$(bpy)_2(dcbpy)$ itself. Since this derivative is undoubtedly a mixture of many derivatives labeled at different lysine groups on lysozyme, it appears that the enviornment on the surface of the protein does not significantly affect the luminescence decay rate. Therefore, an upper limit to the true rate of electron transfer can be obtained by subtractng 1.8×10^6 s^{-1} from the luminescence decay rates shown in Table I. These values are of comparable magnitude to the rates of other electron-transfer reactions with similar distances and driving forces (Axup et al., 1988; Elias et al., 1988). The similarity in the luminescence decay rates of the corresponding ferro- and ferricytochrome *c* derivatives is consistent with the similarity in the ΔE^0 values for the oxidative and reductive electron-transfer reactions. Additional experiments are planned to provide direct evidence on whether the quenching mechanism for the ferro- and ferricytochrome *c* derivatives does indeed involve electron transfer. The new class of derivatives introduced here should allow great flexibility in the design of experiments to determine the effect of driving force, distance, and protein medium on electron-transfer reactions.

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ABC Excinuclease Incises both 5′ and 3′ to the CC-1065–DNA Adduct and Its Incision Activity Is Stimulated by DNA Helicase II and DNA Polymerase I†

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ABSTRACT: CC-1065 is a large molecule that binds covalently to adenine residues of DNA in a sequence-specific manner and lies in the minor groove about four bases to the 5′ side of the adducted residue. Using a reconstituted *Escherichia coli* nucleotide excision repair system, we have obtained data showing that the ABC excinuclease makes incisions both 5′ and 3′ to the CC-1065 adduct and that the incision activity is stimulated by the addition of helicase II and DNA polymerase I (and dNTPs). Our results with the CC-1065 adduct are consistent with the reported in vitro processing of other adducts (e.g., cisplatin, UV photoproducts) but do not agree with a recent study that reported anomalous processing of the CC-1065 adduct by ABC excinuclease and helicase II. Our results also imply that, in binding to damaged DNA, ABC excinuclease does not make important contacts in the minor groove four bases to the 5′ side of the damaged residue.

ABC excinuclease was first described as an enzyme that incises the phosphodiester backbone of DNA on the 3′ and 5′ sides of pyrimidine dimers in vitro (Sancar & Rupp, 1983). The same dual incision pattern was observed later with (acetylamino)fluorene, psoralen (Sancar et al., 1985), and cisplatin (Beck et al., 1985) adducts. However, it was also recognized that occasionally the enzyme incises only on one side of the adduct, i.e., in an "uncoupled" manner. Uncoupled incisions, observed with pyrimidine dimers (Yeung et al., 1983) and psoralen adducts (Van Houten et al., 1986), were usually seen at low frequencies. In contrast, a recent report claimed that the enzyme incised only on the 5′ side of CC-1065 adducts (Tang et al., 1988). Also, as opposed to UV photoproducts and cisplatin adducts (Caron et al., 1985; Husain et al., 1985), with the CC-1065 adduct, helicase II did not stimulate ABC

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excinuclease activity (Tang et al., 1988). The observations made with CC-1065-adducted DNA were believed to reflect unique properties of this substrate. The reported processing of the CC-1065 adduct by ABC excinuclease and helicase II is contrary to the proposed mechanism of excision repair and suggests that the excinuclease in certain instances recognizes the adduct rather than adduct-induced helical anomalies; therefore, we investigated the processing of the CC-1065 adduct in more detail. Our results show the CC-1065 adduct to be "typical" since ABC excinuclease incises on both the 3′ and 5′ sides and the incision activity is stimulated by helicase II plus DNA polymerase I.

Materials and Methods

Repair Enzymes. UvrA, UvrB, and UvrC were purified as described in Thomas et al. (1985). UvrD (helicase II) was the kind gift of Dr. J. Griffith. DNA polymerase I was from Boehringer Mannheim Biochemicals.

Preparation of Substrates. 5′ or 3′ terminally labeled fragments from pBR322 and ^{3}H-labeled pBR322 plasmid were used in these experiments.

To prepare 5′-end-labeled oligomers, the (*Eco*RI–*Bam*HI)$_{381}$ fragment of pBR322 was first treated with bacterial alkaline phosphatase (Bethesda Research Laboratories, 1 h, 65 °C) and then solvent-purified by phenol extraction and ethanol precipitation. The DNA was labeled with T4 polynucleotide kinase (Bethesda Research Laboratories) and [γ-^{32}P]ATP, solvent purified, and cleaved with *Mae*III (Boehringer Mannheim Biochemicals). The two products, (*Eco*RI–*Mae*III)$_{131}$ and (*Mae*III–*Bam*HI)$_{167}$, were gel-purified on a 6% polyacrylamide gel and dialyzed extensively against TEN 7.4 (Tris-HCl, 10 mM, pH 7.4; EDTA, 1 mM; NaCl, 10 mM).

*Mae*III digests of the (*Eco*RI–*Bam*HI)$_{381}$ fragment were 3′ end labeled with the Klenow fragment of DNA polymerase I (Boehringer Mannheim Biochemicals), [α-^{32}P]TTP, and excess cold dGTP to produce a (*Mae*III–*Bam*HI)$_{167}$ fragment labeled only at the *Mae*III end. The labeling protocol resulted in unacceptable length heterogeneity in the (*Mae*III–*Bam*HI)$_{167}$ fragment while the (*Eco*RI–*Mae*III)$_{131}$ fragment, which was used in our studies, had limited length heterogeneity. The (*Mae*III–*Bam*HI)$_{167}$ fragment was also 3′ end labeled with [α-^{32}P]dGTP and cold dATP, dCTP, and TTP and then cleaved with *Fnu*DII, giving a (*Mae*III–*Fnu*DII)$_{135}$ product.

To prepare [^{3}H]pBR322, *Escherichia coli* AB2487 (*recA*$^-$ *thyA*$^-$)/pBR322 was grown in M9 medium containing 2.5 μg/mL thymidine, 0.1 μg/mL thiamin, 1 mM $MgSO_4$, 0.2 mM $CaCl_2$, and 1% casamino acids and 1% glucose to A_{600} = 0.6; then chloramphenicol was added to 0.2 mg/mL. One hour later, [^{3}H]dT (77.9 Ci/mmol) was added to 1 μCi/mL. Cultures were then incubated for 17 h, and the plasmid was purified by Sarkosyl lysis and CsCl–ethidium bromide density gradient centrifugation. The specific activity of the plasmid was 5400 cpm/μg.

CC-1065 was dissolved in dimethyl sulfoxide to 270 mM and further diluted in a solution containing 15 mM NaCl and 1.5 mM sodium citrate, pH 7.4. Drug treatments of DNA were done in this buffer, at room temperature for 2 h, and were stopped by ethanol precipitation; substrates were then resuspended in TEN 7.4. UV treatments of DNA were with germicidal light from a General Electric G8TS lamp. The preparation of psoralen-adducted substrate has been described (Van Houten et al., 1987).

ABC Excinuclease Reaction Conditions. Unless otherwise noted, ABC excinuclease reactions were in 50-μL volumes containing 45 mM Tris, pH 7.4, 45 mM KCl, 10 mM $MgCl_2$, 1.5 mM ATP, 4.4 mM dithiothreitol, and 50 μg/mL BSA. Incubation was at 37 °C.

Processing of Reaction Products. To identify incision sites, CC-1065-adducted, end-labeled fragments were incubated with ABC excinuclease (unless otherwise noted, UvrA, 7.7 nM, UvrB, 52 nM, and UvrC, 60 nM) for 20–25 min, phenol-extracted, ethanol-precipitated, dried, resuspended in formamide plus dyes, heat-denatured at 90 °C for 1–5 min, and electrophoresed on 8% denaturing polyacrylamide gels alongside the corresponding Maxam and Gilbert (1980) sequencing reaction products.

To measure catalytic turnover of the enzyme, the ABC excinuclease activity was measured by its nicking action on CC-1065-modified superhelical [^{3}H]pBR322 DNA. For this assay, the reaction mixture contained 33 μM each of the four dNTPs in ABC excinuclease buffer and DNA polymerase I and helicase II as indicated. Processing of reaction products and data analysis were as described in Sancar et al. (1984).

Results

Identification of Incision Sites Adjacent to CC-1065 Adducts. Products of ABC excinuclease digestion of the CC-1065-adducted, terminally labeled fragments used in this study are shown in Figures 1 and 2 alongside the corresponding negative (nonadducted) and positive (UV-irradiated) controls and Maxam and Gilbert (1980) sequencing reaction products. CC-1065 binds to adenine residues of double-stranded DNA in a sequence-specific manner (see below), and the adduct causes strand breakage when the DNA is heated prior to loading (Reynolds et al., 1985). This property of the adducts allows identification of CC-1065 binding sites in Figures 1 and 2 from the thermal degradation products that appear as doublets with 5′-end-labeled fragments (left panels) and as single bands with 3′-labeled fragments (right panels). (We note that a doublet appears with the 3′-labeled fragment in Figure 2 due to "shadowing" caused by the length heterogeneity of the fragment.) Six CC-1065 binding sites are identified in Figures 1 and 2 and are noted in Figure 3. The adduct at A_{254} comigrates with a contaminant of the 5′-labeled fragment and is identified by the slower moving band of the doublet (5′-labeled fragment) and by the sharp band with the 3′-labeled fragment. Reynolds et al. (1985) reported that CC-1065 commonly binds to the 5′ adenine residue in the sequence 5′-ATTNPu-3′ and the binding sites in Figures 1 and 2 are generally consistent with this pattern, although adenine is more commonly observed at the central position in the five-base sequence.

ABC excinuclease dependent strand breakages are visible on both the 5′ and 3′ sides of CC-1065 binding sites (Figures 1 and 2). Incision patterns, interpreted as before (Sancar & Rupp, 1983; Myles et al., 1987), are illustrated in Figure 3 and are consistent with incision patterns observed with other adducts. ABC excinuclease hydrolyzes the eighth phosphodiester bond 5′ and the fourth or fifth phosphodiester bond 3′ to the CC-1065–adenine adducts. In one exceptional case we fail to see a clear incision to the 3′ side of the adduct at A_{282}, probably due to a high background in the critical area of the gel. Because we do not see a clear incision 3′ to A_{282}, it is possible that the corresponding incision 5′ to A_{282}, visible in Figure 1 (left), was not coupled with a corresponding 3′ incision. Relevant are the results of Tang et al. (1988), who observed ABC excinuclease dependent incisions adjacent to one out of four (or possibly five) CC-1065 adducts. More specifically, Tang et al. (1988) observed incision only on the 5′ side of their adduct. Their 5′ incision of this adduct was clearly visible with a 3′-labeled fragment as well as with the

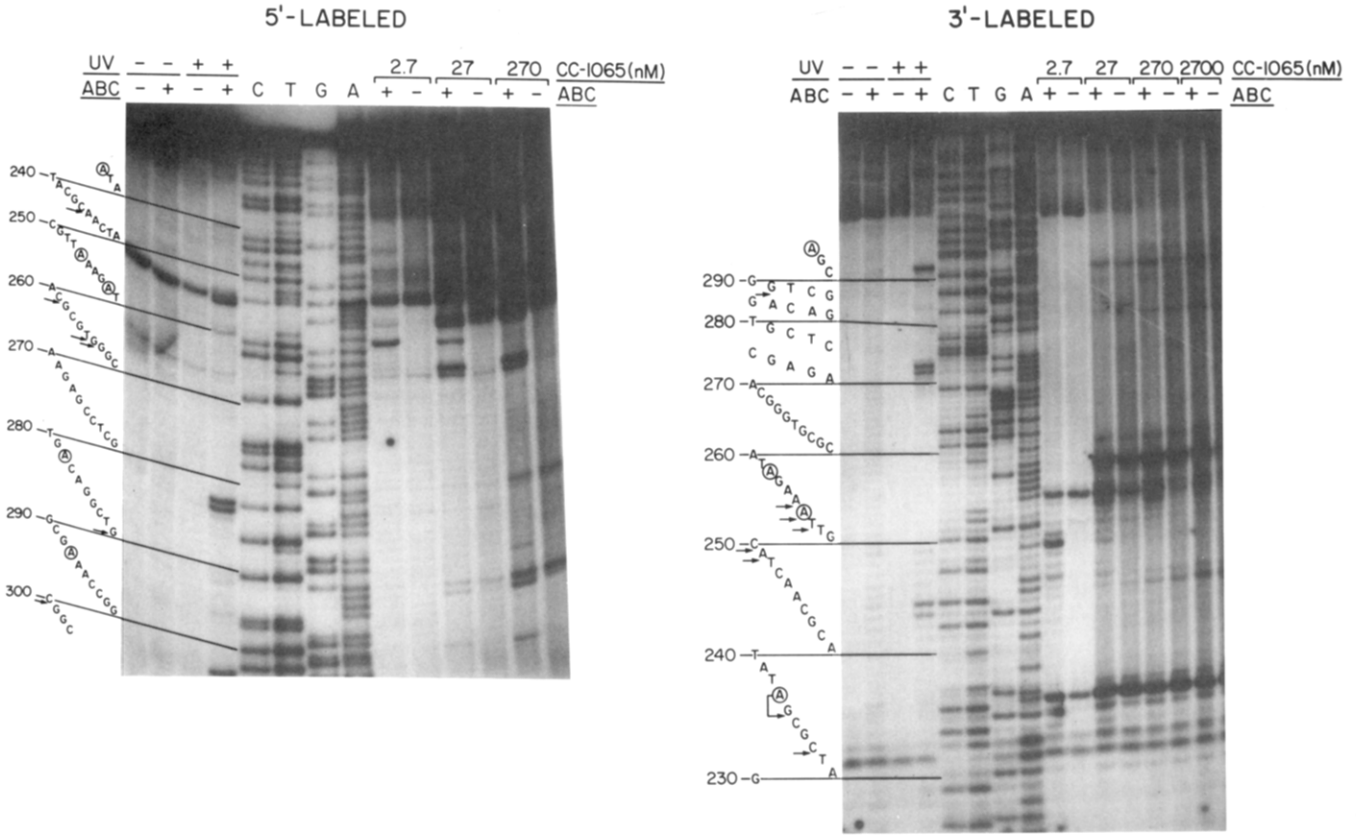

FIGURE 1: CC-1065 binding sites and ABC excinuclease incision of terminally labeled DNA. The 5′-labeled (MaeIII–BamHI)$_{167}$ (left panel) and the 3′-labeled (MaeIII–FnuDII)$_{135}$ (right panel) fragments were treated with the indicated doses of CC-1065. Adducted and control (nonadducted and UV irradiated, 4 kJ/m^2) fragments were incubated with ABC excinuclease (ABC). Reaction products were resuspended in formamide, heat-denatured, and run on 8% polyacrylamide sequencing gels alongside Maxam and Gilbert (1980) reaction products (A + G, A, T + C, and C). The circled bases are the modified adenines, and the arrows indicate the incision sites of ABC excinuclease on the CC-1065-modified DNA. The weakly modified A_{282} residue and the corresponding faint band due to incision 3′ to this adduct are not marked. The G_{236}–A_{237} sequence is reversed in the right panel as indicated by the bracketed arrow due to a gel artifact that did not occur with the 5′-labeled fragment. Reaction conditions were as noted under Materials and Methods except KCl was at 91 mM.

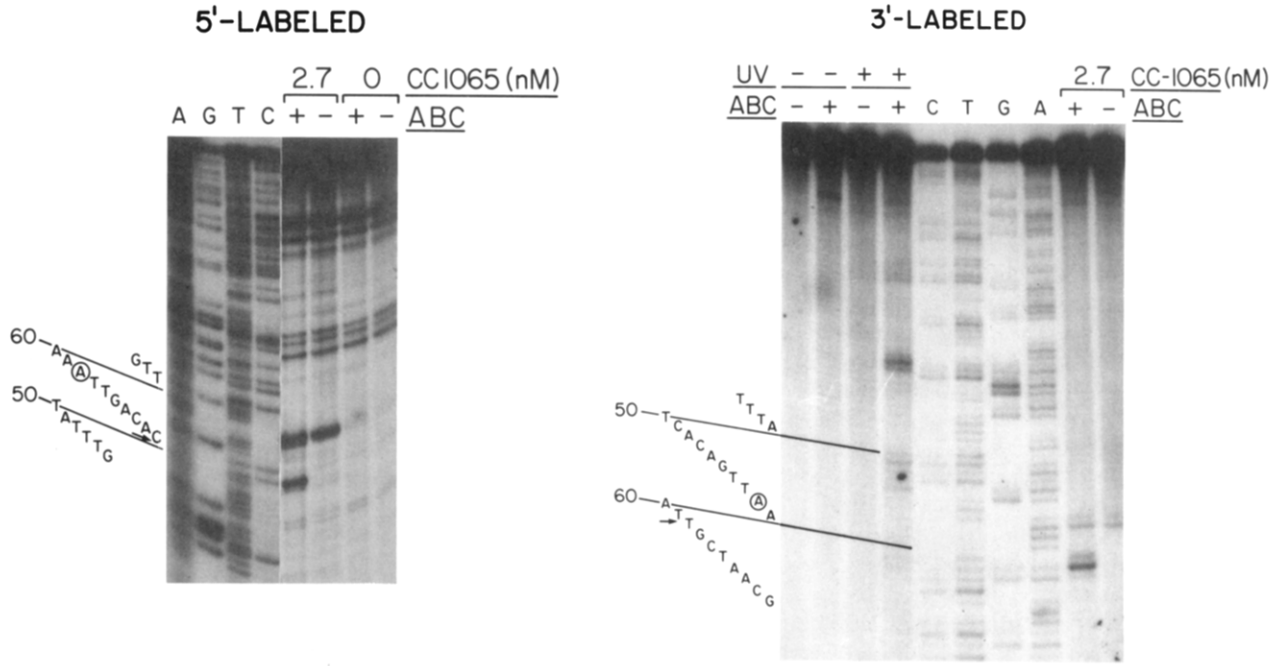

FIGURE 2: CC-1065 binding and ABC excinuclease incision sites on the (EcoRI–MaeIII)$_{131}$ fragment. The figure shows the 5′-labeled (left panel) and 3′-labeled (right panel) (EcoRI–MaeIII)$_{131}$ fragment. The CC-1065-modified base and incision sites are indicated by circles and arrows, respectively. Reaction conditions were as described under Materials and Methods except as follows: dithiothreitol was at 5.3 mM; ATP was at 1.8 mM; (left) UvrA, 9.6 nM, UvrB, 26 nM, and UvrC, 30 nM; (right) UvrA, 9.6 nM, UvrB, 66 nM, and UvrC, 76 nM.

same fragment labeled at the 5′ terminus and therefore was unambiguously uncoupled. Taken together, our results and the results of Tang et al. suggest that uncoupled cutting of CC-1065 adducts is uncommon. We note that, with all other nucleotide adducts studied, uncoupled incisions have been found to be uncommon except under suboptimal reaction

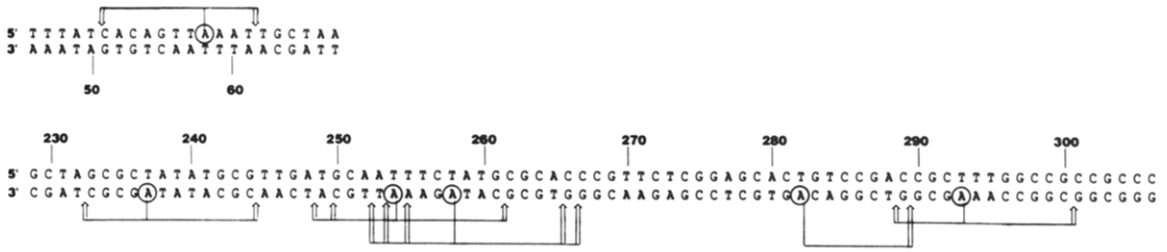

FIGURE 3: ABC excinuclease incisions adjacent to CC-1065 adducts. Incision sites (indicated by arrows) are connected to associated drug binding sites (indicated as circled A residues) and are deduced from Figures 1 and 2. The numbering of bases is according to the conventional pBR322 numbering system.

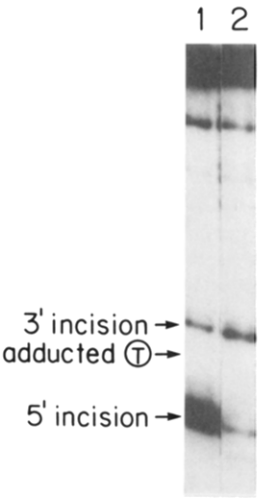

FIGURE 4: Uncoupling of ABC excinuclease incision. The 5′-labeled uniquely psoralen-adducted fragment (Van Houten et al., 1987) was reacted with ABC excinuclease under standard conditions with equimolar amounts of fresh (lane 1) or aged (lane 2) ABC excinuclease preparations. The incision sites and the location of the psoralen-adducted T (thymine) are indicated. Note that because the fragment is 5′-labeled, the band corresponding to 3′ incision is seen only when it is uncoupled from 5′ incision.

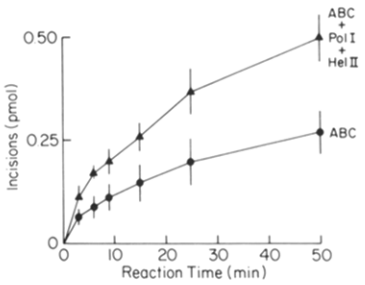

FIGURE 5: Stimulation of ABC excinuclease activity by helicase II and DNA polymerase I. Incision reactions were in 100-μL volumes containing 0.25 pmol of pBR322 with an average of 8.5 adducts/plasmid (i.e., 2.2 total pmol adducts assuming complete reaction of drug with plasmid), 0.15 pmol of UvrA, 0.55 pmol of UvrB, 1.5 pmol of UvrC, 3.0 pmol of helicase II, and 3.7 pmol of *Pol*I. Reactions were initiated by adding UvrC. Samples (12.5 μL) were removed after 0, 3, 6, 9, 15, 25, and 50 min at 37 °C and added to sodium dodecyl sulfate–tracking dye mixtures on ice to stop reactions. Background cutting after 50 min was 0.038-pmol incisions for ABC excinuclease with undamaged plasmid and 0.010-pmol incisions for helicase plus *Pol*I with adducted plasmid and has been subtracted from the values given here. Results are the mean of three experiments and standard errors are shown. The number of incisions was calculated as the product of the nicks per plasmid (calculated from the percent remaining supercoiled form) times the initial quantity of plasmid (0.25 pmol) by using Poisson statistics. ABC, ABC excinuclease; Hel II, helicase II.

conditions such as illustrated in the next section.

Uncoupling of ABC Excinuclease Incisions. Uncoupled ABC excinuclease incisions have been reported previously with various DNA adducts (Yeung et al., 1983; Van Houten et al., 1986; Sancar & Sancar, 1988), occurring usually at low frequency but at higher frequency under suboptimal reaction conditions. To illustrate this latter point, we present the data shown in Figure 4. A 137-bp synthetic DNA fragment containing a psoralen monoadduct at a unique site (Van Houten et al., 1987) was digested with "fresh" ABC excinuclease and with an enzyme that was "aged" by incubating at −20 °C for 2 weeks. The subunits were aged individually, and the UvrC subunit is more sensitive to aging than the other subunits. With fresh enzyme (lane 1) we observe mostly 5′ incisions (which are presumably coupled with 3′ incisions) and a low level of 3′ incisions (which have to be uncoupled to be visible with 5′-labeled DNA). In contrast, even when the reaction was allowed to go to completion, the aged enzyme cut poorly and approximately 80% of the incisions are 3′ incisions, uncoupled from cutting on the 5′ side. It is thus clear that even with a defined adduct at a defined location one can obtain drastically different levels of uncoupling depending on the reaction conditions, and therefore any claims to uncoupled incisions should be made with due precaution.

Stimulation of ABC Excinuclease by DNA Helicase II and DNA Polymerase I. A time course for ABC excinuclease incision of CC-1065-adducted, supercoiled pBR322 is shown in Figure 5. As reported for other adducts (Husain et al., 1985), the enzyme makes approximately stoichiometric incisions (as determined in our case by the amount of UvrA) in the absence of other factors. Inclusion of DNA polymerase I and helicase II results in a doubling of the final level of incision of DNA adducted with CC-1065. Tang et al. (1988) reported that helicase had no effect on ABC excinuclease incision of CC-1065-adducted plasmids. The discrepancy between the two reports may be due to three factors. First, helicase II is thought to stimulate incision by facilitating the turnover of ABC excinuclease. Tang et al. (1988) in most of their experiments used a molar excess of UvrA, UvrB, and UvrC subunits, in which case turnover of the enzyme would have less effect on reaction kinetics than using limiting amounts of subunits such as we have used. Second, Tang et al. did not use DNA polymerase I (with dNTPs), which apparently acts in concert with helicase II to amplify the stimulatory effect of helicase II (Caron et al., 1985; Husain et al., 1985). Third, Tang et al. used $^1/_{2300}$ to $^1/_{120}$ as much helicase II as we used.

Discussion

ABC excinuclease incises the eighth phosphodiester bond 5′ and the fourth or fifth bond 3′ to a variety of DNA adducts in vitro [reviewed in Sancar and Sancar (1988)]. Minor variations in the location of specific incision sites and staggered incision patterns have been noted (Van Houten et al., 1986; Myles et al., 1987). Excinuclease activity is enhanced by the combined action of helicase II and DNA polymerase I, possibly through enhanced turnover of enzyme subunits. Our observations of the ABC excinuclease incision pattern (Figure 3)

and helicase II stimulation (Figure 5) using CC-1065-adducted substrates are consistent with the observations made with other adducts.

Our results and those of Tang et al. (1988) suggest that ABC excinuclease mediated incision on only one side of CC-1065 adducts is uncommon. Their uncoupled 5′ incision may have resulted from a unique secondary structure of their DNA fragment in the vicinity of the adducts that they investigated, or suboptimal conditions of their excinuclease reaction. Possibly relevant are the different ratios of enzyme subunits used by us and by Tang et al. We used UvrA:UvrB:UvrC ratios that ranged from 1:2.7:3.1 to 1:6.7:7.8, while Tang et al. used a ratio of 1:11:1.4. Figure 4 illustrates how suboptimal reaction conditions (in this case involving an aged enzyme preparation) can lead to anomalous incision activity. The failure of Tang et al. to observe stimulation of ABC excinuclease by helicase II in vitro is probably due to the fact that they did not use limiting ABC excinuclease in their experiments and did not include *Pol*I and sufficient amounts of helicase II in the reaction mixtures. Similarly, their observation that uvrD (helicase II) mutations had no effect on the survival of CC-1065-damaged ϕX174 RFI DNA is due to the fact that in transfection assays adducts in incoming DNA do not saturate the constitutive level of ABC excinuclease and therefore there is no need for catalytic turnover of the enzyme to repair all of the repairable plasmids. Indeed *uvrD*$^-$ mutations have no effect on the survival of UV-damaged DNA in the transformation assay (Roberts & Strike, 1981), yet several groups (Husain et al., 1985; Caron et al., 1985; Tang et al., 1988) have demonstrated that helicase II stimulates the incision of UV-damaged DNA by ABC excinuclease in vitro.

CC-1065 is a large molecule that lies in the minor groove of DNA about four bases to the 5′ side of adducted adenine residues. Drug binding inhibits intercalation, alters the ellipticity and DNase I digestion pattern of DNA, and stabilizes DNA against S1 digestion and thermal melting (Swenson et al., 1982; Hurley et al., 1984, 1987). Our observations of "conventional" ABC excinuclease cutting of the CC-1065 adduct suggests that the enzyme does not make important, productive contacts with substrates in the minor groove four bases to the 5′ side of the adduct. We note that incision activity was inhibited at high drug binding ratios (Figures 1 and 2), a phenomenon that may be related to the extensive distortions in heavily adducted DNA. Also, because the incision pattern relative to the CC-1065 lesion (Figure 3) is consistent with the incision pattern of other structurally diverse lesions, it follows that the enzyme recognizes the structural perturbation of the DNA helix caused by CC-1065 rather than by the drug itself. In summary, on the basis of the results with the CC-1065 adduct and all other adducts studied to date, we conclude that both ABC excinuclease and helicase II have a single, common reaction mechanism for processing DNA adducts.

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