Reactions of Type II Restriction Endonucleases with 8-Base Pair Recognition Sites*

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Type II restriction endonucleases usually recognize 4–6-base pair (bp) sites on DNA and cleave each site in a separate reaction. A few type II restriction endonucleases have 8-bp recognition sites, but these seem unsuited for restriction, since their sites are rare on most DNA. Moreover, only one endonuclease that recognizes a target containing 8 bp has been examined to date, and this enzyme, SfiI, needs two copies of this site for its DNA cleavage reaction. In this study, several endonucleases with 8-bp sites were tested on plasmids that have either one or two copies of the relevant sequence to determine if they also need two sites. SgfI, SrfI, FseI, PacI, PmeI, Sse8781I, and SdaI all acted through equal and independent reactions at each site. AscI cleaved the DNA with one site at the same rate as that with two sites but acted processively on the latter. In contrast, SgrAI showed a marked preference for the plasmid with two sites and cleaved both sites on this DNA in a concerted manner, like SfiI. Endonucleases that require two copies of an 8-bp sequence may be widespread in nature, where, despite this seemingly inappropriate requirement, they may function in DNA restriction.

The recognition sequences for the majority of the ~3,000 type II restriction endonucleases identified to date are symmetrical palindromes of DNA, 4–6-bp-long, although ~20 enzymes of this type recognize 8-bp targets (1). The latter are particularly valuable as tools for the analysis of genomic DNA because they usually cleave DNA into larger DNA fragments than the enzymes cutting at 4- or 6-bp sequences, due to the relative rarity of their sites (2). However, doubts exist over whether a restriction enzyme with an 8-bp site could provide a bacterial cell with an effective defense against foreign DNA (3). Restriction demands at least one recognition site on the incoming DNA, and its efficiency increases with the number of sites (4). The probability of DNA escaping restriction, by being modified at all sites before being cleaved at any one site, declines logarithmically with the number of sites. Yet an 8-bp sequence must occur less frequently on DNA than any 4- or 6-bp element from that sequence, so phage or plasmid DNA may often lack an 8-bp site or contain only a small number of such sites. Hence, if the defense against phage infections by a restriction-modification system confers a selective advantage to a bacterial cell, evolutionary pressures should result in a contraction of the length of the recognition sequence (5).

Most of the current information about the mode of action of type II restriction enzymes derives from a relatively small number of enzymes, almost all of which recognize either a 4- or a 6-bp sequence, such as BanIII, EcoRV, MunI, PvuII, or TaqI (Refs. 6–10, and references therein). In each of these examples, the protein is a dimer of identical subunits that interacts symmetrically with a palindromic DNA sequence, so that the active sites in the enzyme are positioned on the scissile phosphodiester bonds in each strand. In the presence of Mg2+, the cofactor for DNA cleavage, the two strands are cut in parallel reactions. The cleavage of both strands is normally completed before the enzyme dissociates from the DNA, although, in some instances, the enzyme dissociates after cutting just one strand and then returns to that site to cut the second strand (11). On DNA with multiple sites, these enzymes usually act in a distributive manner at each individual copy of the recognition sequence. However, they sometimes act processively on a DNA with two or more sites. For example, EcoRI can cleave one site, translocate to another site by an intramolecular process, cut that site, and only then leave the DNA (12, 13). Conversely, the restriction enzymes in the type Ie group, such as EcoRII and NaeI, require two copies of their recognition sequence (14, 15). Both EcoRII and NaeI are reported to be homodimeric proteins that have two distinct DNA-binding sites. One binding site has the catalytic functions for DNA cleavage, but this remains inactive unless a second copy of the recognition sequence binds to an allosteric site elsewhere in the dimer (16–18). The DNA at the allosteric site is not cleaved (19, 20).

To date, a reaction mechanism has been established for only one of the type II enzymes that recognizes a site with 8 specified bp: the SfiI endonuclease from Streptomyces fimbriatus (21–27). In contrast to both the orthodox enzymes such as EcoRV and the type Ie enzymes such as EcoRII, SfiI is a tetrameric protein that has two identical binding sites for its palindromic recognition sequence, each presumably made from two subunits. However, SfiI has no activity when only one DNA-binding site is occupied. Instead, it has to bind two copies of its recognition sequence before being able to cleave DNA. The two sites can be in cis, on the same molecule of DNA, or in trans, on separate molecules of DNA. In the former case, SfiI tethers the intervening DNA in a loop, while, in the latter, it bridges the two DNA molecules. As with other proteins that span two sites (28), SfiI prefers sites in cis over sites in trans. It generally cleaves plasmids with two sites more rapidly than plasmids with one site. Moreover, the turnover of SfiI on plasmids with two sites is normally completed by the liberation of DNA cut in both strands at both sites; only a small fraction of

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1 The abbreviations used are: bp, base pair; kb, kilobase pair(s); MCS, multiple cloning site; SC, supercoiled; OC, open circle; FFL, full-length linear; L1 and L2, linear DNA fragments from the cleavage of a circular DNA with two sites at both sites.

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the DNA is liberated after cutting just one site. The concerted action of SfiI at two recognition sites is reminiscent of the enzymes that mediate DNA rearrangements by site-specific recombination or transposition (29), but a role for SfiI in rearrangements has been rejected (30). Like the orthodox restriction enzymes (31), phosphodiester hydrolysis by SfiI inverts the stereoconfiguration of the phosphate, so its reaction cannot involve a covalent enzyme-DNA intermediate (32). Enzymes that catalyze both DNA breakage and religation normally conserve the energy of the phosphodiester bond, a prerequisite for the religation step, by forming a covalent intermediate (29).

In vivo, SfiI can restrict DNA that has two or more SfiI sites, but it is incompetent at restricting DNA with one site (30). The mode of action of SfiI thus exacerbates the doubts over whether an enzyme recognizing an 8-bp sequence could defend a cell against phage or plasmid DNA. However, the recognition sequence for SfiI is unusual (33) in that it contains 8 specified bp but these are interrupted by an unspecified spacer of 5 bp (Table I). Apart from one isoaschizomer of SfiI, all of the other type II enzymes that cleave DNA at 8-bp sites recognize uninterrupted sequences of 8 consecutive bp (Table I). Hence, while the type II enzymes with continuous recognition sites of 8 bp might act like SfiI, they may differ from SfiI and behave instead like the orthodox enzymes such as BamHI or EcoRV. These possibilities were examined by assaying several restriction enzymes on plasmids that have either one or two copies of the relevant sequence in order to determine whether they act concurrently at two sites or cleave each site in a separate reaction.

**EXPERIMENTAL PROCEDURES**

**Enzymes—**Restriction endonucleases were purchased from the following suppliers: AscI, BseCI, BsiCI, BsmI, BstEII, BclI, and SfiI from New England Biolabs; SfiI from Promega; SsoI from Stratagene; and SfclI from Fermentas; SgrAI from both Roche Molecular Biochemicals and New England Biolabs (with equivalent results). Enzyme concentrations are given in terms of units of enzyme activity, as specified by the supplier. Other enzymes were obtained from New England Biolabs or Roche Molecular Biochemicals.

**DNA—**The plasmids pTA153 (34) and pNEB193 (New England Biolabs) had been degraded before; the latter is identical to the former except for an enlarged multiple cloning site (MC9) with a number of additional restriction sites. Two derivatives of pTA153, pDB7 and pDB8 (Fig. 1a), and one of pNEB193, pAB1 (Fig. 1b), were constructed by standard methods (36). The duplex used in the construction of pDB7 was produced by annealing two 50-base synthetic oligodeoxyribonucleotides that were complementary to each other except for 4 bases at their 5' termini. A resultant 46-bp duplex had 4-base 5'-extensions that matched an EagI terminus at one end and a Styl terminus at the other (Fig. 1a). The SgrAI site in this duplex had the same sequence as the intrinsic site on pTA153, with respect to both its purine/pyrimidine degeneracies (Table I) and to its flanking sequence for 3 bp on either side. The duplex used in the construction of pDB8 was made by the same procedure; it had the same sequence as the duplex for pDB7 except that it lacked the SgrAI site and its 4-base 5'-extensions matched, respectively, EcoRI and HindIII termini (Fig. 1a). The plasmids were used to transform E. coli strains of Escherichia coli, either HB101 (36) or ER2238 (37). The transformants were cultured in M9 minimal medium with 1 mM chlorite [methyl-H]-thymidine, and the covalently closed form of the plasmid was purified by density gradient centrifugations (38). The preparations were largely supercoiled monomeric plasmid, with <10% as either dimeric plasmid or nicked open circle DNA.

**Assays—**Reactions were carried out at 37 °C in 200-μl volumes and were initiated by adding the restriction enzyme (typically 10 units) to the requisite plasmid (10 or 20 nm) in an appropriate buffer. In the first instance, the buffer used with each enzyme was that advised by the supplier. To test the necessity (12), most enzymes were also examined in modified buffers with elevated ionic strengths. For enzymes where the recommended buffer contains NaCl, the modified buffer had double the concentration of NaCl. Similarly, buffers with KOAc were adjusted to twice the advised level of KOAc. At various times after adding the enzyme, aliquots (15 μl) were removed from the reactions and mixed immediately with 10 μl of an EDTA stop mix (38). The samples were analyzed by electrophoresis through agarose under conditions that separated the supercoiled substrate and each of the various products from the reaction (Fig. 2). The segments of the agarose gel that encompassed the substrate and each product were analyzed individually by scintillation counting to yield the concentration of each form of the DNA at each time point (38). For reactions with two recognized linear DNA fragments (L1 and L2) arising from cleavage at both sites were counted together to obtain a single value for the concentration of doubly cut DNA (L12).

**Experimental Strategy—**A distinction between the different modes of action seen among the type II restriction enzymes can be made by analyzing the reaction kinetics of the enzyme on two substrates, one with one copy of its recognition sequence and another with two.

An enzyme that acts like EcoRV will cleave a circular DNA with one copy of its recognition site first in one strand, converting the supercoiled (SC) substrate to the nicked open circle (OC) form of the DNA, and then in the second strand to produce the full-length linear (FLL) form (Fig. 2a). However, the hydrolysis of both phosphodiester bonds is often much faster than the dissociation of the cleaved DNA (8, 13, 39). In such cases, the nicked form exists only as a transient enzyme-bound intermediate, and the sole product that accumulates during a steady-state reaction is the FLL form. A SC DNA with two sites will be cleaved by an orthodox enzyme acting distributively first at one site to yield FLL DNA and then at the other site to give two linear fragments, L1 and L2 (Fig. 2b). The reaction on a two-site DNA should therefore follow a sequential A → B → C pathway, where the concentration of B first falls in a manner specified by the A → B and the B → C steps and where the formation of C is preceded by a characteristic lag phase (40). If the recognition sites on the one- and two-site substrates are all equal to each other, then the initial rate for the utilization of the SC DNA with one site (v1) should equal that for the utilization of the SC DNA with two sites (v2), and the latter should also equal the rate for the conversion of the FLL DNA to L1 and L2 (v2).

A type II restriction enzyme that acts processively on a DNA with multiple sites should also utilize the two-site substrate at the same rate as the one-site substrate. But if the enzyme then travels along the DNA to another site and cuts that site before departing from the DNA, the SC substrate with two sites will be converted quickly to the doubly cut products, without an intervening accumulation of FLL DNA and without a lag phase preceding the formation of L1 and L2. However, the processivity is unlikely to be 100% efficient, and the enzyme will sometimes depart from the DNA before cutting the second site. Nevertheless, processivity will diminish the yield of FLL DNA from the two-site substrate, relative to that from an orthodox enzyme acting distributively. Moreover, the degree of processivity is likely to decrease as the ion strength of the reaction is increased (12, 13). Hence, an enzyme that acts processively at low ionic strength is likely to act in a distributive manner at high ionic strength.

In contrast, a restriction enzyme that follows the mechanism proposed for the type IIE enzymes will utilize the substrate with two sites more rapidly than that with one site, since the interaction with the second site, which is needed to activate the enzyme, will be aided if this is provided in cis rather than in trans. Having cleaved one site on a two-site substrate, a type IIE enzyme would cleave the residual site at a slow rate, so a large amount of FLL DNA should accumulate during the reaction.

An enzyme that acts concurrently at two recognition sites, like SfiI, would also utilize a two-site plasmid more rapidly than a one-site plasmid. However, in contrast to a type IIE enzyme, a concerted reaction on a two-site plasmid will give directly the final products cut at both sites, L1 and L2. A diminished yield of FLL DNA from a SC DNA with two sites could thus be due to either concerted or processive actions, but these can be distinguished by analyzing the reactions at varied ionic strengths. Both the difference in the reaction rates of SfiI on plasmids with one or two sites and the degree of concertedness on plasmids with two sites depend on the concentration of NaCl in the reaction (22). In reactions lacking NaCl, SfiI cleaves DNA with one site almost as readily as DNA with two sites; under these conditions, the protein binds to its recognition sites with sufficiently high affinity so that even the relative ionic strength employed in cis still has a major effect on the reaction rate (27). A small amount of salt prevents the interactions in trans, so SfiI reactions on DNA with one site are blocked at lower levels of NaCl than those on DNA with two sites (22). In high salt, SfiI cleaves DNA with two sites much more rapidly than DNA with one site. But the progressive destabilization of the complex of SfiI and two DNA sites with increasing ionic strength results in the progressive liberation of products from the two-site substrate that have been cleaved in three,
two, or one phosphodiester bonds in place of the product cleaved at both sites in both strands (25, 26).

The reaction kinetics of a restriction enzyme on one-site and two-site substrates thus provide a diagnostic test for the mode of action of the enzyme; for independent reactions at individual sites, \( v_1 = v_2 = v_{SGI} \) for processive action on a DNA with two sites, \( v_1 > v_2 \) for activation by a second site, \( v_1 < v_2 \) for concerted action at two sites, \( v_1 > v_2 \). However, data at one ionic strength that yield a match to one of the above sets of relative values for \( v_1, v_2 \), and \( v_{SGI} \) may be insufficient for the diagnosis. An unambiguous distinction between these possibilities requires data at varied ionic strengths.

A meaningful comparison of the activities on one- and two-site substrates requires the following conditions to be met. First, the plasmids must be isolated from recombination-deficient strains to prevent the one-site DNA from recombining to its dimeric form with two sites; recA strains were used here. Second, the DNA sequences flanking each recognition site on the two substrates must all be the same, since restriction activity is often affected by the flanking DNA (41–43); plasmid substrates were designed to meet this requirement (Fig. 1). Third, the two-site substrate must have an appropriate length of DNA between the sites. Lengths of <300 bp may be inappropriate, because DNA looping between closely spaced sites depends on both the helical periodicity and the bending of the intervening DNA (26). Conversely, on linear DNA, the stability of a loop between sites separated by >300 bp decreases as the separation increases, but this effect is largely nullified by DNA supercoiling (28). On SC DNA, increasing the separation of the sites above 400 bp has at most only a marginal effect on loop stability (44, 45). All of the tests described here used SC plasmids with, in the case of the two-site substrates, >500 bp between the sites. Fourth, the reactions must employ lower concentrations of enzyme than substrate. Otherwise, the enzyme may bind independently to each site on the two-site DNA and cleave this DNA at double the rate of the one-site DNA.

This study used commercial preparations of restriction enzymes, whose concentrations were specified in terms of units of activity rather than molarity. Nevertheless, the reactions are likely to have used lower concentrations of enzyme than DNA. For several enzymes, varied numbers of units were added to the reactions, and in all cases, the reaction velocities increased linearly with the number of units (data not shown). This behavior is characteristic of a steady-state reaction with substrate in excess of the enzyme. If the reactions had contained enzyme in excess of substrate, the rates would not have varied with the amount of enzyme. A further concern arises from the use of SC substrates. If an enzyme is more active on SC DNA than on linear DNA (or vice versa), the rate for the conversion of a SC DNA with two sites to the FLL form will differ from that for the subsequent conversion of FLL DNA to L1 and L2. Several of the enzymes were therefore tested on both SC and linear DNA substrates, the latter being generated by cleaving the plasmid with another restriction enzyme; in all cases, the SC and linearized substrates gave the same reaction rates (data not shown).

**RESULTS**

SgfI, SrfI, SgrAI, and FseI—The above strategy was applied first to a set of four enzymes with 8-bp recognition sites: SgfI, SgrAI, SrfI, and FseI. The first three of these were selected because they, like SfiI, are from *Streptomyces* species (Table I). FseI was chosen because its recognition sequence is the same as that for SfiI except for the absence of the 5-bp interruption in the SfiI site (Table I). Plasmids with one or two recognition sites for each enzyme were constructed from pAT153, which has one site for SgrAI and none for the other enzymes (Fig. 1a).

The first construct, pDB7, contains two sites for SgrAI, separated by 571 bp, and one site for the other enzymes. The second, pDB8, contains two sites for SgfI, SrfI, and FseI, separated in each case by 949 bp. Each enzyme, parallel experiments were carried out on the one-site and two-site plasmids. Samples were withdrawn from the reactions at varied times and analyzed by electrophoresis through agarose. Typical gels, from SgfI reactions on pDB7 and pDB8, are shown in Fig. 2, a and b, respectively (others not shown). With both plasmids, the intact SC, the nicked OC, and the FLL forms of the DNA were isolated from each other, as were the fragments produced by cutting both sites on the two-site DNA, L1 and L2. The concentrations of each form of the DNA, at each time point sampled during the reaction, were then determined (Fig. 3). Rates for the utilization of the one- and two-site substrates, \( v_1 \) and \( v_{2A} \), respectively, were measured from the initial linear decline in the concentration of SC DNA with time, while the rate for the second reaction on the two-site substrates, \( v_{2B} \), was assessed relative to \( v_{2A} \) from the time course for the production and decay of the FLL DNA. If \( v_{2A} = v_{2B} \), the maximal amount of FLL DNA produced during the reaction will be 40% of the total DNA, but if the amount of FLL DNA rises to a maximum of >40% of the total DNA, then \( v_{2A} > v_{2B} \); conversely, a maximum of <40% indicates that \( v_{2A} < v_{2B} \).
Restriction Enzymes with 8-bp Sites

Fig. 2. Reactions of a type II endonuclease on plasmids with one or two recognition sites. The reactions contained 50 units ml⁻¹ SgfI and 20 nM DNA (~95% supercoiled) in 10 mM Tris-HCl (pH 7.9), 100 mM NaCl, 10 mM MgCl₂, and 1 mM dithiothreitol, at 37 °C. The DNA in a was pDB7, which has one SgfI site, and the DNA in b was pDB8, which has two SgfI sites. At timed intervals after adding the enzyme, samples from the reactions were quenched with stop mix and analyzed by electrophoresis through agarose. The schematics in both a and b illustrate the various forms of DNA that can exist during these reactions. The agarose gels in both a and b illustrate the separation of these forms of the DNA; the electrophoretic mobility of each form is marked on the right of the gels. The reaction times (0–120 min) are noted as expanding scales above the gels.

Fig. 3. SgfI on plasmids with one or two recognition sites. The reactions of SgfI on pDB7 (which has one SgfI site) (a) or pDB8 (two SgfI sites) (b) that are shown in Fig. 2, a and b, respectively, were analyzed to obtain the concentrations of the following forms of the DNA at each time point during the reaction: SC, OC; L1, OC; L2, OC; FLL, L1/2, OC; FLL, L1/2. The plasmids were 3H-labeled, and the DNA concentrations were determined by assessing individual segments of the agarose gels (Fig. 2) in a scintillation counter.

The reaction of SgfI on a SC plasmid with one cognate site yielded virtually none of the OC DNA. Instead, almost all of the substrate was converted directly to the FLL product (Fig. 3a). Thus, as with many other restriction enzymes (11), SgfI cuts its recognition site in both strands before dissociating from the DNA. SgfI again yielded virtually none of the OC DNA from the plasmid with two SgfI sites, and the only product formed in significant yield during the initial period of this reaction was FLL DNA; the FLL DNA was subsequently cleaved at the second site to give L1 and L2 (Fig. 3b). Both the initial rise in the concentration of FLL DNA, to a maximum of ~40% of the total DNA, and the lag phase preceding the formation of L1 and L2 denote a sequential A → B → C pathway, with equal rates for the A → B and the B → C steps. Moreover, the rate at which SgfI utilized the one-site substrate (v₁ = 0.41 nM min⁻¹) equaled that for the utilization of the two-site substrate (v₂A = 0.44 nM min⁻¹). The SgfI endonuclease thus clearly cleaves DNA by means of independent reactions at individual sites.

Unlike SgfI, the SrFI and FseI endonucleases initially generated some OC DNA during their reactions on their one-site and two-site substrates, pDB7 and pDB8, respectively, and only later gave FLL DNA. But like SgfI, the rates at which SrFI and FseI utilized the one-site substrate were the same as those on the two-site substrate, and the two sites in pDB8 were cleaved sequentially at equal rates (data not shown). Thus, both SrFI and FseI also cleave DNA through independent reactions at individual sites. Indeed, the initial liberation of OC DNA during the reactions of these two enzymes indicates that they sometimes dissociate from the DNA after cutting one site in one strand.

Fig. 4. SgrAI on plasmids with one or two recognition sites. Reactions at 37 °C contained 50 units ml⁻¹ SgrAI and 20 nM DNA (~90% supercoiled) in 33 mM Tris acetate (pH 7.9), 10 mM magnesium acetate, 0.5 mM dithiothreitol and either 50 mM KOAc (left panels; a and b) or 200 mM KOAc (right panels; c and d). In the two upper panels (a and c), the DNA was pAT153, which has one SgrAI site. In the two lower panels (b and d), the DNA was pDB7, which has two SgrAI sites. Samples taken from the reactions at timed intervals were analyzed as above to obtain the concentrations of the following forms of the DNA: SC (●), OC (□), FLL (▲), and total DNA in the two final products cut at both sites (L1/2) (○); only in b and d.)
result in the cleavage of four phosphodiester bonds. Furthermore, the rate at which SgrAI utilized the two-site substrate (v_{2A} = 1.52 nM min^{-1}) was faster than the one-site substrate (v_{1} = 0.32 nM min^{-1}). The different kinetics of SgrAI on substrates with one or two recognition sites therefore eliminate the possibility that this enzyme acts through independent reactions at individual sites. In addition, while the enhanced reaction rate on pDB7 is consistent with SgrAI being a type IIe enzyme, the lack of accumulation of FLL DNA during this reaction discounts this possibility.

The 5-fold difference in the rates of the SgrAI reactions on two- and one-site substrates is, however, considerably smaller than the 20-fold difference recorded with SfiI (21, 22). Hence, the diminished yield of FLL DNA during the SgrAI reaction on pDB7 (Fig. 4b) might not be due solely to concerted action at two recognition sites, in the manner of SfiI. Instead, it may be due, at least in part, to processivity along the DNA. The reactions of SgrAI on pAT153 and pDB7 were therefore examined at varied ionic strengths; typical reaction records at an elevated ionic strength are shown in Fig. 4, c and d. In parallel, the reactions of an orthodox enzyme, SgfI, were also examined at varied ionic strengths. SgfI is optimally active in the presence of NaCl, but SgrAI is largely blocked by NaCl (data not shown), so the comparison between SgfI and SgrAI was made by using KOAc to vary the ionic strength.

The rates at which SgrI cleaved its two-site and one-site substrates both declined progressively with increasing concentrations of KOAc (data not shown). Nevertheless, the ratio of SgrI activities on the two substrates, v_{2A}/v_{1}, remained at unity at all ionic strengths tested (Fig. 5a). Similarly, the v_{2A}/v_{1} ratios for both SgrI and PacI were unaffected by doubling the ionic strengths of their reaction buffers (data not shown). The rate at which SgrAI utilized its one-site substrate also declined progressively with increasing KOAc concentrations (Fig. 4, a and c). In contrast, the rate at which SgrAI utilized its two-site substrate remained essentially constant at KOAc concentrations of ≤200 mM (Fig. 4, b and d) and was only reduced at ≥250 mM KOAc. Consequently, the ratio of the reaction rates of SgrAI on its two- and one-site substrates increased from 3 to 30 as the KOAc concentration was raised from 0 to 250 mM (Fig. 5b). The diminished yield of FLL DNA during the SgrAI reaction on pDB7 at 50 mM KOAc (Fig. 4b) therefore cannot be due to processivity.

At all concentrations of KOAc tested, SgrAI initially cleaved its one-site substrate in just one strand of the DNA to give the OC form (Fig. 4, a and c). However, although the rate of utilization of the two-site substrate for SgrAI remained constant as the KOAc concentration was increased to 200 mM, the initial products from its reactions on pDB7 at high ionic strengths differed from those at low ionic strength. At KOAc concentrations of ≤100 mM, SgrAI cleaved its two-site substrate in a highly concerted manner, converting almost all of the substrate directly to the final products cut in both strands at both sites (as noted above at 50 mM KOAc; Fig. 4b). In contrast, at concentrations of KOAc of ≥125 mM, SgrAI cleaved the two-site substrate in a sequential series of separate reactions, giving first the OC form and then the FLL form and only later L1 and L2 (in all cases, as in Fig. 4d). Like SfiI (22), both the difference between the reaction rates of SgrAI on one- and two-site substrates and the degree of its concertedness on the two-site substrate vary with the ionic strength of the reaction buffer. The behavior of SgrAI on its one- and two-site substrates matches the expectations for a restriction endonuclease that acts concertedly at two recognition sites.

**Other Restriction Enzymes with 8-bp Sites**—The MCS in pNEB193 contains single copies of the 8-bp sites for AscI, PacI, PmeI, and Sse8387I (Fig. 1b). A derivative of pNEB193, pAB1, was constructed with two copies of the MCS in inverted orientation (Fig. 1b). On pAB1, the distance between the pairs of recognition sites varied from 717 bp for AscI to 803 bp for Sse8387I. The SC forms of pNEB193 and pAB1 were used as one- and two-site substrates for these enzymes. An isoschizomer of Sse8387I, SdaI (Table I), was examined in the same manner. Both Sse8387I and SdaI are from *Streptomyces* species, and they also have in common with SfiI a G:C-rich recognition sequence, as does AscI and all four of the enzymes analyzed above (Table I). The restriction sites that are 8 bp long generally possess a marked preponderance (≥75%) of either G-C bp or A-T bp (1). PacI and PmeI provide two examples of the latter (Table I).

When assayed on pNEB193 and pAB1, Sse8387I, SdaI, PacI, and PmeI all behaved on the one- and two-site substrates in the same manner as SgfI (see Figs. 2 and 3). All four of these enzymes gave the same rates for the utilization of the one- and the two-site substrates (data not shown). All four cleaved the two-site substrate in sequential stages: first at one site to give FLL DNA and then at the second site to give L1 and L2, with the same rates for the two stages. This behavior was observed in both the standard reaction buffer for the enzyme in question and at elevated ionic strengths. Sse8387I, SdaI, PacI, and PmeI thus all cleave DNA via independent reactions at individual recognition sites in the orthodox manner for type II restriction enzymes.

AscI, however, showed a distinctive pattern of behavior on pAB1 (Fig. 6). Instead of cleaving this two-site substrate in sequential stages, first to FLL DNA and then after a lag phase to L1 and L2, the reaction of AscI on pAB1 at a low ionic strength yielded less of the FLL DNA than expected for a pathway involving two kinetically equal steps. It also yielded the doubly cut products directly from the start of the reaction rather than after a lag phase (Fig. 6a). However, at an elevated ionic strength, the reaction profile for AscI on its two-site substrate conformed to the expectations for a two-step sequential pathway, since it now gave rise first to FLL DNA and only later, after a lag phase, the doubly cut products (Fig. 6b).

The reaction of AscI on pAB1 at low ionic strength (Fig. 6a) is consistent with a value for v_{2A}, the rate for cutting the FLL DNA.
Restriction Enzymes with 8-bp Sites

Studies on the kinetics of SacI and SphI have been reported to date. However, SalI, SacI, and SphI all displayed the same activity on the one-site substrate, pNEB193, and the two-site-substrate, pAB1, and they all cleaved the latter by means of independent reactions at individual sites (data not shown).

**DISCUSSION**

Type II restriction enzymes are, conventionally, dimeric proteins that cleave DNA at individual sites (11), but SfiI is a tetramer that cleaves DNA only after binding to two copies of its recognition sequence (25). When the mechanism of SfiI was first characterized (21), no other type II enzyme was known to operate in this manner. SfiI is distinct from the type IIe enzymes such as EcoRII and NaeI (24). It carries out concurrent DNA cleavage reactions at two identical binding sites for its cognate DNA (23, 27), whereas the type IIe enzymes seem to have two dissimilar binding sites, with the DNA at one site acting solely as an activator for catalysis at the other site (15–18). In this study, a screen was developed to search for other endonucleases that require two sites for their catalytic reactions. The kinetics of a restriction enzyme on plasmids that have either one or two recognition sites for the enzyme were shown to provide a clear cut distinction between the following schemes: separate reactions at individual sites; processivity by translocation from one site to another without leaving the DNA; activation by a second copy of the recognition sequence, as in the type IIe systems; and concerted action at two recognition sites, like SfiI. The test was applied to 12 different endonucleases that recognize 8-bp sequences and/or come from *Streptomyces* species in the belief that these would be the most likely to act like SfiI.

Ten of the enzymes behaved in the conventional manner and cleaved individual sites in independent reactions. These included several *Streptomyces* enzymes that recognize either 8-bp sequences (SgfI, SfiI, Ssc8387I, and SdaI) or 6-bp sequences (SacI, SalI, and SphI). The *Streptomyces* enzymes that recognize 8-bp sites all act at G:C-rich sequences (Table I), but conventional behavior was also observed with other enzymes whose 8-bp sites are either G:C-rich, such as FaeI, or A:T-rich, such as PacI and Pmel. Concerted action at two recognition sites is clearly not a universal feature of the restriction enzymes that recognize 8-bp sequences nor of those from *Streptomyces* species. However, two enzymes deviated from the conventional pattern. In one case, AscI, the cleavage of the two-site substrate matched the expectation for a processive enzyme, at least at low ionic strength (Fig. 6a). The translocation of an enzyme from one specific site to another must involve a succession of transient associations with nonspecific DNA. Perhaps AscI dissociates from nonspecific DNA at a slower rate than the other enzymes tested here. The other exception, SgrAI, showed the pattern expected for an enzyme acting concerted at two recognition sites. None of the 12 enzymes followed the pathway proposed for the type IIe enzymes.

The kinetics of SgrAI on one-site and two-site substrates show that this enzyme needs two sites for optimal activity. On a DNA with one site, SgrAI presumably acts in trans, bridging sites on separate molecules, but the resultant complex has too short a lifetime to allow the enzyme to cleave more than one phosphodiester bond before it breaks down (Fig. 4a). On a DNA with two sites, SgrAI would act preferentially in cis, looping out the strength between two sites on the same molecule. At low ionic strength, the lifetime of the complex with sites in cis is long enough to allow the enzyme to cut both strands at both sites before it falls apart (Fig. 4b). The looped complex is likely to have a shorter lifetime at high ionic strength so that the enzyme then has only enough time to cut one phosphodiester bond (Fig. 4d). Nevertheless, as expected given the relative...
stabilities of DNA-protein complexes in *cis* over those in *trans* (28), elevated ionic strengths reduced SgrAI activity on the one-site DNA more severely than that on the two-site DNA (Fig. 5). In these respects, SgrAI behaves like SfiI (22). However, while the ability of SgrAI to cleave four phosphodiester bonds in one turnover suggests a tetrameric structure, this has yet to be established. In further experiments on SgrAI, the ratio of its activities on two- and one-site DNA increased with increasing concentrations of the protein, thus raising the possibility that SgrAI may exist as an inactive dimer and that two DNA-bound dimers associate to form an active tetramer.

The recognition sequence for SfiI contains a 5-bp interruption amid 8 specified bp and thus covers 13 bp, longer than is yet to be established. In further experiments on SfiI, bonds in one turnover suggests a tetrameric structure, but this activity was discovered by assaying cell-free extracts of bacterial cultures for the fragmentation of a test DNA, usually phage λ or adenoviral DNA (11). However, if an endonuclease needs two copies of an 8-bp sequence separated by an appropriate length of DNA, the test DNA may not be a substrate. For example, there are no SfiI sites on phage λ DNA, and the SfiI endonuclease was discovered by assaying extracts from *S. fimбриатус* on adenoviral DNA (33). Fortuitously, adenoviral DNA contains two SfiI sites separated by 1 kb, which is close enough for a looping reaction by SfiI on linear DNA (22). If the SfiI sites on this DNA had been separated by >10 kb, it is unlikely that its activity would have been detected. Hence, concerted action at two DNA sites may be a common feature of the type II restriction endonucleases present in nature but which have yet to be discovered by in vitro assays.

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