Evidence for Multiple Imino Intermediates and Identification of Reactive Nucleophiles in Peptide-Catalyzed β-Elimination at Abasic Sites†

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ABSTRACT: Prior investigations have demonstrated that peptides containing a single aromatic residue flanked by basic ones, such as Lys-Trp-Lys, can incise the phosphodiester backbone of duplex DNA at an AP site via β-elimination. An amine serves as the reactive nucleophile to attack C1′ on the ring-open deoxyribose sugar to form a transient peptide−DNA imino (Schiff base) intermediate, which may be isolated as a stable covalent species under reducing conditions. In the current study, we use this methodology to demonstrate that peptide-catalyzed β-elimination proceeds via the formation of two Schiff base intermediates, one of which was covalently trapped prior to strand incision and the other following strand incision. N-Terminal acetylation of reactive peptides significantly inhibited formation of a trapped Schiff base complex; thus, we demonstrate for the first time that the preferred reactive nucleophile for peptides catalyzing strand incision is the N-terminal α-amino group, not an ϵ-amino group located on a lysine residue as previously postulated. Trapping reactions in which the central tryptophan residue was changed to alanine did not have a significant impact on the efficiency of Schiff base formation, indicating that the presence of an aromatic residue is dispensable for the step prior to peptide-catalyzed β-elimination. Interestingly, the methodology presented here affords a convenient means for covalently attaching an array of peptides onto AP site-containing DNA in a site-specific fashion. We suggest that the generation of such DNA−peptide cross-links may provide utility in studying the repair of biologically significant DNA−protein cross-link damage as DNA−peptide complexes may mimic intermediate structures along a repair pathway for DNA−protein cross-links.

Damage to DNA bases occurs continually within the cell due to numerous endogenous processes and exogenous agents (*1*−*5*). Chemical modification of bases can occur via spontaneous deamination of cytosine to uracil, alkylation, oxidation, photochemical production of cyclobutane pyrimidine dimers, and misincorporation events during DNA replication. The DNA base excision repair (BER) pathway functions as the primary defense against such damage by the action of damage-specific glycosylase enzymes, which catalyze hydrolytic cleavage of the *N*-glycosyl bond to yield an apurinic/apyrimidinic (AP)[1] site as a common repair intermediate (*6*, *7*). In addition, spontaneous base loss contributes significantly to the formation of AP sites in vivo (*8*), and these noncoding lesions must be further processed to restore structural integrity to the DNA.

AP endonucleases are enzymes that specifically recognize AP sites and catalyze cleavage of the phosphodiester backbone. Two families of conserved 5′ AP endonucleases, each with distinct catalytic mechanisms, function to provide the requisite 3′-OH on the damaged strand for repair synthesis by DNA polymerase (*9*). The bifunctional glycosylase/AP lyase enzymes, such as T4-pdg, also generate single-strand nicks at AP sites; however, these enzymes catalyze β-elimination of their base excision products to yield a 3′ break and an α,β-unsaturated aldehyde product. A substantial body of work has now illuminated the mislabeling of the combined glycosylase/AP lyase enzymes as endonucleases because their 3′ products of β-elimination must be further processed by an authentic 5′ endonuclease to yield the essential 3′-OH for repair synthesis to proceed.

When first described, peptide-catalyzed strand incision of DNA at AP sites generated significant interest, because of the observation that particular peptides mimic the substrate specificity and phosphodiester cleavage action of the DNA

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[1] Abbreviations: AP, abasic; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; TBE, Tris-borate-EDTA; SDS, sodium dodecyl sulfate; UDG, uracil DNA glycosylase; Fpg, formamidopyrimidine DNA glycosylase; pdg, pyrimidine dimer glycosylase; $NaCNBH_3$, sodium cyanoborohydride; $NaBH_4$, sodium borohydride; KWK, lysine-tryptophan-lysine; KWKK, lysine-tryptophan-lysine-lysine; KWR, lysine-tryptophan-arginine; HWHH, histidine-tryptophan-histidine-histidine; RWRR, arginine-tryptophan-arginine-arginine; Ac-KWKK, N-terminal monoacetylated lysine-tryptophan-lysine-lysine; Ac-HWHH, N-terminal monoacetylated histidine-tryptophan-histidine-histidine; Ac-RWRR, N-terminal monoacetylated arginine-tryptophan-arginine-arginine; KYK, lysine-tyrosine-lysine; KKWK, lysine-lysine-tryptophan-lysine; PEWKK, proline-glutamate-tryptophan-lysine-lysine; TRWKK, threonine-arginine-tryptophan-lysine-lysine; DPC, DNA−protein cross-link; NER, nucleotide excision repair.

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endonucleases. The most extensively investigated peptide is Lys-Trp-Lys, although other tripeptides comprised of central aromatic residues flanked by basic ones (including Lys-Phe-Lys) can recognize AP sites and catalyze DNA strand incision (*10*, *11*). Prior studies have established that Lys-Trp-Lys catalyzes β-elimination to yield an α,β-unsaturated aldehyde product, catalyzed by a reactive amine (*11*, *12*). The absence of a base at an AP site affords a space that is large enough to accommodate an aromatic side chain moiety that contributes stacking interactions between the adjacent bases to confer specificity in recognition. Such a model for Lys-Trp-Lys binding to DNA has received support from studies in which Lys-Trp-Lys binding to depurinated DNA was accompanied by an increase in the level of fluorescence quenching relative to the level of peptide binding to native DNA (*13*, *14*). The strand cleavage reaction catalyzed by peptides was dramatically inhibited in the presence of increasing NaCl concentrations (*11*); thus, a two-step binding mode has been proposed, mediated first by electrostatic interactions between basic residues and the DNA phosphate backbone, followed by specific base stacking interactions afforded by an aromatic residue (*15*).

β-Elimination proceeds via nucleophilic attack of an amine at C1′ of a reactive aldehyde at an AP site, although the ring-opened reactive aldehyde tautamer, and hydrated aldehyde, comprise only ~1% of the total abasic sites (*16*), while the predominant form of an AP site in solution is a mixture of α- and β-hemiacetals (*17*). An experimental hallmark of the reaction is the formation of an imino intermediate between the polypeptide and AP-containing DNA that may be isolated as a stable covalent species upon reduction by compounds such as sodium borohydride ($NaBH_4$). Several reports in the literature have employed the use of such reducing agents to probe the biochemical mechanism of enzymes possessing AP lyase activity (*18*−*20*). The chemistry is facilitated by the neutral, deprotonated form of the amine; thus, the relative pK_a of the amine and reaction pH are critical. In the active site of an enzyme, the pK_a of the reactive amino group may be sufficiently depressed to favor neutrality of this group at physiological pH, and computer simulations on the ionization state of the N-terminal reactive amine for the enzyme T4-pdg indicate such a decrease from 8.01 to 6.52 upon formation of the enzyme−substrate complex (*21*). In the case of T4-pdg, the α-amine located on the N-terminal threonine residue has been shown to catalyze β-elimination (*22*), although the N-terminal secondary amine of a proline residue for *Escherichia coli* formamidopyrimidine-DNA glycosylase (Fpg protein) has also been shown to catalyze this chemistry (*23*). In the case of Lys-Trp-Lys, it has been postulated that intercalation of the aromatic side chain moiety at an AP site brings the ϵ-amines of the lysine residues into the proximity of the reactive aldehyde to catalyze β-elimination. On the basis of the lower intrinsic pK_a of an α-amine, however, the N-terminal α-amine is predicted to react with greater efficiency.

In this report, we utilize a borohydride trapping methodology to demonstrate that peptide-catalyzed β-elimination at AP sites proceeds via the formation of an analogous Schiff base intermediate as is evidenced for enzymes possessing AP lyase activity. In addition, we demonstrate the formation of a second imine intermediate along the reaction pathway of β-elimination, corresponding to a nicked substrate−peptide complex. We have also generated an array of peptides to both determine the location of the reactive amine and evaluate the role of the aromatic residue in Schiff base formation, monitoring the trapped complex as the primary end point in our assay.

EXPERIMENTAL PROCEDURES

Materials. Lys-Trp-Lys and Lys-Tyr-Lys were obtained as acetate salts from Sigma. All other peptides were prepared by the Protein Chemistry Laboratory (University of Texas Medical Branch, Galveston, TX). Synthetic peptides were analyzed by mass spectrometry following crude synthesis, and the composition of each peptide was verified by observing a major peak corresponding to the predicted molecular mass. Peptides were subsequently purified by high-performance liquid chromatography before use and estimated to be >95% pure. T4-pdg was overexpressed and purified as described previously (*24*). Sodium borohydride and piperidine were obtained from Sigma. Sodium cyanoborohydride was obtained from Aldrich, and [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from DuPont-NEN.

Peptide Quantitation. All peptides were resuspended in a 20:80 acetonitrile/water solution. Peptides containing either a single Trp or a single Tyr residue were quantitated by monitoring absorbance at 280 nm using a Shimadzu BioSpec-1601 spectrophotometer. Concentrations were calculated using 5500 and 1490 M^{-1} cm^{-1} as the Trp and Tyr molar extinction coefficients, respectively (*25*). The peptide Lys-Ala-Lys was quantitated by monitoring peptide bond absorbance at 205 nm as described previously (*26*). Briefly, peptides were dissolved in a 0.01% (v/v) Brij 35 solution (Sigma) in a 20:80 acetonitrile/water solution, and the concentration was calculated using 31 mL mg^{-1} cm^{-1} as the peptide bond extinction coefficient. In the case where peptides were compared in a single experiment, all peptides were quantitated by absorbance at 205 nm. The calculation of the concentration using absorbance measurements at 205 nm was less precise than using measurements taken at 280 nm. However, a comparative analysis of the peptide concentration calculated utilizing Trp absorbance at 280 nm and peptide bond absorbance at 205 nm and weighing on an analytical balance for the peptide Lys-Trp-Lys showed the disparity between these values to be within 12.3%. Additionally, absorbance values measured at 205 nm for Lys-Ala-Lys showed a linear dependence on concentration over the range in which measurements were taken for the calculation of peptide concentration.

Uracil-Containing 26mer Oligonucleotide. An oligonucleotide with the sequence 5′-ACCATGCCTGCAAGAA**U**-TAAGCAATG-3′ was synthesized by the Midland Certified Reagent Co., as well as its complement 3′-TGGTACG-GACGTTCTTAATTCGTTAC-5′. Oligonucleotides were gel purified prior to use, and the U-containing oligonucleotide was γ-^{32}P-labeled on its 5′ end with T4 polynucleotide kinase and annealed to its complement following standard procedures. For experiments requiring single-stranded DNA in trapping reactions, the annealing step was omitted. To prepare AP site-containing substrates for reactions, U-containing 26mer duplex or single-stranded 26mer (11.1 nM) was incubated for 60 min at 37 °C with uracil DNA glycosylase (5 units/pmol of DNA, New England BioLabs) in 50 mM

HEPES (pH 7.0) and 5.0 mM NaCl. Due to the inherent instability of AP DNA, all nicking and trapping assays were carried out immediately following preparation of the substrate. Quantitative conversion of U-containing DNA to AP site-containing DNA was assessed in each experiment by treatment with T4-pdg (18.5 ng/μL) for double-stranded DNA or treatment with 5% piperidine at 90 °C for 3 min for single-stranded DNA. To verify proper annealing of the double-stranded substrate for trapping reactions (shown in Figure 6), single-stranded and double-stranded DNAs were incubated under standard reaction conditions [50 mM HEPES (pH 7.0) and 5 mM NaCl] for 30 min at 37 °C. To each reaction mixture was added 0.2 volume of loading buffer [0.25% (w/v) bromophenol blue and 40% (w/v) sucrose in H_2O], and samples were run on a 7.5% native polyacrylamide gel in 0.5× TBE (45 mM Tris-borate and 1.0 mM EDTA).

Incision of AP DNA by Peptides. AP-containing DNA (1 nM) was incubated with the specified concentration of peptide in 50 mM HEPES (pH 7.0) and 0.5 mM NaCl at 37 °C. Reactions were quenched by the addition of $NaBH_4$ dissolved in H_2O (final $NaBH_4$ concentration of 100 mM). An equal volume of loading buffer [95% (v/v) formamide, 20 mM EDTA, 0.02% (w/v) bromophenol blue, and 0.02% xylene cyanol] was added to each reaction mixture, and samples were heated at 90 °C for 3 min prior to analysis. Samples were loaded onto a 15% polyacrylamide gel (8.3 M urea) in sequencing buffer (134 mM Tris base, 44 mM boric acid, and 10 mM EDTA). The DNAs were separated by electrophoresis for 3 h at 2000 V.

Formation of Covalent Peptide−AP DNA Complexes Using $NaCNBH_3$. For general reactions, AP-containing DNA (1 nM) was incubated with 50 μM peptide (unless otherwise specified) in 50 mM HEPES (pH 7.0), 5 mM NaCl, and 25 mM $NaCNBH_3$ at 37 °C. $NaCNBH_3$ was added to each reaction mixture immediately preceding the addition of peptide, and reactions were quenched by the addition of $NaBH_4$ dissolved in H_2O (final $NaBH_4$ concentration of 100 mM). Samples were analyzed using identical gel conditions as described above.

Quantitation of Schiff Base Complexes. For reactions in which $NaCNBH_3$ was used to accumulate pre-incision Schiff base complexes, the major trapped covalent species is labeled in all figures. Additional complexes were observed, migrating within a denaturing gel to positions corresponding to multiple trapped peptide complexes on a single DNA. Since N-terminal acetylation abolished formation of these complexes, the amount of Schiff base complex was calculated as the total amount of shifted complex as a percentage of the amount of total DNA.

Salt Inhibition of Peptide Trapping. Trapping reactions were carried out as described above, with the addition of equal volume aliquots of NaCl solutions of increasing concentration, to yield the final concentrations as reported. Reaction volumes were adjusted so that the concentrations of peptide, $NaCNBH_3$, and AP site-containing DNA were unchanged. The contribution of Na^+ ion in solution due to Na-HEPES at pH 7.0 was calculated on the basis of a pK_a of 7.5 for HEPES and determined to be 12 mM. The contribution of Na^+ ion in solution due to $NaCNBH_3$ was estimated to be equal to the concentration of $NaCNBH_3$ (25 mM).

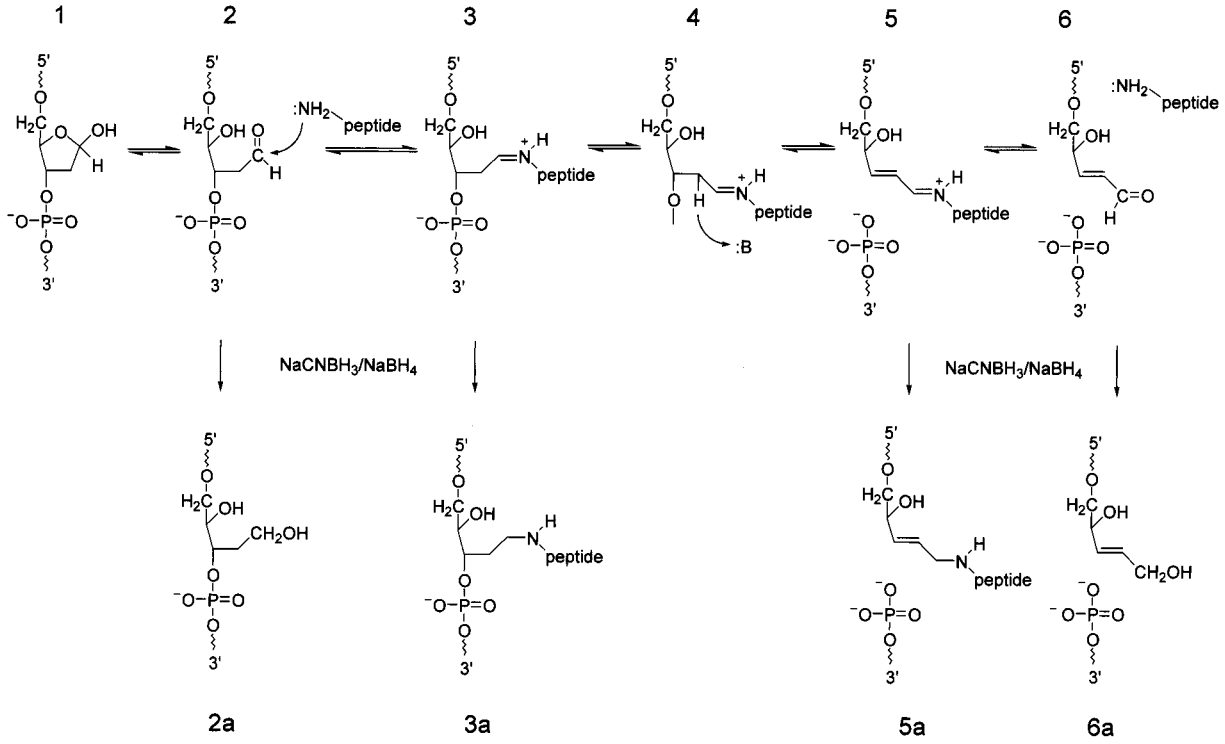
Illustrations. Results were visualized from wet gels by phosphoimager analysis, and product bands were quantitated using ImageQuant (version 5.0) software. The electronic gel files were processed with Adobe Illustrator (version 9.0). Regression analyses were performed using SigmaPlot (2001) software.

RESULTS

Lys-Trp-Lys Nicking at Abasic Sites and Identification of Reaction Intermediates. The first step in Lys-Trp-Lys-catalyzed β-elimination at an AP site involves nucleophilic attack of a peptide amine at C1′ on the deoxyribose sugar, which exists in equilibrium between a ring-closed form and a reactive ring-open aldehyde (Scheme 1, 1 and 2). Along the reaction pathway, multiple intermediates are predicted to occur, which in the presence of a reducing agent lead to the reduced AP site, reduced Schiff base complexes prior to and following strand incision, and the reduced α,β-unsaturated aldehyde product of β-elimination (Scheme 1, 2a, 3a, 5a, and 6a). To test for the formation of each intermediate, increasing concentrations of Lys-Trp-Lys were incubated with a 26 bp DNA containing a single, centrally located AP site, followed by quenching with $NaBH_4$ (Figure 1A). Quenching the reaction with $NaBH_4$ reduced the Schiff base intermediate that formed along the reaction pathway (Figure 1A, a), resulting in a species with retarded mobility as compared to substrate DNA retaining a reduced AP site (Figure 1A, b). We also observed the accumulation of an intermediate product band corresponding to a complex in which the Schiff base complex persists following base-catalyzed abstraction of the 2′-proton and strand break (Figure 1A, c). Because the product of β-elimination catalysis produces an aldehyde, which may itself react with nucleophilic amines on peptides, we tested for the ability of Lys-Trp-Lys to react with the α,β-unsaturated aldehyde product generated by treatment of the 26mer AP site-containing DNA with the bifunctional glycosylase/AP lyase T4-pdg. In this experiment, we observed a trapped complex under reducing conditions that migrated to a position identical to the intermediate product band from the peptide-catalyzed incision reaction (Figure 1A, c), confirming the identity of this species; on the other hand, we observe extremely slow reaction kinetics when monitoring the formation of a post-incision Schiff base when using the α,β-unsaturated aldehyde product of strand incision and peptide as the starting reactants (data not shown). Reaction with Lys-Trp-Lys also gave rise to two concentration-dependent cleavage products consistent with β-elimination and δ-elimination catalysis at an AP site. The α,β-unsaturated aldehyde product of T4-pdg-catalyzed incision was reduced to a primary alcohol in the presence of $NaBH_4$, slightly altering the mobility of this species, and one of the peptide-catalyzed product bands migrated to a position identical to that of the reduced product of β-elimination (Figure 1A, d). Peptide-catalyzed strand incision also produced the δ-elimination product (retaining a 3′-phosphate), which migrated to the same position in a denaturing gel as the product band produced by incubation with piperidine and heat treatment (Figure 1A, e).

Peptide Nicking and Covalent Trapping of Peptides at Abasic (AP) Sites. We next examined other peptides with the general structure Lys-Trp-X (where X represents one or more basic residues) for their ability to incise AP site-

Scheme 1: Overall Reaction for Peptide Trapping and β-Elimination Catalysis at an AP Site[a]



[a] Depicted are the hemiacetal (1) and aldehydic (2) forms of an AP site. Nucleophilic attack by a peptide amine at C1′ of the reactive deoxyribose aldehyde leads to formation of a protonated Schiff base complex (3). β-Elimination proceeds via abstraction of a 2′-proton (4), leading to DNA incision (5) and yielding a trans α,β-unsaturated aldehyde product (6). A subsequent δ-elimination reaction is not depicted in this scheme. Reactions carried out in the presence of $NaBH_4$ or $NaCNBH_3$ yield the following species: reduced AP site (2a), reduced Schiff base complexes prior to and following DNA incision (3a and 5a, respectively), and reduced α,β-unsaturated aldehyde product of β-elimination (6a).

containing DNA and form reaction intermediates equivalent to those observed for Lys-Trp-Lys. In a time course experiment, the peptides Lys-Trp-Lys-Lys and Lys-Trp-Arg produced results similar to those with Lys-Trp-Lys on the 26 bp AP site-containing substrate when quenched with $NaBH_4$ at the end of each incubation period. Each of the two Schiff base intermediates trapped along the reaction pathway migrated with varying mobility, dependent upon the molecular mass of each peptide (Figure 2A, a and c); however, the resulting cleavage products for each reaction migrated to the same positions, consistent with identical β-elimination and δ-elimination products for each peptide (Figure 2A, d and e). In an attempt to accumulate the initial (pre-incision) Schiff base intermediate as an end point in our trapping assay, we conducted experiments in which sodium cyanoborohydride ($NaCNBH_3$) was present throughout the reaction, followed by quenching with $NaBH_4$. In a similar time course experiment as described above, reactions for all peptides showed accumulation of the pre-incision Schiff base complex (Figure 2B, a), while the production of a post-incision Schiff base complex and incision products was virtually eliminated. The disappearance of the AP site-containing oligonucleotide substrate was faster in the presence of $NaCNBH_3$ (Figure 2B) than the corresponding reaction without $NaCNBH_3$ (Figure 2A), despite the use of a lower peptide concentration in the former reactions. We attribute this result to the rapid and continual removal of the pre-incision Schiff base complex from the reaction by the irreversible reduction of this species in the presence of $NaCHBH_3$ (species 3 to 3a, Scheme 1); in effect, this shifts the equilibrium to favor the removal of starting substrate DNA. In contrast, experiments in which $NaBH_4$ was added simultaneously with peptide yielded inefficient trapping of any of the DNA−peptide intermediate complexes (data not shown). Because the reduction of an aldehyde gives rise to an unreactive alcohol moiety at an AP site, the above result suggested preferential reduction of the pre-incision Schiff base complex as compared to reduction of the AP site aldehyde in the presence of $NaCNBH_3$. Consistent with this interpretation, McCullough et al. have shown that the half-life of a nonreduced AP site in the presence of 100 mM $NaBH_4$ is 12 s at pH 6.8 (*20*), and this prompted an experiment aimed at measuring the half-life of a nonreduced AP site in the presence of $NaCNBH_3$. We observed that the half-life of an AP site in the presence of 25 mM $NaCNBH_3$ at pH 5.0 was approximately 9 h (data not shown), and we do not expect this result to vary considerably under our trapping reaction conditions at pH 7.0. From these combined data, we conclude that the choice of reductant can dictate whether one observes an "accumulation" of the pre-incision trapped Schiff base complex or a "snapshot" of the reduced substrate AP site-containing DNA, Schiff base complexes prior to and following strand incision, and β- and δ-elimination products.

Investigation of the Lysine Requirement for Covalent Trapping of Peptides. It has been postulated that the reactive nucleophile for Lys-Trp-Lys in the peptide-catalyzed cleavage of AP site-containing DNA involves an ϵ-amino group on one of the lysine residues, which are brought into the proximity of the AP site upon specific binding conferred by an aromatic residue (*27*). To examine whether a lysine residue is absolutely required for Schiff base formation at

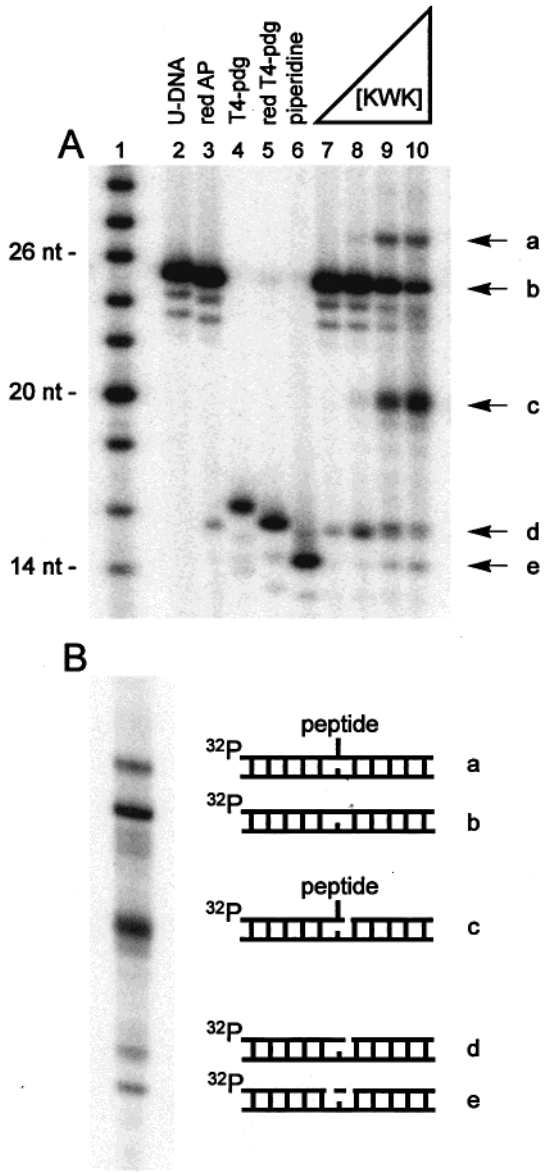


FIGURE 1: Nicking activity of Lys-Trp-Lys on AP site-containing DNA. Substrate AP site-containing 26 bp DNA was prepared immediately prior to use as described in Experimental Procedures. (A) Lane 1, oligonucleotide markers of 8−32 bases; lane 2, 20 fmol of labeled uracil-containing 26 bp DNA (no UDG treatment). Remaining reaction mixtures (lanes 3−10) contained 1 nM labeled AP site-containing DNA in 50 mM HEPES buffer (pH 7.0) and were incubated for 3 h at 37 °C. Incubations were carried out in the presence of 0.5 mM NaCl with DNA alone (lane 3) or in the presence of the enzyme T4-pdg (18.5 ng/μL, lane 5), and reactions were quenched by the addition of 100 mM $NaBH_4$ at the end of the incubation period. Alternatively, incubations were carried out in the presence of T4-pdg (18.5 ng/μL, lane 4) or 5% piperidine (lane 6) and stopped by addition of 95% formamide loading buffer. Nicking reactions were carried out in the presence of 1.0 μM (lane 7), 10 μM (lane 8), 100 μM (lane 9), and 1.0 mM (lane 10) Lys-Trp-Lys and were quenched by the addition of 100 mM $NaBH_4$ at the end of the incubation period. (B) Products obtained from the 1.0 mM Lys-Trp-Lys nicking reaction are shown (labeled single-stranded DNA under denaturing conditions), with the corresponding reduced trapped complexes and products (duplex DNA prior to denaturation) shown as cartoons to the right (a−e).

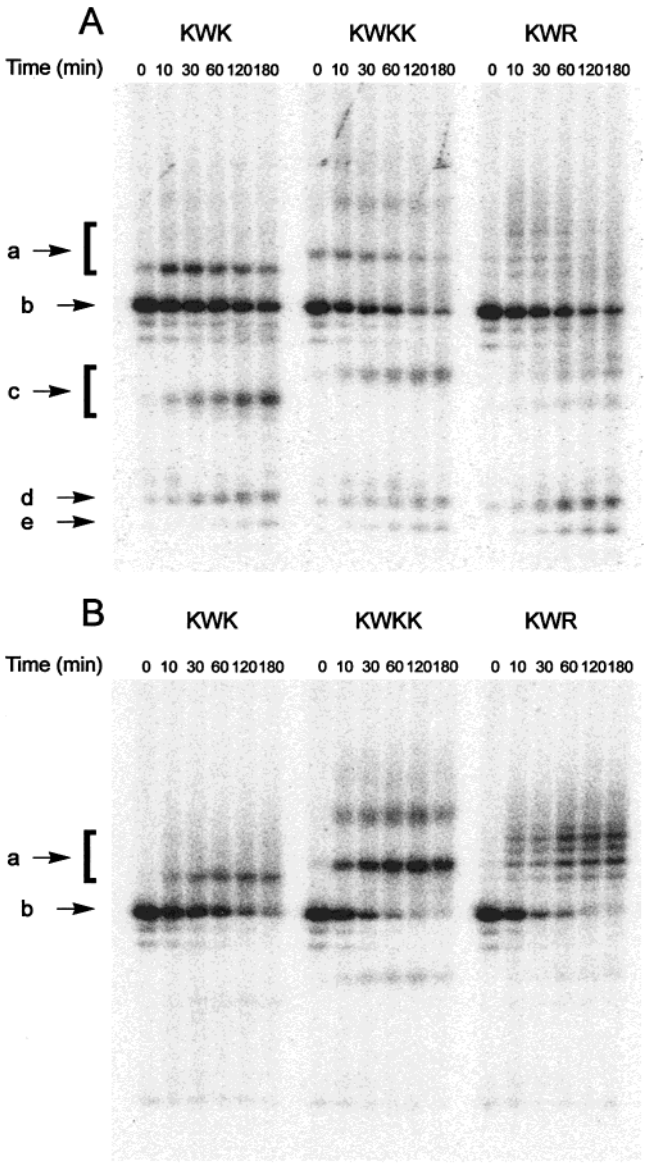


FIGURE 2: Snapshot vs accumulation trapping of peptides onto AP site-containing DNA. Reaction mixtures contained 1 nM labeled AP site-containing DNA in 50 mM HEPES buffer (pH 7.0) and were incubated for 0, 10, 30, 60, 120, or 180 min at 37 °C as indicated. (A) Nicking reactions were carried out in the presence of 0.5 mM NaCl, and the mixture contained 500 μM Lys-Trp-Lys, Lys-Trp-Lys-Lys, or Lys-Trp-Arg as indicated. (B) Accumulation trapping reactions were carried out in the presence of 5.0 mM NaCl, and the mixtures contained 50 μM Lys-Trp-Lys, Lys-Trp-Lys-Lys, or Lys-Trp-Arg and 25 mM $NaCNBH_3$ as indicated. All reactions were quenched by the addition of 100 mM $NaBH_4$ at the end of each incubation period. Labels indicate the positions of the following species: reduced AP site-containing substrate (b), reduced Schiff base complexes prior to and following DNA strand incision (a and c, respectively), reduced α,β-unsaturated aldehyde product of β-elimination (d), and δ-elimination product (e).

an AP site, we synthesized tetrapeptides containing only lysine, histidine, or arginine residues flanking a single tryptophan residue and tested these peptides for the ability to form stable covalent complexes in our accumulation trapping assay. Under our reaction conditions at pH 7.0, arginine residues should retain positively charged side chain moieties, mimicking the electrostatic contribution of lysine in DNA binding, though we cannot confidently predict the ionization state of histidine residues under these conditions. For the peptides Lys-Trp-Lys-Lys and His-Trp-His-His, we observe an accumulation of the trapped pre-incision Schiff base complex that is visualized by a major product band with shifted mobility on a denaturing gel (Figure 3A,B), while

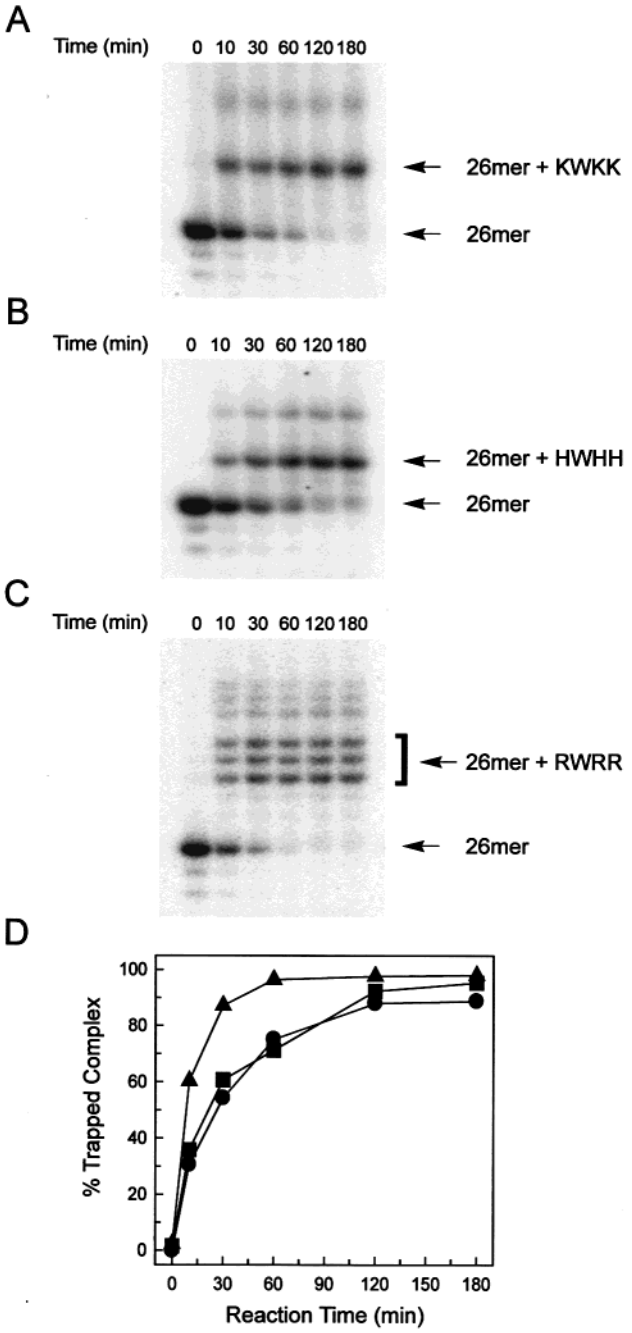


FIGURE 3: Covalent trapping of peptides containing or lacking lysine residues. Reaction mixtures contained 1 nM labeled AP site-containing DNA in 50 mM HEPES buffer (pH 7.0) and were incubated for 0, 10, 30, 60, 120, or 180 min at 37 °C as indicated. All reaction mixtures contained 25 mM $NaCNBH_3$, and the reactions were quenched by the addition of 100 mM $NaBH_4$ at the end of the incubation period. Reactions were carried out in the presence of 5.0 mM NaCl, and the mixtures contained 50 μM (A) Lys-Trp-Lys-Lys, (B) His-Trp-His-His, or (C) Arg-Trp-Arg-Arg. Labels indicate the positions of the reduced AP site-containing substrate (26mer) and the major reduced pre-incision Schiff base complex(es) (26mer + peptide). (D) Kinetics of formation of the trapped complex are plotted for Lys-Trp-Lys-Lys (■), His-Trp-His-His (●), and Arg-Trp-Arg-Arg (▲).

the peptide Arg-Trp-Arg-Arg produces a triplet of complexes stabilized under reducing conditions (Figure 3C). Experiments are under way to evaluate whether the observed multiplet of complexes for Arg-Trp-Arg-Arg trapping arises due to alternative conformational states of the peptide−DNA complex. Since we observed additional minor complexes in our peptide trapping reactions, corresponding to the predicted masses of multiple peptides reacted with one molecule of DNA, we are investigating the possibility that reactions occur in which multiple peptides react with the same DNA. One possibility may be that peptide bond formation occurs at a slow rate due to the high peptide concentrations used in our experiments, leading to the formation of minor trapped complexes containing multimers of peptides. We are currently generating additional 26 bp oligonucleotides (containing multiple uracil residues to form multiple reactive AP sites) to build products consisting of several peptides bound to one DNA molecule. Such products can be used as size markers in the determination of the likely composition of the observed minor products in our peptide trapping experiments; however, the appearance of these minor products does not have a significant impact on the conclusions drawn here, since a major trapped complex predominates. The kinetics of trapping for each of the three peptides above yield comparable profiles (Figure 3D), and we conclude that the lysine residues are dispensable for efficient trapping at AP sites, strongly suggesting that the N-terminal α-amino group is the reactive nucleophile that catalyzes β-elimination.

Interrogation of the Reactive Nucleophile. To probe directly the location of the nucleophile initiating β-elimination chemistry, we modified each of the tetrapeptides described above (Lys-Trp-Lys-Lys, His-Trp-His-His, and Arg-Trp-Arg-Arg) by addition of an N-terminal acetyl moiety. If the active nucleophile for these peptides is found at the N-terminal α-amino group, acetylation should block this reaction by generating an amide at the N-terminus, as well as by steric interference. In each case, we observed that N-terminal acetylation dramatically inhibited the ability to covalently trap these peptides at an AP site in the presence of $NaCNBH_3$, confirming the importance of the amino terminus as the reactive nucleophile (Figure 4A−C). Trapping data for the nonacetylated peptides (identical to those shown in Figure 3) are presented for comparison and show appreciable accumulation of the trapped complex over the same time course (Figure 4A−C). In the case of Arg-Trp-Arg-Arg, N-terminal acetylation impedes formation of all three of the discrete complexes observed in the nonacetylated case, supporting the necessity of a free N-terminal α-amine in the formation of each of these discrete complexes.

Necessity and Positioning of an Aromatic Residue for Covalent Trapping. To achieve comparable efficiency of strand incision at AP sites in a plasmid nicking assay, the concentration of Lys-Ala-Lys must be increased 400-fold when compared to that of Lys-Trp-Lys (*11*). To evaluate the possibility of whether a corresponding aromatic residue requirement is observed in the formation of the pre-incision Schiff base complex, an obligatory intermediate in β-elimination catalysis, covalent trapping at an AP site for the peptides Lys-Ala-Lys, Lys-Trp-Lys, and Lys-Phe-Lys was evaluated. A 10-fold increase in Lys-Ala-Lys concentration gave a quantity of accumulated complex roughly equivalent to that for Lys-Trp-Lys, while an intermediate efficiency of complex formation is observed in the case of Lys-Phe-Lys (Figure 5A). By monitoring the rate of disappearance of substrate AP site-containing DNA in a trapping experiment, we observed a 3.2-fold difference in the rate of formation of the Schiff base complex between Lys-Trp-Lys and Lys-

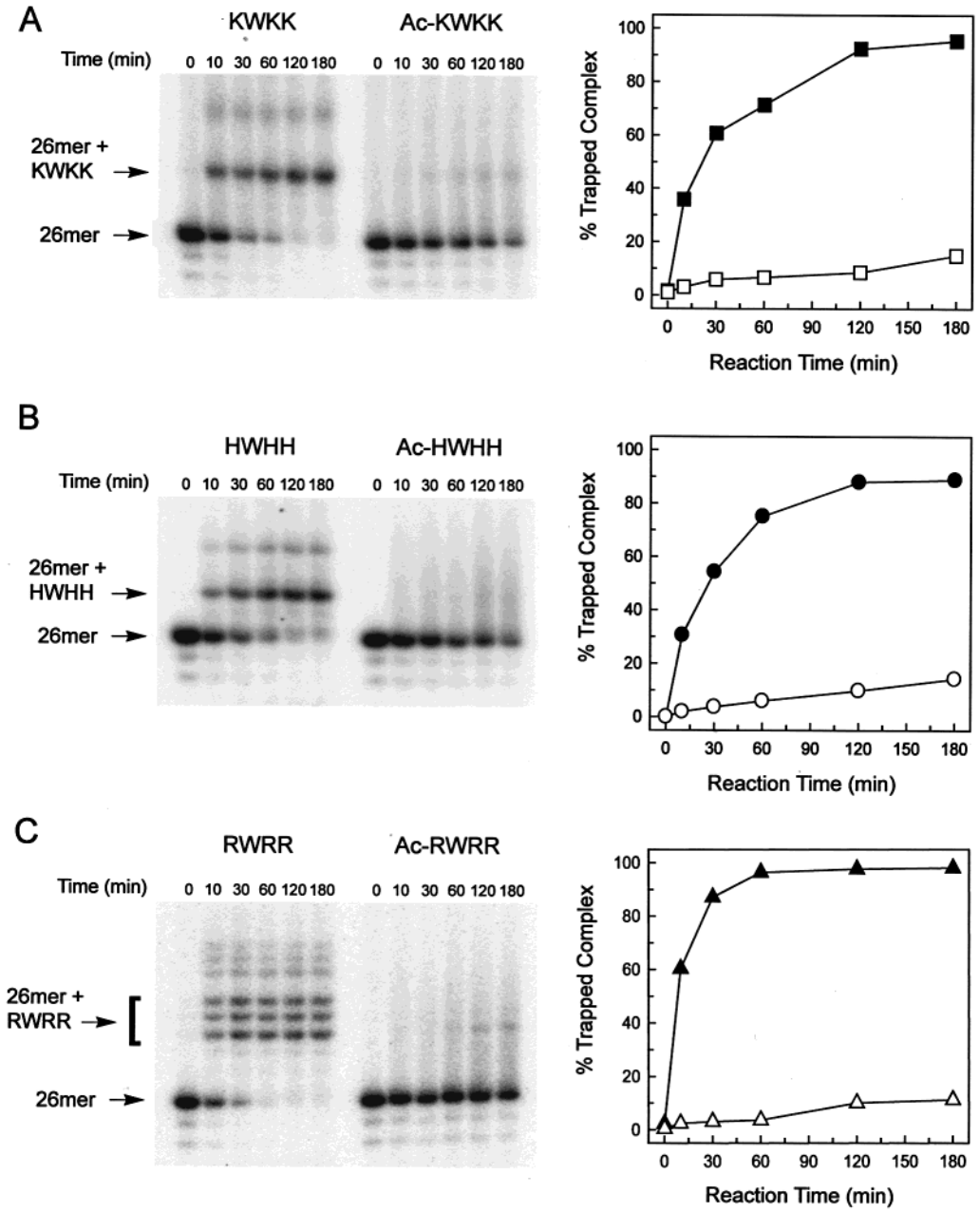


FIGURE 4: Probing the reactive nucleophile in peptide trapping. Reaction mixtures contained 1 nM labeled AP site-containing DNA in 50 mM HEPES buffer (pH 7.0) and were incubated for 0, 10, 30, 60, 120, or 180 min at 37 °C as shown. All reaction mixtures contained 25 mM $NaCNBH_3$, and the reactions were quenched by the addition of 100 mM $NaBH_4$ at the end of the incubation period. Reactions were carried out in the presence of 5.0 mM NaCl, and mixtures contained 50 μM (A) Lys-Trp-Lys-Lys, (B) His-Trp-His-His, or (C) Arg-Trp-Arg-Arg. Peptides retained either a free α-amino N-terminus (KWKK, HWHH, and RWRR) or a monoacetylated N-terminus (Ac-KWKK, Ac-HWHH, and Ac-RWRR) as indicated. Kinetics of formation of the trapped complex are plotted for (A) Lys-Trp-Lys-Lys [(■) nonacetylated and (□) acetylated], (B) His-Trp-His-His [(●) nonacetylated and (○) acetylated], and (C) Arg-Trp-Arg-Arg [(▲) nonacetylated and (△) acetylated]. Labels indicate the positions of the reduced AP site-containing substrate (26mer) and the major reduced pre-incision Schiff base complex(es) (26mer + peptide).

Ala-Lys, evaluated for the time point at which the reaction has proceeded to 50% trapped complex (Figure 5B). Although the rate of formation of the Schiff base complex is facilitated by an aromatic residue, we conclude that the increase in efficiency is greatly diminished as compared to data obtained using DNA strand incision as an end point. We also examined the peptides Lys-Trp-Lys-Lys and Lys-Lys-Trp-Lys to assess the relative importance of the positioning of the aromatic residue with respect to the N-terminal amine (Figure 5A). Each of these peptides gave rise to a comparable concentration dependence for trapping; thus, the positioning of the aromatic residue does not have a significant impact on the ability to form a covalent Schiff base.

Reactivity of Single-Stranded versus Double-Stranded DNA. It has been shown that the rate of incision of depurinated PM2 DNA by Lys-Trp-Lys is 5 times greater for supercoiled DNA than for relaxed DNA, suggesting that single-stranded regions may afford preferential binding sites in peptide-catalyzed DNA strand nicking (*11*). To test whether this assertion is supported by differences in the rate of pre-incision Schiff base formation on single-stranded regions of DNA, we tested for the ability of the peptide Lys-

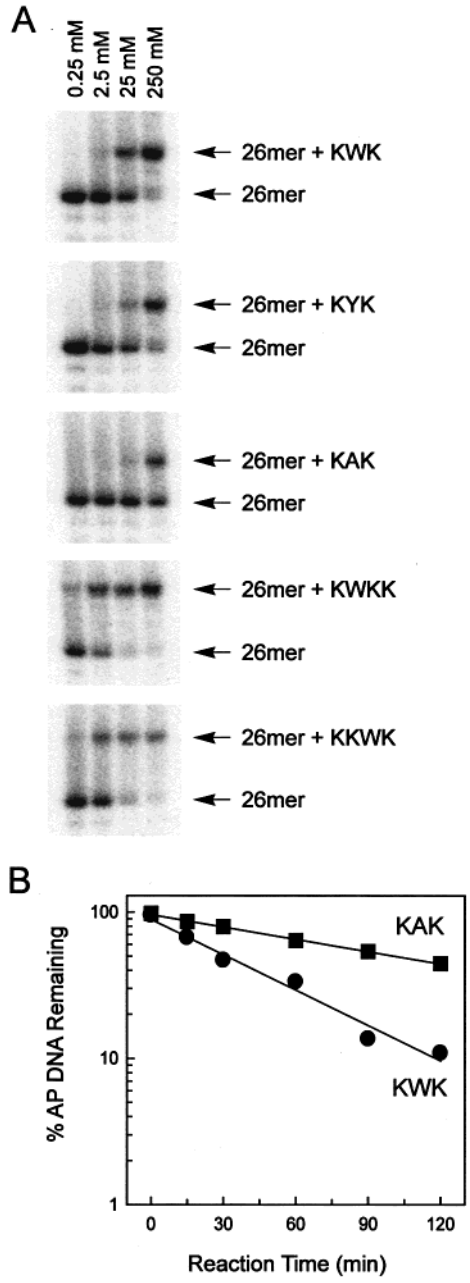


FIGURE 5: Necessity and positioning of an aromatic residue in peptide trapping. Reaction mixtures contained 1 nM labeled AP site-containing DNA in 50 mM HEPES buffer (pH 7.0). All reaction mixtures contained 25 mM $NaCNBH_3$, and reactions were quenched by the addition of 100 mM $NaBH_4$ at the end of the incubation period. (A) Trapping reactions were carried out in the presence of 5.0 mM NaCl and contained 0.25, 2.5, 25, or 250 μM peptide. Reaction mixtures were incubated for 120 min at 37 °C for Lys-Trp-Lys, Lys-Tyr-Lys, Lys-Ala-Lys, Lys-Trp-Lys-Lys, or Lys-Lys-Trp-Lys. Labels indicate the positions of the reduced AP site-containing substrate (26mer) and the major reduced pre-incision Schiff base complex(es) (26mer + peptide). (B) Trapping reactions were carried out in the presence of 5.0 mM NaCl, and the mixtures contained 250 μM Lys-Tyr-Lys or Lys-Ala-Lys. Reaction mixtures were incubated for 0, 15, 30, 60, 90, or 120 min at 37 °C. Kinetics of the percent remaining 26 bp AP site-containing DNA are plotted for Lys-Trp-Lys (●) and Lys-Ala-Lys (■). Straight lines represent least-squares fits to the data. Reaction products were separated by electrophoresis on a 15% denaturing polyacrylamide gel (8.3 M urea) and analyzed as described in the text.

Lys-Trp-Lys to trap onto AP site-containing single-stranded and double-stranded substrates. To ensure the integrity of the duplex for comparison to the single-stranded substrate, the 26mer duplex DNA was incubated for 30 min under standard reaction conditions. Following incubation, native gel analysis showed the starting substrate for duplex trapping reactions to be greater than 89% annealed (Figure 6A). Trapping reactions were carried out over a 1 h time course, and the kinetics of complex formation for trapping of Lys-Trp-Lys-Lys to single-stranded versus double-stranded DNA were virtually identical (Figure 6C). We conclude that Schiff base formation at AP sites shows no preference for single-stranded versus double-stranded DNA.

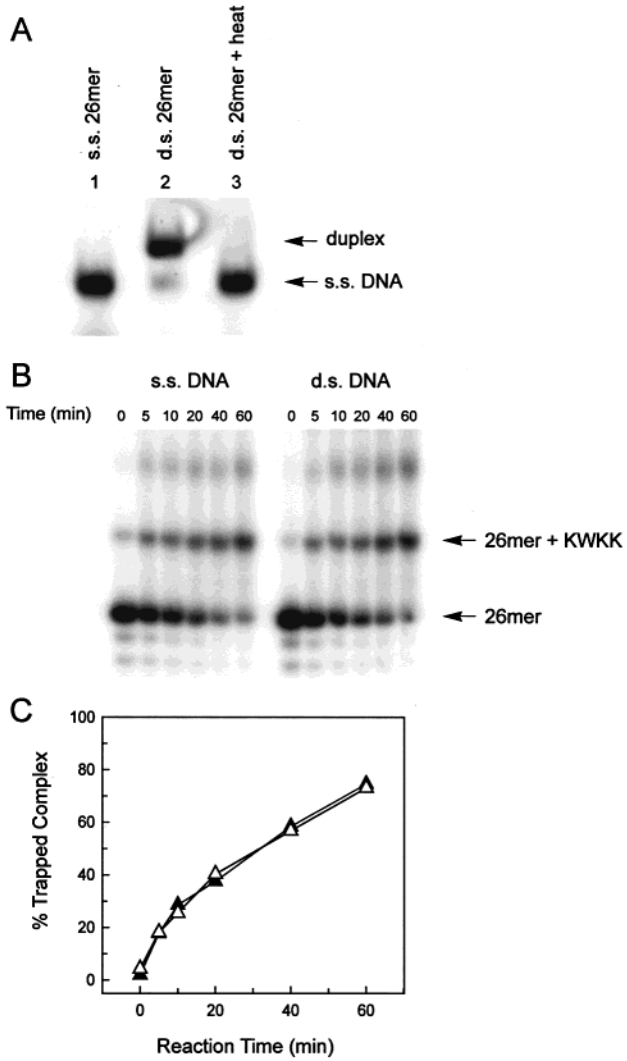


FIGURE 6: Peptide trapping on single-stranded vs double stranded DNA. (A) Labeled single-stranded (lane 1) and double-stranded (lanes 2 and 3) DNA was incubated under standard reaction conditions in 50 mM HEPES buffer (pH 7.0) and 5.0 mM NaCl at 37 °C for 30 min. Double-stranded DNA was heated at 90 °C for 3 min following standard reaction incubation at 37 °C (lane 3). Oligonucleotides were analyzed by electrophoresis on a 7.5% native polyacrylamide gel as described in the text. (B) Reaction mixtures contained 1 nM labeled single-stranded or double-stranded AP site-containing DNA in 50 mM HEPES buffer (pH 7.0) and were incubated for 0, 5, 10, 20, 40, or 60 min at 37 °C as indicated. All reaction mixtures contained 25 mM $NaCNBH_3$, and the reactions were quenched by the addition of 100 mM $NaBH_4$ at the end of the incubation period. Reaction mixtures were incubated in the presence of 5.0 mM NaCl and contained 50 μM Lys-Trp-Lys-Lys. (C) Kinetics of trapped complex formation are plotted for Lys-Trp-Lys-Lys trapping onto single-stranded (▲) and double-stranded (△) AP site-containing DNA.

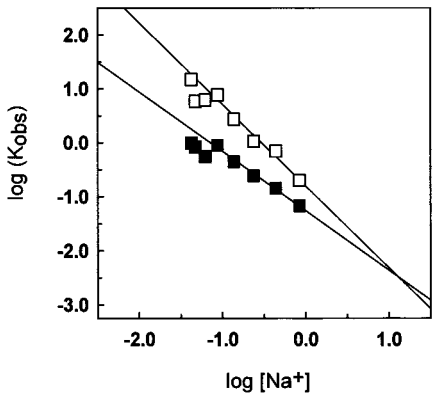


FIGURE 7: Dependence of peptide trapping on Na^+ concentration. Reaction mixtures contained 1 nM labeled AP site-containing DNA in 50 mM HEPES buffer (pH 7.0) and either 50 μM Lys-Trp-Lys or 50 μM Lys-Trp-Lys-Lys. All reaction mixtures contained 25 mM $NaCNBH_3$, and reactions were quenched by the addition of 100 mM $NaBH_4$ at the end of the incubation period. Reaction mixtures were incubated for 120 min at 37 °C in the presence of 5−800 mM NaCl. Data are plotted as the logarithm of the binding constants, K_{obs}, for Lys-Trp-Lys (■) and Lys-Trp-Lys-Lys (□) as a function of log[Na^+]. K_{obs} was calculated as the ratio of the reduced pre-incision Schiff base complex to reduced substrate AP site-containing DNA at the end of the incubation period. The total Na^+ concentration was calculated from [NaCl] + [$NaCNBH_3$] + [Na-HEPES], and the sodium ion concentration calculated from the contribution of Na-HEPES at pH 7.0 and 25 mM $NaCNBH_3$ was 37 mM as described in Experimental Procedures. Straight lines represent least-squares fits to the data at a sodium ion concentration of ≥87 mM. Reaction products were separated by electrophoresis on a 15% denaturing polyacrylamide gel (8.3 M urea) and analyzed as described in the text.

Dependence of Trapping on Na^+ Concentration. Record et al. (*28*) have examined the binding of oligolysine to poly(A)•poly(U) for various chain lengths in investigating the contribution of electrostatic interactions between positively charged ϵ-NH_3^+ groups and the phosphate backbone. A plot of the logarithm of the binding constant (K_{obs}) as a function of log[Na^+] gives a linear slope, directly proportional to the number of ion pairs formed upon peptide binding, and the magnitude of the slope increases linearly with chain length. To assess the contribution of electrostatic interactions for binding to AP site-containing DNA, we evaluated trapping efficiency at various Na^+ concentrations for the peptides Lys-Trp-Lys and Lys-Trp-Lys-Lys. Results for the trapping experiments are shown in Figure 7, in which K_{obs} represents the ratio of trapped covalent complex to free (reduced) AP site-containing DNA. At NaCl concentrations above 50 mM, the slope of the log K_{obs} versus log[Na^+] plot is negative and approximately linear. We observe the slope to be 1.10 in the case of Lys-Trp-Lys trapping, whereas the slope for Lys-Trp-Lys-Lys is 1.51, corresponding to a proportional increase in the number of ion pairs formed for the peptide Lys-Trp-Lys-Lys. We conclude that Lys-Trp-Lys-Lys shows a greater dependence on Na^+ concentration due to the additional ϵ-NH_3^+ group present on the third lysine side chain.

Peptide Mimics of AP Lyase Enzymes. The bifunctional DNA glycosylase/AP lyase enzymes T4-pdg and *E. coli* Fpg both catalyze β-elimination at AP sites, although these two enzymes utilize distinct active site nucleophiles. The reactive nucleophile for the *E. coli* Fpg enzyme has been mapped to the amino-terminal Pro-2 residue, which is immediately followed by a glutamate in the primary sequence and is exposed on the N-terminus following processing of Met-1 (*23*). For the T4-pdg enzyme, the N-terminal α-amine also catalyzes the reaction, although the first two residues in the primary sequence are Thr and Arg (following N-terminal processing of Met-1). To mimic the activities of these two enzymes in our studies, we evaluated trapping for two pentapeptides containing the identical two-residue N-terminal motifs, namely, Pro-Glu-Trp-Lys-Lys and Thr-Arg-Trp-Lys-Lys. In our standard trapping assay, each of these peptides was efficiently trapped as a covalent complex (Figure 8). Although we cannot exclude the possibility that the lysine residues mediate covalent attachment, we conclude this is unlikely based on the preceding N-terminal acetylation data for Lys-Trp-Lys-Lys.

DISCUSSION

Previous studies in which borohydride trapping was employed as a means of probing the biochemical mechanism of bifunctional glycosylase/AP lyases suggested that it should be possible to isolate analogous covalent DNA−peptide intermediates under reducing conditions. However, while peptides such as Lys-Trp-Lys perform β-elimination like these DNA repair enzymes, we observe key differences in the kinetics of the reaction mechanism in the case of peptide-catalyzed β-elimination. As stated, AP lyase enzymes also proceed via the formation of a Schiff base intermediate, yet we are unaware of any published result in which a post-incision Schiff base complex has been unambiguously identified under reducing conditions for an enzyme. Using the borohydride trapping methodology, we observe multiple intermediates along the reaction pathway for peptide-catalyzed β-elimination at AP sites and, in particular, the accumulation of a covalent complex corresponding to peptide bound to nicked DNA (Figure 1A). The formation of this complex may indicate that the peptide, following nucleophilic attack on the deoxyribose sugar, is able to catalyze strand incision, but is not readily dissociated from the DNA following β-elimination. We favor this scenario over the alternative explanation that the α,β-unsaturated aldehyde product of strand incision may react with excess peptide in the reaction, due to the slow kinetics of formation observed for the post-incision Schiff base from these starting reactants. It seems likely that attributes unique to the architecture of bifunctional glycosylase/AP lyases facilitate rapid turnover for these enzymes when compared to the peptides studied here. Others have shown that peptide-catalyzed anti β-elimination proceeds via abstraction of the 2′-*pro-R* proton, whereas strand incision catalyzed by *E. coli* endonuclease III (a glycosylase/AP lyase) proceeds via syn β-elimination to yield abstraction of the 2′-*pro-S* proton (*12*). This difference in stereochemistry has been attributed to a specific enzyme-induced rotational reorientation about the C2′−C3′ bond within the deoxyribose that may serve to favor rapid dissociation of the enzyme. Additionally, in the case of T4-pdg, a cyclobutane pyrimidine dimer-specific DNA glycosylase/AP lyase, the enzyme retains a binding pocket that serves to flip the nucleotide opposite the 5′-pyrimidine of the dimer (*29*). This extrahelical distortion of a nucleotide creates a cavity, which permits access to the damaged strand by the active site residues of T4-pdg (*30*). In contrast, in the absence of enzyme, NMR solution structure determinations

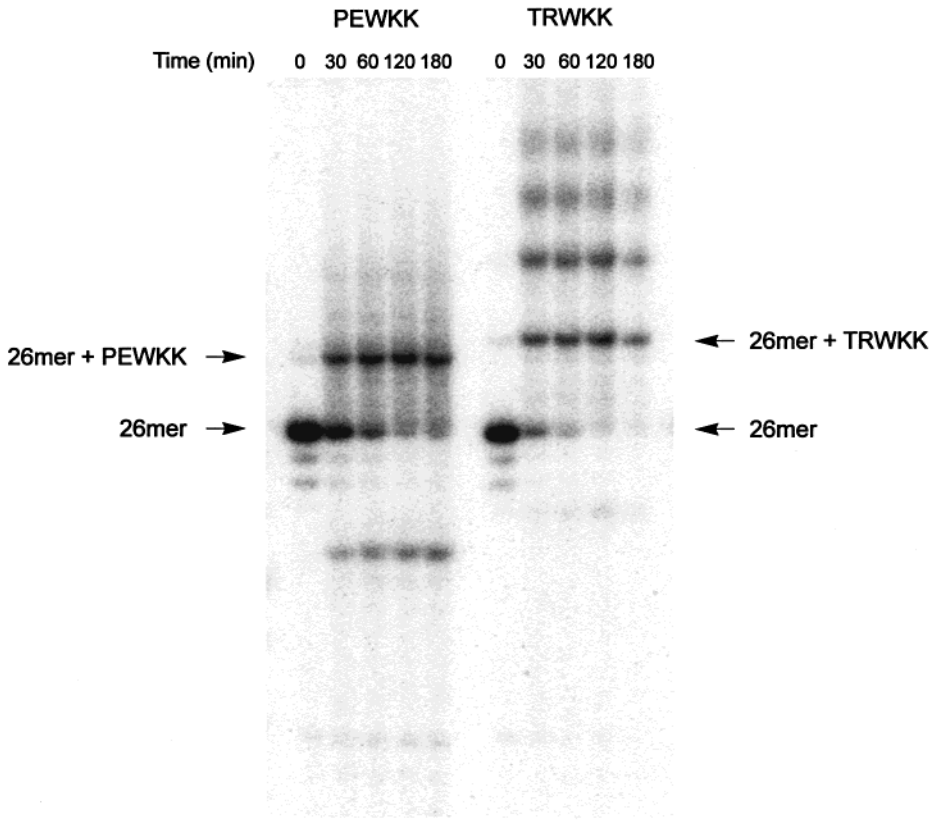


FIGURE 8: Peptide mimics of enzymes with AP lyase activity. Reaction mixtures contained 1 nM labeled AP-containing DNA in 50 mM HEPES buffer (pH 7.0) and were incubated for 0, 30, 60, 120, or 180 min at 37 °C as indicated. All reaction mixtures contained 25 mM $NaCNBH_3$, and reactions were quenched by the addition of 100 mM $NaBH_4$ at the end of the incubation period. Reaction mixtures were incubated in the presence of 5.0 mM NaCl and contained 50 μM Pro-Glu-Trp-Lys-Lys or Thr-Arg-Trp-Lys-Lys. Labels indicate the positions of the reduced AP site-containing substrate (26mer) and the major reduced pre-incision Schiff base complex (26mer + peptide).

indicate that the base opposite an AP site remains predominantly stacked in several cases, irrespective of whether this base is a purine or pyrimidine (*31*−*33*). These data, along with those presented here, may suggest that access to the deoxyribose moiety may be restricted in the case of peptide-catalyzed strand incision, manifested as the observed differences in the various kinetic steps along the β-elimination reaction pathway for peptides and enzymes.

As shown in Scheme 1, the β-elimination reaction requires a neutral amine, which serves as the reactive nucleophile for formation of a Schiff base intermediate prior to strand incision. In the case of an enzyme, pK_a values for ionizing groups may be perturbed, rendering a particular amine more reactive within a local environment. Conversely, in the case of the peptides studied here, the pK_a values should not deviate significantly from those measured in a random coil structure, and we estimate these values to be approximately 7.6 for an α-amine and 10.3 for a lysine ϵ-amine (*34*). Thus, under our reaction conditions at pH 7.0, the population of neutral α-amino groups is significantly greater than the population of neutral lysine ϵ-amines for a given peptide, and the α-amine should exhibit greater reactivity. Consistent with this prediction, and contrary to the prior postulation that specific binding of peptides brings ϵ-amines into the proximity of the AP site to catalyze strand incision, we show that lysines are in fact dispensable for the formation of the Schiff base intermediate (Figure 3). Specific blockage of the α-amine severely inhibits the reactivity of each peptide (Figure 4), suggesting that the overwhelming determinant in the formation of the Schiff base and initiation of β-elimination is the pK_a of the reactive amine.

A substantial body of evidence supports a role for an aromatic residue in the specific binding of Lys-Trp-Lys to AP site-containing DNA when monitoring DNA strand incision as an end point in a nicking assay. Others have demonstrated a 400-fold decrease in efficiency for Lys-Ala-Lys when compared to that of Lys-Trp-Lys in such experiments (*11*). In contrast, the results presented here indicate a dramatic decrease in the dependence on an aromatic residue for formation of the Schiff base intermediate (Figure 5), the first step along the reaction pathway of β-elimination. Because we observed only a nominal decrease in the rate of complex formation for Lys-Ala-Lys as compared to that for Lys-Trp-Lys, our results suggest that the off rate for a peptide that has become complexed to DNA via Schiff base formation is relatively fast in the absence of an aromatic residue. Furthermore, we suggest that the role of the aromatic residue is to stabilize a peptide−DNA Schiff base complex long enough for strand incision to occur.

The experiments presented in this report provide new insight with respect to the kinetics of β-elimination, not only in the case of peptide-catalyzed β-elimination but also in the case of the corresponding reaction carried out by DNA glycosylase/AP lyase enzymes. With respect to utilizing the borohydride trapping methodology as a tool in studying the reaction mechanism, we note important differences in the rate of reduction of each intermediate in the presence of $NaBH_4$ versus $NaCNBH_3$. Specifically, the scheme of

competing reactions and observed results presented here indicate that $NaCNBH_3$ preferentially reduces an imine to give an accumulation of the reduced pre-incision Schiff base complex, while reducing the AP site aldehyde at a much slower rate. In contrast, $NaBH_4$ serves more appropriately as a quencher of the reaction, yielding a snapshot of the reaction intermediates, provided that the formation of the Schiff base complex is the rate-limiting step. These observations are important with regard to the interpretation of data obtained using borohydride as a trap quencher of reactions for both reactive peptides and the bifunctional glycosylase/AP lyase family of enzymes.

One of the possibilities arising from these studies is the option of generating an array of covalent peptide−DNA cross-links at an AP site within a DNA duplex. We suggest that this methodology may be exploited as a strategy in the study of DNA−protein cross-link (DPC) repair. In particular, the methods described in this study will allow for the generation of model DPC substrates, albeit substrates that lack a single base at the site of covalent linkage. While DNA−protein cross-links are induced in biological systems by a variety of chemical and physical agents, many of which are known or suspected carcinogens, the mechanisms of repair for these types of lesions have not been clearly elucidated (*35*−*37*). Nucleotide excision repair (NER) has been implicated in several studies aimed at investigating the repair mechanism(s) for DPCs, and recently, it has been proposed that the active removal of some DPCs in vivo may involve proteolytic degradation of cross-linked proteins (*38*). Specifically, the repair of formaldehyde-induced DPCs in human cells was inhibited upon treatment with lactacystin, a specific proteasome inhibitor (*38*). These results suggest the occurrence of DNA−peptide adducts as intermediate structures along a repair pathway for certain DNA−protein cross-links. The experiments presented herein demonstrate the ability to trap proteins of various size and composition at an AP site within DNA, affording the generation of substrates that may mimic those structures along a pathway of repair for DPCs.

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