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Changes in the Conformation of the Vsr Endonuclease Amino-terminal Domain Accompany DNA Cleavage

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In *Escherichia coli*, T/G mismatches arising from deamination of 5-methylcytosine to thymine are converted to CG base pairs by the very short patch (VSP) repair pathway. DNA Polymerase I removes and resynthesizes the mismatched T starting from a 5′-nick created by the Vsr endonuclease. We used limited trypsinolysis to probe conformational changes in the N-terminal domain of Vsr in response to DNA binding, DNA cleavage and interaction with the polymerase. Our data show that the domain becomes trypsin resistant only under conditions that allow DNA cleavage, while interaction with the polymerase restores trypsin sensitivity. We suggest that the domain changes its conformation as a result of DNA nicking, and that DNA Pol I releases Vsr from the nick by reversing that conformational change.

Key words: DNA polymerase I, DNA repair, limited tryptic digestion, VSP repair, Vsr endonuclease.

Abbreviations: LB, Luria-Bertani medium; IPTG, isopropyl-β-D-thiogalactopyranoside; SSC, saline-medium citrate buffer; SDS, sodium dodecyl sulphate; LC-MS, Liquid chromatography-mass spectrometry.

C5 methylation of selected cytosines is widespread throughout evolution. It is used for a variety of different purposes; particularly in bacteria it is used to distinguish self DNA from non-self DNA, while in eukaryotes it is important in the regulation of gene expression (*1*). However, this process has a considerable cost: it increases C to T mutations at the sites of methylation. Deamination of 5-methylC is up to 21 times more frequent than for its cytosine counterpart (*2*). As a result, all organisms that methylate cytosines have mechanisms to counteract deamination damage and reduce mutation. In *Escherichia coli* and related enterobacteria, this mechanism is very short patch (VSP) repair [reviewed by (*3*)].

VSP repair is initiated by the Vsr endonuclease. Vsr recognizes T/G mismatches within 5′-CTWGG/5′-CCWGG (W is A or T), or closely related, sequences. CCWGG sequences are the substrate for the bacterial DNA cytosine methyltransferase, Dcm. The Vsr endonuclease cleaves DNA at the T/G mismatch, creating a single-strand nick 5′ of T. Genetic evidence suggests that nick translation performed by DNA Polymerase I removes T along with a few 3′-nucleotides and resynthesizes the short patch. Repair is completed by DNA ligase (*4*).

The structure of the Vsr endonuclease was determined first without DNA (*5*). Crystal formation required removal of the first 20 amino acids, suggesting that the N-terminal domain is disordered. When the protein was crystallized with the (uncleaved) DNA substrate, this domain became ordered, with residues 7–15 forming an α-helix which lies across the minor groove (*6*). Met14 and Ile17 reach deeply into the minor groove, leaving only enough room for the sugar phosphate backbones to pass on either side. The DNA in the co-crystal was all in cleaved form.

Co-crystal analysis also showed that Vsr has three aromatic residues (Phe67, Trp68 and Trp86), which intercalate into the major groove, bending the DNA and separating the two strands (*6*, *7*). These three residues are in close proximity to the central AT base pair, the mismatched T and the DNA backbone opposite to the T, respectively. Each of them is essential for DNA binding, strand cleavage and VSP repair (*8*).

In contrast to the aromatic residues, the N-terminal domain is not essential for DNA binding; with the obvious exception of the domain itself, the structure of the Δ14 protein co-crystallized with pre-cleaved DNA is remarkably similar to that involving the wild-type protein. However, while not essential for strand cleavage or VSP repair, it does enhance these functions. Removal of the first 14 amino acids reduces endonuclease activity considerably *in vitro* and decreases repair efficiency *in vivo* (*8*).

What, then, is the function of the N-terminal domain? There are two possibilities: (i) it stabilizes the interaction of the protein with the DNA substrate, thereby enhancing cleavage, or (ii) it stabilizes the interaction of the protein with the cleaved product, thereby protecting the nick from DNA ligase until the arrival of DNA Pol I. To distinguish between these two possibilities, we used limited tryptic digestion to probe the structure of the N-terminal domain in response to DNA binding, strand cleavage and interaction with DNA Pol I. Our data suggest that the second possibility is the more likely one.

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MATERIALS AND METHODS

The procedures for expression and purification of wild-type and mutant Vsr proteins were described previously (*8*). Briefly, pET15b clones of the corresponding genes were freshly transformed into *E. coli* strain Bl21(DE3). Cultures were grown in LB containing ampicillin (100 mg/ml) at 37°C with aeration. Protein expression was induced by 0.1 mM IPTG in mid log phase. Cells were harvested by centrifugation, washed (with 20 mM HEPES pH 7.5, 110 mM NaCl and 10% glycerol, complete EDTA-free protease inhibitor and 10 mM $MgCl_2$) and either used immediately or stored at −80°(C. Cell pellets were dissolved in lysis buffer (20 mM HEPES pH 7.5, 110 mM NaCl and 10% glycerol, complete EDTA- free protease inhibitor and 10 mM MgCl2), and cells were disrupted by two passages through a French press set at 1,260 psi. To remove cell debris, samples were centrifuged for 1 h at 17,600*g*. The His-tagged proteins were purified from the soluble fraction using cobalt affinity resin (Clontech, WN, USA). After washing (with 20 mM HEPES pH 7.5, 110 mM NaCl, 10% glycerol and 10 mM $MgCl_2$), the proteins were eluted in 20 mM HEPES pH 7.5, 110 mM NaCl, 10 mM $MgCl_2$, 10% glycerol and 300 mM imidazole. To remove imidazole, the PD10 desalting columns were used (GE Healthcare, USA); proteins were eluted in buffer containing 20 mM HEPES, 110 mM NaCl, 1 mM DTT and 10% glycerol.

The hexahistidine tag was removed from Vsr by cleavage with thrombin. One milligram of purified protein in HEPES buffer was incubated with 0.25 U of thrombin (Thrombin Cleavage Capture Kit, Novagen, Germany) for 4 h at room temperature. After the reaction was complete, thrombin was removed by streptavidin agarose.

DNA substrates for cleavage assays and proteolysis experiments were prepared by annealing 3′-CCAGCGA CCCCTTG**G**TCCCACCGGTGCCGC-5′ to 5′-GGTCGCT GGGGAAC**C**AGGGTGGCCACGGCG-3′ (homoduplex) and 5′-GGTCGCTGGGGAAC**T**AGGGTGGCCACGGCG-3′ (heteroduplex), both labelled at the 5′-end with IRDye (LI-COR, NE, USA).

For preparation of substrate, 30 nmol of each oligo were mixed in 120 µl SSC buffer (15 mM sodium citrate pH 7.2, 150 mM NaCl), heated to 90°C for 10 min and slowly cooled to room temperature. The final concentration of double-stranded substrate was 250 pmol/µl.

For cleavage assay, **s**amples of fluorochrome-labelled substrate (LI-COR Biosciences, NE, USA) were mixed with Vsr in 20 µl of 20 mM HEPES–KOH, pH 7.8, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.1 mg/ml BSA and incubated at room temperature for defined time periods. The reaction was stopped by transfering the mixture to reaction tubes containing formamide dye mix (0.1% bromophenol blue, 1× TBE, 100 mM EDTA, 7 M urea in formamide) and immediately boiling. The samples were analysed by electrophoresis in 20% denaturing gel (20% acrylamide/bisacrylamide, 1× TBE, 7 M urea).

The cleavage site for Vsr (GGTCC/CTAGG) is placed in the middle of 30-bp heteroduplex that gives a 15-bp product in denaturing gels after cleavage.

For limited proteolysis by trypsin, 300 pmol of Vsr were incubated in reaction buffer (20 mM HEPES, pH 7.8, 110 mM NaCl and 10% glycerol) with 350 pmol homoduplex or heteroduplex DNA in the presence of 5 mM divalent cations (Mg^{+2} or Ca^{+2}) on ice for 40 min. Proteolysis was performed at room temperature. The protein/trypsin ratio was 200:1. Samples were taken at specific time points, and proteolysis was stopped by adding SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer and immediately boiling. One aliquot from each sample was electrophoresed on a 15% SDS–PAGE. This gel was stained with Coomassie blue to determine the distribution of tryptic peptides. The primary trypsin-resistant product was excised from the gel, and its identity determined by LC–MS (UVic Proteomic Center, BC, Canada). A second aliquot was electrophoresed on a 15% SDS–PAGE containing 8 M urea. This gel was scanned with the LI-COR Odyssey Imager to assess the extent of DNA cleavage by Vsr.

The limited proteolysis of Vsr in the presence of Pol I was done as described above, with the addition of 120 pmol Pol I (New England Biolabs, MA, USA). To determine Pol I activity on DNA cleaved by Vsr, 375 pmol of heteroduplex were incubated with 170 pmol Vsr and 20 pmol Pol I in reaction buffer with or without 10 mM dNTPs at 30°C for 60 min. To confirm correction of the T/G mismatch by Polymerase I, DNA was digested with the ScrFI endonuclease. ScrFI cleaves DNA at CCNGG sites; it will cleave repaired CCWGG sites but will not cleave unrepaired C(T/G)WGG sites. Results were visualized after electrophoresis in 15% SDS–PAGE containing 8 M urea.

RESULTS AND DISCUSSION

It had been shown previously that the N-terminal domain of Vsr is sensitive to proteolysis by trypsin (*5*). Limited proteolysis produced a trypsin-resistant fragment, the molecular weight of which corresponded to the protein minus the first 20 amino acids. N-terminus sequencing confirmed cleavage after Arg20. On the basis of this information, we decided to use trypsin to probe for structural changes in the N-terminal domain accompanying DNA binding and/or cleavage, reckoning that formation of the α-helix and close association of the ordered domain with the DNA would protect it from attack by trypsin. This approach has been used successfully to determine the role of the flexible N-terminus domain of human endonuclease III, a glycosylase required for repair of abasic sites (*9*).

As shown in the upper panel of Fig. 1A, the full-length protein was converted to a lower molecular weight species by trypsin as expected. However, mass spec analysis of the trypsin-resistant product showed that cleavage takes place at R15 under our experimental conditions. This was confirmed by the fact that proteolysis of the Δ14 and Δ19 mutants of Vsr (which lack 15 and 19 N-terminal amino acids, respectively) under the same conditions demonstrated no cleavage (data not shown). Rest of the protein was stable and did not cleave further during reaction times of up to 20 min.

Vsr was then incubated with hetero- and homoduplex DNA in the presence of either Mg^{+2} or Ca^{+2}, followed by trypsinolysis for different lengths of time. DNA binding takes place in the presence of both Ca^{+2} and Mg^{+2}, while catalysis takes place only in the presence of

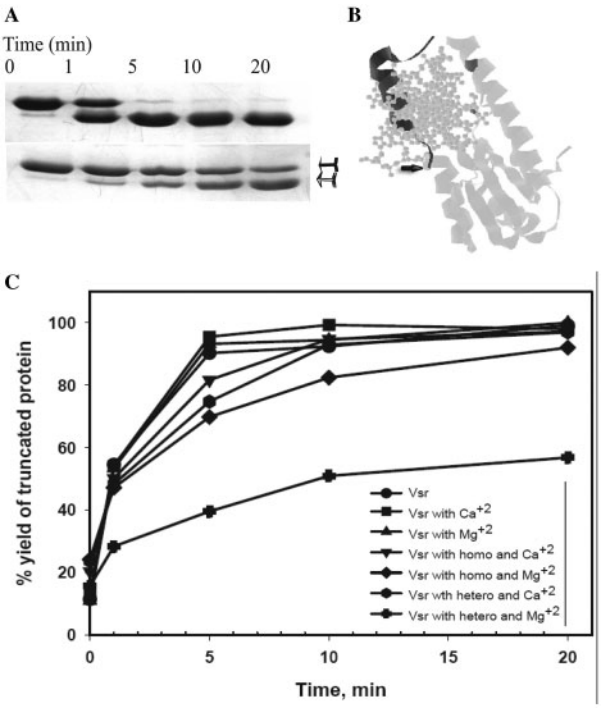

Fig. 1. **Limited trypsinolysis of Vsr: effect of hetero- and homoduplex DNA, and bivalent cations.** (A) Representative SDS–PAGE results showing full-length protein (black arrow) and core fragment (white arrow). Upper panel—Vsr alone, lower panel—Vsr with heteroduplex and Mg^{+2}. (B) Schematical structure of Vsr with DNA. N-terminal domain that is cleaved by trypsin is in black colour. Arrow indicates Arg, which is target for trypsinolysis. (C) Amount of core proteolytic fragment as a function of duration of trypsinolysis reaction, determined by densitometry.

Mg^{+2} (*10*). Trypsin activity is unaffected by the choice of cation (*11*). Figure 1B shows the percentage of the trypsin-resistant product as a function of incubation time in response to these experimental conditions.

Addition of Ca^{+2} or Mg^{+2} does not influence protein stability in the absence of DNA; the N-terminal segment is cleaved completely after 20 min regardless of the choice of cation. Incubating Vsr with homoduplex oligonucleotides, with either Ca^{+2} or Mg^{+2}, shows the same result as protein without any DNA. Surprisingly, the same result was obtained for Vsr incubated with the (T/G) heteroduplex in the presence of Ca^{+2}, conditions under which the protein binds the DNA (*12*). However, the incubation of Vsr with heteroduplex in the presence of Mg^{+2} showed a significant increase in N-terminus stability (Fig. 1A, lower panel; Fig. 1B). About 50% the molecules keep the N-terminus after 10 min of proteolysis, and even after 20 min digestion is not complete.

Visualization of the end-labelled DNA substrate on a denaturing gel showed that Vsr nicks DNA at the T/G mismatch in the presence of Mg^{+2} under our experimental conditions, but that it does not cut in the presence of Ca^{+2} (Fig. 2). Taken together, these results suggest that interaction of the flexible, N-terminal domain of Vsr with DNA does not happen at the time of mismatch binding but instead occurs after DNA cleavage.

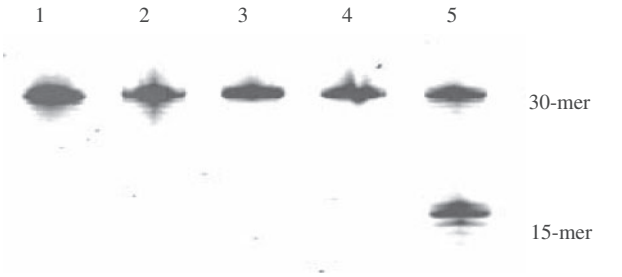

Fig. 2. **Vsr endonuclease activity.** Vsr was incubated with homo- or heteroduplex and bivalent cations on ice for 60 min. Result was visualized by electrophoresis on 15% SDS–PAGE with 8 M urea. 1: heteroduplex alone; 2: homoduplex with Vsr and Ca^{+2}; 3: homoduplex with Vsr and Mg^{+2}; 4: heteroduplex with Vsr and Ca^{+2}; 5: heteroduplex with Vsr and Mg^{+2}.

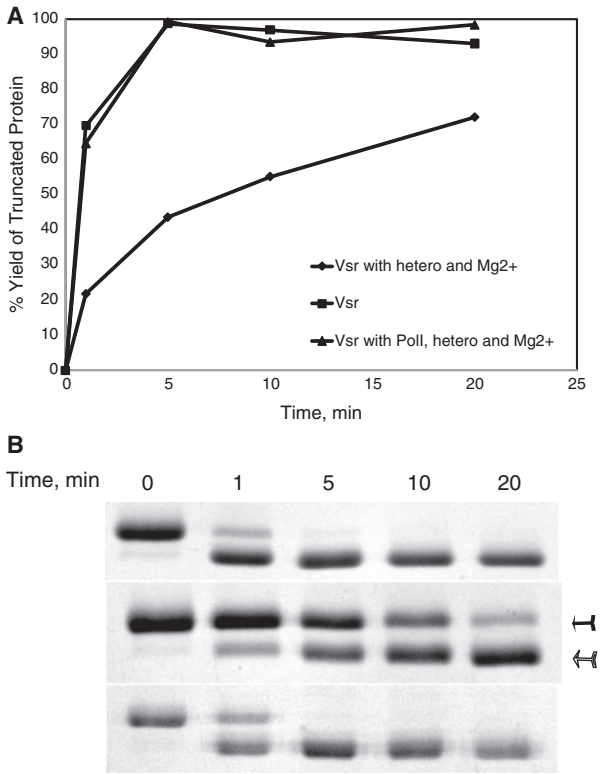

Fig. 3. **Limited trypsinolysis of Vsr in the presence hetero- or homoduplex DNA, and bivalent cations: effect of Pol I.** (A) Amount of core proteolytic fragment as a function of duration of trypsinolysis, determined by densitometry. (B) Representative SDS–PAGE results showing full-length protein (black arrow) and core fragment (white arrow). Upper panel—Vsr alone; middle panel—Vsr with heteroduplex and Mg^{+2}; lower panel—Vsr with heteroduplex and Mg^{+2} in the presence of Pol I.

In vivo, interaction of the N-terminal domain with the minor groove of the DNA following strand cleavage may serve to protect the nick from DNA ligase until the arrival of DNA Pol I. Therefore, we speculated that displacement of Vsr by Pol I would restore the sensitivity of the N-terminal domain to trypsin. As shown in the lower panel of Fig. 3B, addition of Pol I reversed the

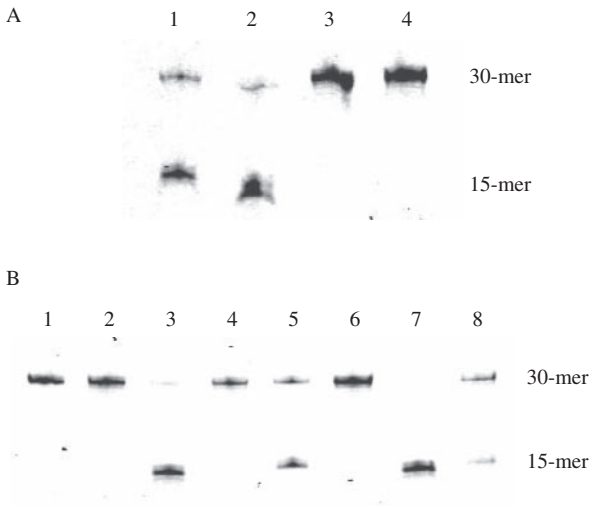

Fig. 4. **Pol I activity.** (A) Ability of Pol I to remove nucleotides after Vsr cleavage and refill nick. 1: heteroduplex with Vsr; 2: heteroduplex with Vsr and Pol I; 3: heteroduplex with Vsr, Pol I and dNTPs; 4: heteroduplex alone. (B) Ability of Pol I to correct T/G mismatch. 1: homoduplex; 2: heteroduplex; 3: heteroduplex with Vsr; 4: heteroduplex with Vsr and Pol I; 5: homoduplex after ScrFI treatment; 6: heteroduplex after ScrFI treatment; 7: heteroduplex with Vsr after ScrFI treatment; 8: heteroduplex with Vsr and Pol I after ScrFI treatment. Results of experiments were visualized by electrophoresis in 15% SDS–PAGE with 8 M urea.

trypsin resistance conferred by heteroduplex DNA in the presence of Mg^{2+} (middle panel) to the levels seen with homoduplex DNA plus Mg^{2+} (upper panel).

To determine whether Pol I does access the nick under these conditions, we tested its ability to remove and resynthesize nucleotides surrounding the nick, and to restore the CCWGG sequence. Figure 4A shows that Pol I removes several bases 5′ of the nick in the absence of added dNTPs (lane 2), then synthesizes new DNA to the 3′-end of the template in the presence of dNTPs (lane 3). Furthermore, this process removes the T/G mismatch and restores the CCWGG sequence, as shown by the fact that the DNA is now cleavable with ScrFI (Fig. 4B, lane 8).

Vsr proteolysis in the presence of heteroduplex DNA showed that the N-terminal domain is only trypsin resistant under conditions where the protein is able to cut the DNA, suggesting that the domain becomes ordered only after the DNA cleavage. This model strongly correlates with the fact that all of the DNA substrate molecules in the Vsr–DNA co-crystals are nicked (*6*). We hypothesize that Vsr scans DNA and interacts with T/G mismatches through residues in its stable core, which include active site residues and the three aromatic amino acids which intercalate into the major groove. Upon DNA cutting or immediately thereafter, the N-terminus forms an α-helix, which slots into the minor groove of the DNA. Interaction with both sides of the DNA keeps Vsr strongly bound to the nicked DNA until it is displaced by DNA Pol I, a step which is accompanied by disruption of the ordered 3D structure of the N-terminal domain.

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CONFLICT OF INTEREST

None declared.

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