Articles

Competition between Anaerobic Covalent Linkage of Neocarzinostatin Chromophore to Deoxyribose in DNA and Oxygen-Dependent Strand Breakage and Base Release†

Lawrence F. Povirk and Irving H. Goldberg*

ABSTRACT: Treatment of poly(dA-dT)·poly(dA-dT) with the nonprotein chromophore of neocarzinostatin in the presence of sulfhydryls resulted in both direct and alkali-dependent base release, indicative of DNA sugar oxidation. Covalent chromophore–DNA adducts were also formed. Under anaerobic conditions, base release was strongly inhibited; however, adduct formation was not inhibited and in some cases was markedly enhanced. In the presence of dithiothreitol, anoxia increased adduct formation by a factor of 2, and a particularly stable adduct species was formed, which was recovered from nuclease digests of the treated DNA as a highly fluorescent compound with structure chromophore–d(TpApT). Acid hydrolysis of chromophore–d(TpApT) released free adenine base and both 3′dTMP and 5′dTMP, leaving a compound that contained only chromophore and the deoxyadenosine sugar. These results conclusively confirm that the chromophore forms a covalent adduct with deoxyribose in DNA. Thus, even in the absence of oxygen, activation of the chromophore by sulfhydryls results in the formation of a species capable of reacting with deoxyribose. Several other adduct species were also formed, some of which were nonfluorescent and relatively hydrophilic, but all of which were produced in increased amounts under anoxia. This inverse relation between sugar oxidation and adduct formation suggests that the two lesions share a common precursor. In the presence of other thiols, the effects of anoxia were somewhat different. With glutathione, anoxia markedly enhanced adduct formation, but the total adduct formed was considerably less than with dithiothreitol. With 2-mercaptoethanol, anoxia had no effect on total adduct formation, but the distribution of adduct species was altered. In particular, the formation of a very labile form of adduct, previously shown to contain an oxidized deoxyribose, was markedly inhibited by anoxia.

Neocarzinostatin is an antibiotic consisting of a protein (M_r 10 700) (Meienhofer et al., 1972) and a tightly but noncovalently bound chromophore (Kappen et al., 1980) with empirical formula $C_{35}H_{33}NO_{12}$, whose structure is known only in part (Hensens et al., 1983). The predominant lesions found in DNA treated with the nonprotein chromophore of neocarzinostatin in the presence of sulfhydryls are strand breaks, resulting from oxidation of thymidine sugar moieties to thymidine 5′-aldehyde (Kappen et al., 1982; Kappen & Goldberg, 1983). Somewhat smaller amounts of free thymine base are also released, presumably also resulting from some as yet uncharacterized form of sugar damage. Although formation of both these lesions is greatly inhibited under anaerobic conditions (Kappen & Goldberg, 1984), the role of oxygen in the reaction is uncertain. In particular, it is not known whether oxygen is involved in the generation of the reactive species that initially attacks deoxyribose or in the conversion of nascent DNA lesions (e.g., carbon-centered deoxyribose radicals) to an irreversibly oxidized form. Kinetic and stoichiometric studies of oxygen uptake (Povirk & Goldberg, 1983) have failed to resolve this question.

Minor products of the reaction between chromophore and DNA are chromophore–DNA adducts (Povirk & Goldberg, 1982a). When 2-mercaptoethanol was used as sulfhydryl cofactor, the predominant adduct formed in poly(dA-dT)·poly(dA-dT) [hereafter referred to as poly(dA-dT)][1] was a highly labile species, which was isolated from nuclease digests as chromophore–d(TpApT). At neutral pH, this compound decomposed, releasing simultaneously chromophore, 3′dTMP, and a modified d(ApT) containing deoxyadenosine 5′-aldehyde (Povirk & Goldberg, 1982b). Formation of these hydrolysis products was taken as indirect evidence for a covalent linkage of the chromophore to the oxidized C-5′ of deoxyadenosine.

When dithiothreitol was used as a cofactor, a similar but much more stable species, again with the structure chromophore–d(TpApT), was isolated, which had sufficiently different chemical properties that we chose to examine it in detail. Rather surprisingly, we find formation of this adduct to be markedly enhanced under anaerobic conditions. In addition, the increased stability of this adduct has allowed conclusive localization of the covalent linkage to the deoxyribose moiety in DNA.

Experimental Procedures

Materials. Radiolabeled poly(dA-dT) was prepared as described by Marshall et al. (1981). [*methyl*-^{3}H]dTTP and [8-^{3}H]dATP were from ICN. [U-^{14}C]dATP was from Amersham. [^{32}P]Poly(dA-dT) was a gift of Dr. Robert L. Charnas. Poly(dA-dT) concentrations are expressed as total moles of phosphate per liter.

The nonprotein chromophore of neocarzinostatin was obtained by methanol extraction of dialyzed clinical neocarzinostatin as described (Povirk et al., 1981). The yield was

† From the Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115. *Received May 8, 1984.* This work was supported by a U.S. Public Health Service research grant (GM12573) from the National Institutes of Health and an award from the Bristol-Myers Co.

[1] Abbreviations: poly(dA-dT), the double-stranded alternating copolymer poly(dA-dT)·poly(dA-dT); HPLC, high-pressure liquid chromatography; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/84/0423-6304$01.50/0 © 1984 American Chemical Society

increased slightly by increasing the extraction time to 4 h. Chromophore stock solutions were stored at −70 °C and contained 0.30 mM chromophore and 16 mM sodium citrate, pH 4, in 80% CH_3OH/20% H_2O.

[*methyl*-^{3}H]Chromophore was prepared biosynthetically. *Streptomyces carzinostaticus* var. F-41 was grown as described by Ishida et al. (1965) but with addition of [*methyl*-^{3}H]-methionine (20 μCi/mL, 1 Ci/mmol; New England Nuclear) to the production medium. The bacteria were removed by centrifugation, and neocarzinostatin was precipitated from the supernatant with ammonium sulfate (Ishida et al., 1965) and dissolved in 20 mM sodium citrate, pH 4. [*methyl*-^{3}H]-Chromophore (0.12 mM, 20 Ci/mol) was extracted as above and had a radiochemical purity greater than 95%. Hydrolysis experiments (J.-L. Giner and I. H. Goldberg, unpublished data) were consistent with the expected presence of approximately equal amounts of [^{3}H]methyl label in the methylamino group of the chromophore sugar moiety and in the methoxy group of the naphthoic acid moiety. All other chromophore methyl groups appear to be derived from acetate condensation.

Anaerobic Reactions. The methanolic chromophore solution was added to an aqueous solution of poly(dA-dT) to give final concentrations of 20% methanol, 1 mM poly(dA-dT), 60 μM chromophore, 3 mM EDTA, and 6 mM sodium citrate, pH 4. The solution, usually 0.4 mL, was lyophilized to remove methanol, dissolved in 0.38 mL of water, and loaded into a Thunberg cuvette (Precision Cells type 26FLS). Ten microliters of concentrated sulfhydryls was added to the solution at 0 °C, and 10 μL of 2.5 M Tris-HCl, pH 8, was placed in the side arm. The cell was then degassed by aspiration for 8 s and filled with argon (oxygen-free grade, <0.5 ppm of O_2), air, or oxygen under slight positive pressure for 8 s. The degassing procedure was repeated 10 times at ambient temperature with constant agitation. The stopcock was then closed, and the reactants were mixed and incubated at 22 °C for 20 min. The concentrated Tris-HCl buffer raises the pH of the solution to 7.8, initiating the activation of the chromophore by sulfhydryls (Povirk & Goldberg, 1983). Because of the known photosensitivity of neocarzinostatin, exposure of chromophore-containing solutions to fluorescent lighting was avoided and all manipulations were performed under dim light.

Base Release. Samples of the above reaction mixtures (50 μL) containing approximately 200 000 cpm were either added to 14 μL of water and kept on ice or added to 7 μL of 1 N NaOH, heated at 90 °C for 30 min, and then neutralized with 7 μL of 1 N HCl. Two 5-μL aliquots of each sample were added to 0.3 mL of water and used for the determination of total radioactivity in the sample. In the case of the [8-^{3}H]-adenine label, these aliquots were lyophilized twice to remove [^{3}H]water formed by slow exchange of the C-8 proton during alkaline treatment (approximately 20% of the total label). The remainder of each sample was subjected to paper chromatography (Povirk et al., 1978) for determination of base release.

Isolation of Chromophore Adducts. Two-tenths milliliter of the above reaction mixtures was diluted with 0.44 mL of 3 M sodium acetate and 0.68 mL of water. To remove noncovalently bound chromophore, the poly(dA-dT) was precipitated with 3.3 mL of ethanol (10 min, −20 °C), centrifuged at 8000*g* for 15 min, washed with 70% ethanol, and redissolved in 0.6 mL of 0.15 M sodium acetate–5 mM EDTA, pH 5. DNase II (100 Kunitz units of Sigma type V) was added, and the solution was incubated at 37 °C for 4 h. Forty microliters of 100 mM $ZnCl_2$ and 30 000 units of endonuclease S1 (Miles) were added, and the incubation was continued for 16 h. Potato

Table I: Effect of Oxygen on Base Release and Adduct Formation in Chromophore-Treated Poly(dA-dT)[a]

	atmosphere		
	argon	air	oxygen
thymine release (%)[b]			
direct	0.05	0.51	0.65
alkali dependent[c]	0.25	1.44	2.04
adenine release (%)[b]			
direct	0.04	0.68	0.99
alkali dependent[c]	0.26	1.74	3.10
total base release per chromophore (%)[d]	4.9	36.4	56.5
ethanol-precipitable [^{3}H]chromophore (%)	35.2	16.8	7.9

[a] [^{3}H]Thymine- or [^{3}H]adenine-labeled poly(dA-dT) (1 mM) was treated under a controlled atmosphere with 60 μM chromophore in the presence of 1 mM dithiothreitol and in some cases subsequently exposed to alkali. Base release was then determined by paper chromatography. Parallel experiments were performed with unlabeled poly(dA-dT) and [*methyl*-^{3}H]chromophore, the poly(dA-dT) was precipitated, and radioactivity in the precipitate and in the supernatant was assayed. [b] As a percent of total [^{3}H]thymine or [^{3}H]adenine label. Base release seen in the absence of chromophore (less than 0.03% in all cases) has been subtracted. [c] Base release after subsequent alkaline treatment minus base release with no alkaline treatment. [d] Total moles of base released divided by total moles of chromophore added.

acid phosphatase (20 μg of Sigma type III) was desalted by centrifugation (Maniatis et al., 1982) and added to the digestion mixture, which was incubated for an additional 6 h. In some cases a preparation of endonuclease Sl (Calbiochem, 0.8 IU per sample) that contained high levels of phosphatase activity was used, and the acid phosphatase treatment was omitted. Essentially all the poly(dA-dT) (excluding chromophore adduct species) was digested to the level of single nucleosides (Povirk & Goldberg, 1982a).

Digestion mixtures were filtered through glass wool and subjected to high-pressure liquid chromatography (HPLC) on a Waters μBondapak C_{18} column preceded by a 2-cm guard column filled with Porasil C_{18}. A convex gradient of methanol in water, both containing 5 mM ammonium acetate and 5 mM acetic acid, was applied to the column at 1 mL/min, and absorbance (254 nm) and fluorescence (excitation at 340 nm, emission at >418 nm) were monitored. In order that fluorescence chromatograms from various experiments could be compared, the sensitivity of the fluorometer (Schoeffel type 970FS) was adjusted with the calibrated range control, while the sensitivity setting was kept constant. In-line absorbance spectra were recorded with a Perkin-Elmer SC85 spectrophotometer. Radioactivity of eluate fractions was assayed in Hydrofluor (National Diagnostics).

Acid Hydrolysis. Aliquots of certain adduct-containing eluate fractions, usually 0.1 mL, were added directly to 0.408 mL of 1 N HCl containing 0.2 A_{260} units of authentic d-(TpApT) (Collaborative Research) and heated at 90 °C in glass screw-capped tubes. The samples were neutralized by addition of 0.25 mL of 3 M sodium acetate and subjected to HPLC as described above, except that a Rainin Microsorb C_{18} column and a linear methanol gradient were used.

Results

Enhanced Production of Chromophore–DNA Adducts under Anoxia in the Presence of Dithiothreitol. Treatment of poly(dA-dT) with chromophore in the presence of dithiothreitol under aerobic conditions resulted in release of both adenine and thymine free base. In both cases, most of the release was dependent on subsequent alkaline treatment, and both direct and alkali-dependent release were severely inhibited under anoxia (Table I). These results are similar to those obtained by Kappen & Goldberg (1984), using various other sulfhydryl compounds and DNA from λ phage. Kappen &

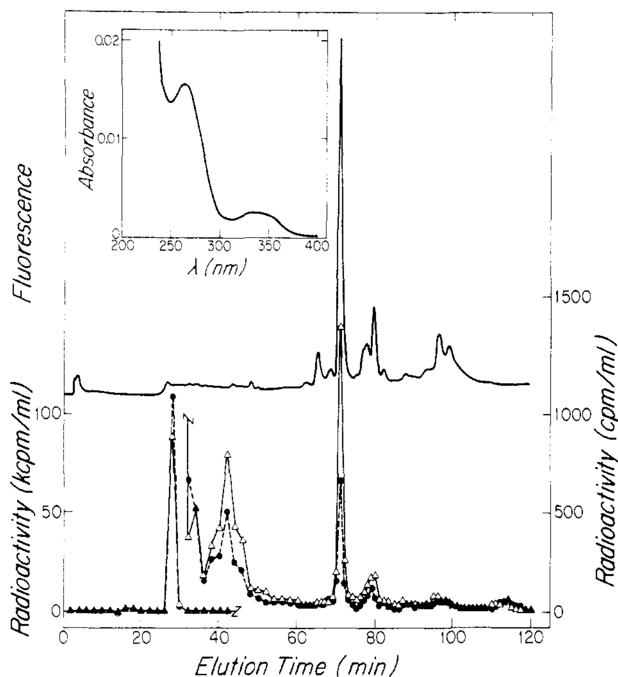


FIGURE 1: HPLC analysis of chromophore–poly(dA-dT) adducts formed in the presence of dithiothreitol. [^{3}H]Thymine-labeled poly(dA-dT) was treated with chromophore in the presence of 1 mM dithiothreitol under aerobic (●) or anaerobic (Δ) conditions, precipitated with ethanol, digested with DNase II, endonuclease S1, and phosphatase, and subjected to HPLC. Radioactivity in 0.5-mL aliquots of 2-mL fractions (0–60-min elution time) or in 1-mL fractions (60–120-min elution time) was assayed, corrected for the difference in counting efficienceis, and expressed as counts per minute per milliliter. The fluorescence profile shown (0.5-μA sensitivity) is that from the aerobic reaction; the profile from the anaerobic reaction was similar but 2-fold greater in magnitude. The elution gradient of methanol in water is shown in Figure 2. Inset: Absorbance profile of the major fluorescent adduct species (anaerobic reaction) eluting at 70 min, recorded on an in-line spectrophotometer.

Goldberg (1983) have also shown that the predominant lesions formed in DNA are nucleoside 5′-aldehydes (necessarily accompanied by phosphodiester cleavage) and that hydrolysis of these lesions accounts for most or all of the alkali-dependent base release. The direct (alkali-independent) base release presumably also results from some as yet uncharacterized form of sugar oxidation. Thus, as with natural DNA, chromophore-induced oxidation of deoxyribose in poly(dA-dT) was found to be strongly oxygen dependent.

By contrast, in reactions performed under identical conditions, irreversible binding of [^{3}H]chromophore to poly(dA-dT) was found to be markedly enhanced by anoxia. Conversely, when reactions were performed under 100% oxygen, base release was significantly enhanced, compared to the aerobic reaction, while adduct formation was inhibited (Table I).

Enhanced production of chromophore–poly(dA-dT) adducts under anoxia was confirmed when adducts were examined by enzymatic digestion, followed by HPLC. The elution profile (Figure 1) was dominated by a single adduct species, eluting at 70 min, which contained thymine radiolabel as well as the majority of the fluorescence attributable to the naphthalene rings of the chromophore. The absorbance spectrum also showed the characteristic broad naphthalene absorbance band (Napier & Goldberg, 1983) centered at 340 nm. In agreement with results presented above, formation of this adduct, as well as a minor adduct species eluting at 80 min, was increased by a factor of approximately 2 under anaerobic conditions. When poly(dA-dT) was digested without precipitation, virtually identical radioactivity profiles were obtained, thus eliminating

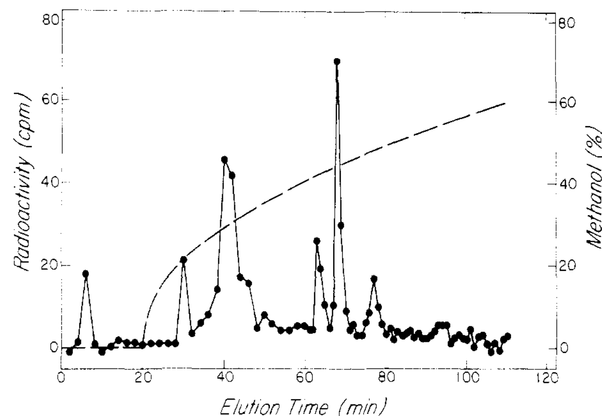


FIGURE 2: Isolation by HPLC of [^{3}H]chromophore-labeled adduct. Poly(dA-dT) (0.5 mM) was treated with [^{3}H]chromophore (60 μM) in the presence of 1 mM dithiothreitol, precipitated, enzymatically digested, and subjected to HPLC with a convex methanol gradient. Aliquots (0.1 mL) of 2-mL fractions (0–60 min) or 1-mL fractions (60–120 min) were removed and assayed for radioactivity. Background of 8 cpm has been subtracted.

the possibility that short fragments of adduct-containing poly(dA-dT) were lost in the precipitation. Under a 100% oxygen atmosphere, the formation of both fluorescent adduct species was inhibited by a factor of approximately 6, compared to the aerobic reaction (not shown).

The major fluorescent peak (eluting at 70 min) proved to be a highly stable species with a structure chromophore–d-(TpApT) (see below) and was examined in detail. The minor peak eluting at 80 min appeared from the fluorescence chromatogram to be heterogeneous and was not further characterized.

There was an additional rather broad [^{3}H]thymine-containing peak eluting at 40–50 min, whose magnitude, like that of the fluorescent peak, was increased 2-fold under anaerobic conditions. This peak coincided with a major peak of [^{3}H]-chromophore radioactivity seen in enzyme digests of ethanol-precipitated [^{3}H]chromophore-treated poly(dA-dT) (Figure 2). Although this nonfluorescent peak accounted for a greater proportion of thymine label than the major fluorescent peak, chromatography on a linear 2-h, 0–70% methanol gradient revealed that it was quite heterogeneous. Four discrete components were resolved. When [U-^{14}C]deoxyadenosine-labeled poly(dA-dT) was treated with [*methyl*-^{3}H]chromophore, it was found that all four components contained both chromophore and deoxyadenosine radiolabel (not shown). Thus, although the structure of these compounds was not further examined, it is clear that each is some form of chromophore–poly(dA-dT) adduct. The great majority of ethanol-precipitable [^{3}H]chromophore radioactivity (approximately 80%) can therefore be unambiguously ascribed to the various covalent adduct species (Figure 2).

In fact, ethanol-precipitable [^{3}H]chromophore radioactivity probably provides an underestimate of adduct formation, since the adducts eluting at 40–50 min appear to have lost the naphthoic acid moiety, which contains one of the two ^{3}H-labeled methyl groups in the chromophore. Loss of naphthoic acid from these species is suggested by (i) their nonfluorescence, (ii) their early elution time, and (iii) the presence of large amounts of a compound that eluted in the position of the free naphthoate derivative of the chromophore, in unprecipitated, enzyme-digested reaction mixtures (not shown). If this factor is taken into account, the estimate of adduct formation under anaerobic conditions (Table I) is increased from 35% to 48%, or nearly equal to the total base release seen under 100% oxygen. Thus, as the oxygen tension is decreased,

the decrease in base release is accompanied by a comparable (although not quite equal) increase in adduct formation. This inverse relationship suggests two competing reaction pathways, both of which begin with the formation of a common dithiothreitol-activated chromophore: an oxygen-dependent pathway leading to DNA sugar oxidation and an oxygen-independent pathway leading to adduct formation.

With [^{3}H]thymine-labeled *Escherichia coli* DNA, adduct formation was likewise increased by a factor of 2 under anoxia in the presence of dithiothreitol. Both nonfluorescent, early-eluting adduct species and fluorescent, late-eluting species were present. However, the HPLC profiles were quite complex, having no single predominant peak (not shown).

Characterization of the Major Adduct Formed in the Presence of Dithiothreitol. When dithiothreitol was used as the reducing agent, the predominant adduct species found in nuclease digests of chromophore-treated poly(dA-dT) was the highly fluorescent species eluting at 70 min and accounting, in the case of the anaerobic reaction, for approximately 1% of the total thymine radiolabel (Figure 1). This compound was found to contain proportionally twice as much thymine as adenine radiolabel, suggesting a structure chromophore–d(TpApT), analogous to the compound recovered from nuclease digests of poly(dA-dT) treated with chromophore in the presence of 2-mercaptoethanol (Povirk & Goldberg, 1982a,b).

In order to examine the structure of chromophore–d(TpApT) formed in the presence of dithiothreitol, it was prepared with six different radiolabels: [*methyl*-^{3}H]thymine base label, [8-^{3}H]adenine base label, [U-^{14}C]deoxyadenosine (base plus sugar) label, [5′-^{32}P]dAMP label, [5′-^{32}P]dTMP label, and [*methyl*-^{3}H]chromophore label. With the exception of the chromophore-labeled material, these were all prepared by synthesizing poly(dA-dT) with the appropriate labeled nucleotides and isolating chromophore–d(TpApT) by HPLC (Figure 1). In order to increase the yield of radiolabeled adduct, the poly(dA-dT) concentration was reduced to 0.5 mM, with the chromophore concentration kept at 60 μM. Under these conditions the major peak contained approximately 0.7% of the total [8-^{3}H]adenine, [U-^{14}C]deoxyadenosine, [5′-^{32}P]dAMP, and [5′-^{32}P]dTMP labels, and 1.3% of the [*methyl*-^{3}H]thymine label, further confirming its structure as chromophore–d(TpApT). This species accounted for 22% of the ethanol-precipitable [^{3}H]chromophore radiolabel (Figure 2).

Treatment of chromophore–d(TpApT) with snake venom phosphodiesterase (5 μg/mL of Sigma type VII) in 55 mM Tris, pH 8.5–5 mM $MgCl_2$ for 5 h at 37 °C released half the thymine label as dTMP, leaving an adduct [presumably chromophore–d(TpA)] that eluted even later than the starting material and that contained the remaining thymine label and all of the adenine label (not shown).

The most definitive information on the structure of chromophore–d(TpApT) was obtained by treatment with hot acid, followed by HPLC (Figure 3). Without hydrolysis, all radiolabels (including both ^{32}P labels, not shown) coeluted with a single asymmetric fluorescent peak at 62 min. After a 5-min hydrolysis, this compound was largely depurinated. Eighty-two percent of the [^{3}H]adenine label (Figure 3A) and 40% of the [U-^{14}C]deoxyadenosine label (not shown) were released as free adenine base. Depurination, however, had little effect on the chromatographic properties of the remaining adduct [presumably chromophore–d(Tp-ribose-pT)], other than a broadening of the peak. All of the [^{3}H]thymine label, 60% of the [U-^{14}C]deoxyadenosine label, and nearly all of the fluorescence coeluted near the position of the starting material.

After 2 h of acid hydrolysis, nearly all of the phosphate and thymine base label was released from the adduct, eluting mainly as dTMP (Figure 3B,C). As expected (Kochetkov & Budovskii, 1972), some of the dTMP (approximately 20%) was hydrolyzed to release free thymine; a corresponding portion of both ^{32}P labels (presumably inorganic phosphate) eluted in the solvent front. The highly fluorescent adduct peak disappeared, and a new, much sharper but less intense fluorescent peak appeared at 80 min, coeluting with a new major peak of [^{3}H]chromophore label. Slightly more than half of the [U-^{14}C]deoxyadenosine label eluted in the position of free adenine, while 24% coeluted with the major peak of both fluorescence and [^{3}H]chromophore label. No ^{32}P label or [^{3}H]thymine label eluted in this position. Since the [8-^{3}H]-adenine label was quantitatively released as the free base, all of the ^{14}C radioactivity eluting at 80 min can be attributed to the deoxyadenosine sugar moiety, from which all other DNA moieties have been removed.

The major coincident peak of ^{3}H and ^{14}C radioactivity at 80 min appeared to be broader than the major fluorescent peak, possibly encompassing several minor fluorescent peaks eluting near the same position. However, chromatography with a more shallow methanol gradient clearly confirmed the coincidence of chromophore fluorescence and ^{14}C radiolabel. None of the ^{14}C label eluted at the position (10–15 min) of the sugar degradation products derived from hydrolysis of unmodified [U-^{14}C]deoxyadenosine-labeled poly(dA-dT) (not shown), suggesting that all of the isolated adduct (even if it is heterogeneous) contains a modified deoxyadenosine sugar. However, the minor hydrolysis products may result from multiple hydrolysis pathways rather than heterogeneity of the starting material. Since the major peak of radioactivity eluting at 80 min contained 49% of the chromophore label and 48% (24%/50%) of the deoxyadenosine sugar label, it seems likely that all five sugar carbons have been retained in this hydrolysis product. In any case, as shown schematically in Figure 4, the formation of a species containing both chromophore and a radiolabeled DNA sugar, but devoid of all other DNA moieties, conclusively shows that the chromophore forms a covalent linkage to deoxyribose in DNA.

Authentic d(TpApT), included in each sample as a marker, was also depurinated and then hydrolyzed by the same acid treatments, releasing ultraviolet-absorbing material that eluted as adenine, dTMP, and thymine. Thus, chromophore–d(TpApT) is not notably more acid labile than the unmodified marker. Similarly, alkaline treatment (0.3 M NaOH, 90 °C, 30 min) did not release free adenine or hydrolyze any of the phosphodiester bonds in either the adduct or the unmodified marker, although the elution time of the adduct was shifted from 60 to 50 min. When either d(TpApT) or chromophore–d(TpApT) was depurinated as described above and then subjected to alkali, most of the thymine label was released as dTMP. In summary, the stability of the chromophore-modified deoxyribose in chromophore–d(TpApT) is not notably different from that of unmodified deoxyribose in DNA. Furthermore, the chromophore–deoxyribose linkage is itself quite stable.

Effect of Anoxia on Adduct Formation in the Presence of Other Thiols. When thiols other than dithiothreitol were used for chromophore activation, considerably less total adduct was formed, as judged by coelution of [^{3}H]thymine label and fluorescence on HPLC (Figure 5, Table II). The [^{3}H]thymine-labeled peak eluting at 40–50 min (Figure 1) was also much less prominent with the other thiols than with dithiothreitol (not shown). Since the other thiols were less effective

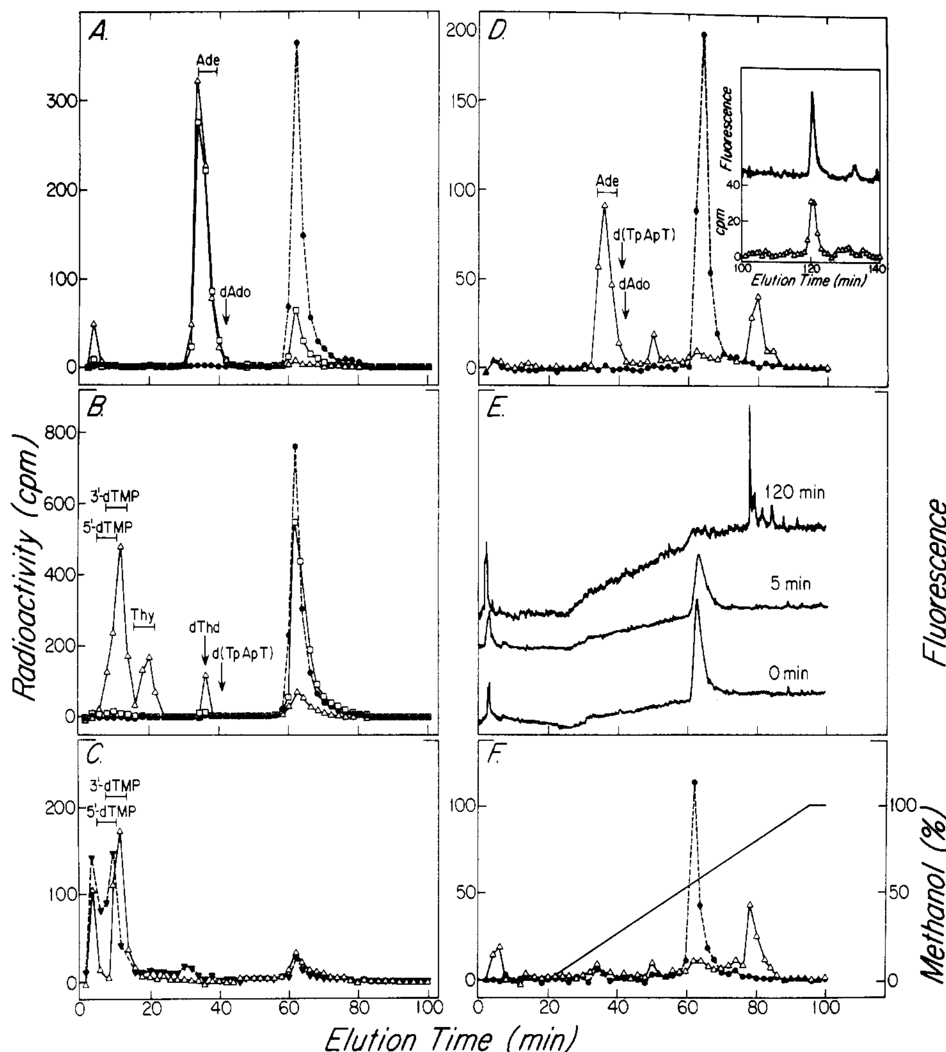


FIGURE 3: Acid hydrolysis of chromophore–d(TpApT). The major fluorescent adduct species formed in the presence of dithiothreitol was prepared with various radiolabels (as in Figure 1) and subjected to acid hydrolysis in 0.8 N HCl at 90 °C for 0 (●), 5 (□), or 120 (△, ▼) min, and the products were analyzed by HPLC. (A) [8-^{3}H]Adenine label, (B) [*methyl*-^{3}H]thymine label, (C) [5′-^{32}P]dTMP (▼) or [5′-^{32}P]dAMP (△) label, (D) [U-^{14}C]deoxyadenosine label, (E) fluorescence profiles from the [^{3}H]adenine samples with the indicated hydrolysis times, and (F) [*methyl*-^{3}H]chromophore label. The fluorometer sensitivity was set at 0.1 μA for the 0- and 5-min samples and at 0.05 μA for the 120-min sample. (Inset in D) [U-^{14}C]Deoxyadenosine-labeled adduct was hydrolyzed for 120 min and subjected to HPLC but with a convex 2-h 0–70% methanol gradient (as in Figure 2); the methanol concentration was 62% at 100 min and 70% at 140 min.

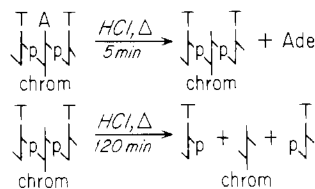


FIGURE 4: Schematic representation of acid hydrolysis of chromophore–d(TpApT). Treatment of chromophore–d(TpApT) with hot acid results in release of adenine free base, followed by hydrolysis of both phosphoester bonds of the depurinated sugar to release 3′dTMP and 5′dTMP. The compound that remains contains only chromophore and the deoxyadenosine sugar moiety, implying a covalent linkage of chromophore to deoxyribose.

than dithiothreitol in chromophore activation, somewhat higher concentrations were used, and it is possible that the high thiol concentrations had a quenching effect on adduct formation. However, there were also qualitative changes in the nature of the adducts formed. With both 2-mercaptoethanol and glutathione, highly complex HPLC elution profiles were obtained, suggesting either multiple forms of adduct or incomplete digestion of adduct-containing poly(dA-dT).

Nevertheless, with glutathione, as with dithiothreitol, anoxia clearly resulted in a dramatic increase in adduct formation.

Table II: Effect of Oxygen on Adduct Formation in the Presence of Various Thiols[a]

reducing agent	atmosphere	[^{3}H]thymine eluting as adduct (%)
dithiothreitol (1 mM)	air	0.76
	argon	1.76
2-mercaptoethanol (10 mM)	air	0.25
	argon	0.25
glutathione (5 mM)	air	0.11
	argon	0.79

[a] Poly(dA-dT) (1 mM) was treated with 60 μM chromophore under either aerobic or anaerobic conditions, and the fraction of [^{3}H]thymine label that coeluted with chromophore fluorescence was determined (Figures 1 and 5). The different concentrations of the sulfhydryl reagents reflect their differential ability in activating the chromophore (Kappen & Goldberg, 1979).

In fact, from the HPLC profiles, some adduct species seemed to be enhanced by at least a factor of 10.

In contrast, anoxia had little effect on total adduct formation in the presence of 2-mercaptoethanol, although there was a change in the elution profile of the fluorescent adducts. In particular, the peak eluting at 86 min was strongly inhibited under anoxia. This result is significant, since this adduct

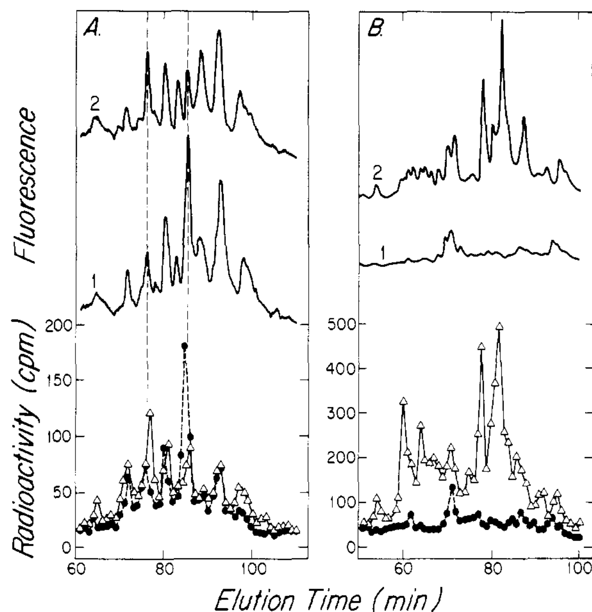


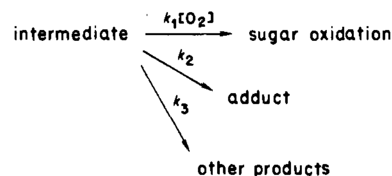
FIGURE 5: Effect of oxygen on formation of chromophore-poly(dA-dT) adducts in the presence of various thiols. [^{3}H]Thymine-labeled chromophore-poly(dA-dT) adducts formed in the presence of 10 mM 2-mercaptoethanol (A) or 5 mM glutathione (B) and under aerobic (●, 1) or anaerobic (△, 2) conditions were enzymatically digested and analyzed by HPLC as in Figure 1. In (A), the total radioactivity of the enzyme digests was 560 000 cpm and the fluorometer sensitivity was 0.1 μA. In (B), the total radioactivity was 820 000 cpm and the fluorometer sensitivity was 0.5 μA.

species has been shown to consist of a compound with the structure chromophore-d(TpApT), in which the deoxyadenosine sugar has been oxidized, possibly to a 5'-acetal (Povirk & Goldberg, 1982a,b). Thus, molecular oxygen is probably involved in this oxidation. The decrease in the formation of this adduct species under anoxia was approximately compensated by the increase in the formation of a species eluting at 72 min. In order to eliminate the possibility that this compound was merely a less digested form of the 86-min peak (e.g., a tetranucleotide), it was subjected to alkaline hydrolysis (0.04 M NaOH, 5 h, 37 °C), which has been shown to quantitatively release free adenine base from the oxidized adduct species (Povirk & Goldberg, 1982a). No detectable [^{3}H]adenine base was released by alkali from the adduct species formed under anaerobic conditions and eluting at 72 min. Instead, radioactivity was found in unidentified products eluting on HPLC after both adenine and deoxyadenosine. Thus, although the structure of this adduct species compound is completely unknown, it is chemically distinct from the major adduct formed under aerobic conditions.

Although base release was not examined in detail with glutathione and 2-mercaptoethanol, it was confirmed, with both reducing agents, that the majority of the thymine release was alkali dependent and that anoxia inhibited thymine release by at least 80%. Interestingly, it was found that, in the presence of 2-mercaptoethanol, thymine release under 100% oxygen was increased by only 7%, compared to the aerobic reaction. This value is much smaller than the 38% increase seen with dithiothreitol (Table I) and may reflect the fact that the competing reaction pathway leading to adduct formation is much less prominent in the presence of 2-mercaptoethanol (Table II), for reasons that are entirely unknown.

Discussion

Our results clearly show that, in the presence of dithiothreitol, the nonprotein chromophore of neocarzinostatin forms a stable covalent linkage to deoxyribose in DNA. When the reaction is performed under anoxia, adduct formation is maximized, while sugar oxidation is greatly suppressed. This inverse relation suggests that there are two competing pathways leading to formation of the two lesions and that, at some point in the reaction, these pathways share a common intermediate. This hypothesis can be expressed as a simple kinetic model of the form



where "other products" includes any outcome not detected as either adduct or base release. Since sugar oxidation has been determined at two oxygen concentrations, the relative values of k_1 and $k_2 + k_3$ can be determined, and the fraction $f = k_1[O_2]/(k_1[O_2] + k_2 + k_3)$ that follows the oxygen-dependent pathway can be calculated. From the data of Table I, we calculate $f = 0.51$ under atmospheric oxygen and $f = 0.84$ under 100% oxygen. Since $k_2/(k_2 + k_3)$ remains constant, the amount of adduct formed should vary in proportion to $(1 - f)$. Thus we would predict that adduct formation under argon, air, and oxygen, respectively, should vary as 1:(1 − 0.51):(1 − 0.84) or 1:0.49:0.16. This is in reasonable agreement with the experimental values of 35.2:16.8:7.9 or 1:0.48:0.22 (Table I). Alternatively, the kinetic equation can be rearranged to predict a linear relationship between 1/(yield of adduct) and $[O_2]$; such a plot gave a correlation coefficient of 0.992 (not shown). The data are thus quantitatively as well as qualitatively consistent with the existence of a common intermediate leading to formation of both lesions.

The most obvious candidate for such an intermediate would be a chromophore-induced carbon-centered deoxyribose radical, which would be expected to react by addition with molecular oxygen, leading to sugar oxidation. Consistent with such a proposal, incorporation of ^{18}O from molecular oxygen into chromophore-induced thymidine 5'-aldehyde has recently been demonstrated (Chin et al., 1984). Such a radical might also, under anaerobic conditions, react by addition with chromophore to form the covalent adduct. Hydrogen abstraction would be a logical mechanism for the formation of the putative carbon-centered radical, and recent studies showing transfer of ^{3}H label from C-5' of thymidine in DNA to a nonexchangeable site on the chromophore clearly support such a mechanism (Charnas & Goldberg, 1984).

An alternative hypothesis, equally consistent with the kinetic model, is that the two reaction pathways diverge before the initial attack on DNA and that there is no common DNA intermediate. Such a hypothesis would, however, require the seemingly unlikely postulate that there are two highly reactive forms of the chromophore, formed in the presence and in the absence of oxygen, both of which have a common precursor and both of which specifically attack deoxyribose in DNA, apparently with nearly the same base sequence specificity (L. F. Povirk and I. H. Goldberg, unpublished results).

Recently, Kappen & Goldberg (1984) have found that misonidazole and other nitroaromatic radiation sensitizers can substitute for oxygen as a cofactor in chromophore-induced DNA strand breakage. However, with misonidazole, little or no nucleoside 5'-aldehyde is formed. Rather, the predominant lesion is spontaneous base release, accompanied by breakdown of the sugar moiety to form strand breaks bearing both 3'- and 5'-phosphate end groups. These results are similar to those

of the present study in that (i) there is an apparent competition between the misonidazole-dependent and the oxygen-dependent pathways, (ii) the nature of the DNA damage resulting from the two pathways is different, but the sequence specificity is the same, and (iii) the maximum yield of the misondiazole-dependent products is approximately the same as that of the oxygen-dependent products. Here again, the data seem most easily explained by postulating a common DNA intermediate, but the alternative hypothesis, i.e., formation of two forms of activated chromophore, cannot be excluded.

Neither of the two model schemes proposed above explains the marked dependence of adduct formation on the nature of the thiol used for chromophore activation. However, there is some evidence that the first step in chromophore activation is an addition reaction with thiol (Hensens et al., 1983; Povirk & Goldberg, 1983). Such a covalently linked thiol could influence the nature of chromophore binding to DNA so as to favor or disfavor covalent adduct formation. Alternatively, various thiols may effect a required second thiol–chromophore reaction with different efficiencies. The fact that dithiothreitol is most effective in producing adducts raises the possibility of some intramolecular reaction involving the second sulfhydryl group of a dithiothreitol–chromophore addition product.

The oxygen-dependent adduct formed in the presence of 2-mercaptoethanol and containing an oxidized deoxyadenosine sugar must be produced by a mechanism quite different from that of the more stable adducts formed in the presence of dithiothreitol. Since this adduct spontaneously decomposes at neutral pH to form a strand break identical with the majority of chromophore-induced strand breaks (i.e., bearing 3′-phosphate and nucleoside 5′-aldehyde end groups), we earlier proposed (Povirk & Goldberg, 1982b) that a similar adduct may be a normal intermediate in strand break formation. Some more recent results tend to support this proposal. First, the formation of this adduct, like production of the strand breaks, is oxygen dependent (Figure 5), suggesting that molecular oxygen may be directly involved in the oxidation of deoxyadenosine that accompanies adduct formation. Second, high concentrations of 2-mercaptoethanol have been found to greatly accelerate the decomposition of this adduct (Povirk and Goldberg, unpublished results). This result agrees with the finding that, besides the thiol necessary for initial chromophore activation, there is additional, oxygen-dependent sulfhydryl consumption associated with the chromophore–DNA reaction that generates 5′-aldehydes (Povirk & Goldberg, 1983). Furthermore, high thiol concentrations have been found to increase both the absolute yield of 5′-aldehyde and the ratio of aldehyde to released free base (L. S. Kappen and I. H. Goldberg, unpublished results). All these results could be explained by postulating that an oxidized adduct species susceptible to thiol-induced decomposition is a normal intermediate in aldehyde formation.

The existence of such an intermediate could also alleviate certain conceptual difficulties in devising a model reaction sequence leading to 5′-aldehydes. If it is assumed that activated chromophore initially abstracts a hydrogen from C-5′ of deoxyribose, the resulting carbon-centered radical on C-5′ would be expected to react by addition with molecular oxygen to form a peroxyl radical, followed by sulfhydryl-induced reduction to a peroxide. Although further sulfhydryl-induced reduction is conceivable (Jocelyn, 1972), reduction of peroxide to the level of aldehyde would result in consumption of more sulfhydryl groups than is observed experimentally (Povirk & Goldberg, 1983). Thus, it seems likely that chromophore may be involved not only in the removal of hydrogen from C-5′ of deoxyribose but also in the subsequent (or simultaneous) addition of O_2 at the same site and in the cleavage of the oxygen–oxygen bond that would be required to produce 5′-aldehyde. An oxygen-dependent, sulfhydryl-sensitive adduct having some form of oxygen linkage between chromophore and C-5′ of deoxyribose would be a logical intermediate in such a process. An oxygen-containing form of activated chromophore would be a possible (but not obligatory) intermediate in the formation of this adduct.

To our knowledge, formation of adducts on deoxyribose in DNA, by any agent, has never been convincingly shown, and consequently virtually nothing is known of the biological effects of these lesions. However, any agent capable of inducing carbon-centered deoxyribose radicals could in principle also induce deoxyribose adducts as a result of addition reactions between these radicals and other molecules. Formation of *N*-ethylmaleimide adducts on deoxyribose in DNA in the presence of ionizing radiation has been proposed to occur by such a mechanism (Ward, 1975). Bulky adducts on DNA sugars obviously could not be removed by repair glycosylases, and it seems unlikely though not impossible that the DNA would be recognized by polymerases as a normal substrate. Furthermore, phosphodiester bonds of adduct-containing DNA sugars appear to be relatively resistant to a variety of nucleases. The manner in which these lesions are repaired and/or bypassed in the cell may prove to be interesting.

Acknowledgments

We thank Jeanne Thivierge for excellent technical assistance, Jose-Luis Giner for preparing [^{3}H]chromophore, and Dr. Zhang Chengbo for preliminary work on adduct characterization.

Registry No. 3′dTMP, 2642-43-5; 5′dTMP, 365-07-1; adenine, 73-24-5; thymine, 65-71-4; dithiothreitol, 3483-12-3; glutathione, 70-18-8; 2-mercaptoethanol, 60-24-2; oxygen, 7782-44-7.

References

Charnas, R. L., & Goldberg, I. H. (1984) *Biochem. Biophys. Res. Commun. 122*, 642.

Chin, D.-H., Carr, S. A., & Goldberg, I. H. (1984) *J. Biol. Chem. 259*, 9975.

Hensens, O. D., Dewey, R. S., Liesch, J. M., Napier, M. A., Reamer, R. A., Smith, J. L., Albers-Schönberg, G., & Goldberg, I. H. (1983) *Biochem. Biophys. Res. Commun. 113*, 538.

Ishida, N., Miyazaki, K., Kumagai, K., & Rikimaru, M. (1965) *J. Antibiot., Ser. A 18*, 68.

Jocelyn, P. (1972) *Biochemistry of the SH Group*, pp 94–100, Academic Press, New York.

Kappen, L. S., & Goldberg, I. H. (1978) *Nucleic Acids Res. 5*, 2959.

Kappen, L. S., & Goldberg, I. H. (1983) *Biochemistry 22*, 4872.

Kappen, L. S., & Goldberg, I. H. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3312.

Kappen, L. S., Napier, M. A., & Goldberg, I. H. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1970.

Kappen, L. S., Goldberg, I. H., & Leisch, J. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 744.

Kochetkov, N. K., & Budovskii, E. I. (1972) *Organic Chemistry of Nucleic Acids*, pp 431–432, Plenum Press, New York.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning*, p 134, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Marshall, L. E., Graham, D. R., Reich, K. A., & Sigman, D.

S. (1981) *Biochemistry 20*, 244.
Meienhofer, J., Maeda, H., Glaser, C. B., Czombos, J., & Kuromiz, K. (1972) *Science (Washington, D.C.) 178*, 875.
Napier, M. A., & Goldberg, I. H. (1983) *Mol. Pharmacol. 23*, 500.
Povirk, L. F., & Goldberg, I. H. (1982a) *Proc. Natl. Acad. Sci. U.S.A. 79*, 369.
Povirk, L. F., & Goldberg, I. H. (1982b) *Nucleic Acids Res. 10*, 6255.
Povirk, L. F., & Goldberg, I. H. (1983) *J. Biol. Chem. 258*, 11763.
Povirk, L. F., Köhnlein, W., & Hutchinson, F. (1978) *Biochim. Biophys. Acta 521*, 126.
Ward, J. F. (1975) *Adv. Radiat. Biol. 5*, 181.

Circular Dichroism and Thermal Melting of Two Small DNA Restriction Fragments of the Same Molecular Weight†

Nancy C. Stellwagen

ABSTRACT: The thermal melting and circular dichroism of two 147 base pair restriction fragments of pBR322 have been studied. The fragment with the higher GC content, 12B, melts at a higher temperature than the other fragment, 12A, as expected. The melting temperatures are proportional to the logarithm of the concentration of NaCl or tris(hydroxymethyl)aminomethane (Tris) buffer, between 1 mM and 0.2 M added salt. In 1 mM Tris buffer, the melting temperatures of the two fragments are nearly equal. The circular dichroism spectra of fragments 12A and 12B in 0.2–10 mM Tris buffer are characteristic of B-form DNA. In 81% ethanol, the circular dichroism spectra of the two fragments are characteristic of A-form DNA. With 1 mM Tris buffer as the supporting electrolyte, fragment 12A exhibits a very sharp B → A transition, with a midpoint at 79% ethanol. However, a biphasic transition is observed for fragment 12B, with midpoints at 73% and 80% ethanol. This biphasic transition may represent the conversion of separate domains of fragment 12B from the B conformation to the A conformation; half of this fragment is much more GC rich than the other half. Methods are also described for preparing polymers of the 12A and 12B fragments.

X-ray diffraction studies of oriented DNA fibers have shown that DNA can adopt a number of different conformations depending on the degree of hydration and the concentration and type of counterions (Langridge et al., 1980a,b). Characteristic circular dichroism (CD)[1] spectra have been correlated with A-form, B-form, and Z-form DNA (Tunis-Schneider & Maestre, 1970; Pohl & Jovin, 1972; Ivanov et al., 1973, 1974). Small DNA restriction fragments have also been found to exhibit these characteristic spectra (Hillen & Wells, 1980; Hillen et al., 1981c; Klysik et al., 1981).

Transitions between A-form and B-form DNA depend on the relative humidity of the sample; in solution, dehydration is effected by adding ethanol or other organic solvent to aqueous solutions of DNA (Ivanov et al., 1973; Malenkov et al., 1975). As the ethanol concentration is increased, the CD spectrum characteristic of B-form DNA changes abruptly to one similar to that of RNA (Brahms & Mommaerts, 1964; Ivanov et al., 1973, 1974). Direct evidence that these circular dichroism changes correspond to the B → A conformational transition has been obtained by X-ray (Gray et al., 1979; Zimmerman & Pfeiffer, 1979) and Raman (Erfurt et al., 1975) measurements. The mechanism of the transition from B-form to A-form DNA has been suggested to be the gradual replacement by ethanol of the spine of water molecules in the minor groove of B-form DNA (Dickerson et al., 1982; Conner et al., 1982). The water molecules around the still hydrated phosphate groups are then proposed to form a network across the major groove, which is quite narrow in A-form DNA (Dickerson et al., 1982; Conner et al., 1982). Since the spine of water molecules down the narrow groove of B-form DNA is disrupted by G residues (Drew & Dickerson, 1981; Dickerson et al., 1982; Conner et al., 1982), fibers of GC-rich DNA are converted more easily into the A conformation than fibers of AT-rich DNA (Pilet & Brahms, 1973; Arnott & Selsing, 1974; Arnott et al., 1974; Pilet et al., 1975).

Previous studies of the ethanol-induced B → A transition in solution have found little or no dependence of the transition on the GC content of the DNA (Pilet & Brahms, 1972; Ivanov et al., 1974, 1983b; Hillen & Wells, 1980). However, most of these studies involved high molecular weight DNAs and/or sheared samples of calf thymus DNA, in which such a dependence might be averaged out. In one study involving DNA restriction fragments (Hillen & Wells, 1980), the midpoint of the B → A transition was found to occur at 72 ± 2% ethanol, independent of DNA sequence and/or molecular weight. However, in this study, the concentration of low molecular weight electrolyte apparently was not kept constant but varied with ethanol concentration. Unfortunately, the midpoint of the B → A transition is very sensitive to electrolyte concentration (Ivanov et al., 1973, 1974). Recently, Ivanov et al. (1983a) have found that the midpoint of the B → A transition of the decanucleotide $(CCCTGCAGGG)_2$ increased from 74% to 80% when the GC base pairs at residues 3 and 8 were replaced by AT base pairs. Hence, base pair sequence

† From the Department of Biochemistry, University of Iowa, Iowa City, Iowa 52242. *Received April 10, 1984.* This work was supported by Grant GM-29690 from the National Institute of General Medical Sciences.

[1] Abbreviations: bp, base pair(s); EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; CD, circular dichroism; T_m, melting temperature; A, adenine; T, thymine; G, guanine; C, cytosine.

0006-2960/84/0423-6311$01.50/0 © 1984 American Chemical Society