One recognition sequence, seven restriction enzymes, five reaction mechanisms

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ABSTRACT

The diversity of reaction mechanisms employed by Type II restriction enzymes was investigated by analysing the reactions of seven endonucleases at the same DNA sequence. NarI, KasI, Mly113I, SfoI, EgeI, EheI and BbeI cleave DNA at several different positions in the sequence 5′-GGCGCC-3′. Their reactions on plasmids with one or two copies of this sequence revealed five distinct mechanisms. These differ in terms of the number of sites the enzyme binds, and the number of phosphodiester bonds cleaved per turnover. NarI binds two sites, but cleaves only one bond per DNA-binding event. KasI also cuts only one bond per turnover but acts at individual sites, preferring intact to nicked sites. Mly113I cuts both strands of its recognition sites, but shows full activity only when bound to two sites, which are then cleaved concertedly. SfoI, EgeI and EheI cut both strands at individual sites, in the manner historically considered as normal for Type II enzymes. Finally, BbeI displays an absolute requirement for two sites in close physical proximity, which are cleaved concertedly. The range of reaction mechanisms for restriction enzymes is thus larger than commonly imagined, as is the number of enzymes needing two recognition sites.

INTRODUCTION

Type II restriction endonucleases recognize specific DNA sequences and cleave DNA at specified locations within or adjacent to their recognition sites, in reactions that normally require only Mg^{2+} as a cofactor (1–3). In many cases, the recognition sequence is a symmetrical palindrome of 4–8 consecutive base pairs, though some recognize discontinuous palindromes, interrupted by a segment of specified length but unspecified sequence (4,5). Many of the enzymes that act at palindromic sites are dimers that interact symmetrically with their targets, positioning one active site on each strand of the DNA: e.g. EcoRI, EcoRV and BamHI (6). They normally cut both strands within the lifetime of the enzyme–DNA complex (7–9). The phosphodiester bonds cleaved by these enzymes are usually located within the sequence: in some cases, near the 5′ ends, to leave duplexes with 5′ single-strand extensions; in others, at the middle of the site, to leave flush-ended duplexes; in further cases, near the 3′ ends. However, a subset of the Type II systems, called Type IIS (5), recognize asymmetric sequences and cleave both strands at specific locations on one side of the recognition site. Another subset, the Type IIB enzymes, also cut the DNA at specified positions distant from their recognition sites, but on both sides of the sequence.

The myriad applications of these enzymes in molecular biology (10) have driven extensive searches for new nucleases with novel specificities. At present, more than 3500 Type II enzymes have been identified from many different species of bacteria, and these encompass about 240 distinct recognition sequences (4). Hence, in many instances, two or more enzymes from different species have a common recognition site. In these instances, the first enzyme found to recognize a particular sequence is known as the prototype, while others that cleave the same sequence are called isoschizomers. Some isoschizomers cleave at different positions from the prototype and these are called neoschizomers (5). With the exceptions of certain isoschizomers, the Type II endonucleases show little similarity in amino acid sequence (11), apart from some residues that bind the Mg^{2+} ions needed for catalysis (12–14). Nevertheless, restriction enzymes with dissimilar amino acid sequences often have similar core structures (3,6). Isoschizomers can, however, be virtually identical to each other, with >50% amino acid identity (15,16), but some isoschizomers lack any similarity in primary sequence (17). Neoschizomers are expected to deviate from the prototype, since they must position their active sites against the DNA differently (12), but this re-positioning need not involve radically different architectures. For example, EcoRV and BglI cleave DNA to leave flush-ended or 3′-extended fragments, respectively, yet the individual subunits in these two enzymes are very similar: they differ only at the subunit interface (18) and operate by similar mechanisms (19).

The restriction enzymes often taken to represent the Type II systems, such as EcoRV and BamHI, act at individual copies of their recognition sites (2,3). But many restriction enzymes become active only after interacting with two copies of the target sequence (2,20,21). The Type II enzymes that need two sites include some that cleave at a palindromic sequence (22–30), others (the Type IIS enzymes) that act adjacent to asymmetric sites (31–34), and still others (the Type IIB enzymes) on both sides of their targets (35). A number of the restriction enzymes that interact with two sites, the Type IIE systems, use one copy of the sequence to activate

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the enzyme to cleave a second copy: these enzymes are usually dimers but with two functionally distinct DNA-binding clefts; one with the catalytic functions for DNA cleavage and one for the activator (22,25). After interacting with two sites, a Type IIE enzyme cuts just one of the sites (31,36). For another group of enzymes that interact with two sites, the Type IIF systems, the interaction leads to the concerted cleavage of the DNA at both sites (20,21). The Type IIF enzymes usually act as tetramers (37), with two identical DNA-binding clefts, each made of two subunits, but they seem to have virtually no activity until both clefts are filled with cognate DNA (38).

The Type I and the Type III restriction endonucleases need to interact with two copies of their recognition site for most—or all—of their DNA cleavage reactions (21,39), since these often require the collision of two enzyme molecules bound to separate sites after they have pushed the intervening DNA into expanding loops (39). The same applies to most enzymes that restrict methylated DNA (39). Amongst the Type II enzymes, the majority of the Type IIS enzymes (31), and almost all of the Type IIB enzymes (J. J. T. Marshall, D. M. Gowers and S. E. Halford, unpublished data), display full activity only after binding two copies of their recognition site. But it has yet to be established what fraction of the Type II enzymes with palindromic sites need two sites. To address this question, we examined seven Type II endonucleases that all cleave DNA at one palindromic sequence, 5′-GGCGCC-3′. The reason for selecting these enzymes is that this particular sequence is cleaved at a greater variety of positions than any other hexanucleotide site for the Type II enzymes (4): the cleavage points in this sequence include four of the five internal phosphodiester bonds. The seven enzymes were tested against DNA substrates with one or two copies of this sequence, to determine whether they need to bind to two sites and, if so, how many phosphodiester bonds are cleaved per turnover.

MATERIALS AND METHODS

Enzymes

Restriction enzymes were obtained from the following suppliers and stored at −20°C: BbeI, Takara Biomedicals, Japan; EgeI and Mly113I, SibEnzyme, Russia; EheI, MBI Fermentas, Lithuania; KasI, NarI and SfoI, New England Biolabs, USA. Their concentrations are noted in terms of units of enzyme activity per millilitre, as specified by the supplier. Immediately prior to use, the enzymes were diluted to the requisite concentration in the reaction buffer advised by the supplier supplemented with 50% (v/v) glycerol. The resolvase from the transposon Tn21 was purified and used as before (40). All other enzymes were from New England Biolabs.

DNA

The plasmid pUC19 has a single copy of the GGCGCC sequence, at position 235 (41). A plasmid with two copies, pDG5, was constructed from pMLE1 (36), a derivative of pUC19, by using standard procedures (10) to clone at a BcgI site (that at position 1287) a duplex made from two oligodeoxyribonucleoetides. The duplex contained the sequence TACCGCATCAGGCGCCATTCGCCATT (site

underlined) so that both NarI sites in pDG5 have the same 10 bp flanking sequences as the site in pUC19.

Transformants of *Escherichia coli* HB101 (10) with pUC19 or pDG5 were cultured in M9 minimal media containing 37 MBq/l [*methyl*-³H]thymidine (Amersham Biosciences) and the plasmids purified by CsCl density-gradient centrifugations (36). The preparations generally contained 85–95% supercoiled monomeric plasmid, with 5–15% as open-circle (OC) and dimeric forms. The supercoiled form of pDG5 was converted into a catenane by reactions with 10-fold molar excess Tn21 resolvase, as described previously (42). DNA concentrations were assessed from A_{260} measurements.

Reactions

Reactions were conducted initially in the reaction buffer advised by the supplier of the enzyme: for NarI, My113I, EgeI and BbeI, buffer A—10 mM Tris–HCl (pH 7.4), 10 mM MgCl₂, 1 mM dithiothreitol and 100 µg/ml bovine serum albumin; for KasI and SfoI, buffer B—10 mM Tris–HCl (pH 7.4), 10 mM MgCl₂, 1 mM dithiothreitol, 50 mM NaCl and 100 µg/ml albumin; for EheI, buffer C—33 mM Tris–acetate (pH 7.9), 10 mM magnesium acetate, 66 mM potassium acetate and 100 µg/ml albumin. Some further experiments used the recommended buffer supplemented with additional NaCl.

The reactions, usually at 37°C, contained 5 nM DNA (³H-labelled) in 200 µl of the appropriate buffer. An aliquot (10 µl) was removed, to act as a pre-reaction control, before initiating the reaction by adding 2–4 µl of enzyme that had been freshly diluted to the requisite concentration. At timed intervals, aliquots were removed and quenched immediately by mixing with an equal volume of 100 mM EDTA in 0.1 M Tris–HCl (pH 8.0), 40% sucrose and 0.1% bromophenol (27,31). The aliquots were analysed by electrophoresis through agarose under conditions that gave the optimal separation of the various cleaved forms of the DNA from each other and from the initial substrate (36,42). The segments of the gels that encompassed each form were analysed by scintillation counting (19,29), to determine the concentration of each form at each time point sampled. The concentrations shown are the means from four independent experiments: for clarity, the error bars for the standard deviations (typically <10% of the mean values) have been omitted. The data were fitted to the appropriate function(s) (43) by non-linear regression in GRAFIT (Erithacus Software, Slough, UK) or by numerical integration in SCIENTIST (MicroMath Scientific Software, Salt Lake City, UT).

RESULTS

Experimental strategy

The mode of action of a restriction endonuclease can be deduced from its reactions on two substrates: one with a single copy of its recognition sequence and one with two copies (19,29,31,36). Given identical—or at least functionally equivalent—flanking sequences around each site (44), an enzyme that acts at solitary sites should cleave the substrate with one site at the same rate as that with two sites, and will cleave each site in the two-site substrate in separate but

kinetically equal reactions. But if the enzyme acts processively on the two-site DNA, the maximal amount cut at one site generated during the reaction will be lower than the level expected for a distributive reaction, 40% of the total DNA. On the other hand, an enzyme that binds two sites is almost certain to have a higher affinity for DNA with two sites than DNA with one site, as the former permits the interaction *in cis* and the latter only *in trans* (21). If the DNA is at a concentration below its K_m, the difference in affinity will result in the two-site DNA being cleaved faster than the one-site DNA. Conversely, if the DNA concentration is above the K_m, the one- and the two-site substrates may be cleaved at equal rates, but raising the salt concentration of the reaction buffer can often raise the K_m. Hence, several restriction enzymes cleave one- and two-site substrates at equal rates in reactions at low salt but cleave the two-site DNA more rapidly than the one-site DNA at high salt (29,33,45). The initial products from the two-site substrate reveal the nature of the interaction with two sites: for a Type IIE enzyme such as NaeI, DNA cut at one site; for a Type IIF enzyme such as SfiI or NgoMIV, DNA cut at both sites, bypassing species cut at just one site (36).

Further information on the mode of action of a restriction enzyme interacting with two sites can be obtained from its reaction on a catenane consisting of two interlinked circles of covalently closed DNA, with one site in each ring (42,46,47). On such a catenane, a protein cannot translocate from one ring to the other by a one-dimensional (1-D) process, whereas bridging interactions between the rings can occur through three-dimensional (3-D) space as readily as those between two sites in one circle.

NarI, KasI, Mly113I, SfoI, EgeI, EheI and BbeI all cleave DNA in the sequence GGCGCC, at a variety of positions (Figure 1A). The plasmid pUC19 has a single copy of this sequence and a plasmid with two copies, pDG5 (Figure 1B), was constructed from a derivative of pUC19 (36). The pDG5 sites both had the same flanking sequences as the pUC19 site, thus excluding variations between sites. The two sites in pDG5 are interspersed with two *res* sites from the transposon Tn*21*, so site-specific recombination *in vitro*, by the resolvase from Tn*21* (40), converts this DNA into a catenane made up of two interlinked circles of covalently-closed DNA, with one copy of the target sequence in each ring (Figure 1B).

The various mechanisms noted above can be distinguished by steady-state reactions on these three substrates, with the enzyme at a lower concentration than the DNA, but not by single-turnover reactions with excess enzyme. The reactions at low enzyme concentrations reveal the forms of the DNA liberated from the enzyme whereas the products observed in reactions with excess enzyme may still be bound to the enzyme (31,36). However, the concentrations of all of the enzymes tested here are in terms of units of enzyme activity rather than molarity. Consequently, to determine whether steady-state conditions pertained, the reactions of almost all of these enzymes were examined across a range of enzyme concentrations. In all cases tested, the reaction velocities increased linearly with the number of units of enzyme activity (data not shown). If the reactions had been under single-turnover conditions, the velocities would most likely have remained constant, regardless of the number of units added. The reactions were thus conducted under steady-state conditions. Even though the enzyme concentrations in these
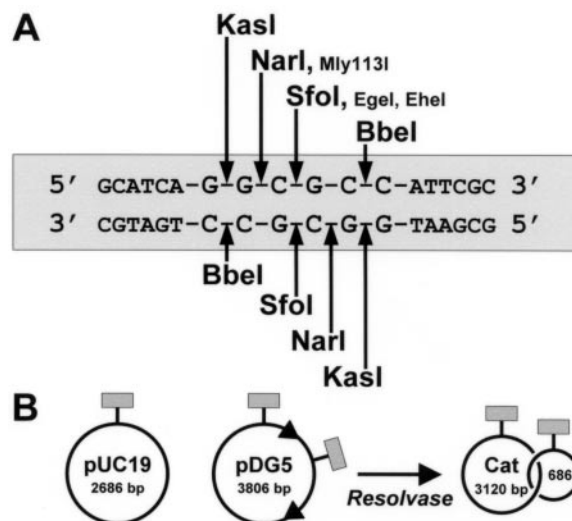

Figure 1. Enzymes and substrates. (**A**) Shown, within the grey box, are the sequence GGCGCC and the sequences flanking this site in pUC19. The enzymes KasI, NarI, Mly113I, SfoI, EgeI, EheI and BbeI cleave GGCGCC at the positions shown. (**B**) The plasmids pUC19 (2686 bp) and pDG5 (3806 bp) contain, respectively, one and two copies of the sequence in (A), indicated by the grey box(es). In pDG5, the two GGCGCC sites are separated by 1063 (or 2743 bp) and are interspersed with two *res* sites from Tn*21* (arrowheads). The action of Tn*21* resolvase at the *res* sites generates a catenane consisting of two interlinked circles of covalently closed DNA, one large (3120 bp) and one small (686 bp). Both circles carry one copy of the GGCGCC sequence (grey box).

reactions were almost certainly lower than the DNA concentration, it is impossible to give a precise value for the molarity of the enzyme in the commercial preparations used here, since these were of indeterminate purity.

NarI at GG↓CGCC

NarI is the prototype of the restriction enzymes that cleave DNA at the sequence GGCGCC: it cleaves the bond after the second nucleotide (Figure 1A). It is inactive at some NarI sites but can be activated to cleave resistant sites by adding a duplex with the recognition sequence (23,24). NarI may therefore need to interact with two copies of its site. This possibility was examined by testing NarI against plasmids that carry either one or two copies of the GGCGCC sequence (Figure 2).

NarI cleaved the plasmid with one site in just one strand, converting the supercoiled (SC) substrate almost quantitatively to the open-circle form (Figure 2A). Cleavage of the opposite strand, to convert the OC DNA to the full-length linear (FLL) form, took much longer and was <50% complete even after 18 h. The SC plasmid with two sites was also converted first to OC DNA, cut in one strand at one or both sites. The rate of utilization of the SC plasmid with two sites was almost 20 times faster than that for the plasmid with one site (Table 1). Moreover, with the two-site plasmid, the OC form was converted at a relatively rapid rate to the FLL form, in which both strands are cut at one of the two sites (Figure 2B).

NarI thus differs from the orthodox Type II restriction enzymes like EcoRI and EcoRV in at least two respects.
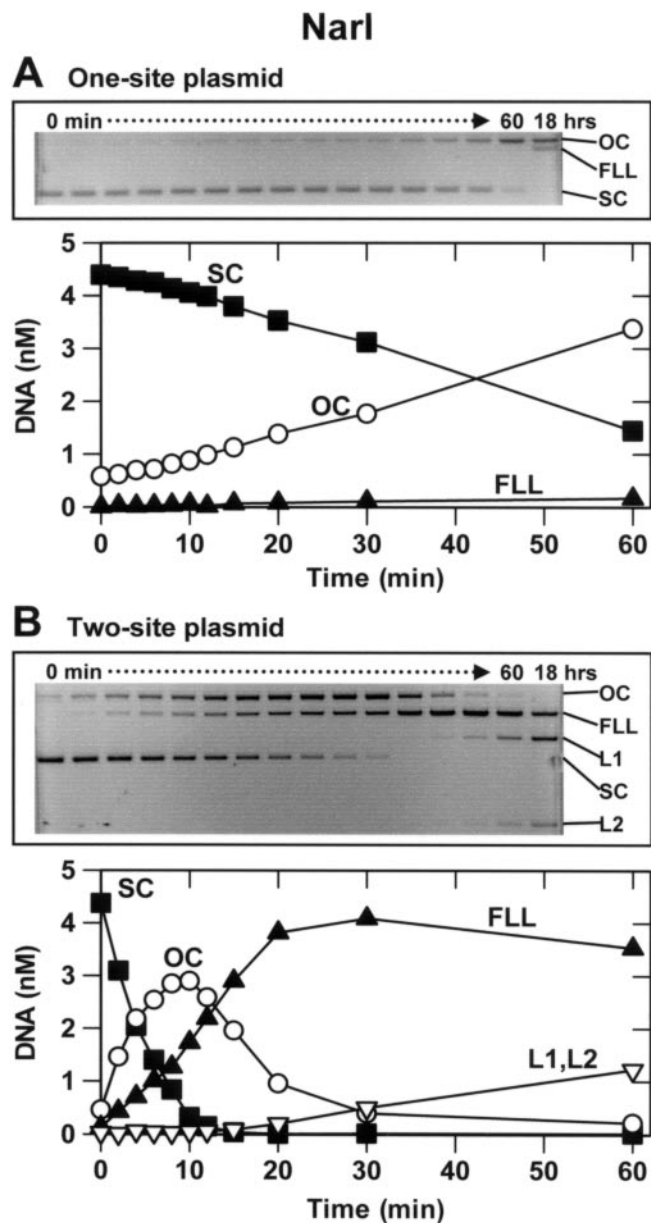
NarI

A One-site plasmid

B Two-site plasmid

Figure 2. NarI on one- and two-site plasmids. Reactions contained 40 U/ml NarI and 5 nM supercoiled (SC) DNA in buffer A at 37°C. The DNA was in (**A**), pUC19, with one NarI site; in (**B**), pDG5, with two NarI sites. In both cases, the upper panel shows an agarose gel of samples taken from the reaction at the times indicated: the electrophoretic mobilities of the open-circle (OC), full-length linear (FLL) and SC DNA are marked on the right: in (B), the two linear products from cutting both sites (L1 and L2) are also marked. The lower panels show the concentrations of the following forms of the DNA during the first 60 min of the reaction: SC substrate, black squares; OC DNA, white circles; FLL DNA, black triangles; (in B alone), the mean of the two final products (L1,L2), white inverted triangles.

First, instead of acting at a solitary site (19), it needs to interact with two copies of its recognition sequence for full activity. Second, after binding to a solitary site, the orthodox enzymes generally cut both strands before dissociating from the DNA (7–9) but NarI, even when bound to two sites, still cuts only one phosphodiester bond before leaving the DNA.

The reactions of NarI were characterized further by using as the substrate a catenane derived from the two-site plasmid (Figure 3). The catenane contains one large (3120 bp) and one small (686 bp) ring of SC DNA, with one copy of the target sequence in each (Figure 1B). Catenanes not only reveal the nature of communications between distant DNA sites, but can also yield insights into the cleavage of the individual phosphodiester bonds in the substrate. The formation of OC DNA from a SC plasmid with two recognition sites (Figure 2B) could be due to cutting any one of four phosphodiester bonds, or to one bond at each site. But a catenane gives rise to distinct products from nicking the large ring, the small ring or both (Figure 3A). Similarly, the formation of FLL DNA from the two-site plasmid fails to reveal which of the two sites is cut in both strands, or whether the second site is nicked, but again distinct products are formed from the catenane after cutting any two or three phosphodiester bonds.

NarI gave the same rate of substrate utilization on the catenane as on the two-site plasmid: i.e. much faster than the one-site plasmid (Table 1). Hence, NarI must interact with two sites through 3-D space and not by following the 1-D contour between the sites. The reaction on the catenane also permitted evaluations of the amounts of the intact DNA and the DNA that had been cleaved at one, two, three and four phosphodiester bonds (Figure 3B). The experimental data for the formation and/or decay of all five of these species (Figure 3B) were compared to the reaction scheme in Figure 3A. These were generated by numerical integration in SCIENTIST, using various values for the rate constants for the individual steps in this scheme: for each model, the rate constant(s) were varied until the sum-of-squares deviation from the experimental data reached a minimum. The first model tested used a single rate constant for all of the steps (48), but the rate constant that gave the minimal deviation yielded theoretical curves that deviated wildly from the experimental data (Supplementary Material, Figure S1A). Two further models employed two rate constants: either one for all of the steps *in cis* and another for all steps *in trans*; or one for all of the reactions at an intact recognition site and another for all of the reactions at a nicked site. The optimal fits upon varying two rate constants gave theoretical progress curves that lay closer to the experimental data than the single rate constant model, but which still deviated substantially from the experimental data (Supplementary Material, Figure S1B). The simplest scheme that matched the data required four rate constants: one for reactions *in cis* at intact sites; a second, twice as slow, for reactions *in cis* at nicked sites; a third, six times slower, for reactions *in trans* at intact sites; the fourth, 15 times slower, for reactions *in trans* at nicked sites (Supplementary Material, Figure S1C).

The NarI endonuclease thus acts most readily at intact copies of its recognition sequence, on DNA molecules that carry a second copy of the sequence, but it then cleaves just one strand at one site. In a separate reaction, it then cleaves the intact strand of the nicked site at a slow rate. These reactions were also studied at various pH values between 9.7 and 5.6 (data not shown). While the reactions at alkaline pH values liberated large amounts of OC DNA (viz. Figure 2), the maximal amounts of OC DNA formed during the

Table 1. Enzymes at GGCGCC

| Enzyme | Source | Reaction buffer | Enzyme (U/ml) | Initial rates of substrate utilization (nM DNA/min) | | | Ratio (two-site rate/one-site rate) |
				One-site[a]	Two-sites[b]	Catenane[c]	
NarI	*Nocardia argentinensis*	A	40	0.04	0.71	0.75	18
KasI	*Kluyvera ascorbata*	B	40	0.91	0.73	1.02	1.0
Mly113I	*Micrococcus lylae*	A	5	0.07	0.08	0.11	1.3
Mly113I	—	A + 0.15 M NaCl	5	0.72	2.87	nd	4
Mly113I	—	A + 0.2 M NaCl	5	0.29	3.63	nd	13
SfoI	*Serratia fonticola*	B	12	2.7	2.1	nd	0.8
EgeI	*Enterobacter gergoviae*	A	5	1.43	1.24	nd	0.9
EheI	*Erwinia herbicola*	C	5	1.63	1.37	nd	0.8
BbeI	*Bacillus brevis*	A	30	0.003	0.92	1.05	330

Reactions contained the enzyme at the concentration listed, in the buffer indicated, and 5 nM DNA at 37°C (except for SfoI, at 21°C). Rates of substrate utilization were evaluated from the initial linear decline in substrate concentration with time. For ratios of rates, the two-site rate denotes, wherever possible, the mean of the rates on pDG5 and the catenane.
[a]The DNA was pUC19, with one GGCGCC site.
[b]The DNA was pDG5, with two sites.
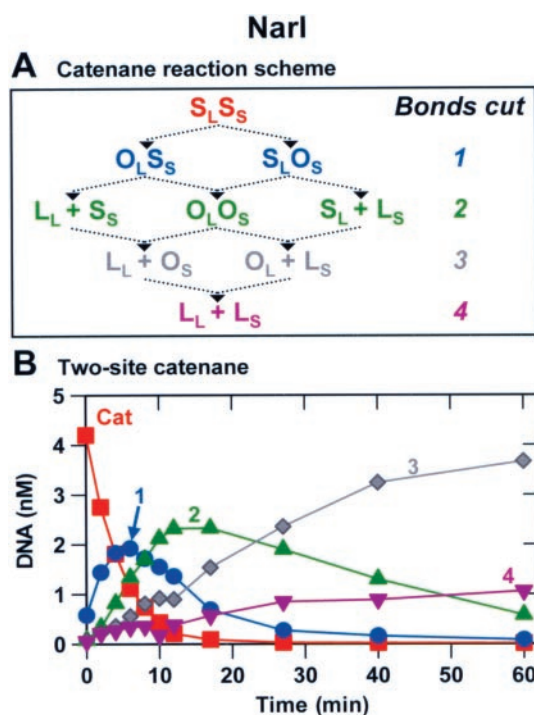[c]The catenane was derived from pDG5, with one site in each ring.
nd—not determined.

Figure 3. NarI on catenated DNA. (**A**) The scheme shows all of the distinct products from cleaving a catenane in one site in each ring: S, O and L denote supercoiled, open-circle and linear DNA respectively. Shown in red; the intact catenane containing two interlinked rings of SC DNA (S_LS_S), one large (3120 bp) and one small (686 bp), marked, respectively, by the subscripts 'L' and 'S'; in blue, the products after cutting one phosphodiester bond, two interlinked rings nicked in either the large (O_LS_S) or the small (S_LO_S) ring; in green, the products after cutting two bonds, either one linear and one supercoiled species ($L_L + S_S$ from cutting both strands in the large ring, or $S_L + L_S$ from cutting both in the small) or two interlinked rings of OC DNA (O_LO_S); in grey, the products after cutting three bonds, one linear and one OC form ($L_L + O_S$ or $O_L + L_S$); in purple, the large (L_L) and the small (L_S) linear products after cutting all four bonds. (**B**) The reaction contained 40 U/ml NarI and 5 nM Cat (the catenane from pDG5; Figure 1B) in buffer A at 37°C. Samples from the reactions were analysed by electrophoresis through agarose to separate, wherever possible, all of the species in (A). The concentrations of the following forms were assessed: intact catenane, red squares; the sums of the products cleaved in one (blue circles), two (green triangles), three (grey diamonds) and four (purple inverted triangles) phosphodiester bonds. (In cases where the product contains unlinked species, e.g. $L_L + S_S$, the mean of the two concentrations is noted.)

reactions declined at lower pH values, in a manner consistent with a pK_a of 6.4. At pH values below 6, NarI cleaved both strands without liberating the OC form. Since the observed pK_a is close to the secondary pK_a of phosphate at 6.8, the reluctance of NarI to cleave a nicked site at alkaline pH may be due to the second negative charge on the 5′ phosphate.

KasI at G↓GCGCC

KasI is a neoschizomer of NarI: it cuts the same sequence but after the first rather than the second nucleotide (Figure 1A). Like NarI, KasI cleaved the plasmid with one copy of the GGCGCC sequence first in one strand, to release the OC form, and then, in a separate reaction, in the other strand, to generate the FLL DNA (Figure 4A). When the formation and decay of the OC form was fitted to the equation for B in the reaction scheme A→B→C (43), the best fit was obtained with a 25-fold higher value for the first rate constant over the second. Hence, after cutting one strand of the DNA at an intact copy of its recognition site at a relatively rapid rate, KasI cleaves the second strand at a much slower rate. But unlike NarI, this ratio did not alter with the pH of the reaction (data not shown). The reluctance of KasI to cleave the intact strand of a nicked site is unlikely to be due to the negative charge on the 5′ phosphate in the nicked strand.

KasI also cleaved the two-site DNA in sequential stages, cutting first one strand at one (or both) sites to generate the OC DNA, then cutting both strands at one site to give the FLL form and finally both strands at both sites, to yield the two linear fragments L1 and L2 (Figure 4B). However, in marked contrast to NarI, no significant difference was observed between the rates at which KasI consumed the one- and two-site plasmids (Table 1). Nor was any difference observed as the salt concentration was increased from 50 to 200 mM (data not shown). Hence, it is most unlikely that KasI needs to interact with two recognition sites before cleaving the DNA. It almost certainly acts at individual sites.

From the catenane (Figure 3A), KasI generated substantially more of the intermediates cleaved at two phosphodiester bonds than at any other stage. Moreover, under conditions

where the rate of utilization of the catenane was similar in the KasI and NarI reactions (Table 1), the intermediates cleaved at two bonds persisted for much longer with KasI (Figure 4C) than with NarI (Figure 3B). Further analysis of the intermediates cut by KasI at two bonds revealed a large preponderance
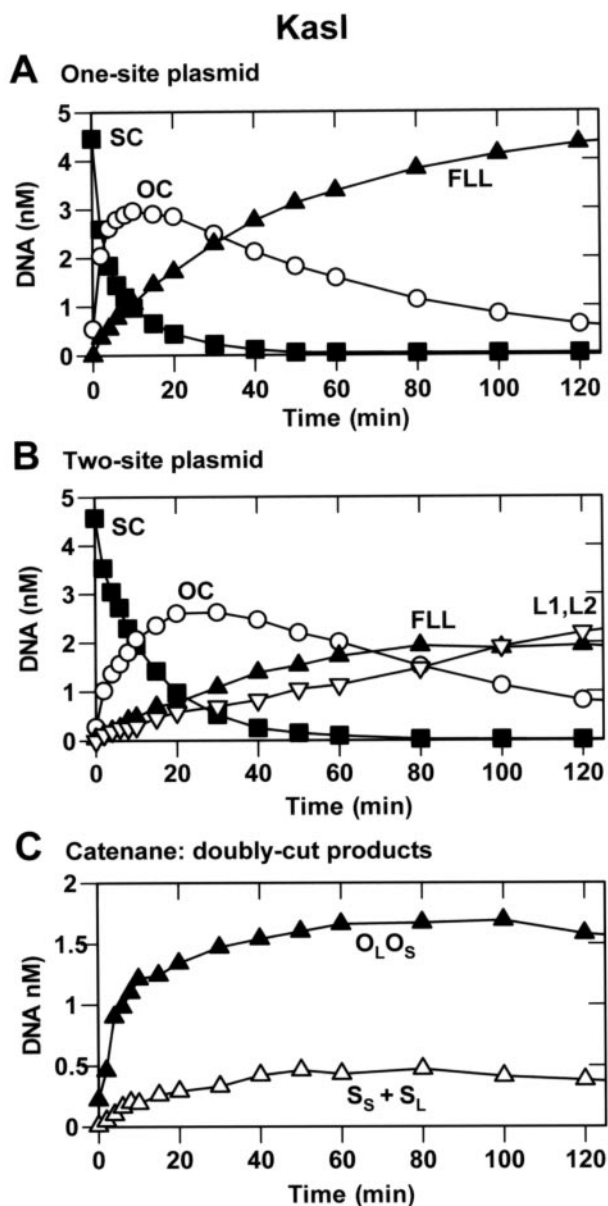
of the doubly nicked species ($O_L O_S$) over the species carrying a double-strand break at one site, in either the small ring (to produce $L_S + S_L$) or the large (to yield $L_L + S_S$). Hence, given a choice between cutting one strand at each of two separate copies of its intact site, or cutting both strands at one site, KasI carries out separate reactions at both intact sites. The primary activity of KasI is thus to make single-strand breaks at its recognition sites rather than double-strand breaks.

Mly113I at GG↓CGCC

Mly113I cleaves the GGCGCC sequence in the same position as the prototype NarI and might thus be expected to act like NarI. In the reaction buffer recommended for Mly113I, that lacks NaCl, Mly113I cleaved the SC plasmid with one recognition site directly to the FLL form (Figure 5A), without any accumulation of the OC intermediate that had been formed in the NarI reaction (Figure 2A). The ability of Mly113I to cleave both strands during one DNA-binding event thus differs from NarI. It matches instead the behaviour of the orthodox Type II restriction enzymes like EcoRV and EcoRI (7,8). In the buffer without NaCl, Mly113I cleaved the two-site plasmid (Figure 5B) and the catenane with one site in each ring at similar rates to the one-site plasmid (Table 1). So in this respect as well, Mly113I acts like an orthodox Type II enzyme acting at individual sites (19,29). However, under these conditions, the two-site plasmid was cleaved directly to the final products, the two linear fragments—L1 and L2—from cutting
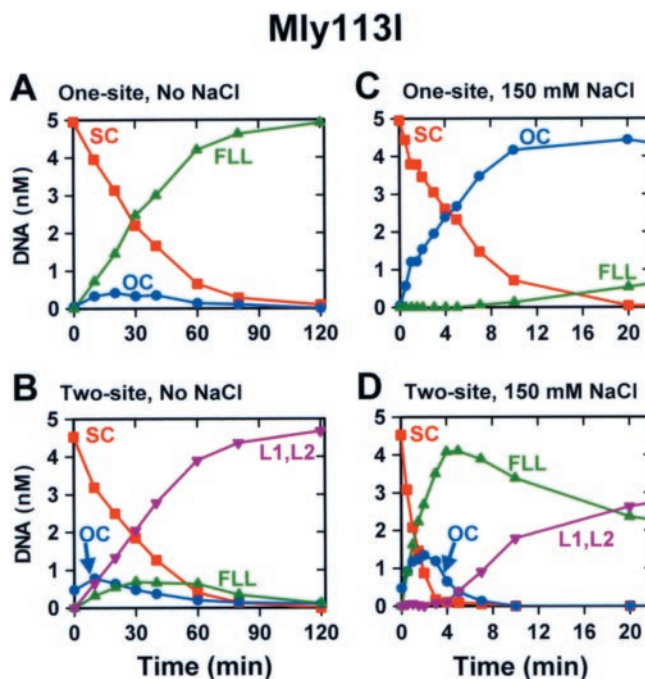

Figure 4. KasI on one- and two-site DNA. Reactions contained 40 U/ml KasI and 5 nM SC DNA in buffer B at 37°C. The DNA was in (**A**), pUC19, with one KasI site; in (**B**), pDG5, with two KasI sites; in (**C**), Cat (Figure 1B), with one KasI site in each ring. Samples taken from the reactions were analysed by electrophoresis through agarose. For the reactions in (A) and (B), the following were assessed: SC substrate, black squares; OC DNA, white circles; FLL DNA, black triangles; (in B alone), the mean of the two final products (L1,L2), white inverted triangles. For the reaction in (C), the concentrations of the various products from a catenane with one recognition site in each ring (Figure 2A) were assessed as in Figure 2B. Shown here are the products cleaved at two phosphodiester bonds: the DNA cleaved in one strand at each site (i.e., the catenane containing two interlinked rings of nicked DNA, $O_L O_S$), black triangles; the sum total of the two species cut in both strands at one site (in either the small ring, to release S_L, or the large ring, to release S_S), white triangles.

Figure 5. Mly113I on one- and two-site DNA at varied salt. Reactions at 37°C contained 5 U/ml Mly113II and 5 nM SC DNA in either buffer A (**A** and **B**) or buffer A + 150 mM NaCl (**C** and **D**). The DNA was either pUC19, with one Mly113I site (A and C), or pDG5, with two Mly113I sites (B and D). Samples were taken from the reactions at various times and were analysed by electrophoresis through agarose. The concentrations of the following forms of the DNA were assessed: SC substrate, red squares; OC DNA, blue circles; FLL DNA, green triangles; (in B and D) the mean of the two final products (L1,L2), purple inverted triangles.

both strands at both sites, without liberating the FLL form cut at one site (Figure 5B). Similarly, the catenane (S_LS_S; Figure 3A) was cleaved directly to the two final products, $L_L + L_S$, bypassing almost completely all of the intermediates en route (data not shown).

The direct conversion of a two-site substrate to the products cut at both sites could be due to processivity: maybe after cutting one site, Mly113I translocates efficiently to the other site by an intramolecular process and so cleaves both sites before leaving that individual molecule of DNA (48). Alternatively, it could be due to a concerted reaction at both sites, in the manner of a Type IIF enzyme like SfiI, though the identical rates on the one- and the two-site plasmids imply that this enzyme can achieve its V_{max} rate not only upon interactions with two sites *in cis* but also with sites *in trans* (29,45). To distinguish these possibilities, the Mly113I reactions on the two plasmids were analysed in the presence of various NaCl concentrations, in the range 0–200 mM (Figure 5C and D; Table 1). The rates for the utilization of the one- and two-site substrates both varied with the NaCl concentration but more so on the two-site plasmid than on the one-site plasmid (Table 1). Consequently, the 1:1 ratio of the two-site:one-site rate in the absence of NaCl changed to nearly 2:1 in the presence of 100 mM NaCl (data not shown), 4:1 in 150 mM salt and 13:1 in 200 mM salt (Table 1). In addition, increased NaCl concentrations caused Mly113I to dissociate from the one-site plasmid after cleaving just one phosphodiester bond, to liberate the OC form (Figure 5C), and to dissociate from the two-site plasmid mainly after cleaving two phosphodiester bonds, to liberate the FLL form (Figure 5D).

While a processive scheme could account for the increased yield of partially cleaved products at high salt, processivity can never by itself result in the initial reaction on the two-site DNA becoming faster than that on the one-site DNA. Hence, the fact that Mly113I cleaves the two-site plasmid more rapidly than the one-site at high salt shows that it must interact with two sites before cleaving DNA. The Mly113I sites can be either *in trans*, on two separate molecules of DNA, or *in cis*, on the same DNA, but its complex with sites *in trans* is less stable than that with sites *in cis*. Consequently, elevated salt concentrations disrupt the *trans* complex more than the *cis* complex, so that the *trans* complex not only fails to achieve the V_{max} rate, it also falls apart after cutting just one phosphodiester bond. In contrast, the lifetime of the *cis* complex at elevated salt still allows the enzyme to cut at least two phosphodiester bond before falling apart [viz. (45)].

SfoI, EgeI, EheI at GGC↓GCC

SfoI, EgeI and EheI all cut the central phosphodiester bond of the GGCGCC sequence to leave flush-ended DNA (Figure 1A). SfoI reactions were first carried out at the recommended temperature of 37°C but these reactions stopped before all of the DNA was cleaved (data not shown). The amount of DNA cleaved increased as the temperature was reduced, probably as a result of the enzyme surviving for longer, and at 21°C the reaction continued through to completion (Figure 6). At 21°C, SfoI cleaved the one-site plasmid directly to the FLL form, without releasing any of the nicked OC DNA (Figure 5A). Hence, it cuts its recognition sequence in both strands within a single DNA-binding event. On the
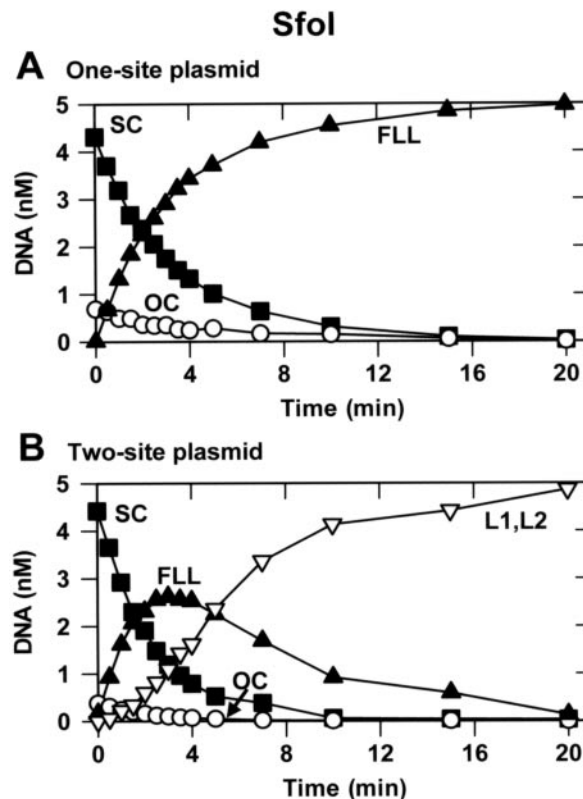

Figure 6. SfoI on one- and two-site DNA. Reactions contained 12 U/ml SfoI and 5 nM SC DNA in buffer B at 21°C. The DNA was: in (**A**), pUC19, with one SfoI site; in (**B**), pDG5, with two SfoI sites. Samples were taken from the reactions at various times and analysed to assess the following: SC substrate, black squares; OC DNA, white circles; FLL DNA, black triangles; [in (B) alone], the mean of the two final products (L1, L2), white inverted triangles.

two-site plasmid, SfoI again bypassed the OC form cut in one strand and instead proceeded directly to the FLL form cut in both strands at one site at 21°C: the FLL form was subsequently cleaved at the residual site, in both strands, to yield the two end products, L1 and L2 (Figure 6B). The formation and decay of the FLL form during the reaction on the two-site plasmid matched that expected (43) for a two-step consecutive reaction, A→B→C, with equal rates for first and second steps. Moreover, the rate of cutting either site on the two-site plasmid fell close to that for cleaving the single-site plasmid (Table 1). SfoI must therefore cleave a DNA with multiple recognition sites by means of separate and independent reactions at each site, each resulting in the cleavage of both strands at that site.

In their reactions at 37°C, both EgeI and EheI behaved in the same way as SfoI. They also cleaved the two-site substrate in two separate but kinetically equal reactions (data not shown), and they again gave similar rates on the one- and two-site plasmids (Table 1).

BbeI at GGCGC↓C

The final enzyme in this series, BbeI, cleaves the fifth phosphodiester bond within the GGCGCC sequence (Figure 1A), and is the only one to leave 3′ single-strand extensions. Under its recommended reaction conditions, BbeI showed virtually
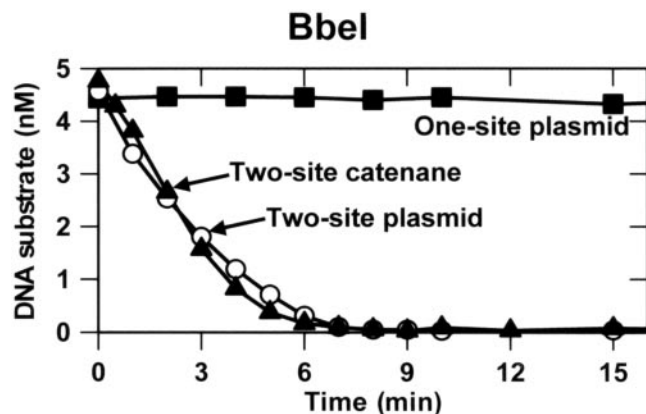
Bbel

Figure 7. BbeI on one- and two-site DNA. Reactions in buffer A at 37°C contained 30 U/ml BbeI and 5 nM SC DNA. Samples were taken from the reactions at various times and analysed to assess the residual concentration of the substrate. The substrates were: pUC19, with one BbeI site, black squares; pDG5, with two BbeI sites, white circles; Cat (Figure 1B), with one BbeI site in each ring, black triangles.

no activity against the plasmid with a single copy of this sequence (Figure 7). However, under the same conditions and at the same enzyme concentration, BbeI readily cleaved both the plasmid with two sites and the catenane with one site in each ring (Figure 7). The ratio of the rates on the two-site to the one-site DNA, nearly 300-fold, is thus larger than any recorded previously with a Type II restriction enzyme. Moreover, the reactions on the two-site substrates were largely concerted, for the most part proceeding directly to the final products cut at both strands in both sites (data not shown). For example, the initial products from the catenane ($S_L S_S$, Figure 3A) were mainly the two linear fragments from cutting both rings, $L_L + L_S$, rather than any of the intermediates cut at less than four phosphodiester bonds.

BbeI thus has virtually an absolute requirement for two copies of its recognition sequence for its DNA cleavage reactions, but the two sites cannot be *in trans*, on two separate molecules of DNA. Instead, they must be tethered to each other, either by being *in cis* on the same DNA chain or by the interlinking of two catenane rings.

DISCUSSION

The seven enzymes examined here all cleave DNA at the same sequence, albeit at different positions within this sequence (Figure 1A). At present, neither structural nor primary sequence data are available for any of these enzymes (4), so whether the common recognition sequence stems from common features of amino acid sequence or 3-D structure remains to be determined. It has been suggested that restriction enzymes with related recognition sites are more alike in primary sequence than those with unrelated sites (50). However, sequence similarities amongst restriction enzymes seem largely uncorrelated to similarities in reaction mechanism. For example, Cfr10I, NgoMIV and SgrAI have related sequences (14) and all three interact with two sites before cleaving DNA, but they do so by different mechanisms (28,30,37). In contrast, two further members of the NgoMIV

group, Acc11I and Kpn2I, cleave DNA at individual sites, like the orthodox Type II enzymes, and are most likely functional as dimers rather than tetramers (A. Welsh and S. E. Halford, unpublished data). Nevertheless, regardless of possible similarities in protein structure, the seven enzymes examined here displayed a total of five distinct reaction mechanisms.

The only enzymes that used the same mechanism were SfoI, EgeI and EheI, which all cleave the third phosphodiester bond in the GGCGCC sequence. These three enzymes may be very similar to each other. On the other hand, two other enzymes that cleave at a common position (the second phosphodiester bond), NarI and Mly113I, clearly operate by very different mechanisms. Different mechanisms for cleaving the same bond at the same sequence have been noted before: e.g. several enzymes that cleave ↓CC(A/T)GG act differently from the prototype, the Type IIE enzyme EcoRII (13,14,51).

The five distinct mechanisms for the restriction enzymes cleaving GGCGCC can be classified on the basis of the number of recognition sites that the enzyme binds before cleaving the DNA and the number of bonds cleaved per DNA-binding event.

One site, one bond

Under all conditions tested, KasI cleaved substrates with two copies of the recognition sequence at the same rate as the DNA with one copy (Figure 4; Table 1). Though KasI might conceivably cleave the one-site plasmid by means of a *trans* reaction, by bridging two separate molecules of the plasmid, it is much more likely that it acts at individual sites, like the orthodox Type II enzymes such as EcoRI, EcoRV and SfoI (see Figure 6). However, while EcoRV and EcoRI normally cut the target in both strands before dissociating from the DNA (7,8), Kas I cuts the recognition site in just one strand before dissociation and then returns to the DNA, to cut the second strand at a slow rate. Indeed, on a DNA with two sites, it nicks both sites long before making a double-strand break at either site (Figure 4C). The ratio of the rates for first and second strand cleavages is, however, invariant with pH, so the slow rate for cutting the second strand is unlikely to be due to the negative charge on the 5′ phosphate at the nicked site.

The orthodox enzymes make double-strand breaks by virtue of their dimeric structures: one active site in the dimer cuts one strand and the other active site the second strand (7–9). The primary activity of KasI is to make single-strand rather than double-strand breaks, so it is possible that it is active as a monomer rather than a dimer. (The unknown purity of the commercial preparations of KasI, and of the other enzymes examined here, preclude subunit structure determinations by gel filtration or analytical ultracentrifugation.) Alternatively, if it is a dimer, one of the two active sites must have a faster catalytic rate than the other.

A restriction enzyme that makes mainly single- rather than double-strand breaks might seem to be incompetent for the restriction of foreign DNA *in vivo*, as single-strand breaks can be repaired by DNA ligase before they progress to double-strand scissions (52,53). However, in *E.coli*, the repair of nicks induced by either EcoRI or EcoRV involves not only DNA ligase but also recombination and other SOS activities (54). It has yet to be established whether such functions operate efficiently in *Kluyvera ascorbata*, the source of KasI. Another

possibility is that the difference between the rates at which KasI cleaves each strand is much smaller *in vivo* than *in vitro*.

One site, two bonds

SfoI, EgeI and EheI all consumed the one-site substrate at the same rate as the two-site substrate, and cleaved each site in the latter by means of two independent but kinetically equal reactions (Figure 6; Table 1). Their reactions at each individual site lead to the cleavage of both strands at that site before dissociation from the DNA. The profiles of their reactions on the one- and the two-site plasmids thus match exactly those observed previously for orthodox Type II restriction enzymes making double-strand breaks at individual sites (19,29). These three enzymes thus bind to solitary sites, almost certainly as dimers of identical subunits with one active site in each subunit, one of which cleaves one strand of the DNA and the other the second strand.

Two sites, one bond

The enhanced rate at which NarI cleaves either the plasmid or the catenane with two sites, relative to the DNA with one site (Table 1), shows that this enzyme displays its optimal activity only after interacting concurrently with two copies of the site. The reason why the one-site DNA is cleaved slowly is probably because this requires an interaction with two sites *in trans*, spanning two sites on two separate molecules of the plasmid, which is intrinsically disfavoured to interactions with sites *in cis* (21). However, the interaction with two sites leads to the cleavage of just one strand of one site during each DNA-binding event, at least at pH values ⩾6.4 (Figures 2 and 3). This pattern differs from the Type IIE enzymes such as NaeI or FokI, which interact with two sites but which then make a double-strand break at one site (31,36). It also differs from the tetrameric Type IIF enzymes such as SfiI or Cfr10I, that likewise interact with two sites but which then normally act concertedly to cut both strands at both sites (27,28).

The reaction profile of NarI is, however, reminiscent of BfiI (34). The BfiI endonuclease is a homodimer in which each subunit comprises two domains: a catalytic unit that forms the dimer but which has only one active site, at the dimer interface; a DNA-recognition unit, one attached to each catalytic domain (55). The two recognition units must each bind a copy of the target sequence but the single active site in the dimer then cleaves one phosphodiester bond at a time (34), moving sequentially from one strand to the other (56). Like NarI, the difference in the rates at which BfiI cuts the two strands at one site also varies with pH but while high pH increases the difference with NarI, it reduces the difference with BfiI (56). At the nick, a 5′ phosphate with two negative charges, as would occur at pH values >6.8, seems to encourage BfiI to move its active site to the second strand and so complete the double-strand break, whereas it causes NarI to dissociate from the DNA and thus fix the single-strand break. Despite this, the mechanistic similarities of NarI and BfiI are unlikely to be accompanied by any structural similarities. While NarI cleaves within a palindromic sequence, BfiI is a Type IIS enzyme that recognizes an asymmetric sequence and cleaves the DNA at fixed positions downstream of the site. The two proteins are thus likely to have radically different architectures. Moreover, BfiI is unique amongst restriction enzymes in not requiring Mg^{2+} ions (57) but when

NarI was tested for DNA cleavage activity in buffers without Mg^{2+}, no activity was detected (data not shown).

Two sites, four bonds

In the absence of NaCl (Figure 5A and B), Mly113I cleaved the plasmids with one and two copies of its recognition site at the same rate (Table 1) but the two-site plasmid was cleaved directly to the products cut in both strands at both sites, without liberating the intermediates en route to these products. Hence, Mly113I may need to interact with two copies of its site to cleave DNA. If so, at zero NaCl it must be able to bridge two sites *in trans* readily enough to achieve the same reaction rate as that from sites *in cis*. This was confirmed by reactions at raised salt concentrations (Figure 5C and D), where Mly113I cleaved the two-site plasmid considerably faster than the one-site plasmid (Table 1). The Type IIF enzymes SfiI (45,48) and SgrAI (29,37) both show exactly the same changes in reaction profile with salt as those observed here with Mly113I. This is clearly diagnostic of a Type IIF system. Mly113I can therefore be added to the rapidly growing Type IIF subset. It is likely to be active as a tetramer (27,28,48) with two DNA-binding clefts (30), both of which bind a copy of the recognition sequence and cleave it in both strands before releasing the end products (Figure 5B). The appearance of the intermediate products cut at one, two or three phosphodiester bonds in the reactions at elevated salt (Figure 5D) is due to premature dissociation from the DNA before cutting all four bonds (45).

BbeI also cleaved the plasmid with two sites directly to the final products cut in both strands at both sites but, unlike Mly113I, it had virtually no activity on the one-site DNA (Figure 7). The difference in cleavage rates on the two- and the one-site plasmids by BbeI, more than 300-fold, is larger than any observed previously among the Type II restriction enzymes. Bridging interactions spanning two DNA sites through 3-D space will almost always occur more readily with sites *in cis* than *in trans* (21) but an enzyme that bridges sites *in cis* by a looping interaction must also be able to bridge two sites *in trans,* even though this may be at reduced efficiency. Hence, a looping mechanism ought to result in reduced activity on sites *in trans*, as seen with NarI or Mly113I, but not the near-zero activity seen with BbeI. An absolute requirement for sites *in cis* can be accounted for by a tracking mechanism in which the enzyme translocates along the DNA between the sites: viz. the Type I restriction enzymes (39,47). However, BbeI cleaved the catenane with one site in each ring as fast as the two-site plasmid, which eliminates tracking schemes. BbeI therefore does not need covalent continuity between the sites, but rather it demands sites tethered together in 3-D space. Why this enzyme has essentially no activity on sites *in trans* has yet to be elucidated.

Orthodox versus unorthodox

Of the seven enzymes examined here, it had been suggested previously that NarI might need to interact with two copies of its recognition sequence before cleaving DNA (23,24). But no indication had been given before that any of the other six might behave in any way other than in the orthodox manner; for a Type II restriction enzyme, the orthodox being typified by enzymes like EcoRI, EcoRV and BamHI. Yet only three out of the seven acted in the orthodox manner: SfoI, EgeI

and EheI. Obviously, seven is not a statistically significant sample of the 3500 Type II restriction enzymes identified to date. Nevertheless, this, together with numerous other studies (20–38), shows that the Type II restriction endonucleases do not all act like EcoRI or EcoRV. Instead, they display a much more diverse range of reaction mechanisms than had generally been anticipated (1), most of which involve long-range inter-actions between distant DNA sites (21). The latter include the Type I and the Type III systems and also the Type IV systems that restrict methylated DNA (39). These three types probably constitute ∼50% of the restriction-modification systems that exist *in vivo* (58). They also include most of the Type IIS and the Type IIB enzymes (31–35), and, from this and other studies, a significant fraction of the Type II enzymes with palindromic sites. Hence, the so-called orthodox enzymes that act at solitary sites, like EcoRV and BamHI, may constitute a minority grouping of restriction enzymes, perhaps <25% of the total.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

ACKNOWLEDGEMENTS

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