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Structural Determinants for Specific Recognition by T4 Endonuclease V*

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DNA glycosylases catalyze the scission of the N-glycosyl bond linking either a damaged or mismatched base to the DNA sugar phosphate backbone. T4 endonuclease V is a glycosylase/apurinic (AP) lyase that is specific for UV light-induced cis-syn pyrimidine dimers. As a proposed transition state analog/inhibitor for glycosylases, a phosphoramidite derivative containing a pyrrolidine residue has been synthesized. The binding of endonuclease V to this duplex was analyzed by gel mobility shift assays and resulted in a single stable complex of reduced mobility and an apparent $K_d$ of 17 nM. To assess the importance of the positive charge for specific binding, studies using other non-cleavable substrate analogues were performed. Wild type T4 endonuclease V shows an 8-fold decreased affinity for a tetrahydrofuran as compared with the pyrrolidine residue, demonstrating the significance of the positive charge for recognition. A 2-fold increase in binding affinity for a reduced AP site was observed. Similar assays using catalytically compromised mutants (E23Q and E23D) of endonuclease V demonstrate altered affinities for the pyrrolidine as well as tetrahydrofuran and reduced AP sites. This approach has provided insight into the structural mechanism by which specific lesions are targeted by the protein as well as the structural determinants of the DNA required for specific recognition by T4 endonuclease V.

Base excision repair provides organisms a major line of defense against a multitude of base damage including UV-induced cyclobutane pyrimidine dimers, alkylation, and misincorporation (1). The initiating cascade of events in base excision repair includes specific site location, damage recognition, and excision of the improper or modified base by a DNA glycosylase. All DNA glycosylases catalyze the breakage of the N-C$_1$' glycosyl bond linking the damaged base to the sugar phosphate backbone. A subset of these glycosylases has a concomitant apurinic (AP) lyase activity, which catalyzes a $\beta$-elimination reaction leaving a 3' $\alpha$-$\beta$-unsaturated aldehyde and a 5' phosphate. This product is then further processed by an endonuclease to create a 3'-OH for polymerization and ligation.

T4 endonuclease V has served as the prototype for mechanistic studies of the molecular basis of recognition and catalysis for the glycosylase/AP lyase enzymes, including determination of the active site (2, 3), the residues necessary for DNA binding (4), and the chemical basis of catalysis (5). T4 endonuclease V is a 16-kDa cyclobutane pyrimidine dimer-specific glycosylase with an associated AP lyase activity. It has been shown that T4 endonuclease V binds DNA through electrostatic interactions and then scans along the helix in a salt-dependent one-dimensional search until a pyrimidine dimer is encountered (6, 7). The enzyme then cleaves the N-glycosyl bond between the base and the sugar on the 5' side of the dimer and subsequently cleaves the phosphodiester bond between the pyrimidines.

The x-ray crystal structure of T4 endonuclease V has been solved and has revealed the presence of a glutamic acid residue (Glu-23) near the active site nucleophile (Thr-2), thus implicating this acidic residue in the reaction chemistry (8, 9). Recently, building on the vast amount of information obtained on T4 endonuclease V, a unified catalytic mechanism for DNA glycosylases and glycosylase/AP lyases, has been proposed (10). In this model, the initial catalytic process proceeds by a nucleophilic attack at the sugar C$_1$' of the damaged base (Fig. 1, structure 1). It is proposed that the glycosylase/AP lyase enzymes use an amino group as the attacking nucleophile, resulting in an imino intermediate that can be trapped experimentally by reduction with NaBH$_4$ (Fig. 1, structure 3). Enzymes with only the glycosylase activity use a nucleophile from the medium such as an activated water molecule. Recently, the co-crystal structure of a catalytically inactive T4 endonuclease V mutant (E23Q) with dimer containing DNA has been solved (11). Interestingly, the crystal structure revealed a sharp kink in the DNA helix at the thymine dimer, and the adenine base opposite the 5'-thymine of the dimer is flipped out of the DNA duplex and into a pocket on the protein surface (11).

Although the biochemistry and structural information on T4 endonuclease V have led to the proposed catalytic mechanism and the identification of two active site groups (the N-terminal amino acid and Glu-23), there are still many gaps in our understanding of glycosylase mechanisms. One of the barriers to obtaining a detailed picture of the interactions of glycosylases with DNA is the relatively fleeting nature of the complexes that are formed. Thus, the production of stable long-lived complexes between DNA repair enzymes and DNA would facilitate an understanding of the interactions that occur prior to catalysis, including site-specific recognition and base flipping. In an effort to design molecules that bind DNA repair proteins in stable long-lived complexes suitable for structural analysis, Schärer et al. (12) presented data on a pyrrolidine-based inhibitor for AlkA, an *Escherichia coli* DNA glycosylase. The pyrrolidine residue contains a positively charged nitrogen in place of the endocyclic oxygen mimicking a proposed transition state for glycosylases, where a positive charge is accumulated at the O$_1$/C$_1$' (Fig. 1, structure 2). Recently, Schärer et al. (12) have shown that AlkA binds very tightly to this duplex with an apparent $K_d$ of 16 pM and that this transition state analog also

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§The abbreviations used are: AP, apurinic; BSA, bovine serum albumin; bp, base pair(s).
serves as a potent inhibitor for the AlkA-catalyzed reaction.

This study utilizes the pyrrolidine inhibitor to make comparative measurements of binding affinities for T4 endonuclease V on a series of related DNA structures to gain insight into the mechanism of specific site recognition. This approach may prove useful to 1) examine the catalytic distinction between glycosylases and glycosylase/AP lyases, 2) determine the structural features of the DNA that mimic the transition state for UV-specific glycosylases, 3) determine the molecular architecture of the reaction intermediates, and 4) determine the structural features of the protein required for differential recognition and catalysis.

EXPERIMENTAL PROCEDURES

T4 Endonuclease V—T4 endonuclease V (wild type) was purified from E. coli AB2480 (recA−, uvrA−) cells transformed with a denV expression vector as described previously (13). E23Q and E23D endonuclease V mutants were created by site-directed mutagenesis and purified as described (14).

Oligonucleotide Substrates—DNA oligonucleotides containing a site-specific pyrrolidine residue, reduced abasic site residue, or a propanediol residue were synthesized (15). DNA containing a site-specific tetrahydrofuran residue was a generous gift from Francis Johnson (SUNY, Stonybrook, NY). Complementary sequences were synthesized using standard procedures, and the deprotected oligonucleotides were electrophoretically purified on 20% denaturing polyacrylamide gels. The bound and free substrate bands were visualized by autoradiography of the wet gels using Hyperfilm-MP.

Binding Assays—The pyrrolidine, reduced abasic site, tetrahydrofuran, and propanediol-containing oligonucleotides were 5′-end labeled and annealed to the complementary strand (32P)ATP and annealed to a complementary strand with the indicated base opposite the modification (Table I).

Determination of Nonspecific Binding Affinity—Standard gel mobility shift binding assays were performed in the presence of increasing amounts of nontarget DNA. A control 25-base oligonucleotide containing a cytosine at position 13 was 5′-end labeled and annealed to its complementary strand. In a separate experiment, a pBKS plasmid (3 kilobases) was used as a nontarget DNA competitor. T4 endonuclease V was incubated with increasing amounts of T4 endonuclease V (40 nM) and pyrrolidine containing duplex (100 pM) were incubated in the presence of various amounts of nonspecific DNA as indicated. Following 30 min at 25 °C, the samples were loaded onto a 7.5% native polyacrylamide gel. The bound and free substrate bands were visualized and quantitated as described above.

Thymine Dimer-specific Nicking Activity and Inhibition of T4 Endonuclease V—The CS-49 duplex substrate (250 pM) was incubated with T4 endonuclease V in the standard reaction buffer (25 mM sodium phosphate (pH 6.8), 100 mM KCl, 100 μg/ml BSA) in a total volume of 20 μl. The potential inhibitors were added simultaneously with the substrate where indicated, prior to the addition of limiting enzyme (25–50 nM). Reactions were incubated at 25 °C for 5 min and terminated by placing the reaction mixture on a dry ice-ethanol bath. An equal volume of loading buffer (95% (v/v) formamide, 20 μM EDTA, 0.02% (w/v) bromophenol blue, 0.02% (w/v) xylene cyanol) was added, and the samples were heated to 90 °C for 5 min prior to loading on 15% denaturing polyacrylamide gel (8 μM urea) in a 1 × TBE buffer (90 mM Tris borate, 2 mM EDTA, pH 8.0). The DNA was separated by electrophoresis for 3 h at 800 volts. Bands were visualized by autoradiography of the wet gels using Hyperfilm-MP x-ray film (Amersham). The appearance of the 20-base product band was quantitated using a Molecular Dynamics PhosphorImager and ImageQuant software (Sunnyvale, CA). The data were plotted using Kaleidagraph (Synergy Software, Reading, PA).

RESULTS

Experimental Rationale—To assess the relative binding affinities of T4 endonuclease V for specifically modified oligonucleotides representing a series of proposed transition state analogs or substrate analogs, gel mobility shift analysis was utilized. This analysis has provided a detailed picture of the apparent equilibrium binding affinities (Kd) and the architecture of the DNA necessary for optimal discrimination between nontarget and target sites.

As shown in Fig. 2, the DNAs chosen include a proposed...
transition state analog (pyrrolidine) and DNA containing abasic site analogs either incapable of sugar ring opening (tetrahydrofuran) or incapable of ring closure (reduced apurinic site). A structure completely lacking the sugar ring but retaining the phosphodiester backbone (propanediol) was also included in the study.

Determination of Active Molecules of Endonuclease V—The fraction of active molecules of wild type endonuclease V was determined by active site titration analysis on dimer-containing DNA. The 5'-end-labeled CS-49 was annealed to the complementary strand, and 1 nM was incubated with increasing amounts of endonuclease V (0–10 nM) in the standard reaction buffer in the presence of NaBH₄, a reducing agent previously shown to covalently trap endonuclease V on pyrimidine dimer-containing DNA (5). Following incubation at 25 °C, the reactions were analyzed for the number of endonuclease V molecules covalently trapped on the DNA, indicating the number of active sites available (data not shown). Under stoichiometric conditions, 50% of the T4 endonuclease V molecules were active, and thus, all reported concentration values reflect this activity. As it was not possible to determine the number of active sites for catalytically compromised mutants, the binding constants are reported assuming 100% active protein, and thus may be underestimating the true affinity of the mutants for these DNAs.

Wild Type Endonuclease V Binding to Substrate Analogs—Binding of T4 endonuclease V to the pyrrolidine-containing duplex was monitored by gel mobility shift analysis. The interaction of T4 endonuclease V with this DNA results in the formation of a single stable complex in a 7.5% native polyacrylamide gel. This complex is specific for the pyrrolidine-containing duplex DNA, since it was resistant to competition with nonspecific competitor DNA. Either nontarget 25-base duplex DNA or nondamaged plasmid DNA (0–4 μM, circles) were examined. Dixon plot analysis was performed for the determination of the apparent dissociation constants for both the linear 25-bp duplex and the plasmid DNAs (Fig. 4). Using the Kₐ for T4 endonuclease V binding to the pyrrolidine-containing 25-bp DNA (determined in Fig. 3), the following equation (17) was utilized to determine the nonspecific binding affinity (Kₑₛ):

\[
x \text{ intercept } = -K_a(1 + S/K_a) \quad (\text{Eq. 2})
\]

where \( S \) is substrate DNA concentration and \( K_a \) is the binding affinity for substrate DNA as determined by gel mobility shift assays. Using this analysis, the apparent equilibrium binding affinity of T4 endonuclease V for the 25-bp duplex and the plasmid DNA are \( 1.5 \times 10^{-6} \) M and \( 1.2 \times 10^{-6} \) M, respectively.

Catalytically Compromised T4 Endonuclease V Mutants Binding to Substrate Analogs—Equilibrium binding studies were performed using gel mobility shift assays as described above. Two previously described catalytically compromised en-
Specific Recognition by T4 Endonuclease V

TABLE I

Sequences of site-specific analog-containing duplexes

| DNA duplex        | Endo V | E23Q | E23D |
|-------------------|--------|------|------|
| Pyrrolidine       | 5'-GGAAGTGTGCGA (PYR) GAGCTCAAGCGC-3' |
| Reduced AP site   | 5'-GTAACCTGAGC (RAP) TATGCGTAAAC-3' |
| Propanediol       | CACCTAGAGCTGCAATGAGCTTCG |
| Tetrahydrofuran   | 5'-GCCGAGTGGAGCCGG (PD) CAGGGTGCCACG-3' |
|                   | TGGCGGTGCTCAGGG (THF) TGGCGGTGCTCAGGG |

FIG. 4. Dixon plot analysis of nonspecific binding of T4 endonuclease V. Endonuclease V (40 nm) was incubated with the pyrrolidine DNA (100 pm) in the standard binding buffer (25 mM sodium phosphate (pH 6.8), 100 mM KCl, 5% glycerol, and 100 µg/ml BSA) in the presence of increasing amounts of nonspecific competitor plasmid DNA (closed circles) or 25-bp DNA (closed squares) as indicated. Following a 30-min incubation at 25°C, the reactions were loaded onto a 7.5% native polyacrylamide gel, and the bound and free substrate bands were quantitated as described.

specific binding of T4 endonuclease V to pyrrolidine-containing analog/inhibitor as well as other noncleavable abasic site analogs. This glycosidase/AP lyase shows relatively tight binding in the nanomolar range for these DNAs. In the studies using the pyrrolidine-containing DNAs, the presence of a positive charge enhanced the binding of T4 endonuclease V 8-fold as compared with a neutral sugar ring-closed structure (tetrahydrofuran), while the stable ring-opened form (reduced AP) enhanced the binding 16-fold. Thus, both the positive charge and the ring-opened structure facilitate tighter T4 endonuclease V binding. As seen in Fig. 1, the proposed chemistry would predict that both of these structures resemble intermediates along the reaction pathway. However, it has yet to be determined whether the formation of the covalent intermediate occurs before or after ring opening of the sugar. As reported previously, AlkA, an E. coli DNA glycosylase, also bound to the pyrrolidine DNA with high affinity; however, unlike T4 endonuclease V, it showed a 3 × 10^4-fold enhancement of binding for the posi-
tively charged pyrrolidine as compared with the tetrahydrofuran-containing DNA (12). Other glycosylases and glycosylase/AP lyases have also been shown to bind specifically to the pyrrolidine DNA. Interestingly, of those examined, only interactions of AlkA and endonuclease III with the analog/inhibitor were highly specific for the presence of the positive charge (15) while 2,6-dihydroxy-5- N-formamidopyrimidine DNA glycosylase, adenine DNA glycosylase, and alkyl-N-purine DNA glycosylase behaved similarly to T4 endonuclease V, exhibiting only 10-fold or less discrimination between the pyrrolidine and the tetrahydrofuran. It appears from those studied to date that there is no correlation between the enzyme's substrate specificity and the relative discrimination between a positively charged analog as compared with a neutral abasic site analog.

To determine the specificity of T4 endonuclease V, binding affinities for linear and circular duplex nontarget DNA were determined and found to be approximately $1 \times 10^{-6}$ M. Consequently, T4 endonuclease V discriminates between undamaged DNA and a pyrrolidine-containing DNA by a factor of 100, while for the tetrahydrofuran or propanediol the discrimination decreases to only 10- and 5-fold, respectively. This high affinity for nontarget DNA is expected, since T4 endonuclease V uses a nontarget scanning mechanism for specific site location.

During the course of these studies, it was noted that the base opposite the pyrrolidine residue does not affect T4 endonuclease V affinity for binding (data not shown). Using the 25-base oligonucleotide containing the pyrrolidine, complementary strands containing a cytosine, adenine, thymine, or guanine at the position opposite the pyrrolidine were synthesized. No difference in equilibrium binding affinities was observed as measured by gel mobility shift assays. This was unexpected as the co-crystal structure has shown that the adenine opposite the 5'-thymine of the thymine dimer is flipped out of the DNA helix and into a "pocket" in the enzyme (11). In the case of uracil DNA glycosylase, the pocket is highly specific for uracil. Preliminary fluorescence experiments utilizing the pyrrolidine residue with a 2-aminopurine in the complementary strand suggest that the base opposite the pyrrolidine is flipped extrahelical.2 The binding results suggest that the T4 endonuclease V protein "pocket" may not be very specific for the flipped residue.

2 A. McCullough and R. S. Lloyd, unpublished observations.
out base as both purines and pyrimidines may be accommodated. This is supported by the co-crystal structure in which the flipped base is arranged between two amino acids, not forming any hydrogen bonds with protein residues, and unpublished data cited by Vassylyev et al. (11), demonstrating that the base opposite the 5'-thymine does not affect T4 endonuclease V glycosylase activity (11).

**Examination of the Structural Features of T4 Endonuclease V Required for Differential Recognition and Catalysis**—The x-ray crystal structure of T4 endonuclease V revealed the presence of Glu-23 near the active site (8, 9, 11), and site-directed mutagenesis of this residue has demonstrated its involvement in the catalytic mechanism of the enzyme (3, 14). E23Q demonstrates an increased affinity for both the reduced AP and the tetrahydrofuran as compared with the wild type endonuclease V but has the same affinity for the pyrrolidine DNA. In addition, E23Q retains relatively tight binding to a cis-syn thymine dimer substrate. Thus, the presence of the carboxyl group, though critical for catalysis, is not necessary for specific binding of the enzyme. In fact, the presence of a neutral residue at this position actually increases the DNA binding affinity of the enzyme 3.5× for the tetrahydrofuran and 4.5× for the reduced AP. The E23D mutant exhibited the same trend in binding affinities as did the E23Q mutant; however, the values were much higher indicating a lower affinity for all the DNAs investigated. These data are consistent with the E23D mutant being devoid of any glycosylase activity and having a diminished AP lyase activity despite it being a relatively conservative mutation. Thus, the position of a side chain in the active site is important for proper damage recognition by the enzyme, most probably due to a structural fit that may stabilize the complex. The decrease in specific binding by E23D may contribute to its diminished AP lyase activity previously reported (14). As with the E23Q, E23D showed the same affinity for the dimer-containing DNA as the pyrrolidine DNA; however, it bound with a 3-fold higher affinity to the reduced AP DNA and with only slightly less affinity to the tetrahydrofuran as compared with the pyrrolidine. Thus, for both mutants, the presence of the positive charge makes only a slight difference in the binding affinity (1.5–2-fold).

**Use of Transition State and Substrate Analogs for Structural and Mechanistic Studies on DNA Glycosylase/AP Lyases**—Base excision repair is initiated by a complex cascade of events leading to damage recognition and catalysis. These events include target site location, specific binding, active site positioning (which may involve a conformational change in the enzyme and base flipping), and catalytic chemistry. This study has demonstrated that stable enzyme-DNA complexes can be formed between a DNA glycosylase/AP lyase and a proposed transition state analog as well as other structurally related substrate analogs. These DNAs, as well as the catalytically compromised mutants, may provide a detailed examination of the steps preceding catalysis by allowing numerous structural and mechanistic studies on the stable enzyme-DNA complex, including NMR and x-ray crystallography. The structural characteristics of the analogs investigated in this study have revealed potential structures for a series of second generation transition state analogs, such as a positively charged sugar ring-opened structure that may form an even more stable complex with T4 endonuclease V. This approach will not only make structural studies more feasible but will provide a means of dissecting the pre-catalysis mechanism for T4 endonuclease V as well as other glycosylases (this study and Ref. 15).

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