Characterization of Amino Acid Substitutions That Severely Alter the DNA Repair Functions of *Escherichia coli* Endonuclease IV†

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ABSTRACT: *Escherichia coli* endo IV is a bifunctional DNA repair protein, i.e., possessing both apurinic/apyrimidinic (AP) endonuclease and 3′-diesterase activities. The former activity cleaves AP sites, whereas the latter one removes a variety of 3′-blocking groups present at single-strand breaks in damaged DNA. However, the precise reaction mechanism by which endo IV cleaves DNA lesions is unknown. To probe this mechanism, we have identified eight amino acid substitutions that alter endo IV function in vivo. Seven of these mutant proteins are variably expressed in *E. coli* and, when purified, show a 10−60-fold reduction in both AP endonuclease and 3′-diesterase activities. The most severe defect was observed with the one remaining mutant (E145G) that showed normal protein expression. This mutant has lost the ability to bind double-stranded DNA and showed a dramatic 150-fold reduction in enzymatic activities. We conclude that the AP endonuclease and the 3′-diesterase activities of endo IV are associated with a single active site, that is perhaps remote from the DNA binding domain.

Apurinic/apyrimidinic (AP)[1] sites are produced in cellular DNA both naturally and by many chemical agents. AP sites are clearly highly mutagenic, and must be repaired by AP endonucleases in order to prevent genetic mutations (*1*, *2*). Endonuclease (endo) IV, encoded by the *nfo* gene, was first identified as a minor AP endonuclease in *Escherichia coli* (*3*, *4*). This enzyme cleaves the sugar−phosphate bond 5′ to the AP site to produce a 3′-hydroxyl group and a 5′-deoxyribose phosphate (*5*, *6*). The 5′-deoxyribose phosphate is then removed by a 5′-deoxyribose phosphodiesterase to create a single-nucleotide gap, followed by DNA polymerase-mediated DNA resynthesis, and rejoining via DNA ligase (*7*, *8*). Endo IV also has a second enzymatic activity, a 3′-diesterase, which removes a multitude of 3′-blocking groups (e.g., 3′-phosphoglycolate and 3′-phosphate) that are present at single-strand breaks in DNA induced by oxidative agents, including the antitumor drug bleomycin and the chemical oxidant hydrogen peroxide (*5*).

E. coli also has a second dual-function enzyme, exonuclease III (encoded by the *xth* gene), that cleaves AP sites, as well as removes 3′-blocking groups at the ends of single-strand breaks in DNA (*9*). This enzyme is unique, as it possesses a 3′-5′-exonuclease activity (*1*). Despite the enzymatic similarities that exist between endo IV and exonuclease III, the two enzymes are structurally unrelated, share no amino acid identity, and can be readily distinguished from each other in crude *E. coli* extracts (*1*, *10*). Endo IV is a Mg^{2+}-independent enzyme, whereas exonuclease III is Mg^{2+}-dependent and very sensitive to inhibition by the metal chelator EDTA (*10*). Although endo IV has no metal ion requirements, it does contain 2.4 atoms of Zn^{2+} and 0.7 atom of Mn^{2+} (*11*). The role of these metal ions in endo IV function remains unclear (*11*).

Several lines of evidence clearly indicate that endo IV can recognize and cleave DNA lesions that are refractory to cleavage by exonuclease III: (i) *E. coli* single mutants, deficient in either endo IV or exonuclease III, showed strikingly dissimilar patterns of hypersensitivity toward DNA-damaging agents (*12*). For example, endo IV-deficient mutants are specifically hypersensitive to the oxidants bleomycin and *tert*-butylhydroperoxide, whereas the exonuclease III-deficient mutants are hypersensitive to H_2O_2, MMS, and mitomycin C (*12*, *13*). (ii) Overproduction of endo IV fully restores drug resistance to the exonuclease III-deficient mutant, but the converse has not been observed, suggesting that endo IV is capable of processing a broader array of DNA lesions (*13*). This notion was further supported by the findings from two direct in vitro studies: (i) purified endo IV, but not purified exonuclease III, cleaves the α-anomer of deoxyadenosine, an oxidative DNA lesion produced by hydroxyl attack on the anomeric hydrogen atom at the C1′ position of deoxyribose (*14*); and (ii) endo IV was shown to be more effective than exonuclease III at repairing bleomycin-damaged chromosomal DNA (*15*). The broad substrate specificity of endo IV may account for its conservation in eukaryotes such as the yeast *Saccharomyces cerevisiae* and the nematode *Caenorhabditis elegans* (*2*).

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[1] Abbreviations: AP, apurinic/apyrimidinic; EMSA, electrophoretic mobility shift assay; endo IV, endonuclease IV; IPTG, isopropyl β-D-thiogalactoside; MMS, methyl methanesulfonate; PCR, polymerase chain reaction; PMSF, phenylmethylsufonyl fluoride; SDS, sodium dodecyl sulfate.

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The mechanism by which endo IV recognizes and cleaves the phosphodiester bonds at sites of DNA damage is unknown. In the case of exonuclease III, X-ray crystal analysis predicts that catalysis of phosphodiester bond cleavage is driven by an aspartic/histidine amino acid pair, and facilitated by a single metal ion attached to a glutamate residue (*16*). However, these corresponding amino acid residues are absent in endo IV (*2*, *17*). In this report, we identify by chemical mutagenesis several essential amino acid substitutions that alter endo IV function in the repair of both AP sites and DNA strand breaks with blocked 3′-termini. All of the mutants showed a reduction of AP endonuclease/3′-diesterase activities, ranging from 10- to 150-fold. One of these substitutions prevented endo IV from binding to DNA, whereas the others destabilized the protein and were deemed essential for the proper maintenance of the protein tertiary structure.

EXPERIMENTAL PROCEDURES

Bacteria and Transformation. The bacterial strains used in this work were the parent AB1157 and the mutant BW528 [Δ(*xth-pnc*), *nfo1::kan*)], kindly provided by B. Weiss (Emory, Atlanta, GA). Strains were transformed with the indicated vector or plasmids (see below) by the $CaCl_2$ method (*13*).

Construction of pDR24. The plasmid pRPC124 (kindly provided by Dr. R. Cunningham, Albany, NY) contains the entire coding region of the *E. coli nfo* gene and the flanking 5′- and 3′-untranslated DNA sequences (*12*). This plasmid was used as the template to amplify, via the polymerase chain reaction (PCR), the sequence from −21 to +937 (numbering with respect to the first nucleotide of the *nfo* start codon) using the primers DR1 (5′-GGGTTTAACAGGGTACCCG-C**ATG**AAATAC-3′, start codon shown in boldface) and DR2 (5′-TTTCGTTCGGCTGGATCCGCGGGTTACGCC-3′) bearing the restriction sites (underlined) for *Kpn*I and *Bam*HI, respectively. The PCR reaction contained 100 ng of pRPC124 and 1 μg each of primers DR1 and DR2 in a final reaction of 100 μL. The conditions for amplification were 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 2 min for a total of 30 cycles. The PCR product (958 bp), bearing the entire *nfo* coding region and ∼150 bp of the 3′-untranslated region, was digested with both *Kpn*I and *Bam*HI and subcloned next to the *lac* promoter in the *E. coli* expression vector pKEN2 (kindly provided by Dr. G. Verdine, Harvard University) to generate the plasmid pDR24. The *Kpn*I/*Bam*HI fragment from pDR24 was isolated and subcloned into pBluescript (KS), and both DNA strands of the *nfo* gene were sequenced by the dideoxy chain termination method (*18*) to verify the nucleotide sequence.

Chemical Mutagenesis and Mutant Selection. The pDR24 plasmid (5 μg) was resuspended in 25 μL of 250 mM sodium acetate (pH 4.3) containing 1 M sodium nitrite and incubated at room temperature for 30 min (*19*). After the treatment, 100 μL of TE buffer [10 mM Tris-HCl (pH 7.5) + 1 mM EDTA] and 100 μL of 2.5 M sodium acetate, pH 7.0, were added to neutralize the reaction. The DNA was precipitated with 3 volumes of 95% ethanol, washed twice with 70% ethanol, and resuspended in 50 μL of TE buffer at a final concentration of 100 ng/μL. The nitrous acid treated pDR24 plasmid (100 ng) was used for amplifying the *nfo* gene using 1 μg each of the primers DR1 and DR2 in a PCR reaction (100 μL) under the same conditions described above. The mutated PCR-amplified 958-bp fragment was digested with *Kpn*I and *Eco*RV (*Eco*RV cuts the *nfo* fragment 50 nt upstream of the stop codon) to generate a 850-bp fragment. The resulting 850-bp *Kpn*I/*Eco*RV fragment was ligated into pDR24, which was previously digested with *Kpn*I/*Eco*RV to remove the same length of the native fragment. This step replaced 850 bp out of 900 bp of the native *nfo* gene with the mutated version. The ligated mixture was transformed directly into strain BW528, and ampicillin-resistant colonies were directly streaked onto (i) Luria broth **(**LB) agar, (ii) LB agar containing 9 μM MMS, and (iii) LB agar containing 0.5 μg/mL bleomycin. Colonies that were unable to grow on either drug plates were scored as bearing mutation in the *nfo* gene.

Site-Directed Mutagenesis. Site-directed mutations within the *nfo* gene were generated by the QuikChange site-directed mutagenesis kit (Stratagene). The oligonucleotides used for site-directed mutagenesis were all 30-mer in length, and each carried the indicated nucleotide substitution(s) in the center of the oligonucleotide (see Table 2 for the amino acid changes).

Gradient Plate Assay. This assay was performed as described previously (*13*). Briefly, cells were replicated as a thin line along the drug gradient, and after 24 h of growth at 37 °C, the distance (millimeters) of growth of each strain is expressed as a percentage of the parent. Growth all along the gradient is considered to be 100%.

Immunoblot Analysis. The conditions used for Western blot analysis were as previously described (*20*), except the anti-endo IV polyclonal antibodies were used at a dilution of 1:5000 and immunoreactive polypeptides were detected by chemiluminescence (Dupont−NEN).

Crude Extract Preparation. Total extract was prepared as previously described (*13*). Briefly, plasmids bearing cells were grown in 5 mL of LB containing 100 μg/mL ampicillin to an OD_{600} of ∼0.8. For induction of the lac promoter, IPTG was added at a final concentration of 0.1 mM for 4 h. Cells were harvested, washed once, and resuspended in extraction buffer [50 mM Tris-HCl, pH 7.5, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamide, and 1 μg/mL each of aprotinin, leupeptin, and pepstatin A]. Cells were lysed by two passes through a French pressure cell at 10 000 psi, and the debris was removed by centrifugation at 12000*g* for 15 min at 4 °C.

Endo IV Purification. Native endo IV and the mutant polypeptides were purified from total extracts derived from 2 L of cells grown as described above. The endo IV proteins were purified as previously described (*5*), except the heating step after gel filtration was omitted in order to prevent any heat-induced changes in the endo IV mutant polypeptides.

Enzyme Assays. To prepare the substrates used in the enzymatic assays, [α-^{32}P]dCTP (250 μCi, NEN) in 50% ethanol was dried and resuspended in 50 μL of freshly prepared 3 M $NaNO_2$ in 1 N acetic acid, followed by incubation for 4 h at room temperature. This condition deaminated the dCTP to produce [α-^{32}P]dUTP (*21*). The reaction was neutralized by addition of 62−70 μL of freshly prepared 10% triethylamine (*21*). It is important to empirically check the volume of 10% triethylamine required to neutralize the deaminated dCTP. The entire [α-^{32}P]dUTP was

added to a 3-mL reaction consisting of 67 mM potassium phosphate, pH 7.4, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 20 μM poly[d(A-T)] (Boehringer Mannheim, stock adjusted to 10 absorbance units/mL at 260 nm wavelength in 10 mM Hepes·KOH, pH 7.4, 1 mM EDTA), 150 μg of BSA, 0.5 mM dATP, 0.5 mM dTTP, 40 μCi of [^{3}H]dUTP (10−16 Ci/mmol), and 50 units of DNA polymerase (Klenow fragment). The reaction mixture was incubated at 37 °C for 18−24 h, at which point incorporation of [^{3}H]dUTP should be ~80%, as determined by GF/C filtering. The labeled poly[d(A-T)], which contained both [α-^{32}P]dUTP and [^{3}H]dUTP, was extracted once with phenol and once with phenol/chloroform, followed by extraction with dry *sec*-butanol to reduce the volume to 1 mL, and extraction with ether. The polymer was heated at 70 °C for 3 min and then loaded on a 30 cm AcA54 gel filtration column that was preequilibrated with HE buffer containing 150 mM NaCl. A 1 mL fraction was collected, and the major portion of the label polymer was eluted in the first few fractions, which were pooled and ethanol-precipitated with 3 volumes of 95% ethanol. The pellet was air-dried and resuspended in 500 μL of HE.

To generate substrate containing AP sites, 200 μL of the dUTP-labeled polymer was treated with uracil−DNA glycosylase (BRL) in a 500 μL reaction that contained 35 mM HE (pH 7.6), 50 mM NaCl, 2 mM EDTA, and 25 units of uracil−DNA glycosylase, followed by incubation at 37 °C for 30 min. To generate the 3′-blocked termini, another 200 μL of uracil-containing polymer was treated in a 500 μL reaction with uracil−DNA glycosylase as above, followed by the addition of 25 units of endonuclease III (kindly provided by Dr. Melamede, University of Vermont) at 37 °C for 60 min. Endonuclease III cleaves AP sites to produce the α,β-unsaturated aldehyde product, 4*R*-4-hydroxy-*trans*-2-pentenal, which is efficiently removed by endonuclease IV (*22*). Crude extracts or purified endo IV polypeptides were assayed for both AP endonuclease and 3′-diesterase activities using the above substrates that contained AP sites and 3′-α,β-unsaturated aldehyde, respectively (*6*). A typical assay contains 1 pmol of substrate in a 25 μL reaction mix containing 50 mM Hepes·KOH (pH 7.6), 50 mM KCl, 1 mM EDTA, 100 μg of BSA, and the indicated concentrations of protein extracts or purified protein. In the case of AP endonuclease assay, and after incubation at 37 °C for 20 min, the samples were treated with 6 μL of 50 mM EDTA and 7.5 μL of 1 N NaOH, followed by incubation at 70 °C for 45 min to release the label acid-soluble 5′-deoxyribose phosphate. All reactions were stopped by the addition of 50 μL of Norit carrier (0.5 mg/mL BSA, 25 mM potassium phosphate, 20 mM sodium pyrophosphate), 200 μL of 0.8 M TCA, and 100 μL of acid wash Norit, vortexed, and incubated on ice for 10 min. Samples were centrifuged for 10 min at 12 000 rpm in an Eppendorf centrifuge, and the Norit-nonabsorbable reactivity was counted using 10 volumes of scintillation fluid (Beckman Ready value). One unit of enzyme cleaves 1 pmol of the substrate per minute under the standard reaction conditions (*6*, *23*).

Electrophoretic Mobility Shift Assay. A 5′ end-labeled ^{32}P-41-mer double-stranded oligonucleotide (*10*) was used as a probe for the EMSA. Approximately 0.1 ng of the labeled probe (corresponding to 1.5×10^4 cpm) was incubated with 15 ng of wild-type and mutant endonuclease IV proteins in buffer D (10 mM Hepes−NaOH, pH 7.9, 20 mM KCl, 10% glycerol, 0.1 mM EDTA, 0.5 mM DTT, 1 mM PMSF) for 20 min at room temperature. The formation of protein−DNA complexes was resolved by electrophoresis through a 6% native polyacrylamide gel in high ionic strength Tris−glycine buffer (50 mM Tris, pH 8.8, 380 mM glycine, 2 mM EDTA) (*24*). The gel was migrated at 12 V/cm for 6 h at 4 °C, dried, and revealed by autoradiography. Supershift experiments were carried out as above, except the DNA−protein complexes were incubated with 30 μg of either preimmune or rabbit antiserum raised against the endonuclease IV protein for 30 min at room temperature prior to being loaded on the gel.

RESULTS

Isolation of Endo IV Mutants by Lack of Complementation of the DNA Repair-Deficient Strain BW528. The *E. coli* strain BW528 (*xth*$^-$ *nfo*$^-$) lacks the two bifunctional DNA repair enzymes endo IV and exo III. These enzymes are required to repair AP sites produced, for example, by the alkylating agent methyl methanesulfonate (MMS), as well as single-strand breaks with blocked 3′-termini produced by oxidants, such as bleomycin and H_2O_2 (*9*, *13*). As a consequence, strain BW528 is hypersensitive to MMS and to bleomycin, as compared to the parent strain AB1157 (*Xth Nfo*) (Figure 1) (*13*). These drug hypersensitivities can be readily visualized on gradient plate assays, on which DNA repair-proficient strains grow all along the drug gradient while repair-deficient strains grow only a short distance (Figure 1). The plasmid pDR24 was previously constructed to express endo IV by placing the coding region of the *nfo* gene under the control of the IPTG-inducible promoter *lac* in the vector pKEN2 (*20*). When this plasmid was introduced into strain BW528, it conferred full MMS and bleomycin resistance (Figure 1). pDR24 also conferred to strain BW528 resistance to H_2O_2 and *tert*-butylhydroperoxide (data not shown). The empty vector pKEN2 restored no drug resistance to strain BW528 (Figure 1). Since no IPTG was included in the drug gradient, it would appear that under the noninducing condition, the *lac* promoter is capable of directing the expression of enough endo IV required to repair both MMS- and bleomycin-induced DNA lesions (Figure 1) (*13*).

The plasmid pDR24 was therefore used to identify base-substitution mutations within the *nfo* gene that would alter drug resistance to strain BW528. Briefly, pDR24 was first treated with nitrous acid under conditions that generate a single mutation per plasmid (*19*), and mutations within the *nfo* coding region were permanently fixed by PCR amplification (see Experimental Procedures). The amplified *nfo* gene was used to replace the cognate fragment in pDR24, which was not previously exposed to the chemical treatment, and directly introduced into strain BW528. At least 6000 independent transformants were initially tested for drug sensitivities by streaking the colonies onto a solid LB agar plate (plate assay) containing either MMS (9 μM) or bleomycin (0.5 μg/mL), which permitted growth of strain BW528/pDR24, but not strain BW528/pKEN2 (data not shown). The preliminary assay identified 39 transformants of strain BW528 that no longer grew in the presence of either MMS or bleomycin (see below). It should be noted that no transformant was found that selectively showed resistance either to MMS or to bleomycin. We presumed that these 39

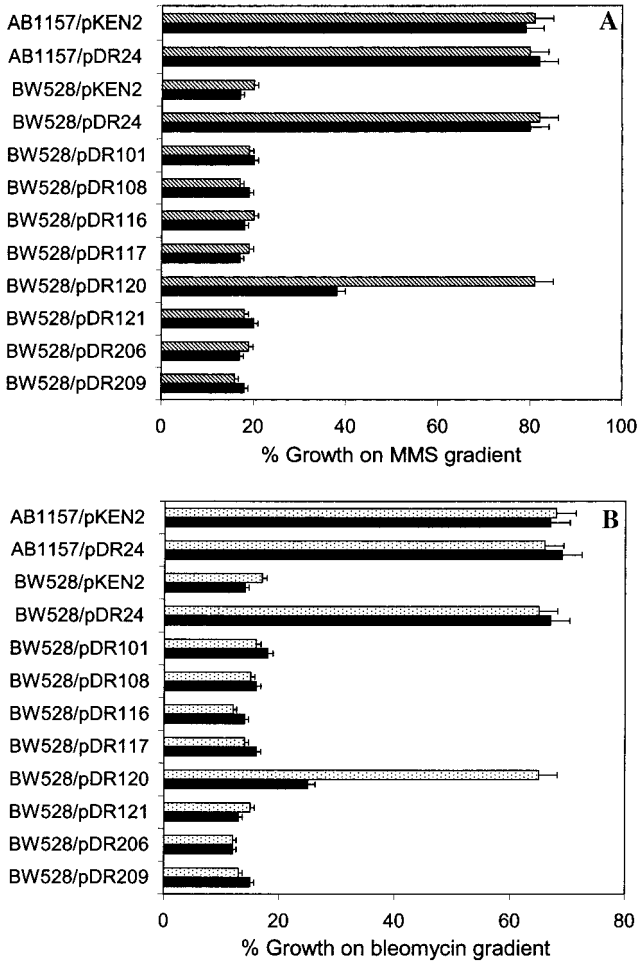

FIGURE 1: Drug resistance conferred to *E. coli* strains bearing the native and mutant *nfo* genes. AB1157 (xth^+, nfo^+) is the parent of BW528 (xth^-, nfo^-). The results were obtained from gradient plate assays where the bottom layer contained either 0.4 mmol of methyl methanesulfonate (MMS, panel A) or 50 μg of bleomycin (panel B). Growth all along the gradient is considered to be 100%. Black bars and hatched or dotted bars represent results obtained when the gradient plate assays were performed in the absence and the presence of 0.1 mM IPTG, respectively.

transformants harbored mutations specifically within the *nfo* gene carried by plasmid pDR24. Such mutations could either (i) prevent expression of the endo IV polypeptide due to an early stop codon, or (ii) produce inactive truncated proteins, or (iii) generate highly unstable protein, and or, most interestingly, (iv) create stable full-length polypeptides that are functionally inactive.

To directly screen for mutants producing functionally inactive full-length endo IV polypeptide, crude extracts were prepared from all 39 colonies and examined for expression of the protein by Western blot analysis using anti-endo IV polyclonal antibodies raised against purified endo IV. Only eight colonies, designated BW528/pDR101; BW528/pDR108; BW528/pDR116; BW528/pDR117; BW528/pDR120; BW528/pDR121; BW528/pDR206; and BW528/pDR209, were found to express a polypeptide with comparable size as the native endo IV (Figure 2, top panel). These 8 plasmid-bearing colonies were chosen for further studies, as the remaining 31 colonies either expressed no visible or various truncated endo IV polypeptides (data not shown). There appears to be some microheterogeneity that subsisted among two of the eight mutant polypeptides; i.e., pDR116 and pDR117 produced polypeptides that migrated slightly faster on the SDS gel (Figure 2, top panel). However, these forms are not due to premature stop codons (see below) and may reflect some structural deformation. More importantly, five of the plasmids (pDR101, pDR108, pDR116, pDR117, and pDR121) expressed significantly less (2.5-, 2.5-, 3.0-, 5.0-, and 2.0-fold, respectively) of endo IV polypeptide, as compared to the level produced by pDR24 (Figure 2, top panel). Two of the remaining three plasmids (pDR120 and pDR209) expressed an intermediate level (1.5-fold lower) of endo IV polypeptide, whereas the final plasmid, pDR206, appeared to express a comparable protein level (1.25-fold lower) as pDR24 (Figure 2, top panel). Since each plasmid is derived from the same vector background, the difference in protein expression might be a reflection of structurally altered endo IV polypeptides that are more susceptible to the action of proteases. In fact, the endo IV polypeptide produced by pDR117 was often accompanied by the appearance of faintly detected lower molecular weight polypeptide species, which could originate from the full-length protein by proteolytic processing (Figure 2, bottom panel). Incubation of the crude extracts at 37 °C for 15 min did not induce any significant turnover either of the native or of any of the endo IV mutant proteins (data not shown). This suggests that the processing of some of the mutant endo IV polypeptides may actually occur in vivo.

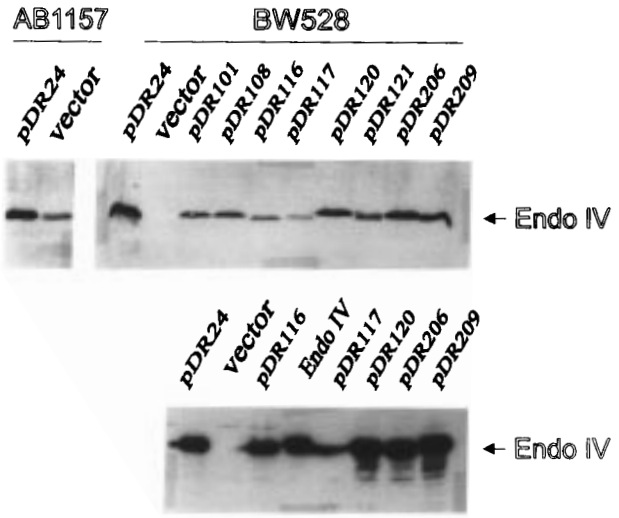

FIGURE 2: Comparison of the expression levels of native endo IV and its mutant forms by Western blot analysis. Each lane contains 20 μg of total extracts derived from the indicated plasmid-bearing strains. Arrows indicate the positions of purified endo IV (31 kDa). The top and bottom panels show extracts derived from log-phase cells untreated or treated with 0.1 mM IPTG for 4 h, respectively.

Level of Drug Resistance Conferred by Mutant Plasmids. It was difficult to assess by the plating assay whether any of the eight chosen plasmids conferred partial drug resistance to strain BW528. A rapid semiquantitative analysis was therefore performed using the gradient plate assay, as described above. Among the eight plasmids, only pDR120 showed a modest resistance to both MMS and bleomycin, as compared to strain BW528 harboring the empty vector (Figure 1A,B). Since a higher level of the endo IV polypeptide can be attained from the IPTG-inducible lac promoter (Figure 2, bottom panel), we tested whether the plasmids can provide enhanced drug resistance when IPTG is included in the gradient plate assay. As shown in Figure 1A,B, among

Table 1: Levels of AP Endonuclease and 3′-Diesterase in Crude Extracts Derived from Plasmid-Bearing Strains[a]

		AP endonuclease (units/mg of protein)		3′-diesterase (units/mg of protein)	
host strain	plasmid	−IPTG	+IPTG	−IPTG	+IPTG
AB1157	pKEN2	4.0	5.0	2.0	2.0
	pDR24	29.0	369.0	13.8	132.2
BW528	pKEN2	<0.04	<0.04	<0.02	<0.02
	pDR24	20.3	301.4	11.5	123.2
	pDR101	<0.04	<0.04	<0.02	<0.02
	pDR108	<0.04	<0.04	<0.02	<0.02
	pDR116	<0.04	<0.04	<0.02	<0.02
	pDR117	<0.04	<0.04	<0.02	<0.02
	pDR120	0.4	5.0	0.12	1.8
	pDR121	<0.04	<0.04	<0.02	<0.02
	pDR206	<0.04	<0.04	<0.02	<0.02
	pDR209	<0.04	<0.04	<0.02	<0.02

[a] Crude extracts were prepared as described and quantified for endo IV activities. EDTA was added in the assay at 5 mM to suppress exonuclease III detection.

the eight mutant plasmids, only pDR120 conferred full parental drug resistance to strain BW528 in the presence of IPTG. This finding can be rationalized if pDR120 produces a protein with a suboptimal level of enzymatic activities that can be compensated for by enhanced protein production.

Levels of AP Endonuclease and 3′-Diesterase in Crude Extracts Derived from the Strains Carrying the nfo Mutant Plasmids. The inadequacy of the *nfo* mutant plasmids to complement strain BW528 drug sensitivities could be a direct reflection of the level of expressed AP endonuclease and 3′-diesterase. As such, crude extracts were prepared from the plasmid-bearing strains and quantified for endo IV activities. Total extracts derived from strain BW528 carrying the empty vector expressed extremely low, but detectable, levels of AP endonuclease and 3′-diesterase activities, as compared to the parent strain AB1157 (Xth, Nfo) (Table 1) (*13*). It should be noted that in the parent strain any contribution of AP endonuclease activity from the Mg^{2+}-dependent enzyme, exonuclease III, should be completely suppressed by inclusion of the Mg^{2+} chelator EDTA. Extracts derived from strain BW528 carrying plasmid pDR24 expressed nearly 5-fold higher level of activities than the parent strain with the empty vector (Table 1, 20.3 vs 4.0 units/mg of protein). This finding is consistent with the higher production of endo IV polypeptide from the pDR24 plasmid, as compared to the normal expression in the parent strain AB1157 (Figure 2, top panel). Among the eight *nfo* mutant plasmids, only pDR120 expressed a very low level of both AP endonuclease and 3′-diesterase activities, approximately 10% of the parental (AB1157) level (Table 1). Like pDR24, IPTG-inducible activities were observed with plasmid pDR120, but not with the seven other plasmids (Table 1). The lack of IPTG-inducible activities from the plasmids pDR101, pDR108, pDR116, pDR117, pDR121, pDR206, and pDR206 cannot be related to lack of protein production, as these plasmids showed at least 15-fold induction of endo IV polypeptide (Figure 2, bottom panel). Two conclusions can be drawn from these results: (i) The partial drug resistance conferred by pDR120 to strain BW528 is the result of lower levels of endo IV DNA repair activities; and (ii) the seven plasmids, excluding pDR120, appear to express protein with more severe enzymatic deficiency.

Table 2: Amino Acid Substitutions Altering Endo IV Biological Functions

mutant number	base pair substitutions	single amino acid change	region mutated	drug resistance MMS/BLM	*x*-fold reduction
120	$T^{259}CG-cCG$	S87P	I	P/P	10
117	$T^{292}GC-cGC$	C98R	I	−/−	55
116	$GT^{428}G-GaG$	V143E	II	−/−	60
206	$GA^{434}A-GgA$	E145G	II	−/−	150
121	$TGC^{543}-TGg$	C177W	III	−/−	30
209	$GA^{536}T-GgT$	D179G	III	−/−	40
108	$A^{574}CT-gCT$	T192A	III	−/−	25
101	$CT^{779}C-CcC$	L260P	V	−/−	30
		Site-Directed Mutants			
301	$GA^{434}A-GgA$	E145G*	II	−/−	
302	$TGC^{543}-TGg$	C177W*	III	−/−	
303	$GA^{536}T-GgT$	D179G*	III	−/−	
304	$CT^{779}C-CcC$	L260P*	V	−/−	
305	$T^{541}GC-gcC$	C177A*	III	−/−	
306	$GA^{536}T-GcT$	D179A*	III	−/−	
307	$T^{553}GC-gcC$	C181A*	III	F/F	
308	$AA^{662}A-Acc$	K221T*	IV	F/F	

[a] Normal nucleotides are shown in uppercase letters, and the substituted bases are marked by lowercase letters. The nucleotide numbering is relative to the first nucleotide of the ATG start codon of the *nfo* gene. Asterisks denote amino acid changes generated by site-directed mutagenesis. The designations −/−, P/P, and F/F represent no, partial, and full complementation of BW528 drug resistance, respectively. The fold reduction is relative to the native enzyme.

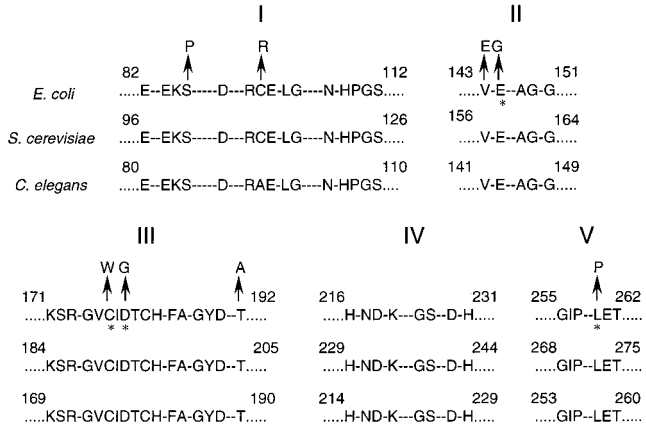

FIGURE 3: Complete conservation of five regions of the endo IV family of AP endonuclease/3′-diesterase DNA repair enzymes. The arrows indicate the single amino acid changes in each of the eight mutants isolated by chemical treatment. The asterisks denote mutants generated by site-directed mutagenesis. Numbers mark the corresponding position of the amino acid residues at the beginning and ending of each conserved region for the indicated members with respect to the first methionine of the open reading frame.

The Endo IV Mutants Bear Single Amino Acid Change. To identify the mutations that affected endo IV enzymatic activities, the entire *nfo* coding region of each plasmid was sequenced in both directions. All of the plasmids were found to contain a single base pair substitution in the *nfo* gene leading to a single amino acid change (Table 2). For simplicity, the mutations in the *nfo* gene will hereafter be referred to by the amino acid change (Table 2). Most of the mutations were localized to four of the five highly conserved regions that exist among the endo IV functional and structural homologues, including *S. cerevisiae* Apn1 and *C. elegans* CeApn1 (Figure 3) (*2*). Despite its high conservation, no mutation was identified in region IV (Figure 3). It is possible that either region IV is refractory to mutation by the chemical

Table 3: Level of Drug Resistance Conferred by the Native and Endo IV Mutants[a]

	% growth on drug gradient			
	MMS		bleomycin	
strain BW528	−IPTG	+IPTG	−IPTG	+IPTG
native endo IV	81	82	67	68
none	16	17	13	11
E145G	18	19	11	14
E145G*	17	20	14	16
C177W	18	16	12	13
C177W*	17	20	16	15
C177A*	16	18	13	14
D179G	19	16	15	12
D179G*	18	17	14	11
C181A*	79	81	65	69
K221T*	68	82	53	67
L260P	17	18	17	18
L260P*	18	16	15	13

[a] The results represent averages of data derived from two independent gradient plate assays. Growth along the full length of the gradient is taken to be 100%.

treatment or it tolerates mutations that do not substantially interfere with endo IV biological functions.

To confirm that these single amino acid substitutions were responsible for altering endo IV function, we recreated four of the endo IV mutants by site-directed mutagenesis and verified their ability to restore drug resistance to strain BW528. These four site-directed mutants, denoted by an asterisk, E145G*, C177W*, D179G*, and L260P*, mirrored the identical pattern of drug resistance to strain BW528 as the mutants obtained by chemical mutagenesis (Table 3). Because the C177W substitution represents a bulky amino acid change (cysteine to tryptophan), we predict that this may cause steric hindrance that could affect, for example, the enzyme catalytic activities, and hence the inability to restore drug resistance to strain BW528. However, replacing tryptophan for alanine to create the C177A* mutant also conferred no drug resistance to BW528 (Table 3), suggesting that Cys 177 is absolutely required for full enzymatic activities of endo IV. Cys 177 is unlikely to constitute a disulfite bridge, as endo IV is not inactivated by the strong reducing agent dithiothreitol and perhaps Cys 177 may provide an attachment site for one of the Zn^{2+} atoms contained by endo IV. Replacement of the immediate downstream Cys 181 with alanine (C181A*) conferred complete drug resistance to BW528 (Table 3), suggesting that Cys 181 is not crucial for endo IV biological function. Since no chemically derived mutants were obtained in region IV, we tested if site-directed mutagenesis in this region might nonetheless interfere with endo IV biological function. As such, Lys 221 was replaced with Thr, but this substitution, K221T*, conferred upon BW528 a slightly reduced level of drug tolerance, as compared to the native enzyme (Table 3). However, normal resistance was achieved if K221T* expression was induced by IPTG (Table 3). Whether other substitutions within region IV have a more drastic effect on endo IV activities was not tested. In any case, the above findings consistently demonstrate that single amino acid substitutions that alter the AP endonuclease activity also affect the 3′-diesterase activity of endo IV, suggesting that both activities are linked to a common catalytic center.

Characterization of Endo IV Mutants. It is possible that some of the mutants engender altered physical properties,

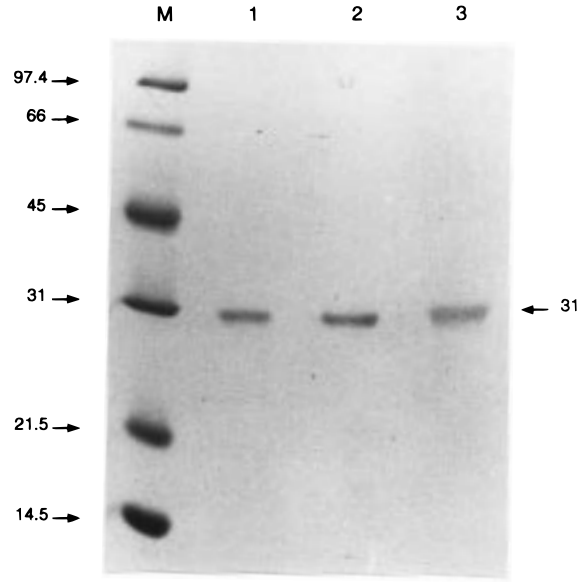

FIGURE 4: Purification of the native and mutant forms E145G and D179G of endo IV. The proteins were purified as described under Experimental Procedures. Lane M, protein standard (kDa); lanes 1 to 3 each contained 1 μg of purified protein fraction of native endo IV, E145G, and D179G, respectively. Proteins were analyzed by 12.5% SDS−PAGE and detected by Coomassie Blue staining.

such as binding to DNA and metal ions, that affect their ability to recognize and process damaged DNA. To explore these possibilities, we first examined if there are differences during the purification to homogeneity of the four chosen mutants, C98R, E145G, D179G, and L260P, respectively, belonging to regions I, II, III, and V, as compared to the native enzyme. Briefly, the proteins were purified from strain BW528 carrying either plasmid pDR24 (native), or pDR117 (C98R), or pDR206 (E145G), or pDR209 (D179G), and/or pDR101 (L260P), grown in the presence of IPTG to induce the production of endo IV protein. Crude extracts were prepared and subjected to a sequential four-step purification that includes ammonium sulfate precipitation, gel filtration, binding to single-stranded DNA, and ion exchange, and the purity of three of these proteins, native, E145G, and D179G, was assessed by Coomassie staining (Figure 4). There was no distinct difference during the first two steps of purification of the mutant proteins with respect to yield, as compared to the native protein (data not shown). However, in the third step of purification, only the E145G mutant was observed to bind very weakly to single-stranded DNA (Figure 5A). While the majority of native, or C98R, or D179G, and/or L260P protein eluted at a NaCl concentration of ∼250 mM, the E145G mutant protein eluted at a much lower salt concentration (∼160 mM) as determined by Western blot analysis (Figure 5A; for simplicity, data shown for native and E145G). Moreover, the amount of the E145G mutant protein retained by the single-stranded DNA agarose column was less than 25% of the amount of the recovered native endo IV protein (Figure 5A). Most of the E145G protein was recovered in the flow through fraction (Figure 5A). This finding suggests that relative to the native endo IV, E145G has weak affinity for single-stranded DNA. Despite this defect, a sufficient quantity of E145G mutant protein could be obtained by repeat loading of protein fractions on the column. It should be noted that the recovery of C98R, D179G, and L260P mutant proteins from the single-stranded

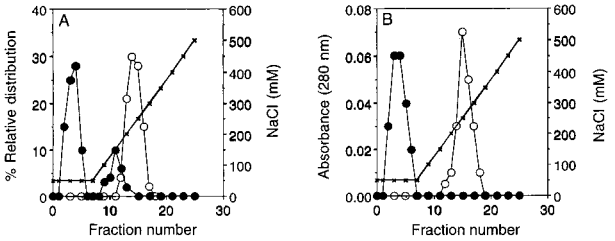

FIGURE 5: Elution profile of the native endo IV and the mutant E145G from single-stranded and double-stranded DNA agarose columns. Panel A: the gel filtration fraction was applied onto the single-stranded DNA column, and endo IV or endo IV mutant E145G polypeptide elution was monitored by Western blot analysis using anti-endo IV polyclonal antibodies. Panel B: a fixed amount (3 μg) of each of the purified endo IV proteins was subjected to chromatography on a double-stranded DNA agarose column, and protein elution was determined by the absorbance at $A_{280\ nm}$. Open circles, native endo IV: closed circles, mutant E145G; crosses, linear salt gradient.

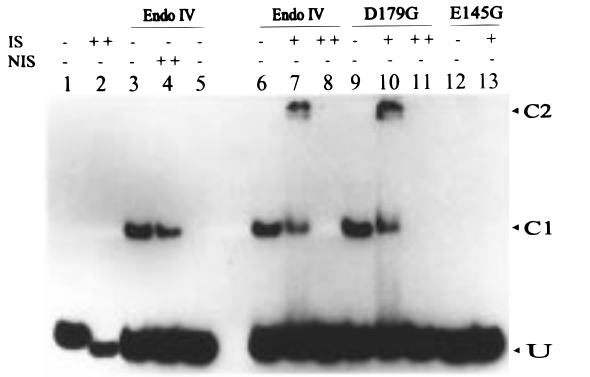

FIGURE 6: Mobility-shift analysis of a 41-mer-labeled double-stranded oligonucleotide by native endo IV and the mutants E145G and D179G. Lanes 3−13 contained 10 ng of the purified protein. Lane 5, the purified endo IV was preincubated with 1 mM EDTA for 48 h at room temperature before addition of the 41-mer-labeled double-stranded oligonucleotide. The single + (30 μg) or double ++ (90 μg) represent the amount of immune serum (IS) or nonimmune serum (NIS) preincubated with the indicated protein after addition of the probe. Neither IS (lane 2) nor NIS alters the mobility of the probe (data not shown).

DNA agarose was not different from the native endo IV protein (data not shown).

Based on the above finding, we predict that the E145G mutant would also bind relatively poorly to double-stranded DNA. To test this, a fixed amount (3 μg) of either purified native endo IV or purified E145G was subjected to chromatography on a double-stranded DNA agarose column. As shown in Figure 5B, the major portion of endo IV eluted at a NaCl concentration of ~250 mM, as determined by the optical density at 280 nm. In contrast, when the same amount of the E145G mutant protein was applied to the double-stranded DNA column, virtually none of the protein was withheld and found exclusively in the flow through fractions (Figure 5B). This finding is consistent with the notion that E145G harbors a defect in binding DNA.

The DNA binding defect of the E145G mutant was not restricted to the type of double-stranded DNA, as this mutant protein was also unable to bind to a random 41-mer ^{32}P-labeled double-stranded oligonucleotide when assessed by the electrophoretic mobility-shift assay (Figure 6). In this assay, the purified native endo IV or the purified D179G mutant protein shifted the mobility of the oligonucleotide to produce complex C1 (lanes 6 and 9, respectively). In contrast, purified E145G did not form any complex with the oligonucleotide (lane 12). The foregoing data suggest that Glu 145 of endo IV may be required to coordinate binding of the protein to double-stranded DNA, although we cannot exclude the possibility that E145G could cause a conformational change in the protein thereupon preventing proper interaction with the DNA. Premixing either purified native endo IV or purified D179G protein with the oligonucleotide, and following addition of anti-endo IV polyclonal antibodies, resulted in the formation of a new super-shifted complex C2 (Figure 6, lanes 7 and 10, respectively). The formation of the latter complex was solely dependent on the endo IV polypeptide and the anti-endo IV antibody, as no such complex was formed in the absence of endo IV (lane 2) or with the nonimmune serum (lane 4). Surprisingly, addition of a 3-fold excess of the anti-endo IV antibody completely blocked the appearance of both C1 and C2 complexes, suggesting that the antibody may mask the endo IV DNA binding site. However, the excess antibody did not inhibit endo IV enzymatic activities (Table 4). These two latter observations can be accounted for by the assumption that endo IV has at least two distinct domains, a DNA binding domain and a catalytic domain.

Table 4: Effect of Immune Serum and EDTA on Endo IV AP Endonuclease Activity

purified endo IV (ng)	immune serum (μg)	EDTA (mM)	% activity (AP endonuclease)
10	0	0	100
10	20	0	100
10	50	0	100
10	100	0	100
10	200	0	90
none	50	0	0
none	200	0	0
10	0	1 mM/1 h	100
10	0	1 mM/12 h	80
10	0	1 mM/24 h	40
10	0	1 mM/48 h	2

[a] Endo IV was incubated with the indicated amount of either immune serum for 30 min on ice or EDTA at room temperature before measuring the AP endonuclease activity.

Since phosphodiesterases are known to use divalent ions to facilitate interaction and cleavage of DNA (*25*, *26*), it is conceivable that endo IV could use one of its tightly associated metal ions to coordinate binding to the DNA. As such, the E145G mutant could have a reduced affinity for the metal ions, thereby causing the DNA binding defect. To test this, purified E145G was extensively dialyzed against buffers containing either 1.0 mM $ZnCl_2$ or $MnCl_2$ or a combination of both divalent ions. Under these conditions, the E145G protein still did not bind to the double-stranded DNA (data not shown). Thus, it is unlikely that E145G has a reduced affinity for metal ions. However, we cannot rule out the possibility that the E145G mutant partially or completely lost the ability to bind the key divalent ion essential for contacting the DNA (see Discussion).

The Endo IV Mutants Are Highly Inefficient at Processing AP Sites and Blocked 3′-Termini. To quantify the enzymatic efficiencies of the endo IV mutants, the purified proteins were quantified for both AP endonuclease and 3′-diesterase activities using synthetic substrates (*6*, *23*). While the purified

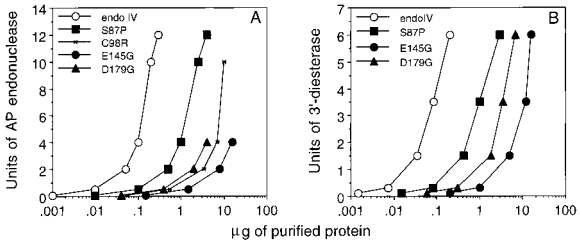

FIGURE 7: Measurement of AP endonuclease and 3′-diesterase activities of native endo IV and its mutant forms. Purified proteins were assayed for enzymatic activities using synthetic substrates.

native endo IV was proficient at cleaving the AP site, the mutants S87P, C98R, E145G, and D179G were highly inefficient, showing 10-, 55-, 150-, and 40-fold reduction in AP endonuclease activity, respectively (Figure 7A). These endo IV mutants also showed a parallel reduction of the 3′-diesterase activity (Figure 7B; data shown only for native, S87P, E145G, and D179G). The remaining four mutant proteins, V143E, C177W, T192A, and L260P, also exhibited a residual level of enzymatic activities, manifesting a decrease of 60-, 30-, 25, and 30-fold, respectively (Table 2). It is clear that none of the eight endo IV mutants was completely devoid of enzymatic activities. Altogether, these data confirm that the lack of drug resistance conferred by the endo IV mutants is a direct result of diminished enzymatic activities.

DISCUSSION

Chemical and site-directed mutageneses were employed to identify several amino acid substitutions that sharply interfere with the ability of endo IV to perform its normal biological function in repairing damaged DNA. Each of the eight mutants showed parallel decreases in both AP endonuclease and 3′-diesterase activities, raising the possibility that the activities are governed by a single active site. If endo IV possesses a single active site, then no mutants should be expected that show preferential resistance to either one of the DNA damaging agents. Indeed, our search for endo IV mutants did not reveal any that shows selective sensitivity to either MMS or bleomycin. It is documented that members of the exonuclease III family manifest a single catalytic center for both AP endonuclease and 3′-diesterase (*27*, *28*). For example, replacement of any one of the human Hap1 active site amino acid residues (i.e., Glu 96, Asp 283, and His 309 as predicted from the X-ray crystal structure of exonuclease III) (*16*) drastically reduces to varying extents (400−2000-fold) both AP endonuclease and 3′-diesterase activities of the protein (*27*, *29*). Thus, it would appear that these dual-function enzymes, belonging to the endo IV and the exo III families, possess a single catalytic center. However, there are two independent reports where mutants have been identified which show normal resistance to MMS, and either no or partial resistance to oxidants. In one study, a single endo IV mutant, G149D, was isolated by chemical mutagenesis and found to fully complement the MMS, but not H_2O_2, sensitivity of a *xth*$^-$ *nfo*$^-$ double mutant strain (*30*). Moreover, the G149D mutant protein was purified and shown to fully repair AP sites, but not H_2O_2-induced DNA lesions (*30*). In the second study, Rrp1 (a *Drosophila* homologue of exonuclease III) mutants T462A and K463A restored MMS resistance, but not *tert*-butylhydroperoxide resistance, when expressed in a bacterial *xth*$^-$ *nfo*$^-$ double mutant strain (*28*). The differential drug resistances and the corresponding DNA repair defect observed by these latter mutants can be explained if the mutants bear subtle structural alterations that prevent proper recognition of single-strand breaks with blocked 3′-ends.

From our data, no assignments can be made with regard to the amino acids that are directly involved in the reaction mechanism of phosphodiester bond cleavage, as no stable substitutions were identified that completely abrogate the enzyme activities. Instead, at least five (C98R, V143E, C177W, T192A, and L260P) of the eight substitutions apparently render structural changes that cause destabilization of the endo IV polypeptide. Such structural defects may also account for the reduced enzymatic activities. Thus, in the natural protein Cys 98, Val 143, Cys 177, Tyr 192, and Leu 260 are essential for preservation of the proper tertiary structure of the protein. Because a majority of the substitutions affected the stability of endo IV, it is plausible that replacement of the active site amino acid residues may actually produce mutants that are even more unstable in vivo. Such mutants will not be detected by our screening procedure, which scored mutants on the basis of endo IV polypeptide expression. Evidently, correct prediction of the active site amino acids of endo IV will be greatly simplified once its X-ray crystal structure is unraveled.

Among all the mutants, E145G shows the most severe defect. It completely lost the ability to bind double-stranded DNA and shows a significant reduction in DNA repair activities, as much as 150-fold. The enzymatic deficiency of E145G is not surprising considering endo IV may require proper contact with the DNA before the ensuing catalytic function. Thus, Glu 145 could be the key amino acid in DNA recognition, or in making direct contact with DNA. If Glu 145 facilitates endo IV binding to DNA, how might this occur? Several enzymes are known to use metal cofactors to either directly or indirectly bind DNA (*31*−*35*). It is reasonable to assume that endo IV could use the side chain of Glu 145 to bind DNA via one of its Zn^{2+} ions. If so, the E145G endo IV mutant would be expected to have a lower metal ion content, as opposed to the native enzyme. Although this experiment has not been undertaken here, a related mutant, E145Q, was recently generated by site-directed mutagenesis and found to contain a reduced Zn^{2+} content, but this mutant remains uncharacterized particularly with respect to its ability to bind DNA (B. Haas and R. P. Cunningham, personal communication). The need for metal ions to contact the DNA is further supported by the finding that extensive treatment of native endo IV with the divalent metal ion chelator EDTA abolishes binding of the apoprotein to DNA (Figure 6, lane 5).

The fact that the E145G mutant lacks the ability to bind DNA while retaining enzymatic activities, albeit very low, may suggest that binding of endo IV to DNA is not a prerequisite for phosphodiester bond cleavage. Consistent with this interpretation is the observation that while anti-endo IV antibody can block endo IV from interacting with DNA, it does not interfere with the enzymatic activities. From these preliminary accounts, it would appear that the DNA binding region of endo IV is separate from its catalytic domain. Alternatively, we cannot preclude the possibility that the poor enzymatic activities of E145G arise if Glu 145 also

has a role in facilitating the catalytic mechanism. In such a case, Zn^{2+}-mediated Glu 145 contact with DNA may act to polarize the phosphodiester bond for nucleophilic attack by the enzyme, as proposed for exonuclease III (*16*). This latter enzyme uses the side chain of Glu 34 bound to Mg^{2+} to enhance cleavage of the phosphodiester bonds (*16*).

In summary, we provide compelling evidence that the AP endonuclease and 3′-diesterase activities of endo IV are linked to a common catalytic center, and that Glu 145 may be required to mediate DNA contact or recognition. Identification of the precise amino acid residues involved in the catalytic mechanism would necessitate determination of the high-resolution X-ray crystal structure of endo IV. Such information would also be useful to delineate the feature(s) that empower(s) endo IV with broader substrate specificity, and perhaps a compromise of the 3′- to 5′-exonuclease activity, as compared to exonuclease III.

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