Mutational Analysis of the Archaeal Tyrosine Recombinase SSV1 Integrase Suggests a Mechanism of DNA Cleavage in \textit{trans}*

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The only tyrosine recombinase so far studied in archaea, the SSV1 integrase, harbors several changes in the canonical residues forming the catalytic pocket of this family of recombinases. This raised the possibility of a different mechanism for archaeal tyrosine recombinase. The residues of IntSSV tentatively involved in catalysis were modified by site-directed mutagenesis, and the properties of the corresponding mutants were studied. The results show that all of the targeted residues are important for activity, suggesting that the archaeal integrase uses a mechanism similar to that of bacterial or eukaryotic tyrosine recombinases. In addition, we show that IntSSV exhibits a type IB topoisomerase activity because it is able to relax both positive and negative supercoils. Interestingly, \textit{in vitro} complementation experiments between the inactive integrase mutant Y314F and all other inactive mutants restore in all cases enzymatic activity. This suggests that, as for the yeast Flp recombinase, the active site is assembled by the interaction of the tyrosine from one monomer with the other residues from another monomer. The shared active site paradigm of the eukaryotic FLP protein may therefore be extended to the archaeal tyrosine recombinase IntSSV.

Tyrosine recombinases form a large family of site-specific recombinases comprising more than 150 members, most of which were identified on the basis of sequence similarities (1, 2). Within this family, several subfamilies can be defined such as the \textit{\lambda}-phage integrase family, the Xer recombinases family, or the yeast plasmid recombinases family (1). The hallmark of tyrosine recombinases is the conservation of six noncontiguous residues: Arg\textsubscript{G}, Lys\textsubscript{p}, His\textsubscript{p}, Arg\textsubscript{G}, His/Trp, Tyr (Table I). This motif is directly involved in catalysis of DNA strand cleavage and strand exchange (for review, see Ref. 3). Five of the six residues are located within the highly conserved boxes I and II found in tyrosine recombinases (1, 4, 5), whereas the sixth residue, Lys\textsubscript{p}, was identified by alignments with the eukaryotic topoisomerases IB (6). Two different structural organizations of this motif have been described from crystallographic data. In prokaryotic tyrosine recombinases XerD (7), Cre (8), HP1 integrase (9), and \lambda-Int (10, 11), the six active site residues come from a single monomer, whereas the eukaryotic FLP recombinase presents a shared active site, where the catalytic tyrosine is provided by one monomer, and the five other residues are from another monomer (12). In this latter case, the active site is created by dimer association. As a consequence of this organization, Flp realizes \textit{trans} cleavage (13, 14), whereas prokaryotic recombinases act in \textit{cis} (8, 9, 15–17). \textit{Cis} cleavage is the result of \textit{cis} activation/\textit{cis} cleavage where the tyrosine of the bound monomer attacks the nearby activated phosphate. In \textit{trans} cleavage, binding of a monomer to its site leads to activation of the adjacent phosphodiester that will be attacked by a nucleophile (here a tyrosine) provided in \textit{trans} by a partner monomer, a mechanism that can be described as \textit{cis} activation/\textit{trans} cleavage. In both cases the chemistry of the reaction is conserved and is similar to that used by topoisomerases IB (18). The Arg\textsubscript{G}, His\textsubscript{p}, and Arg\textsubscript{G} side chains coordinate the scissile phosphate, activating it for nucleophilic attack by the tyrosine and stabilizing the resulting transient penta-coordinated phosphate. In some proteins, the His/Trp side chain forms a hydrogen bond to the nonbridging oxygen of the scissile phosphate (19), whereas in Flp this residue is more likely involved in protein-protein interactions (20). The Lys\textsubscript{p} residue is critical for activity of topoisomerases IB and tyrosine recombinases (6, 21, 22). Enzymatic analysis of vaccinia topoisomerase IB mutants revealed that this residue is the general acid catalyst that protonates the 5’-oxygen of the leaving strand (21). Crystal structures of Cre (8), Flp (12), \lambda-Int (10), and human topoisomerase IB (19) reveal that this residue contacts the base adjacent to the cleavage site in the minor groove. Other structural data show that Lys\textsubscript{p} is located on a loop displaying a high conformational flexibility (9, 23). Therefore the Lys\textsubscript{p} residue was proposed to serve similarly as a general acid in the reaction mechanism catalyzed by tyrosine recombinases (21).

So far, the only studied archaeal member of the tyrosine recombinases family is the SSV1 integrase (Int\textsubscript{SSV})\textsuperscript{1} encoded by SSV1, a virus of the extremely thermophilic archaeon \textit{Sulfolobus shibatae}. Int\textsubscript{SSV} catalyzes the site-specific integration of the viral DNA into the host chromosome using viral and chromosomal attachment sites attP and att\textsubscript{F} (24, 25). In a previous work we have shown that Int\textsubscript{SSV} exhibits a cleavage mechanism dependent on Tyr\textsubscript{314}, leading to the formation of a 3’-phosphoprotein intermediate like other tyrosine recombinases (26). However, Int\textsubscript{SSV} harbors substitutions at several conserved positions (Fig. 1 and Ref. 2) and cannot be classified phylogenetically in any subgroup of the tyrosine recombinases family. Whether the recombination reaction catalyzed by Int\textsubscript{SSV} would follow the general mechanism described for tyrosine recombinases or would be different in archaea remained an open question (27). To characterize further the site-specific recombination mechanism in archaea we have generated Int\textsubscript{SSV} mutants and analyzed their enzymatic properties. Eight

\textsuperscript{1} The abbreviations used are: Int\textsubscript{SSV}, SSV1 integrase; BSA, bovine serum albumin; LMC, low mobility complex.

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positions were targeted, including the putative six conserved catalytic residues identified by sequence alignments (Fig. 1). In the absence of a full recombination assay available in vitro, we have tested the different mutants for their binding to target sequence, covalent complex formation, and cleavage/religation properties. We found that IntSSV has a type IB topoisomerase activity, a property shared by some other tyrosine recombinases (28-31). All of the mutants generated alter the enzymatic properties of IntSSV, indicating that the residues targeted are likely involved in the catalytic process. The conservative changes of some catalytic residues in IntSSV, with regard to the consensus, may reflect an adaptation of the active site to thermophily and/or may be the signature of the primitive active site that evolved to give the tyrosine recombinase and topoisomerase IB catalytic pockets (18, 32). Importantly, in vitro complementation assays between Y314F and all other IntSSV inactive mutants restored both cleavage and DNA recombination, indicating that the IntSSV active site could be assembled by dimerization activities, suggesting that as for the yeast Flp recombinase, IntSSV Arg240 rather than Lys243 was considered to be the Lys required for the catalytic process. The others mutations were introduced by M13 kit (Stratagene) according to the supplier vector, and the resulting plasmid pGEM-SN180 was used as nonspecific substrate in the relaxation assay. The 180-bp fragment SN180, containing a 5-attP and a 3-attB, was then 5-end labeled on both strands using T4 phosphatase and terminal nucleotidyl transferase or 5-end labeled by [γ-32P]ATP and terminal nucleotidyl transferase or 5-end labeled by [γ-32P]ATP and T4 polynucleotide kinase. Unincorporated nucleotides were removed by spin dialysis. For cleavage experiments, the XT oligonucleotide (5'-TTCCGGCTTCCGGACCCGGA-3') was either 3'-end labeled using [γ-32P]ATP and terminal nucleotidyl transferase or 5'-end labeled by [γ-32P]ATP and T4 polynucleotide kinase. Unincorporated nucleotides were removed by spin dialysis, and the labeled oligonucleotide was hybridized to TE buffer with a 2-fold excess of unlabeled complementary strand, XB (5'-ACCCGCGTCTCCGGAGGGCA-3'). The resulting substrate is referred to as XTB (26).

**DNA Binding Reactions and Electrophoretic Mobility Shift Assay—** The DNA binding reactions were carried out in 20 μl of a mixture containing 10 nM 5'-end-labeled P207 substrate, 0.2 μM wild-type or mutant IntSSV, in a binding buffer composed of 50 mM Tris, pH 7.5, 125 mM NaCl, and 1 mM EDTA. Incubation was performed for 1 h at room temperature. 5 μl of 5× loading buffer (10 mM Tris, pH 7.5, 20% glycerol, 1 mM EDTA, 0.1 mg/ml BSA, and 0.1% xylene cyanol) was then added to the reaction mix. The DNA binding reactions were loaded onto an 8% nondenaturating acrylamide gel containing 5% glycerol. Electrophoresis was performed in 1× TGE buffer (50 mM Tris, pH 7.5, 8 mM glycine, 0.1 mM EDTA) at 4 °C for 4 h at 7.5 V/cm. The DNA-protein complexes were visualized by autoradiography and phosphorimaging.

**Filter Binding Assay—** 12.5 nM 5'-end-labeled XTB substrate was incubated at room temperature for 1 h with 2 μM wild-type or mutant IntSSV in the binding buffer described previously. Each reaction mixture was spotted under vacuum on a nitrocellulose membrane (Protran 0.2 μm, Schleicher & Schuell) and the spot washed three times by 20 μl of binding buffer. The membrane was heated 2 h at 80 °C, and the protein-DNA complexes were visualized by phosphorimaging. The binding efficiency of each protein was quantified using the ImageQuant version 1.2 software. The results presented are the mean of three independent experiments.

**Cleavage Assays—** For covalent complex formation, 12.5 nM 5'-end-labeled XTB substrate was incubated with 1 μM wild-type or mutant IntSSV in the binding buffer described previously. The reaction mixtures were visualized by autoradiography and phosphorimaging. Through 12% SDS-polyacrylamide gel, and covalent complex formation was visualized by autoradiography and phosphorimaging of the gel. The determination of the cleavage position was performed by incubating 1 or 2 μM wild-type or mutant IntSSV with 12.5 nM 5'-end-labeled XTB substrate in the conditions described above. Reactions were stopped by the addition of 0.1× Laemmli buffer (final concentrations: 40 mM Tris, pH 6.8, 5% SDS, 8% glycerol, 250 mM β-mercaptoethanol, 0.005% bromophenol blue) and heating for 5 min at 98 °C. The reaction products were analyzed by electrophoresis through 12% SDS-polyacrylamide gel, and covalent complex formation was visualized by autoradiography and phosphorimaging of the gel.
The catalytic conserved residues of tyrosine recombinases (Tyr Rec) and topoisomerases IB (Topo IB) are in bold letters. Residues Asp/Glu (box I) and His (box II) are conserved at 80% within the tyrosine recombinases family. The corresponding residues in toposomerasers IB (Lys and Thr) are also indicated. The corresponding residues in IntSSV as well as the mutations introduced for this study are presented on the last two lanes of the table. This table was adapted from Ref. 3.

### Table I

| Tyr Rec consensus | Arg<sub>1</sub> | Asp/Glu | Lys<sub>1</sub> |
|-------------------|-------------|---------|--------------|
| Cre               | Arg<sup>173</sup> | Glu<sup>176</sup> | Lys<sup>201</sup> |
| λ-Int             | Arg<sup>191</sup> | Asp<sup>194</sup> | Lys<sup>235</sup> |
| XerD              | Arg<sup>248</sup> | Glu<sup>252</sup> | Lys<sup>172</sup> |
| Flp               | Arg<sup>278</sup> | Asp<sup>274</sup> | Lys<sup>235</sup> |

| Topo IB consensus | Arg | Lys | Lys<sub>1</sub> |
|------------------|----|-----|---------------|
| Vaccinia Topo IB | Arg<sup>130</sup> | Lys<sup>175</sup> | Lys<sup>147</sup> |
| Human Topo IB    | Arg<sup>488</sup> | Lys<sup>485</sup> | Lys<sup>532</sup> |

| Int<sup>SSV</sup> Mutations | Arg<sup>211</sup> | Glu<sup>214</sup> | Arg<sup>240</sup> | Lys<sup>240</sup> | His | Leu | Leu | Leu | Lys<sup>282</sup> | Arg<sup>291</sup> | Tyr<sup>214</sup> |
|-----------------------------|------------------|------------------|------------------|------------------|-----|-----|-----|-----|------------------|------------------|------------------|
| Leu                         | Glu              | Asn              | Lys              | Lys              | His  | Leu | Leu | Leu | Arg              | His              | Tyr              |

at 52 V/cm. Cleavage products were visualized by autoradiography and phosphorimaging of the gel.

DNA Relaxation Assay—12.5 ng plasmid pGEM-P207, pGEM-SV80, or pKSSattB was incubated with the indicated amount of Int<sup>SSV</sup> (wild-type or mutant) in 30 mM Hepes, pH 7.5, 50 μg/ml BSA, and 125 mM NaCl for 3 h at 65 °C. poly(dI-dC)poly(dI-dC) was used as nonspecific competitor when indicated. After incubation, samples were treated with SDS (0.5% final) and 10× loading buffer (100 mM EDTA, 5% SDS, 40% glycerol, and 0.35% bromophenol blue) was added. Reaction mixes were loaded on a 2% agarose gel, and electrophoresis was performed in 1× TEP buffer (90 mM Tris phosphate, 1 mM EDTA) at room temperature for 4 h at 3.5 V/cm. DNA was visualized by staining with ethidium bromide.

Relaxation assays with calf thymus topoisomerase IB (Invitrogen) were done as follows: 12.5 ng plasmid was incubated for 30 min at 37 °C with 1 μl of topoisomerase (corresponding to 5–15 units) in 50 mM Tris, pH 7.5, 50 mM KCl, 10 mM MgCl<sub>2</sub>, 0.5 mM dithiothreitol, 0.1 mM EDTA, and 30 μg/ml BSA in a final volume of 50 μl. Reactions were stopped and analyzed as described above.

To monitor the activity of Int<sup>SSV</sup> on positively or negatively supercoiled substrates, the reaction products were analyzed by two-dimensional agarose gel electrophoresis (34). After the first dimension was performed as described above, the second dimension was run in a perpendicular direction in TEP buffer containing 3 μg/ml chloroquine for 15 h at 0.8 V/cm. Chloroquine was removed by washing the gel in water for at least 3 h. The distribution of topoisomers was then visualized by staining with ethidium bromide.

**RESULTS**

**Mutagenesis Strategy and Production of Int<sup>SSV</sup> Mutants**

The signature of tyrosine recombinases is the conservation of two short regions of similarity in their C-terminal part (1, 2, 4, 5). Within these regions, six residues are strictly conserved and were shown to be directly involved in catalysis by biochemical studies and crystal structures of tyrosine recombinases and topoisomerases IB (for a review, see Ref. 3). Alignment of Int<sup>SSV</sup> with members of the tyrosine recombinase family suggested that they correspond to residues Arg<sup>211</sup>, Arg<sup>240</sup>, Lys<sup>278</sup>, Arg<sup>281</sup>, Arg<sup>304</sup>, and Tyr<sup>314</sup> in Int<sup>SSV</sup> (Fig. 1 and Table I). All of these positions were subjected to site-directed mutagenesis, as well as two other less conserved positions Glu<sup>214</sup> and Lys<sup>262</sup>, which correspond to positions conserved at more than 80% within the tyrosine recombinases conserved boxes I and II (Fig. 1). The latter residues were shown to play a role in the catalytic process mediated by the Flp recombinase (35–37). Within Int<sup>SSV</sup> three of the six conserved residues are divergent from the tyrosine recombinases consensus, namely Arg<sup>211</sup>, Lys<sup>278</sup>, and Arg<sup>304</sup>. We have replaced all divergent residues with the consensus residues, and in addition we have introduced either aliphatic or residues with an opposed charge at these positions (Table I). Residues Glu<sup>214</sup> and Lys<sup>262</sup> were targeted with the same rationale.

None of the mutations introduced altered the level of expression nor the purification behavior of proteins (Fig. 2), although two mutants showed an increased sensitivity to proteolysis (R281L and R304L). All mutant proteins were therefore purified as described previously for wild-type Int<sup>SSV</sup> (26) with the addition of a mixture of inhibitors in all buffers up to the chromatographic step.

All Int<sup>SSV</sup> Mutants Are Still Able to Bind the att Sites—Int<sup>SSV</sup> activity depends on its ability to recognize and bind specific DNA sites. We analyzed the binding efficiency of Int<sup>SSV</sup> on a substrate that carries the POP<sup>+</sup> core sequence of the attP site (Fig. 3A) by electrophoretic mobility shift assay (Fig. 3B). In the absence of competitor DNA, three protein-DNA complexes of retarded electrophoretic mobility can be observed, referred to as CI, CII, and LMC (low mobility complexes), the major species observed corresponding to the CII complex (Fig. 3B, lane 2). Adding increasing amounts of nonspecific DNA (SN180) to the reaction leads to the disappearance of the LMC to the benefit of CII (Fig. 3B, lanes 3 and 4). At a molar ratio of up to 1:20 (specific:nonspecific), the CI and CII complexes are also competed (lane 5). The apparent affinity of Int<sup>SSV</sup> for its core site is relatively low but in the same order of magnitude as that observed for binding of the λ-Int to its POP<sup>+</sup> site in the absence of IHF (38). A simple interpretation of this profile is that CI could reflect the binding of one monomer of Int<sup>SSV</sup> to one “core-type” site (P or P<sup>+</sup>) and CII the binding of a dimer to both core-type sites (P and P<sup>+</sup>). The heterogeneous third complex, LMC, could either represent higher order structures of CI and/or CII or more likely be generated by nonspecific binding of more than two monomers of Int<sup>SSV</sup> along the substrate.

To verify whether the Int<sup>SSV</sup> mutants were still able to bind DNA, we analyzed their ability to generate protein-DNA complexes with attP. Given the apparent low affinity of Int<sup>SSV</sup> for this substrate, the experiment was realized in the absence of competitor DNA. The DNA binding profile of most of the mutants is similar to that of the wild-type protein (Fig. 3C). However, the ratio of complexes over the free substrate depends on the mutation introduced. Mutants E214N, R240K, R281L, R282L, R304H, R304L, and Y314E behave as the wild-type protein with regard to the amount of CII formed. Mutants K278H, K278L, and R282HI generate a somewhat reduced amount of CI and CII. At the same time the LMC disappear as the free substrate amount increases. The last class of mutants (R211L, R211Q, E214K, R240L, and Y314F) generate less CI.
and CII but about the same amount of LMC as the wild-type protein. Among these, some mutants retain a large amount of substrate in the wells, possibly by nonspecific aggregation (see E214K, R240L, and R281L in Fig. 3C). Nevertheless, all of the mutants generally conserved the property to generate CI and CII and CII is systematically the most abundant, suggesting that it is the more stable IntSSV-DNA association.

**IntSSV Has a Topoisomerase IB Activity**—Previous work on IntSSV described an in vitro test for recombination (24, 39). Unfortunately, like others, we were unable to reproduce the published data. While attempting to set up new conditions for an in vitro recombination assay, we found that IntSSV has a DNA relaxation activity (Fig. 4) as some other tyrosine recombinases (28–31). As for recombination, DNA relaxation requires strand cleavage and strand rejoining steps. The optimum for relaxation activity is observed when incubating 2 μM IntSSV with 12.5 nM supercoiled DNA substrate for 2 h at 65 °C in the presence of 125 mM NaCl, without ATP or MgCl₂. Unlike XerC and XerD (29), the relaxation activity is not stimulated by the presence of glycerol in the assay. Incubation of purified IntSSV with the plasmid pGEM-P207, containing the attP site, led to the appearance of DNA topoisomers (Fig. 4A, lane 3). Neither phenol extraction nor proteinase K treatment of the samples altered the migration pattern, indicating that there were no protein-DNA covalent complexes present at the end of the reaction (data not shown). The appearance of topoisomers reflects that IntSSV is able to promote relaxation of a supercoiled DNA. On time course experiments, the amount of each topoisomer increased, but their distribution did not vary, and gels containing chloroquine revealed that the totality of substrate DNA was at least partly relaxed (data not shown). This strongly suggests that IntSSV is more distributive than processive for DNA relaxation.

The influence of the substrate topology on IntSSV relaxation activity was also tested. Interestingly, IntSSV relaxes both negatively and positively supercoiled plasmids (Fig. 4C) even though the relaxation of both substrates is incomplete (compare II and IV in Fig. 4C). Relaxation of negative and positive supercoils is specific for type IB topoisomerases, ruling out any possible contamination by an *E. coli* topoisomerase. So far, only IntSSV and λ-Int (30) have been shown to behave specifically as type IB topoisomerases, although a type I activity was demonstrated for both Xer proteins (29) and Cre (28). This is consistent with the idea that tyrosine recombinases and type IB topoisomerases evolved from an ancestral common catalytic module.

The IntSSV DNA relaxation activity is not site-specific because it can also be observed with plasmid pGEM-SN180, which does not contain any att site (Fig. 4B, lane 3). Once again, this behavior is similar to that of λ-Int (30). Addition of poly(dI-dC)-poly(dI-dC) as nonspecific competitor DNA (Fig. 4, A and B, lanes 4–7) was slightly more inhibitory when no att
site was present on the substrate (compare lanes 4–7 in the A and B panels). This most likely reflects a better affinity of IntSSV for pGEM-P207 than for pGEM-SN180 rather than a site-specificity for the topoisomerase activity.

Although no strand exchange occurs, the topoisomerization reaction requires strand cleavage and strand religation, as in a complete recombination process. In the absence of a reliable recombination assay we decided to use the relaxation assay, in addition to the binding and cleavage assays, as a reporter of activity for IntSSV and its mutants.

Most IntSSV Mutants Are Deficient in DNA Relaxation—The ability to promote strand cleavage and religation was evaluated for all mutants by using the relaxation assay. As expected, the Y314F mutant is unable to relax supercoiled DNA, once again ruling out a topoisomerase contaminant in the protein samples. Although most IntSSV mutants are inactive in the relaxation assay, three mutants (R240K, K278H, and R304H) retain a low but significant level of activity (Fig. 5). Interestingly, these mutants correspond to the restoration of the tyrosine topoisomerase IB; lane 8; plasmid incubated with calf thymus topoisomerase IB; lane 9; plasmid linearized by HincII. Supercoiled plasmid (FI), open circular plasmid (FII), and linearized plasmid (FIII) are indicated by arrows on A. C, DNA relaxation activity of IntSSV on topologically different substrates. Negatively supercoiled (I and II) or positively supercoiled (III and IV) pKSattB were incubated without (I and III) or with 2 μM IntSSV (II and IV) for 3 h at 65 °C. The reaction products were analyzed by two-dimensional gel electrophoresis (33). The left part of the arch corresponds to negative topoisomers and the right part to positive topoisomers.

catalysis than thermostability. Moreover, none of the three mutations restoring the consensus at a catalytic residue (R240K, K278H, R304H) shows a better activity at lower temperature. One possible interpretation is that thermal adaptation of the catalytic pocket requires more than one of these adjustments in the charge relay system.

Most IntSSV Mutants Are Impaired in Covalent Complex Formation but Not in Their Ability to Bind a Minimal Substrate—We have shown previously that incubation of IntSSV with a synthetic minimal 19-bp substrate (referred as to XTB) leads to the formation of a covalent complex, resulting from cleavage of a phosphodiester bond and creation of a phosphotyrosine intermediate (26). This property was used to evaluate the effects of the mutations on the cleavage step of the reaction. Only one mutant, R304H, is still able to generate protein-DNA covalent complex, although with a low efficiency corresponding to about 9% of the wild-type activity (see Fig. 7B). All other IntSSV mutants are unable to catalyze covalent complex formation (see Fig. 7B), although in some cases a faint signal with a lower mobility than the protein-DNA covalent complex can be detected (see for example mutant E214K in Fig. 7B). Strikingly, mutants that retained a low DNA relaxation activity were deficient in covalent complex formation, suggesting that the cleavage of a small synthetic substrate may be a more stringent assay than the relaxation assay. Indeed, it was shown previously for the Flp recombinase that some mutants showing activity on large substrates were deficient in cleavage activity when tested on small synthetic substrates (40).

Because the lack of covalent complex formation could arise from defective binding to the substrate, we checked whether IntSSV mutants were able to bind XTB by using a filter binding assay. The results presented in Fig. 6 show that most mutants bind the synthetic substrate quite efficiently. Two mutants showed an important defect in substrate binding (R240K and...
K278L), the latter being totally unable to retain XTB on a filter assay. Quite surprisingly, K278L was able to bind a larger DNA substrate although less efficiently than the wild-type enzyme (see Fig. 3C). This suggests that the K278L substitution alters the DNA binding properties of IntSSV and that this effect is more drastic on the short 19-bp substrate. The other substitution made on the same position (K278H) does not significantly alter XTB binding but still affects the cleavage activity, as it is the case for most of the mutants. Remarkably, all other mutants are unable to promote covalent complex formation (Fig. 7B) even though they can bind significantly to the synthetic substrate (Fig. 6). This indicates that all of the residues targeted are likely involved in the cleavage reaction, suggesting that IntSSV follows the mechanism proposed for tyrosine recombinases and topoisomerases IB (32).

Complementation between Mutants Restores Both Strand Cleavage and DNA Relaxation Activity—Several lines of evidence suggest that at moderate ionic strength IntSSV behaves as a dimer in solution. This led us to test whether the active species of IntSSV could be dimeric rather than monomeric, in other words, whether IntSSV could follow the shared active site model (13, 14). We therefore set up in vitro complementation assays with the collection of defective mutants (Fig. 7).

One fully inactive mutant of each targeted residue was co-incubated with the Y314F mutant in the relaxation assay (Fig. 7A). The activity was restored in all cases, indicating that Y314F can complement all of the mutants affected on any other position. On the other hand, coinubcation of Y314F with Y314E (Fig. 7A) and all others mutant combinations did not restore the relaxation activity (data not shown). Furthermore, the addition of 1 mM tyrosine in the relaxation assay with Y314F did not produce topoisomers (data not shown) making it unlikely that a loose tyrosine residue from a monomer may interfere in the reaction. This strongly suggests that, for one considered catalytic site, Tyr314 is brought by a monomer, and all the other residues are provided by the second one. In the complementation experiment three different types of dimers can be formed, two homodimers and a heterodimer. Only the heterodimer is expected to be a catalytically active species with one active site restored. It is thus not surprising to observe that the combination of mutants involving 2 μM total proteins in the reaction is less efficient in relaxation than 1 μM wild-type integrase (Fig. 7A).

Covalent complex formation is also restored when any mutant is mixed with the Y314F mutant (Fig. 7B). Interestingly, complementation is efficient between Y314F and K278L, which is the only mutant unable to bind the XTB substrate (Fig. 6). Heterodimer formation may therefore compensate for the binding defect of K278L. This suggests that protein-protein interactions established between two monomers contribute to stabilize the dimer at least on the synthetic substrate. Four mutants, E214N, R240K, R304H, and R304L, restore the wild-type level of cleavage when complemented by Y314F (Fig. 7B). These mutants bind P207 as efficiently as the wild-type (Fig. 3) and except for R240K also bind efficiently the XTB substrate (Fig. 6), suggesting again that protein-protein contacts are important for proper protein-DNA interactions and therefore catalysis. The behavior of Lys282 mutants is more puzzling. Both mutants of this position bind the XTB substrate with a good efficiency (Fig. 6) and efficiently produce the CII complex (Fig. 7B). The corresponding residue in Flp is His309. The Flp
structure reveals that this residue is not involved in DNA contacts. His^{309} from one Flp monomer rather interacts with His^{345} from the second monomer, which brings the catalytic Tyr^{343}. The His^{309}-His^{345} interaction was proposed to allow a correct positioning of Tyr^{343} toward the scissile phosphate in the Flp catalytic pocket (12). If the mode of dimer assembly is conserved between Flp and IntSSV, the Lys^{282} residue would then be critical for stabilizing the dimer in an active conformation. Heterodimers K282L/His-Y314F could then have a loose interface that may alter their stability and thus their catalytic efficiency.

The complementation reaction was analyzed further by verifying that the cleavage specificity was not altered when mixing two mutants (Fig. 7C). The small synthetic substrate XTB was 3′-end labeled on the top strand and incubated with wild-type, mutants, and mutant mixes. As was pointed out previously (26), the amount of products observed with the 3′-end-labeled substrate is lower than that obtained for the 5′-end-labeled substrate in all cases. Analysis of the reactions on a denaturing gel shows that the cleavage product obtained in the mixing experiments is the same as that obtained with the wild-type IntSSV (Fig. 7C), indicating that complementation between active sites mutants leads to the correct cleavage site specificity. The simplest interpretation for these results is that IntSSV active site assembly proceeds through monomer association. The results obtained in the different complementation assays are consistent with the hypothesis that IntSSV requires protein-protein interactions to assemble active sites, either by assembling two shared active sites within a dimer or, like Flp, four active sites within a tetramer (12).

**DISCUSSION**

We report here the characterization of 15 IntSSV mutants. The targeted residues were comprised in the canonical active site motif common to tyrosine recombinases and topoisomerases (IB (Table I)). Crystallographic data and mechanistic analysis support the hypothesis that these two enzyme families would have evolved from an ancestor catalytic domain with a strand transferase activity (41). In a previous work we have shown that like other virus-encoded integrases, IntSSV cleavage
sites specifically target the border of the anticondor loop of a tRNA gene and that cleavage is mediated via a 3'-phosphotyrosine intermediate (26). However, several discrepancies in the conserved catalytic residues raised the possibility that the catalytic process would be different in the archaeal kingdom (27). The results presented here provide evidence to unravel this point.

First, we have shown that the wild-type IntSSV is able to promote DNA relaxation. The relaxation activity does not require ATP or MgCl₂ and is monitored both on positively or negatively supercoiled DNA, a property specific for topoisomerase IB. As for the α-Int (30) and unlike Cre or XerC/XerD (28, 29) the relaxation activity is not dependent on a specific DNA site. Detection of relaxation activity for Cre, Flp, α-Int, and Xer proteins is possible only under conditions where (almost) no recombination occurs (28–31). For IntSSV no recombination products would be expected from the substrates used in the relaxation assay. These data are consistent with the idea that the DNA relaxation activity of tyrosine recombinases is more likely a bypass of the recombination reaction.

The second point of evidence for mechanism conservation in IntSSV is provided by the properties of the mutants studied here. None of the mutations abolished the DNA binding properties of IntSSV, although one mutant (K278L) was totally defective for binding to a small synthetic substrate, and some mutants may be impaired in establishing protein-protein interactions (K282L, K282L). However, mutations at any of the conserved positions of the catalytic pocket strongly alter IntSSV activities. All mutants but one (R304H) are unable to generate a protein-DNA covalent complex on a small synthetic substrate. This is consistent with the proposed role in catalysis for these conserved residues (4, 22, 35, 37, 42–47). Hence, Arg₂¹¹, Lys³₇₈, and Arg²₈¹ in IntSSV could coordinate the scissile phosphate, thus activating it for nucleophile attack by Tyr₃₁₄. Residue Arg²₄⁰ corresponding to the conserved Lys₃₁₄ is also critical for IntSSV activity. Replacement of Arg²₄⁰ by a leucine abolishes the relaxation activity, whereas the R240K mutation only reduces it. Furthermore, complementation of R240K by Y₃₁₄F on the small synthetic substrate allows more covalent complex to be formed than that observed for R240L. This indicates that a positive side chain at this position is important for catalysis. Mutants made on equivalent residues in vaccinia topoisomerase IB (48), XerD (6), and more recently Flp (20) have the same behavior, i.e. binding to substrate but deficiency in cleavage and relaxation or recombination, suggesting that Arg²₄⁰ could fulfill the role of Lys₃₁₄ in the IntSSV active site. As for tyrosine recombinases (20) and topoisomerase IB (45, 47), residue Arg²₄⁰ corresponding to the conserved His/Trp is essential for IntSSV activity. Although the R304L mutant is unable to cleave a synthetic substrate and relax a supercoiled substrate, the R304H mutant retains a slight level of cleavage (Fig. 7B) and has a low relaxation activity (Fig. 5). The increased sensitivity of R304L toward proteolysis as well as altered fluorescence spectra for R304H and R304L suggest that this position is important for proper folding of the protein. However, whether Arg²₄⁰ is involved in docking the helix (or structural motif) providing the catalytic tyrosine as for Flp (20) or in forming a hydrogen bond to the nonbridging oxygens of the scissile phosphate as for the human topoisomerase IB (19) cannot be inferred from our experiments. Mutants at position Glu₁¹⁴ bind the different substrates with almost the same efficiency as wild-type, indicating that a negatively charged side chain at this position is not required for substrate recognition. Furthermore, the complementation experiment indicates that E214K is less efficient in cleavage than E214N, suggesting that the length of the side chain at this position may be important for correct dimer assembly. Even though the strong conservation of this acidic residue in tyrosine recombinases and different mutational analysis (35, 43) suggested that it is involved in catalysis, crystal structures indicate that its role would rather be architectural by maintaining the active site geometry via interactions with the nearby conserved Arg⁵. Finally, mutants at residue Lys²₈² behave as mutants at the equivalent positions in the R and Flp recombinases (36, 37). Furthermore, the behavior of the Lys²₈² mutants in the complementation assays is consistent with the proposed role of this residue in Flp (12), i.e. correct positioning of the catalytic tyrosine mediated by interactions with a residue located on the helix that delivers the tyrosine.

Changes of conserved residues within the putative IntSSV active site may be the signature of a chemical adaptation to thermophily. Indeed the conserved histidine residues of tyrosine recombinases are replaced by arginine or lysine residues in IntSSV. The pKₐ decrease of bases at high temperatures, correlated with the pKₐ variability of buried histidines (49) could account for these replacements in IntSSV catalytic pocket. Around 80 °C lysines and arginines could therefore have a pKₐ close to the pKₐ of histidines at lower temperature, thus being chemically equivalent for catalysis. It should be noted however that thermophilic Xer-like proteins do not have this signature, although there is no biochemical evidence for an enzymatic activity of these proteins (50). Another possible interpretation for the His → Arg/Lys replacements in IntSSV is that they could be the remnant of the primitive catalytic module that evolved to give the topoisomerase IB and tyrosine recombinase catalytic pockets.

Finally, the results obtained in the complementation assays strongly suggest that IntSSV would follow the shared active site paradigm. The shared active site model has been controversial for a long time (51–54). For Flp, the recent crystal structures (12, 55) are consistent with trans delivery of the catalytic tyrosine, which was inferred from complementation assays (13, 56). The trans activity of Cre (57) was ruled out by crystallographic data (8, 23), and further bulk experiments provided evidence for cis cleavage (58). In the case of α-Int, two different enzymatic analyses showed divergent results with regard to the cis versus trans origin of the catalytic tyrosine (17, 59). The first structural data did not allow discrimination because the crystal obtained was disordered in the region of the catalytic tyrosine (11). More recently, the structure of α-Int bound on a suicide substrate shows that on a substrate allowing binding of only one monomer the catalytic tyrosine is delivered in cis (10).

It was suggested that trans cleavage may be artifactual (54), either because the substrates used were suicide substrates, and may thus alter the protein-DNA interactions, or because mixing two mutant proteins would allow another residue to play the role of a surrogate nucleophile in the cleavage reaction. As was pointed out recently, the use of an assay scoring not only cleavage but also the strand-joining event can avoid artifacts in the determination of cis or trans cleavage (60). We show here that cleavage specificity, covalent complex formation, and relaxation activities of IntSSV can be restored when mixing mutants of each conserved residues with the Y314F mutant. This is the only combination allowing complementation, making it unlikely that another residue may be a surrogate nucleophile in the assays. Furthermore, the relaxation activity requires not only cleavage of the DNA substrate but also strand religation. The level of complementations observed are also consistent with a "half of the sites" activity. Indeed, the wild-type level of plasmid relaxation is never reached when mixing two mutants. By contrast, full complementation can be observed in the assay measuring covalent complex formation. In this latter case, the
rationale of the experiment consisted in measuring a stoichiometric activity with a large excess of protein with respect to the 19-bp oligonucleotide. Some mixing reactions give lower amounts of products than wild-type, suggesting that on small synthetic substrates the stability of the IntSSV dimer alone or in complex with DNA is important for activity. The simplest interpretation of these results is that the IntSSV active site is assembled at a dimer interface with Tyr$^{344}$ provided by one protomer and all other residues targeted here by another protomer. However the exact architecture of the intasome, i.e., IntSSV assembled on DNA for efficient recombination, cannot be assessed by the assays used in this study. Therefore, a cis-acting mechanism for the full recombination process cannot be totally excluded. A final answer to this question will be brought by structural analysis of IntSSV. If indeed an archaeal tyrosine recombinase is following the shared active site assembly in the eubacterial kingdom, the helix swapping may have occurred by the addition of a C-terminal module improving contacts between protomers, whereas in the eukaryal kingdom may have occurred by the addition of a C-terminal module improving contacts between protomers.

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Mutational Analysis of the Archaeal Tyrosine Recombinase SSV1 Integrase Suggests a Mechanism of DNA Cleavage in trans
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