The Role of Base Flipping in Damage Recognition and Catalysis by T4 Endonuclease V*

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The process of moving a DNA base extrahelical (base flipping) has been shown in the co-crystal structure of a UV-induced pyrimidine dimer-specific glycosylase, T4 endonuclease V, with its substrate DNA. Compared with other enzymes known to use base flipping, endonuclease V is unique in that it moves the base opposite the target site extrahelical, rather than moving the target base itself. Utilizing substrate analogs and catalytically inactive mutants of T4 endonuclease V, this study investigates the discrete steps involved in damage recognition by this DNA repair enzyme. Specifically, fluorescence spectroscopy analysis shows that fluorescence changes attributable to base flipping are specific for only the base directly opposite either abasic site analogs or the 5′-thymine of a pyrimidine dimer, and no changes are detected if the 2-aminopurine is moved opposite the 3′-thymine of the pyrimidine dimer. Interestingly, base flipping is not detectable with every specific binding event suggesting that damage recognition can be achieved without base flipping. Thus, base flipping does not add to the stability of the specific enzyme-DNA complex but rather induces a conformational change to facilitate catalysis at the appropriate target site. When used in conjunction with structural information, these types of analyses can yield detailed mechanistic models and critical amino acid residues for extrahelical base movement as a mode of damage recognition.

The initiating events in base excision repair are performed by a class of enzymes, DNA glycosylases. These damage-specific enzymes are responsible for recognizing and binding to damaged bases and catalyzing the cleavage of the N–C_1' glycosydic bond linking the damaged base to the sugar phosphate backbone. Glycosylases thus provide the specificity to base excision repair. The precise mechanism by which these enzymes discriminate between nontarget and target DNA bases is beginning to be elucidated for a few glycosylases due to insights from high resolution x-ray crystallographic structures (reviewed in Ref. 1). In particular, uracil DNA glycosylases and a catalytically inactive mutant of T4 endonuclease V have been

co-crystallized with their product or substrate DNAs, respectively, and shown to move a base extrahelical (nucleotide), placing the base in a pocket within the enzyme.

The mechanism of base flipping is not limited to DNA repair enzymes. In fact, it appears to be a generalized mechanism for catalytic DNA binding proteins (reviewed in Refs. 2 and 3). The first evidence that an enzyme flips a base extrahelical was revealed in the crystal structure of a DNA cytosine 5-methyltransferase, *Hha*I, complexed with its substrate DNA, in which the target cytosine was flipped out of the DNA helix and into the active site pocket of the enzyme where the methylation reaction can occur (4). A similar methyltransferase, *Hae*III, has also been shown to move a base extrahelical (5), suggesting that this may be a conserved mechanism for this class of enzymes. As mentioned above, the two co-crystal structures for the glycosylases demonstrate a base (nucleotide) is flipped extrahelically; however, the mode of base flipping between the two enzymes is quite different. Uracil DNA glycosylases, like the methyltransferases, flip out the target base uracil into a uracil-specific pocket, thus providing the basis for specificity (6–8). T4 endonuclease V is unique in that it flips out the base in the opposite strand from the target site, suggesting a related but distinct mode of recognition, relying on the contacts with the nondamaged strand and the conformation of the phosphate backbone (9).

The catalytic mechanism of T4 endonuclease V has been extensively studied (reviewed in Refs. 10 and 11). It is a highly specific glycosylase that cleaves the glycosydic bond of the 5′-pyrimidine of a *cis-syn* cyclobutane pyrimidine dimer, a major photoproduct induced in DNA by exposure to ultraviolet light. This glycosylase also possesses a concomitant AP lyase activity, resulting in the production of a 3′ α,β-unsaturated aldehyde and a 5′-phosphate via a β-elimination reaction at the glycosylase generated abasic (AP) site. Biochemical and structural analyses have demonstrated the involvement of two key residues in the catalytic activity of the enzyme, Glu-23 and the N-terminal Thr-2 (12–18).

The co-crystal structure of a catalytically inactive mutant of T4 endonuclease V (E23Q) bound to thymine dimer-containing DNA has been solved (9). Interestingly, the structure revealed not only that the adenine base opposite the 5′-thymine of the pyrimidine dimer was flipped out of the DNA duplex and into a pocket on the protein surface but that the DNA substrate was sharply bent (60°) at the thymine dimer (9). Fig. 1 shows the overall structure of the co-crystal complex with the key active site residues and the extrahelical adenine indicated. Interestingly, the adenine in the pocket does not form hydrogen bonds with any protein residues but rather is held in place by weak van der Waals interactions (9). The thymine opposite the adenine remains within the helix although the base stacking interactions are disrupted. Thus, the enzyme appears to induce a conformational change in the DNA to allow the active site

[1] The abbreviations used are: 2-Ap, 2 aminopurine; AP, abasic site; rAP, reduced abasic site; BSA, bovine serum albumin; bp, base pairs.

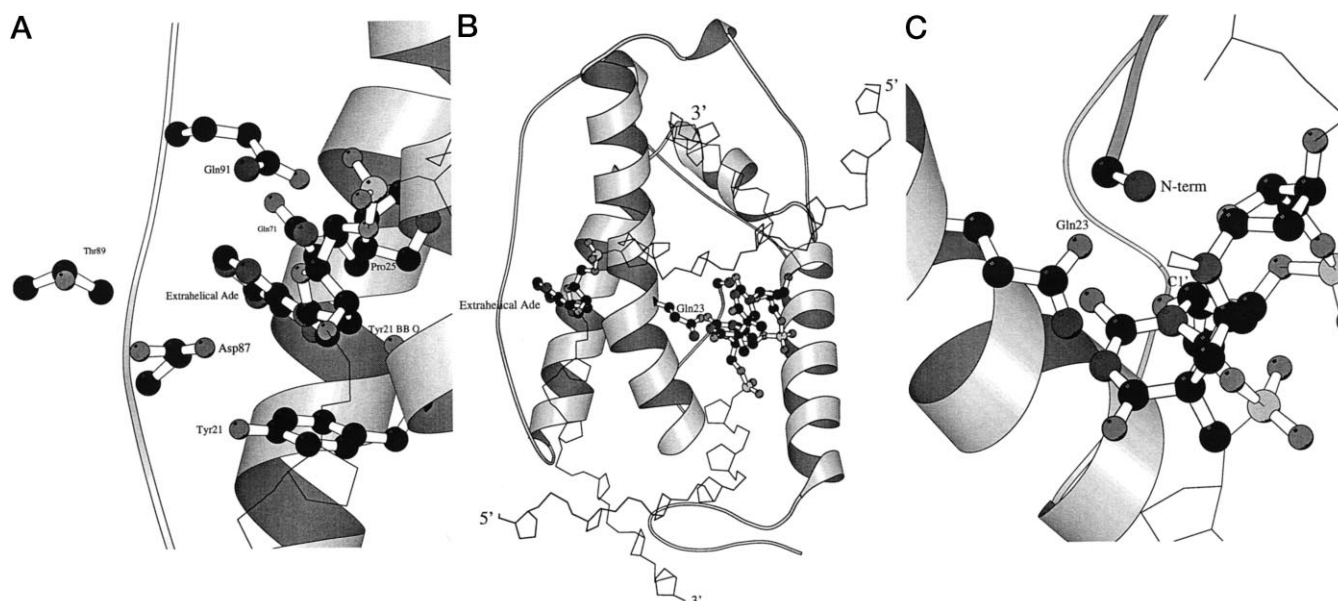**This paper is available on line at http://www.jbc.org**

FIG. 1. **Structure of E23Q bound to 12-bp duplex containing a thymine dimer.** *A*, view of extrahelical base binding pocket. *B*, co-crystal structure. *C*, view of active site in co-crystal. Images were generated in Molscript (38) using the x-ray crystal coordinates (9).

residues access to the glycosidic bond to catalyze the reaction. Interesting questions arise from the structural data; at what step in the enzyme reaction does the extrahelical capture of the base occur? Is it simultaneous and/or necessary for specific binding? These questions can be addressed using the well-characterized T4 endonuclease V system that provides an opportunity for studying the pre-catalytic events involving specific damage recognition and the role of base flipping in establishing specificity.

In this study we utilize the fluorescent properties of the base analog, 2-aminopurine (2-Ap), to monitor the binding of the enzyme and, more importantly, the relative helical position of the 2-Ap when it is in complex with the T4 endonuclease V. The use of 2-Ap in examining local changes in DNA structure has been well documented for several enzyme systems (19–25). It is an effective fluorophore for probing enzyme-induced conformational changes in the DNA helix as it forms a Watson-Crick base pair with thymine (26), is sensitive to the surrounding environment (27), and is substantially quenched when present in duplex DNA as compared with single-stranded DNA (21), thus providing an ideal system to analyze local DNA structure.

By using modified oligonucleotides previously shown to form stable-specific complexes with T4 endonuclease V (28), and by replacing the adenine with 2-Ap placed directly opposite the modification in the complementary strand, this study demonstrates that the movement of a base extrahelically upon binding of T4 endonuclease V can be monitored by fluorescence spectroscopy. This movement is specific for only the base directly opposite either the abasic site analogs or the 5′-thymine of a pyrimidine dimer. Furthermore, the process of base flipping, specific binding, and catalysis can be dissected using this technique in combination with various substrate analogs and catalytic mutants of the enzyme.

EXPERIMENTAL PROCEDURES

T4 Endonuclease V—T4 endonuclease V (wild type) was purified from *Escherichia coli* AB2480 (*recA⁻*, *uvrA⁻*) cells transformed with a denV expression vector as described previously (29). E23Q endonuclease V mutants were created by site-directed mutagenesis and purified as described (18).

Oligonucleotide Substrates—The duplex DNAs that were used for this study are shown in Table I. DNA oligonucleotides containing a site-specific pyrrolidine residue or a reduced abasic site residue (rAP) were synthesized as described previously (30). Complementary se-

TABLE I
Duplexes containing 2-aminopurine
2, aminopurine; P, pyrrolidine; and R, reduced abasic site.

	Duplex sequence	Modification	Excitation maximum
			nm
I.	GCACGAATTAAG |||||||||||| CGTGCTT**2**ATTC	Controls	312
a, b,	. . . . .A**2**. . .		
II.	GCACGAA<u>TT</u>AAG |||||||||||| CGTGCTT**2**ATTC	Pyrimidine dimer	307
a, b,	. . . . .A**2**. . .		
III.		Pyrrolidine cytosine	
a,	. . . . .P.		305
b,	GGATAGTGTCCACGTTACTCGAAGC ||||||||||||||||||||||||| CCTATCACAGGT**2**CAATGAGCTTCG		307
IV.	GTGAACCTGAGCRTAGCTCAGTAAC ||||||||||||||||||||||||| CACTTGGACTCG**2**ATCGAGTCATTG	Reduced abasic site	305

quences were synthesized using standard procedures, and the deprotected oligonucleotides were purified electrophoretically on 20% denaturing polyacrylamide gels. The 2-Ap phosphoramidite was purchased from Glen Research (Sterling, VA) and incorporated into the designated oligonucleotide sequences (Table I) following standard synthesis procedures. Complementary oligonucleotides were annealed by mixing the DNAs, heating to 75 °C, and slow cooling to room temperature. The non-2-Ap strand was present in 2-fold excess to ensure that the majority of the fluorophore was present in duplex DNA. The presence of duplex DNA was confirmed by electrophoresis through a native polyacrylamide gel.

The 49-mer containing a *cis-syn* cyclobutane thymine dimer (CS-49) used as substrate for activity assays was prepared as follows: a 12-mer containing a thymine dimer (<u>TT</u>), GCACGAA<u>TT</u>AAG (a gift from J-S. Taylor, Washington University), was 5′-end-labeled (1:10 γ-^{32}P-ATP: unlabeled ATP) and annealed to a complementary 49-mer. The 12-mer was extended to a 36-mer in a reaction containing 0.3 mM dNTPs, Sequenase (13 units, U. S. Biochemical Corp.), 40 mM Tris-HCl, pH 7.5, 20 mM MgCl$_2$, and 50 mM NaCl incubated at 25 °C for 2 h, followed by the addition of another 13 units of Sequenase and 0.2 mM dNTPs and

incubation at 25 °C for another 24 h upon which gel analysis indicated the extension of the 12-mer to a 36-mer was essentially complete. A 13-mer was then ligated to this 36-mer annealed to the complementary 49-mer forming fully duplex 49-bp DNA. The resulting 49-mer was then purified by gel electrophoresis through a 15% denaturing polyacrylamide gel (8 M urea), and the oligonucleotides recovered were allowed to re-anneal.

All oligonucleotide strands containing the base analogs, thymine dimer, and a control sequence were γ-^{32}P-labeled on the 5′-end with T4 polynucleotide kinase (New England Biolabs) following standard procedures and annealed to their complementary strands as shown in Table I. The DNA concentrations were determined by measuring the absorbance at 260 nm.

Enzyme Activity Assays—For the 25-bp duplexes, 1.25 nM duplex, with the indicated strand labeled, was incubated with enzyme (6.25 nM) in a standard reaction buffer (25 mM sodium phosphate, pH 6.8, 100 μg/ml BSA, 100 mM KCl) in a total volume of 20 μl for 1 h at 25 °C. For the 12-bp DNAs, 6.24 nM duplex and 25 nM enzyme were incubated as described above. An equal volume of loading buffer (95% (v/v) formamide, 20 mM EDTA, 0.02% (w/v) bromphenol blue, 0.02% (w/v) xylene cyanol) was added, and the samples were heated to 90 °C for 5 min prior to loading on a 15% denaturing polyacrylamide gel (8 M urea) in 1 × TBE buffer (90 mM Tris borate, 2 mM EDTA, pH 8.0). The DNAs were separated by electrophoresis for 3 h at 800 V. Bands were visualized by autoradiography of the wet gels using Hyperfilm-MP x-ray film (Amersham Corp.). The appearance of the product band was quantitated using a Molecular Dynamics PhosphorImager and ImageQuant software (Sunnyvale, CA).

Binding Assays—Binding of T4 endonuclease V wild type or E23Q to the duplexes described above was assayed using gel mobility shift analysis. The reactions contained 25 mM sodium phosphate, pH 6.8, 100 mM KCl, 5% glycerol, 100 μg/ml BSA, 2 nM (25 bp) or 0.5 nM (12 bp) DNA duplex, and 200 nM T4 endonuclease V in a total volume of 20 μl. Following a 30-min incubation at 25 °C, the free DNA and enzyme-bound DNA were separated by electrophoresis through a 7.5% (10% for 12 bp) native polyacrylamide gel in 0.5 × TBE buffer (45 mM Tris borate, 1 mM EDTA) for 2 h at 120 V. Bands were visualized by autoradiography of the wet gels using Hyperfilm-MP x-ray film (Amersham Corp.).

Fluorescence Assay—Steady state fluorescence spectra were collected on a SPEX Industries model FL221 fluorometer (Edison, NJ). Fluorescence emission spectra were obtained by exciting the sample at a predetermined maximum excitation wavelength as shown in Table I and scanning from 340 to 420 nm emission wavelengths at 0.5-nm increments. The excitation and emission monochromators were set at slit widths of 1.5 mm for the reduced abasic site duplex or 2.0 mm for all other duplexes. The reduced abasic site containing duplex had a higher intrinsic fluorescence than the other duplexes, thus decreasing the slit width decreased the signal to be approximately the same values as the other duplexes studied. The photomultiplier voltage was set at 950 V. The data were collected using the DM3000 software (version 3.3) and imported into Excel (Microsoft, Redmond, WA) and Kaleidagraph (Synergy Software, Reading, PA) for graphic representation. Fluorescence emission spectra for the reaction buffer and the enzymes (each concentration) at the appropriate excitation wavelength were obtained and subtracted as background from all reactions. Photobleaching of this fluorophore was determined to be minimal under the time scale of these experiments; however, as a precaution, all reactions were incubated in the dark to avoid the complication of photobleaching. After obtaining the emission scan for the DNA alone (200 nM in 25 mM sodium phosphate, pH 6.8, 100 mM KCl, in a total volume of 200 μl), enzyme was added at the indicated concentrations, mixed, allowed to bind for 5 min at 25 °C, and the emission scan observed from 340 to 420 nm. The reactions were held at 25 °C during the scan time (approximately 3 min).

Acrylamide Quenching Assays—Quenching of the enhanced fluorescence signal produced upon endonuclease V binding to the pyrrolidine/2-Ap duplex was investigated using acrylamide as a quencher. The fluorescence emission scan for the DNA alone (200 nM), with endonuclease V (200 nM), and both with the addition of increasing amounts of acrylamide (10, 20, 30, 40, 50, 60, 75, 100, 200, 300 mM) were examined using the standard procedure described above. 200 nM single-stranded 2-Ap DNA was also incubated with and without acrylamide.

Activity assays of wild type endonuclease V in the presence of acrylamide on the CS-49 substrate were performed by incubating the DNA (1 nM) and endonuclease V (20 nM) in the standard reaction buffer for 30 min at 25 °C. Where indicated, acrylamide at the following concentrations, 10, 50, 100, 200, and 400 mM, was added to DNA prior to the

addition of T4 endonuclease V. Preincubation of 400 mM acrylamide with the enzyme (20 nM) for 10 min at 25 °C prior to the addition of the DNA was also performed. The reaction products were separated through a 15% denaturing polyacrylamide gel (8 M urea) gel. Bands were visualized by autoradiography of the wet gels using Hyperfilm-MP x-ray film (Amersham Corp.). The appearance of the product band was quantitated using a Molecular Dynamics PhosphorImager and ImageQuant software (Sunnyvale, CA).

RESULTS

Binding and Activity of Endonuclease V on 2-Aminopurine-containing Duplexes—DNAs were constructed for binding analysis of endonuclease V in which the adenine opposite the modification or target site was replaced with the 2-Ap fluorophore. To demonstrate the utility of the 2-Ap-containing duplexes for use in the fluorescence assay, we first examined if the enzyme binds specifically to these DNAs. Previously, it has been shown that endonuclease V binds with relatively high affinity to duplexes containing a site-specific pyrrolidine or rAP residue with an unmodified base in the opposite strand (28). Gel mobility shift analyses were used to demonstrate that duplexes containing a 2-Ap residue placed opposite these modified abasic sites are specifically bound by endonuclease V.

As shown in Fig. 2A, both wild type and mutant E23Q enzyme bind to the 2-Ap 25-bp duplexes (*lanes 1–6*) in a manner similar to the non-2-Ap duplex (*lanes 7–9*, and see Ref. 28). Fig. 2A also shows that E23Q binds to the 12-bp duplexes containing a thymine dimer with a 2-Ap opposite either the 5′- or 3′-thymine (*lanes 10–13*) but not to a control duplex containing a normal thymine opposite the 2-aminopurine base (*lanes 14–15*). The higher molecular weight bands seen in *lanes 3, 6, and 9* upon E23Q binding have been observed previously under conditions of excess enzyme and determined to be due to more than one enzyme molecule bound to a DNA molecule (18). To ensure that the 2-Ap does not disrupt the catalytic activity of the enzyme on thymine dimer ($\underline{TT}$)-containing DNA, wild type endonuclease V was incubated with $\underline{TT}$/2-Ap-containing 12-bp duplex (Table I, part II), and the products were analyzed by denaturing gel electrophoresis. As shown in Fig. 2B, placing the 2-Ap residue opposite either the 5′-thymine (*lanes 1–3*) or the 3′-thymine (*lanes 4–6*) of the $\underline{TT}$ did not affect catalysis by endonuclease V as evidenced by the complete conversion of the labeled 12-mer to the expected product at approximately the 7-mer position (*lanes 3 and 6*). No cleavage products were observed with the catalytically inactive E23Q enzyme and the same duplexes as expected (*lanes 2 and 5*). DNA not containing a dimer opposite 2-Ap was not processed by endonuclease V (*lanes 7–9*). These data establish the ability to use 2-Ap opposite a dimer as an appropriate substrate for endonuclease V.

The duplexes containing abasic site analogs and 2-Ap were incubated with wild type and mutant endonuclease V to ensure that these oligonucleotides were not cleaved by the enzymes which could generate misleading results in the fluorescence studies. As shown in Fig. 2, *C and D*, none of the 2-Ap/abasic site analog paired duplexes are substrates for endonuclease V. (Note: the light bands present in Fig. 2D, *lanes 4–6*, are background degradation of the DNA as evidenced by the appearance in *lane 4*, the no enzyme control; thus, this band is not a product band.) Thus, any enhancement in fluorescence will not be attributable to release of a base from the duplex or cleavage of the DNA backbone.

Fluorescence Changes Induced by Wild Type Endonuclease V upon Binding to 2-Aminopurine-containing Duplexes—Once it was determined that these substrates and substrate analogs would be suitable for analyses, the relative fluorescence of the duplexes containing 2-Ap was examined. Prior to the addition of enzyme, the relative fluorescence and emission and excitation maxima were determined for each duplex. The excitation
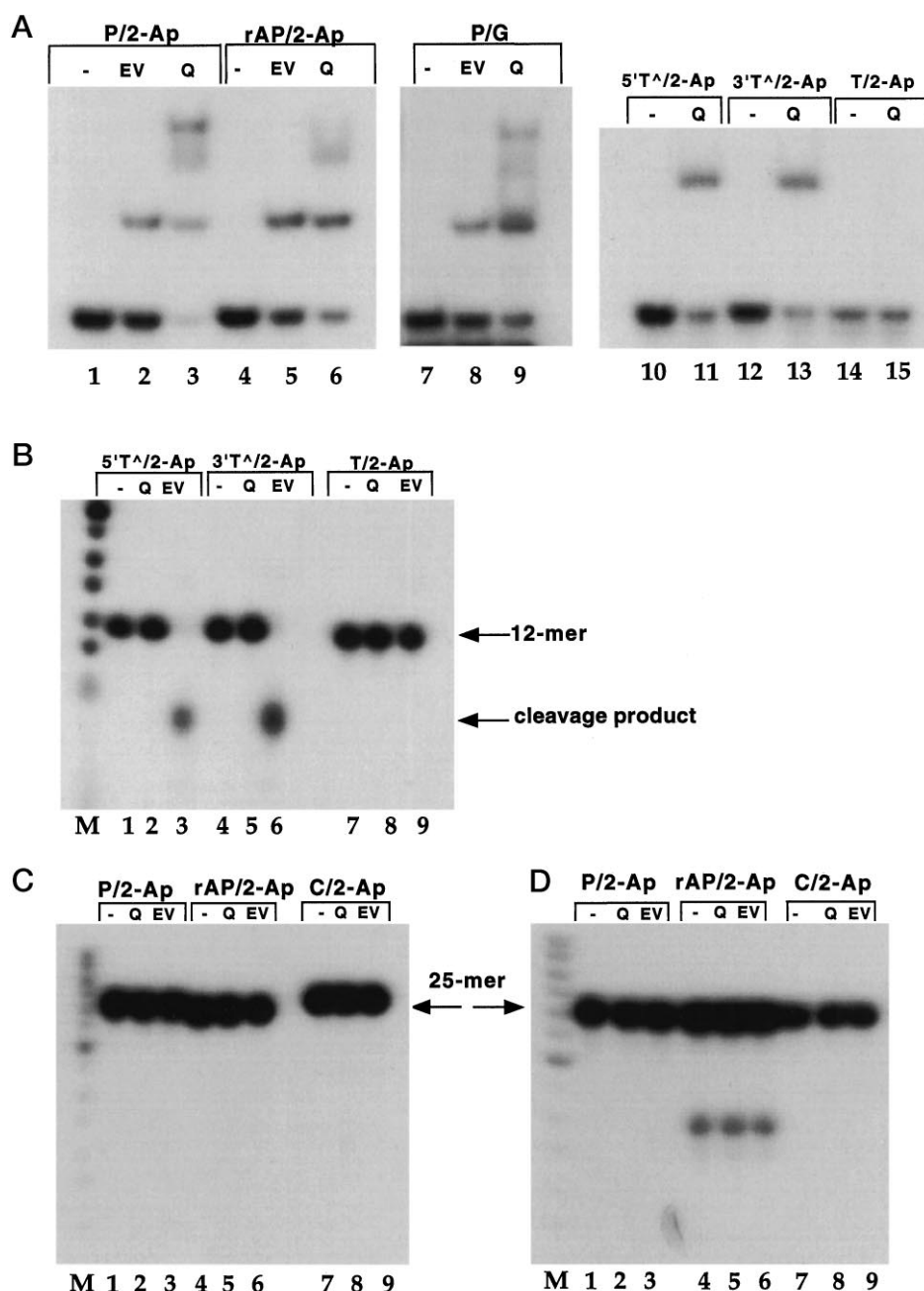
FIG. 2. **Binding and cleavage by T4 endonuclease V on 2-aminopurine containing duplexes.** *A*, specific binding of endonuclease V to 2-Ap containing duplexes. 2 nM 5′-γ-³²P-end-labeled duplex containing pyrrolidine (*P*)/2-Ap (*lanes 1–3*), reduced apurinic site (*rAP*)/2-Ap (*lanes 4–6*), pyrrolidine (*P*)/G (*lanes 7–9*), or 0.5 nM 5′ T̲/2-Ap (*lanes 10–11*), 3′ T̲/2-Ap (*lanes 12–13*), or T/2-Ap (*lanes 14 and 15*) were incubated with 200 nM wild type endonuclease V (*EV, lanes 2, 5,* and *8*) or 200 nM E23Q (*Q, lanes 3, 6, 9, 11, 13,* and *15*) in 25 mM sodium phosphate, pH 6.8, 100 mM KCl, 5% glycerol, and 100 μg/ml BSA. Following a 30-min incubation at 25 °C, the complexes were separated by electrophoresis through a 7.5% (*lanes 1–9*) or 10% (*lanes 10–15*) native polyacrylamide gel for 2.5 h at 120 V. *B,* cleavage activity of endonuclease V on dimer/2-Ap duplexes. 6.24 nM 5′-γ-³²P-end-labeled duplex DNA was incubated with either 25 nM endonuclease V wild type (*EV, lanes 3, 6,* and *9*) or E23Q (*Q, lanes 2, 4,* and *8*), or in the absence of enzyme (*lanes 1, 4,* and *7*) in the standard reaction buffer (25 mM sodium phosphate, pH 6.8, 100 mM KCl, and 100 μg/ml BSA) for 1 h at 25 °C. Reaction products were separated by electrophoresis on a 15% denaturing polyacrylamide gel (8 M urea). 5′ T̲/2-AP (*lanes 1–3*); 3′ T̲/2-AP (*lanes 4–6*); T/2-AP (*lanes 7–9*). *C* and *D*, cleavage activity of endonuclease V on abasic analogs/2-Ap containing duplexes. 1.25 nM 5′-γ-³²P-end-labeled DNA duplexes with the 2-Ap containing labeled strand (*C*) or abasic analog containing labeled strand (*D*) were incubated with 6.25 nM enzyme in the standard reaction buffer for 1 h at 25 °C. The reaction products were separated on 15% denaturing polyacrylamide gels (8 M urea). Pyrrolidine (*P*)/2-AP (*lanes 1–3*); reduced apurinic site (*rAP*)/2-AP (*lanes 4–6*); cytosine (*C*)/2-AP (*lanes 7–9*).

maxima are given in Table I and correspond well with previously published excitation maxima for 2-Ap in various duplex sequence and size contexts (21, 23, 24, 31). As several of the duplexes lacked a base opposite the fluorophore (pyrrolidine and reduced AP), the relative intensity of the fluorescence of these DNAs was higher than those with a base opposite the fluorophore, indicative of the quenching effects of a base paired to 2-Ap. The fluorescence signal of 2-Ap was quenched by

duplex formation for the pyrrolidine/2-Ap duplex (Fig. 3*A*). Thus, even though there is no base pairing, the location of the 2-Ap in a double helical environment does quench the signal as compared with the single-stranded 2-Ap oligonucleotide.

Once the intrinsic fluorescence of the pyrrolidine/2-Ap DNA was determined (Fig. 3*A*), wild type endonuclease V was added to the reaction, and an emission scan was taken (Fig. 3, *A* and *B*). As shown in Fig. 3*B*, the increase in fluorescence intensity

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A

B
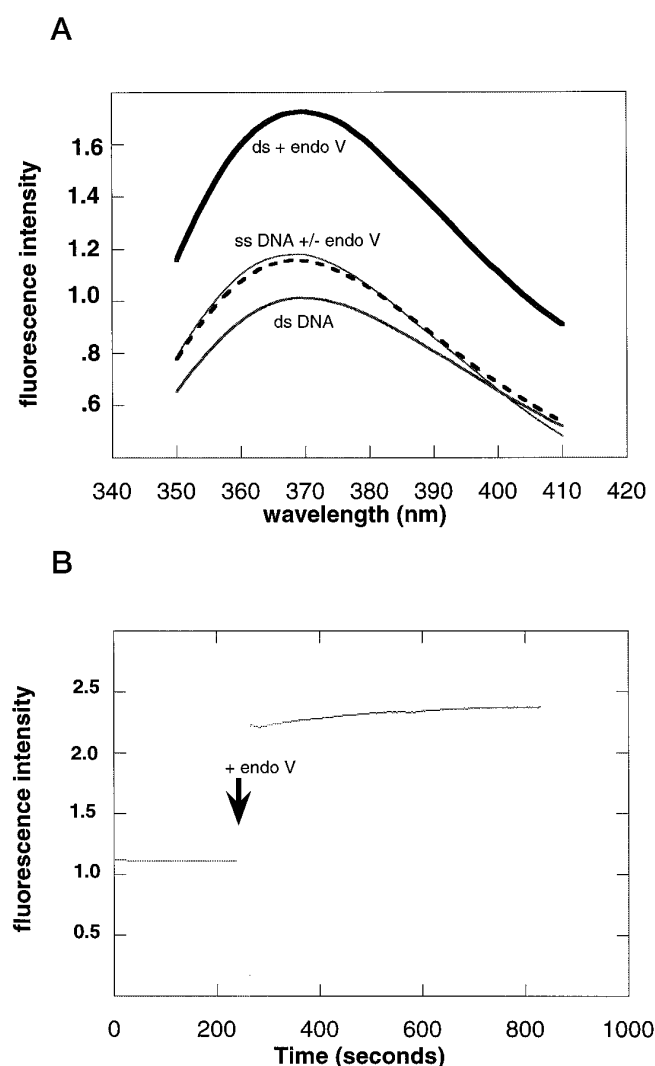
FIG. 3. **Fluorescence of 2-aminopurine/pyrrolidine duplex.** Steady state fluorescence emission spectra were obtained using a SPEX Industries model FL221 fluorometer at a fixed excitation wavelength of 305 nm. Reactions contained 200 nM DNA in 25 mM sodium phosphate, pH 6.8, 100 mM KCl, ±200 nM endonuclease V at 25 °C. All buffer and enzyme fluorescence emissions were subtracted from these scans. *A,* effect of duplex formation and endonuclease V binding on fluorescence of 2-Ap containing DNA. Fluorescence emission spectra of the single-stranded 2-Ap containing 25-mer was quenched when annealed to the complementary strand. Upon addition of endonuclease V no change is observed for the single-stranded DNA fluorescence, whereas the duplex DNA fluorescence is enhanced 2-fold upon enzyme binding. *B,* time scan of fluorescence emission at 370 nm following the addition of endonuclease V. The reaction contained 200 nM duplex 2-Ap/pyrrolidine DNA in 25 mM sodium phosphate, pH 6.8, and 100 mM KCl. The emission scan was initiated and after approximately 200 s, 200 nM endonuclease V was added to the cuvette, mixed, and the emission scan continued for 900 s with 1-s increments.

occurred within seconds. The reaction was followed for 15 (Fig. 3*B*) and 30 min (data not shown), and after the initial enhancement, no significant increase in fluorescence was observed. Thus, all subsequent enzyme binding reactions were incubated for 5 min for consistency. This enhanced fluorescence was dependent on duplex DNA, since no increase was observed when endonuclease V was incubated with single-stranded 2-Ap DNA (Fig. 3*A*). This was expected as it has been shown previously that endonuclease V does not bind significantly to single-stranded DNA (32). The enhanced fluorescence was also dependent on DNA as no interaction was observed between free 2-Ap and endonuclease V (data not shown).

A

B
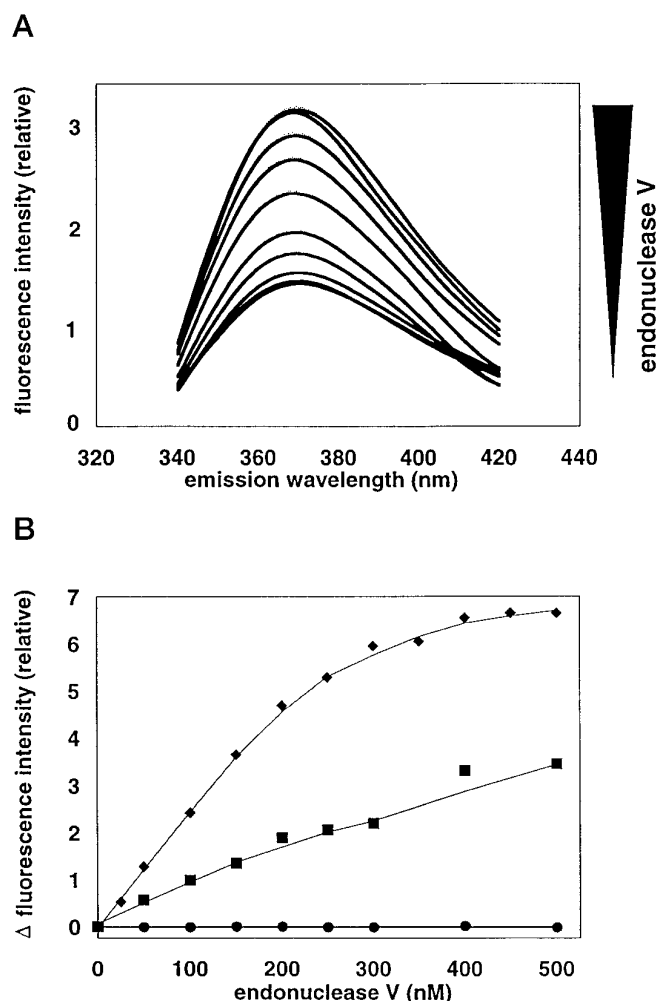
FIG. 4. **Enhanced fluorescence of 2-aminopurine/abasic analog duplexes as a function of endonuclease V concentration.** *A,* representative emission scans of duplex 2-Ap/pyrrolidine DNA in the presence of endonuclease V. Reactions contained 200 nM DNA in the standard buffer of 25 mM sodium phosphate, pH 6.8, 100 mM KCl at 25 °C. The fluorescence emission scans (at excitation of 305 nm) of the DNA alone (*bottom curve*) and in the presence of increasing endonuclease V from 50–500 nM as indicated on the *right axis* are shown. All buffer and enzyme fluorescence emissions were subtracted from these scans. *B,* graphical representation of fluorescence emission intensity at λmax of 370 nm. Reactions were performed as in *A* for pyrrolidine/2-Ap (*squares*), rAP/2-Ap (*diamonds*), and C/2-Ap (*circles*). The peak emission fluorescence at 370 nm was plotted as a function of enzyme concentration.

The intensity of the signal was dependent on the enzyme concentration, as increasing amounts of endonuclease V resulted in increased fluorescence of the pyrrolidine/2-Ap DNA (Fig. 4). Enhanced fluorescence was also observed when wild type endonuclease V was incubated with the reduced abasic site/2-Ap duplex (Fig. 4*B*). However, no increase in fluorescence was observed when a non-modified base (cytosine) was placed directly opposite the 2-Ap base (Fig. 4*B*). The enhanced fluorescence is thus directly associated with a specific binding event. The enhanced fluorescence observed for endonuclease V binding was typically 2–5-fold for the pyrrolidine/2-Ap duplex and 2–7-fold for the reduced AP/2Ap duplex. These fluctuations appear to be dependent on the preparation of the substrate and the relative enzyme activity.

As evidenced by the emission scans (Fig. 4*A*), there does not appear to be any spectral shift associated with endonuclease V binding to these duplexes. The increased fluorescence observed upon enzyme binding is more substantial than the fluorescence

of the single-stranded 2-AP oligonucleotide (Fig. 3*A*), suggesting it is not just enzyme induced local unwinding of the helix resulting in a region of single-stranded DNA surrounding the 2-Ap (see below for discussion).

Correlation of Enhanced Fluorescence with the Extrahelical Base of the Co-crystal Structure of E23Q Bound to Dimer-containing DNA—To support further the idea that the enhanced fluorescence observed with the wild type enzyme on the non-cleavable substrates was indeed due to the 2-aminopurine being moved extrahelically by the enzyme, fluorescence studies were conducted using the E23Q enzyme and TT/2AP duplexes. The duplexes containing a site-specific thymine dimer with a 2-Ap base placed opposite either the 5′-thymine (5′ T/2-Ap; Table I, part IIa) or the 3′-thymine (3′ T/2-Ap; Table I, part IIb) showed similar excitation and emission scans. Likewise, the fluorescence of the 2-Ap base was quenched significantly upon duplex formation (Fig. 5*A*). Following an emission scan of the 5′ T/2-Ap at the optimum excitation wavelength, E23Q was titrated into the binding reaction at the indicated concentrations (Fig. 5*B*). As seen in the emission scans, a significant increase in the fluorescence was observed in a concentration-dependent manner upon E23Q binding to the duplex containing the 2-Ap directly opposite the 5′-thymine of the TT. This increase was typically 3–4-fold over the base intensity of the duplex DNA in the absence of enzyme. At high concentrations of E23Q, the enhanced fluorescence begins to be quenched (Fig. 5*B, dotted lines*) suggesting saturation of the specific binding sites. This is most likely due to the presence of multiple enzyme molecules bound to the DNA, which has been shown to occur on this substrate as evidenced by gel mobility shift analysis (Fig. 2*A*).

The most convincing evidence that the enhanced fluorescence upon endonuclease V binding is due to movement of a base extrahelically and not to general disturbances in the local helical structure is shown in Fig. 5*C*. The placement of the 2-Ap base opposite the 3′-thymine of the dimer (*i.e.* one base 5′ to the previous duplex, see Table I, part IIb) resulted in no apparent increase in fluorescence intensity of the duplex upon E23Q binding (Fig. 5*C*). As shown in Fig. 2*A*, this duplex is bound by the enzyme; thus, the enhanced fluorescence due to E23Q binding to TT/2Ap DNA is highly specific for only the base that is predicted to be moved extrahelically based on the co-crystal structure. Binding of E23Q to a control duplex containing a normal thymine opposite the 2-Ap produced no increase in fluorescence intensity, indicating the enhanced fluorescence is due to a specific binding event and does not occur on nontarget DNA as detected by this method.

Interestingly, when the effect of E23Q binding to the pyrrolidine/2-Ap and the reduced AP/2-Ap duplexes was investigated, no increase in fluorescence was observed (Fig. 5*C*). This was not expected as E23Q binds to these substrates with relatively high affinity similar to wild type endonuclease V (Fig. 2*A* and Ref. 28), and the wild type enzyme does increase the fluorescence upon binding to these two duplexes (Fig. 4). Thus, despite the specific binding to the pyrrolidine and reduced AP site-containing duplexes, the E23Q does not appear to move the 2-Ap extrahelically in these duplexes. Thus, specific binding can be achieved without a stable base flipping event.

Quenching of the Enzyme-enhanced 2-Aminopurine Fluorescence—To assess the environment of the extrahelical 2-Ap, quenching studies were performed using acrylamide as the probe. The fluorescence emission scans were obtained for the pyrrolidine/2-Ap duplex, the single-stranded 2-Ap oligonucleotide, and the pyrrolidine/2-Ap with endonuclease V bound. Acrylamide was added at increasing concentrations as indicated, and emission scans were observed. The data were ana-
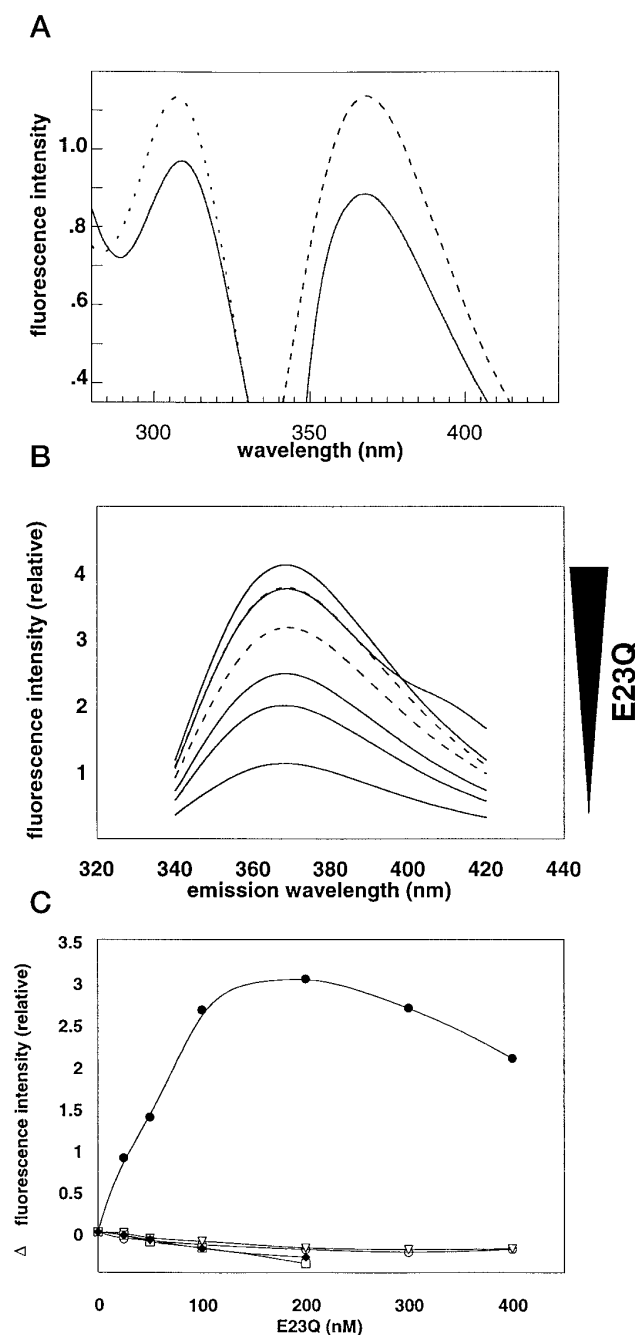
FIG. 5. **Enhanced fluorescence of 2-aminopurine/thymine dimer duplexes as a function of E23Q concentration.** Steady state fluorescence emission spectra were obtained using a SPEX Industries model FL221 fluorometer at a fixed excitation wavelength of 307 nm. Reactions contained 200 nM DNA in 25 mM sodium phosphate, pH 6.8, and 100 mM KCl at 25 °C. All buffer and enzyme fluorescence emissions were subtracted from these scans. *A*, effect of thymine dimer containing- duplex formation on the fluorescence of a 2-Ap containing 12-base oligonucleotide. Fluorescence emission spectra of the single-stranded 2-Ap containing 12-mer (*dotted line*) was quenched when annealed to the complementary strand (*solid line*). *B*, representative emission scans of 5′ T/2-Ap DNA in the presence of E23Q mutant endonuclease V. Reactions contained 200 nM DNA in the standard buffer of 25 mM sodium phosphate, pH 6.8, 100 mM KCl at 25 °C. The fluorescence emission scans of the DNA alone (*bottom curve*) and in the presence of increasing E23Q from 25–400 nM as indicated on the *right axis* are shown. The *dotted lines* represent emission scans at the highest concentration of enzyme used (300 nM and 400 nM). *C*, graphic representation of fluorescence emission intensity at λ_{max} of 370 nm. Reactions were performed as in *B* for 5′ T/2-Ap (*closed circles*), 3′ T/2-Ap (*open circles*), pyrrolidine/2-Ap (*inverted triangles*), rAP/2-Ap (*squares*), and C/2-Ap (*diamonds*). The peak emission fluorescence at 370 nm was plotted as a function of enzyme concentration.
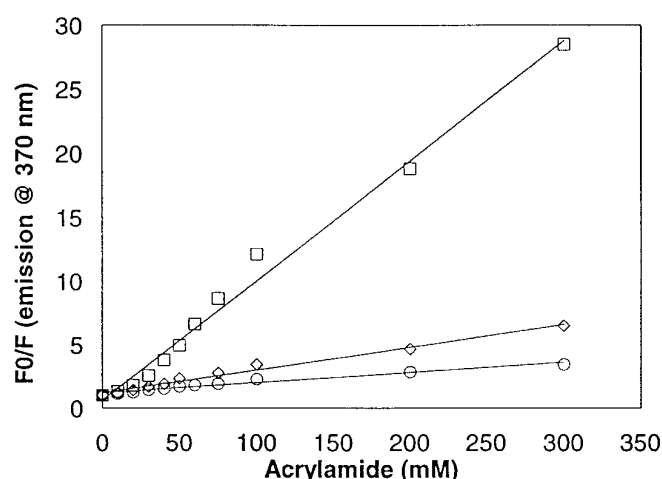
FIG. 6. **Acrylamide quenching of endonuclease V-induced fluorescence enhancement of 2-Ap containing DNA.** Reactions contained 200 nM DNA in the standard buffer of 25 mM sodium phosphate, pH 6.8, 100 mM KCl at 25 °C. The fluorescence emission scans (at fixed excitation of 305 nm) of the DNA alone either single-stranded (*diamonds*), duplex (*circles*), or duplex in the presence of 200 nM endonuclease V (*squares*) were observed in the presence of increasing amounts of acrylamide as indicated. The change in emission fluorescence at λ_{max} 370 nm was plotted as a function of quencher concentration.

lyzed as the ratio F_0/F, where F_0 is the fluorescence in the absence of quencher and F is the fluorescence in the presence of quencher. The data are presented in Fig. 6.

In the absence of bound endonuclease V, the acrylamide quenches the 2-Ap fluorescence both in the single-stranded DNA and the duplex DNA. When the fluorescence is enhanced by the binding of enzyme, the acrylamide quenching of the fluorescence signal is quite substantial. The linearity of the Stern-Volmer plots for the DNA alone is indicative of a single population of fluorophore within the DNA (33), whereas the slight curvature seen with the enzyme-DNA complex may reflect a combination of acrylamide quenching the 2-Ap in the enzyme complex, as well as quenching occurring in DNA molecules without enzyme bound (*i.e.* all molecules may not be bound under these conditions). An alternative explanation is that the curvature may be indicative of a static quenching component. The mechanism underlying this observation can be ascertained through lifetime measurements.

To ensure that the decreased fluorescence signal in the presence of acrylamide was not due to acrylamide inhibition of the enzyme, the effect of acrylamide on enzyme activity was investigated. No effect on the enzyme's activity was observed (data not shown); thus, the decreased fluorescence is due to acrylamide quenching of the signal. This result, in conjunction with the fluorescence enhancement data in Fig. 4, indicates that the base opposite an abasic site analog is positioned extrahelically when bound by the wild type enzyme.

DISCUSSION

The movement of bases extrahelically as a means of promoting specificity for DNA repair enzymes is a process that is difficult to ascertain in the absence of x-ray crystallographic structures. This study has demonstrated that the movement of the base opposite the target site by a repair enzyme may be measured by fluorescence techniques. More significantly, the discrete steps along the pathway leading to catalysis, such as specific binding and base flipping, can be investigated by using a combination of modified oligonucleotides that form stable, pre-catalytic complexes representing intermediates along the reaction pathway. The evidence that the wild type enzyme exhibits base flipping on the pyrrolidine and reduced AP DNAs,

both abasic site analogs, suggests that base flipping does occur for endonuclease V on both the abasic site target as well as the thymine dimer target. However, these data await confirmation by x-ray crystallographic studies on the pyrrolidine DNA-endonuclease V complex.[2] These studies will provide evidence as to whether this base flipping event does occur when endonuclease V encounters abasic sites in the DNA (not associated with glycosylase action of the enzyme). It is interesting that the catalytic mutant E23Q, which does move the adenine opposite the thymine dimer extrahelically, is not capable of stably "trapping" the same base extrahelically, when it is placed opposite an abasic site analog. Interestingly, another catalytic mutant E23D also exhibits no detectable base flipping on the pyrrolidine/2Ap duplex (data not shown). However, due to the E23D mutant having general lower binding affinity to these substrates, it is difficult to determine if the lack of flipping is due to steric interference in the case of E23D, if the charge difference on E23Q has any effect on base flipping opposite an abasic site, or if subtle structural differences at the location of the mutant residues destabilize the extrahelical base conformation. A more detailed analysis is necessary to understand the differences between these catalytically inactive mutants which appear to exhibit some differences in base flipping as compared with the wild type enzyme.

The wild type endonuclease V binds tightly to both the pyrrolidine/2Ap and the reduced AP/2Ap and moves the opposing base extrahelically, and yet these are not catalytic substrates for the enzyme. Therefore, using these substrate analogs, it appears that base flipping may occur in the absence of catalysis, *i.e.* base flipping by T4 endonuclease V is not sufficient for catalysis to occur. Likewise, using the E23Q catalytic mutant, we can observe wild type levels of binding to the pyrrolidine or reduced AP, yet no movement of the opposing base is detected. Thus, we can separate tight, specific binding from base flipping (*i.e.* base flipping does not result from all specific binding events), as well as base flipping from catalysis. This implies that base flipping does not add to the stability of the specific enzyme-DNA complex but rather induces a conformational change to facilitate catalysis at the appropriate target site. The fact that this assay cannot detect base flipping associated with nonspecific binding events is evidence that a stable extrahelical conformation is not maintained; however, this does not address the possibility that endonuclease V may use base flipping as a means of scanning nontarget DNA (34, 35), transiently moving bases extrahelically to probe for the target base. These transient species would not be detected using this method. Therefore, it is still plausible that although base flipping does not occur with every specific binding event on these substrate analogs, endonuclease V may still utilize this process as a means to discriminate target from nontarget DNA.

The quenching study reveals that the protein residues in close proximity to the flipped 2-Ap are not interfering with the ability of the relatively large acrylamide molecule to quench the enzyme-induced fluorescent enhancement. This approach may prove useful in revealing the microenvironment of the extrahelical base. These data taken together with the enhanced fluorescence and the x-ray crystallographic structures for T4 endonuclease V indicate that the fluorescence approach is indeed measuring a base flipping mechanism. We should point out, however, that caution should be taken when interpreting these types of fluorescence analyses in the absence of independent corroborating data. There is much evidence in the literature for enhanced 2-Ap fluorescence upon binding of enzymes

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[2] G. Verdine and T. Ellenberger (Harvard University), work in progress.

which is not attributable to bases being moved out of the DNA helix. For example, changes in 2-AP fluorescence have been used to demonstrate helicase activity (21) and nucleotide incorporation and exonucleolytic removal of bases by polymerases (19). These studies give similar enhanced fluorescence signals as seen for T4 endonuclease V, yet when taken together with other biochemical and structural data, the mechanisms of the enhanced fluorescence are quite distinct.

In this regard, while these studies were in progress, Allan and Reich (25) presented data using a similar assay to study the *Eco*RI DNA methyltransferase binding to its target adenine. In their system, the enhanced fluorescence by the methyltransferase was much greater and accompanied by a blue spectral shift. This is interesting because it implies an altered environmental state around the extrahelical base unlike that of T4 endonuclease V. Another difference is that they observe adjacent base effects, *i.e.* when they place the fluorophore one base away on the same strand as the adenine that is moved extrahelically, they still observed an enhanced fluorescence, whereas with endonuclease V, there is no fluorescence enhancement observed when the fluorophore is moved by one position. Thus, the local changes in DNA structure may be more rigid for T4 endonuclease V as shown in the co-crystal structure, where there is no distortion of the adenine opposite the 3′-thymine, or it is possible that the protein residues surrounding that base may quench any increase in signal. Since the methyltransferase studies were done without knowledge of the co-crystal structure, it will interesting to compare their findings with a structure when one becomes available.

T4 endonuclease V is the only enzyme for which it has been structurally demonstrated that the base opposite the target site is moved, bringing the active site to the base rather than the base to the active site. This suggests that there may be at least two generalized mechanisms for base flipping: one in which the target base is flipped into the enzyme's active site, and the other in which the base that is complementary to the target base is flipped to allow the enzyme's active site access to the target glycosydic bond for the catalytic reaction. The crystal structures of other repair enzymes, for which active sites have been determined but were solved in the absence of the DNA substrate, also suggest they may utilize a base flipping mechanism to gain access to the catalytic site. Interestingly, it has been speculated, based on the crystal structure of a DNA photolyase, another repair enzyme which recognizes cyclobutane pyrimidine dimers, that unlike T4 endonuclease V, the target pyrimidine dimer is flipped and moved into a cavity on the enzyme (36). It remains to be determined if other repair enzymes demonstrate an extrahelical movement of the nontarget base as does endonuclease V.

The pathways or mechanisms employed for moving a base extrahelically and returning the base into the B-form helix remain unclear. Recent evidence suggests that the human uracil DNA glycosylases inserts a conserved amino acid residue, Leu-272, into the minor groove, promoting the movement of the uracil extrahelically (37). In the case of T4 endonuclease V, the sharp bend in the DNA substrate at the target site associated with unusual phosphate conformations appears to promote the movement of the base extrahelically, rather than the insertion of a specific protein residue. It remains to be determined which specific amino acid residues are involved in this drastic DNA conformational change associated with T4 endonuclease V binding as well as which residues play critical roles in the trapping of the extrahelical base. Likewise, it is still unclear what promotes the release of the base and its movement back into the helix. T4 endonuclease V, having two very distinct substrates, a pyrimidine dimer and an abasic site, presents a

unique opportunity for examining whether the DNA conformational change and the base movement occur for both DNA modifications to a similar extent and at a similar rate. Using these types of analyses, combined with structural information, detailed mechanistic models for extrahelical base movement as a mode of damage recognition and/or active site positioning used by DNA repair enzymes can be established.

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ENZYMOLOGY:

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