Tn5 Transposase Active Site Mutations Suggest Position of Donor Backbone DNA in Synaptic Complex*

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Tn5 transposase (Tnp), a 53.3-kDa protein, enables the movement of transposon Tn5 by a conservative mechanism. Within the context of a protein and DNA synaptic complex, a single Tnp molecule catalyzes four sequential DNA breaking and joining reactions at the end of a single transposon. The three amino acids of the DDE motif (Asp-97, Asp-188, and Glu-326), which are conserved among transposases and retroviral integrases, have been shown previously to be absolutely required for all catalytic steps. To probe the effect of active site geometry on the ability to form synaptic complexes and perform catalysis, single mutations at each position of the DDE motif were constructed. The aspartates were changed to glutamates, and the glutamate was changed to an aspartate. These mutants were studied by performing in vitro binding assays using short oligonucleotide substrates simulating the natural substrates for the synaptic complex formation and subsequent transposition steps. The results indicate that the aspartate to glutamate mutations restrict synaptic complex formation with substrates resembling the natural transposon prior to transferred strand nicking. This suggests a structural model in which the donor backbone DNA, prior to nicking, occupies the same space that is invaded by the longer side chains present in the aspartate to glutamate mutants. Additionally, catalytic assays support the previous proposal that the active site coordinates two divalent metal ions.

The naturally isolated Tn5 transposon transposes at a very low frequency, most likely because frequent transposition would be detrimental to the survival of the cell, constituting a selective disadvantage to a highly active transposon. The low level of transposition can be attributed to both the low activity of the Tnp protein and the suboptimal 19-bp end sequences. The low level of protein activity has been increased by the introduction of two point mutations, E54K and L372P (5, 6). This mutant is termed EK/LP and is the standard for this and other studies of Tn5 Tnp. The rate of transposition is further increased by replacing the naturally occurring end sequences with a 19-bp site termed the mosaic end (ME) (7). The combination of Tnp EK/LP and ME sequences has allowed the development of an efficient series of in vitro assays used to study the individual steps of the transposition process via gel shift (3) and catalytic assays (2, 6, 8, 9).

Each Tnp molecule possesses a single active site, responsible for carrying out all four catalytic steps, at a single 19-bp end (4, 10). In the first and third steps, transferred strand nicking and hairpin resolution, a water molecule acts as a nucleophile to hydrolyze a DNA phosphate bond, whereas in the second and fourth reactions, hairpin formation and strand transfer, the 3′-hydroxyl group of the transferred strand acts as the nucleophile (Fig. 1). The active site contains a triad of acidic residues (Asp-97, Asp-188, and Glu-326) termed the DDE motif. This motif is believed to be conserved among all transposases and retroviral integrases. The DDE residues serve to coordinate divalent metal ions that are required for catalysis (11–14). A previous study has suggested that Asp-97 and Glu-326 contribute to the coordination of one metal ion, whereas Asp-97 and Asp-188 coordinate a second ion (15). All three of the DDE residues are required for all four catalytic steps of transposition (9).

In this study point mutations were introduced at each of the DDE residues in the EK/LP background, creating three separate single mutants. These mutations consist of changing the two aspartates to glutamates and the glutamate to an aspartate, yielding D97E, D188E, and E326D. These alterations were chosen because they leave the catalytically essential carboxyl groups in place at each of the three positions, while slightly altering their spatial location within the active site. By studying the ability of these mutants to form paired ends complexes (PECs), the equivalent of synaptic complexes with short oligonucleotide substrates, we were able to predict the spatial position assumed by the nontransposon DNA adjacent to the transposon end sequences, termed donor backbone (dbb), in synaptic complexes prior to the first catalytic step of transposition, transferred strand nicking. Additionally, use of these mutants in catalytic studies lends support to the previous proposal that the DDE motif serves to coordinate two divalent metal ions in the active site (15). To our knowledge, no studies of similar chemically conservative mutations of the DDE motif
in other transposases or retroviral integrases have been reported in the literature.

EXPERIMENTAL PROCEDURES

Cultures—All bacterial cultures for cloning and protein purification were grown in Luria broth (16). Ampicillin was purchased from Sigma and added to agar or liquid media at 100 μg/ml. Restriction enzymes were purchased from New England Biolabs and Promega. T4 DNA ligase was purchased from Promega. Pfu polymerase was purchased from Stratagene. T4 DNA polynucleotide kinase was purchased from New England Biolabs. Oligonucleotides were purchased from Integrated DNA Technology. Sequencing was performed using the PerkinElmer “Big Dye” kit with the aid of the University of Wisconsin-Madison Biotechnology Center.

Site-directed Mutagenesis—The plasmid pRZPET2, which contains the hyperactive EKLP Tn5 tnp (6), was used as a template for site-directed mutagenesis using the single end overlap procedure (17). PCR products bearing the desired mutations were cloned into pRZPET2. D97E was cloned at the XbaI and NotI sites, D188E was cloned at the NotI and NheI sites, and E326D was cloned at the NheI and HindIII sites. The constructs for protein expression were created by swapping the mutated regions from the pRZPET2 derived plasmids into the expression vector pGRTYB35, which encodes for the EKLP Tn5 Tnp as a carboxyl-terminal fusion to intein and chitin binding domains as described previously (2). The pRZPET2 plasmids containing the D97E, D188E, and E326D mutations were digested with BglII, and the overhangs filled in with Pfu followed by digestion with XbaI. The fragments containing the mutated tnp genes were cloned into the pGRTYB35 Smal and XbaI sites to yield pWSR5021 (D97E), pWSR5022 (D188E), and pWSR5018 (E326D). The presence of mutations was confirmed by DNA sequencing.

Bacterial Strains—Expression of Tnp mutant proteins for purification was performed in Escherichia coli strain ER2566 (F- fhuA2 [Jon] ompT lacZ::T7 gene1 gal sula11 (ΔmerC-merR)114::IS10 (mer::73:: miniTn10-TesS::Rsp8-210::Tn10)(TesS) enda1 I (cem)) (New England Biolabs). All plasmid propagation utilized DH5α (endA1 hsdR17(rk+)

The following oligonucleotides were used for hairpin PEC formation and 3’ nicking assays (ME shown in bold): 5’-CTCAACCAGCCA-GCTCCCAACACTGCTCTCTTTAGACATCTTCACATCCGTGAATGAGACCA-TGCGATGT-3’ (nontransferred strand) and 5’-ACATGCATGCTCACTC-GTCAAGATGTGTTTAAAGAGACACGTTGTTGGGAGCTCGAACTGAGCA-G-3’ (transferred strand).

For prenicked Tnp expression and hairpin formation assays, the nontransferred strand oligonucleotide was replaced by the following two oligonucleotides: 5’-TGTTGGGAGCTCGAACTGAGCA-G-3’ (donor backbone compliment) and 5’-ACATGCATGCTCACTC-GTCAAGATGTGTTTAAAGAGACACGTTGTTGGGAGCTCGAACTGAGCA-G-3’ (transferred strand).

For prenicked Tnp expression and hairpin formation assays, the nontransferred strand oligonucleotide was replaced by the following two oligonucleotides: 5’-TGTTGGGAGCTCGAACTGAGCA-G-3’ (donor backbone compliment) and 5’-ACATGCATGCTCACTC-GTCAAGATGTGTTTAAAGAGACACGTTGTTGGGAGCTCGAACTGAGCA-G-3’ (transferred strand).

In Vitro Catalytic Reactions—All catalytic reactions were performed in 10-μl reaction volumes containing 235 nM Tnp, 0.1 mM potassium glutamate, 25 mM HEPEs, pH 7.5, 50 μg/ml bovine serum albumin, 1 mM β-mercaptoethanol, 1 mM MnCl₂, and 75 μg/ml tRNA. DNA substrates were added to a concentration of 25 nM. Reactions were incu-
results

Three single mutants were constructed in the EK/LP background, a hyperactive version of Tnp containing mutations E54K and L372P. These mutations were made at the catalytically critical DDE motif residues. The substitution mutations (D97E, D188E, and E326D) are chemically conservative, differing by only one carbon atom in the length of the side chain. Using these mutants, which have slightly perturbed active site geometry, we were able to probe the spatial requirements for formation of paired ends complexes and catalytic activity in Tn5 transposition. The ability of each of the three active site mutants to form PECs with oligonucleotides simulating the natural substrate at all steps of transposition and to catalyze the three reactions comprising cleavage of the transposon from donor backbone (dbb) DNA was tested in vitro.

It is possible to examine the individual steps of the transposition process in isolation by approximating the naturally occurring DNA substrates at different stages of the transposition mechanism using short radiolabeled oligonucleotides containing the 19-bp ME sequence, which is recognized by Tnp and defines the end of the transposon. Incubation of DNA substrates with Tnp in the absence of divalent metal ions, required for catalytic activity, allows the formation of PECs containing two DNA molecules and two Tnp molecules (3). These PECs are analogous to the synaptic complexes that are the context of transposition. The PECs can be distinguished from free DNA by the decreased mobility of complexes in native polyacrylamide electrophoresis. Because coordination of divalent metal ions is required for catalytic activity, PEC assembly represents the ability of Tnp to form the nucleoprotein complexes within which the transposition reactions occur but not the reactions themselves. Incubation of Tnp with the same radiolabeled oligonucleotides in the presence of divalent metal ions results in Tnp-mediated processing of substrate DNA. Denaturing polyacrylamide electrophoresis of the products allows an analysis of the catalytic reactions (2).

Unnicked Substrate—An unnicked substrate analogous to naturally occurring Tn5 DNA, containing both transposon DNA with an embedded ME sequence and dbb DNA, was used to investigate PEC formation and transferred strand nicking. This 60-bp substrate was radiolabeled with 32P at the 5‘-end of the transferred strand. PEC formation was investigated by incubating the unnicked substrate with Tnp and determining the percentage of DNA shifted on a native gel because of decreased PEC mobility (Fig. 2). Two of the mutants, D97E and D188E, show a marked decrease in the ability to form PECs with the unnicked substrate. Without replacing the chemical group at either position, a carboxyl, the change from aspartate to glutamate in both mutants has the effect of increasing the length of the respective amino acid side chains by one additional carbon atom. This extends the carboxyl group by about 1.5 angstroms. Both of these mutants show a severalfold decrease in the ability to bind the unnicked substrate. D97E forms PECs with the unnicked substrate 30% as efficiently as EK/LP, whereas D188E forms PEC only 22% as well. The opposite change in mutant E326D, which withdraws the carboxyl group by one carbon atom by replacing the glutamate with an aspartate, shows no decrease in PEC formation ability and actually seems to increase preference for the unnicked substrate, forming PECs 170% as efficiently as EK/LP (Table I).

The activity of the first catalytic step of transposition, transferred strand nicking, was investigated by incubating the unnicked substrate with Tnp and Mn2+. Introduction of a nick by Tnp produces a labeled 40-mer. Those nicked molecules that undergo hairpin formation result in a labeled 80-mer. Finally, those hairpins that are resolved produce a labeled 40-mer (Fig. 3). Therefore, the amount of DNA that undergoes transferred strand nicking can be determined by quantitating the percentage of total DNA that migrates on a denaturing gel at both the 40-mer and 80-mer positions. The D97E mutant shows no ability to nick the substrate, whereas both D188E and E326D show decreased nicking, performing the reaction 9 and 34% as

PEC Formation Assays—PEC assays were performed in 10-μl reaction volumes containing 235 nM Tnp, 0.1 M potassium glutamate, 25 mM HEPES, pH 7.5, 50 μg/ml bovine serum albumin, 1 mM β-mercaptoethanol, and 75 μg/ml tRNA. DNA substrates were added to a concentration of 25 nM. Reactions were incubated at 37 °C for 1 h prior to addition of glycerol loading dye. Samples were electrophoresed in an 8% native polyacrylamide gel and visualized with a PhosphorImager. Radiolabeled DNA was quantitated using ImageQuant software. PEC formation is presented as the percentage of total counts per lane shifted as a result of decreased PEC mobility. All values represent the average of three independent reactions.

FIG. 2. PEC formation assay with unnicked substrate. The efficiency of each mutant to form PECs with short radiolabeled oligonucleotides was determined by gel shift assays. Incubation of Tnp with unnicked DNA substrate results in the formation of PECs that have decreased mobility in a native polyacrylamide gel. The diagram (top) graphically depicts the complexes that are formed, and an example is shown (bottom) of the shifts produced by the three DDE mutants, EK/LP, and a no-protein negative control (−) as visualized on a native gel.
PEC formation with DDE mutants

The ability of DDE mutants to form PECs was tested with un-nicked, pre-nicked, hairpin, and pre-cleaved substrates. Values represent the percentage of total counts/lane shifted because of decreased PEC mobility. All values are the average of three independent reactions. Standard deviations are shown in superscript. The numbers in parentheses refer to PEC formation as a percentage of EK/LP PEC formation.

<table>
<thead>
<tr>
<th>Donor Backbone Position</th>
<th>EK/LP</th>
<th>D97E</th>
<th>D188E</th>
<th>E326D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Un-nicked</td>
<td>28²±⁵ (100)</td>
<td>8²±² (30)</td>
<td>6³ (22)</td>
<td>47³ (170)</td>
</tr>
<tr>
<td>Pre-nicked</td>
<td>6² (100)</td>
<td>77³ (125)</td>
<td>67³ (108)</td>
<td>81³ (131)</td>
</tr>
<tr>
<td>Hairpin</td>
<td>29±⁴ (100)</td>
<td>51± (176)</td>
<td>41±³ (140)</td>
<td>68± (236)</td>
</tr>
<tr>
<td>Pre-cleaved</td>
<td>36±⁴ (100)</td>
<td>43±³ (121)</td>
<td>53±² (149)</td>
<td>32± (89)</td>
</tr>
</tbody>
</table>

**Table II**

Catalytic reactions with DDE mutants

The ability of the DDE mutants to catalyze the three DNA cleavage steps of transposition was tested. Values represent reaction products as a percentage of total counts/lane. All values are the average activity of three independent reactions. Standard deviations are shown in superscript. The numbers in parentheses refer to activity as a percentage of EK/LP activity.

<table>
<thead>
<tr>
<th>Catalytic Reaction</th>
<th>EK/LP</th>
<th>D97E</th>
<th>D188E</th>
<th>E326D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicking</td>
<td>31³ (100)</td>
<td>0³ (0)</td>
<td>3² (9)</td>
<td>10³ (34)</td>
</tr>
<tr>
<td>Hairpin formation</td>
<td>44³ (100)</td>
<td>3³ (8)</td>
<td>8³ (18)</td>
<td>16² (37)</td>
</tr>
<tr>
<td>Hairpin resolution</td>
<td>51³ (100)</td>
<td>0³ (0)</td>
<td>16³ (22)</td>
<td>14³ (27)</td>
</tr>
</tbody>
</table>

**Fig. 3. Transferred strand nicking assay.** The ability of each mutant to catalyze the first step of transposition, transferred strand nicking, was assayed by incubating Tnp with unnicked DNA substrates in the presence of Mn²⁺. Nicking produces a labeled 40-mer, whereas the following two steps, hairpin formation and resolution, produce a labeled 80-mer and 40-mer, respectively. Adding the amount of substrate that migrates as 40 and 80 mers on a denaturing polyacrylamide gel accounts for all of the substrate that underwent transferred strand nicking. The diagram (top) graphically depicts the labeled oligonucleotides that are present at each reaction step, and an example is shown (bottom) of the reaction with the DDE mutants, EK/LP, and a no-pec protein negative control (--) as visualized on a denaturing gel.

**Discussion**

The DDE motif (residues 97, 188, 326) has been shown by crystallographic studies to coordinate the divalent metal ions necessary for the catalytic action of Tn5 Tnp (4, 15). It has also been shown that all three residues are required for every catalytic step of transposition. Replacing any of them with an alanine (9) or switching them to their amide equivalents, aspartate to asparagine and glutamate to glutamine (data not shown), destroys all activity. Although the DDE residues are required for catalysis, they are not required for PEC formation. None of the alanine or amide substitutions substantially depresses PEC formation. In this study we analyzed the effects of substituting glutamate for Asp-97 and Asp-188, while changing Glu-326 to an aspartate. The only difference between aspartate and glutamate is an additional carbon in the glutamate side chain. This should amount to an ~1.5-angstrom difference in the position of the catalytically important carboxyl group in any of the three single mutants. D97E and D188E should result in a slightly deeper insertion of the carboxyl into the active site at the altered residue, whereas in the case of E326D the carboxyl should be withdrawn by a similar amount. The obvious consequence of these changes is to increase crowding in the D97E and D188E
mutants, while providing more space in the E326D mutant.

Crystallographic studies reveal the position of DNA substrate in the active site. However, all existing structural data depicts Tnp complexed with precut transposon DNA or with DNA containing only one base of dbb DNA. Consequently, we have no knowledge about the position of dbb DNA in synaptic complexes. By allowing a comparison of how these mutations perturb PEC formation, these experiments suggest the position of dbb DNA in synaptic complexes prior to transferred strand nicking.

Both of the mutations that increase side chain length of the altered residue, D97E and D188E, dramatically impair PEC formation with the 60-bp unnicked substrate, which resembles the natural substrate for synaptic complex formation, and for the first catalytic step, transferred strand nicking. Not surprisingly, the E326D mutant, in which the side chain is withdrawn from the active site, forms PECs proficiently. This suggests that the interference with PEC formation by D97E and D188E is a result of increased crowding. This observation makes it appear likely that unnicked dbb DNA is positioned such that it experiences steric interference from the mutationally extended side chains present in these two proteins.

Other possible explanations, such as a general decrease in ability to interact with DNA, seem unlikely in light of the undiminished ability of these mutants to bind precut, hairpin, and precleaved substrates. Both D97E and D188E bind precut and hairpin substrates at high levels. The precut substrate has the same number and sequence of base pairs as the unnicked substrate. The only difference between these two substrates is the introduction of a transferred strand nick by annealing two oligonucleotides to the nontransferred strand instead of one. The introduction of the nick should have the effect of making the DNA more flexible. Increased flexibility, which could allow the dbb to avoid steric conflict introduced by either of the mutated side chains, is consistent with the observation that PEC formation is unimpaired with the precut substrate for all three of the mutants. The unhindered ability of all three mutants to form PECs with the hairpin and precleaved substrates, which have no dbb DNA, is also consistent with the suggestion that it is steric interactions between the dbb and the mutated residues at positions 97 and 188 that impair PEC formation with the unnicked substrate.

The possibility that the mutated 97 and 188 residues clash sterically with dbb DNA suggests a model in which the dbb DNA of the unnicked substrate normally occupies space that is invaded by the extended side chains in D97E and D188E. Fig. 5 depicts the active site with the three DDE mutations superimposed in red over the naturally occurring residues in green. Tnp is depicted in blue, DNA from the 19-bp terminal repeat is shown as gray bases and purple ribbons, and Mn⁴⁺ ions as dark blue spheres. The glutamate mutations at positions 97 and 188 impair PEC formation with unnicked substrate. All three mutations impair transferred strand nicking, hairpin formation, and hairpin resolution, with D97E showing the most severe inhibition in all three cases.
tion of dbb DNA are also constrained to the space that can be occupied by the longer glutamate side chains but not the shorter aspartate side chains at positions 97 and 188. This indicates that dbb DNA lies directly adjacent to the active site prior to transferred strand nicking.

A relative wealth of structural information is available for Tn5. Recent crystallographic evidence indicates that the DDE residues coordinate two divalent metal ions to the active site (15). The results of the catalytic reactions performed as a part of this study support this two metal model. Although all three mutations to the DDE motif retain the carboxyl that is critical for metal ion coordination, it is reasonable to expect that slightly altering their spatial positions in the active site would decrease the efficiency with which they perform this function, which is what was observed. None of the mutants is as catalytically competent as EK/LP at the nicking, hairpin formation, or hairpin resolution steps. The elimination of function at the nicking and hairpin resolution steps and the relatively more severe reduction of function at the hairpin formation step caused by the D97E mutation supports the model of an active site that coordinates two divalent metal ions.

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REFERENCES