DNA Deaminating Ability and Genotoxicity of Nitric Oxide and its Progenitors

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Nitric oxide (NO), a multifaceted bioregulatory agent and an environmental pollutant, can also cause genomic alterations. In vitro, NO deaminated deoxynucleosides, deoxynucleotides, and intact DNA at physiological pH. That similar DNA damage can also occur in vivo was tested by treating *Salmonella typhimurium* strain TA1535 with three NO-releasing compounds, including nitroglycerin. All proved mutagenic. Observed DNA sequence changes were >99% C→T transitions in the *his*G46 (CCC) target codon, consistent with a cytosine-deamination mechanism. Because exposure to endogenously and exogenously produced NO is extensive, this mechanism may contribute to the incidence of deamination-related genetic disease and cancer.

MUCH HUMAN GENETIC DISEASE (*1–3*) and cancer (*4–6*) is thought to arise from deamination of DNA, yet "spontaneous" (hydrolytic) deamination is extremely unfavorable energetically—with a half-life for cytosine residues estimated as 30,000 years in the duplex (*7*). Certain toxic agents may catalyze this reaction and increase the incidence of the indicated disorders. One candidate is nitric oxide (NO), a cigarette smoke constituent (*8*), air pollutant (*8*), and recently discovered bioregulatory agent (*9, 10*) produced in numerous cell types in amounts that can total many millimoles per day per person (*11*). We present evidence that this widely studied bioeffector molecule can accelerate base deamination in vitro and cause deamination-induced genetic changes in the living cell.

The prediction that NO might damage DNA (*12, 13*) followed from the recognition that it can diazotize primary arylamines ($ArNH_2$) nitrosatively in an oxidizing environment (*14*) (Eq. 1). Since hydrolysis of the diazonium ion product would replace the nitrogen substituent by oxygen (Eq. 2), it was reasoned that diazotization and hydrolysis of cytosine and other DNA bases as in Eqs. 3 to 6 in vivo might lead to heritable genetic alterations.

$$ArNH_2 + NO \xrightarrow[(Ox)]{-e^-} ArN_2^+ + H_2O \quad (1)$$

$$ArN_2^+ + H_2O \rightarrow ArOH + N_2 + H^+ \quad (2)$$

$$dC + NO \xrightarrow[pH\ 7.4]{-e^-} dU + N_2 + H^+ \quad (3)$$

$$m^5dC + NO \xrightarrow[pH\ 7.4]{-e^-} dT + N_2 + H^+ \quad (4)$$

$$dG + NO \xrightarrow[pH\ 7.4]{-e^-} dX + N_2 + H^+ \quad (5)$$

$$dA + NO \xrightarrow[pH\ 7.4]{-e^-} dI + N_2 + H^+ \quad (6)$$

where Ox is oxidation, m^5dC is methyl-2′-deoxycytidine, dX is 2′-deoxyanthosine, and dI is deoxyinosine. We tested the chemical aspects of this hypothesis using the nucleoside dC (Eq. 3) as a model compound. Results are summarized in Table 1, runs 1 to 15. Deamination was negligible under anaerobic conditions (run 1) but was significant in air (run 2), showing that the reaction requires both NO and an oxidant. Percentage yield at a given NO dose was essentially independent of dC concentration (runs 2 to 4). Similar deamination levels were observed for the cytosine residues in intact DNA (run 5). Nitrite (NO_2^-) and nitrate were major by-products, typically in yields of 95 and 3%, respectively. Nitrite in the absence of NO induced negligible transformation (run 6), as expected (*15*) for neutral solutions. Nitrite and thiocyanate (SCN^-) increased NO deamination efficiency (runs 7 and 8); plots of NO dose versus amount of dU formed in runs 7 and 8 were linear (Fig. 1A), suggesting that similar deamination efficiencies would be observed at low levels of NO exposure when suitable nucleophilic catalysts are present. 5-Methylated dC was similarly deaminated (runs 9 to 11, Eq. 4), as were purine nucleosides (runs 12 and 13, Eqs. 5 and 6) and nucleotides (runs 14 and 15).

The chemical results are consistent with the mechanism of Fig. 1B, in which NO is initially oxidized to a reactive species (Y) capable of transferring an $(NO)^+$ moiety to available nucleophiles, including the bases' exocyclic amino groups. The latter N-nitro-

Table 1. NO-induced deamination of DNA and its constituents. Conditions were as described in Fig. 1A. Deoxynucleosides were determined by high-performance liquid chromatography (HPLC) with ultraviolet detection (*28*), except that the mobile phase contained 50 mM KH_2PO_4 in 3% methanol. DNA was hydrolyzed enzymatically (*29*). Deoxynucleotides were analyzed by HPLC with an Alltech Adsorbosphere Nucleoside/Nucleotide 7U column equilibrated with 50 mM KH_2PO_4 and 1.75 mM tetrabutylammonium dihydrogen phosphate (*30*). Except as indicated for runs 1 and 6, NO gas was bubbled into the aerobic reaction mixtures until 1 mol/liter was absorbed.

Run	Substrate, initial concentration	Catalyst, initial concentration	Deamination product, final concentration	Bases altered per 10^3
1*	dC, 100 mM	None	ND†	ND†
2	dC, 100 mM	None	dU, 0.5 mM	5
3	dC, 10 mM	None	dU, 0.03 mM	3
4	dC, 1 mM	None	dU, 0.004 mM	4
5	Calf thymus DNA, 2 mg/ml	None	dU	5
6‡	dC, 10 mM	NO_2^-, 0.5 M	ND	ND
7	dC, 10 mM	NO_2^-, 0.5 M	dU, 0.075 mM	7.5
8	dC, 10 mM	SCN^-, 1 M	dU, 0.8 mM	80
9	m^5dC, 10 mM	None	dT, 0.08 mM	8
10	m^5dC, 10 mM	NO_2^-, 0.5 M	dT, 0.12 mM	12
11	m^5dC, 10 mM	SCN^-, 1 M	dT, 1.0 mM	100
12	dG, 1 mM	SCN^-, 1 M	dX, 0.012 mM	12
13	dA, 1 mM	SCN^-, 1 M	dI, 0.038 mM	38
14	dC 5′-monophosphate, 100 mM	SCN^-, 1 M	dU 5′-monophosphate, 1.8 mM	18
15	dG 5′-monophosphate, 100 mM	SCN^-, 1 M	dX 5′-monophosphate, 1.3 mM	13

*An anaerobic dC solution was exposed to NO for the same length of time used in the aerobic reactions. †ND, not detected (detection limit, 0.3 nucleosides per 10^3). ‡This aerobic reaction mixture was not exposed to NO.

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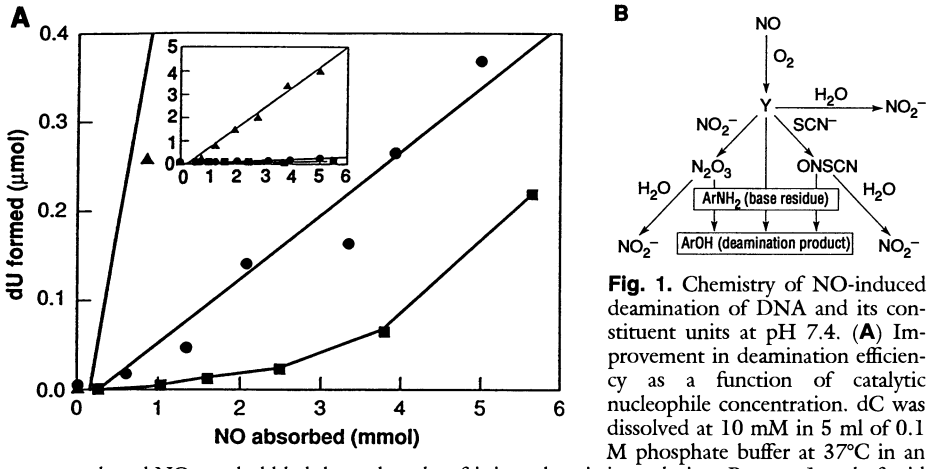

Fig. 1. Chemistry of NO-induced deamination of DNA and its constituent units at pH 7.4. (**A**) Improvement in deamination efficiency as a function of catalytic nucleophile concentration. dC was dissolved at 10 mM in 5 ml of 0.1 M phosphate buffer at 37°C in an open vessel, and NO was bubbled through a glass frit into the stirring solution. Because 1 mol of acid formed per mole of NO autoxidized in solution and the amount of NO absorbed overall was in tenfold molar excess relative to buffer, a pH-stat was used as the primary means of controlling hydrogen ion concentration. The buffer's function was to eliminate the extremes in pH that the automated titrator would otherwise have engendered. The combination held the pH in the range from 6.8 to 7.6. Progress of NO absorption was determined from the amount of base delivered by the pH-stat. dU concentration was monitored by HPLC as described in Table 1. With no added nucleophile, the plot (■) showed curvature indicating increasing efficiency of deamination as progressive NO absorption increased the nitrite concentration. Catalysis by nitrite was confirmed by repeating the reaction in the presence of added $NaNO_2$ at an initial concentration of 0.5 M (●). Even greater efficiency of NO-induced deamination was seen in the presence of 1 M KSCN (▲); the inset shows the same data with an expanded ordinate to illustrate the catalytic power of thiocyanate over a greater extent of reaction. (**B**) Proposed mechanism of NO-induced DNA deamination at physiological pH. Oxidation of NO to N_2O_3 or N_2O_4 as characterized in the gas phase does not account for the substantial increase in deamination efficiency on addition of nitrite and other features of the chemistry observed here. Rather, the nucleophile effects of Fig. 1A and the other chemical findings summarized in the text suggest that deamination is initiated by autoxidation of NO to a reactive intermediate, Y (possibly the peroxynitrite radical, ONOO·), which partitions among available nucleophilic $(NO)^+$-acceptors. Reaction with the most abundant nucleophile, water, produces $(H_2ONO)^+$, which rapidly deprotonates to NO_2^- and does not react further at this pH. Some Y may directly transfer the $(NO)^+$ moiety to the nucleoside's exocyclic amino group, producing an intermediate that on loss of water yields the diazonium ion; the latter, unstable species reacts with water to give N_2 and the deaminated base (Eqs. 3 to 6). When NO_2^- or SCN^- are present, their reactions with Y produce N_2O_3 or ONSCN, respectively; these agents play important catalytic roles in nitrous acid–induced N-nitrosation (*15*) and appear from the present work to be key intermediates in NO-induced deamination of deoxynucleosides when they are present at physiological pH.

sation results in deamination, the products of which are those seen in nitrous acid– (*16*) and nitroprusside-induced (*17*) diazotizations of deoxynucleosides. The chemical observations including catalysis by nucleophiles were those expected of nitrite-induced nitrosation, diazotization, and deamination reactions (*15*), with the critical difference that the transformations of Fig. 1 proceeded smoothly at pH 7.4. In this respect, NO-induced base changes represent a novel mechanism of DNA damage, chemically distinct from previously described hydrolytic [catalyzed (*18*) or spontaneous (7)] and nitrite-induced (*16, 18*) DNA deamination routes.

To confirm that small concentrations of dissolved NO can induce deamination similar to that observed with NO gas, we exposed 0.2 M dC to $Na(O_2N_2\text{-}NEt_2)$, a compound whose utility for the controlled biological delivery of NO has recently been illustrated (*19*). The reaction was run at 37°C and physiological pH in 1 M phosphate buffer containing 1 M KSCN. Deamination occurred to the extent of 0.8 nucleosides per thousand when a total of 0.1 mol of $Na(O_2N_2\text{-}NEt_2)$ per liter was added in aliquots such that the solubility limit of NO was not exceeded.

The possibility that such nitrosative deamination could cause genetic alterations in living cells was addressed by exposing *S. typhimurium* strain TA1535 (*rfa, ΔuvrB, his*G46) (*20*) to the same compound. The $Na(O_2N_2\text{-}NEt_2)$ was more mutagenic than nitrite (Fig. 2). Nitroglycerin, a therapeutic agent that generates NO metabolically (*10*), was also weakly mutagenic in TA1535 (Fig. 2). The spermine-NO complex, a second compound developed for the spontaneous biological release of NO (*19*), gave the highest mutant yields (Fig. 2). Base substitutions arising from treatment with this compound were characterized with an oligonucleotide probing method (*21*) (Table 2). Virtually all of the mutants analyzed (113 out of 114) contained C→T transitions.

Induction of C→T mutations is consistent with the predicted deaminative mechanism. Deamination of unmethylated cytosine residues produces uracil (Eq. 3). Removal of uracil by uracil glycosylase (*22*) without restoration of cytosine would leave an abasic site, a lesion commonly misrepaired by insertion of adenine opposite the site during replication (*23*). Misrepair would produce the observed G:C→A:T transition. If uracil is not removed, it could pair with incoming adenine to give the same mutation on replication (*22*). If the cytosine is methylated, deamination would yield thy-

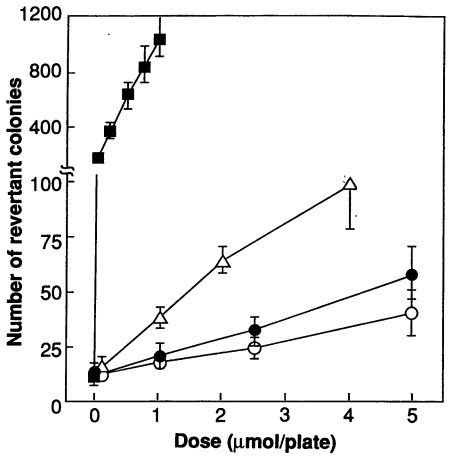

Fig. 2. Histidine reversion of *S. typhimurium* TA1535 following exposure to nitroglycerin (●), a nitrite control (○), $Na(O_2N_2\text{-}NEt_2)$ (△), and the spermine-NO complex (■). Aliquots in 0.1 ml of dimethylsulfoxide (for nitroglycerin) or 1 mM NaOH (for the remaining test agents) were applied to standard mutagenicity test plates (*20*). The nitroglycerin sample was 10.1% nitroglycerin on lactose, USP. Lactose and dimethylsulfoxide at doses comparable to those present in the nitroglycerin solutions yielded typical TA1535 background levels of 10 to 15 revertant colonies per plate. Values are means ± SD; $n = 4$ for $Na(O_2N_2\text{-}NEt_2)$ and the nitrite control, $n = 15$ for the spermine-NO complex, and $n = 6$ for nitroglycerin.

Table 2. Sequence changes in His^+ revertants of *S. typhimurium* TA1535 on treatment with an NO-releasing compound. The spermine-NO test agent was synthesized by reacting spermine with NO to form the adduct, $H_2N\text{-}(CH_2)_3\text{-}NH_2^+\text{-}(CH_2)_4\text{-}N[N(O)NO]^-\text{-}(CH_2)_3\text{-}NH_2$ (*19*). From a spermine-NO treatment plate (0.1 μmol per plate) (*20*) containing 202 *S. typhimurium* colonies (induction factor, 18 times background), 114 colonies were isolated. Control plates contained an average of 11 colonies per plate; most of these (68% of 111 analyzed) contained CTC mutations. All six classes of mutations listed were recovered among the controls. Sequence determination was by colony hybridization (*21*).

Number (%) with codon change (CCC→)					
CTC	TCC	ACC	CAC	GCC	CCC*
90(79)	23(20)	0	1(1)	0	0

*Suppressor (sequence change not in triplet).

mine directly (Eq. 4) (*24*). This change is especially significant genetically in mammalian DNA (*1–6*), whose methylation patterns are critical to gene regulation and differentiation (*25*).

The results show that NO induces a mutation in bacteria that in people has been linked to a variety of disorders suspected to result from base deamination. The list of conditions in which such G:C→A:T transitions have been implicated includes hemophilia (*2*), familial Alzheimer's disease (*3*), colon cancer (*5*), retinoblastoma (*6*), and Gerstmann-Sträussler syndrome (*26*), to name just a few.

Nitrosative deamination of DNA by NO may represent an important mechanism of genomic alteration. Given the numerous sources of human exposure to endogenously as well as exogenously produced nitric oxide (*8–11, 14*), and recent evidence for NO-induced DNA damage in mammalian cell lines (*12, 27*), this pathway's possible involvement in the above-mentioned and other genetic changes thought to arise from deamination of nucleic acids should be well-worth exploring.

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24. This lesion is responsible for certain mutational hotspots in *Escherichia coli* [C. Coulondre, J. H. Miller, P. J. Farabaugh, W. Gilbert, *Nature* **274**, 775 (1978)]; G:T mismatch repair enzymes exist, but are not 100% efficient [T. C. Brown and J. Jiricny, *Cell* **50**, 945 (1987)]. Two of many other mechanisms by which NO might conceivably cause G:C→A:T transitions include deamination of dG (Eq. 5), an apparently nonreparable lesion [T. Lindahl, *Mutat. Res.* **238**, 305 (1990)], and formation of nitrosamine carcinogens [C. D. Leaf, J. S. Wishnok, S. R. Tannenbaum, *Carcinogenesis* **12**, 537 (1991)] that can O^6-methylate guanine [see S. Sukumar, V. Notario, D. Martin-Zanca, M. Barbacid, *Nature* **306**, 658 (1983)].
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Prediction of a Crystallization Pathway for Z-DNA Hexanucleotides

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Crystallization of macromolecules for structural studies has long been a hit-or-miss process. The crystallization of hexanucleotides as Z-DNA was studied, and it was shown that the cation concentration for crystal formation could be predicted from solvation free energy (SFE) calculations. Solution studies on the conformation and solubilities of the hexanucleotides showed that a critical concentration of the DNA in the Z-conformation must be present in solution to effect crystallization. The SFE calculations therefore predict the propensity of the hexanucleotides to adopt the left-handed conformation and the driving force required to reach this critical concentration relative to the intrinsic solubility of Z-DNA for crystallization.

SINGLE-CRYSTAL STUDIES ON OLIGONUCLEOTIDES have provided a wealth of structural information on the currently recognized forms of DNA and RNA and have the potential to expand our understanding of other important conformations. The general application of x-ray diffraction is limited to cases where diffraction-quality single crystals can be obtained. The "science" of crystal growth has, at best, been a hit-or-miss proposition.

The crystallization of polynucleotides is further complicated by a number of factors. The dense packing of DNA and RNA into a crystal necessarily requires high salt concentrations to counterbalance the negatively charged phosphate backbone. At least for DNA, varying the ionic condition also affects the conformation of the molecule in solution. DNA structures are of interest because of their high degree of polymorphism and because sequence and environment can affect their conformations. These same factors, however, significantly hinder DNA crystallization by introducing a high degree of conformational heterogeneity to the oligomers in solution. In this report, we describe studies that address the critical steps in the crystallization of hexanucleotides as Z-DNA, including the stabilization of the Z-conformation under solutions that are relevant to crystallization and the intrinsic solubility of the Z-conformation at these conditions. From this, a pathway is predicted that allowed us to crystallize the previously unstudied sequence d(CICGCG) as Z-DNA.

In order to gain insight into the factors that stabilize Z-DNA at the molecular level, the structures for a number of hexanucleotides have been studied by x-ray diffraction at atomic resolution (*1*). The crystals of these hexanucleotides are all isomorphous,

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