Active Site Plasticity of Endonuclease V from *Salmonella typhimurium*†

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ABSTRACT: Base deamination is a major type of DNA damage under nitrosative stress. Endonuclease V initiates repair of deaminated base damage by making a nucleolytic incision one nucleotide away from the 3′ side of the lesion. Within the endonuclease V family, the substrate specificities are different from one enzyme to another. In this study, we investigated deamination lesion cleavage activities of endonuclease V from the macrophage-residing pathogen, *Salmonella typhimurium*. *Salmonella* endonuclease V exhibits limited turnover on cleavage of deoxyinosine- and xanthosine-containing DNA. Binding analysis indicates that this single-turnover property is caused by tight binding to nicked products. The nicking activity is similar between the double-stranded deoxyinosine- and deoxyxanthosine-containing DNA. Cleavage rates are not affected by bases opposite the deoxyinosine or deoxyxanthosine lesions. The enzyme is also active on single-stranded deoxyinosine- and deoxyxanthosine-containing DNA. Unlike endonuclease V from *Thermotoga maritima*, *Salmonella* endonucleae V can only turnover deoxyuridine-containing DNA to a limited extent when substrate is in excess. Binding analysis indicates that *Salmonella* endonuclease V achieves tight binding to deoxyuridine-containing DNA, a property that distinguishes it from *Thermotoga* endonuclease V. Cleavage analysis on mismatch-containing DNA also indicates that the active site of *Salmonella* endonuclease V can accommodate pyrimidine-containing mismatches, resulting in more comparable cleavage of pyrimidine- and purine-containing mismatches. This comprehensive DNA cleavage and binding analysis reveals the plastic nature in the active site of *Salmonella* endonuclease V, which allows the enzyme to enfold both purine and pyrimidine deaminated lesions or base pair mismatches.

Endonuclease V is a DNA repair enzyme which hydrolyzes the second phosphodiester bond 3′ from a base lesion (*1*, *2*). Dependent on the sources, some homologues of this enzyme have broad endonuclease activity against deoxyinosine, deoxyxanthosine, uracil, urea, AP site, and mismatches (*1*−*6*). When Mn^{2+} is used as the metal cofactor, the *Escherichia coli* endo V is also capable of cleaving insertions/deletions and FLAP and pseudo Y structures (*7*). The endonuclease V homologue from *Archaeoglobus fulgidus* (Afu),[1] however, has very narrow substrate specificity. Afu endo V demonstrates cleavage activity only on deoxyinosine-containing DNA, suggesting that endonuclease V homologues from different species have significantly different substrate specificity (*8*). The involvement of *E. coli* endonuclease V in repair of deamination DNA base lesions and N^6-hydroxylaminopurine has been substantiated by genetic studies (*9*−*11*). Structural and functional analysis of thermostable endonuclease V from *Thermotoga maritima* (Tma) has identified amino acid residues that affect binding, catalysis, and enzymatic turnover (*12*). For practical applications, the mismatch cleavage property of *E. coli* and Tma endonuclease V has been exploited for development of mutation identification or scanning methods (*13*, *14*).

Endonuclease V is a ubiquitous protein in nature, as its homologues have been found in eubacteria, archaea, and eukaryotes including *Schizosaccharomyces pombe*, *Caenorhabditis elegans*, *Arabidopsis thaliana*, rats, mice, and humans. This suggests that endonuclease V-mediated repair is an ancient pathway, and repair of base deamination caused by nitrosative stress is an important process that has been retained during evolution in many organisms. Many thermophilic bacteria and archaea contain an endo V orthologue, suggesting its role in deoxyinosine repair due to accelerated adenine deamination at high temperatures (*15*).

Endonuclease V homologues have been identified in several pathogenic bacteria including *Salmonella typhimurium* and *Yersinia pestis*. Yet, the biochemical properties of endonuclease V in microbial pathogens are unknown. *S. typhimurium* is a leading cause of human gastroenteritis (*16*). Its ability to reside in macrophages, which impose oxidative and nitrosative stress, makes it an interesting model that could be used to understand the biochemical properties of endonuclease V in pathogenic organisms. It is well-known that nitrosative stress causes DNA base deamination (*17*−*19*). We herein report a comprehensive biochemical analysis of

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[1] Abbreviations: Afu, *Archaeoglobus fulgidus*; BSA, bovine serum albumin; dNTP, deoxyribonucleoside triphosphate; DTT, dithiothreitol; 6-Fam, 6-carboxyfluorescein; *E. coli*, *Escherichia coli*; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; I, deoxyinosine; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; Tet, 4,7,2′,7′-tetrachloro-6-carboxyfluorescein; Sty, *Salmonella typhimurium*; Tma, *Thermotoga maritima*; U, deoxyuridine; wt, wild type; X, deoxyxanthosine.

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Salmonella endonuclease V. To the best of our knowledge, this is the first report of a detailed kinetic analysis of endonuclease V using double-stranded and single-stranded deoxyuridine-, deoxyinosine- and deoxyxanthosine-containing DNA substrates. This work demonstrates that endonuclease V from *S. typhimurium* (Sty) has broad substrate specificities against various deamination base lesions. Its enzymatic properties are compared with other endonuclease V family proteins. Sty endonuclease V shows unique biochemical properties that are distinct from endonuclease V homologues from *A. fulgidus*, *T. maritima*, and *E. coli*. A major finding is that Sty endo V exhibits tight binding not only to purine deaminated base lesions but also to the pyrimidine deaminated base lesion deoxyuridine. These results indicate that the active site of this enzyme has adapted a high degree of plasticity to accommodate all deaminated base lesions.

MATERIALS AND METHODS

Reagents, Media, and Strains. All routine chemical reagents were purchased from Sigma Chemicals (St. Louis, MO), Fisher Scientific (Suwanee, GA), or VWR (Suwanee, GA). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). DNA sequencing kits were purchased from Applied Biosystems (Foster City, CA). BSA, dNTPs, and pGEMT-EASY were purchased from Promega (Madison, WI). *Taq* DNA polymerase was purchased from Eppendorf (Hamburg, Germany). The protein assay kit, the horseradish peroxidase substrate Opti-4CN for western blot, and PVDF membrane were purchased from Bio-Rad (Hercules, CA). Anti-His (C-term)-HRP antibody was purchased from Invitrogen (Carlsbad, CA). HiTrap chelating, SP, and Q columns were purchased from Amersham-Pharmacia Biotech (Piscataway, NJ). Oligodeoxynucleotides were ordered from Integrated DNA Technologies Inc. (Coralville, IA). LB medium was prepared according to standard recipes. Sty endoV sonication buffer consisted of 20 mM HEPES−KOH (pH 7.4), 1 mM EDTA (pH 8.0), 0.5 mM DTT, 0.1 mM PMSF, and 50 mM NaCl. PBS buffer (pH 7.4) consisted of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2PO_4, and 2 mM KH_2PO_4. GeneScan stop buffer consisted of 80% formamide (Amresco, Solon, OH), 50 mM EDTA (pH 8.0), and 1% blue dextran (Sigma Chemicals). TB buffer (1×) consisted of 89 mM Tris base and 89 mM boric acid. TE buffer consisted of 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA. *E. coli* host strain BL21-(DE3) [F^-, *omp*T, *hsd*S_B, (r_B-, m_B-), *dcm*, *gal*, λ (DE3)] and JM109 [*end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17(r_k-,m_k+), *rel*A1, *sup*E44, Δ (*lac-proAB*), [F′, *tra*D36, *proAB*, *lacI*^{q}ZΔ-M15]] were from our laboratory collection.

Plasmid Construction, Cloning, and Expression of Sty Endo V. The putative endo V gene from *S. typhimurium* strain 14028 was amplified by PCR using the forward primer EV.Sty.01 (5′ GTC GGA TCC CAT ATG GAT CTC GCG TCG CTA CG 3′; the *Nde*I site is underlined) and the reverse primer EV.Sty.02R (5′ CGC GGA TCC AAG CTT GCG CTG AAT TTC TTG CCA AC 3′; the *Hin*dIII site is underlined). The PCR reaction mixture (100 μL) consisted of 10 ng of *S. typhimurium* strain 14028 genomic DNA (a kind gift from Dr. Ferric Fang), 400 nM forward primer EV.Sty.01 and reverse primer EV.Sty.02R, 1× *Taq* PCR buffer (Eppendorf), 125 μM each dNTP, and 2 units of *Taq* DNA polymerase. The PCR procedure included a predenaturation step at 94 °C for 4 min, 32 cycles of three-step amplification with each cycle consisting of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s and extension at 72 °C for 1 min, and a final extension step at 72 °C for 15 min. The PCR product was sliced from 1% agarose gel and purified by routine phenol extraction and ethanol precipitation. The purified PCR product was directly ligated with pGEMT-Easy according to the manufacturer's instruction manual. The ligation mixture was transformed into *E. coli* strain JM109 competent cells prepared by a $CaCl_2$ method (*20*). The resulting plasmid pGEMT-StyV was digested with *Nde*I and *Hin*dIII. The Sty endo V gene was recovered from 0.7% agarose gel and ligated to pET21a+, which was digested with the same pair of restriction enzymes. The Sty endo V gene in the resulting plasmid (pET-Sty) was confirmed by DNA sequencing. To express the C-terminal His-6-tagged Sty endo V, pET-Sty was transformed into *E. coli* strain BL21(DE3) by standard protocol (*20*).

An overnight *E. coli* culture containing pET-Sty was diluted 100-fold into LB medium supplemented with 50 μg/mL ampicillin and 0.2% glucose. The *E. coli* cells were grown at 37 °C while being shaken at 250 rpm until the optical density at 550 nm reached about 0.6. IPTG was added to a final concentration of 1 mM. The culture continued to grow under the above condition for an additional 3.5−4 h. The cells were collected by centrifuging at 4000 rpm at 4 °C and washed once with precooled PBS buffer. The cell paste was stored at −20 °C prior to use.

To purify the Sty endo V protein, the cell paste from a 1 L culture was suspended in 10 mL of sonication buffer and sonicated at output 5 for 3 × 1 min with 5 min rest on ice between intervals. The sonicated solution was clarified by centrifuging at 12000 rpm at 4 °C for 20 min. The supernatant was transferred into a fresh tube and loaded into a 5 mL HiTrap chelating column. The bound protein in the column was eluted with a linear gradient (10 column volumes) of 0−1 M imidazole in chelating buffer B [20 mM Tris-HCl (pH 7.6) and 50 mM NaCl] using a Bio-Rad BioLogic chromatographic system. Fractions containing the Sty endo V protein as seen on 15% SDS−PAGE were pooled and dialyzed against HiTrap Q column buffer A [20 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.2 mM DTT] overnight at 4 °C. The dialysis sample was then loaded onto a 5 mL HiTrap Q column. The Sty endo V protein did not bind to the Q column and was eluted in the flow-through fraction. The flow-through fraction was dialyzed against HiTrap SP buffer A [20 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 0.2 mM DTT] at 4 °C overnight. The sample was loaded onto a 1 mL HiTrap SP column and eluted with a linear gradient (10 column volumes, 0−1 M) of NaCl in HiTrap SP buffer B (HiTrap SP buffer A containing 1 M NaCl). The putative Sty endoV protein was eluted at 300−400 mM NaCl. The homogeneity of the protein was examined by 15% SDS−PAGE analysis. The Sty endo V protein concentration was determined on SDS−PAGE using BSA as a standard.

Western blot analysis was carried out using an antibody raised against the C-terminal His tag to confirm that the protein was overexpressed in *E. coli* cells. The protein samples were first separated on 15% SDS−PAGE and then transferred onto a PVDF membrane by electroblotting at 100

V for 1 h using a Bio-Rad Mini Trans-Blot apparatus. The membrane was blocked with 1% low-fat milk. Anti-His (C-term)-HRP antibody (1 μL) diluted in 5 mL of 1% BSA solution was added onto the membrane sealed in a plastic bag at room temperature for 1 h while shaking gently. The color reaction was developed using Opti-4CN as a substrate.

Site-Directed Mutagenesis. To exclude the possibility of contamination of *E. coli* proteins in the activity assay, an active site alanine mutant at D35 of Sty endo V was constructed. An overlapping extension PCR procedure was used for site-directed mutagenesis (*21*). The first round of PCR was carried out using pEV.Sty as template DNA with two pairs of primers, EV.Sty 01 and SV.D35A.B1 (5′ CTC AAA ACC GAC GGC CGC CCC GCC GAT AAA CGC TGG 3′) and EV.Sty 02R and SV.D35A.T1 (5′ TTT ATC GGC GGG GCG GCC GTC GGT TTT GAG CAG GGC 3′), respectively. The PCR mixture (50 μL) contained 10 ng of pEV.Sty DNA as a template, 200 nM each primer, 50 μM each dNTP, 1× *Taq* DNA polymerase buffer, and 1 unit of *Taq* DNA polymerase. The PCR procedure was composed of a predenaturation step at 95 °C for 2 min, 25 cycles with each cycle consisting of denaturation at 95 °C for 30 s, annealing, and extension at 60 °C for 4 min, and a final extension step at 72 °C for 5 min. The resulting two expected DNA fragments were used for overlapping PCR to introduce the desired mutation. This second run of PCR reaction mixture (100 μL), which contained 1 μL of each of the first run PCR products, 50 μM each dNTP, 1× *Taq* DNA polymerase buffer, and 2 units of *Taq* DNA polymerase, was initially carried out with a predenaturation at 95 °C for 2 min, five cycles with each cycle of denaturation at 95 °C for 30 s and annealing and extension at 60 °C for 4 min, and a final extension at 72 °C for 5 min. Afterward, 100 nM outside primers (EV.Sty01 and EV.Sty02R) were added to the above PCR reaction mixture to continue the overlapping PCR reaction under the same reaction condition. The PCR product was first cloned into pGEMT-Easy and then subcloned into pET21a+ as described above. The recombinant plasmid (pET-Sty-D35A) containing the desired mutation gene was confirmed by DNA sequencing and transformed into *E. coli* strain BL21(DE3). The Sty endo V D35A mutation protein was expressed and purified as described above for the wild-type Sty endo V protein.

Oligodeoxynucleotide Substrates. The fluorescently labeled oligodeoxynucleotide substrates were prepared as described (*2*). The sequence of a typical deoxyinosine substrate is as follows: 5′-TACCCCAGCGTCTGCGGTGTTGCGTCAGT-TGTCATAGTTTGATCCTCTAGTCTTGTTGCGGGTTCC-3′ and 3′-GGGGTCGCAGACGCCACAACGCAITCAA-CAGTATCAAACTAGGAGATCAGAACAACGCCC-Fam. Oligodeoxynucleotides containing deoxyinosine or deoxyuridine were ordered from IDT and purified by PAGE. The oligonucleotides were dissolved in TE buffer at a final concentration of 10 μM. The two complementary strands with the unlabeled strand in 1.2-fold molar excess were mixed, incubated at 85 °C for 3 min, and allowed to form duplex DNA substrates at room temperature for more than 30 min. To construct deoxyxanthosine-containing oligonucleotide, deoxyxanthosine triphosphate (dXTP) was generated by treating dGTP with acidified sodium nitrite as described (*22*, *23*). Two oligonucleotides (10 μM EV37N.BU, 5′-Fam-CCC GCA ACA AGA CTA GAG GAT CAA ACT ATG ACA ACT-3′, and 15 μM EV38N.BD, 5′-AC GCA ACA CCG CAG ACG CTG GGG-3′) were annealed with 15 μM EV35C.T (5′-TA CCC CAG CGT CTG CGG TGT TGC GTC AGT TGT CAT AGT TTG ATC CTC TAG TCT TGT TGC GGG TTC C-3′) to form a one-nucleotide gap DNA substrate (opposite the underlined C base). The fill-in reaction mixture (200 μL) containing 5 μM DNA, 0.03 unit/μL Klenow fragment, 1× EcoPol buffer, 5 mM $MgCl_2$, and 40 μM dXTP was incubated at 37 °C for 2 h to add an deoxyxanthosine at the 37th base pair position. The incorporation of the dXTP was confirmed by running a 1/1000 dilution of the reaction on a 7 M urea−10% polyacrylamide gel with a 36 and 37 base pair marker (Figure 3A). The extended oligonucleotide was purified by electrophoresis using a 7 M urea−16% denaturing polyacrylamide gel. The oligonucleotide bands were excised from the gel and eluted by shaking overnight in elution buffer (7.5 M ammonium acetate, 1 M magnesium acetate) at 37 °C. The sample was desalted by chromatography using a C_{18} Sep-Pak column and dried by Speedvac. The purified 37-mer was reannealed with the template strand EV35C.T. The deoxyxanthosine-containing 37-mer was extended to full length by incubating with 0.03 unit/μL Klenow fragment, 1× EcoPol buffer, 5 mM $MgCl_2$, and 0.1 mM each dNTP at 37 °C for 2 h in a final volume of 100 μL. The full-length 62-mer was purified, desalted, and dried as described above and dissolved in TE. Double-stranded deoxyxanthosine-containing substrates were formed by annealing the 62-mer with various top strand 66-mers.

Sty Endo V Cleavage Assays. The cleavage reaction mixtures (20 μL) containing 10 mM HEPES−KOH (pH 7.4), 1 mM DTT, 2% glycerol, 5 mM $MgCl_2$ unless otherwise specified, 10 nM oligonucleotide DNA substrate, and 10 nM Sty endo V protein unless otherwise specified were incubated at 37 °C for 30 min. The reactions were terminated by addition of an equal volume of GeneScan stop buffer. The reaction mixtures were then heated at 94 °C for 3 min and cooled on ice. Samples (3.5 μL) were loaded onto a 10% denaturing polyacrylamide gel containing 7 M urea. Electrophoresis was conducted at 1500 V for 1.6 h using an ABI 377 sequencer (Applied Biosystems). Cleavage products and remaining substrates were quantified using GeneScan analysis software version 3.0.

Gel Mobility Shift Assays. The binding reaction mixtures contained 100 nM fluorescently labeled oligonucleotide DNA substrates, 5 mM $MgCl_2$ or $CaCl_2$ or 5 mM EDTA, 20% glycerol, 10 mM HEPES−KOH (pH 7.4), 1 mM DTT, and an indicated amount of Sty endo V protein. The binding reactions were carried out at 37 °C for 30 min. Samples were electrophoresed on a 6% native polyacrylamide gel in 1× TB buffer supplemented with 5 mM $MgCl_2$ or $CaCl_2$ or EDTA. The bound and free DNA species were analyzed using a Typhoon 9400 imager (Amersham Biosciences) with the following settings: PMT at 600 V, excitation at 495 nm, and emission at 535 nm.

RESULTS

Deoxyinosine Substrates. Cloned endonuclease V from *S. typhimurium* strain 14028 is a 223 amino acid protein that shows an identical sequence to the endo V homologue sequence of *S. tyhimurium* LT2 at the protein level (*24*). Sty

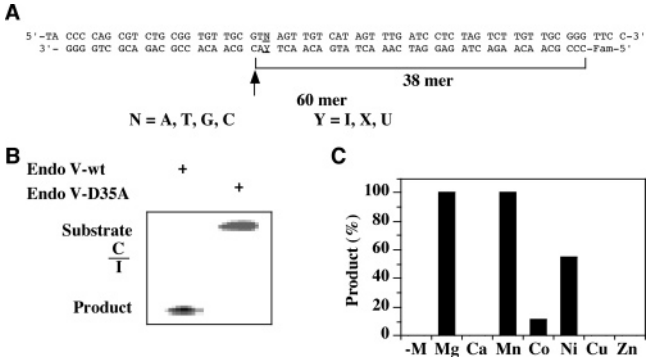

FIGURE 1: Cleavage activity assay of Sty endo V. (A) Sequences of lesion-containing oligodeoxynucleotide substrates. The arrow indicates the site of cleavage. (B) Cleavage activity of wt and D35A on inosine-containing substrate (C/I). Cleavage reactions were performed as described in Materials and Methods with 100 nM purified wt or D35A Sty endo V protein. (C) Cleavage activity of Sty endo V on C/I substrate in the presence of 100 nM wt Sty endo V and various metal ions (5 mM).

endo V shares 93% and 43% amino acid sequence identities with *E. coli* and Tma endo V, respectively. Previous studies indicate that NAD^+-dependent ligases from the *Thermus* species have significantly different biochemical properties despite high sequence homology (*25*). At the DNA level, the only differences are at position 52, in which the Leu residue is encoded by TTG in strain 14028 instead of CTG, and at position 176, in which the Thr residue is encoded by ACG in strain 14028 instead of ACA. The C-terminal His-tagged *Salmonella* endo V was purified by metal chelating and other chromatographic methods and confirmed by Western blot using a C-terminal His-tag specific antibody (data not shown). Unlike *E. coli* endo V (*26*), we did not experience solubility problems during purification, and the purified Sty endo V was highly soluble. The cleavage assays were performed using duplex lesion-containing oligodeoxynucleotide substrates as shown in Figure 1A. The bottom strand is labeled with Fam fluorophore, and various base lesions were placed at position 37 from the 5′ side. We initially tested cleavage activities using deoxyinosine-containing DNA substrates. The wild-type *Salmonella* endo V showed strong deoxyinosine endonuclease activity when the enzyme:substrate ratio (E:S) was 10:1, resulting in complete cleavage of the deoxyinosine substrate (Figure 1B). The cleavage site was located one nucleotide away from the 3′ side of the deoxyinosine, consistent with previous studies of *E. coli* and Tma endo V (*1*, *2*). Sty endo V was most active in low salt reaction conditions (data not shown). To rule out the possibility that the observed activity was due to contamination by the endogenous *E. coli* host endo V, we constructed an active site mutation (D35A). D35 in *Salmonella* is located in an equivalent position to D43 in Tma endo V. Previous biochemical studies have identified D43 in Tma endo V as an active site residue (*12*). The D35A mutant protein was purified to homogeneity by a chromatographic procedure similar to that of the wt enzyme and confirmed by Western blot analysis (data not shown). The D35A mutant showed no sign of deoxyinosine cleavage activity, affirming that the observed DNA cleavage was authentic to cloned *Salmonella* endo V (Figure 1B).

To examine the metal ion dependency, we tested a series of divalent metal ions. *Salmonella* endo V was most active

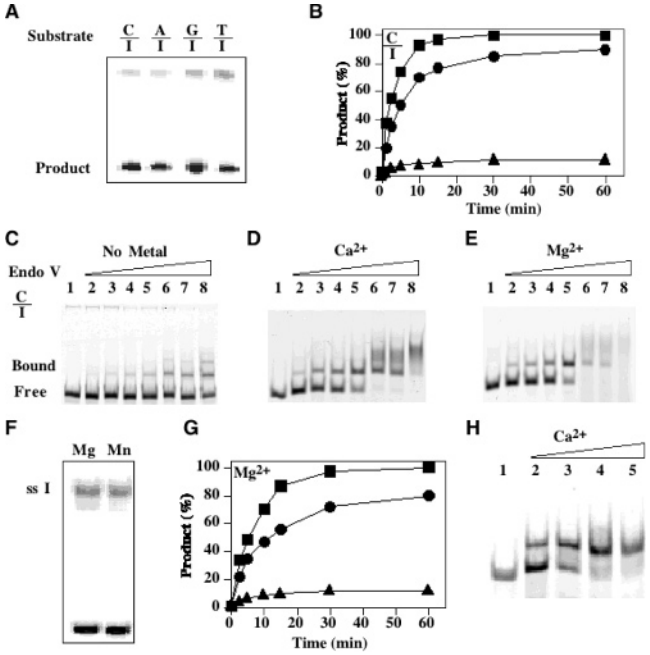

FIGURE 2: Cleavage and binding analysis of Sty endo V on deoxyinosine-containing DNA substrates. (A) Representative GeneScan gel picture of cleavage activity of Sty endo V on deoxyinosine-containing double-stranded DNA substrates with an E:S (enzyme:substrate) ratio of 1:1. (B) Time course analysis of cleavage activity on C/I substrate: (▲) E:S = 1:10; (●) E:S = 1:1; (■) E:S = 10:1; $S = 10$ nM. (C−E) Gel mobility shift analysis of binding of Sty endo V to double-stranded deoxyinosine-containing substrate (C/I) without metal ion, with 5 mM $CaCl_2$, or with 5 mM $MgCl_2$. Lanes: 1, $E = 0$ nM; 2, $E = 10$ nM; 3, $E = 20$ nM; 4, $E = 50$ nM; 5, $E = 100$ nM; 6, $E = 250$ nM; 7, $E = 500$ nM; 8, $E = 1$ μM. (F) Cleavage of single-stranded deoxyinosine-containing substrate in the presence of 5 mM $MgCl_2$ or 5 mM $MnCl_2$. (G) Time course analysis of cleavage of single-stranded deoxyinosine-containing substrate in the presence of 5 mM $MgCl_2$: (▲) E:S = 1:10; (●) E:S = 1:1; (■) E:S = 10:1; $S = 10$ nM. (H) Gel mobility shift analysis of binding of Sty endo V to single-stranded deoxyinosine-containing substrate with 5 mM $CaCl_2$. Lanes: 1, $E = 0$ nM; 2, $E = 50$ nM; 3, $E = 100$ nM; 4, $E = 250$ nM; 5, $E = 500$ nM.

Table 1: Apparent Rate Constants for Cleavage of I, X, and U Substrates (min^{-1})[a]

	top strand				
bottom strand	G	C	A	T	
I	0.18	0.18	0.20	0.19	0.10
X	0.23	0.19	0.18	0.21	0.45
U	0.08	0.16	0.04	0.14	0.08[b]

[a] The reactions were performed as described in Materials and Methods with 10 nM endo V and 5 mM $MgCl_2$. Data are an average of two independent experiments. [b] The single-stranded deoxyuridine cleavage reactions were performed with 5 mM $MnCl_2$.

with Mg^{2+} or Mn^{2+} as the metal cofactor but was also active with Ni^{2+} and Co^{2+} (Figure 1C). It was reported that *E. coli* endo V was active with Mg^{2+}, Mn^{2+}, and Co^{2+} (*1*). Like *E. coli* and Tma endo V, Sty endo V cleaves all four deoxyinosine base pairs (C/I, A/I, G/I, and T/I) (Figure 2A). The apparent rate constants for deoxyinosine cleavage ranged from 0.18 min^{-1} for the G/I base pair to 0.20 min^{-1} for A/I (Table 1). When the substrate was in excess (E:S = 1:10), Sty endo V only achieved limited turnover, resulting in about 10% cleavage (Figure 2B). When the E:S ratio was raised to 1:1 or 10:1, essentially all of the deoxyinosine substrate

was converted to the cleavage product (Figure 2A,B). These observations are consistent with previous studies on Tma endo V (*2*).

Sty endo V bound to the deoxyinosine-containing substrate in the absence of a metal cofactor (Figure 2C). At higher enzyme concentrations, multiple enzyme molecules apparently bound to the DNA sequence to form E_nS complexes, resulting in multiple retarded bands (Figure 2C). Since Ca^{2+} supports DNA binding but not cleavage for endo V (*2*, *12*), we therefore can use it to study binding without triggering catalysis. Sty endo V exhibited higher affinity to the deoxyinosine substrate in the presence of Ca^{2+} (Figure 2C, compare lane 5). The apparent K_d was about 63 nM in the presence of Ca^{2+}. In the absence of a metal cofactor, the apparent K_d could not be determined with reasonable accuracy due to weak binding. Since the highest enzyme concentration used in the assay was 1 μM (Figure 2C, lane 8), we estimated that the apparent K_d in the absence of a metal cofactor is greater than 1 μM. Thus, Ca^{2+} enhanced the binding affinity greater than 16-fold. In the presence of the catalytically active metal, Mg^{2+}, Sty endo V should have cleaved the deoxyinosine substrate to product; yet, a distinct retarded band was still observed (Figure 2C, lanes 1−5). This result indicates that Sty endo V, similar to Tma endo V, retains strong binding to the nicked product. The apparent K_d determined in the presence of Mg^{2+} was about 36 nM, a value comparable with that determined in the presence of Ca^{2+} (Figure 2D,E). These results suggest that Sty endo V has similar binding affinity to the deoxyinosine substrate and nicked product. However, nonspecific cleavage may have occurred at very higher enzyme concentrations, resulting in disappearance of DNA bands, consistent with results obtained from Tma endo V (*2*).

Sty endo V was active toward single-stranded deoxyinosine in the presence of Mg^{2+} or Mn^2 (Figure 2F). The apparent rate constant for single-stranded deoxyinosine cleavage was about 0.10 min^{-1} (Table 1). The enzyme achieved only limited turnover when the substrate was in excess (Figure 2G, E:S = 1:10). Close to complete turnover was observed when the E:S ratio was raised to 1:1 or 10:1 (Figure 2G). The apparent K_d for the single-stranded deoxyinosine substrate was estimated to be 52 nM in the presence of Ca^{2+} (Figure 2H), a value comparable to that for the double-stranded deoxyinosine substrate.

Deoxyxanthosine Substrates. Deoxyxanthosine is a deamination product of guanosine. The deoxyxanthosine-containing DNA substrates were constructed by enzymatic incorporation of dXTP to an oligonucleotide as detailed in Materials and Methods (Figure 3A). Like the deoxyinosine substrates, all four deoxyxanthosine base pairs are good substrates for Sty endo V (Figure 3B). The extent of turning over C/X was limited to 20% with E:S ratio of 1:10 (Figure 3C). However, complete turnover was observed when the enzyme was in excess. The cleavage rates for deoxyxanthosine substrates were quite comparable to those for deoxyinosine substrates, with the apparent rate constants ranging from 0.18 min^{-1} for A/X to 0.23 min^{-1} for G/X (Table 1).

As for single-stranded deoxyxanthosine cleavage, Sty endo V was active with either Mg^{2+} or Mn^{2+} as the metal cofactor (Figure 3D). The enzyme showed a greater turnover even when the E:S ratio was 1:10 (Figure 3E), suggesting that the Sty endo V may not bind to the single-stranded

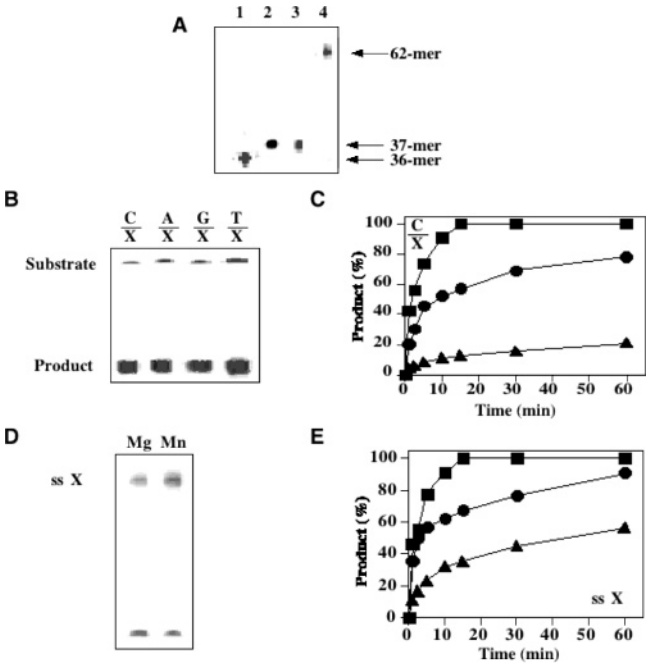

FIGURE 3: Cleavage activity of Sty endo V on deoxyxanthosine-containing DNA substrates. (A) Incorporation of dXTP into DNA. Lanes: 1 and 2, 36- and 37-mer markers; 3, single-nucleotide fill-in product; 4, full-length extension product. (B) Representative GeneScan gel picture of cleavage activity of Sty endo V on deoxyxanthosine-containing double-stranded DNA substrates with an E:S ratio of 1:1. (C) Time course analysis of cleavage activity on C/X substrate: (▲) E:S = 1:10; (●) E:S = 1:1; (■) E:S = 10:1; S = 10 nM. (D) Cleavage of single-stranded deoxyxanthosine-containing substrate in the presence of 5 mM $MgCl_2$ or 5 mM $MnCl_2$. (E) Time course analysis of cleavage of single-stranded deoxyxanthosine-containing substrate in the presence of 5 mM $MgCl_2$: (▲) E:S = 1:10; (●) E:S = 1:1; (■) E:S = 10:1; S = 10 nM.

deoxyxanthosine cleavage product as tightly as the single-stranded deoxyinosine cleavage product. The apparent rate constant of 0.45 min^{-1} for the single-stranded deoxyxanthosine substrate was about 2-fold higher than that for the double-stranded deoxyxanthosine substrates (Table 1).

Deoxyuridine Substrates. Deoxyuridine is the deamination product of cytidine. The deoxyuridine cleavage activities of Sty endo V varied greatly depending on the base pairs. Cleavage of the A/U base pair was minimum with Mg^{2+} (Figure 4A). The deoxyuridine endonuclease activity was enhanced with Mn^{2+}, but the A/U activity remained at least 50% lower than other deoxyuridine base pairs (Figure 4B). A detailed time course study was conducted to compare the deoxyuridine cleavage activities. Cleavage of deoxyuridine in the A/U base pair was below 5% using an E:S ratio of 1:10 or 1:1 and reached about 50% when the enzyme was in 10-fold excess (Figure 4C). Cleavage of deoxyuridine in G/U, C/U, and T/U base pairs was complete when the enzyme was in 10-fold excess (Figure 4C−F)). The apparent rate constants were 0.04, 0.08, 0.14, and 0.16 min^{-1} for A/U, G/U, T/U, and C/U base pairs, respectively (Table 1). The low A/U cleavage activity of endo V bears resemblance to the activity of some uracil DNA glycosylases (*27*−*30*).

To assess the binding affinity to the deoxyuridine-containing base pairs, we performed gel mobility shift assays with either Ca^{2+} or Mg^{2+} as the metal cofactor. Sty endo V failed to show any distinct binding pattern to A/U (Figure

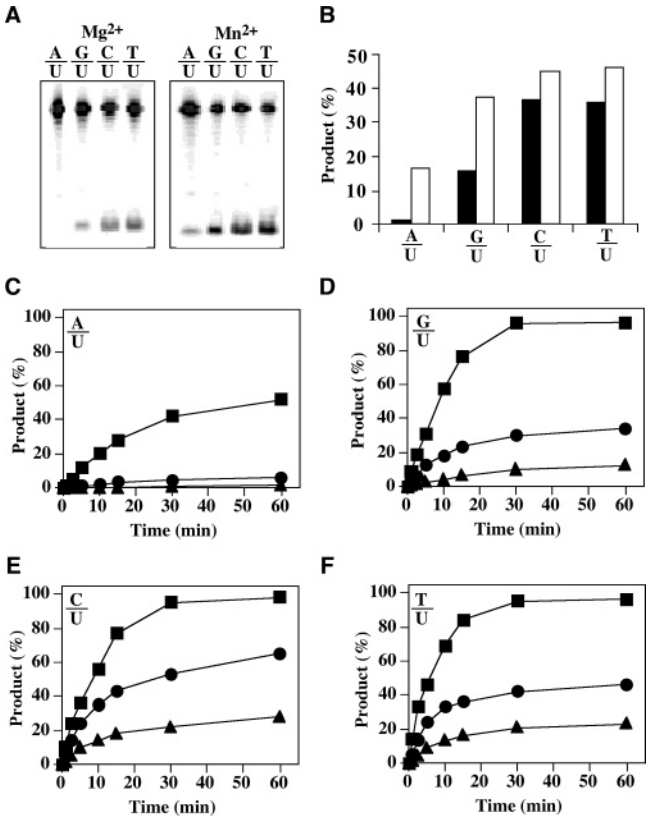

FIGURE 4: Cleavage activity of Sty endo V on deoxyuridine-containing DNA substrates. (A) Representative GeneScan gel picture of cleavage activity of Sty endo V on deoxyuridine-containing double-stranded DNA substrates with an E:S ratio of 1:1 in the presence of 5 mM $MgCl_2$ or $MnCl_2$. (B) Quantitative comparison of cleavage activity of Sty endo V on deoxyuridine-containing double-stranded DNA substrates as shown in (A). (C−F) Time course analysis of Sty endo V of cleavage activity in the presence of 5 mM $MgCl_2$ on A/U, G/U, C/U, or T/U substrate: (▲) E:S = 1:10; (●) E:S = 1:1; (■) E:S = 10:1; S = 10 nM.

5A), suggesting that the enzyme does not form a stable complex with the substrate that can be detected by gel mobility shift analysis. However, distinct banding was observed with G/U, C/U, and T/U substrates in the presence of Ca^{2+} (Figure 5B−D). The relatively tight binding to the G/U, C/U, and T/U substrates was also observed in the presence of Mg^{2+} (Figure 5B−D, Mg^{2+}, lane 3). Under the same assay conditions, Tma endo V showed no detectable retarded band to the T/U substrate (ref *2* and data not shown). These results indicate that Sty endo V, but not Tma endo V, can form stable complexes with some double-stranded deoxyuridine-containing DNA substrates.

Sty endo V also showed single-stranded deoxyuridine cleavage activity (Figure 6A). Mn^{2+} appeared to be a better metal cofactor for single-stranded deoxyuridine cleavage, but overall the activity was quite low even when the enzyme was in excess (Figure 6B), resulting in an apparent rate constant of 0.08 min^{-1} as assayed using Mn^{2+} as the metal cofactor (Table 1). The low single-stranded deoxyuridine endonuclease activity was at least in part attributable to weak binding affinity to the single-stranded deoxyuridine substrate (Figure 6C).

Base Pair Mismatch Substrates. To test mismatch endonuclease activities, oligonucleotide substrates, with the top strand labeled with Fam fluorophore and the bottom strand labeled with Tet fluorophore, were used (Figure 7). The 12 mismatch substrate set employed here was identical to that used in a previous study on Tma endo V (*2*). In the presence of Mg^{2+}, cleavage of C/C and G/T by Sty endo V was the weakest (Figure 7A). Sty endo V did not show a strong preference for a purine base in a mismatch base pair as previously demonstrated in Tma endo V (Figure 7A) (*2*). The lack of preference for a purine mismatch was more obvious when the assay was carried out in the presence of Mn^{2+} (Figure 7B). Cleavage of all 12 mismatches was detected (Figure 7B). By contrast, Tma endo V shows very little cleavage of A/C and T/C and especially C/C cleavage under the same assay conditions (*2*). As with Tma endo V (*1*, *2*), some nonspecific cleavage products were visible during cleavage of mismatch base pairs. For Sty endo V, mismatch cleavage was more prominent at the strands that were close to the 3′ end (Figure 7). This is different from a previous report indicating that *E. coli* endo V preferentially cleaves the strands with a mismatch closer to the 5′ end (*3*). Tma endo V, on the other hand, shows no obvious preference for either strand (*2*).

DISCUSSION

This work reports the biochemical properties of endo V from the intracellular pathogen, *S. typhimurium*. This study was prompted by the different DNA repair activities demonstrated by endo V from different organisms. Our primary objective was to understand the substrate specificity of Sty endo V in deamination DNA repair. This work presents an extensive biochemical analysis of endonuclease V activity on the three deaminated base lesions in DNA, deoxyinosine, deoxyxanthosine, and deoxyuridine. Data obtained from this work demonstrate that Sty endo V possesses unique biochemical properties that have not been observed in other endo V homologues, which provides insight into the active site plasticity of endo V family proteins.

Deoxyinosine Endonuclease Activity. Deoxyinosine endonuclease activities are a hallmark of the endo V family. All of the endo V enzymes examined thus far contain robust deoxyinosine cleavage activities except for a cloned mouse homologue which shows very low activity (*1*, *2*, *6*). Like Tma and *E. coli* endo V, Sty endo V shows DNA cleavage activities against all deoxyinosine base pairs. As previously demonstrated in *E. coli* endo V (*31*), the Sty enzyme can bind to deoxyinosine-containing DNA without a metal cofactor. However, unlike *E. coli* endo V, which shows similar binding affinity with or without a metal cofactor (*31*), DNA binding is enhanced in the presence of a metal ion for Sty endo V, suggesting that the ternary protein−DNA−M^{2+} complex is more stable than the binary protein−DNA complex (Figure 2). The tight binding to the nicked deoxyinosine product underlies the observed single-turnover property when the substrate was in excess (Figure 2B). As suggested, tight binding to the nicked lesion-containing products may help to protect further DNA degradation by nucleases, and the enzyme−DNA complex may serve as a sensor for recruiting other repairasome components, a requirement for a highly ordered repair process (*2*, *29*, *32*−*36*).

Deoxyxanthosine Endonuclease Activity. Deoxyxanthosine is a deamination product of guanosine. Previously, xanthine was considered unstable and readily depurinated to an AP

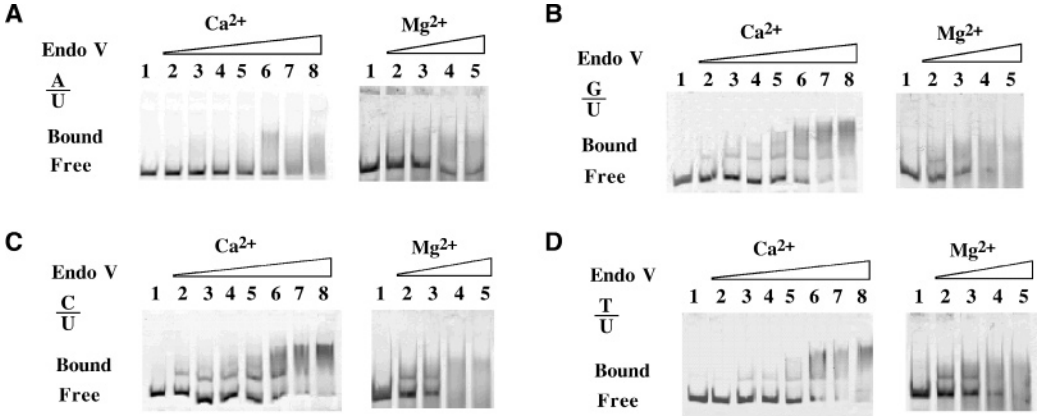

FIGURE 5: Binding analysis of Sty endo V on deoxyuridine-containing DNA substrates. (A−D) Gel mobility shift analysis of binding of Sty endo V to A/U, G/U, C/U, or T/U substrate with 5 mM $CaCl_2$ or 5 mM $MgCl_2$. Lanes (Ca^{2+}): 1, $E = 0$ nM; 2, $E = 10$ nM; 3, $E = 20$ nM; 4, $E = 50$ nM; 5, $E = 100$ nM; 6, $E = 250$ nM; 7, $E = 500$ nM; 8, $E = 1$ μM. Lanes (Mg^{2+}): 1, $E = 0$ nM; 2, $E = 50$ nM; 3, $E = 100$ nM; 4, $E = 250$ nM; 5, $E = 500$ nM.

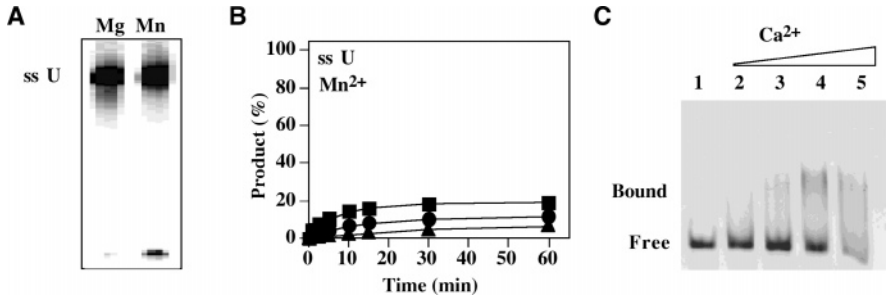

FIGURE 6: Cleavage activity of Sty endo V on deoxyuridine-containing single-stranded DNA substrate. (A) Cleavage of single-stranded deoxyuridine-containing substrate in the presence of 5 mM $MgCl_2$ or 5 mM $MnCl_2$. (B) Time course analysis of cleavage of single-stranded deoxyuridine-containing substrate in the presence of 5 mM $MnCl_2$: (▲) E:S = 1:10; (●) E:S = 1:1; (■) E:S = 10:1; $S = 10$ nM. (C) Gel mobility shift analysis of binding of Sty endo V to single-stranded deoxyuridine-containing substrate with 5 mM $CaCl_2$. Lanes: 1, $E = 0$ nM; 2, $E = 50$ nM; 3, $E = 100$ nM; 4, $E = 250$ nM; 5, $E = 500$ nM.

site (*37*). However, a more recent study indicates that deoxyxanthosine is a relative stable lesion under physiological conditions with a half-life of 2.4 years in double-stranded DNA (*38*). Biochemical and genetic investigations show that *E. coli* endo V is involved in repair of deoxyxanthosine (*9*, *39*). We conducted a detailed kinetic analysis on deoxyxanthosine DNA cleavage. This work demonstrates that Sty endo V has robust deoxyxanthosine endonuclease activity, which results in essentially complete cleavage of double-stranded deoxyxanthosine-containing DNA when the E:S ratio is 1:1 or above (Figure 3C). The cleavage efficiency of deoxyxanthosine is similar to deoxyinosine (Table 1). Like the *E. coli* enzyme (*39*), Sty endo V cleaves all four deoxyxanthosine-containing base pairs efficiently (Figure 3B). However, Sty endo V also possesses single-stranded deoxyxanthosine endonuclease activity (Figure 3D,E). A previous study indicates that *E. coli* endo V does not cleave single-stranded deoxyxanthosine (*39*). Another notable similarity in cleavage kinetics is that the Sty endo V exhibits a single-turnover property to both double-stranded deoxyinosine and deoxyxanthosine substrates (Figures 2B and 3C), indicating that the enzyme maintains tight binding to the nicked deoxyxanthosine products.

Deoxyuridine Endonuclease Activity. Hydrolytic deamination of cytidine to deoxyuridine is a frequent event in a living cell (*37*). Cells are equipped with multiple repair enzymes to deal with cytidine deamination (*40*). Deoxyuridine endonuclease activities have been observed in *E. coli* and Tma endo V but not Afu endo V (*2*, *8*, *41*, *42*). However, genetic analysis does not implicate *E. coli* endo V as an enzyme involved in deoxyuridine repair (*10*). Sty endo V shows some unique features of deoxyuridine endonuclease activities. In a previous study, we demonstrate that Tma endo V can rapidly turn over T/U-containing double-stranded DNA even when the substrate is in excess (*2*). The lack of tight binding to the nicked deoxyuridine product may account for the enhanced turnover (*2*). Sty endo V, on the other hand, appears to turn over double-stranded deoxyuridine-containing substrates to a very limited extent when the substrates are in excess (Figure 4). Binding analysis indicates that Sty endo V can bind to the G/U, C/U, and T/U with good affinity (Figure 5). Tighter binding to these substrates is consistent with low turnover when the substrates are in excess (Figure 4). These results suggest that some deoxyuridine substrates can be retained in the active site of Sty endo V, in a fashion similar to deoxyinosine or deoxyxanthosine.

Recognition Mechanism and Active Site Plasticity. This study, along with previous analyses of *E. coli* and Tma endo V proteins, demonstrates that endo V family proteins possess broad substrate specificity for deamination base lesions, with the exception of the orthologue from *A. fulgidus* which is deoxyinosine-specific (*1*−*5*, *7*, *8*, *39*). The general aspects of the substrate recognition mechanism have been discussed in previous reports on the basis of results obtained from *E. coli* and Tma endo V, which emphasize the role of the 6-keto (or 4-keto in uracil) and N^7 positions of a purine base in substrate recognition (*2*, *4*, *5*). Data obtained from this work in general are consistent with previous analyses. However,

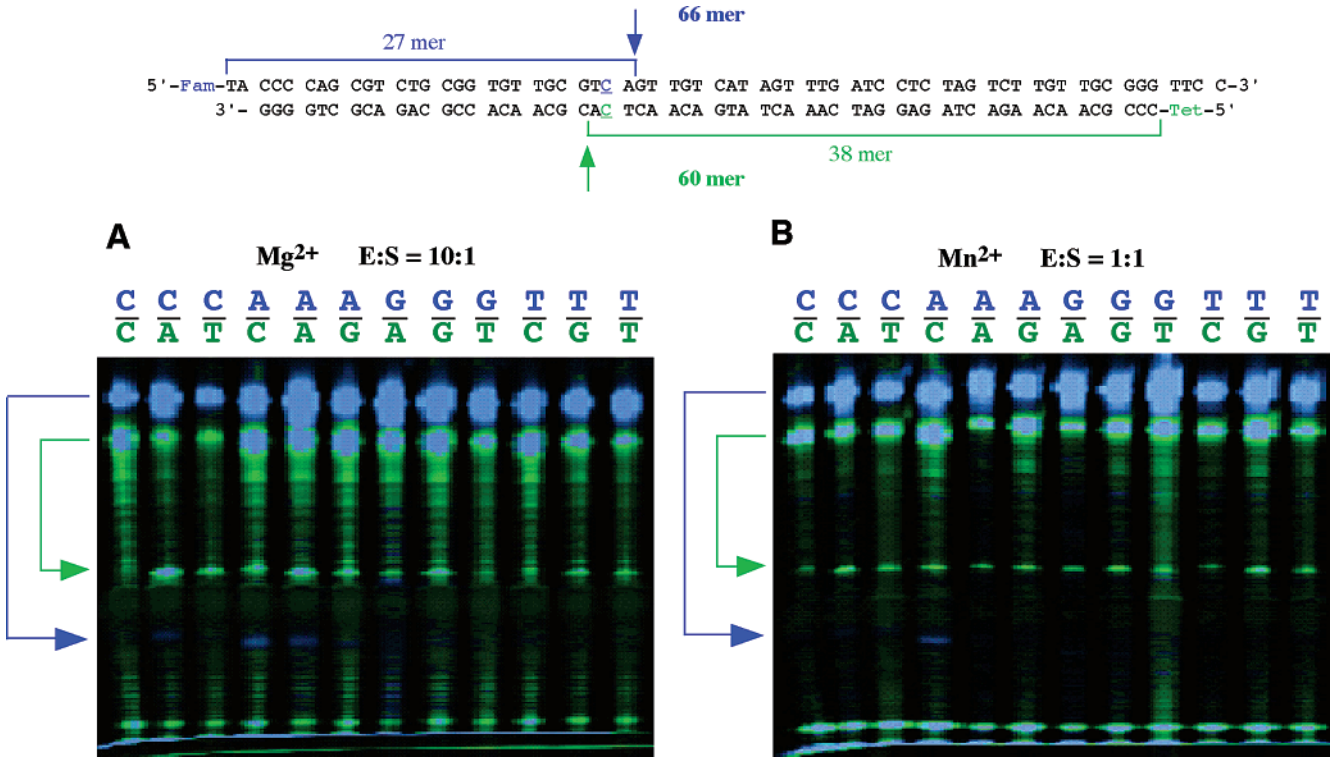

FIGURE 7: Cleavage activity of Sty endo V on base pair mismatch DNA substrates. The location of mismatch base pairs is underlined. Arrows indicate corresponding cleavage products. (A) Cleavage of mismatch substrates with E:S = 10:1 in the presence of 5 mM $MgCl_2$. (B) Cleavage of mismatch substrates with E:S = 1:1 in the presence of 5 mM $MnCl_2$.

a distinct difference between Sty and Tma endo V is their binding affinity to deoxyuridine. Gel mobility shift analysis consistently fails to demonstrate distinct binding to deoxyuridine-containing DNA by Tma endo V (ref *2* and data not shown). However, binding to the deoxyuridine-containing DNA by Sty endo V was readily detected by the same technique (Figure 5). These results indicate that the active site of Sty endo V exhibits a greater degree of plasticity. This property allows the Sty enzyme not only to bind to purine deamination products such as deoxyinosine and deoxyxanthosine with high affinity but also to maintain a stable complex with the pyrimidine deamination product deoxyuridine.

In contrast, the endo V from *A. fulgidus*, as a proposed primordial ancestor of endonuclease family, may have a rigid active site that can only accommodate deoxyinosine (*8*). The active site of Tma endo V obviously is more plastic than that of the Afu enzyme, as demonstrated by cleavage activities of deoxyinosine, deoxyxanthosine, deoxyuridine, AP site, and preferential cleavage of purine mismatches (*2*). However, Tma endo V fails to retain deoxyuridine-containing DNA in the active site to form a stable complex. Consequently, no distinct binding pattern can be observed with Tma endo V. On the other hand, the lack of tight binding to the deoxyuridine cleavage products allows the enzyme to achieve enhanced turnover, as compared with the inosone cleavage (*2*).

The active site plasticity is also reflected by mismatch base pair cleavage activities. Tma endo V seems to prefer purines within a mismatch base pair (*2*). *E. coli* endo V shows very poor cytosine-containing strand cleavage activity in C/A, C/T, and C/C base pairs (*3*). The active site of Sty endo V appears to be able to accommodate mismatch base pairs better than other endo V proteins. Both cytidine-containing strands in C/A and C/T base pairs were cleaved at rates comparable to to those of other mismatch base pairs, suggesting that even a pyrimidine base such as cytosine in a mismatch base pair environment is also well recognized.

Recent biochemical studies suggest that some uracil DNA glycosylases in fact can efficiently remove the purine deamination product hypoxanthine (*43*, *44*). Thus, active site plasticity is an emerging common theme in some deamination DNA repair enzymes. Further comparative structural and biochemical investigations should shed more light on the evolution of active site plasticity of DNA repair enzymes.

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