Protein–protein interaction between monomers of coliphage HK022 excisionase

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Abstract **Excisionase (Xis) is an accessory protein that is required for the site-specific excision reaction of the coliphages HK022 and λ. Xis binds in a strong cooperative manner to two tandem binding sites (X1 and X2) located on the P arm of the attachment (*att*) sites on the phage genome. As a result of crosslinking experiments in vivo and in vitro of Xis-overexpressing cells, by gel filtration of purified Xis and by FRET analyses we show that Xis monomers of HK022 interact and form dimers that are not dependent on the single Cys residue of the protein and on the presence of DNA. The formation of the dimers may explain the strong binding cooperativity of Xis to its sites on DNA.**

Keywords: Bacteriophage HK022; Excisionase; Protein–protein interaction; Site-specific recombination

1. Introduction

The integration of coliphages HK022 into the genome of its host (*Escherichia coli*) results from a site-specific recombination reaction between the *attP* site located on the circular phage chromosome and the *attB* site located on the host chromosome, a mechanism that is identical to that of coliphage λ. In both phages *attB* is small, composed of a core that is 21 base pairs (bp) long whose central 7 bp (the overlap) are the site of the DNA exchange. *attP* is much longer, it is composed of a similar core and two arms (P and P′) that carry binding sites for the catalytic Integrase (Int) recombinase and for accessory proteins that are required for the reaction. The integration of either phage leads to the formation of the prophage that is flanked by the recombinant sites *attL* and *attR*, which are the substrates of the reverse excision reaction. Integration (*attP* × *attB*) and the reverse excision reaction (*attL* × *attR*) are catalyzed by the phage-encoded Int recombinase with the assistance of the host-encoded accessory DNA-bending protein integration host factor (IHF). However, excision also requires the presence of the phage-encoded excisionase (Xis) protein, an additional DNA bending accessory protein [1] that belongs to a family of proteins known as the recombination directionality factors (RDF) [2]. The factor for inversion stimulation (FIS) is a third accessory protein that is host-encoded and in λ it has been shown that it can replace Xis when the latter is in a low concentration (reviewed in [3,4]). Though λ and HK022 share an identical mechanism of site-specific recombination, the Int proteins of the two phages show a partial homology and each Int is specific in recognizing its own *att* sites. In contrast, the Xis proteins of the two phages are identical except for one amino acid difference such that each Xis protein can be used by either phage [5]. Xis is composed of 72 amino acids and it binds to two sites, each of 13 bp (X1, X2), located in tandem on the P arm of *attP* and *attR*. The protein carries two domains, an amino-terminal DNA binding domain and a carboxyl-terminal domain that interacts with Int. In either phage, the binding nature of Xis to its two adjacent binding sites (X1, X2) shows an extremely strong cooperativity, suggesting that oligomers of Xis may bind directly to both sites [6,7]. The structure of the N-terminal domain of Xis-λ (residues 1–55) bound to its X2 DNA binding site has been resolved, whereas its C-terminal domain (residues 56–72) that is responsible for the Xis–Int interaction remained disordered [8]. The structure of the N-terminal domain has revealed a complex of two proteins, one that interacts with the X2 binding site and the other not-specifically bound. Such a structure may result from a protein–protein interaction between two Xis monomers [8]. In the present communication, we show that intact Xis molecules of HK022 interact to form dimers in solution as well as within the bacterial cells. The dimerization of Xis occurs independently of its DNA binding sites.

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Abbreviations: β-ME, β-mercaptoethanol; bp, base pairs; DSG, disuccinimidyl glutarate; DSS, disuccinimidyl suberate; FITC, fluorescein-5-isothiocyanate; TRITC, tetramethylrhodamine-5- (and 6) isothiocyanate; FRET, fluorescence resonance energy transfer; IHF, integration host factor; Int, integrase; IPTG, isopropyl β-D-thiogalactopyranoside; PBS, phosphate buffer saline; SDS–PAGE, polyacrylamide gel electorphoresis in the presence of sodium dodecyl sulphate; RDF, recombination directionality factor; Xis, excisionase

2. Materials and methods

2.1. Bacterial strains and plasmids

Escherichia coli strain BL21(DE3) pLys [9] was used to overexpress Xis that was cloned under the T7 promoter. Plasmid pPG1 carries xis of HK022 cloned in pETI1 [10]. Plasmid pPG15 caries the His-tagged *xis* of HK022 clone in pET14m [7]. Plasmid pPG123 carries the substrate of Xis attR-t1t2-attL cloned in pBluescript [7]. pPG165 carries the His-tagged *xis* C28S mutant cloned in pET14m [11].

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2.2. Protein purification

Xis was purified as described [11] from an IPTG-induced culture of strain BL(DE3)(pLys) transformed with plasmids pPG15 or pPG165.

2.3. Cross-linking experiments

For in vivo crosslinking experiments 1 ml cultures of strain BL21(DE3), transformed with plasmid pPG1, pPG15 or pPG165, were induced at the logarithmic growth phase with 1mM IPTG and pelleted after 2 h. The pellet was resuspended in 20 μl of a phosphate buffer saline (PBS) containing 2 mM disuccinimidyl suberate (DSS) at room temperature. The controls were resuspended in PBS buffer without DSS. After 30 min the cells were centrifuged and resuspended in 30 μl containing loading buffer (1 M Tris, 5% glycerol, 4% SDS and 5% bromphenol) that quenches the reaction (Pierce). β-Mercaptoethanol was added to the relevant samples to a final concentration of 2 mM and the samples were boiled for 3 min. Aliquots were subjected to 15% SDS–PAGE and the protein was detected by a Western blot.

For in vitro crosslinking experiments, the cells were resuspended in PBS buffer sonicated and centrifuged at 4 °C. A 20 μl sample of the supernatant was treated with 2 mM DSS for 30 min at room temperature. In the control samples, DSS was omitted. Loading buffer was added to a final concentration as above and the samples were subjected to 15% SDS–PAGE and a Western blot.

For crosslinking experiments of the purified Xis, the purified protein was dialyzed against PBS and 20 μl samples (10 μg) were treated with 2 mM DSS for 30 min at room temperature. SDS–PAGE and the Western blot were as above.

2.4. Immunoblots

These were done as previously described [12].

2.5. Gel filtration chromatography

Seven milligrams of purified Xis was loaded on a Superdex 75 PC column (Pharmacia Biothech Inc.) that was equilibrated in 50 mM Tris buffer containing 200 mM NaCl, 2 mM DTT and calibrated with Ovalbumin (45 kDa), Cytochrome *C* (12.4 kDa) and Bacitracin (1.45 kDa). Half ml fractions were collected, samples of 30 μl of each fraction were separated on 15% SDS–PAGE and the protein was detected by a Western blot.

2.6. In vitro excision assay

The reaction mixture (10 μl) was as previously described [7] using plasmid pPG123 as substrate. Reaction products were cut by the restriction enzymes *Xho*I and the restriction was inactivated for 10 min at 75 °C. The products were separated on 1% agarose gel and stained with ethidium-bromide.

2.7. Fluorescence resonance energy transfer (FRET) analysis

950 μl of purified and dialyzed Xis protein (2 mg/ml) was conjugated with 50 μl (1 mg/ml) of the fluorescence probe fluorescein-5-isothiocyanate (FITC) and another 950 μl of Xis was conjugated with 50 μl (2 mg/ml) tetramethylrhodamine-5- (and 6-) isothiocyanate (TRITC). Protein dialysis and the conjugation procedure were as described previously [11]. The degree of labeling was 30% for both Xis-FITC and Xis TRITC.

3. Results

3.1. Formation of Xis dimers in vitro

We have noticed on a denaturing SDS–PAGE gel that purified Xis tends to form with time dimers that become the dominant species after a few days. These dimers result from the oxidation of the single Cys28 residue in the monomers forming a Cys–Cys bridge between two monomers because they are completely resolved when treated with a reducing agent (data not shown). However, due to the reducing nature of the cellular milieu [13] it is unlikely that such dimers are formed within the cell.

To test the existence of another form of Xis oligomers, we treated a fresh preparation of purified His-tagged Xis with disuccinimidyl suberate (DSS; Pierce), a crosslinking agent between proteins. DSS is a bifunctional *N*-hydroxysuccimide ester that reacts with side chain amines. The DSS-treated protein was run on an SDS–PAGE that subsequently underwent an immunoblot using antiserum raised against purified Xis (Fig. 1). In the presence of β-mercaptoethanol, the purified protein shows the formation of cross linked dimers as well as a higher oligomer whose size agrees with that of tetramers. The band that is seen in all lanes is an unknown non-specific protein that reacts with the polyclonal antibodies raised against Xis and co-purifies with it.

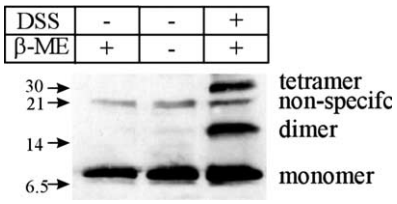

Fig. 1. Western blot of crosslinked purified Xis protein. β-ME.

To confirm that Xis forms dimers, we exposed freshly purified protein to gel filtration in the presence of the reducing agent DTT (Fig. 2A). Two peaks were eluted, whose molecular weights correspond to monomers and dimers of Xis. An immunoblot of the eluted fractions shows that these two peaks cross-react with the Xis antiserum (Fig. 2B). An Xis activity assay of the fractions of each peak and the fraction between them (Fig. 2C) indicates that the protein in each of the two peaks is active. A gel filtration of the C28S mutant protein showed a profile that was similar to that of the wild-type protein (not shown).

3.2. Formation of Xis-dimers in vivo

Because cells are permeable to DSS, this crosslinker can be used to monitor protein–protein interactions within the cells cells [14,15]. Strains that overexpress the native Xis and a His-tagged Xis were induced (with IPTG) and were treated with DSS. Proteins extracted from the cells were run on an SDS–PAGE acrylamide gel and the subsequent immunoblot shows that only the induced cells that were treated with DSS have revealed the presence of Xis dimers (Fig. 3). These results indicate that a part of the overexpressed Xis can be trapped within the cells in a dimeric form that is not a Cys–Cys dimer. The formation of the dimers in Fig. 3B is not due to the presence of the His-tag because dimers are also evident in cells that overexpress an untagged native protein (Fig. 3A). The covalent dimers appear more extensively when the cells are broken open prior to the treatment with the cross linker (Fig. 3C).

To verify that the single Cys residue of Xis does not play any role in the intracellular Xis–Xis interaction, we repeated the experiments shown in Fig. 3 using a strain that overexpresses an Xis mutant whose single Cys residue was replaced by Ser. Fig. 4 shows that despite the C28S mutation, dimers and tetramers are formed as in the case of the wild-type protein. Thus, the single Cys residue is not involved in the formation of these oligomers.

3.3. Protein–protein interaction between Xis molecules

To confirm that Xis molecules interact with each other in solution in the absence of DNA, we performed FRET assays using a mixture of purified Xis conjugated with FITC and purified Xis conjugated with TRITC. A significant FRET result is consistent with a direct physical interaction between proteins that are in proximity of less than 100 Å apart [16]. An

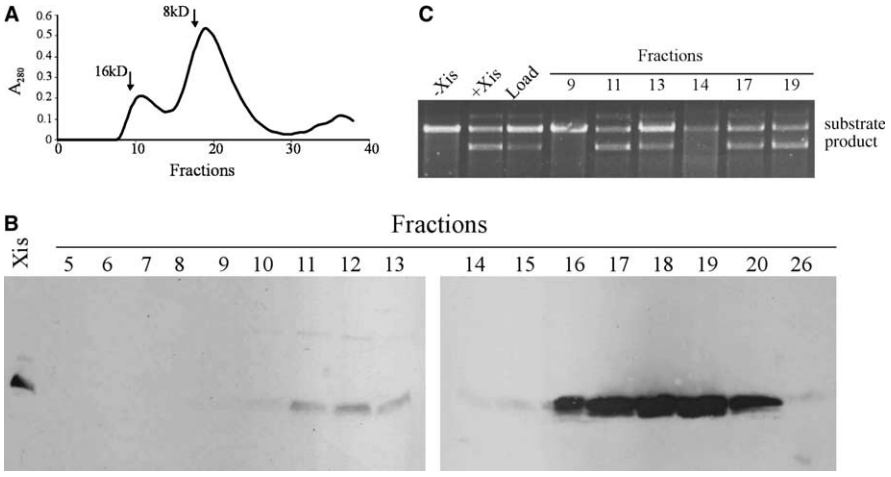

Fig. 2. (A) Spectophotometric profile of purified wild type Xis separated by gel filtration. The arrows indicate the position of molecular weight markers (see 2). (B) Western blot of the different fractions eluted from the gel filtration column. (C) Xis activity of indicated fractions. −Xis, reaction in the absence of enzyme showing only the substrate. +Xis, full reaction using purified Xis.

equimolar (2 μM) mixture of purified FITC-conjugated wild-type Xis with TRITC-conjugated wild-type Xis (excitation/emission wavelengths of 490/518 and 550/580, respectively, with a spectral overlap of an R_0 value of about 55 Å [17]) was subjected to excitation of the Xis-FITC molecules at 490 nM and the emission of the Xis-TRITC molecule at 580 nm as a result of energy transfer was recorded (Fig. 5A). At different times a decline in the emission of the labeled Xis-FITC at 518 nm was coupled with a corresponding increase in the emission of Xis-TRITC at 580 nm, showing the transformed energy from the FITC-conjugated Xis molecules to the TRITC-conjugated ones and indicating a very close proximity of Xis molecules as a result of Xis–Xis interactions. Similar results were obtained with the C28S mutant protein (Fig. 5B).

4. Discussion

The P arm of the attachment sites *attP* and *attR* of phages HK022 and λ carry two adjacent, tandem and similar binding sites (X1, X2) that are not completely identical between the two phages. Nevertheless, both phages code for practically identical Xis proteins that can recognize the sites of either phage [5]. Gel retardation experiments with *attP*-λ sites [6] and with *attR*-HK022 sites [7] have shown that at the lowest amount of added Xis at which DNA retardation could be observed, the major DNA–protein complex was the saturated one, i.e., at a low concentration of Xis, the DNA fragment became complexed at both binding sites. This was interpreted as a strong cooperativity, such that Xis, first bound to X1 efficiently stimulates binding to X2 [7]. An alternative possibility is that preformed dimers can bind directly to both sites [6]. The results presented in this work support the alternative possibility because it provides evidence that non-covalent Xis dimers exist in the intact cells as well as in the preparation of the purified protein. The interaction between Xis monomers in the formation of dimers became evident from three independent analyses: crosslinking, gel filtration and FRET. The dimerization is independent of the single Cys residue of the protein and is also independent of the presence of the DNA that carries the Xis-binding sites. In the crosslinking experiments in which the relative yield of the dimers was high, we have also observed a cross-reacting band whose size is in agreement with a tetrameric form (Figs. 1 and 4B and C). Since the two binding sites of Xis (X1 and X2) are arranged in tandem, it is reasonable to assume that the bound dimers are also in tandem orientation, in which case tetramers can also be formed by the interaction of two dimers. Similar results were

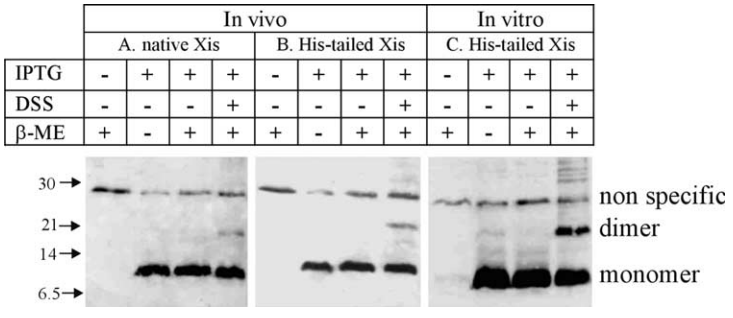

Fig. 3. Western blot of induced and uninduced Xis-expressing strains. (A) and (B) are in vivo crosslinking experiments performed with a strain that carries plasmids pPG1 and pPG15, respectively. (C) is an in vitro crosslinking experiment performed with a cell extract of strain that carries plasmid pPG15. The cells were induced with 1 mM IPTG and the samples in the different lanes were treated with 2 mM DSS and 2 mM β-mercaptoethanol as indicated in the above table. Arrows on left indicate molecular weight markers.

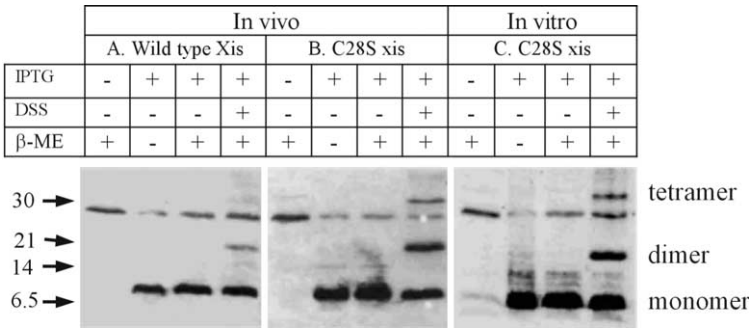

Fig. 4. Western blot of induced and uninduced Xis-expressing strains. (A) and (B) are in vivo crosslinking experiments performed with the wild-type strains that express the wild-type Xis (pPG15) and the mutant Xis (pPG165), respectively. (C) is an in vitro crosslinking experiment performed with the mutant strain. The treatments were as in Fig. 3.

obtained when we used discuccinimidyl glutarate (DSG), a crosslinker that is similar to DSS but is shorter (not shown).

The results of the experiments reported above cannot prove whether dimers are an ultimate requirement for the activity of Xis. The activity of the monomeric as well as the dimeric fractions shown in Fig. 2C does not exclude that each of these fractions contains both forms. In any case, the results indicate the existence of self-recognition sites within the protein. In agreement, the crystal structure of the DNA–protein complex shows that two N-terminal subunits of Xis interact in a tandem arrangement, and it has been suggested that two monomers interact via the α1 helix of one of them with the N-terminus of the second [8]. The dimerization assays presented above may help to test this hypothesis.

Finally, it must be taken into account that all the experiments reported here were performed either with cells that overexpress Xis or with the purified protein. Confirmation that these dimers play a biological role in excision must come either from in vitro experiments with purified dimers and monomers or from experiments with induced lysogenic cells.

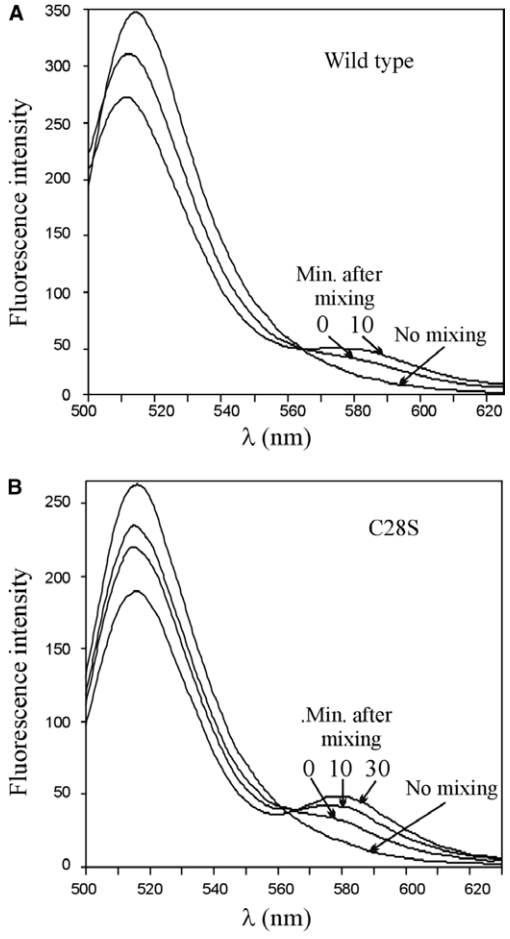

Fig. 5. Xis–Xis interaction assayed by FRET analyises. (A) Wild-type Xis. (B) C28S mutant Xis.

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