Biochemical and Biophysical Research Communications 400 (2010) 1–6

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Mini Review

Structural and functional views of salt-bridge interactions of λ integrase in the higher order recombinogenic complexes visualized by genetic method

Sang Yeol Lee

Department of Life Science, Kyungwon University, San 65, Bokjeong-Dong, Sujeong-Gu, Seongnam-Si, Kyeonggi-Do 461-701, Republic of Korea

ARTICLE INFO

Article history:
Received 5 August 2010
Available online 12 August 2010

Keywords:
Protein–protein interactions
DNA–protein interactions
Multimeric complexes
Site-specific DNA recombination

ABSTRACT

The integrase protein encoded by bacteriophage λ (Int) catalyzes site a specific DNA recombination by which the viral chromosome is inserted into and excised out of the host genome through the formation of higher order recombinogenic nucleoprotein complexes. Genetic and biochemical studies on the Int carried out by isolating "multimer-specific" mutants had revealed informative functional characteristics of specific electrostatic interactions occurring among the functional domains of Int. The λ Int recombination system shows the usefulness of structural and functional investigation of multimeric protein complexes through genetic studies on the electrostatic interactions of proteins comprising multimeric complexes. This approach is especially powerful when combined with NMR and X-ray crystallography results providing biological evidences of specific molecular interactions among proteins.

1. Introduction

The λ integrase protein (Int)[1] catalyzes site-specific DNA recombination by which a viral genome of *Escherichia coli* phage λ is inserted into and excised out of its host chromosome [1]. This reaction occurs at specific DNA loci; *att*P/*att*B for 'integrative' and *att*L/*att*R for 'excisive' recombination (Fig. 1A) [2]. Int belongs to the large λ Int family of recombinases, which use transient covalent phosphor-tyrosine intermediates to exchange DNA strands for DNA recombination [3,4]. λ Int is a heterobivalent protein, which simultaneously binds to two distinct DNA sites (arm-type *att* site and core-type *att* site) and acts as a bridge connecting the two DNA sites (Fig. 1B) [5–7]. Int is composed of three functional domains and these domains perform their unique jobs (Fig. 1C). The amino-terminal and the CB domains specifically bind to the arm-type *att* site and the core-type *att* site DNAs, respectively. The catalytic domain has an RHR triad (arginine, histidine, and arginine), which activates the scissile phosphate on the DNA backbone, and a tyrosine residue, which attacks the scissile phosphate and cuts the DNA strand [8–10].

λ Int performs a reaction in a higher order multimeric complex with recombining DNA strands, which is called a recombinogenic complex [5,6,9,11–13]. The site-specific DNA recombination by the λ Int requires highly coordinated homomeric and heteromeric protein–protein interaction networks for the allosteric regulation of Int to achieve serial activation and inactivation [14–16]. This tight regulation is critical for the harmonized DNA cleavage, exchange and ligation that finally forms a recombined DNA product [17,18].

Electrostatic interactions are ion pairs between oppositely charged residues (asp/glu with arg/lys/his) and play important roles in protein folding, binding, flexibility, stability, and function [19]. A pair of oppositely charged residues can be defined as a salt-bridge interaction. The salt-bridge interaction occurs when the two oppositely charged residues are within 4 Å. Since DNA strands bound by Int provide pivotal anchors for the protein–protein contacts (arm-type DNA for the amino-terminal domains of Int and core-type/HJ DNA for the CB/catalytic domains of Int), λ Int is a good model for studying the functional role of a specific protein–protein interaction.

The salt-bridging surface residues of Int were found in the amino-terminal domain and the CB domain and their functional roles were elucidated. Each interaction has highly specific functional roles. Mutant Int's, which are lacking each specific interaction, provided useful information regarding the mechanisms of the regulation of Int activation/inactivation, and the DNA transactions (cleavage, strand exchange and ligation) during the site-specific DNA recombination.

2. Strategy of searching for salt-bridge interactions

The protein–protein interfaces of each domain of λ Int have been explored by alanine scanning analysis of the amino-acid residues (especially, charged residues for fishing out salt-bridge interactions) on the Int surface [16,20]. The monomeric NMR structure of the amino-terminal domain [22] and X-ray crystal structures of

E-mail address: leesaye@kyungwon.ac.kr

[1] *Abbreviations used:* Int, integrase; HJ, Holliday-junction.

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doi:10.1016/j.bbrc.2010.08.030

catalytic and CB-catalytic domains [21,23] were inspected and a number of candidate residues (positively or negatively charged) were changed into alanine using site-directed mutagenesis. Alanine substitution will extinguish the charge dependent electrostatic interaction in the multimeric Int complex.

In searching for the salt-bridging residues, three types of DNA molecules were designed and utilized (Fig. 2) [16]. A half-*att* site suicide substrate can be cleaved by an Int monomer and provides a single Int-binding site. A full-*att* site suicide substrate and Holliday junction DNA has multiple binding sites for the Int protomers, and thus provides possible interacting interfaces of Int protomers (Fig. 2). Those multimer-specific mutants which have normal catalytic activity, indistinguishable from wild-type on the half-*att* site DNA, but had severely depressed activity as multimeric Int (i.e., on the full-*att* site and HJ), were selected among the alanine substituted mutants. The amino-acid residues from the multimer-specific alanine mutants were then changed into the oppositely charged residues (i.e., R30D, D71R, E153R, and R169D) and the multimer-specific depression of catalytic activity was checked. A pair of interacting residues is confirmed with a double mutation. If switching the charge on the two interacting candidate residues restores Int's activity in the multimeric state, then it is likely that they formed a salt-bridge. Based on this logic, two salt-bridges were identified: R30–D71, in the amino-terminal domain, and E153–R169 in the CB domain interaction.

3. R30–D71 interaction of amino-terminal domain of Int

Int shares a high degree of similarity in the mechanisms of DNA cleavage, strand exchange and ligation with Cre and Flp, the other tyrosine recombinase family members [24,25]. However, Int possesses an additional amino-terminal domain which specifically binds to its cognate arm-type *att* biding sites. Interaction between the amino-terminal domains and their cognate arm-type *att* sites are known to play important roles in the regulated directionality of Int. Surprisingly, a Cre chimera, which was generated by the addition of the amino-terminal domain of Int to Cre, is endowed with the Int-like regulated directionality of recombination [26]. The regulatory function of the amino-terminal domain of Int is a result of interactions with its cognate arm-type *att* site, and the homomeric interactions with its neighboring partners [20].

R30 in the amino-terminal domain and D71 in the adjacent linker region of λ Int electrostatically interact with each other. This interaction is dependent on the arm-type *att* site DNA [20,27]. There are two consecutive arm-type binding sites (P′1 and P′2) for the amino-terminal domain of λ Int on the P′ arm of *att* site DNA. This provides nests for the two adjacent amino-terminal domains of Int with a head to tail orientation.

This intermolecular salt-bridge is found to occur across the cleaving planes of Holliday junction DNA intermediate. The R30–D71 interaction directs HJ resolution bias, which determines the resolution plane of four-way HJ intermediate (E–W or N–S, Fig. 3A). Although the cyclic arrangement of four Int molecules on the HJ allows four possible interactions among the Int protomers on the HJ, only the two interactions on each arm-type DNA across the center of HJ branches are required. This interaction contributes to the two-fold symmetry delineating active and inactive partners within the tetrameric recombinogenic complex (Fig. 3B) [20].

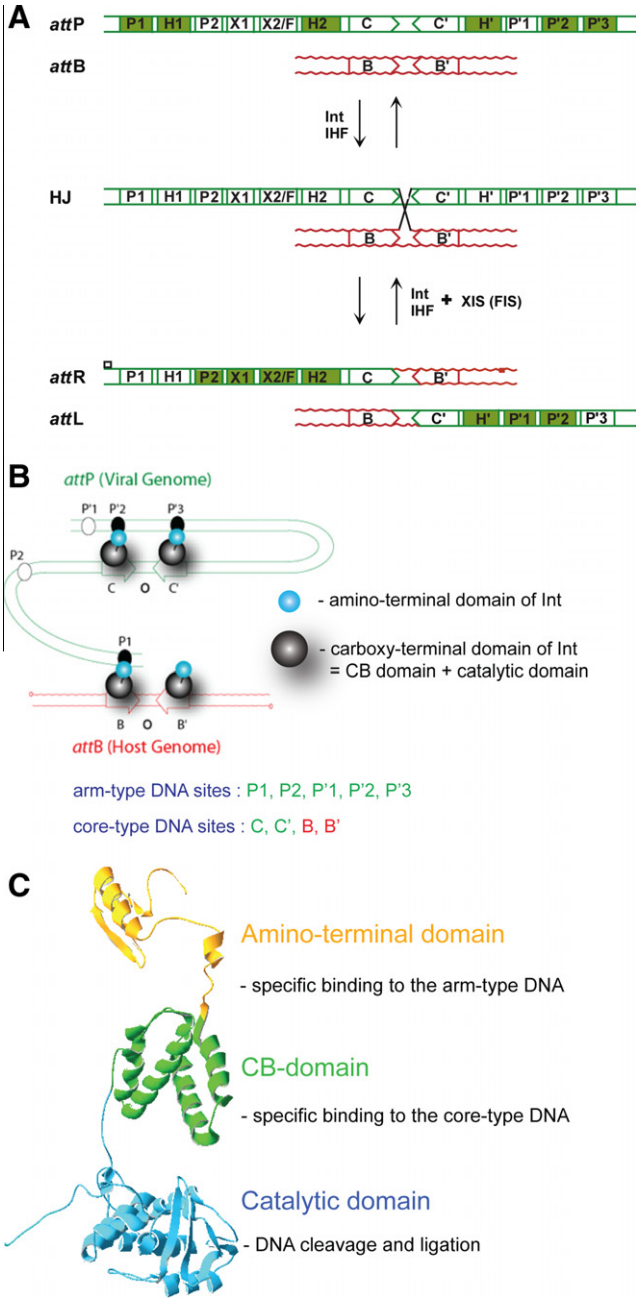

Fig. 1. Reaction path and structural elements of λ Int-dependent recombination. (A) Diagram of the site-specific recombination reaction. Integrative recombination between the phage *att*P and bacterial *att*B sites requires the phage encoded Int protein and the host-encoded IHF to generate recombined *att*L and *att*R sites in the prophage. Excisive recombination between *att*L and *att*R sites requires additional accessory DNA-binding proteins, Xis (phage-encoded) and Fis (host-encoded) proteins. Int has four core-type binding sites (C/C′/B/B′) that flank the 7 bp overlap region as inverted repeats. Int cleavage, strand swapping, and ligation at the left boundary of the overlap region generates a four-way Holliday junction intermediate. HJ is resolved to form recombinant products by the same sequence of events at the right boundary of the overlap region. There are five arm-type Int-binding sites: two single sites in the P arm (P1/P2) and three adjacent sites in the P′ arm (P′1/P′2/P′3). There are six binding sites for the three accessory proteins: IHF (H1/H2/H′), Xis (X1/X2), and Fis (F). Fis binding site overlaps with the X2 site. Filled rectangles indicate protein-binding sites occupied in the arms of *att*P during integrative recombination and in the arms of *att*L and *att*R during excisive recombination. (B) Illustration of the molecular bridge. Heterobivalent Int protein simultaneously binds to the distant arm-type (P/P′) and core-type *att* sites (C/C′/B/B′) and thus acts as a molecular bridge connecting two distant DNA sites. (C) Domain organization of λ Int. λ Int is composed of three functional domains. The amino-terminal domain (yellow color) binds specifically to arm-type *att* site, the CB domain (green color) binds specifically to core-type *att* site and catalytic domain (blue color) possesses active tyrosine residue and RHR triad to perform DNA cleavage and ligation. Illustration is based on the crystal structure [23]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

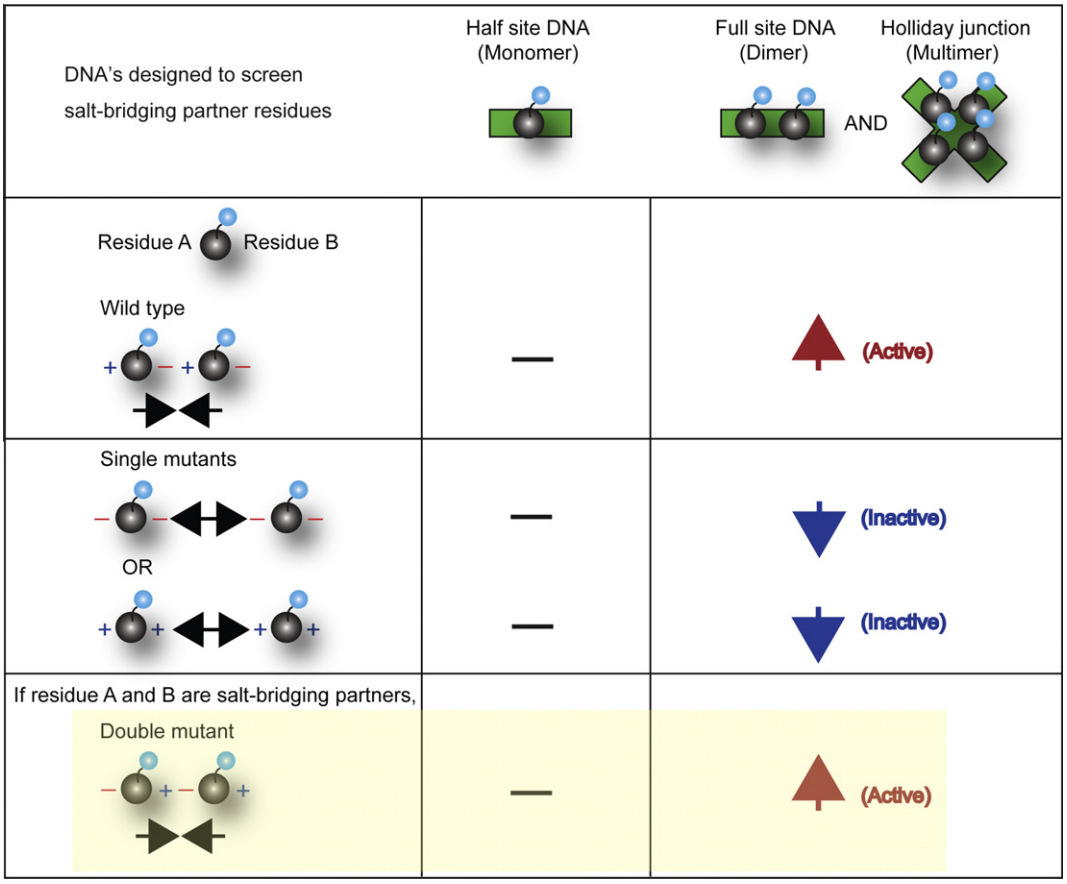

Fig. 2. Strategy of searching for the salt-bridge interactions. Three DNA substrates were designed to select multimer-specific mutants of Int. Half-*att* site DNA has only one core-type *att* site accommodating single Int protomer. Full-*att* site DNA has two core-type *att* sites flanking 7 bp overlap sequence. HJ DNA has four core-type *att* sites, thus Int can form a tetrameric complex on the HJ DNA. If a particular pair of oppositely charged residues interact with salt-bridge each other, single mutation one of the interacting residue into oppositely charged residues (D/E to K/R/H or K/R/H to D/E) or alanine will lose an electrostatic salt-bridge interaction. The catalytic activity of charge-reversal single mutant and wild-type Int will not be different on the half-*att* site since mutant and wild-type Int act as a monomer. However, the catalytic activity of single charge-reversal mutants, if the mutated residue is involved in the salt-bridge interaction, will not be stimulated on the full-*att* site and HJ while the catalytic activity of wild-type Int is stimulated by the multimer-specific activation. If switching the charge on the two interacting candidate residues restores Int's catalytic activity through the multimer-specific activation, then it is likely that the two residues form a salt-bridge.

The R30–D71 interaction is highly dependent on the arm-type DNA binding. The allele-specific suppression is noticeably diminished in the absence of arm-type DNA and this indicates that the interaction is dependent on the arm-type DNA binding of the amino-terminal domains of λ Int. Together with the twofold symmetries of the amino-terminal domains, the arm-type *att* site DNA provides asymmetrical constraints on the recombinogenic complex [28].

The arm-type DNA stimulates Int dimerization as well as the cleavage reaction of core-type *att* site DNA in the presence of heterologous DNA [29]. Since the P′1,2 arm-type DNA carries two consecutive binding sites for the amino-terminal domain of Int, arm-type DNA may stabilize the dimer complex of Int on the core-type *att* site DNA. Since Int binds weakly to core-type *att* site DNA, positioning of Int on the core-site may be supported by strong Int-arm-type DNA binding. This drives multimer-specific activation and leads to the stimulated cleavage reaction of Int. However, this arm-type DNA stimulation is observed only in the presence of heterologous DNA. In the absence of heterologous carrier DNA, arm-type DNA is not stimulatory but inhibitory for the cleavage activity of Int [27]. There may be two possible explanations for the observation. The arm-type DNA may simply compete with core-type DNA for the active site pocket of Int, or arm-type DNA binding may inhibit the activity of one Int out of two Int molecules bound to it.

The possible answer to this question was provided by the investigation of the charge-reversal single mutants and the allele-specific suppressor mutant. In case of the wild-type and the allele-specific suppressor mutant, they both showed 50% attenuated cleavage activity with arm-type DNA compared to the cleavage activity without arm-type DNA. The two single charge-reversal mutants responded oppositely to the arm-type DNA. Actually they exhibited the two extremes of the effects of the arm-type DNA on Int activation. The D71R mutant did not show any attenuated cleavage activity with arm-type DNA, and the R30D mutant showed only 10% cleavage activity with arm-type DNA, which is only 1/5 of wild-type Int. These unusual responses of the single charge-reversal mutants to arm-type DNA were diminished by restoration of the R30–D71 salt-bridge, which was able to reform in the double charge reversal of the allele-specific suppressor mutant. This suggests that the depressed Int activity by the arm-type DNA is the result of the selective attenuation of Int by the amino-terminal domain interactions on the arm-type DNA.

There are three recently published crystal structures of Int multimers bound to DNA. The first one shows a dimer of Int, whose amino-terminal domain is truncated, bound to core-type *att* site [23]. In the structure, the active site tyrosine residue from one Int protomer is covalently attached to the scissile phosphate of the cleaved DNA strand via a phospho-tyrosine linkage. The second structure shows a full-length Int dimer bound to core-type and

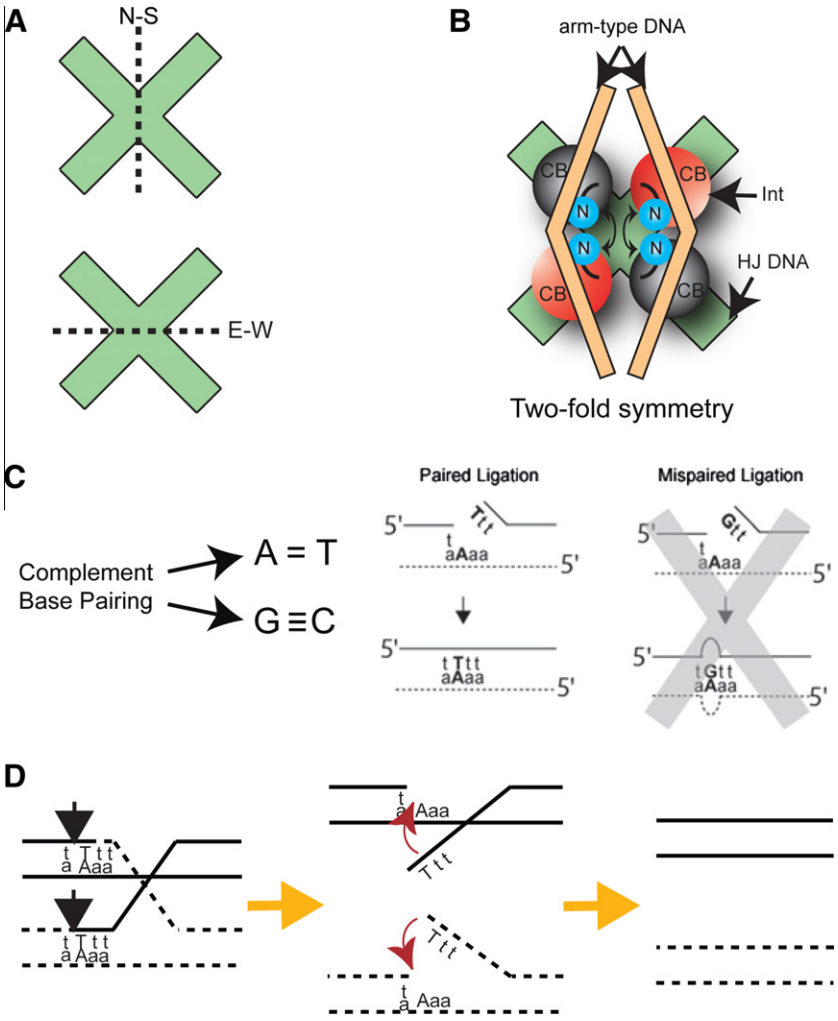

Fig. 3. Functional roles of R30–D71 and E153–R169 salt-bridges. (A) The direction of HJ intermediate resolution determines the direction of the recombination, integrative or excisive recombination. R30–D71 salt-bridge is involved in the determination of the HJ resolution and thus contributes to the regulated directionality of recombination. (B) A twofold symmetric model for the recombinogenic complex of HJ, two arm-type *att* site oligonucleotides, and four Int protomers based on the results from the investigation of R30–D71 salt-bridge. The crystal structure reported later indicated that amino-terminal domains bound to the P′1,2 arm-type *att* site oligonucleotides in trans and form cyclically permutated organization. Thus the amino-terminal domain of one Int protomer located over the carboxy-terminal domain (CB + catalytic domains) of the neighboring Int protomer. (C) Sensitivity to homology at the sites of DNA ligation of multimeric Int is dependent on E153–R169 salt-bridge. E153–R169 interaction is critical for the paired DNA ligation through the appropriate purine–pyrimidine base pairing by multimeric complex of Int. (D) E153–R169 salt-bridge contributes to the strand exchange during HJ resolution by guiding single-strand DNA to the desired position to be reconnected through the paired ligation with proper purine–pyrimidine base pairing.

arm-type *att* site [30]. This represents the complex structure in the stage right after the first exchange of DNA strands. The third structure is composed of four full-length Int protomers bound to HJ and two arm-type DNA molecules, which represents a complex active for the bottom strand cleavage.

The two complex structures with full-length Int show the dynamic nature of the R30–D71 salt-bridge. In the crystal structure of two full-length Int dimers bound to core-type and arm-type *att* site DNAs, the distance between R30 and D71 is 5.61 Å representing the lack of R30–D71 salt-bridge (Fig. 4B). The two sets of R30–D71 charge pairs on the arm-type site oligonucleotides from the complex structure in the HJ show the asymmetric nature of the R30–D71 salt-bridges. The distances between the two residues on the same arm-type *att* site oligonucleotides are 2.66 Å and 8.82 Å, indicating that only one R30–D71 salt-bridge exists at a time (Fig. 4C). The R30 residue of the Int protomer active for the bottom strand cleavage is satisfied with the salt-bridge interaction with D71 residue from the inactive Int protomer. These differences may indicate the transient nature of R30–D71 salt-bridge interaction. It may be required only at the time when a particular Int protomer needs to be activated for the cleavage reaction. The requirement for an arm-type *att* site for the salt-bridge interaction is also shown in the complex structure with HJ. The distances between the two residues (R30 and D71) from the two amino-terminal domains bound to different arm-type *att* site DNA molecules are about 19.59 Å and 11.68 Å, showing that the R30–D71 salt-bridge interaction may occur *in cis* (between the residues from the two neighboring amino-terminal domains bound the same arm-type *att* site DNA).

The asymmetric interactions of the amino-terminal domains may be closely related with the asymmetric requirement for the arm-type *att* sites in the real reaction. In the crystal structure of a complex with HJ, amino-terminal domains on the P′1,2 arm-type sites were bound to two P′1,2 arm-type sites *in trans* forming a cyclically permutated organization [30]. Since the crystal structure lacks the accessory DNA-bending proteins and their cognate sites, it does not exactly reflect biologically active integrative and/or excisive recombinogenic complex. The model based on the crystal

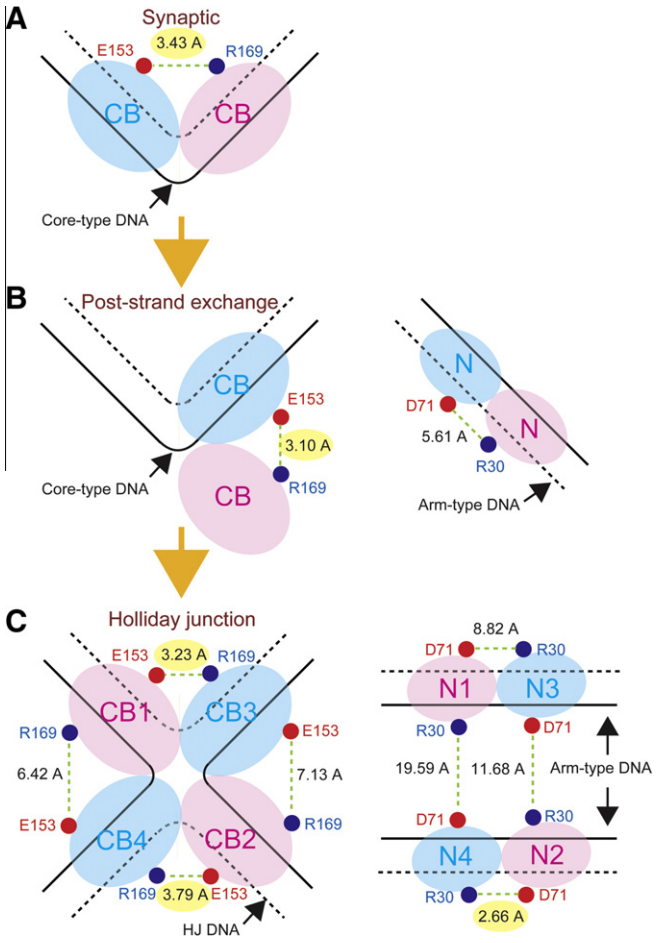

Fig. 4. Salt-bridge interactions in different steps of recombination reaction. (A) In the crystal structure of amino-terminally truncated Int dimer bound to core-type *att* site oligonucleotide, the distance between E153 from one Int protomer and R169 from neighboring Int protomer is about 3.43 Å indicating the existence of salt-bridge interaction. (B) In the crystal structure of full Int dimer bound to core-type and P′1,2 arm-type *att* site oligonucleotides, E153 and R169 residues from each Int protomers are placed within 4 Å and thus they form a salt-bridge. In case of the amino-terminal domains on the arm-type *att* site DNA, the distance between R30 and D71 residues from each Int protomers is 5.61 Å indicating the two residues are not forming salt-bridge. (C) R30–D71 and E153–R169 interaction found in the crystal structure of full Int tetramer bound to HJ and two P′1,2 arm-type att site oligonucleotides. Two pairs of E153–R169 salt-bridges were found and only one pair of R30–D71 is within 4 Å range of salt-bridge interaction. Illustrations are based on the investigation of crystal structures [23,30].

structure predicts there may be additional "non-canonical" arm-type sites to complete the twofold symmetry from the crystal structure while it had been known that only three "canonical" arm-type sites are bound by Int for each integrative and excisive recombination. However, the data from research based on the biotin interference assay to probe specific DNA binding sites and DNA protection experiments do not support the existence of a "non-canonical" arm-type site and suggest the asymmetry of the amino-terminal domains and arm-type sites in each integrative and excisive recombination may reflect the regulation of directionality of recombination [31].

The amino-terminal domain of Int also interacts with Xis DNA-bending protein. The E47 residue is involved in this interaction [22]. This heteromeric Int-Xis interaction may also contribute to the asymmetry of amino-terminal domains in integrative and excisive recombinations.

Nonetheless, the requirement for the two R30–D71 salt-bridges in HJ resolution may indicate that amino-terminal domain interactions across the HJ are required for the DNA cleavage and strand exchange at some point of the reaction. The asymmetric arm-type site requirements, along with the accessory proteins, may regulate the directionality of recombination, while the R30–D71 salt-bridges contribute to the selection of which Int protomer is activated for the reaction.

4. E153–R169 interaction of core binding-domain of Int

E153 in the CB domain, and R169 in the adjacent linker region (connecting the CB and C-terminal domains of λ Int), electrostatically interact with each other [16]. The E153–R169 interaction is consistent with the ~3.2 Å distance between these side-chains in the crystallized Int complex structures (Fig. 4A–C).

As in the investigation of the R30–D71 interaction, a single charge-reversal mutant, R169D, showed the important mechanical functions of the E153–R169 salt-bridge interaction in addition to its catalytic activation of Int.

The R169D mutant is more effective than wild-type Int at exchanging DNA strands with illegitimate base pairings [18]. This single mutant shows us that the E153–R169 ion pair is critical for the proper DNA ligation based on the correct purine-pyrimidine pairing (Fig. 3C). This means that the E153–R169 interaction aids correct positioning of the single-strand DNA, formed by Int DNA cleavage, to achieve proper strand exchange in HJ resolution, and in the formation of the final recombined DNA product (Fig. 3D).

Since Int requires the E153–R169 salt-bridge in the multimeric state for full activation, the existence and the disappearance of the interaction in the crystal structure may represent a dynamic movement of the Int complex during the serial activation and inactivation of Int protomers responsible for the sequential formation and resolution of the HJ.

In looking at the E153–R169 and R30–D71 ion pairs together, we can find a correlation between the two molecular interactions on the HJ DNA. The two Int protomers interacting via the E153–R169 salt-bridge interaction on the HJ have their amino-terminal domains on the same arm-type DNA molecule (Fig. 4D). The E153 of CB1 (CB domain of Int 1) and the R169 of CB3 (CB domain of Int 3) are placed within the salt-bridging distance and the N1 (amino-terminal domain of Int 1) and N3 (amino-terminal domain of Int 3) are on the same arm-type DNA. In the case of Int 2 and Int 4, the same molecular organization was found. However, the amino-terminal domains on the different arm-type DNA molecules do not appear to be interacting via their R30–D71 salt-bridge and their CB domains are also not enjoying the E153–R169 salt-bridge interactions. This implies that the activated Int protomers with E153–R169 salt-bridges on the HJ DNA are regulated by the selective attenuation function of the amino-terminal domain interactions on the arm-type DNAs.

5. Conclusion

Each of the two salt-bridge interactions of λ Int has its unique role during the site-specific DNA recombination reaction. Now there are several Int structures from NMR and X-ray crystallography, so it's possible to find the ion pairs involved in the salt-bridge interactions. However, this does not necessarily lead to a complete understanding of the functional roles of a specific molecular interaction in the reaction pathway. Investigation of protein–protein interaction using charge reversal and allele-specific suppressor mutants not only provides genetic evidence for the existence of a specific salt-bridge between the two oppositely charged residues, it also shows us the functional roles of the salt-bridges in the reaction.

Thus genetic studies within the complex structure provide information about the overall protein domain interactions

represented by the specific residue-residue interactions, which may be visualized as one snapshot of the dynamic complex in the X-ray crystal structure. In addition, these studies suggest functional roles of the specific salt-bridge interactions. The combination of NMR/X-ray crystallography results with genetic methods is a powerful approach to understanding the structural and functional characterization of an enzyme reaction, especially for reactions involving multimeric enzyme complexes.

Acknowledgment

This work was supported by the Kyungwon University Research Fund in 2010.

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