH⁻ form) with stirring at 4°C until a fixed pH value, about pH 11 in this case, was obtained. After the resin was removed by filtration, the filtrate was lyophilized.

Assay of DNA Strand Breaking Activity The reaction mixture (100 μ l) containing 1 μ g of plasmid pBR322 ccc-DNA and various concentrations of sample compounds in 50 mM Tris-HCl buffer (pH 7.2) was incubated at 37°C. At intervals, 10 μ l of the reaction mixture was mixed with 3 μ l of 22.5 mM EDTA (pH 8.2) containing 1.5% (w/v) sodium dodecyl sulfate, 25% (w/v) sucrose and 0.22% (w/v) bromophenol blue to stop the reaction. The resulting mixture was directly layered on 0.8% agarose gels in a horizontal slab gel apparatus. Electrophoresis was done in 40 mM Tris-acetate buffer (pH 8.2) containing 5 mM sodium acetate and 1 mM EDTA at 100 V per gel for 30 min at room temperature. After electrophoresis, the gels were stained with ethidium bromide (0.5 μ g/ml) for 30 min, and washed with running water for 3 min. The stained DNA bands, ccc-DNA, nicked open circular (oc)-DNA and linear DNA, were made visible using an ultraviolet lamp (302 nm) and then photographed with Polaroid film. For quantitative analysis of DNA on the gel, the photographic negatives were scanned with a Shimadzu CS-930 TLC scanner for the optical density (OD) measurement.

Spectrometry UV absorption spectra in water were obtained with a JASCO 670 UV-VIS spectrophotometer. Positive-ion fast atom bombardment (FAB) mass spectra were obtained with a JEOL JMS-HX110 double-focusing mass spectrometer/JMA-DA700 data system. The fast atom beam used was generated from xenon ions, which were accelerated to 6 kV. Glycerol was used as the liquid matrix. ¹H- and ¹³C-nuclear magnetic resonance (NMR) spectra were recorded on a JEOL GX-400 spectrometer, with tetramethylsilane (TMS) as an internal standard.

RESULTS

Comparison of DNA Breaking Activity of HCl-Free D-Glucosamine Sample with That of D-Glucosamine Hydro-

chloride Figure 1 shows the course of the reaction of pBR322 ccc-DNA with HCl-free D-glucosamine sample compared with that of D-glucosamine hydrochloride in the presence or absence of 1 mM CuCl₂. In this case, the amounts of remaining ccc-DNA were used as a marker of DNA breaking activity. In the HCl-free D-glucosamine sample, the amounts of ccc-DNA decreased much faster than that of the original D-glucosamine hydrochloride. The presence of Cu²⁺ promoted their activities much more and, when ccc-DNA was treated with 10 mM HCl-free D-glucosamine sample in the presence of 1 mM CuCl₂, all the ccc-DNA was converted into linear DNA *via* oc-DNA after only 10 min of reaction. Figure 2 shows the effect of the concentrations of HCl-free D-glucosamine sample and D-glucosamine hydrochloride on their ccc-DNA strand breaking activities in the presence or absence of 1 mM Cu²⁺. In both cases, their DNA strand breaking activities were dependent on their concentrations and increased, with the increase being especially remarkable in the presence of Cu²⁺.

UV Absorption Spectrum of HCl-Free D-Glucosamine Sample To examine the reason why the DNA strand breaking activity was heightened by removing the hydrochloride from D-glucosamine hydrochloride, the UV absorption spectrum of HCl-free D-glucosamine sample in water was measured at 200–400 nm and compared with that of D-glucosamine hydrochloride. HCl-free D-glucosamine sample gave an absorption maximum at 274 nm which was not observed in the D-glucosamine hydrochloride, suggesting that some products other from D-glucosamine may be generated in the HCl-free D-glucosamine sample. Moreover, the absorption maximum of HCl-free D-glucosamine sample was similar to that of fructosazine,^{8,9)} one of the condensed dimers of D-glucosamine generated in the aqueous solution. However, either in the presence or absence of Cu²⁺, fructosazine showed a much lower DNA strand breaking activity as compared with that of HCl-free D-glucosamine sample (data not shown). Therefore, fructosazine itself was not considered to be one of the substances essential for the HCl-free D-glucosamine sample to cause a strong DNA strand breaking activity.

Fig. 1. Time Course of ccc-DNA Breakage by D-Glucosamine Hydrochloride and HCl-Free D-Glucosamine Sample

Plasmid pBR322 ccc-DNA was incubated with D-glucosamine HCl (○) or HCl-free D-glucosamine sample (●) in 50 mM Tris-HCl buffer (pH 7.2) at 37°C without (A) or with (B) 1 mM CuCl₂. (A) 100 mM Sugar without CuCl₂, (B) 10 mM sugar with 1 mM CuCl₂.

Fig. 2. Effect of Sugar Concentration on ccc-DNA Breakage by D-Glucosamine Hydrochloride and HCl-Free D-Glucosamine Sample

Plasmid pBR322 ccc-DNA was incubated with various concentrations of D-glucosamine HCl (○) or HCl-free D-glucosamine sample (●) in 50 mM Tris-HCl buffer (pH 7.2) at 37 °C for 3 h without CuCl₂ (A) or for 10 min with 1 mM CuCl₂ (B).

Fig. 3. Positive-Ion FAB Mass Spectra of D-Glucosamine Hydrochloride (A), Fructosazine (B) and HCl-Free D-Glucosamine Sample (C)

Positive-Ion FAB Mass Spectrum of HCl-Free D-Glucosamine Sample To confirm the generation of products other than D-glucosamine and fructosazine in the aqueous solution of HCl-free D-glucosamine sample, the positive-ion FAB mass spectrum of HCl-free D-glucosamine sample was measured using glycerol as a liquid matrix and compared with those of D-glucosamine hydrochloride and fructosazine. As shown in Fig. 3, D-glucosamine hydrochloride (A) showed peaks at m/z 180 corresponding to $[M(\text{D-glucosamine}) + \text{H}]^+$ as a base peak, m/z 162 corresponding to $[M + \text{H}^+ - \text{H}_2\text{O}]$ and m/z 359 corresponding to $[2M + \text{H}]^+$. Fructosazine (B) showed a peak at m/z 321 corresponding to $[M(\text{fructosazine}) + \text{H}]^+$. On the other hand, HCl-free D-glucosamine sample (C) showed peaks at m/z 180 and 162 due to D-glucosamine and m/z 321 corresponding to fructosazine formed from

D-glucosamine, and m/z 305 corresponding to deoxyfructosazine,¹⁰⁾ although the intensity of all these peaks was low. A base peak at m/z 323 was also detected. Therefore, a product other than D-glucosamine and fructosazine in the HCl-free D-glucosamine sample (C) was considered to be of fundamental significance. Since this product showed an ion peak at m/z 323, which was greater than m/z 321 of fructosazine for 2-protons, on the positive-ion FAB mass spectrum, it was believed to be dihydrofructosazine [2,5-bis(D-arabino-tetrahydroxybutyl) dihydropyrazine], the 2-proton adduct of fructosazine and regarded as a precursor of fructosazine (Fig. 4). Moreover, the elemental composition of m/z 323 ion, measured by high-resolution FAB mass spectrometry, was $\text{C}_{12}\text{H}_{23}\text{N}_2\text{O}_8$, which agreed with that of dihydrofructosazine ($\text{C}_{12}\text{H}_{22}\text{N}_2\text{O}_8$) + proton (H).

Fig. 4. Formation of Dihydrofructosazine, Fructosazine and Deoxyfructosazine from D-Glucosamine

Relationships between the Peak Intensity of Dihydrofructosazine on Positive-Ion FAB Mass Spectrum and the DNA Strand Breaking Ability of HCl-Free D-Glucosamine Sample To confirm that dihydrofructosazine was involved in the DNA strand breaking activity of HCl-free D-glucosamine sample, the relationship between the intensity of the peak at m/z 323 on the positive-ion FAB mass spectrum and the DNA strand breaking activity of the HCl-free D-glucosamine sample was examined. HCl-free D-glucosamine sample was dissolved in sterile water in an Erlenmeyer flask sealed with a serum cap and kept at 37 °C with shaking; at intervals an aliquot of this solution was lyophilized. As shown in Fig. 5, this sample showed a high DNA strand breaking activity for several days, while the intensity of the peak at m/z 323 on positive ion FAB mass spectrum was maintained at a fixed high level. However, with the passage of incubation time, the peak intensity gradually decreased, and the intensity reduction was always accompanied by a reduction in DNA breaking activity, indicating that dihydrofructosazine was involved in the DNA strand breaking activity of the HCl-free D-glucosamine sample.

Attempt to Isolate Dihydrofructosazine from HCl-Free D-Glucosamine Sample by High-Performance Liquid Chromatography (HPLC) Preparative analyses of dihydrofructosazine generated in HCl-free D-glucosamine sample were carried out by HPLC. The three separated fractions were lyophilized to give colorless cloudy syrup, which showed respective proportions of 0.5, 1, and 2 of the peak intensity (m/z 323/ m/z 321) on FAB mass spectra. This syrup thus evidently contained dihydrofructosazine. On $^1\text{H-NMR}$ spectra, however each syrup fraction showed only the signal pattern in accord with fructosazine. The syrup recovered from the NMR sample showed only the

Fig. 5. Relationship between the Amounts of Dihydrofructosazine Generated from HCl-Free D-Glucosamine Sample and DNA Breakage Activity

HCl-free D-glucosamine sample was kept at 37 °C with shaking in sterilized water. At intervals the relative amount of dihydrofructosazine (%) in the incubation mixture was determined from its relative peak-intensity on the positive-ion FAB mass spectrum as follows: dihydrofructosazine (%) = $\{[A/(A+B+C+D)]/[A_0/(A_0+B_0+C_0+D_0)]\} \times 100$, where A , B , C and D are the peak-intensity of dihydrofructosazine (m/z 323), D-glucosamine (m/z 180), deoxyfructosazine (m/z 305) and fructosazine (m/z 321), respectively, and A_0 , B_0 , C_0 , and D_0 are those at 0 d ($A_0/(A_0+B_0+C_0+D_0)=0.52$). The DNA breaking activity (relative amount of remaining ccc-DNA (%)) was measured by incubating the sample (1 mm equivalent) with pBR322 ccc-DNA in 50 mM Tris-HCl buffer (pH 7.2) at 37 °C for 1 h in the presence of 1 mM CuCl_2 .

peak (m/z 321) due to fructosazine on FAB-mass spectra, while the m/z 323 due to dihydrofructosazine diminished to noise level intensity. It can thus be concluded that dihydrofructosazine changed to give fructosazine by dehydration during the isolation and measurement process.

DNA Strand Breaking Activity of Some Dihydropyrazine Derivatives The DNA strand breaking activity of 2,5-

Table 1. pBR322 ccc-DNA-Breakage by Some Dihydropyrazine Derivatives

Sample	Concn. (mM)	Rel. amts. of remaining ccc-DNA (%)	
		Without Cu ²⁺ 3 h	With 1 mM Cu ²⁺ 1 h
2,5-Dihydro-3,6- dimethylpyrazine	1	89	8
	10	58	0
2,3-Dihydro-5,6- dimethylpyrazine	1	94	76
	10	84	6

dihydro-3,6-dimethylpyrazine¹¹⁻¹⁴⁾ and 2,3-dihydro-5,6-dimethylpyrazine,¹⁵⁾ having a dihydropyrazine structure just like dihydrofructosazine, was then examined. As Table 1 shows, the activity of these dihydropyrazines was comparable to that of HCl-free D-glucosamine sample, supporting the idea that dihydrofructosazine would be the principal agent for the HCl-free D-glucosamine sample to break the DNA strand of plasmid pBR322.

DISCUSSION

In this study we found that a D-glucosamine sample dehydrochlorinated with anion exchange resins shows much higher DNA strand breaking activity than the original D-glucosamine hydrochloride. The increase in this activity was remarkable, especially in the presence of Cu²⁺. The true substance in the HCl-free D-glucosamine sample causing breakage of DNA strand was suggested to be dihydrofructosazine, which was formed by the condensation of two molecules of D-glucosamine, considering the measurement of UV absorption, positive-ion FAB mass spectra and NMR spectra of HCl-free D-glucosamine sample. Then, we tried to isolate dihydrofructosazine from HCl-free D-glucosamine sample by HPLC but were unsuccessful due to its instability, and it was converted to fructosazine during the isolation and measurement process. More stable compounds having a dihydropyrazine skeleton were therefore investigated. The fact that some dihydropyrazine derivatives such as 2,5-dihydro-3,6-dimethylpyrazine and 2,3-dihydro-5,6-dimethylpyrazine showed a high DNA strand breaking activity comparable to that of HCl-free D-glucosamine sample indicated that dihydrofructosazine must be one of the strongest candidates causing display of the high DNA strand breaking activity.

This dihydrofructosazine was thought to be formed by the reaction of amino group with carbonyl group (Maillard reaction) between two molecules of D-glucosamine. Several facts support the possibility that dihydrofructosazine was the most important factor in the DNA strand breaking activity: considerable amounts of D-glucosamine hydrochloride exist in HCl-free form in its neutral and/or slightly alkaline aqueous solution³⁾; 2-acetamido 2-deoxy-D-glucose (N-acetyl-D-glucosamine) and methyl 2-amino-2-deoxy- α -D-glucopyranoside did not show any DNA strand breaking activity, because the C-2 amino and C-1 aldehyde groups were modified respectively, and therefore the condensation between the two molecules could not take

place³⁾; and the DNA strand breaking activity of D-glucosamine hydrochloride was stimulated by phosphate ion, one of the promoters of Maillard reaction.^{3,4)} The DNA strand breaking activity of D-glucosamine hydrochloride was exhibited much better in its alkaline condition, under which HCl tended to be removed from it.³⁾ In practice, when the aqueous solution of D-glucosamine hydrochloride was treated with NaOH, dihydrofructosazine was produced in the lyophilized reaction mixture. In this case, however, the relative amounts of dihydrofructosazine were much lower as compared with the sample prepared by treatment with anion exchange resin; moreover, treatment with NaOH led to drastic decomposition of the sugar chain. Therefore, we used anion exchange resin to remove HCl from D-glucosamine hydrochloride for a milder preparation of HCl-free D-glucosamine sample.

In general, amino sugars having free amino group are unstable in aqueous solutions, and D-glucosamine has been reported to undergo autoxidative rearrangement and degradation at physiological pH as free base, consequently forming fructosazine, D-arabinose, 5-(hydroxymethyl)-2-furaldehyde and other unidentified products.⁶⁾ In these degradation products, 5-(hydroxymethyl)-2-furaldehyde was reported to show a mutagenic activity and DNA strand breaking activity.¹⁶⁾ However, 5-(hydroxymethyl)-2-furaldehyde was not detected in the HCl-free D-glucosamine sample in our experiments. Moreover, the authentic sample commercially available did not show any DNA strand breaking activity under our experimental conditions (data not shown). In the earlier stage of Maillard reaction of reducing sugar with amino acid, it was also suggested that some free radicals such as pyrazine cation radical could be generated *via* dihydropyrazine.¹⁷⁻¹⁹⁾ The facts shown in our previous experiments⁴⁾ that the pBR322 DNA-breakage by D-glucosamine hydrochloride in the presence of Cu²⁺ occurred at sequence-specific (pyrimidine-purine (5'→3')) sites but not uniformly at all nucleotide sites suggested the involvement of a factor other than $\cdot\text{OH}$. In the DNA strand breaking activity, therefore, the involvement of this kind of free radical, generated *via* dihydrofructosazine from the HCl-free D-glucosamine samples other than $\cdot\text{OH}$, which was described in our previous paper,⁵⁾ remains a possibility and is currently being investigated.

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