Characterization of Bacteriophage Lambda Excisionase Mutants Defective in DNA Binding

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The bacteriophage λ excisionase (Xis) is a sequence-specific DNA binding protein required for exciscive recombination. Xis binds cooperatively to two DNA sites arranged as direct repeats on the phage DNA. Efficient excision is achieved through a cooperative interaction between Xis and the host-encoded factor for inversion stimulation as well as a cooperative interaction between Xis and integrase. The secondary structure of the Xis protein was predicted to contain a typical amphipathic helix that spans residues 18 to 28. Several mutants, defective in promoting excision in vivo, were isolated with mutations at positions encoding polar amino acids in the putative helix (T. E. Numrych, R. I. Gumport, and J. F. Gardner, EMBO J. 11:3797–3806, 1992). We substituted alanines for the polar amino acids in this region. Mutant proteins with substitutions for polar amino acids in the amino-terminal region of the putative helix exhibited decreased excision in vivo and were defective in DNA binding. In addition, an alanine substitution at glutamic acid 40 also resulted in altered DNA binding. This indicates that the hydrophilic face of the α-helix and the region containing glutamic acid 40 may form the DNA binding surfaces of the Xis protein.

Site-specific recombination by bacteriophage λ is a complex process that requires the formation of nucleoprotein complexes composed of specific DNA sites in the phage and bacterial chromosomes and host- and phage-encoded proteins. Integrative recombination between specific attachment sites, attP and attL, on the bacterial chromosome, generates recombinant attR and attL sites flanking the prophage DNA (14). Excision of the prophage is accomplished by site-specific recombination between the attR and attL sites to regenerate the intact host and phage genomes. Both reactions are catalyzed by the phage-encoded protein integrase (Int), assisted by accessory proteins. The host-encoded integration host factor (IHF) is a protein required for both reactions. Excision requires an additional phage-encoded protein called excisionase (Xis). Excision is stimulated by the factor for inversion stimulation (FIS) supplied by the host. The directionality of recombination is determined by the amount of Xis present in the cell. During prophage induction, enough active Xis is present to promote excision (7, 26). The amount of Xis protein present during establishment of lysogeny is limited due to instability of the protein (26). The integration reaction is inhibited by Xis in vitro (1, 17).

Xis is a sequence-specific DNA binding protein of 72 amino acids (1). Xis recognizes two direct, imperfect 13-bp repeats, designated X1 and X2, on the attR site (see Fig. 1) (28). The FIS binding site, designated F, partly overlaps the X2 site (24, 25). Xis binds DNA cooperatively at the X1 and X2 sites (4) and also binds to X1 cooperatively with FIS at the F site (19, 24). Both Xis and FIS bind DNA upon binding to their specific sites (23). In addition, occupation of X1 by Xis facilitates binding of Int to the P2 site that lies adjacent to the X1 site, presumably through protein-protein interactions (4, 19, 27). The P2 site is a relatively weak Int binding site and is required for excision but not for integration (2, 25).

Xis protein is multifunctional despite its small size. It binds to DNA and interacts with FIS and Int. However, little structural information on the Xis protein is available. Numrych et al. (19) carried out an extensive mutational analysis of Xis and isolated amino acid substitution mutants of Xis with decreased DNA binding affinity in the bacteriophage P22 challenge-phage assays. Their mutations are located in the amino-terminal half of the protein. In contrast, other mutations resulting in defective interaction with Int are at the carboxyl end of the protein (19, 27). No mutants that bound DNA but failed to interact with FIS were isolated. Of the 15 amino acid substitution mutations that decrease DNA binding, 8 change polar residues. Four of these substitutions are clustered between glutamic acid 19 and arginine 26. In addition, another substitution mutant, E40K, showed decreased DNA binding in a challenge-phage assay. Because these polar residues may interact specifically with DNA or FIS, we constructed alanine substitution mutants of each and analyzed their DNA binding properties and their ability to promote excision in vivo.

MATERIALS AND METHODS

Bacterial and phage strains. Escherichia coli strain DH5α [supE44 ΔlacU169 (de80 lacZΔM15) hisD17 recA1 endA1 g69 thi-1 relA1] was used for cloning and purification of variant Xis proteins. BL21(DE3)F−ompT ΔdpsΔIΔrpsLΔrpsN Δmgl (a chs57 ind1 Sam7 ninS lacUV5-T5gene1) dam was used for purification of wild-type His-tagged Xis protein. The E. coli strain LE292 (HfrH argE [Am] rpoB galT::Δ[amt–FII]) and its fis::kan derivative were used for the red colony test. LE292 (rps2::kan) was constructed by generalized transduction using phage P1vir grown on E. coli strain MO (fs::kan) (19). A crude extract containing the wild-type Xis protein was prepared from RJ1529 (fs::kan) harboring pPS2-3ΔRS (19). The challenge phage P22xix2B and its derivatives containing variant X1-X2-F sites were used as templates for preparation of DNA in gel-shift assays (see Fig. 1) (18). As a nonspecific DNA for gel-shift assays, a challenge phage, P22 P’123(II), was used (15).

Media, chemicals, and enzymes. The media and buffers were described previously (19). Antibiotics (Sigma) were added to the media as follows: ampicillin to 50 μg/ml, spectinomycin to 100 μg/ml, and kanamycin to 50 μg/ml. For the red-colony test, timetin (SmithKline Beecham Pharmaceuticals) was used instead of ampicillin at a concentration of 50 μg/ml to prevent the growth of ampicillin-sensitive satellite colonies. Isopropyl-β-D-thiogalactopyranoside (IPTG) was obtained from Sigma and used at the indicated concentrations. T4 DNA ligase, T4 polynucleotide kinase, and restriction endonucleases were obtained from New England Biolabs, Inc. (Beverly, Mass.). Site-directed mutagenesis was performed using the QuikChange kit (Stratagene, La Jolla, Calif.) according to the manufacturer’s instructions.

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from Bethesda Research Laboratories or New England Biolabs. Tag DNA polymerase was obtained from Promega.

**Plasmid constructions.** PCR was used to isolate and amplify the xis gene from phase X DNA. The upstream primer Xis-1 contained an Nhel restriction site preceding the initiation codon of the xis gene, and the downstream primer Xis-2 carried an EcoRI site following the xis translation termination codon (Table 1). PCR was carried out using Taq DNA polymerase in a solution containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, and 200 μM concentrations of each deoxynucleoside triphosphate. The fragment was digested with Nhel and EcoRI and ligated into the plasmid pET28a (Novagen), which was previously prepared with the same set of enzymes. After transformation into E. coli strain DH5α, a clone harboring the insert was confirmed by sequencing (22). The resulting clone, pYL-XisHP, was transformed into E. coli BL21(DE3) for expression and purification of the Xis protein.

For excision assays in vivo, the XbaI-HindIII fragment containing the His-tagged xis gene of the plasmid pYL-XisHP was subcloned into the XbaI-HindIII backbone of the plasmid pCRK101 (15). The resulting plasmid, pYL-XisHR, carried the His-tagged xis gene under control of the P₃₄ promoter.

**Site-directed mutagenesis.** The plasmids containing alanine substitution mutants, pXisE19α, pXisR22α, pXisR23α, pXisR26α, pXisE27α, and pXisE40α, were constructed via site-specific mutagenesis using the modified megaplasmid PCR method (12). Ten picomoles of the mutagenic oligonucleotide and Xis-2 primer (Table 1) were added to the template pYL-XisHR DNA. This reaction was carried out using Taq DNA polymerase with various concentrations of each deoxynucleoside triphosphate. The fragment was digested with EcoRI and cloned into the EcoRI site of the plasmid pYL-XisHR. Each mutation was confirmed by DNA sequencing.

**Expression and purification of His-tagged Xis and its derivatives.** E. coli strains DH5α, harboring derivatives of the pYL-XisHR plasmid, and BL21(DE3), with the pYL-XisHP plasmid, were used for expression of the Xis mutants and the wild-type Xis protein, respectively. Cells were grown to mid-log phase in Luria-Bertani medium containing the appropriate antibiotics. Production of His-tagged proteins was induced by adding IPTG to a final concentration of 1 mM, followed by growth for 3 h at 37°C. After centrifugation, harvested cells were disrupted by treatment in a French pressure cell in 50 mM sodium phosphate buffer, pH 7.4, with 300 mM NaCl. Sonication was used to disrupt the bacterial cell wall. The cleared sonic extract was used as a source of labeled, nonspecific DNA. A pair of synthetic oligodeoxynucleotides, Omnt and α-Omnt (Table 1), were used as primers for amplification. They were annealed to the sequences encompassing the Xis binding sites and mutant derivatives. The amplified short fragments containing desired base-pair changes, which in turn encodes a His tag at the amino terminus of the protein. The xis gene under control of the P₃₄ promoter.

**Excision assay.** The red- colony test (11) was used to assay the ability of Xis derivatives to promote excisive recombination in vivo. The galT gene of E. coli strain LE292 contains a λ prophage that lacks the int and xis genes. When the Int and Xis proteins are supplied from plasmids, the inserted prophage is excised and excised recombinant plasmid is purified for analysis.

**Secondary structure predictions.** The secondary structure of the Xis protein was predicted using various algorithms available at the Network Protein Structure Database.

**RESULTS AND DISCUSSION**

**His-tagged Xis retains the binding specificity and functions of wild-type Xis.** In order to simplify the purification of Xis and its mutant derivatives, we constructed a modified gene that encodes a His tag at the amino terminus of the protein. The xis gene was then amplified by PCR using phase P2x2x2B or its derivatives as templates (Table 1). The phage P2x2x2B (12), which contains the att site, and the pET-15b vector were used for expression of Xis under the control of the P₃₄ promoter. His-tagged Xis retains the binding specificity and functions of wild-type Xis.

**FIG. 1.** The attR site of bacteriophage λ and nucleotide sequences of wild-type and variant Xis binding sites. The two arm-type binding sites, P1 and P2, and the two core-type sites, C and B', on the attR region are recognized by Int. Fig. 1A depicts the att site and the attR region. The illustration of the mutant Xis binding sites 2B10, 2B29, and 2B22 has been described by Nummrich et al. (18). Deletion of the indicated base.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence*</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xis-1</td>
<td>d(CTAGGCTGACCTGATCGTCCGATTCGAGG)</td>
<td>Upstream primer for xis</td>
</tr>
<tr>
<td>Xis-2</td>
<td>d(CTAGGATCTCTGATCTTTCCTTCTCCAT)T</td>
<td>Downstream primer for xis</td>
</tr>
<tr>
<td>PCKR-p</td>
<td>d(CTAGGATTCTCTGATCTTTCCTTCTCCAT)</td>
<td>Upstream primer for mutagenesis</td>
</tr>
<tr>
<td>E19α</td>
<td>d(AGAAGCTTTGACCACTTGTCG)</td>
<td>Mutagenic primer for E19α</td>
</tr>
<tr>
<td>R22A</td>
<td>d(TGAACAGTCGTCACGATCT)</td>
<td>Mutagenic primer for R22A</td>
</tr>
<tr>
<td>R23A</td>
<td>d(AAACAGTTCGTCACGATCT)</td>
<td>Mutagenic primer for R23A</td>
</tr>
<tr>
<td>R26A</td>
<td>d(TCGAAGTCGTCACGATCT)</td>
<td>Mutagenic primer for R26A</td>
</tr>
<tr>
<td>R27A</td>
<td>d(TGCTGGTCGTCACGATCT)</td>
<td>Mutagenic primer for R27A</td>
</tr>
<tr>
<td>E40A</td>
<td>d(GATGATTTGACCACTTGTCG)</td>
<td>Mutagenic primer for E40A</td>
</tr>
<tr>
<td>Omnt</td>
<td>d(GGGATCCCTTACGATCG)</td>
<td>Used in synthesizing gel-shift DNA</td>
</tr>
<tr>
<td>α-Omnt</td>
<td>d(GATGATTTGACCACTTGTCG)</td>
<td>Used in synthesizing gel-shift DNA</td>
</tr>
</tbody>
</table>

* Underlined sequences are complementary to the xis gene. Bold-faced sequences represent the alanine codons.
gene from phage λ was amplified and cloned into the 

Nei-I EcoR1 backbone of plasmid pET28a as described in Materials and Methods. The resulting clone, pYL-XisHP, was trans- 

formed into E. coli BL21(DE3) for expression and subsequent purification of the His-tagged Xis. After isolating the wild-type His-tagged protein, we analyzed its sequence-specific DNA binding properties in vitro by gel-shift assays. The DNA frag-

ments used in the binding reactions contained either the wild-

type X1-X2-F sites or the variant sites shown in Fig. 1. The 

mutant site 2B10 carries a 4-bp deletion in the X2 site. The other two variant sites, 2B29 and 2B22, have a single-nucleo-

tide substitution in X1 and X2, respectively. Variants were 

isolated as mutants defective for Xis binding using the chal-

lenge-phage system (18).

The purified wild-type His-tagged Xis bound DNA containing 

the wild-type X1-X2-F sites (Fig. 2A, lanes 2 and 3). Addition of FIS to the reaction facilitated binding of His-tagged Xis to its binding site, indicating that His-tagged Xis interacts cooperatively with FIS in DNA binding (Fig. 2A, lanes 5 and 6). Similar results showing weak cooperativity between FIS and Xis have been reported previously (19, 25). The Xis protein did not bind the deletion variant site 2B10, but FIS protein shifted 

DNA fragments containing the variant 2B10 (Fig. 2A, lanes 7 to 

12). The mutant site 2B29 was bound by His-tagged Xis but with lower affinity than the wild-type site (Fig. 2A, lanes 13 to 

18). Mutant 2B22 was bound only when FIS was present (Fig. 2A, lanes 19 to 24).

The binding patterns of the fusion protein were the same as 

those of the wild-type Xis protein without the His tag (Fig. 2B), 

showing that the positively charged His tag was not affecting 

binding. When crude extracts containing the wild-type Xis pro-

tein were added to DNA with Xis binding sites, Xis-DNA complexes were detected only with the wild-type binding site, 

not with the mutant binding sites 2B29 and 2B22 (Fig. 2B, 

lanes 1, 4, and 7). If FIS protein was supplied to the reaction, 

Xis-FIS-DNA complexes were detected in the reactions with 

all three Xis binding sequences. However, the amounts of 

ternary complexes formed with the variant sites, 2B29 and 

2B22, were significantly less than with wild-type DNA (Fig. 2B, 

lanes 3, 6, and 9). Thus, the His-tagged Xis protein binds 

specifically to its binding sites and interacts cooperatively with 

the FIS protein.

To measure the recombination activity of the protein in vivo, 

DNA encoding the His-tagged xis gene was subcloned down-

stream of the Pm promoter as described in Materials and 

Methods. The resultant plasmid, pYL-XisHR, was trans-

formed into LE292 containing the Int-producing plasmid 

pIntB1 (19). Excision was assayed by the red-colony test (11).

The Xis protein produced from the plasmid pYL-XisHR func-

tioned as efficiently as the wild-type Xis protein without the 

His tag in the excision reaction. Colonies turned red within 

24 h on MacConkey-galactose plates containing 1 mM IPTG. 

It took another 12 h if the host carried a defective fis gene. 

Taken together with the gel-shift data, this led us to conclude 

that the His tag does not significantly affect the functions of Xis 

protein, including DNA binding and cooperative interactions 

with FIS. All the following data were obtained using His-

tagged versions of Xis.

**Amino acid residues from Leu 18 to Glu 27 may form an**

**amphipathic α-helix.** The carboxyl-terminal region of Xis is 

required for cooperative binding of Int, presumably through 

protein-protein interactions (19, 27). A nonsense mutant was 

isolated that encodes an Xis protein containing the amino-

terminal 53 amino acids. It bound to DNA containing the 

X1-X2-F sites and interacted cooperatively with the FIS pro-

tein (19). The precise regions of Xis involved in DNA binding or 

FIS interaction were not localized. Secondary structure pre-

diction algorithms indicated that three regions of the Xis pro-

tein could form α-helices (5, 8, 9, 10, 13, 16, 20). Amino acids 

from residues 5 to 10 were predicted to form the first helix, and 

the region spanning leucine 18 to glutamic acid 27 was pre-

dicted to form the second helix. The third postulated helix was 

in the region proposed by Numrych et al. (19) to be involved in 

cooperative interactions with Int. However, Xis lacked recog-

nizable DNA binding motifs, for example, a helix-turn-helix 

motif (6). We observed that although Xis does not form a 

canonical helix-turn-helix motif, the second helical region 

could form a typical amphipathic helix (Fig. 3). Furthermore,
Numrych et al. (19) found that several substitutions for the hydrophilic residues in this region resulted in a loss of Xis function in vivo. Those substitutions included the changing of glutamic acid 19 to a lysine, arginine 22 to a histidine, arginine 23 to a glutamine, and arginine 26 to a tryptophan. Those results are consistent with the hypothesis that this region forms an \( \alpha \)-helix and the surface-exposed hydrophilic residues may be in direct contact with DNA or FIS.

Substitutions for polar residues in the putative amphipathic helix change DNA binding. To gain more information on the function of each of the polar side chains on the putative amphipathic helix, we constructed alanine substitution mutants of the hydrophilic residues. Glutamic acid 19, arginines 23 and 26, and glutamic acid 27 were individually replaced with an alanine residue. The proteins were designated E19A, R23A, R26A, and E27A, respectively. We also constructed a mutant with an alanine substitution at position 22, but we could not detect the protein on a sodium dodecyl sulfate-polyacrylamide gel. This mutant was not analyzed further. Each of the Xis variants was tested for DNA binding and ability to promote excision in vivo. Gel-shift assays were performed using partially purified proteins as described in Materials and Methods.

The mutant E27A protein bound DNA containing the X1-X2-F sites with an affinity similar to that of the wild-type protein. It also discriminated between the wild-type and variant Xis binding sites as did the wild-type protein (data not shown). Variants R23A and R26A, however, failed to form specific complexes with DNA in the absence of the FIS protein, even when 50-fold more protein was added to the reactions than in reactions using the wild-type Xis. Mutants R23A and R26A formed a complex with DNA containing wild-type Xis binding sites only in the presence of FIS (Fig. 4A). However, they failed to bind the variant site 2B10 under the same conditions (Fig. 4B). These results indicate that although the latter two mutants have decreased DNA binding affinity, they retain the ability to interact with the FIS protein. They bind to the specific Xis binding site only when FIS is present.

Another variant, E19A, bound DNA differently. The E19A protein did not form a discrete complex with DNA containing the X1-X2-F sites as the wild-type protein did. Instead, it formed complexes that migrated slowly only at high protein concentrations (Fig. 5B, lane 1). Multiple, discrete complexes were formed when the protein was present at lower concentrations (Fig. 5B, lanes 2 and 3). We suggest that the rapidly migrating bands are complexes with the mutant protein bound nonspecifically to DNA. To test if the E19A protein could bind to a sequence lacking the Xis or FIS binding sites, we used DNA containing the \( \lambda \) Int arm-type binding sites of \( \lambda \). As shown in Fig. 5B, lanes 4 to 6, the E19A protein also formed multiple discrete complexes with nonspecific DNA. We interpret the multiple bands to be nonspecific complexes with various amounts of the Xis E19A protein bound to a single DNA fragment. We note that the binding affinity of E19A for nonspecific DNA was greater than that of the wild-type Xis (Fig. 5). At a 31 nM concentration, wild-type Xis formed neither specific nor nonspecific complexes (Fig. 5A, lanes 3 and 7). Specific complexes of the wild-type Xis with DNA fragments containing the Xis binding site were detected when 125 nM protein or more was added (Fig. 5A, lanes 1 and 2). Under the same conditions (125 and 500 nM Xis), small amounts of nonspecific complexes were also detected (Fig. 5A, lanes 1, 2, 5, and 6). In contrast, the E19A protein bound nonspecifically to DNA at a concentration of 31 nM (Fig. 5B, lanes 3 and 7).

The ability of each mutant to promote the excision reaction of \( \lambda \) in vivo was assessed by the red-colony test. The results are shown in Table 2. The E27A protein promoted excision as

![FIG. 3. Helical wheel projection of the putative \( \alpha \)-helix spanning amino acid residues 18 to 27 in Xis. The residues named in the boxes are the amino acids for which substitutions resulted in defective excision in vivo (19).](http://as.asm.org/)

![FIG. 4. DNA binding of XisR23A and XisR26A. The final concentration of wild-type Xis was 0.1 \( \mu \)M, and that of the mutant Xis proteins was 5 \( \mu \)M. Each reaction contained 2.5 nM labeled DNA and 50 ng of sonicated calf thymus DNA. Where indicated, 6.3 ng of FIS was present. Electrophoresis was performed at 4°C.](http://as.asm.org/)
efficiently as the wild-type protein when expression of proteins was induced by IPTG at a concentration of 1 mM. Two variants, R23A and R26A, could excise DNA only in the presence of FIS but not as well as the wild type did. These results are consistent with the gel-shift data. It was surprising to find that the E19A protein also promoted excision, albeit with lower efficiency than the wild-type Xis. One explanation for this result is that although the E19A protein binds to nonspecific DNA significantly better than the wild-type Xis, it might continue to form a bent DNA complex when bound to the specific site, thereby stimulating excision. The fact that the E19A protein also promotes excision better in the presence of the FIS protein also indicates that it interacts cooperatively with the FIS protein. Thus, with help of FIS, the E19A protein may be effectively recruited to the Xis binding site to bend DNA and to promote the excision reaction.

**Mutant with a substitution of an alanine for glutamic acid 40 binds to nonspecific DNA.** Numrych et al. (19) also isolated a mutant in which glutamic acid 40 was replaced by a lysine. To study the function of this residue in DNA recognition in more detail, we made a His-tagged construct of it. Gel-shift assays showed that the E40A protein, like the E19A protein, bound to both specific and nonspecific DNA fragments (Fig. 5C, lanes 5 to 8). However, the binding pattern for the fragment with specific sequences was different from that for the fragment with nonspecific DNA sequences (Fig. 5C). Although we do not understand the cause of the difference, it might indicate that the E40A protein distinguishes the specific Xis binding sites from random DNA to some extent. The fact that the E40A mutant promoted excision and showed cooperativity with FIS (Table 2) supports this hypothesis. As discussed above for the E19A protein, the E40A protein may also bend DNA when bound to a specific Xis binding site and provides a functional substrate for excision. However, the sequence specificity of the wild-type Xis was significantly relaxed by the substitution for glutamic acid 40, indicating that this residue may also participate, either directly or indirectly, in sequence-specific DNA recognition.

In summary, we constructed mutant Xis proteins with alanine substitutions of polar residues on the putative amphipathic α-helix and glutamic acid 40. Three of the four alanine substitutions, E19A, R23A, and R26A, altered the DNA binding patterns of Xis. One substitution, E27A, which resulted in a change at the carboxyl end of the helix, did not change the DNA binding specificity of Xis. This behavior is consistent with the hypothesis that Xis forms an amphipathic α-helix from leucine 18 to glutamic acid 27, although the latter amino acid residue may not interact with DNA. This study suggests that the amino-terminal, hydrophilic face of the amphiphatic helix may be in close contact with DNA. In particular, glutamic acid 19 appears to play a role, direct or indirect, in conferring DNA binding specificity, and arginines at positions 23 and 26 are required to bind to DNA with high affinity. The finding that a substitution for glutamic acid 40 also increased binding affinity to nonspecific DNA suggests that the region containing glu-
tamic acid 40 may form an additional DNA binding surface on the Xis protein.

We note that the putative helical region from amino acid 18 to 28 and the region carrying glutamic acid 40 are separated by an unusual amino acid sequence containing three consecutive prolines. Thus, the proline residues may play a role in positioning the flanking amino acid residues in a conformation that allows them to interact simultaneously with DNA. We look forward to comparing our analysis to the emerging structural studies. The combination of the two approaches may reveal the exact nature of the functional interactions between the amino acids and binding-site DNA that lead to excision.

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