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FokI restriction endonuclease

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ABSTRACT

The FokI restriction endonuclease recognizes an asymmetric DNA sequence and cuts both strands at fixed positions upstream of the site. The sequence is contacted by a single monomer of the protein, but the monomer has only one catalytic centre and forms a dimer to cut both strands. FokI is also known to cleave DNA with two copies of its site more rapidly than DNA with one copy. To discover how FokI acts at a single site and how it acts at two sites, its reactions were examined on a series of plasmids with either one recognition site or with two sites separated by varied distances, sometimes in the presence of a DNA-binding defective mutant of FokI. These experiments showed that, to cleave DNA with one site, the monomer bound to that site associates via a weak protein–protein interaction with a second monomer that remains detached from the recognition sequence. Nevertheless, the second monomer catalyses phosphodiester bond hydrolysis at the same rate as the DNA-bound monomer. On DNA with two sites, two monomers of FokI interact strongly, as a result of being tethered to the same molecule of DNA, and sequester the intervening DNA in a loop.

INTRODUCTION

Type II restriction endonucleases recognize specific DNA sequences, generally 4–8 bp long, and cut both strands at fixed positions within or close to the site (1). Most [but not all (2)] need Mg^{2+} as a reaction cofactor (3,4). Many restriction enzymes are dimers of identical subunits and their recognition sites are generally symmetric, with the same 5′–3′ sequence in each strand (1). In these instances, the contacts between one protein subunit and one DNA strand are duplicated by the other subunit on the complementary strand (5). This arrangement leaves the catalytic centres from the two subunits each placed to cleave one strand: the two centres exhibit equal rate constants for phosphodiester bond hydrolysis (6–9). The restriction enzymes historically considered as the orthodox Type II systems, such as EcoRI, EcoRV and BamHI, all act in this manner (3–5). A few, such as BbvCI, recognize asymmetric sequences by employing two different subunits, one for each DNA strand, but otherwise act as above (10,11). The dimeric enzymes generally cleave each recognition site in an independent reaction, though they can act processively on DNA with multiple sites (8,12).

The so-called orthodox Type II endonucleases are, however, a minority group amongst these enzymes. Most restriction enzymes have to bind two recognition sites before they can cleave DNA (3,13–16). Some, the Type IIE enzymes (17) such as EcoRII, NaeI and Sau3AI (18–20), form dimers with two (or more) DNA-binding clefts. One cleft contains the catalytic moieties from both subunits, to cut both strands of the cognate DNA. The other cleft lacks catalytic functions but the enzyme is inactive unless this cleft is also filled with cognate DNA. Consequently, Type IIE nucleases bind two copies of the recognition site but cleave only one (21). Others, the Type IIF enzymes (17), such as SfiI, Cfr10I, NgoMIV and SgrAI, also interact with two sites but cleave both sites in both strands in a concerted reaction (22–25). The Type IIF enzymes form tetramers in which two subunits bind one copy of the recognition sequence (25,26) but no activity ensues until the other two subunits bind a second copy (27). Most proteins that bind two sites have higher affinities for sites in the same molecule of DNA than for sites on separate molecules, since the local concentration of one site in the vicinity of another is usually higher *in cis* than *in trans* (15,28). The restriction enzymes that need two sites conform to these principles: both Type IIE and Type IIF systems normally cleave plasmids with two sites faster than DNA with one site (20–25). They trap loops on two-site DNA (23,29,30) while they synapse two molecules of one-site DNA (14,18,27).

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

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In all of the above examples, two subunits of the protein contact the target DNA, usually in symmetric fashion, and contribute two active sites, one for each strand. However, many Type II systems recognize asymmetric sequences and cut the DNA at fixed positions away from the site (1). The Type IIS enzymes make double-strand breaks on one side of the site while the Type IIB enzymes do so on both sides (17). Two protein subunits cannot interact symmetrically with an asymmetric DNA sequence. In principle, such a sequence can be recognized in its entirety by a single subunit. But if a restriction enzyme uses a single subunit to recognize an asymmetric sequence and if that subunit has only one active site, how does it cut both strands of the DNA? This problem is compounded by the fact that the majority of the Type IIS and Type IIB enzymes require two recognition sites for full activity [(31,32) and J. J. T. Marshall, personal communication]. Some act like Type IIE enzymes: for example, FokI, MboII and BfiI all cleave two-site substrates more rapidly than DNA with one site, but cut only one site at a time, or even just one strand (2,31,33). In contrast, the Type IIS enzyme BspMI and the Type IIB enzyme BcgI cleave their two-site substrates directly to the final products cut at both sites, like Type IIF enzymes (32,34). While a monomeric protein may be able to cleave both DNA strands, by having two catalytic centres in one polypeptide chain (35) or by moving a single centre from one strand to the other (36), it is improbable that a monomer could interact with two separate sites at the same time.

To date, the best characterized of the restriction enzymes that recognize asymmetric sequences, in terms of structural data, is the Type IIS enzyme FokI (37–40). FokI cleaves DNA 9 and 13 bases downstream of an asymmetric sequence, as follows:

5′-G-G-A-T-G-N-N-N-N-N-N-N-N-N↓N-N-N-N-N-3′
3′-C-C-T-A-C-N-N-N-N-N-N-N-N-N-N-N-N-N↑N-5′.

It exists in solution and binds DNA as a monomer (41,42). Indeed, FokI remains a monomer in free solution even at micromolar concentrations (40,41), far above the nanomolar concentrations typically used in enzyme assays (31). The monomer has two domains (43): an N-terminal DNA-binding domain that spans the entire recognition sequence and a C-terminal catalytic domain that possesses a single active site (37). To cleave both strands at one site, two monomers have to associate to give a dimer with two active sites (38,39). Dimerization occurs via the catalytic domains: the catalytic domain can by itself associate with the wild-type enzyme and mutations at one particular surface in this domain severely diminish activity (39). Even so, DNA substrates with a single site are cleaved very slowly and full activity is observed only on DNA with two or more sites (31). However, upon binding an oligoduplex with the specific sequence, two monomers can assemble into a synaptic complex with two duplexes (40). Hence, on a plasmid with two sites, two monomers may be able to bind to the separate recognition sites via their DNA-binding domains and to each other via their catalytic domains. This would give the dimeric form of the protein with the two active sites needed to make a double-strand break, with the intervening DNA trapped in a loop. On the two-site DNA, the monomers are tethered close to each other and so might interact more strongly with each other than with an untethered monomer in free solution.

In this study, the modes of action of FokI on plasmids with either one or two recognition sites were analysed from the kinetics of its DNA cleavage reactions. On the plasmid with one site, dimerization was driven by adding a mutant of FokI that had a defective DNA-binding domain but an unaltered catalytic/dimerization domain (39). The association of two wild-type monomers was also investigated by carrying out single-turnover reactions on the one-site plasmid with progressively increasing concentrations of the native enzyme. On plasmids with two sites, the length of DNA between the sites was varied, to see if FokI loops out the intervening DNA. DNA looping was first demonstrated by finding that the ability of a protein to interact with two sites *in cis* varied cyclically with the length of DNA between the sites, with a periodicity that matched the helical repeat of the DNA, ~10.5 bp (28). The periodicity arises because both DNA sites must present the same surface to the protein. On a DNA without topological nodes (30), the requisite surfaces will be on the same side of the DNA when separated by integral number of helical turns but will be on opposite sides when separated by a further half-turn. This strategy was used previously to examine DNA looping by SfiI (29), a Type IIF tetramer, and will be used here on FokI, a monomer that falls into both Type IIE and IIS categories.

MATERIALS AND METHODS

Proteins

An *Escherichia coli* strain that over-produces wild-type FokI endonuclease was a gift from Dr W. E. Jack (New England Biolabs). The strain contains an ampicillin-resistance plasmid carrying the gene for the nuclease expressed from a P_{tac} promoter gene and a kanamycin-resistance λ lysogen with the gene for the FokI methyltransferase (44). Growth of this strain, induction of gene expression, cell harvesting and disruption, and chromatography on phosphocellulose (Whatman P11) were all as described previously (44). FokI eluted from the P11 column at ~0.3 M NaCl. The fractions containing the FokI protein were diluted 3-fold into 10 mM potassium phosphate (pH 6.8), 1 mM EDTA, 10% (v/v) glycerol, loaded onto a Mono S cation exchange column (GE Healthcare) and eluted with a linear gradient of 0.1–0.8 M NaCl in 10 mM potassium phosphate (pH 6.8), 1 mM EDTA and 10% glycerol. The peak fractions, which contained FokI at a purity of >95%, were pooled and stored at −20°C.

The N13Y variant of the FokI endonuclease was purified from a strain supplied by Dr J. Bitinaite (New England Biolabs). The strain encodes a fusion protein comprised of the mutant FokI attached to an intein and a chitin-binding domain. The FokI protein was purified by the IMPACT-CN system (New England Biolabs) exactly as described before (39). The mutant protein was also >95% pure, as judged by SDS–PAGE.

Concentrations of purified FokI proteins were determined from A_{280} readings using an extinction coefficient of 72 520 $M^{-1}cm^{-1}$ and are given in terms of the monomer (39). Prior to reactions, enzymes were diluted to the requisite concentration in 10 mM Tris–HCl (pH 7.4), 0.1 mM EDTA, 100 mM NaCl, 10% glycerol and 0.2% (v/v) Triton X-100.

DNA

Plasmid pSKFokI is a 2953 bp derivative of pBluescriptSK(−) that contains a single FokI recognition site located 103 bp away from its multiple cloning site (MCS) (39). A 2979 bp plasmid with two FokI sites in inverted orientation 181 bp apart, pIF181, was constructed by cutting pSKFokI at its BamHI and XbaI sites in the MCS and ligating the resulting vector to a duplex made by annealing two oligodeoxyribonucleotides (MWG Biotech, London):

GATCAATCGCCTTGCAGCACATCCCCCTTGG
TTAGCGGAACGTCGTGTAGGGGGAACCGATC.

The FokI site in this duplex (underlined) has the same flanking sequences as the site in pSKFokI, for 5 bp upstream and for 15 bp downstream of the site (the latter spans the points of cleavage). The ligation mixture was used to transform *E.coli* HB101 (45), and transformants tested for the desired construct by restriction analysis. To confirm its validity, the plasmid was sequenced across the region spanning the two FokI sites (University of Dundee Sequencing Service), as were all the other plasmids constructed here.

To make additional plasmids with two FokI sites different distances apart, pIF181 was cleaved at individual restriction sites between its FokI sites and the DNA then treated with either T4 DNA polymerase (to remove 3′ single-strand extensions) or Klenow polymerase (to fill in 5′ extensions) (29,45). The resultant blunt-ended DNA was circularized with T4 DNA ligase and used to transform *E.coli* HB101. Transformants carrying the desired plasmids were identified as above. The initial derivatives from pIF181 were then used for multiple rounds of equivalent manipulations to yield eventually a series of plasmids, pIF170–pIF199, which all had the two FokI sites from pIF181 but with different distances between the sites. (The nomenclature of these plasmids denotes that they have inverted FokI sites separated by the number of base pairs indicated.)

The transformants were cultured in M9 minimal media with 37 mBq/l [methyl-^{3}H]thymidine and the covalently closed DNA purified by density-gradient centrifugations (21,22). Typically, >90% of the DNA in each preparation was the supercoiled form of the monomeric plasmid, with <10% as dimeric or nicked open-circle forms.

DNA cleavage reactions

Steady-state reactions contained 10 nM DNA (one of the above plasmids, ^{3}H-labelled) in 200 µl Reaction Buffer [20 mM Tris–acetate (pH 7.9), 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM DTT and 100 µg/ml BSA] at 37°C, supplemented in some cases with the N13Y mutant of FokI at a concentration between 20 and 250 nM. An aliquot of wild-type FokI (3–5 µl) was then added to a final concentration of either 0.5 or 2 nM. Samples (15 µl) were removed at timed intervals (a zero-time point was taken before adding the enzyme), mixed immediately with 10 µl of EDTA Stop-Mix (24,31), and subjected to electrophoresis through agarose under conditions that separated the supercoiled substrate from the reaction products. The segments of agarose that contained the supercoiled, open-circle and linear forms of the DNA were analysed by scintillation counting, to assess the concentration of each form at each time-point (11). Reaction velocities were evaluated by fitting the initial phase of substrate utilization to a linear slope.

Single-turnover reactions were generally carried out by first adding wild-type FokI (final concentration in the range 6.25–250 nM) to ^{3}H-labelled pSKFokI (2.5 nM) in 500 µl Reaction Buffer lacking magnesium acetate. The mix was incubated on ice for 30 min before transfer to 37°C. The reaction was then initiated by adding magnesium acetate to a final concentration of 10 mM. An aliquot (30 µl) of the mixture was removed before adding the Mg^{2+} and further 30 µl aliquots removed at timed intervals thereafter. The samples were mixed immediately with 20 µl of Stop-Mix, prior to electrophoresis through agarose and analysis as above. Some single-turnover reactions were initiated by adding FokI to a solution of pSKFokI in Reaction Buffer with magnesium acetate: these yielded the same results as those initiated by adding Mg^{2+} to the enzyme–DNA mix.

Apparent rate constants were evaluated from the single-turnover reactions by using SCIENTIST (MicroMath software, Salt Lake City, UT) to fit simultaneously the concentrations of each form of the DNA to the differential equations for the relevant reaction scheme, as described previously (11). All other fitting procedures were by non-linear regression in GRAFIT (Erithacus Software, Slough, UK).

RESULTS AND DISCUSSION

Experimental strategy

The FokI endonuclease is a monomeric protein (40,41) that cleaves plasmids with two FokI sites faster than plasmids with one site (31). A scheme that can account for these properties is shown in Figure 1. It proposes that the FokI monomer bound to its recognition site on a DNA with one site associates weakly with a second monomer from free solution, so that only a small fraction of the DNA-bound protein forms the active dimer (Figure 1a). It further proposes that, on DNA with two sites, two monomers tethered close to each other associate strongly to give a higher fraction of dimer, concomitantly trapping the intervening DNA in a loop (Figure 1b). To validate this scheme, it is necessary to determine firstly how readily the monomer of FokI associates to a dimer on a one-site DNA and, secondly, whether it traps loops on two-site substrates. The first question was examined in two ways. One used a mutant of FokI defective for specific DNA binding and which has no activity by itself; this mutant has however an intact catalytic/dimerization domain so it can still interact with wild-type FokI and enhance its activity (38,39). The association of mutant and wild-type enzymes was assessed from the rate at which the wild-type enzyme cleaved a one-site plasmid in the presence of progressively increasing concentrations of the mutant. The other way measured the wild-type/wild-type association from single-turnover reactions with FokI in excess of the DNA.

The second question was examined by testing whether the activity of FokI on DNA with two sites varied cyclically with the length of DNA between the sites, with a periodicity matching the helical repeat of DNA. The cyclical dependence of a DNA-looping interaction is observed most readily with sites 100–200 bp apart. DNA loops <100 bp in length are energetically disfavoured on account of the energy required to bend the

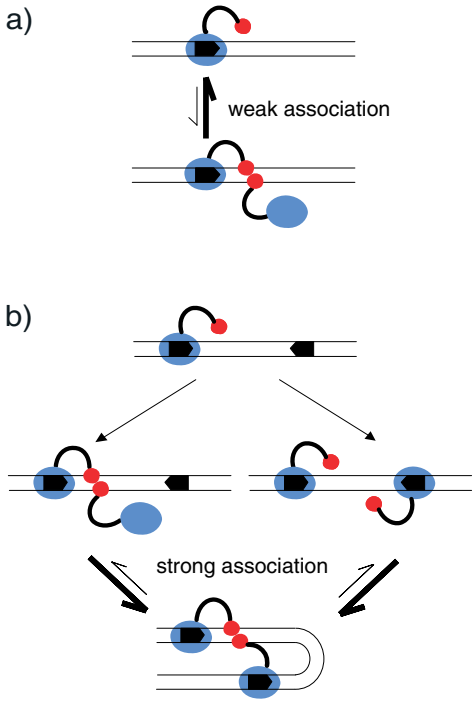

Figure 1. Schematic representation for the FokI monomer on DNA with one (**a**) or two (**b**) recognition sites. In both, the N-terminal DNA-binding domain of the FokI protein (43) is shown as a blue oval, its C-terminal catalytic/dimerization domain as a red circle and the linker between the domains as a black curve. The two strands of the DNA are shown as parallel lines and the FokI recognition sites as filled arrows (to mark their orientations). In (a), the monomer bound to the solitary recognition site associates weakly with a second monomer via its C-terminal domain to give an unstable dimer. The two catalytic domains each engage one strand of the DNA downstream of the recognition site (the assignment of target strands to monomers is arbitrary) but only one of the two DNA-binding contacts the recognition sequence. In (b), the DNA carrying a monomer of FokI at one of its two sites binds a second monomer; either to form a dimer at the initial site (left-hand pathway) or as a monomer at the second site (right-hand pathway). Both routes then lead to the dimer bound to both recognition sites via their N-terminal domains and to both strands of the DNA at one (arbitrary) cleavage site, via their C-terminal domains, with the intervening DNA trapped in a loop. The tethering of both monomers to the same chain strengthens their association and results in a stable dimer.

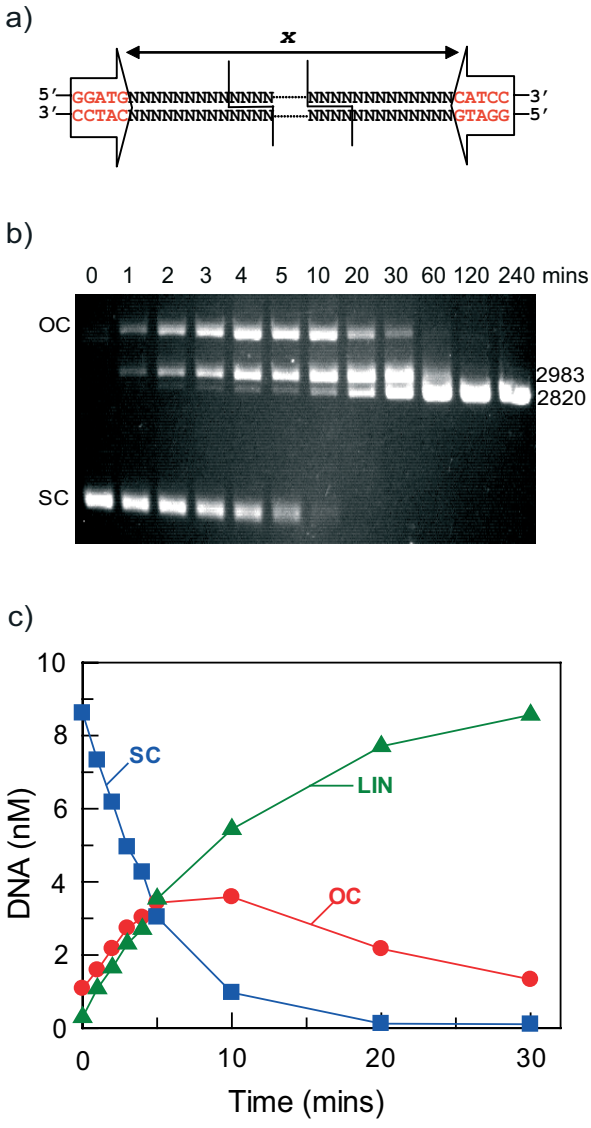

Figure 2. Plasmids with two FokI sites. (**a**) The plasmids constructed here, pIF170–pIF199, contained two copies of the recognition sequence for FokI (in red), in inverted (head-to-head) orientation (as indicated by the arrows encompassing the sequences). The positions where FokI bound to either left- or right-hand recognition sites cleave the DNA are marked with jagged lines. The distances between the sites are measured from recognition sequence to recognition sequence (noted as ***x***) rather from the cleavage positions. (**b**) The reaction contained 2 nM FokI and 10 nM ^{3}H-labelled pIF185 (90% in its SC form, 10% as OC) in Reaction Buffer at 37°C. After adding the enzyme, aliquots were removed from the reaction at the times indicated above each lane, mixed immediately with Stop-Mix, and subsequently subjected to electrophoresis through agarose. The electrophoretic mobilities of the SC and the OC forms of pIF185 are indicated on the left: on the right, the 2983 bp product from cutting pIF185 at one FokI site and the 2820 bp product from cutting both sites (the other product from cutting both sites is 163 bp and migrates off the end of the gel). (**c**) The concentrations of the following forms of DNA from the reaction in (b) were determined by scintillation counting: supercoiled DNA substrate (blue squares, marked SC); open circle DNA (red circles, marked OC); sum of the linear products cut at one and at two FokI sites, (green triangles, marked LIN).

DNA, while loops of >300 bp are largely independent of the helical periodicity as the change in twist is then dissipated over many base pairs (15,28). Starting from a plasmid with a single FokI site, pSKFokI (39), a series of plasmids was constructed with two FokI sites separated by 170–199 bp, pIF170-pIF199. As enzymes that bridge two asymmetric sequences can be affected by their relative orientation (46,47), all of these plasmids carried their FokI sites in inverted (head-to-head) orientation (Figure 2a). In this arrangement, the sites of DNA cleavage by FokI are 22 bp closer together than the recognition sites, as a result of FokI acting 9 and 13 bp downstream of its recognition sequence. The distances noted here are the numbers of bp separating the recognition sites rather than the cleavage sites (Figure 2a). Their cleavage sites are separated by 148–177 bp.

Steady-state reactions

The reactions of wild-type FokI endonuclease on the above plasmids were first carried out under steady-state conditions, with the enzyme at a lower concentration than the DNA. Samples were withdrawn from the reactions at timed intervals and subjected to electrophoresis through agarose, to separate the supercoiled substrate from the nicked and the linear DNA

products (Figure 2b). The DNA was ^{3}H-labelled and the concentrations of each form at each time point measured by scintillation counting (Figure 2c).

As noted previously (31), FokI cleaves the supercoiled (SC) form of a plasmid with one recognition site to give mainly the linear (LIN) form cut in both strands; a small fraction of the SC substrate was converted initially to the open-circle (OC) form cut in one strand but this was subsequently cleaved in its intact strand to give more of the LIN product (data not shown). Hence, after binding to a DNA with one site, the FokI endonuclease usually cuts both strands before dissociating from the DNA, a process that requires either two active sites or one site used twice over (36).

The only substrate with two FokI sites examined previously in kinetic experiments had sites in directly repeated (head-to-tail) orientation (31). This study used a series of plasmids with two sites in inverted orientation separated by varied distances. Nonetheless, these plasmids were all cleaved by FokI in the same manner as the plasmid with repeated sites, as shown here with a representative example, pIF185 (Figure 2). The SC form of pIF185 was converted first to its OC form, by cutting one strand at either one or both sites. Some of the OC DNA observed during the reaction may remain bound to the enzyme, as a reaction intermediate prior to generating the products cut in both strands. But since the concentration of OC DNA rose to a level above that of the enzyme, some OC DNA must be liberated from the enzyme into free solution after the nuclease has cut just one strand. The DNA was then converted into its full-length linear form cut in both strands at either one of the two sites and eventually, in a much slower reaction, to the final product cut in both strands at both sites (the smaller of the two products, 163 bp in the case of pIF185, migrates off the end of the gel). As the 2983 bp product from cutting pIF185 at one FokI site was only partially separated on the gel from the principal (2820 bp) product from cutting both sites (Figure 2b), the concentrations of these two species were assessed together as LIN (Figure 2c). On both one- and two-site plasmids, reaction velocities were evaluated from the utilization of the SC DNA rather than from the formation of any individual product.

The velocities of FokI reactions on one- or two-site substrates can be compared only at the same enzyme concentration, as its reaction rates increase disproportionately with enzyme concentration rather than linearly (31,39). At the concentration of FokI tested (Figure 3a), its turnover number on the representative two-site plasmid, pIF185, was about 10 times faster than that on the one-site plasmid, pSKFokI; 0.42 mol DNA/mol enzyme/min compared with 0.046 mol/mol/min. Many restriction enzymes that act at individual sites, such as EcoRI or EcoRV, have turnover rates on plasmid substrates of around 1 mol/mol/min (3,6–8,11). The turnover rate of FokI on the one-site plasmid is thus very much slower than is usual for a Type II restriction enzyme, though it cleaves the two-site plasmid at a normal rate for a restriction reaction.

In an earlier study, FokI cleaved a plasmid with two directly repeated sites 211 bp apart about 20 times faster than the one-site plasmid (31). The reason why it cleaved this two-site plasmid more rapidly than pIF185 might be due to the different orientations, as was observed with another Type IIS enzyme BspMI (47). Alternatively, with closely spaced sites, the different orientations result in considerably different distances

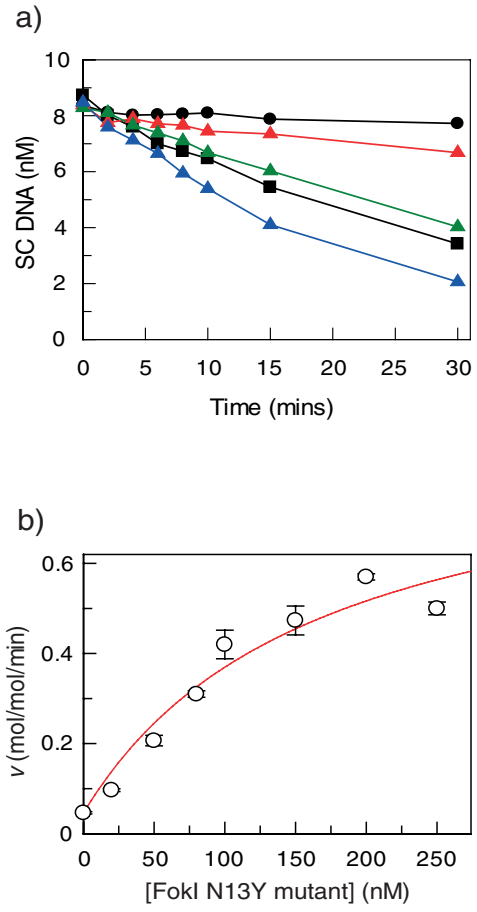

Figure 3. FokI cleavage of DNA with one or two sites. (**a**) Reactions contained 0.5 nM wild-type FokI endonuclease and 10 nM DNA in Reaction Buffer at 37°C: either pSKFokI, a plasmid with one FokI site (black circles); or pIF185, a plasmid with two FokI sites (black squares). Samples were removed from the reactions at timed intervals and the residual concentrations of the SC substrate measured as in Material and Methods. Additional reactions of wild-type FokI on pSKFokI were carried out as above except for the addition of the N13Y mutant of FokI at one of the following concentrations; 20 nM (red triangles), 50 nM (green triangles), 100 nM (blue triangles). (**b**) Initial rates for the utilization of the SC substrate were measured from reactions containing 0.5 nM wild-type FokI and 10 nM pSKFokI, as above, in the presence of the N13Y mutant at the concentration indicated on the *x*-axis. Each data point is the mean of ⩾2 independent reactions; error bars indicate SDs. The velocities on the *y*-axis are given in terms of mol DNA consumed/mol wild-type FokI/min. The red line is the optimal fit of the data to Equation 1, which gave: $v_0 = 0.046 \pm 0.003$ mol/mol/min; $v_\infty = 0.91 \pm 0.19$ mol/mol/min; $K_{Dm} = 166 \pm 73$ nM.

between the scissile phosphodiester bonds: with inverted (head-to-head) sites, 22 bp shorter than the site-to-site distance; with repeated (head-to-tail) sites, the same as the site-to-site distance. In addition, though the plasmids in the pIF170-pIF199 series all carry FokI sites in inverted orientation, they are not cleaved by FokI at a uniform rate (see below, Figure 5a). The turnover rates of FokI on two-site plasmids, relative to the one-site DNA pSKFokI, thus vary from one two-site substrate to the next, from ~5 times faster with some to ~20 times faster with others.

Wild-type/mutant dimerization at a single site

The replacement of Asn13 in FokI, a pivotal base-specific contact (37), with a bulky tyrosine residue is likely to disrupt

the entire interface between the protein and the recognition sequence (39). The substitution is, however, distant from the C-terminal catalytic/dimerization domain. The N13Y mutant of FokI has no specific DNA cleavage activity, presumably due its inability to bind to its recognition sequence. Under the conditions used here, prolonged incubation of 200 nM N13Y with 10 nM DNA, resulted in low levels of non-specific cleavage, as judged by smeared products in agarose gels (data not shown). Nevertheless, qualitative experiments had revealed that the addition of N13Y could enhance the rate at which wild-type FokI cleaves DNA (39).

To examine quantitatively the ability of the N13Y mutant to stimulate the native enzyme, reactions were carried out on the one-site plasmid with a fixed (low) concentration of wild-type protein to which varied concentrations of the mutant were added: the reaction velocities were then measured at each concentration of mutant (Figure 3). In these experiments, the native enzyme is the only protein present that can actually cleave DNA but, because it was present at a 20-fold lower concentration than the plasmid, it gave a very slow rate of DNA cleavage (Figure 3a). The slow rate can be accounted for by the fact that, at a 20:1 ratio of plasmid to enzyme, only a small fraction of the DNA molecules will carry the two monomers of FokI needed for the cleavage reaction. The addition of the mutant enzyme enhanced markedly the rate at which this concentration of wild-type enzyme cleaved the DNA. As the concentration of N13Y was increased, the rate first increased up to and then beyond the level observed on the representative two-site plasmid, pIF185 (Figure 3a).

The increase in reaction velocities with the concentration of the mutant enzyme followed a hyperbolic curve (Figure 3b), from a basal level (v_0) observed with the wild-type enzyme alone to a maximal value (v_∞) at saturation with the mutant. The hyperbolic response can be accounted for by assigning the basal velocity to the dimer formed from the low level of wild-type FokI in the reaction without N13Y and the maximal velocity to the situation when all of wild-type enzyme has dimerized with the mutant. The observed velocity (v) is then related to the concentration of the mutant ([M]) through the equation:

$$v = v_0 + (v_\infty - v_0)\left\{\frac{[M]}{(K_{Dm} + [M])}\right\}, \qquad 1$$

where K_{Dm} is the equilibrium dissociation constant for the wild-type/mutant dimer. At saturation, the mutant caused a ~20-fold increase in the rate at which native FokI cleaved the one-site DNA, from 0.046 to 0.91 mol/mol/min. The maximal rate on the one-site DNA thus matches the steady-state rate observed on the optimal two-site substrate for FokI found to date (31). However, to achieve the maximal rate on the one-site DNA, high concentrations of the mutant protein had to be added, which shows that the association of wild-type and mutant enzymes is indeed weak, with a K_D value (the point at which one half of the native enzyme has formed a dimer) of 166 ± 73 nM (Figure 3b). The relatively large error margins on this K_D value are due to the paucity of data at protein concentrations above the K_D, but the low level of non-specific DNA cleavage by N13Y precluded measurements at higher concentrations of the mutant.

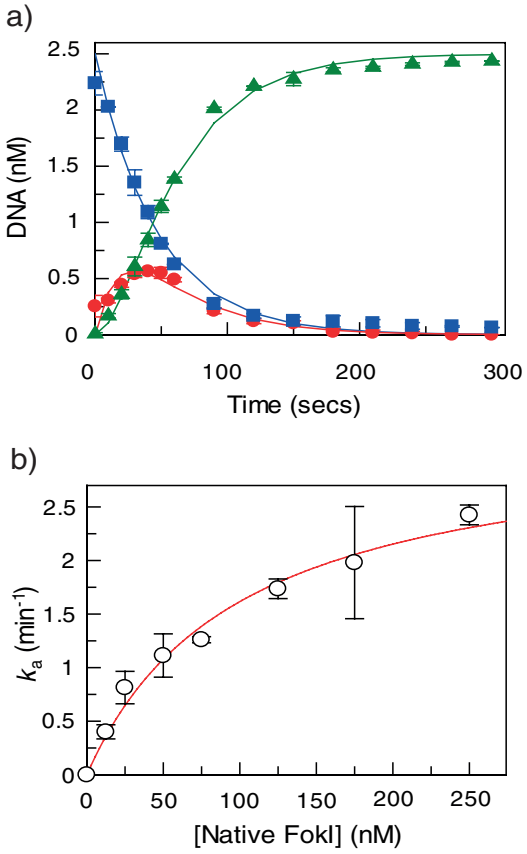

Figure 4. Single-turnovers of wild-type FokI. (**a**) FokI endonuclease was incubated with pSKFokI (a plasmid with one FokI site) before adding magnesium acetate, to give a reaction that contained 50 nM FokI and 2.5 nM ^{3}H-labelled DNA (~95% SC) in Reaction Buffer at 37°C. Samples were removed from the reactions at timed intervals and analysed as in Materials and Methods to determine the concentrations of the following forms of the DNA: SC DNA, blue squares; OC DNA, red circles; LIN DNA, green triangles. The changes in the concentrations of all three forms of the DNA were fitted globally to the kinetic equations for a two-step reaction scheme (Equation 2), to give values for the apparent rate constants k_a and k_b: the lines shown are the best fits. (**b**) Reactions at varied concentrations of FokI endonuclease were carried out as in (a) and, in each case, values for k_a and k_b determined as above. The dependence of k_a on the enzyme concentration was fitted to Equation 4. The best fit (red line) was obtained with K_{Dw} = 99 ± 19 nM and k_2 = 3.2 ± 0.3 min^{-1}. In both (a) and (b), each data point is the mean of ⩾2 independent repeats: error bars indicate SDs.

Wild-type/wild-type dimerization at a single site

To carry out an enzyme reaction under steady-state conditions, the enzyme must be present at a lower concentration than the substrate (48). If the wild-type/wild-type dimer of FokI has the same equilibrium dissociation constant as the wild-type/mutant dimer, then to convert more than half of the enzyme to dimer, its concentration has to be >166 nM, which in turn requires >>166 nM plasmid to achieve steady-state conditions. Hence, at any practicable concentration of one-site plasmid, only a tiny fraction of the native enzyme can ever be in its dimeric state during a steady-state reaction. The wild-type/wild-type dimerization was therefore examined by carrying out single-turnover reactions of FokI, with the enzyme at higher concentrations than the one-site plasmid (Figure 4a). Single turnovers of Type II restriction enzymes generally have

half-times of <1 s and are too fast to monitor without rapid-mixing equipment (6–9,11). However, the single-turnover reactions of FokI on pSKFokI had much longer half-times and could be carried out by hand-mixing the reagents: samples were taken from the reactions at ⩾10 s intervals, added to the Stop-Mix and analysed as before to measure the amounts of SC, OC and LIN DNA at each time point sampled (Figure 4a).

The changes in the concentrations of the SC, OC and LIN forms of the DNA were fitted globally to a two-step reaction scheme,

$$\mathrm{SC} \xrightarrow{k_a} \mathrm{OC} \xrightarrow{k_b} \mathrm{LIN}, \qquad \mathbf{2}$$

to give values for the two apparent rate constants, k_a and k_b, respectively, at each enzyme concentration tested (Figure 4a). The apparent rate constants for the first step, the k_a values, increased with the concentration of FokI in a hyperbolic manner (Figure 4b), while those for the second step showed no systematic variation: at virtually all FokI concentrations tested, the values for k_b fell in the range 3.5 ± 1.1 min^{-1} (see Figure 4a for one FokI concentration, other concentrations not shown). [The large scatter in the values for k_b is a consequence of the relatively low yield of OC DNA during these reactions, with the result that the apparent rate constants for the OC→LIN step are ill-defined.]

The single turnovers were mostly done by first equilibrating the enzyme with the DNA in the absence of Mg^{2+}, conditions where FokI can bind to its recognition site but where it has no catalytic activity; DNA cleavage was then initiated by adding Mg^{2+}. However, at all FokI concentrations examined, reactions started by adding enzyme to DNA and Mg^{2+} gave the same rate constants as those started by adding Mg^{2+} to the enzyme–DNA mix (data not shown). Hence, the single-turnover rates cannot be limited by the initial association of the FokI monomer with the DNA but must instead reflect a step in the reaction pathway after the DNA-binding step. The following scheme can accommodate both the increases in k_a with increasing FokI concentrations, and the invariance of k_b:

$$\mathrm{E.SC} + \mathrm{E} \xrightleftharpoons{K_{Dw}(=k_{-1}/k_1)} (\mathrm{E})_2\mathrm{SC} \xrightarrow{k_2} (\mathrm{E})_2\mathrm{OC} \xrightarrow{k_3} (\mathrm{E})_2\mathrm{LIN}. \qquad \mathbf{3}$$

E and $(E)_2$ are the monomeric and dimeric forms of the FokI enzyme and K_{Dw} the equilibrium dissociation constant for the dimerization of the wild-type enzyme on the DNA. Provided that the protein is in excess of the DNA and that the equilibration between monomer and dimer is rapid compared with the first hydrolytic step in the reaction pathway, the apparent rate constants for the two steps, k_a and k_b (2), are related to the intrinsic rate constants for the individual steps in the reaction mechanism, k_2 and k_3 (3), as follows (11):

$$k_a = \frac{k_2 \times [E_0]}{(K_{Dw} + [E_0])}, \qquad \mathbf{4}$$

$$k_b = k_3, \qquad \mathbf{5}$$

where $[E_0]$ is the total concentration of FokI endonuclease.

Equation 3 thus predicts that the values of k_a should increase with the enzyme concentration in a hyperbolic manner, governed by the monomer/dimer equilibration, until reaching a maximal value when all of the enzyme-bound DNA is dimeric. By fitting the increases in k_{cat} to Equation 4, the equilibrium dissociation constant of the wild-type/wild-type dimer at the recognition site, K_{Dw}, was evaluated at 99 ± 19 nM (Figure 4b). This value lies within the error margins from that for the wild-type/mutant dimer, 166 ± 73 nM (Figure 3b). The N13Y mutant of FokI thus associates with the wild-type enzyme at an individual recognition site just as readily as a second monomer of the wild-type enzyme, even though this mutant is blocked from specific DNA binding. In the wild-type/mutant dimer at an individual site, only the wild-type subunit can be bound to the recognition site. Hence, in a wild-type/wild-type dimer at an individual site, only one of the two subunits can contact the recognition sequence, while the DNA-recognition domain from the second subunit must remain detached from the DNA.

Equation 3 also indicates that as the FokI concentration is increased, the values for the apparent rate constant k_a should approach that for the intrinsic constant k_2. Extrapolation to infinite enzyme yielded a value of 3.2 ± 0.3 min^{-1} for k_2 (Figure 4b). Equation 3 indicates further that the values of k_b ought not to vary with the enzyme concentration, as this step is preceded by an irreversible step in the reaction pathway, the cleavage of the first strand: the invariant value for k_b, 3.5 ± 1.1 min^{-1}, corresponds directly to k_3. The intrinsic rate constants at which the FokI dimer bound to an individual site cuts the two strands of the DNA adjacent to that site are thus indistinguishable, within error limits. However, the two rate constants for the DNA cleavage steps are both considerably larger than the steady-state turnover rate of the wild-type enzyme, even when all of the wild-type had been converted to dimer by saturating it with the N13Y mutant, i.e. the value for v_∞, 0.91 min^{-1} (Figure 3b). Hence, the turnover rate is limited not by the DNA cleavage steps in the reaction pathway but by some subsequent step, most likely the final dissociation of the product cut in both strands, as is the case with many other restriction enzymes (3,6–8).

With dimeric restriction enzymes at palindromic recognition sequences, for instance EcoRV or EcoRI, the interactions between one protein subunit and one half of the DNA are duplicated by the second subunit with the other half of the DNA (5). The active sites in each subunit are positioned identically and they exhibit the same rate constants for hydrolysing their target phosphodiester bonds, one in each strand (6–9). In contrast, in the dimeric form of FokI at an individual recognition site the two monomers are not positioned identically: one subunit is bound to the recognition sequence while the other is attached to the DNA-bound monomer by a protein-protein interaction. The active site in the DNA-bound subunit of the FokI presumably attacks one specified strand of the DNA, probably 13 bp away in the antisense (CATCC) strand, and the protein-bound subunit the other strand, the sense (GGATG) strand (40). Yet, despite the lack of equivalence of the two subunits, the rate constants for the two hydrolytic reactions are indistinguishable from each other. The catalytic domains of the two subunits are thus likely to be in equivalent configurations, even if the two DNA-binding domains are not equivalent.

DNA looping with two sites

Under steady-state conditions, FokI cleaves plasmids with two recognition sites more rapidly than plasmids with one site though the velocity on the one-site DNA was elevated to that on the two-site DNA by adding a large excess of FokI protein (Figure 3a). This suggests that FokI has the same intrinsic activity on one-site and two-site DNA but that it dimerizes more readily on the two-site substrate; perhaps two monomers bind to the separate recognition sites via their N-terminal domains and to each other via their C-terminal domains (40). If so, the DNA between the two sites is trapped in a loop. To test for looping by FokI, its activity was examined on a series of plasmids with two sites separated by varied distances, to see if it varies cyclically with the distance. Reactions were carried out on each plasmid in the series pIF170–pIF199. These plasmids possess two FokI sites in inverted orientation, separated by the number of base pairs indicated (Figure 2a). All of the reactions contained a 20-fold lower concentration of enzyme than DNA recognition sites (as in Figure 2c), conditions that disfavour the dimerization of FokI at an individual site.

The velocity of the reaction on each plasmid was measured from the linear decline in the concentration of the supercoiled substrate, over the initial portion of the reaction. The velocities varied from one plasmid to the next, but not in a systematic manner (Figure 5a). A Fourier transform of the reaction velocities failed to reveal any cyclical dependence on the length of DNA between the sites (data not shown). However, it was shown above that the rate-limiting step of the FokI reaction occurs after the DNA cleavage step and is probably the release of the cleaved product. Consequently, even if the change in distance between the FokI sites affects its ability to bridge the sites, it will not necessarily affect the steady-state velocities.

The SC DNA of each plasmid in this series was cleaved initially to give the OC form nicked at either one or both sites: then the LIN DNA with a double-strand break at one site; finally the end product with double-strand breaks at both sites (Figure 2b). During these reactions, the concentration of OC DNA rose to a maximum value that was higher than the enzyme concentration, and then declined as the reaction progressed towards completion. The OC DNA formed in excess of the enzyme cannot be an enzyme-bound intermediate en route to the products cut in both strands, but must instead be nicked DNA that has dissociated from the enzyme into free solution, before the enzyme cuts the second strand. The magnitude of this excess provides a measure of the instability of the enzyme–DNA complex (29). An unstable complex may have a lifetime that is too short to allow the enzyme to cut both strands before it falls apart: in this case, the enzyme will often dissociate from the DNA after cutting just one strand and liberate a large amount of OC DNA. Conversely, a stable complex will often remain intact for sufficient time to allow the enzyme to cut both stands before dissociating, so only a small amount of OC DNA is released during the reaction.

The maximal amount of OC DNA produced during the reactions on each plasmid was measured, and the excess of OC DNA over enzyme plotted against the inter-site spacing (Figure 5b). The reactions on the substrates with sites separated by 170, or 180 or 190 bp all resulted in the release of relatively small amounts of OC DNA, indicative of relatively stable DNA–protein complexes. In contrast, the substrates with spacings in between those noted above, e.g. 174 or 185 or 196 bp, all yielded larger amounts of OC DNA, indicative of unstable complexes. The amount of OC DNA liberated during these reactions varied cyclically with the spacing, in the manner of a sine wave. The best fit to a sine function was obtained with a periodicity of 9.9 bp, close to the value for the helical repeat of DNA under similar solution conditions, 10.5 bp (49). [For a DNA looping interaction, the amplitude of the cyclical response diminishes with increasing cycle number (28) but as the range of site separations examined here covers only three helical repeats, a sine

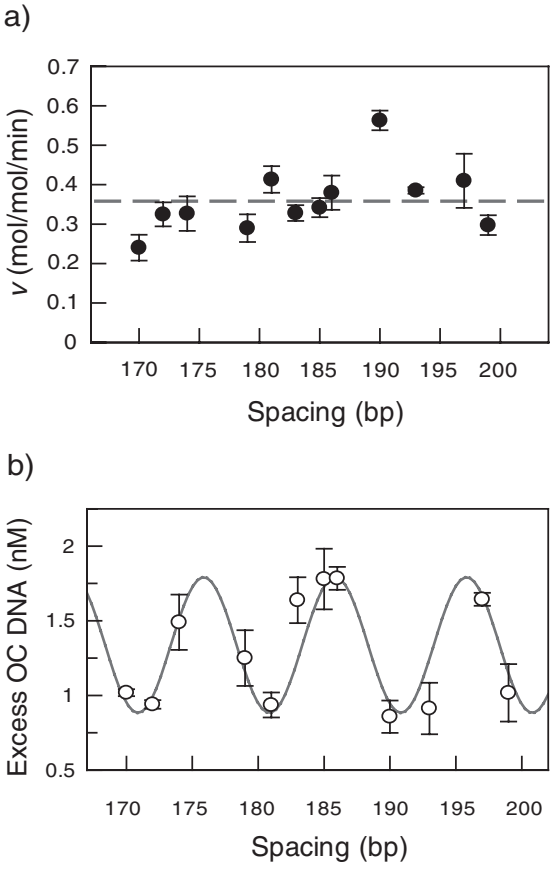

Figure 5. Varied spacings between two FokI sites. Reactions contained 2 nM wild-type FokI endonuclease and 10 nM plasmid (⩾85% supercoiled) in Reaction Buffer at 37°C. The DNA was one from the series pIF170–pIF199, which each contain two FokI sites in inverted orientation separated by the number of base pairs indicated. (**a**) Reaction velocities were determined from the decline in the concentration of each supercoiled substrate with time and are plotted against the inter-site spacing for that plasmid. The dashed line indicates the mean of the velocities across the series. (**b**) The maximal concentration of OC DNA generated during each reaction was measured and the concentration of the enzyme subtracted from these values, to give the excess of OC DNA over the enzyme. The excess is plotted against the inter-site spacing and the data fitted to the equation for a sine wave, $y = A.\sin(\omega.x + P) + \text{offset}$ (where A, ω and P denote the amplitude, periodicity and phase, respectively). The line shows the optimal fit, which was obtained with a periodicity of 9.9 ± 0.4 bp. In both (a) and (b), each data point is the mean from more than three independent measurements; error bars indicate SE of means.

function with a constant amplitude still provides an approximate account.]

Since the amount of OC DNA liberated during these reactions is related to the stability of the complex, the stability of the reactive complex of FokI with a two-site substrate depends on whether or not the sites are positioned on the same face of the DNA helix. Hence, to achieve its maximal activity on a two-site substrate, the FokI enzyme must bind to both sites concurrently and trap the intervening DNA in a loop. The monomeric form of FokI encompasses a single recognition sequence (37) so the concurrent binding to two sites must involve the dimeric form of the protein, with presumably one subunit bound to each recognition site. In principle, the looping interaction could be established by first forming the dimer at one site, as on a one-site DNA, and then using the subunit that is not bound to the DNA to capture the second site (Figure 1b). Alternatively, the loop could be trapped by two monomers each binding to a separate site and then associating with each other. It has yet to be determined which of these pathways is used by FokI, or whether both contribute.

CONCLUSIONS

Most restriction enzymes are maximally active only after interacting with two copies of their recognition sequence (3,15). For the Type I and the Type III endonucleases, the interaction with two sites involves the translocation of the intervening DNA into loops that expand with time, until the enzymes collide with each other (13,15). In contrast, the Type II endonucleases that need two sites generally possess two separate binding surfaces for their cognate DNA, both of which have to be occupied before the enzyme becomes fully active (14–16). The two copies of the recognition sequence can be located either on separate DNA molecules, in which case the enzyme bridges the two molecules; or they can be on the same molecule, when the concurrent binding of the enzyme to both sites holds the intervening DNA in a loop. A protein that has to bind to two DNA sites to fulfil its function will generally engage two sites in the same molecule more readily than sites in two separate molecules of DNA, as the physical separation between sites *in cis* is almost always less than that between sites *in trans* (15,28). Consequently, under almost all conditions, these Type II enzymes cleave two-site substrates more rapidly than one-site substrates. The only exceptions are situations, typically low salt, where even though the K_m value for the one-site DNA is larger than that for the two-site DNA, it is still sufficiently low to yield the V_{max} rate (24,34,50).

Under standard reaction conditions, the FokI endonuclease also cleaves plasmids with two copies of its recognition sequence more rapidly than plasmids with a single copy (Figure 3a), but for reasons that differ from many other Type II restriction enzymes. It is however unlikely that the scheme for FokI (Figure 1) is unique to this enzyme, as several other Type IIS restriction enzymes are almost certain to act in the same way as FokI (31,33). Instead of the DNA–protein association being more favourable with the two-site substrate as is the case with many Type II enzymes, FokI displays a more favourable protein-protein association on the two-site DNA. Indeed, FokI does not need to interact with two copies of its recognition sequence in order to achieve its full activity. It can achieve the same turnover rate in its steady-state reactions on a DNA with one site as that on the optimal two-site substrates (Figure 3a) but to do so, protein concentrations >>100 nM have to be employed (Figures 3b and 4b). At the enzyme concentrations typically used in restriction assays, usually ⩽1 nM, FokI cleaves one-site substrates at remarkably slow rates, much slower than is usual for Type II restriction enzymes and much slower than FokI itself on a DNA with two sites. The monomer/dimer equilibrium constant measured here shows that the association is indeed weak, as had been proposed, and has a K_D of ~100 nM (38) (Figure 1a). Hence, at ~1 nM enzyme, only a small fraction of the monomers bound to solitary sites will have associated with a second monomer to give the active dimer, which results in a reaction velocity far below its maximum.

Nevertheless FokI dimerizes much more readily when one of the two subunits is bound to the DNA than when both subunits are free in solution: the free protein in the absence of cognate DNA remains a monomer even at protein concentrations >1 μM (40,41). The DNA-bound subunit must therefore be in a conformation that favours dimerization relative to the free protein.

Only one of the subunits in the FokI dimer at a single site is bound directly to the recognition site in the DNA. The two subunits are therefore in different environments, in contrast to an orthodox (dimeric) restriction enzyme at a palindromic site where the two subunits are in identical environments (5). It is therefore remarkable that the two subunits of the FokI dimer at its asymmetric site display the same rate constants for phosphodiester bond hydrolysis (Figure 4), in the manner of an orthodox enzyme at a symmetrical site (6–9). On the other hand, when bound to a DNA with two FokI sites, the DNA between the sites is held in a loop (Figure 5), which shows that both subunits of the FokI dimer must then bind to a copy of the recognition sequence. The binding of two subunits to separate sites in the same molecule of DNA tethers the subunits in close proximity of each other, so that they then associate strongly to the dimer (Figure 1b). This in turn leads to the enhanced reaction velocity on the two-site substrates.

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