論文の内容の要旨

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論文題目 Two crystal structures of restriction DNA glycosylase R.PabI in complex with DNA reveal its mechanism of searching for and interrogating the recognition sequence
(制限 DNA グリコシラーゼ R.PabI による認識部位探索の構造基盤解明)

Introduction

R.PabI was discovered as a type II restriction enzyme from the hyperthermophilic archaeon *Pyrococcus abyssi*. It recognizes the 5'-GTAC-3' sequence and belongs to the HALFPIPE superfamily. Until now, two crystal structures of R.PabI have been reported in its free form and in complex with a dsDNA containing the 5'-GTAC-3' sequence. The R.PabI-specific dsDNA complex structure indicates that R.PabI flips the G and A bases of the recognition sequence out of the DNA duplex and hydrolyzes the *N*-glycosidic bond of the flipped adenine in a similar manner to DNA glycosylases, although most restriction enzymes cleave phosphodiester bonds at specific sites by hydrolysis. Thus, R.PabI has been termed a restriction DNA glycosylase, which recognizes the specific DNA sequence like a type II restriction enzyme but uses glycosylase activity to facilitate the cleavage of the sequence. The objective of this study is to reveal the mechanisms by which R.PabI efficiently searches and interrogates the recognition sequence. I have solved two new crystal structures of R.PabI. The first structure, an inactive R.PabI mutant (R32A/E63A) in complex with a 20-bp dsDNA which does not contain the 5'-GTAC-3' sequence, should reflect a structural state of R.PabI searching its recognition sequence, while the second structure, a weakly active R.PabI mutant (Y68F/K154A) in complex with a 23-bp dsDNA containing two 5'-GTAC-3' sequences, should reflect a structural state of R.PabI interrogating the recognition sequence.

Chapter 1.

1-1. The crystal structure of the R.PabI (ΔN/R32A/E63A)-nonspecific dsDNA complex

The structure of an inactive R.PabI mutant (ΔN/R32A/E63A) in complex with a 20-bp dsDNA (5'-GCACTAGTTCGAACTAGTGC-3') was solved at 1.9 Å resolution. The complex structure shows that the R.PabI mutant binds to the DNA in an apparent tetrameric form. The 20-bp DNA is sandwiched by the half-pipe regions of two R.PabI homodimers. R70 and D71 in a protomer form two salt bridges with D71' and R70' of a protomer in the other homodimer, respectively. In total, the two R.PabI homodimers are connected by four salt bridges. Due to the interaction with the tetrameric R.PabI, the DNA is bent by about 20° in the middle portion, where the minor and the major grooves are expanded and narrowed by about 2 Å, respectively. The two β8-β9 loops in a homodimer, which were shown to play pivotal roles in unwinding DNA and flipping out the bases, are positioned within the major and the minor grooves. In addition, the side chain of R26 is positioned within the minor groove. It is assumed that these loops and the side chain of R.PabI could play important roles in searching for the target sequence.

1-2. The electrophoretic mobility shift assay and glycosylase activity assay of R.PabI mutants

To examine the importance of the tetrameric structure of R.PabI in searching for the target sequence, the electrophoretic mobility shift assay (EMSA) and the glycosylase activity assay were performed. The same 20-bp dsDNA was used for the EMSA and the crystallization, while 24-, 500-, and 3,000-bp dsDNAs were used for the glycosylase activity assay as substrates. Two single mutations that would inhibit the tetramer formation (R70D and D71R) and a double mutation that would restore the tetramer formation (R70D/D71R) were produced and used in these experiments. The results of the EMSA experiments showed that the D71R mutant reduced the ability to form the tetrameric structure, which was probably due to the electrostatic repulsion and steric hindrance between R70 and R71', whereas the R70D and R70D/D71R mutants retained the ability to form the tetrameric structure on the 20-bp DNA.

In the glycosylase activity assay, all of the three mutants showed comparable activities to WT in cleaving the 24-bp DNA. However, when longer substrate DNAs were used, the cleavage efficiencies varied in the following orders: WT > R70D/D71R = R70D > D71R in cleaving the 500-bp DNA, and WT > R70D/D71R > R70D = D71R in cleaving the 3000-bp DNA. These results indicated that all of the three mutants retain comparable glycosylase activity and the tetramer formation of R.PabI on DNA is important for efficient scanning of the target sequence. In addition, the importance of the side chain of R26 was evaluated by the glycosylase activity assay. The R26A mutant had comparable activity to WT in cleaving the 24-bp DNA, and the cleavage efficiency became lower when longer substrate DNAs were used. These data supports the proposal that the side chain of R26 could play an important role in searching for the target sequence.

1-3. Discussion

The crystal structure of an inactive R.PabI mutant (ΔN/R32A/E63A) in complex with a 20-bp dsDNA had an apparent tetrameric structure in which two R.PabI homodimers sandwich the DNA and four salt bridges are formed between the two homodimers. The tetrameric structure of R.PabI was shown to be important for efficient scanning of the target sequence by the DNA binding and the DNA glycosylase assays.

Chapter 2.

2-1. The crystal structure of the R.PabI (ΔN/K154A/Y68F)-specific dsDNA complex

The structure of a weakly active R.PabI mutant (ΔN/K154A/Y68F) in complex with a 23-bp dsDNA (5’-TCAGCAGTACTAAGTACTGCTGA-3’, the double underlines indicate the two recognition sequences of R.PabI, "GTAC", spaced by three bases) was solved at 2.4 Å resolution. The complex structure shows that two homodimers of the R.PabI mutant separately bind to one DNA and independently recognize the two target sequences on the DNA. The DNA shows characteristic structures: (1) each recognition sequence is substantially bent, (2) the base pairs in the recognition sequence are significantly untwisted (the average twist degree is 21°, much lower than that of the B-form DNA, 36°), and (3) the minor groove at the binding sites is expanded by approximately 9 Å. The β2-β3 loop of each R.PabI protomer protrudes into the expanded minor groove of the DNA and stabilizes the expansion by forming hydrogen bonds and van der Waals interactions with the backbone atoms but not with the bases of DNA. Notably, the hydroxy group of T28 in the β2-β3 loop in one protomer of each homodimer forms two hydrogen bonds with the bases of G and T in the second “GTAC”. Although the DNA is bent in a similar manner to that in the reported R.PabI (K154A)-specific DNA complex structure, the new complex structure shows unique features both in protein and DNA; (1) the β8-β9 loops in both protomers are positioned away from the DNA groove and (2) no base is flipped out and the double helix of the DNA remains intact. In the new complex structure, the positions and angles of all the base pairs in the DNA was calculated, indicating that the T-A step in the target sequence is bent with the biggest roll degree among all of the base pairs (42°, cf. the roll degree between bases in the B-form DNA equals 0°). Since the T-A step has the lowest stacking energy among all kinds of dinucleotides, the flexibility of the T-A step in the recognition sequence "GTAC" may facilitate the sequence recognition of R.PabI.

2-2. The glycosylase activity assay of R.PabI mutants

The comparison with the R.PabI (ΔN/R32A/E63A)-nonspecific DNA complex and the R.PabI (ΔN/K154A/Y68F)-specific DNA complex indicates that the protrusion of the β2-β3 loop into the minor groove should occur before that of the β8-β9 loop, suggesting that the β2-β3 loop may play an important role in deforming the target sequence and recognizing it using the side chain hydroxy group

of T28 in the β2-β3 loop. To prove the importance of the β2-β3 loop and the side chain of T28, the glycosylase activity assays of the P27G/T28G, T28G and T28W mutants were performed using a 24-bp dsDNA as the substrate. The results indicated that the glycosylase activity of the three mutants were substantially lower than WT, confirming that the β2-β3 loop and T28 of R.PabI do play important roles in cleaving the recognition sequence.

2-3. Discussion

The crystal structure of a weakly active mutant (ΔN/K154A/Y68F) of R.PabI in complex with a 23-bp dsDNA containing two "GTAC" sequences has been solved. The complex structure shows that the recognition sequences in the DNA are substantially bent and the minor groove at these sequences are significantly expanded as in the reported R.PabI (K154A)-specific DNA complex structure. However, the new complex structure exhibits a significant difference from the formerly reported structure. The G and A bases in the recognition sequence are flipped out and that of A is cleaved in the former structure, whereas no base is flipped out of the DNA double helix in the new structure.

Thus, the new complex structure would be most likely formed when R.PabI encounters its recognition sequence and starts to transform into the recognition complex. The above results suggest that after arriving at its recognition sequence, R.PabI sharply bends the DNA and opens the minor groove, the β2-β3 loop is protruded into the expanded minor groove to maintain the expansion. The bending of the DNA and the opening of the minor groove would later facilitate the flipping and cleavage of the bases.

Conclusions

In this research, I have solved two new crystal structures of restriction DNA glycosylase R.PabI in complex with DNA. The structural and biochemical analyses have revealed the efficient searching and interrogating mechanisms for the recognition sequence by R.PabI. Since R.PabI recognizes the specific target sequence like a type-II restriction enzyme while cleaves DNA in a similar manner to a glycosylase, the elucidation of the searching and interrogating mechanism of R.PabI would enlarge our understanding on the interaction between protein and DNA. In addition, since R.PabI is the only protein whose crystal structures have been elucidated among the HALFPIPE superfamily members, the new crystal structures would contribute to better understanding of the DNA recognition mechanism by the previously unstudied superfamily.

Publication

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