Products of the Reaction between a Diazoate Derivative of 2′-Deoxycytidine and L-Lysine and Its Implication for DNA−Nucleoprotein Cross-Linking by NO or HNO_2

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Recently, we have reported that a stable diazoate intermediate (dCyd-diazoate) is produced upon the reaction of dCyd with nitrous acid and nitric oxide [Suzuki, T., Nakamura, T., Yamada, M., Ide, H., Kanaori, K., Tajima, K., Morii, T., and Makino, K. (1999) *Biochemistry* **38**, 7151−7158]. In this work, the reaction of dCyd-diazoate with L-Lys was investigated. When 0.4 mM dCyd-diazoate was incubated with 10 mM L-Lys in sodium phosphate buffer (pH 7.4) at 37 °C, two unknown products were formed in addition to dUrd. By spectrometric measurements, the products were identified as dCyd−Lys adducts with C4(dCyd)−N^{α}(Lys) and C4(dCyd)−N^{ϵ}(Lys) linkages (abbreviated as dCyd−αLys and dCyd−ϵLys, respectively). The yields at the reaction time of 72 h were 28.0% dCyd−αLys, 13.4% dCyd−ϵLys, and 11.1% dUrd with 33.9% unreacted dCyd-diazoate. When 0.4 mM dCyd-diazoate was incubated with 22 mg/mL poly(L-Lys) at pH 7.4 and 37 °C for 24 h, 82% of the free dCyd-diazoate disappeared, indicating adduct formation with the polymer. At pH 7.4 and 37 °C, dCyd−αLys and dCyd−ϵLys were fairly stable and gave rise to no product after incubation for 7 days. At pH 4.0 and 70 °C, both adducts disappeared with the same first-order rate constant of 1.7×10^{-6} s^{-1} ($t_{1/2} = 110$ h), which was $\sim 1/3$ of that of dCyd. These results suggest that if dCyd-diazoate is formed in DNA in vivo, it may react with free L-Lys and the side chain of L-Lys in nucleoproteins, resulting in stable adducts and DNA−protein cross-links, respectively.

Introduction

Nitrous acid (HNO_2) and nitric oxide (NO) under aerobic conditions react with the amino group of dCyd, resulting in dUrd, a deamination compound, in vitro (*1*, *2*). It has been thought that the formation of dUrd is one of the major causes of mutations induced by HNO_2 and NO in vivo, since dUrd can adopt the same base pairing scheme as dThd in DNA (*3*, *4*). Because the spontaneous hydrolytic deamination of dCyd also occurs at biologically significant rates in cells (*5*), a specific repair enzyme, uracil-DNA glycosylase, exists abundantly in cells and excises Ura from DNA by cleaving the *N*-glycosidic bond (*6*, *7*). dUrd produced by HNO_2 or NO should be also excised by the same enzyme. However, a recent study employing several mutant strains of *Escherichia coli* has shown that a defect in uracil-DNA glycosylase has little impact on cell killing and mutagenesis by HNO_2 (*8*). On the other hand, strains defective in Uvr nuclease, which excises pyrimidine dimers and various bulky adducts (*9*), exhibited pronounced sensitivity to killing and significantly stimulated induction of rifampicin resistance under the same conditions. The results indicate that HNO_2 induces a particular class of DNA lesions that are not recognized by uracil-DNA glycosylase but are by UvrABC nuclease. We have recently shown that 2′-deoxyoxanosine (dOxo)[1] which is produced from dGuo by the reaction with HNO_2 and NO in addition to 2′-deoxyxanthosine (dXao), a deamination product, reacts with Gly, resulting in a stable adduct (*10*, *11*). This result suggests that if dOxo formed in vivo in DNA it will react with Gly and other free amino acids in the vicinity, resulting in adducts which may be recognized by UvrABC nuclease.

Recently, we have also isolated and characterized the intermediate produced from dCyd by HNO_2 and NO treatments (*12*). By spectrometric measurements, the product was identified as a diazoate derivative of dCyd, 1-(β-D-2′-deoxyribofuranosyl)-2-oxopyrimidine-4-diazoate (dCyd-diazoate) (Figure 1). The time course of the concentration change of dCyd-diazoate exhibited a profile characteristic of a reaction intermediate, and the dCyd-diazoate concentration was greater than that of dUrd at the initial stage of the reaction. dCyd-diazoate was also

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[1] Abbreviations: dCyd-diazoate, diazoate derivative of dCyd, 1-(β-D-2′-deoxyribofuranosyl)-2-oxopyrimidine-4-diazoate; dCyd−αLys, dCyd−Lys adduct with a C4(dCyd)−N^{α}(Lys) linkage; dCyd−ϵLys, dCyd−Lys adduct with a C4(dCyd)−N^{ϵ}(Lys) linkage; dXao, 2′-deoxyxanthosine; dOxo, 2′-deoxyoxanosine; RP-HPLC, reversed phase HPLC; t_R, retention time; APCI, atmospheric pressure chemical ionization.

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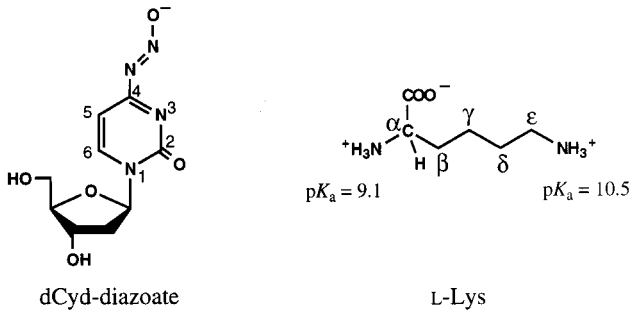


Figure 1. Structures of dCyd-diazoate [1-(β-D-2′-deoxyribofuranosyl)-2-oxopyrimidine-4-diazoate] and L-Lys.

generated from dCyd in neutral solution by NO treatment under aerobic conditions. At pH 7.4 and 37 °C, dCyd-diazoate was fairly stable ($t_{1/2}$ = 330 h). Uracil-DNA glycosylase did not excise the diazoate residue from an oligodeoxynucleotide containing this damage. These results show that the diazoate is generated as a stable intermediate in the reactions of dCyd with HNO_2 and NO and that the major product is not dUrd but the diazoate in the initial stage of the reactions. Thus, once formed in vivo, the diazoate persists for a long time in DNA and may directly exert major cytotoxic and/or genotoxic effects. In addition, dCyd-diazoate is likely to react with various intracellular nucleophiles under physiological conditions since it is an intermediate of diazotization. The resulting adducts of dCyd in DNA may also lead to mutagenic or lethal events of cells and be recognized by UvrABC nuclease. Until now, however, there has been no information about the reaction of dCyd-diazoate with cellular molecules.

We report herein identification and characterization of the reaction products of dCyd-diazoate with L-Lys at physiological pH and temperature.

Materials and Methods

Materials. The dCyd-diazoate was prepared as described previously (*12*). dCyd, dUrd, N^{α}-BOC-L-Lys, and poly(L-Lys) hydrobromide (average molecular weight of 35 000) were obtained from Sigma (St. Louis, MO). All other reagent grade chemicals were purchased from Wako Pure Chemicals (Osaka, Japan) or Nacalai Tesque (Osaka, Japan) and used without further purification. Water was purified with a Millipore Milli-QII deionizer.

HPLC Conditions. The HPLC system consisted of Hitachi L-6000 and L-6200 pumping systems. On-line UV spectra were obtained with a Hitachi L-3000 UV/vis photodiode array detector. For analysis and separation of nucleosides by RP-HPLC, a Cosmosil 5C18-MS octadecylsilane column (4.6 mm × 150 mm, particle size of 5 μm, Nacalai Tesque) was used. The eluent was 100 mM triethylammonium acetate buffer (pH 7.0) containing acetonitrile. The acetonitrile concentration was increased from 0 to 20% for 20 min with a linear gradient mode. RP-HPLC analyses were performed at room temperature and a flow rate of 1.0 mL/min.

Spectrometric Measurements. NMR spectra were measured using a Brucker ARX-500 spectrometer in H_2O/D_2O (9:1, v/v) at 10 °C for **1** or DMSO-d_6 at 27 °C for **2**. The chemical shifts (parts per million) were referenced to TSP-d_4 for H_2O/D_2O or TMS for DMSO-d_6 as the internal standard. The signal assignments were determined by DQF-COSY or TOCSY (40 ms).

Spectrometric Data of 1 (dCyd−αLys). ^{1}H NMR (500 MHz, 9:1 H_2O/D_2O at 10 °C): δ 7.76 (d, J_{56} = 7.6 Hz, 1H, H-6), 7.20 (d, J_{NHCH} = 7.4 Hz, 1H, NH), 6.28 (dd, 1H, H-1′), 6.06 (d, 1H, H-5), 4.42 (m, 1H, H-3′), 4.35 (m, 1H, H-α), 4.04 (ddd, 1H, H-4′), 3.79 (ABX, 2H, H-5′,5″), 2.98 (m, 2H, H-ϵ), 2.38 (ddd, 1H, H-2″), 2.31 (ddd, 1H, H-2′), 1.88 (m, 1H, H-β or -β′), 1.78 (m, 1H, H-β or -β′), 1.69 (m, 2H, H-δ), 1.46 (m, 2H, H-γ). UV: λ_{max} = 273 nm (pH 7.0).

Spectrometric Data of 2 (dCyd−ϵLys). ^{1}H NMR (500 MHz, DMSO-d_6 at 27 °C): δ 7.81 (dd, J_{NHCH_2} = 5.2 and 5.2 Hz, 1H, ϵNH), 7.72 (d, J_{56} = 7.5 Hz, 1H, H-6), 6.15 (dd, 1H, H-1′), 5.76 (d, 1H, H-5), 5.35 (br, 1H, 3′- or 5′-OH), 5.04 (br, 1H, 3′- or 5′-OH), 4.19 (ddd, 1H, H-3′), 3.74 (ddd, 1H, H-4′), 3.54 (ABX, 2H, H-5′,5″), 3.21 (m, 2H, H-ϵ), 3.14 (m, 1H, H-α), 2.09 (ddd, 1H, H-2″), 1.92 (ddd, 1H, H-2′), 1.72 (m, 1H, H-β or -β′), 1.69 (m, 1H, H-β or -β′), 1.46 (m, 2H, H-δ), 1.37 (m, 2H, H-γ). UV: λ_{max} = 273 nm (pH 7.0).

Quantitative Procedures. The concentrations of products were evaluated from integrated peak areas of HPLC chromatograms and molar extinction coefficients at 260 nm. The values of ϵ_{260} for dCyd−αLys and dCyd−ϵLys were estimated to be 1.26 × 10^4 and 1.24 × 10^4 M^{-1} cm^{-1}, respectively, from integration of the H-5 and H-6 proton signals and RP-HPLC peak area detected at 260 nm relative to those of dCyd. The ϵ_{260} values of 7.3 × 10^3, 7.4 × 10^3, and 1.01 × 10^4 M^{-1} cm^{-1} were used for dCyd-diazoate, dCyd, and dUrd, respectively (*12*, *13*). In the quantitative analysis of the reaction products, the initial RP-HPLC peak area was used as a standard.

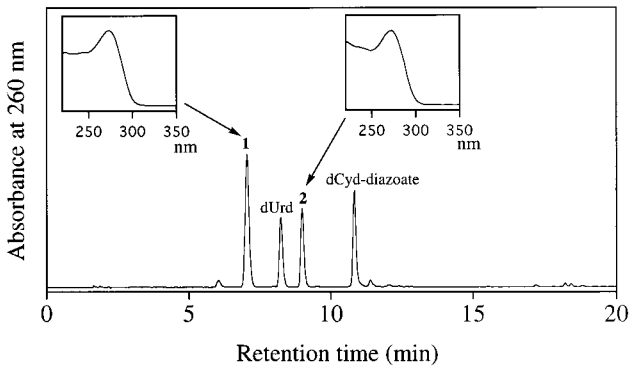
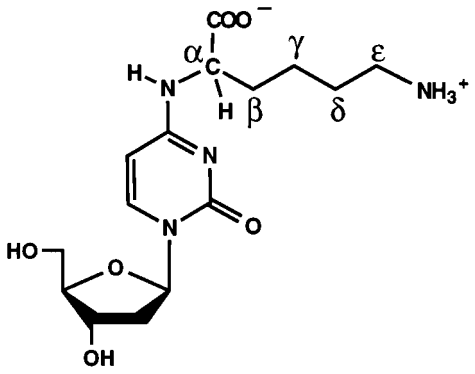


Figure 2. RP-HPLC chromatogram of dCyd-diazoate incubated with L-Lys. dCyd-diazoate (0.4 mM) was incubated with 10 mM L-Lys in 100 mM sodium phosphate buffer (pH 7.4) at 37 °C for 72 h. The sample was separated with an ODS column (4.6 mm × 150 mm). The eluent was 100 mM triethylammonium acetate buffer (pH 7.0) containing CH_3CN, and the flow rate was 1.0 mL/min. The CH_3CN concentration was increased from 0 to 20% for 20 min in a linear gradient mode. Insets are on-line-detected UV spectra of **1** and **2**.

Results

Detection and Identification of Reaction Products of dCyd-Diazoate with L-Lys. When 0.4 mM dCyd-diazoate was incubated with 10 mM L-Lys in 100 mM sodium phosphate buffer (pH 7.4) for 72 h at 37 °C, two unknown products were eluted as major peaks designated as **1** (t_R = 7.1 min) and **2** (t_R = 9.0 min) in addition to dUrd, a deamination product (t_R = 8.2 min), and the starting dCyd-diazoate (t_R = 10.7 min) in the RP-HPLC chromatogram (Figure 2). These products exhibited similar UV spectra with λ_{max} values of 273 nm (Figure 2 insets). The products were isolated by preparative RP-HPLC and subjected to structural assignment. The ^{1}H NMR spectrum of **2** was recorded in DMSO-d_6, and on the other hand, that of **1** was recorded in H_2O/D_2O (9:1) since **1** precipitated in DMSO-d_6. Both of the ^{1}H NMR spectra of **1** and **2** exhibited two aromatic protons, H-5 and H-6, and a set of signals of the intact 2′-deoxyribose moiety originated from dCyd in addition to four sets of methylene protons (H-β, H-γ, H-δ, and H-ϵ)



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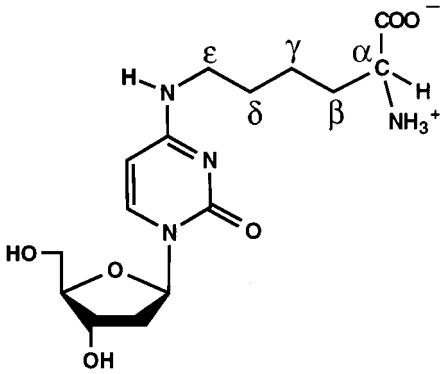

2 (dCyd-εLys)

Figure 3. Structures of **1** (dCyd−αLys) and **2** (dCyd−ϵLys).

and one methine proton (H-α) from L-Lys. Additionally, a signal of imino proton was observed in both the spectra of **1** and **2**. The imino proton signal of **1** was a doublet (J = 7.4 Hz) and correlated with H-α in the DQF-COSY spectrum, whereas that of **2** was a doublet of a doublet (J = 5.2 and 5.2 Hz) and correlated with H-ϵ in the TOCSY spectrum. Moreover, when N^{α}-BOC-L-Lys whose α-amino group is protected by a butoxycarbonyl group was incubated with dCyd-diazoate under the same conditions, only two products appeared in the RP-HPLC chromatogram, one of which was dUrd. The HPLC fraction containing the other product was collected and further analyzed. The treatment of the collected product with 50% trifluoroacetic acid at 37 °C for 15 min afforded a single product peak of **2** in RP-HPLC analysis (data not shown). Combining these data, we have concluded that **1** and **2** are products of dCyd with C4 substitution ate α- and ϵ-amino groups of L-Lys, respectively (Figure 3). Compounds **1** and **2** are denoted as dCyd−αLys and dCyd−ϵLys, respectively.

Reaction Kinetics for Adduct Formation. Figure 4 shows the time courses of concentration changes in dCyd−αLys, dCyd−ϵLys, and dUrd along with conversion of dCyd-diazoate when 0.4 mM dCyd-diazoate was incubated with 10 mM L-Lys in 100 mM sodium phosphate buffer (pH 7.4) at 37 °C. The concentrations of the products were quantified by the combination of RP-HPLC peak areas and molar extinction coefficients. The values were calibrated using the initial dCyd-diazoate concentration as a standard. Throughout the reaction, the ratio between dCyd−αLys and dCyd−ϵLys was ca. 2:1, and the yield of dUrd was comparable to that of dCyd−ϵLys. The

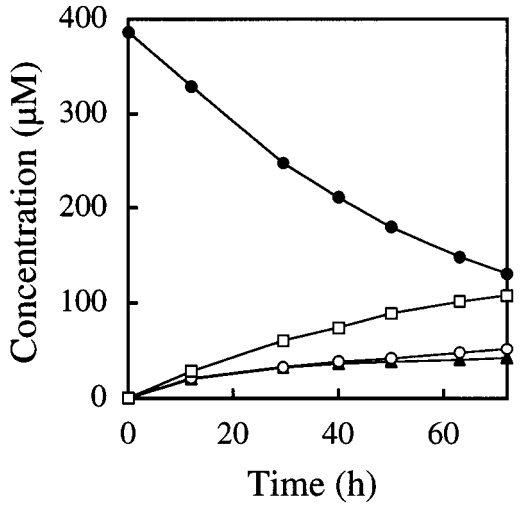

Figure 4. Time course of concentration changes obtained for dCyd−αLys (□), dCyd−ϵLys (○), and dUrd (▲) together with that for dCyd-diazoate (●). dCyd-diazoate (0.4 mM) was incubated with 10 mM L-Lys in 100 mM sodium phosphate buffer (pH 7.4) at 37 °C. The concentration was determined by RP-HPLC analysis.

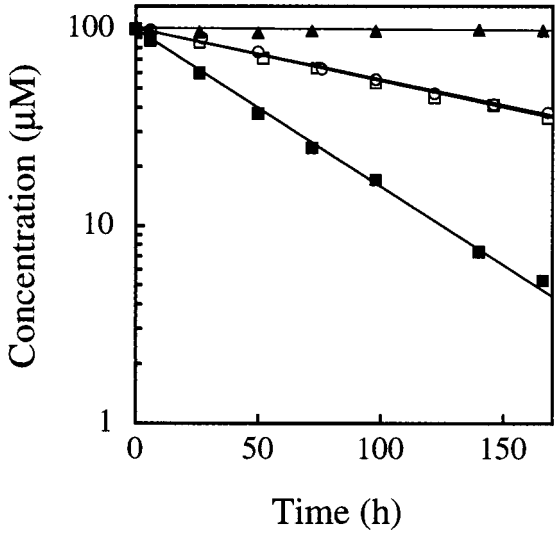

Figure 5. Exponential plots of the concentration changes obtained for dCyd−αLys (□), dCyd−ϵLys (○), dCyd (■), and dUrd (▲) as a function of incubation time. The solution of dCyd−αLys, dCyd−ϵLys, dCyd, or dUrd (0.1 mM each) was incubated in 100 mM sodium acetate buffer (pH 4.0) at 70 °C. The concentration was determined by RP-HPLC analysis.

yields at the incubation time of 72 h were 28.0% dCyd−αLys, 13.4% dCyd−ϵLys, and 11.1% dUrd with 33.9% unreacted dCyd-diazoate.

Stability of dCyd−αLys and dCyd−ϵLys. To evaluate the stability of the products, the isolated dCyd−αLys, dCyd−ϵLys, dCyd, and dUrd (0.1 mM each) were incubated in neutral solution (pH 7.4, 37 °C, 100 mM sodium phosphate buffer) and mild acidic solution (pH 4.0, 70 °C, 100 mM sodium acetate buffer). The reactions were monitored by RP-HPLC. At pH 7.4 and 37 °C, dCyd−αLys and dCyd−ϵLys gave rise to no product after incubation for 7 days (data not shown). At pH 4.0 and 70 °C, dCyd−αLys and dCyd−ϵLys disappeared with incubation time. The plot of concentrations of the adducts against the incubation time followed first-order kinetics (Figure 5). The rate constant was determined to be 1.7 $\times$ 10^{-6} s^{-1} ($t_{1/2}$ = 110 h) for both adducts. On the other

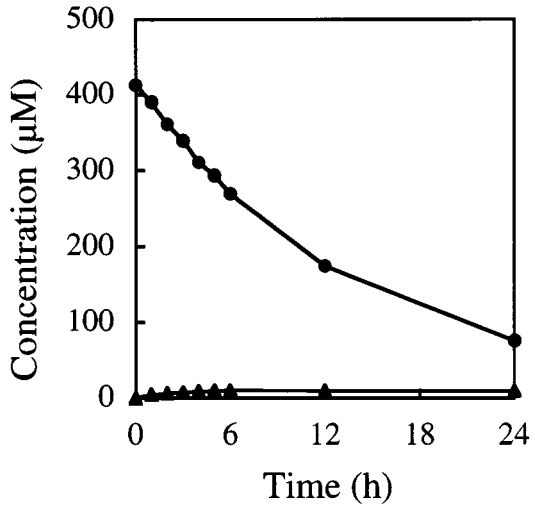

Figure 6. Concentration changes obtained for free dCyd-diazoate (●) and dUrd (▲) in the presence of poly(L-Lys). dCyd-diazoate (0.4 mM) was incubated with 22 mg/mL poly(L-Lys) in 100 mM sodium phosphate buffer (pH 7.4) at 37 °C for the indicated time. The sample was filtered with a Millipore Cosmonice Filter W to remove poly(L-Lys) and dCyd−poly(L-Lys) adducts. The concentration of dCyd-diazoate and dUrd in the filtrate was determined by RP-HPLC analysis.

hand, dCyd disappeared with a first-order rate constant of 5.0×10^{-6} s^{-1} ($t_{1/2} = 39$ h), and dUrd was stable.

Reaction of dCyd-Diazoate with Poly(L-Lys). To elucidate whether dCyd-diazoate reacts with the amino group of the L-Lys side chain in protein, dCyd-diazoate was incubated with poly(L-Lys). dCyd-diazoate (0.4 mM) was incubated with 22 mg/mL poly(L-Lys) in 100 mM sodium phosphate buffer (pH 7.4) at 37 °C for ≤24 h. Aliquots of the solution were filtered by a Millipore Cosmonice Filter W (pore size, 0.45 μm) to remove the polymer and analyzed by RP-HPLC. Before chromatography, it was confirmed that the filter did not retain dCyd-diazoate, dCyd, and dUrd. The concentration of dCyd-diazoate in the filtrate decreased with an increase in incubation time, and a small amount of dUrd appeared (Figure 6). At the incubation time of 24 h, 82% of the dCyd-diazoate disappeared but the yield of dUrd in the filtrate was 4%. When dCyd and dUrd were incubated with poly(L-Lys) for 24 h under the same conditions, the concentration of the compounds did not change before and after filtration.

Discussion

Protonated NO_2^- (i.e., HNO_2) and NO under aerobic conditions generate several reactive nitrogen species, including N_2O_3 that reacts with amines resulting in nitrosamines (*14*, *15*). The nitrosated primary amines are spontaneously converted to several intermediates such as diazohydroxide (R−N=N−OH), diazoate (R−N=N−O^-), diazonium (R−N≡N^+), and alkyl cation (R^+) in neutral solutions (*14*). The alkyl cation or its precursor (diazo species) results in the corresponding hydroxylate compound as a result of nucleophilic attack of a water molecule. In the nitrosation of dCyd, the most stable intermediate is the diazoate derivative, i.e., dCyd-diazoate (*12*). dCyd-diazoate is converted exclusively to dUrd by attack of a water molecule in neutral solution. In this study, we found that the incubation of dCyd-diazoate with L-Lys resulted in adducts, in which C4 of dCyd was linked to the α- or ϵ-amino group of L-Lys. An additional product of this reaction was dUrd formed by hydrolysis of the diazoate group. The reaction leading to the dCyd−Lys adducts is likely to be initiated by nucleophilic attack of the amino groups of L-Lys on the cation or the precursor diazo species of dCyd.

Under the conditions described here, incubation of 10 mM L-Lys with dCyd-diazoate resulted in the dCyd−αLys adduct, and its yield was 2-fold greater than that of dUrd. Since the intracellular concentration of L-Lys is 1.4 mM in healthy human muscle (*16*), it is possible that a biologically significant amount of the adduct can be produced by the reaction of dCyd-diazoate in DNA with intracellular L-Lys. In addition to L-Lys, cells contain various free amino acids. The total concentration of the naturally occurring 20 free amino acids is 34 mM in human muscle cells (*16*). The average of the pK_a values for the α-amino groups of the 20 amino acids is 9.5 which is essentially comparable to that of the α-amino group of L-Lys (pK_a = 9.1) (*17*). In the first approximation, the rate of the reaction between the amino acids and dCyd-diazoate should primarily depend on the pK_a value of the α-amino group since the reaction is initiated by nucleophilic attack of the α-amino group. Thus, the rate of adduct formation with the naturally occurring amino acids will be comparable to that for L-Lys (α-amino group). Accordingly, dCyd-diazoate in a free form or in DNA may preferentially react with amino acids yielding the adducts rather than with water yielding dUrd in cells.

In this study, the reaction with the ϵ-amino group of L-Lys resulted in another adduct with a yield similar to that of dUrd. The pK_a value of the ϵ-amino group of L-Lys is 10.4 (*17*) and about 1 unit higher than that of the α-amino group of L-Lys. The yield of dCyd−ϵLys was ∼$^1/_2$ of that of dCyd−αLys and was higher than that simply expected from the difference in pK_a values of the α- and ϵ-amino groups. The α-amino group of L-Lys has the bulky carboxyl group in the vicinity, whereas the ϵ-amino group is free of steric hindrance. The difference in accessibility of the amino group to the electrophilic carbon, i.e., C4 of the cation (or precursor diazo species) derived from dCyd-diazoate, may affect the reactivity. After incubation of dCyd-diazoate with poly(L-Lys) and subsequent removal of poly(L-Lys) by filtration, most of dCyd-diazoate (82%) disappeared from the filtrate, although the concentration of dCyd and dUrd treated in the same manner did not change. This result suggests that dCyd-diazoate reacted with the ϵ-amino groups of the side chain in poly(L-Lys) and was trapped by the polymer. Therefore, it is possible that dCyd-diazoate formed in DNA may react with the amino group of the L-Lys side chain in nucleoproteins, resulting in DNA−protein cross-links, although the rate of cross-link formation with nucleoproteins would be lower than that with poly(L-Lys) because of the relatively low abundance of L-Lys residues on the surface of nucleoproteins and the possible pK_a variation of the ϵ-amino group of L-Lys.

Among nucleoproteins, histone should be one of the proteins most likely forming DNA−protein cross-links since it is in direct contact with DNA and has L-Lys rich domains. Consistent with this notion, it has been reported that HNO_2 treatment of nucleohistone gives rise to DNA−histone cross-links (*18*). There are two possible mechanisms for the cross-link formation caused by nitrosation of DNA. The first one involves formation of a

covalent bond between the L-Lys side chain on histone and the reactive aldehyde group in the DNA abasic site. Abasic sites are known to be generated by spontaneous depurination of 2′-deoxyxanthosine (dXao), a deamination product of dGuo caused by nitrosation, since the *N*-glycosidic bond of dXao is very labile (*19*, *20*). The second mechanism involves cross-links between the L-Lys side chain and nitrosation products themselves. 2′-Deoxyoxanosine (dOxo), a byproduct of dGuo nitrosation (*10*), reacts with the α-amino group of Gly, resulting in a stable adduct (*11*). Therefore, it is possible that dOxo formed in DNA may react with the ϵ-amino group of L-Lys on histone, resulting in DNA−histone cross-links, although there is currently no direct evidence for the reaction. This study has suggested a new pathway in the second mechanism where dCyd-diazoate plays an essential role. During the course of the reaction, the nitrosated dCyd exists as the diazoate intermediate for a certain period of time before being converted to dUrd. When the fact that isolated dCyd-diazoate is fairly stable in water ($t_{1/2}$ = 330 h at pH 7.4 and 37 °C) (*12*) is considered, the lifetime of the diazoate in cellular DNA would also be rather long. Combining this and the present data for the reactivity of the diazoate with the ϵ-amino group of L-Lys, we found that covalent bond formation between dCyd-diazoate and an L-Lys residue would be a feasible pathway causing DNA−histone cross-links.

At physiological pH and temperature (pH 7.4 and 37 °C, respectively), dCyd−αLys and dCyd−ϵLys were fairly stable and no changes in their concentrations were observed after incubation for 7 days. Thus, if the adducts and DNA−protein cross-links are once formed in vivo, they may persist for a long period, eventually exerting mutagenic and lethal effects. A recent study employing several *E. coli* mutants has shown that defects in UvrABC nuclease lead to a pronounced sensitivity to cell killing and an increased frequency of mutation via HNO_2 treatment (*8*). The UvrABC nuclease acts on a broad spectrum of types of DNA damage, including pyrimidine dimers, abasic sites, various adducts, and even DNA interstrand cross-links (*9*). In view of these results and the previous study (*8*), UvrABC nuclease may repair the amino acid adducts of DNA or DNA−protein cross-links produced by the reaction between dCyd-diazoate in DNA and intracellular free amino acids or nucleoproteins.

In conclusion, it has been shown that dCyd-diazoate reacts with L-Lys, resulting in two stable adducts at physiological pH and temperature. This result also implicates a novel mechanism of the formation of DNA adducts and DNA−protein cross-links generated by HNO_2 and NO.

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References

(1) Schuster, H., and Schramm, G. (1958) Bestimmung der biologish wirksamen einheit in der ribosenucleinsäure des tabakmosaikvirus auf chemischem wege. *Z. Naturforsch.* **13b**, 697−704.
(2) Wink, D. A., Kasprzak, K. S., Maragos, C. M., Elespuru, R. K., Misra, M., Dunams, T. M., Cebula, T. A., Koch, W. H., Andrews, A. W., Allen, J. S., and Keefer, L. K. (1991) DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. *Science* **254**, 1001−1003.
(3) Takahashi, I., and Marmur, J. (1963) Replacement of thymidylic acid by deoxyuridylic acid in the deoxyribonucleic acid of a transducing phage for *Bacillus subtilis*. *Nature* **197**, 794−795.
(4) Warner, H. R., and Duncan, B. K. (1978) In vivo synthesis and properties of uracil-containing DNA. *Nature* **272**, 32−43.
(5) Frederico, L. A., Kunkel, T. A., and Shaw, B. R. (1990) A sensitive genetic assay for the detection of cytosine deamination: Determination of rate constants and activation energy. *Biochemistry* **29**, 2532−2537.
(6) Lindahl, T. (1974) An *N*-glycosidase from *Escherichia coli* that releases free uracil from DNA containing deaminated cytosine residues. *Proc. Natl. Acad. Sci. U.S.A.* **71**, 3649−3653.
(7) Krokan, H., and Wittwer, C. U. (1981) Uracil-DNA glycosylase from HeLa-cells: general properties, substrate specificity and effects of uracil analogs. *Nucleic Acids Res.* **9**, 2599−2613.
(8) Hartman, Z., Henrikson, E. N., Hartman, P. E., and Cebula, T. A. (1994) Molecular models that may account for nitrous acid mutagenesis in organisms containing double-stranded DNA. *Environ. Mol. Mutagen.* **24**, 168−175.
(9) Van Houten, B. (1990) Nucleotide excision repair in *Escherichia coli*. *Microbiol. Rev.* **54**, 18−51.
(10) Suzuki, T., Yamaoka, R., Nishi, M., Ide, H., and Makino, K. (1996) Isolation and characterization of a novel product, 2′-deoxyoxanosine, from 2′-deoxyguanosine, oligodeoxynucleotide, and calf thymus DNA treated by nitrous acid and nitric oxide. *J. Am. Chem. Soc.* **118**, 2515−2516.
(11) Suzuki, T., Yamada, M., Ide, H., Kanaori, K., Tajima, K., Morii, T., and Makino, K. (2000) Identification and characterization of a reaction product of 2′-deoxyoxanosine with glycine. *Chem. Res. Toxicol.* **13**, 227−230.
(12) Suzuki, T., Nakamura, T., Yamada, M., Ide, H., Kanaori, K., Tajima, K., Morii, T., and Makino, K. (1999) Isolation and characterization of diazoate intermediate upon nitrous acid and nitric oxide treatment of 2′-deoxycytidine. *Biochemistry* **38**, 7151−7158.
(13) Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Plainview, NY.
(14) Zollinger, H. (1994) *Diazo Chemistry I: Aromatic and Heteroaromatic Compounds*, pp 39−64, VCH, Weinheim, Germany.
(15) Williams, D. L. H. (1988) *Nitrosation*, pp 10−16, Cambridge University Press, Cambridge, U.K.
(16) Roth, E., Mühlbacher, F., Karner, J., Hamilton, G., and Funovics, J. (1987) Free amino acid levels in muscle and liver of a patient with glucagonoma syndrome. *Metabolism* **36**, 7−13.
(17) Brown, T. A. (1991) *Molecular Biology Labfax*, p 29, BIOS Scientific Publishers, Oxford, U.K.
(18) Potti, N. D., and Bello, J. (1971) Cross-linking of nucleohistone by nitrous acid. *Mutat. Res.* **12**, 113−119.
(19) Burnotte, J., and Verly, W. G. (1971) A kinetic approach to the mechanism of deoxyribonucleic acid cross-linking by HNO_2. *J. Biol. Chem.* **246**, 5914−5918.
(20) Suzuki, T., Matsumura, Y., Ide, H., Kanaori, K., Tajima, K., and Makino, K. (1997) Deglycosylation susceptibility and base-pairing stability of 2′-deoxyoxanosine in oligodeoxynucleotide. *Biochemistry* **36**, 8013−8019.

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